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Fax: 36 (1) 210 2959
E-mail: actamih@net.sote.hu

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"DIFFICULT TO TREAT INFECTIONS" PHARMACOKINETIC AND PHARMACODYNAMIC FACTORS

A REVIEW

J. M. SCHIERHOLZ*, J. BEUTH, G. PULVERER

Institute for Medical Microbiology and Hygiene of the University of Cologne, Goldenfelsstr.19–21, 50935 Cologne, Germany

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"Difficult to cure infections" are characterized by poor penetration of antibiotics into infected vegetations, altered metabolic state of bacteria within the vegetation, absence of adequate host defense/cellular response.

These infections typically include endocarditis, urinary tract infections (infected urinary tract stones), abscesses, infected fibrin clots (septic thromboemboli, haematomas, catheter-related infections) and foreign body infections. Four main aspects are discussed for the influence on human therapy:

1. the kinetics of antibiotic diffusion into vegetations
2. the specificity of some pharmacodynamic aspects and pharmacokinetic regimes
3. fibrin as one of the main constituents associated with infectious processes and
4. synergistical activities of antibiotic combinations on bacterial vegetations.

Keywords: antimicrobial therapy, pharmacokinetic factors

Although most microorganisms are highly susceptible to many antibiotics an efficient therapy must be given in order to achieve a bactericidal effect and it must be maintained for an appropriate time to eradicate these infections.

The poor effect of mono-antibiotic treatment may be due to several factors. Usually, the diffusion of antibiotics into bacterial vegetations, abscesses or biofilms is poor. The generation time of bacteria may be significantly prolonged, thus influencing the results of treatment. The local milieu, e.g. pH, protein concentration, concentration

* Corresponding author

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of various ions, bacterial enzymes and leukocyte enzymes may also inhibit the effect of antibiotics. The bacteria may develop resistance or be protected against certain antibiotics. Fibrin as integral component of intravascular thrombus, surgical wounds, haematomas, vegetations and foreign bodies has been shown to be a predisposing factor for infection. Fibrin may further act as a protective environment for microorganisms since fibrin clots are hard to penetrate by antibiotics. Infectious processes in endocarditis vegetations involve local activation of coagulation resulting in deposition of fibrin. In total, it is still unexplained how the vegetations infected with a high density of bacteria are able to escape both the humoral and the cellular immune defense forces.

Pharmacokinetics of antibiotic diffusion into vegetations

In an experimental endocarditis model equilibrium between antibiotic concentration in the serum and the vegetation was found and the concentrations in infected vegetations were higher than in noninfected ones [1]. Thus, the difficulty encountered in sterilizing the vegetation contrasts with the apparently adequate concentration of antibiotics in the vegetation. The diffusion of the antibiotics into large vegetations may be one explanation. For teicoplanin, a measurable concentration could be shown at the periphery of the vegetations, however, no diffusion into the core [2]. For penicillin and cephalosporines a high diffusion gradient could be verified between the periphery and the core after one half-time of the drugs in contrast to the initially progressively diffusion into the vegetation. A homogeneous diffusion throughout the vegetation was observed with aminoglycosides, chinolones and daptomycin [3, 4]. The concentration gradient between the periphery and the core of vegetation could explain the need for local concentrations significantly exceeding the MICs and MBCs. The failure of teicoplanin to diffuse into the core of vegetations could be one explanation for the failures observed with this antibiotic in the therapy of human staphylococcal endocarditis [5]. Even after increasing the teicoplanin dose to about 5 times the MIC of *S. aureus* and about 10 times the MICs of most strains of *Staphylococcus* species therapeutic failures have been observed [5]. The decreased activity of teicoplanin in the presence of a high inoculum as well as the deleterious influence of polysaccharides can also contribute to these failures. However, other factors such as the killing rate by the antibiotics, the possible inactivation of the antibiotic by local, physicochemical conditions and the metabolic state of the bacteria must be taken into account.

Pharmacodynamic factors

Several general principles of the therapy of bacterial vegetations are known. A bactericidal effect must be obtained for a long period of time and there is a good correlation between killing kinetics by defined antibiotics *in vitro* and their *in vivo* efficacy [6]. In severe infection, the kinetics of the bactericidal effect and the duration of the persistent suppression of bacterial growth can bring the antibiotic concentration to a subinhibitory local level (called the post-antibiotic effect, PAE) which controls the antibiotic efficacy as well as the dose and the dosing interval [7]. Antibiotics with a slow bactericidal killing rate (i.e. with a time-dependent effect) and no PAE, such as betalactams, must be administered at short intervals in order to maintain local inhibitory levels throughout the entire application interval. In contrast, antibiotics with a rapid killing rate (i.e. with a concentration dependent effect) and with a PAE such as aminoglycosides and chinolones can be administered at longer intervals. Some pharmacodynamic factors are specific for the antibiotic activity towards vegetations:

a, the number of bacteria in vegetations,

b, the metabolic state of bacteria and,

c, the local secretion of exopolysaccarides [8].

Mechanism of biofilm drug resistance

Drug resistance of adherent bacteria is based on multifactorial interactions of different physicochemical and pharmacological properties. Several mechanisms are thought to be dominant:

- the suppression of bacterial growth within the biofilm,
- the physicochemical interaction of the glycocalyx with defined antibiotics (via dipole-dipole-, H-, ionic-bonds- complexes),
- changes of the cell envelope and cell wall alterations subsequent to cell density transcriptional activation following adhesion to hard and soft tissues,
- oxygen limitation,
- microenvironment at the infection site,
- age and corresponding biomass of the biofilm.

Bacteria deep inside vegetations have reduced metabolic activities as assessed by measurement of H-L-alanine incorporation after incubation of the infected vegetation *in vitro* [8]. These resting metabolically nonactive colonies could explain relapses after therapy and the necessity of long-term therapy. Metabolic modifications

due to nutritional limitations are associated with a dramatic increase in bacterial density and the development of a thick exopolysaccharide layer. These factors in addition to the uneven diffusion of antibiotics inside vegetations can modify the local effective level. Also the time required to sterilize bacterial vegetations might be increased with a consequent need to lengthen the duration of treatment.

The role of fibrin clots

The tropism of many pathogens for fibrin may favour bacterial growth leading to infection [9]. As penetration of antibiotics into fibrin is limited, therapy of septic phlebitis, bacterial endocarditis or infection of intravascular devices, where fibrin is an integral part of the infection, is often ineffective or necessitates long-term therapy.

Fibrin is one of the main constituents of the inflammatory response associated with most infectious processes [10]. Fibrin plays a major role in tissue repair and covers grafts and prostheses that are implanted. While fibrin can be considered as a natural barrier against bacterial invasion, it may also create a protective environment for microbes embedded in these fibrin clots favouring the localization of infection and the formation of abscesses, while limiting in some specific circumstances the propagation of infection. Pharmacodynamics describe the relationship between changes in drug concentration and the measurable effects of drug treatment and allows the simultaneous evaluation of the pharmacokinetic parameters and the *in vivo* activity of an antimicrobial agent over a determined period of time.

The affinity of some bacteria to fibrin strengthens the idea that this molecule plays a central role in the pathophysiology of post-surgical infections and endocarditis. *In vitro* and *in vivo* experiments have shown that, once fixed to fibrin, bacteria have the ability to grow into fibrin clots located in an appropriate environment.

Human fibrinogen can agglutinate certain strains of *S. aureus*. The interaction between fibrinogen and its receptor, the clumping factor, is strong and has been located on the carboxy terminal segment of the y chain of the fibrinogen [11]. Some strains of *Streptococcus sanguis* and *S. mutans* grown in sucrose-rich media have the capacity to produce an extracellular substance called dextran, which plays a distinguished role in the adherence to fibrin [6, 12]. These strains are frequently implicated in the etiology of dental caries and are frequently associated with endocarditis. Compared with dextranase-treated streptococci, dextran-producing strains adhere more rapidly to fibrin-coated surfaces [13]. The presence of platelets further increases the capacity of pathogens to adhere to fibrin. Fibronectin, a large dimeric protein which has shown great affinity for *S. aureus* and streptococci, may also participate in the fibrin clot-

bacterial interaction [14]. In fact, it has a great affinity for fibrin and fibrinogen. Fibronectin is also present in the granules of platelets (as fibrinogen) and is partially released when they are activated. Thus, platelet-derived fibronectin can play a role in platelet adhesion to fibrin. Fibronectin could bridge bacteria and fibrin; in the same way, platelets involved in the thrombus could bind fibronectin-bacteria complexes to fibrin. *S. aureus* seems to bind to its 27 kDa amino terminal segment.

Laminin and collagen found in the subendothelium and in the basement membrane are also two extracellular matrix proteins which may be implicated in bacterial adhesion. *S. aureus*, *S. pyogenes* and *S. sanguis* bind to surfaces coated with laminin [9]. Determinants of bacterial adherence to fibrin clots are known for cardiac streptococci, since these pathogens have been most commonly associated with the type of infections where adherence to components of the coagulation process is a determinant factor of pathogenicity. For other pathogens, including Gram-negative bacteria and anaerobes which are frequently involved in post-surgical infections, incorporation of bacteria in infected clots is a key process in the development of infection and is the most likely mechanism responsible for initiation of these infections. In fact, unlike staphylococci and streptococci, Gram-negative pathogens do not seem to have specific mechanisms for binding to surfaces and are not frequently associated with foreign body infections. Nevertheless, for Gram-positive bacteria the interaction with ligands remains hypothetical, however, the affinity between these bacteria and thrombus or vegetation constituents seems to be strong. This is most probably the result of a combination of diverse factors described above.

Fibrin, the solid state of the coagulation process, in collaboration with platelets facilitates the infectious process by trapping bacteria. The fibrin clot is also an ideal environment for bacterial growth as the presence of fibrin lowers the number of pathogens needed to produce experimental intraperitoneal infections. In contrast with the direct introduction of bacteria into the peritoneum, which induces septicaemia. Introduction of infected fibrin clots intraperitoneally leads to abscess formation without septicaemia and reduces the mortality rate compared with intraperitoneal infections induced with fibrin [15]. Thus, in this model, fibrin protects against sepsis, but also protects the pathogens against the activity of antibiotics as drugs diffuse poorly into fibrin [16]. In contrast, in bacterial endocarditis or in infected prostheses, the vegetations or the infected fibrin deposits on the prosthesis are the site of dissemination of infection as infected microscopic platelet aggregates are constantly released into the circulation. Host defense mechanisms, including macrophages and polymorphonuclear leukocytes, are often absent within infected fibrin clots or vegetations.

In vivo synergy

A combined penicillin/gentamicin regimen was more effective in eradication of *S. aureus* from endocardial vegetation than either drug alone [12]. An enhanced *in vivo* bactericidal activity was also found with naphcillin/gentamicin. Conflicting results have been reported with combinations including rifampin. The reason might be the excellent extravascular and intracellular *in vivo* diffusion capacity of rifampin not always overcoming the trend towards antagonism observed *in vitro* in combination with other antibiotics [17, 18].

In experimental endocarditis caused by penicillin susceptible *S. aureus* strains the combination of penicillin with rifampin was less effective than penicillin alone [12]. In another experiment, however, rifampin plus cloxacillin was more effective than cloxacillin alone in experimental endocarditis caused by *S. aureus*, even though bactericidal titers in serum suggested antagonism [19]. There is a concern about the use of quinolones in combination with rifampin for the treatment of staphylococcal infections. Despite the antagonism observed between the 2 agents against staphylococci *in vitro* [20, 21] pharmacokinetic studies have documented the excellent extravascular and intracellular penetration for both drugs [22]. In an endocarditis model caused by an MRSA strain the combination of vancomicin with rifampin was significantly more effective than the single drug regimens in terms of reducing MRSA in vegetations, the rate of sterilization of vegetations, and the rate to cure endocarditis [23, 24]. Similar efficacy was observed with combinations of rifampin with glycopeptide antibiotics [25], in contrast to *in vitro* time-kill studies in which rifampin reduced the bacterial activity of the glycopeptide compound. The excellent pharmacokinetic properties of rifampin *in vivo* are able to overcome the trend towards antagonism observed *in vitro*.

The emergence of resistance to rifampin during therapy for severe staphylococcal infections is a well-known problem might be best prevented by a combination with another antibiotic.

In comparison to the variety of antimicrobial substances investigated, lipophilic rifampin with a high antistaphylococcal activity may be the drug showing most of these properties [26]. Investigations on lipophilic drug combinations indicated that the addition of rifampin to bacteriostatic agents such as erythromycin and fusidic-acid resulted in antimicrobial activities being more effective than the single agents towards stationary growth phase bacteria in PBS [27]. The addition of bactericidal mupirocin to bacteria in the stationary state was also effective. The molecular level of additive and synergistic antimicrobial effects of rifampin with erythromycin, mupirocin and fusidic-acid was assumed to be a sequential activity of these substances on DNA- and protein-synthesis level. Due to the high lipophilic nature of the drugs, intracellular

accumulation may enhance the post-antibiotic effect and thus the killing efficacy. Lipophilic drug combinations containing rifampin were tested in this study and may be beneficial for the outcome of implant infections with staphylococci under stationary growth phase. Recently, Zimmerli et al. presented a randomized clinical trial on the antimicrobial efficiency of infected orthopedic implants [28]. Among patients with stable implants, those who tolerates 6 months therapies with rifampin-ciprofloxacin experienced cure of the infections without removal of the implant.

With regard to the low resistance rate of staphylococci to fusidic-acid, lipophilic drug combinations of rifampin with fusidic acid may be a beneficial alternative for the outcome of difficult to treat infections.

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ISOLATION OF RARE OPPORTUNISTIC PATHOGENS IN HUNGARY: CASE REPORT AND SHORT REVIEW OF THE LITERATURE

I. *RHODOCOCCUS EQUI*

B. SZABÓ[1], C. MISZTI[1], L. MAJOROS[1], Z. NÁBRÁDI[2], SZ. GOMBA[3]

[1]Department of Microbiology, [2]2nd Department of Surgery and [3]Department of Pathology, University Medical School of Debrecen, Nagyerdei krt. 98, H-4012 Debrecen, Hungary

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Rhodococcus equi is a well-established pathogen in foal pneumonia and is increasingly recognized as a pathogen in immunocompromised humans. We have isolated a Gram-positive coccobacillus from 8 blood samples and lung tissues of a renal transplant patient. Colony morphology, growth in Löwenstein-Jensen medium, 21 biochemical reactions, the characteristic morphological cycle (coccus-rod-coccus) and the CAMP test established the *R. equi* diagnosis. Histological studies of 2 lung biopsy specimens revealed numerous microabscesses with aggregates of polymorphonuclear leukocytes surrounded by abundant foamy macrophages. Our isolates proved to be sensitive to majority of antibacterial drugs. The appropriate therapy (amoxicillin-clavulanate) proved to be effective, however six months later a relapse was observed. Data show that in spite of its rare occurrence, *R. equi* infection represents a diagnostic and therapeutic challenge. The taxonomical, epidemiological, clinico-pathological, diagnostic and therapeutic data of *R. equi* are discussed.

Keywords: opportunistic pathogen, *Rhodococcus equi,* renal transplantation

Rhodococcus equi, a weakly acid-fast Gram-positive coccobacillus was originally isolated in 1923 by Magnusson as *Corynebacterium equi* [1]. The organism is a causative agent of pneumonia of foals and is occasionally isolated from pathological lesions of other domestic animals [2]. The first human case was reported in 1967 [3] and since then *R. equi* is becoming increasingly recognized as a pathogen for immunocompromised persons. In the majority of cases *R. equi* – like in horses – causes pneumonia [4], but other sources of *R. equi* isolates have also been reported,

including pelvic abscess [5], brain abscess [6], paraspinal abscess [7], subcutaneous abscess [8], pleural effusion [9], empyema [10], osteomyelitis [11], endophtalmitis [12] and lymphadenitis [13]. However, *R. equi* is still considered as a rare human pathogen, because the frequency of isolations is extremely low. Up to 1994 65 human cases were reported [4]. Here we describe our *R. equi* isolations from a renal transplant patient. To our knowledge up to 1998 three additional isolations were registered in Hungary; two isolates were obtained from pneumonia of AIDS patients and one from blood of an immunodeficient infant.

Patient, materials and methods

A 48-year-old male renal transplant recipient presented fever and cough with a localized nodular infiltrate by chest X-ray. The suspect of mediastinal tumor indicated serial biopsy. Parallel with this sampling haemocultures were also performed. The automatized Bact Alert 120 haemoculture system (Organon Teknika, Turnhout, Belgium) was used for isolation of *R. equi* from blood samples of the patient. The biopsy specimens were enriched and plated on sheep blood, chocolate and eosin-methylen blue agar. The identification of species was performed by API Coryne strip (24 reactions) of ATB system (Bio Mérieux, Lyon, France).

The Löwenstein-Jensen medium and 5% sheep blood agar with colonies of *Staphylococcus aureus* (for CAMP test) were also inoculated. The Gram stain of culture was performed from broth culture of rhodococci at 0, 6, 12 and 24 h, after inoculation. Ziehl-Neelsen stainings were also performed. Disc diffusion antibiotic sensitivity studies were done using 5% blood enriched 0.8 mm thick Mueller-Hinton agar.

The histopathology of biopsy specimens was evaluated after May-Grünwald-Griemsa, Periodic-acid-Schiff (PAS) and tissue Gram stainings.

Results

The necrotizing lung tissue showed dense histiocytic infiltrate with PAS-positive cytoplasms. The tissue Gram staining revealed abundant Gram-positive intracellular coccobacilli.

The 1–3 mm moist buff to salmon pink colonies on blood and chocolate agar appeared within 24 hours from six haemocultures and 2 lung biopsy specimens. Older colonies showed more distinct salmon-pink color. The mucoid colonies can be

considered as type B because of the abundant slime production (Fig. 1). Our figures (Figs 1–3) including legends are shown on web site of Department of Microbiology, University Medical School of Debrecen, Debrecen, Hungary: http://www.microb.dote.hu/rhodococc.htm Successive Gram stains revealed a change from cocci to bacillary form and then again to cocci within a 24 h cycle. Yellowish colonies on Löwenstein-Jensen medium appeared within 24 h (Fig. 2). The Ziehl-Neelsen staining revealed slightly acid-fast coccobacilli (Fig. 3). The CAMP test with *S. aureus* proved to be positive. The nitrate reduction, pyrazinamidase, alkaline phosphatase, alpha-glucosidase and catalase reactions proved to be positive, the urease production was slow. The pyrrolidonyl arylamidase, beta-glucuronidase, beta-galactosidase, N-acetyl-β-glucosaminidase, esculin (β-glucosidase), gelatine hydrolysis, glucose fermentation, ribose fermentation, xylose, mannitol, lactose, sucrose, glycogen tests were negative.

The antibiotic sensitivity of our 8 *R. equi* isolates showed the same pattern (Table I).

The patient was treated with amoxicillin-clavulanate for two weeks, and chest X-ray showed improvement, so he left the hospital. Six months later, however, he was admitted again, *R. equi* was cultured from his blood. The appropriate therapy resulted in remission again and the patient was released.

Discussion

The taxonomical, epidemiological, clinico-pathological, diagnostic and therapeutic comments and conclusions can be summarized as follows.

The Rhodococci have had a long and confused taxonomic pedigree. We try to stress only the clinically relevant "episodes". The original assignment of *R. equi* to the genus *Corynebacterium* [1] is mainly of diagnostic importance because of possibility of its overlooking as a contaminant diphteroid rod. The genus *Rhodococcus* was provisionally assigned to the genus *Mycobacterium* [14]. Indeed, many features of *R. equi* resemble to that of this taxon (weakly acid fastness, culture on Löwenstein-Jensen medium and, more importantly, similarities in pathogenesis, see below). At present, however, the genus should be restricted to actinomycetes because of the peptidoglycan consisting of N-acetylglucosamine and N-glycoil muramic acid, furthermore, the whole-organism sugar pattern type A [15].

R. equi normally resides in soil. The bacterium can be isolated from intestinal tract of cows, horses, sheep and pigs, and very frequently from herbivore dung [16]. However, besides the soil of agricultural areas the urban parks can also serve as a

source of *R. equi* isolates [17]. The route of transmission to susceptible person is usually the respiratory tract [18]. The typical presentation of the disease – as it happened in our case – consists of fever, chills, and cough occurring in an immunocompromised host with a necrotizing cavitary pneumonia.

Table I

Antibiotic sensitivity and resistance of R. equi *strains*

Penicillin G, V	R	Nitrofurantoin	S
Oxacillin	R	Nalidixic acid	M
Ampicillin	S	Oxolinic acid	M
Carbenicillin	S	Norfloxacin	S
Mezlocillin	S	Pefloxacin	S
Piperacillin	R	Ciprofloxacin	S
Amoxicillin+ Clavulanate	S	Trimethoprim	R
Ampicillin+ Sulbactam	S	Trimethoprim + Sulfamethoxazol	S
Imipenem	S	Vancomycin	S
Meropenem	S	Teichoplanin	S
Cefazolin	R	Erythromycin	S
Cefalexin	R	Clarithomycin	S
Cefaclor	R	Josamycin	S
Cefuroxime	S	Roxithromycin	S
Cefamandole	R	Clindamycin	R
Ceftriaxone	S	Gentamicin	S
Cefoperazone	S	Tobramycin	S
Ceftazidime	R	Netilmicin	S
Cefotaxime	S	Amikacin	S
Ceftibuten	S	Chloramphenicol	S
		Tetracycline	S

S: sensitive
M: moderately sensitive
R: resistant

The PAS stains revealed intensely positive histiocytes in microabscesses of necrotizing cavitary lung tissue, resembling those seen in Whipple's disease [4]. Intracellular Gram-positive coccobacilli were easily demonstrated by tissue Gram-

stain. The adhesion, penetration and intracellular growth of *R. equi* plays a pivotal role in the pathogenesis and clinical course of infection. We have reported our studies on pathogenesis of *R. equi* infection elsewhere [19]. Comparative analysis of virulence of human and equine isolates suggested that rhodococci of human origin were more virulent. The cytokines (mainly tumor necrosis factor and interferon gamma) play an important role in the defense mechanisms against *R. equi* infection. The mycolic acid content of cell wall of *R. equi* is also responsible for the similarities with pathogenesis of mycobacteria [20].

R. equi is usually first noted by the laboratory when isolated from a sterile source [biopsies or blood]. The colony morphology and growth on Löwenstein-Jensen's media may help draw attention to identification of *R. equi.* However, misinterpretation of acid fast forms in the cases of patients with fever and cavitary lung lesion may result in the misdiagnosis of mycobacterial infection [21]. Another, above-mentioned diagnostic misinterpretation is a consideration of *R. equi* as a contaminating diphterioid. From this point of view very important is the site of the isolation (should be sterile), the reproducible results and, if possible, the demonstration of intracellular coccobacilli by tissue Gram staining.

The therapy of *R. equi* infection is not easy, however the majority of isolates – including ours – are usually sensitive to many antibiotics. The principles of treatment are similar to that of the mycobacterial infections (combined, long-lasting and specific antibiotic therapy). Unfortunately, lethal cases are also reported in spite of the treatment with the appropriate drug [22]. Our case also demonstrates that the hidden nature of *R. equi* represents a serious therapeutic obstacle. Furthermore, recently emerging resistance of *R. equi* strains was reported [22].

Summarizing this report: *R. equi* is a rare pathogen, but may represent a serious therapeutic challenge, mainly if the diagnosis is not adequate. Furthermore, the *R. equi* may serve as a useful model for study of pathogenesis of intracellular infection.

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EFFECT OF AMINOGLYCOSIDES ON SURFACE HYDROPHOBICITY OF *ACINETOBACTER BAUMANNII*

ANNA HOŠTACKÁ

Institute of Preventive and Clinical Medicine, Limbova 14, SK–83301 Bratislava, Slovakia

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Effects of amikacin, gentamicin, netilmicin and tobramycin at subinhibitory concentrations (sub-MICs) (1/4, 1/8, 1/16 or 1/32 of their MICs) on the cell surface hydrophobicity of two *Acinetobacter baumannii* strains (7194 and 16265) were evaluated. Hydrophobicity was determined by two different methods – by adherence of bacteria to hydrocarbon (xylene) and by aggregation of bacteria in ammonium sulphate solutions at various concentrations. The adherence of *A. baumannii* strains to xylene decreased, mainly, after treatment with netilmicin at 1/4, 1/8 or 1/16 of the MIC (to 6.4%, 17.0% or 24.5% of the control value) (strain 7194) and after treatment with amikacin and gentamicin at 1/4 of their MICs (to 58.4% or 54.4%) (strain 16265). A decrease in surface hydrophobicity of exposed strains under these conditions was shown in salting-out test, too. Tobramycin reduced hydrophobic properties of *A. baumannii* strains at all tested sub-MICs to only a small extent.

Keywords: *Acinetobacter baumannii*, effect of aminoglycosides on surface hydrophobicity

Though members of the genus *Acinetobacter* are regarded as ubiquitous saprophytic bacilli which can be find frequently in soil, drinking and surface waters, sewage and they may persist as commensals on the human skin, they also have emerged as potential or true nosocomial pathogens [1, 2]. Several species of clinical importance have been identified but the majority of clinical isolates belongs to *Acinetobacter baumannii*. *A. baumannii* is implied in a wide spectrum of nosocomial infections, including pneumonia, septicemia, urinary tract and wound infections, secondary meningitis and endocarditis [2–4]. With the increased use of broad-spectrum antibiotics, many acinetobacters are becoming resistant to the major groups of antibiotics [5–7]. This trend poses a serious threat to hospitalized patients, because these bacteria have a significant capacity for long-term survival in the hospital

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environment [2]. Though antibiotics at subinhibitory concentrations (sub-MICs) do not kill bacteria, they can modify different bacterial properties connected with their virulence [8–12].

In this study, the changes in cell surface hydrophobicity of two *A. baumannii* strains after treatment with amikacin, gentamicin, netilmicin and tobramycin at sub-MICs were studied.

Material and methods

Bacterial strains

Two *A. baumannii* strains (7194 and 16265) isolated from respiratory tract of patients were studied. The strains were identified by the BBL Crystal Enteric/Non-fermenter ID system (Becton-Dickinson, USA).

Antibiotics and MIC

The antibiotics used in this study were amikacin (Bristol-Myers SQUIBB, Co. USA), gentamicin (ICN Biochemicals, USA), netilmicin (Schering-Plough, USA) and tobramycin (Biogal, Hungary). MIC determination was made by the macrodilution broth method (Mueller-Hinton broth supplemented with 25 mg of $CaCl_2$ and 12.5 mg of $MgSO_4$ per 1-MHB) by using serial twofold dilutions of the antibiotic. The lowest dilution of antibiotic allowing no visible growth after a 24 h incubation at 37 °C was considered as MIC. The MIC values were 3.12 mg/l both for amikacin and gentamicin, 6.25 mg/l for netilmicin and 0.78 mg/1 for tobramycin (strain 7194) and 1.56 mg/l for amikacin, gentamicin, netilmicin and 0.78 mg/l for tobramycin against strain 16265.

Treatment of strains with antibiotics

Bacterial suspension (0.2 ml, A_{600}=0.5) in MHB (9.7 ml) together with antibiotic (0.1 ml) at concentration 1/4, 1/8, 1/16 or 1/32 of their MICs were incubated for 24 h at 37 °C. Control cultures were not exposed to antibiotics. The sediments obtained after centrifugation of bacterial suspensions were washed, adjusted at an OD_{400} of 1.0 and used for determination of the cell-surface hydrophobic properties of the tested strains. Bacterial hydrophobicity was determined using two methods – adherence to hydrocarbon and aggregation in salt solutions.

Adherence to hydrocarbon (BATH)

The method of Rosenberg et al. [13] was used. Four ml of adjusted, treated as well as control bacterial suspensions were vortexed with xylene (1 ml) for 60 s and then incubated for 30 min. After phase separation, the optical density of the lower aqueous phase was measured at 400 nm. The results were expressed as a percentage decrease in the optical density of the aqueous phase compared with the optical density of the initial cell suspension.

Salting-out test (SAT)

The procedure outlined by Lindhal et al. [14] was applied. Bacterial suspensions of control and treated cultures (20 µl) were mixed with a series of dilutions of ammonium sulphate (20 µl) ranging from 0.25 to 2.5 mol/1. The resulting mixture was then gently shaken on a glass for 2 min. The lowest concentration of ammonium sulphate at which bacterial aggregation was visible was determined.

Results and discussion

Adherence represents one of the important factors in the pathogenesis of medically relevant microorganisms. In addition to specific factors, it is also mediated by different nonspecific factors, including hydrophobicity [15]. Our results showed that aminoglycosides at the tested sub-MICs reduced to different extent the surface hydrophobicity of *A. baumannii* strains evaluated by adherence of bacteria to xylene as well as by aggregation of bacteria in salt solutions. Adherence of strain 7194 to xylene after exposure to netilmicin at 1/4, 1/8 or 1/16 of the MIC decreased to 6.4%, 17.0% or 24.5% compared with the control value (without antibiotic) (Table I). Amikacin and gentamicin at the tested sub-MICs reduced adherence of this strain to 80.6–97.4% or to 81.3–98.2% of the control values. On the other hand, amikacin and gentamicin at 1/4 of their MICs effectively decreased hydrophobicity of strain 16265 (to 58.4% or 54.4%) (Table I). Netilmicin was less efficient. Adherence of treated strain was in the range of 81.4% to 94.1% of the control value. A decrease in surface hydrophobicity of the tested strains after exposure to antibiotics was also observed in the SAT assay, i.e. higher concentrations of ammonium sulphate were needed to aggregate the treated bacteria in comparison with unexposed ones. Hydrophobicity of both bacterial strains after treatment with tobramycin was slightly reduced at all tested sub-MICs (to 90.1–99.1% of the control values).

It is known that bacterial structures – lipopolysaccharides (LPSs), outer membrane proteins, lipoproteins, phospholipids and fimbriae affect cell surface hydrophobicity [16, 17]. The aminoglycoside antibiotics can also influence LPS synthesis in bacteria [18]. Bacteria lacking O-antigen repeating units of LPS and the constitutive capsular polysaccharide were more hydrophobic than the wild type [19].

Table I

The cell-surface hydrophobic properties of A. baumannii *strains treated with sub-MICs of aminoglycosides*

Antibiotic	Fraction of MIC	Strain	BATH[a]	SAT[c]
Netilmicin	0	7194	94.3±0.4 (100)[b]	0.5
	1/32		92.6±0.2 (98.2)	0.5
	1/16		23.1±1.4 (24.5)	1.25
	1/8		16.0±1.0 (17.0)	1.25
	1/4		6.0±1.0 (6.4)	1.25
Amikacin	0	16265	95.0±0.2 (100)	0.5
	1/32		94.9±0.1 (99.9)	0.5
	1/16		85.6±0.3 (90.1)	0.5
	1/8		83.7±0.7 (88.1)	0.75
	1/4		55.5±0.8 (58.4)	1.0
Gentamicin	0	16265	93.9±0.3 (100)	0.5
	1/32		92.5±0.4 (98.5)	0.5
	1/16		92.3±0.6 (98.3)	0.5
	1/8		83.3±1.1 (88.7)	0.75
	1/4		51.1±1.0 (54.4)	1.0

[a]Percentage decrease in absorbance of the lower aqueous phase compared with that of the original suspension. The values represent means from four measurements ± SD (standard deviation)

[b]Percentage hydrophobicity in parentheses

[c]The lowest molar concentration of ammonium sulphate causing visible bacterial aggregation

Our results showed that the tested antimicrobials belonging to the same class of antibiotics, differently affected the cell surface hydrophobicity of *A. baumannii strains*. Similar results were published by Tateda et al. [20] with two 14-membered macrolide antibiotics – erythromycin and oleandomycin for *P. aeruginosa* S-6. Though the

studied aminoglycosides share similar structures, there are certain differences regarding substitution mainly on NH_2 groups of aminosugars. The mode of action of aminoglycosides is pleiotropic and includes inhibition of ribosomal function, membrane damage and suppression of the initiation of DNA replication [21]. It is possible that the various degree of changes in surface hydrophobicity of *A. baumannii* strains induced by these antibiotics is associated with the modifications in fine chemical structures of aminoglycosides. They may be related to different alterations in bacterial surface components, which affect hydrophobic properties. Changes in LPS and in outer membrane proteins in connection with decreased hydrophobicity and increased serum sensitivity in *P. aeruginosa* S-6 treated with sub-MICs of erythromycin were reported by Tateda et al. [22].

Virulent strains in some pathogens have been found to be more hydrophobic than avirulent strains [23]. It is possible that a decrease in hydrophobicity of bacterial strains treated with sub-MICs of some antibiotics in *in vitro* experiments, observed also in this study, would contribute to the reduction of infection *in vivo*. Further experiments are needed to answer this question properly.

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A NEW POWERFUL ANTIBACTERIAL SYNERGISTIC COMBINATION OF TRIMETHOPRIM AND TRIMEPRAZINE

A. GUHA THAKURTA[1], S. K. MANDAL[1], KUMKUM GANGULY[1], SUJATA G. DASTIDAR[1], A. N. CHAKRABARTY[2]

[1]Department of Pharmaceutical Technology, Division of Microbiology, Jadavpur University, Calcutta 700 032, India
[2]Department of Medical Microbiology and Parasitology, Calcutta University College of Medicine, Calcutta 700 020, India

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The antihistaminic phenothiazine trimeprazine (Tz) was found to exhibit significant antibacterial activity on the basis of *in vitro* and *in vivo* tests. For the study of synergism due to a combination between Tz and trimethoprim (Tm), drug soaked filter paper discs were placed on young culture lawns of sensitive bacteria on nutrient agar plates. Calculation of the area of inhibition zones for determining the degree of synergism between Tz and Tm showed the increase to be statistically significant ($p<0.01$) when compared with their individual effects. By the checkerboard assessment procedure, the fractional inhibitory concentration (FIC) index was found to be 0.18, confirming synergism. The protective capacity of this combination was then assessed in Swiss white mice using *S. typhimurium* as the challenge bacterium, and the level of bacterial load was determined from infected autopsied animals. Statistical analysis of the data by students 't' test finally proved that a combination of Tz+Tm was highly synergistic.

Keywords: synergistic combination, trimethoprim, trimeprazine, antibacterial activity

It is now known that the antibiotics and antibacterial chemotherapeutics that had been active against a wide range of bacteria have gradually lost the battle, as mutants resistant to such drugs have quickly developed all over the world. Significant antimicrobial action of a large variety of drugs called non-antibiotics (2–12, 14, 16, 17) has opened up a new avenue for fighting the ever increasing problem of drug resistance. These non-antibiotics possess most of the properties of antibiotics and their

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antimicrobial function can often be further potentiated by suitable combination resulting in synergism (2, 4, 7, 18).

The present study describes augmentation of antimicrobial activity of a known chemotherapeutic drug, trimethoprim by combining with a recently recognized non-antibiotic, trimeprazine [10].

Materials and methods

Drugs

All the drugs were received through the courtesy from their respective manufacturers in India in dry powder form and were stored at 4 °C. Trimethoprim (Tm) was obtained from Burroughs Wellcome (India) Ltd., trimeprazine (Tz) and fluphenazine (Fz) were procured from Sarabhai Chemicals, trifluoperazine was sent by Smith Kline Beecham Pharmaceuticals (India) Ltd., promethazine (Pz) was given by Rhone-Poulenc (India) Ltd., and promazine (Pr) was obtained from Wyeth Laboratories (India) Ltd. A 20 mg/ml stock solution was prepared for every drug and sterilized by glass filtration (G5); Tm was dissolved in minimal amount of propylene glycol and the rest of the volume was adjusted with 5.0% dextrose [19]. All the other drugs were soluble in distilled water.

Bacteria

The strains of bacteria used are described in Table I. These were maintained in the laboratory in freeze-dried ampoules.

Media

Liquid media used for this study were peptone water (PW 1.0% bacteriological peptone, Oxoid, plus 0.5% NaCl, pH 7.2–7.4), nutrient broth (NB, Oxoid) and Mueller-Hinton broth (MH, Oxoid). Solid media were: peptone agar (PA), nutrient agar (NA) and Mueller-Hinton agar (MHA), obtained by solidifying the above liquid media with 1.0% agar (Oxoid No. 3), NA was used for tests on Gram-positive bacteria, while PA and MHA were used for the rest of the bacteria as required.

Determination of minimum inhibitory concentration (MIC) of different drugs

The MIC of Tm, Tz, Fz, Tf, Pz and Pr with respect to different test bacteria (Table I) was accurately determined by spotting (in triplicate) about 10^5 colony forming units (CFU) on plates containing a drug at the following concentrations (μg/ml):

0 (control), 10, 25, 50, 100, 200, 400, 800, 1000 and checking for growth up to 48 h with extended incubation when necessary.

Assessment of combination of antimicrobial affects

This was done by 2 methods: (i) disc diffusion technique [15] for determining single and combined effects of the drugs. Filter paper discs containing a particular concentration of a drug were placed on PA/NA in such a way that the inhibitory circles of 2 drugs just touched each other tangentially, when the reaction between them was one of indifference. In case of synergism the inhibitory circles enlarged and merged together [13]. The increase in surface area ($\pi r2$) due to the combination vis-à-vis those due to single effects was statistically evaluated for the level of significance. (ii) The effects observed above were examined by the checkerboard dilution test for corroboration as well as for determination of the fractional inhibitory concentration (FIC) index according to Krogstad and Moellering [15] using doubling dilutions of the pair of test agents in MH broth. This effect was further confirmed by the checkerboard test using doubling dilutions (μg/ml); 10 to 640 against Tz and 10 to 1280 against Tm.

In vivo *test*

This was performed on a Swiss strain of white mice, each weighing 18–20 g. The median lethal dose of *Salmonella typhimurium* NCTC 74 (sensitive *in vitro* to both Tm and Tz) was first determined, and then the challenge dose (50 MLD) corresponding to 8.5×10^9 CFU was standardized using a fixed point of optical density at 640 nm (in a Klett-Summerson colorimeter). Tz was administered at 1.5 μg/g body weight and Tm at 3.5 μg/g body weight (being calculated from available pharmacological data, [1]).

The lethal dose of Tz was 3.0 μg/g [10] and that of Tm was 7 μg/g of a mouse [1]. These were injected (ip) as 0.1 ml sterile solution containing 30 μg of Tz and 70 μg of Tm into each mouse (or as 0.1 ml saline in the control) 3 h before the challenge. For effects of combination of Tz+Tm, 24 mice were divided into 4 groups, all of which received the challenge dose 3 h after administration of the drug(s). The 1st group received only 30 μg of Tz, and; the 2nd group had 70 μg of Tm alone, the 3rd group was given 30 μg of Tz plus 70 μg of Tm, and the remaining group (control) received only saline. All the animals were autopsied after 18 h, their livers and spleens were removed aseptically along with 0.2–0.4 ml of blood and CFU counts were determined individually from each sample. The drug concentration was also determined at 0 h in all the mice in a separate experiment.

Table I

Determination of MIC of trimethoprim (Tm) and different phenothiazines

Strain	MIC μg/ml						Source
	Tm	Tz	Pr	Tf	Pz	Fz	
S. aureus NCTC 8530	200	50	400	100	50	100	S. P. Lapage, London
Sh. dyenteriae 7 519/66	100	100	200	20	400	20	K. P. Carpenter, London
Sh. boydii 10 386/67	200	20	50	20	100	200	K. P. Carpenter, London
Sh. sonnei 17	200	200	100	20	200	200	J. D. Abbott, Manchester
V. cholerae ATCC 14033	400	100	200	20	100	100	S. Mukerjee, Calcutta
S. typhimurium NCTC 74	200	100	400	100	100	100	A. K. Ghosh, London

Tz, trimeprazine; Pr, promazine; Tf, trifluperazine; Pz, promethazine; Fz, fluphenazine

Table II

Synergism between trimethoprim (Tm) and trimeprazine (Tz) with respect to various Gram-positive and Gram-negative bacteria

Strain	Diameter of the inhibition zone in mm				Percentage increase on the basis of πr^2	
	Single (A)		Combined (B)		Tm 400	Tz 300
	Tm 400*	Tz 300	Tm 400	+ Tz 300		
S. aureus 8530	26.2	28.3	28.6	32.8	19.15	34.24
Sh. dysenteriae 7	15.6	19.7	18.5	22.4	40.63	29.28
Sh. boydii 10	17.8	21.3	20.4	24.6	31.35	33.38
Sh. sonnei 17	14.6	18.5	17.4	20.2	42.03	19.22
V. cholerae 14033	13.4	16.2	15.2	18.5	28.67	30.32
S. typhimurium 74	15.1	19.2	17.6	23.6	26.39	33.81

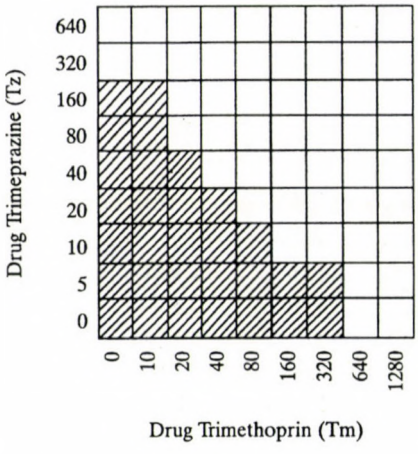
*Amount (μg) of the drug/disc

Mean surface area of the inhibition zones (mm^2) was calculated as πr^2 on the basis of their mean diameter (2r) and % increase was calculated as B-A/A×100, which was highly significant ($p<0.01$)

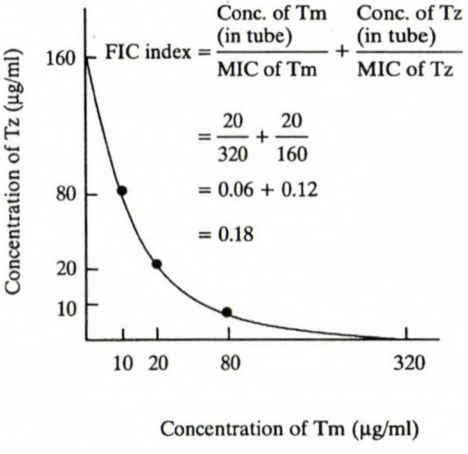
Results

Determination of antimicrobial activity of the agents tested. An analysis of the results presented in Table I shows that the MIC of Tm varied between 100 and 400 μg/ml with respect to all the 6 test bacteria. However, the phenothiazines were found to be much more inhibitory, as their MIC varied from 20–200 μg/ml.

Effect of combination of Tm and Tz in vitro. The data presented in Table II show that when Tm (400 μg) and Tz (300 μg) discs were placed individually on a young culture lawn of *S. aureus* 8530, the average zones of inhibition were 26.2 and 28.3 mm respectively, which increased to 28.6 and 32.8 mm respectively when the discs were placed for combined effects. The increase percents were calculated to be 19.15 and 34.24 against Tm and Tz, respectively. A similar synergistic effect was noted with respect to the other test bacteria. The other phenothiazines also proved to be synergistic when tested with Tm. The synergism between Tm and Tz was further scrutinized by the 'checkerboard' experiment using doubling dilutions [15] (Fig. 1). The MIC (μg/ml)



Arrangements of drug dilutions. Shading= visible growth after 18 hours of incubation at 37 °C.



Isobologram plotted from the results of checker board test.

Fig. 1. Determination of FIC index of Tm+Tz combination by checkerboard technique using *Sh. boydii* 10

Table III

CFU/ml of S. typhimurium 74 in organ homogenates and blood samples of mice

No. of mouse	Drug (μg/mouse)	CFU/ml		
		Blood	Liver	Spleen
1	Tz (30)	2.0×10^6	2.5×10^6	1.5×10^6
2		3.5×10^6	2.8×10^6	2.0×10^6
3		1.8×10^5	9.0×10^5	8.5×10^5
4		7.6×10^5	8.7×10^5	7.0×10^5
5		8.5×10^5	9.5×10^5	7.5×10^5
6		8.5×10^5	1.5×10^5	$6.9*10^5$
1	Tm (70)	8.0×10^5	7.6×10^5	2.2×10^5
2		3.0×10^6	8.1×10^5	8.0×10^5
3		9.5×10^5	2.4×10^6	4.5×10^5
4		7.5×10^5	6.5×10^5	6.5×10^5
5		6.8×10^5	7.5×10^5	5.8×10^5
6		5.8×10^5	3.1×10^6	6.8×10^6
1		8.8×10^3	2.5×10^4	8.5×10^3
2	Tz (30)	3.5×10^4	3.8×10^3	9.5×10^3
3	+	5.0×10^4	6.7×10^3	4.5×10^4
4	Tm (70)	9.6×10^3	7.8×10^3	4.0×10^4
5		7.9×10^3	6.5×10^3	5.5×10^4
6		2.5×10^4	1.5×10^4	5.8×10^4
1		6.5×10^7	4.5×10^7	1.2×10^8
2		7.0×10^7	2.5×10^7	6.8×10^7
3	Saline	7.5×10^7	1.1×10^8	4.5×10^7
4		1.2×10^8	9.5×10^6	7.2×10^7
5		4.8×10^7	5.0×10^7	2.1×10^8
6		5.5×10^7	5.5×10^7	8.5×10^7

All the mice received a challenge dose of 8.5×10^9 CFU of *S. typhimurium* 74, 3 h after administration of the drug(s) or saline. Animals were killed 18 h after challenge, their blood samples taken and livers, spleens homogenized and checked for viable bacteria. Statistical analysis of combined effect (Tm+Tz) versus single effect (Tm or Tz) when compared by *t*-test revealed $p<0.01$.

of Tm and Tz were 320 and 160; respectively, while in combination the values became 20. The FIC index for *Sh. boyii* 10 386/67 was found to be 0.18, which again confirmed the synergism.

In vivo *activity*. The studies of blood and organ homogenates of normal mice obtained from the same experimental stock yielded no *S. typhimurium* or any other salmonellae. The drugs Tm (3.5 μg/g of a mouse) along with Tz (1.5 μg/g of a mouse) significantly reduced the CFU/ml of the challenge strain in blood, liver and spleen samples (Table III).

Discussion

A comprehensive study of the combined effects of phenothiazines and Tm showed that synergism was present with respect to Tm and Tz *in vitro*, since significant increase of inhibition was evident in case of both Gram-positive and Gram-negative test bacteria. This was corroborated by the FIC index. *In vivo* studies clearly showed that the two agents act synergistically in the animal model as well. It may be pointed out here that the amount of Tz to protect an animal was much less than Tm. Since both these drugs have been used satisfactorily for long in clinical medicine for different purposes, their safety for human application is unquestionable. Therefore, with suitable structural modifications it may be possible to obtain far more powerful drugs with more accurate synergistic combinations thereby creating a new generation of non-antibiotic antimicrobials.

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PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR β-AMYLASE OF *BACILLUS MEGATERIUM* B_6

RINA RANI RAY

Department of Microbiology, Bose Institute, Calcutta 700054,
Department of Zoology, Barasat Govt. College, Barasat, North 24-Pgns. West Bengal, India*

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The extracellular β-amylase from starch induced *Bacillus megaterium* B_6 was purified to homogeneity in a very convenient way; through molecular sieving as demonstrated by the presence of a single band of protein in SDS-PAGE and single peak in gel scanning. The molecular mass of the purified enzyme (monomer) was found to be unusually high, around 105,000 Da. The pH and temperature optima of the purified β-amylase were at 6.9 and 60 °C, respectively. Mn^{2+} and exogenous thiols were found to play a remarkable role in reactivation of thermally and chemically denatured enzyme. The purified enzyme could saccharify both pure and low quality starches, where maltose could be detected as the major end product.

Keywords: *Bacillus megaterium*, extracellular β-amylase

Out of different starch degrading enzymes, β-amylase (EC.3.2.1.2,α-1, 4-D-glucan malto hydrolase) hydrolyses starch and allied substrates to produce β-maltose and limit dextrin. In comparison to that of other amylases, few reports are available on purification of microbial β-amylases and most of them involved laborious multiple steps, like ethanol precipitation followed by ion exchange chromatography [1]; ammonium sulphate precipitation followed by lead acetate treatment, column chromatography in SE Sephadex and gel filtration [2], raw starch adsorption followed by successive anion and cation exchange chromatography, molecular sieving and electrofocussing [3]. From industrial point of view these energy and time consuming, expensive processes of enzyme purifications are not at all recommended. Therefore, urge is made for a new source of microbial β-amylase, which can be purified easily, preferably in a single step process. In the present work, a convenient method of

* Corresponding address

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purification of β-amylase from *B. megaterium* B_6 up to homogeneity is described and general biochemical attributes of this purified β-amylase are discussed.

Materials and methods

Bacterial strain and culture conditions

The cells of *Bacillus megaterium* B_6 a potent producer of extracellular saccharifying β-amylase [4], was grown at 37 °C in a medium composed of (g/l): peptone, 0.9; $(NH_4)_2HPO_4$, 0.4; KCl, 0.1; $MgSO_4.7H_2O$, 0.1; $NaH_2PO_4.2H_2O$, 0.5 and starch 1.0 (pH-7.0), on a rotary shaker (160 rev/min) for 9 h. The culture was centrifuged (10,000 × g for 10 min at 4 °C) and the cell free supernatant was used as the source of β-amylase.

Enzyme assay

The assay solution (1 ml) containing equal volume of crude enzyme and starch solution containing 1% (wt/vol) starch in 50 mM phosphate buffer (pH 6.9) was incubated at 60 °C for 5 min. The amount of reducing sugar liberated was quantified by the dinitrosalicylic acid method [5], using maltose as standard. One unit of β-amylase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar (maltose) per min per ml. Protein concentration was estimated by the method of Lowry et al. [6], with BSA as standard.

β-amylase purification

The crude β-amylase was concentrated by passing through an ultrafiltration membrane (Spectrum, Texas, USA). The concentrated enzyme (100 kDa) was then applied to a Superose-12 gel filtration column (2×30 cm, Pharmacia), attached with Fast Protein Liquid Chromatography (FPLC) system (Pharmacia, Sweden) previously equilibrated with 50 mM phosphate buffer (pH 6.9) adjusting the flow rate to 0.5 ml/min. The eluted fractions with active peak, showing β-amylase activity, were pooled together and concentrated through ultrafiltration (membrane of molecular cut off 100 kDa) and rechromatographed in Superose-12 gel filtration column, equilibrated with the same buffer at same flow rate.

Electrophoresis

Polyacrylamide gel electrophoresis of the purified β-amylase was performed on 7% polyacrylamide tube gel at 4 °C following the method of Davis [7], and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of purified β-amylase was performed according to the method of Laemmli [8]. The gels were stained with Coomassie Brilliant blue for 30 min and destained by a solution containing acetic acid/methanol/water (3:2:35/vol). The properly destained gels were scanned in 2202 Ultroscan, Laser Densitometer (LKB).

Determination of molecular weight

The molecular mass (MM) of the purified enzyme was estimated by gel filtration [9] on a Superose-12 column (2×30 cm) controlled with FPLC system, at a flow rate 0.5 ml/min previously calibrated by standard molecular mass marker proteins. The molecular mass was also estimated by SDS-PAGE using standard molecular mass markers [10], β-galactosides (116,000), phosphorylase-b (97,400), BSA(66,000), Albumin, egg (45,000), and carbonic anhydrase (29,000).

Thin Layer Chromatography (TLC)

The end products of starch hydrolysis formed by the action of purified β-amylase were detected on TLC using the solvent system butanol/pyridine/water (6:4:3/vol) and by spraying anisaldehyde. Maltose, maltooligosaccharides, and glucose were used as standards.

Effect of pH

The relative β-amylase activity using 1.0% (wt/vol) starch was determined at various pHs in various buffers, 50 mM citrate phosphate (pH 4.5–6.5), 50 mM phosphate (pH 6.5–7.5), 50 mM Na_2HPO_4-NaOH (pH 7.5–10.5). To determine pH-stability, the purified β-amylase was preincubated at 29 °C for 30 min under standard assay conditions, and was expressed as percentage of maximum value (control).

Effect of temperature

Assay solution (1 ml) containing equal volume of properly diluted enzyme solution and starch solution containing 1% (wt/vol) dissolved in 50 mM phosphate buffer (pH 6.9) was incubated at various temperatures (25–75 °C) for 5 min. To determine the thermoinactivation kinetics of purified β-amylase, properly diluted enzyme samples were preincubated at various temperatures (30–70 °C) for 10–60 mins

and after cooling down to 0 °C, their residual activities were measured under standard assay conditions.

Effects of metal ions and other chemicals

Enzyme assays were performed in the presence of various metal ions (1 mM) and other reagents (0.001% wt/vol). The relative activities of the enzyme were compared with the enzyme activity obtained in 50 mM phosphate buffer (pH 6.9).

Substrate specificity

The relative activity of purified β-amylase was determined in presence of various gelatinized (cooked) substrates (1% wt/vol) under standard assay conditions.

Kinetic determinations

Initial rates of starch hydrolysis were determined at various substrate concentrations (2.5 mg/ml to 20 mg/ml) under standard assay condition. The kinetic constants K_m and V_{max} were estimated by the method of Lineweaver and Burk [11].

Table I

Summary of purification of β-amylase from the culture filtrate of B. megaterium B_6

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture filtrate	2170	1728	1.26	1	100
Ultra filtration	569.7	53.55	10.64	8.4	26.25
Superose 12	236.6	5.92	39.94	31.7	10.90
Rechromatography in Superose 12	218.4	0.04	5200	4.1×10^3	10.06

The substrate was soluble starch (1% wt/vol). The initial culture volume was 1000 ml.

Results and discussion

Purification of β-amylase. The results of purification of β-amylase from *B. megaterium* B_6 was summarized in Table I. The enzyme could be purified up to homogeneity by successive ultra filtration and gel filtration. Figure 1 shows the gel filtration pattern of ultrafiltered β-amylase from Superose 12 column. The proteins were fractioned into four peaks, of which peak No. 2 coincided with the peak of β-amylase activity. The active peak (2nd peak, fraction No. 10–14) rechromatographed on Superose 12 column, gave a sharp protein peak of β-amylase which was found to be 4.1×10^3 fold purified. The homogeneity of this purified enzyme could be demonstrated by the single band in SDS-PAGE (Fig. 2) and a single peak that could be recorded in scanning of the gel.

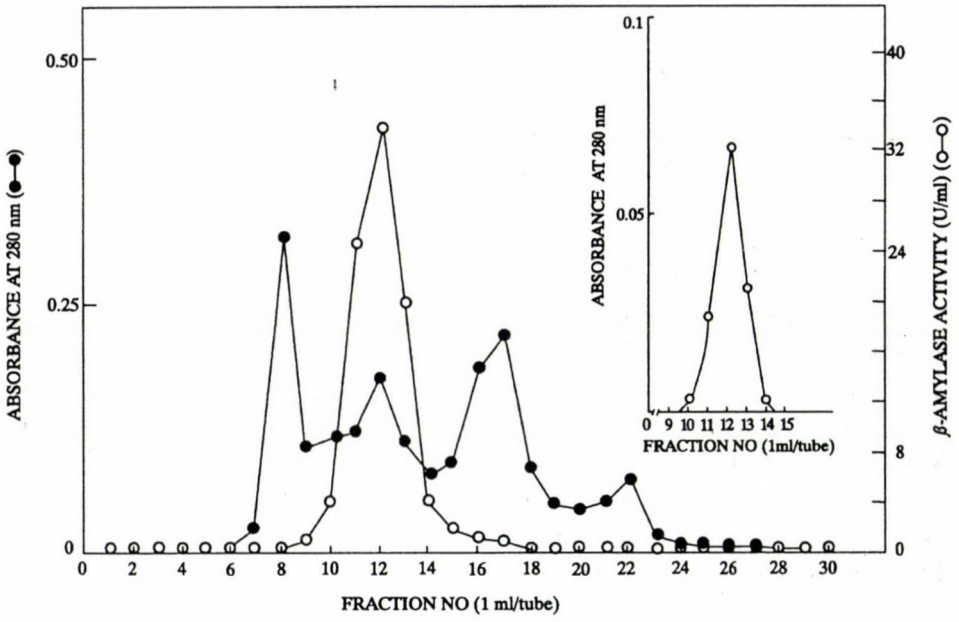

Fig. 1. Purification of β-amylase of *Bacillus megaterium* B_6 by Superose 12 gel-filtration chromatography. Inset: Purification profile of the 2nd peak after rechromatographed on Superose 12 column.

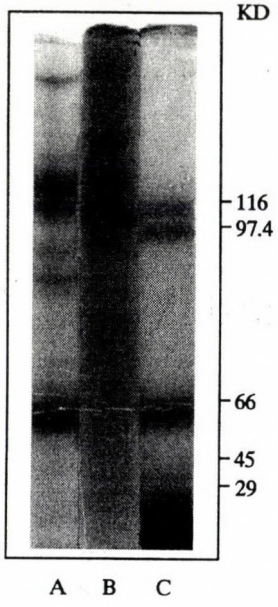

Fig. 2. SDS-PAGE of purified β-amylase from *B. megaterium* B_6. Molecular weight markers are indicated (crude and purified enzyme loaded in each tube = 50 μg)
Lane A: crude β-amylase, Lane B: purified β-amylase, Lane C: molecular mass markers

Molecular mass. The molecular mass of the purified enzyme was estimated to be 105,000 and 102,000 by Superose 12 gel filtration and SDS-PAGE, respectively. The identical molecular mass of the enzyme protein determined by the two aforementioned methods clearly indicates that the β-amylase in question is a monomer. Therefore, it could be concluded that the molecular weight of β-amylase from *B. megaterium* B_6 was exceptionally large in comparison to β-amylases from some already reported microbial strains [12–14]. This large molecular mass of the enzyme (monomer) made its purification more convenient.

Effect of pH. The optimum pH for the activity of purified β-amylase was 6.9 and 90% stability was found between pH 6.5 to 7.5. The pH profile is very close to β-amylases from other bacteria [14] and actinomyceta [13].

Effect of temperature. The temperature optimum was found to be at 60 °C, which resembles the temperature optimum of some fungal [15] and actinomycetes [13] β-amylases. The purified β-amylase was found to lose its activity when exposed to 60 °C and the failure of reversal of enzyme activity after bringing to ambient temperature indicates the irreversible nature of thermodenaturation. The plot of thermoinactivation

kinetics of purified β-amylase from *B. megaterium* B_6 preincubated at 60 °C was distinctly biphasic, with an initial rapid and later slow phase. The pattern of thermoinactivation kinetics remains identical to that of its partially purified state [16]. The accelerated thermoinactivation in presence of oxidizing agent like Cu^{2+} and restoration of enzyme activity in presence of reducing agent like reduced glutathione (GSH) indicated that the inactivation might have been caused by the oxidation of thiols present at the active site of the enzyme [17]. The thermostabilization of the purified β-amylase could be imparted by the addition of exogenous thiols like dithiothreitol (DTT), β-mercaptoethanol (β-ME), cysteine HCl and metals like Mn^{2+} and through crosslinking its amino groups by a bifunctional agent like glutaraldehyde [16].

Table II

Effect of various chemicals on the activity of purified β-amylase of B. megaterium B_6

Chemical	Concentration	β-amylase activity (%)
None	–	100
Phyticacid	1–5 mM	100
NaCl	1 mM	103
KCl	1 mM	103
$MnCl_2$	1 mM	198
$CaCl_2$	1 mM	138
$SnCl_2$	1 mM	91
$SrCl_2$	1 mM	137
$BaCl_2$	1 mM	143
$CoCl_2$	1 mM	165
$CuCl_2$	1 mM	7
$PbCl_2$	1 mM	100
$FeCl_3$	1 mM	84
$HgCl_2$	1 mM	7
$Al_2(SO_4)_3$	1 mM	43
$ZnSO_4$	1 mM	46
$AgNO_3$	1 mM	6
$Bi(NO_3)_3$	1 mM	46
$Mg(NO_3)_3$	1 mM	101

(Continued on p. 36.)

Table II (continued)

EDTA	1 mM	100
Urea	1 mM	96
Guanidine HCl	1 mM	106
Cysteine-HCl	1 mM	108
Dithiothreitol (DTT)	1m M	99
β-mercaptoethanol (β-ME)	1 mM	191
α-cyclodextrin	1 mM	95
β-cyclodextrin	1 mM	97
γ-cyclodextrin	1 mM	80
p-chloromercuribenzoate (pCMB)	1 mM	3
pCMB (1mM)+Cysteine HCl	(5 mM)	55
pCMB (1mM)+Cysteine HCl	(10 mM)	66
pCMB (1mM)+DTT	(1 mM)	79
pCMB (1mM)+DTT	(5 mM)	110
pCMB (1mM)+ -ME	(1 mM)	68
pCMB (1mM)+ -ME	(5 mM)	109
pCMB (1mM)+ -ME	(10 mM)	120
Triton X_{100}	0.001%	92
Tween 20	0.001%	84
Tween 40	0.001%	85
Tween 80	0.001%	97
Sodium dodecyl sulphate	0.001%	43
Sodium deoxy cholate	0.001%	85
Sodium taurocholate	0.001%	100

Effect of metal ions and other additives. Out of different metal ions tested (Table II) Mn^{2+} showed the best result while Cu^{2+}, Ag^{2+}, Hg^{2+} probably through formation of mercaptide of SH groups of the enzyme strongly inhibited the activity of β-amylase.

Though thiol inhibitors like p-chloromercuribenzoate (pCMB) and n-ethylamaleimide (NEM) caused a remarkable reduction in enzyme activity, addition of exogenous thiol brought about a reactivation of the denatured enzyme (Table II) confirming the existence of thiols at the active site of the enzyme, an observation similar to that of Freer [18]. On the other hand, the enzyme was not inhibited by Schardinger dextrins (α, β and γ-cyclodextrin), an observation similar to the β-amylase from *Clostridium thermosulphurogense* [1]. Except SDS, other surfactants and bile salts had no negative effect on β-amylase activity (Table II).

Table III

Substrate specificity of purified β-amylase of B. megaterium B_6

Substrate	β-amylase activity (%)
Starch	100
Maize starch	100
Tapioca starch	90
Amylose	79
Amylopectin	88
Dextrin	13
Xylan	2
Pullulan	0
Cellulose	0
CM-Cellulose	0

Affinity to various substrates. The purified β-amylase showed highest activity to starch, the amylose and amylopectin, an apparently amazing behaviour similar to few other amylases [18, 19]. It did not hydrolyze cellulose, carboxymethyl cellulose, xylan and pullulan (Table III).

Kinetic constants. The Lineweaver Burk plots of data prepared from hydrolysis of starch by purified β-amylase indicated the K_m and V_{max} values at 60 °C to be 4.34 mg/ml and 7.1 mg maltose/min/mg protein, respectively.

End product analysis. Maltose could be detected as major end product of starch hydrolysis in TLC. Though traces of maltro-oligo saccharides were found, no glucose could be detected.

From an overview of catalytic properties of β-amylases isolated from various strains of *B. megaterium* (Table IV), it becomes evident that although much of the characteristics of β-amylase from B_6 are similar to that of other strains of *B. megaterium*, its molecular weight was very large. The large molecular weight of the enzyme and complete absence of other contaminating amylases [4, 26] in the culture filtrate made its purification procedure very simple and the enzyme could be purified up to homogeneity only through molecular sieving. This convenience in purification of β-amylase makes the enzyme suitable for application in industrial scale.

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Table IV

Catalytic properties of β-amylase from various strains of B. megaterium

Strains	Temperature (°C)		PH		Substrate	Incubation time (min)	Molecular weight (D)	Additives		End	References
	Optima	Stability range	Optima	Stability range				Activators	Inhibitors		
B. megaterium 32	40–50	below 55	6.5	5.0–7.5	1% starch	30	–	Cysteine	pCMB	β-Maltose	[2]
B. megaterium NCIB 7581	50	–	7.0	5.0–9.0	1% starch	30	–	Cysteine	pCMB	β-Maltose	[20]
B. megaterium AJ 3355	60	–	7.0	–	0.5% starch	30	–	–	Hg^{2+}	Maltose	[21]
B. megaterium	75	60–80	5.5	5.0–8.0	2% starch	10	55,000	–	Hg^{2+}, Cu^{2+}	Oligo-saccharides	[22]
B. megaterium G-2	60	20–55	7.0	5.0–8.0	2% starch	10	60,000	–	Hg^{2+}, pCMB, Cu^{2+} etc.	α-Maltose	[23]
B. megaterium NCIB 9376	–	–	–	–	–	–	32,000	–	–	–	[24]
B. megaterium	–	–	–	–	–	–	58,000	–	–	–	[25]
B. megaterium B_6	60	below 65	6.9	6.5–7.5	1% starch	5	105,000	Cysteine, Mn^{2+}	Hg^{2+}, Hg^{2+}, pCMB NEM	β-Maltose	This work

pCMB=p-chloro-mercuribenzoate

NEM = n-ethyl maleimide

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GROWTH BEHAVIOUR AND INDOLE ACETIC ACID (IAA) PRODUCTION BY A *RHIZOBIUM* ISOLATED FROM ROOT NODULES OF *ALYSICARPUS VAGINALIS* DC

R. N. BHATTACHARYYA*[1] and B. R. PATI[2]

[1]P. G. Department of Botany, Darjeeling Government College, Darjeeling - 734 101, West Bengal,
[2]Department of Botany and Forestry, Vidyasagar University, Midnapur, West Bengal, India

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From the root nodules of *Alysicarpus vaginalis* DC, the symbiont was isolated and identified as a *Rhizobium* sp. The bacteria produced a high amount (107 μg/ml) of indole acetic acid (IAA) in culture from tryptophan supplemented yeast extract mannitol medium. The isolate preferred L-isomer of tryptophan for maximum IAA production. The production was maximum when the bacteria reached its stationary phase of growth. The production of IAA could be increased up to 70% over yeast extract glucose medium by supplementing $ZnSO_4$, $7H_2O$ (0.5 μg/ml). L-asparagine (0.2%) and sodium dodecyl sulfate (1.0 μg/ml). The possible relationship between the rhizobial IAA production and legume-rhizobia symbiosis is discussed.

Keywords: *Alysicarpus vaginalis*, IAA production, *Rhizobium*, root nodule

The formation of root nodule is a complex developmental process involving sequential exchange of chemical signals between the bacterial microsymbiont and the host plant [1, 2]. From the very beginning of nodule research, it was claimed that nitrogen fixation was the only function of root nodule. But later the hormone content of the nodules received importance due to its involvement in the formation [3] and development [4, 5] of the nodules. *Rhizobium* spp. are well known to produce IAA from tryptophan in culture [6–8]. But though the family Leguminosae (Fabaceae) has more than 14,000 species, only 8–9% have been examined for nodulation and even less than 0.5% have been studied in relation to their symbiotic relationship with nodule bacteria [9]. Again most of the information came from the *Rhizobium* spp. isolated from the root nodules of leguminous pulses due to its immense agriculturally

* Corresponding author

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importance [6, 10]. Such reports on the non-agricultural herbs are very scanty. *Alysicarpus vaginalis* DC is such a non-agricultural suberect herb, nothing is reported about its nodular hormone content or about its microsymbiont. This plant is suitable for hay, makes a good pasturage, and is an excellent soil improver [11].

The purpose of the present study was to check the IAA-synthesizing capacity of the *Rhizobium* sp. isolated from neglected leguminous herb, and to direct an effort to optimize the IAA production by different chemical supplements to have an insight on this part of legume-rhizobia symbiosis, in addition to nitrogen fixation.

Materials and methods

Isolation of microsymbiont

For the isolation of microsymbiont, the fresh and healthy root nodules of *Alysicarpus vaginalis* DC were taken, surface sterilized by 95% ethanol and 0.1% $HgCl_2$ followed by washing with sterile distilled water. The nodules were cut open and were crushed between two sterile glass slides and the fluid coming out of the crushed nodules were streaked aseptically on yeast extract mannitol (YEM) [12] agar plates. The bacterial growth obtained from the streaks was diluted serially in order of 10 using sterile distilled water. From the dilution tubes of higher order, 0.1 ml aliquots were plated on YEM agar plates. From the dilution plates, single colony was isolated on YEM slants. The microsymbiont was identified to be *Rhizobium* sp. following the methods given in Manual of Microbiological Methods [13] and also following Jordan [9].

Medium and growth conditions

The basal medium for incubation was YEM medium of Skerman [12] having 0.01% $CaCl_2$, $2H_2O$ instead of NaCl and $CaCO_3$ and supplemented with different isomers of tryptophan. The bacteria were incubated in 20 ml medium in 100 ml Erlenmeyer flasks with three replicates at 30±2 °C on a rotary shaker at 150 rpm for 28 h (the optimum time for growth and IAA production). Growth of bacteria was measured turbidimetrically in a spectrophotometer at 540 nm.

Determination of IAA production

For the determination of IAA production, the medium was centrifuged at 5000 g after the incubation time and the cell free supernatant was used for IAA production [8]. Equal volume of 1N $NaHCO_3$ was added to the supernatant and acidified with 9N

H_2SO_4 to pH 3.0. Then it was extracted thrice, each time with an equal volume of chilled peroxide-free diethyl ether. The pooled ether phase was evaporated, the residue was eluted with 95% ethanol and subject to thin layer chromatography. The Rf zone corresponding to that of authentic IAA (Sigma, USA) was eluted with ethanol, dissolved in water and used for colorimetric measuring [14]. In cultural studies, at the beginning different carbon sources were added separately to the tryptophan supplemented basal medium omitting mannitol. Then the different chemicals were added individually to the tryptophan supplemented basal medium having most suitable carbon source and the individual effect of the chemical on IAA production was measured. To find the maximum production of IAA by the *Rhizobium* sp. in culture, the medium was enriched with the supplements, which individually increased IAA production to maximum.

Statistical analyses were done following Panse and Sukhatme [15]. The critical difference (CD) was found to be significant.

Results

The symbiont isolated from the root nodules of *A. vaginalis* was identified as a *Rhizobium* sp. The bacteria produced a good amount of IAA when the YEM medium was supplemented with different isomer of tryptophan (Table I). The optimum growth and IAA production (107.0 μg/ml) was obtained with L-tryptophan (2.0 mg/ml) supplemented YEM medium (Table I). The growth and IAA production phase of the bacteria started simultaneously and reached their respective stationary phase at 28 h (Fig. 1).

The effects of carbon sources, minerals, nitrogen sources and cell wall affecting chemicals on IAA production by this *Rhizobium* was checked individually. It was found that glucose (Table II), $ZnSO_4$, $7H_2O$ (Table III), L-asparagine (Table IV) and Sodium dodecyl sulphate, SDS (Table V) had the maximum promoting effect on IAA production. To check the maximum IAA production in culture, the chemicals, which individually increase the maximum IAA production, were added to the medium. The production of IAA was increased from 126.6 μg/ml (in tryptophan supplemented yeast extract glucose medium) to 215.22 μg/ml (Table VI).

Discussion

The isolated Rhizobium produced a high amount of IAA in culture when the YEM medium was supplemented with different isomers of tryptophan (Table I). The bacteria preferred L-isomer for this high amount (107 μg/ml) of IAA (Table I) in culture and also for growth (Table I). Similar preference of isomer for IAA production was found by other authors [6, 16] in other *Rhizobium* spp. The bacteria could not sufficiently utilize D-tryptophan for these functions because of its toxic effects probably through interaction with Na^+ [17, 18]. Again, the racemase required for D-tryptophan metabolism [17, 19] might be lacking in this bacterium. The low amount of IAA produced from D-tryptophan could not be explained. A small amount of IAA was also produced from DL-tryptophan as it contained the L-isomer. The bacteria utilized 2 mg/ml L-tryptophan for its maximum production (Table I). The production of IAA was much less than the amount of tryptophan in the medium.

Table I

Effects of L-, DL- and D-isomer of tryptophan on growth and IAA production by the Rhizobium *sp. of the root nodules of* A. vaginalis *in culture*

Isomer of tryptophan	Concentration (mg/ml)	Growth O.D.	IAA production (μg/ml)
L	1	1.30	66.20
	2	2.90	107.00
	3	1.93	103.40
DL	1	1.20	59.82
	2	1.70	83.30
	3	1.75	84.00
D	1	1.20	7.21
	2	1.50	17.10
	3	1.58	16.98
Critical difference at P = 0.05		0.04	2.81

Growth and IAA production was checked in yeast extract mannitol (YEM) medium after 28 h of shake culture at 30 ± 2°C.

The growth and IAA production phase of the bacteria started immediately after inoculation, and reached their respective stationary phase at 28 h (Fig. 1). But after 28 h of incubation, the level of IAA was declined which indicates the release of IAA degrading enzyme in the medium by this *Rhizobium*. Such IAA oxidase activity was also reported in some other *Rhizobium* spp. either at free living [6] or at bacteroid state [20].

Table II

Effect of carbon sources on growth and IAA production of the Rhizobium *sp.*

Carbon sources	Growth O.D:	IAA production (μg/ml)
Control	0.70	14.68
Lactose	2.25	90.87
Fructose	2.40	99.36
Galactose	1.90	100.60
Sucrose	2.30	116.84
Mannose	1.55	100.61
Maltose	1.90	103.50
Myo-inositol	2.20	122.79
Arabinose	2.45	103.85
Xylose	2.55	123.33
Glucose	2.45	126.59
Mannitol	1.90	107.10
Critical difference at P = 0.05	0.09	2.05

The bacteria were grown in L-tryptophan (2.0 mg/ml) supplemented yeast extract mineral medium along with carbon sources at 1% level individually. In the control, the medium was without any carbon sources. Other conditions were the same as in Table I.

The replacement of mannitol of the basal YEM medium with other ten carbon sources (at 1% level) revealed that the *Rhizobium* sp. could utilize all eleven carbon sources for both growth and IAA production (Table II). Maximum growth and IAA production (126.59 μg/ml) was obtained in xylose and glucose, respectively, and Table II shows that carbon source played a vital role in the growth and rhizobial IAA production. The optimum concentration of glucose for maximum IAA production was

1% (data not given to reduce Table number). Role of carbon source in the rhizobial IAA production was also reported earlier [16, 21].

The effect of minerals on the IAA production by this *Rhizobium* was checked. Table III shows that only Zn^{2+} and Fe^{2+} had a promotive effect on IAA production, and only Zn^{2+} had a promotive effect on growth. The optimum concentration of Zn for IAA production was 0.5 μg/ml (data not given). It was also reported earlier that $ZnSO_4$ was most suitable for both growth and IAA production by a *Rhizobium* sp. [16]. The promotive effect of Zn^{2+} on IAA production might be due to better growth of the organism (Table III).

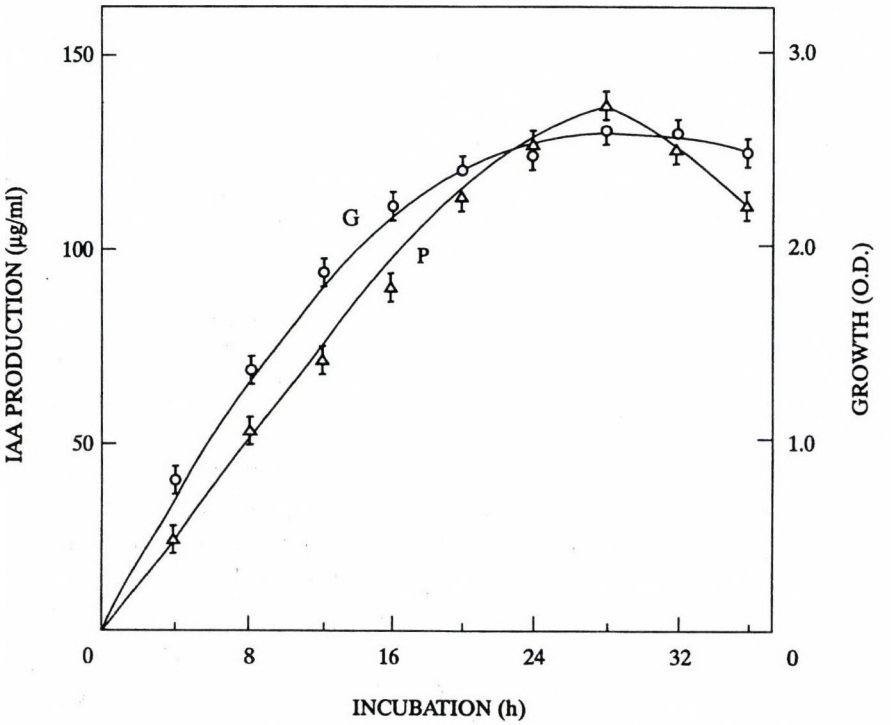

Fig. 1. Growth (G, O.D. value) and IAA production (P, μg/ml) by the isolate in L-tryptophan (2.0 mg/ml) supplemented yeast extract glucose medium. Bars on the points indicate ± SE

The effect of different inorganic and organic nitrogen sources on growth and IAA production by this *Rhizobium* was also studied. Out of seven nitrogen sources, L-asparagine was most effective for the IAA production, whereas maximum growth was obtained in KNO_3 (Table IV). The 0.2% L-asparagine gave maximum IAA production (data not given). *Rhizobium* spp. can utilize several nitrogen compounds for growth, which might be responsible for higher IAA production [18]. In the other hand, it was also reported that the presence of amino acids like alanine inhibits the conversion of tryptophan to IAA by *R. meliloti* [7].

Increase in permeability by affecting the cell wall or membrane yielded an increased amount of amino acids in the medium [22]. In a similar experiment, the presence of SDS to the medium promoted better IAA production by this *Rhizobium* (Table V), however maximum growth was obtained by the presence of EDTA followed by SDS.

Table III

Effect of metal ions on growth and IAA production by the Rhizobium *sp.*

Salts of metal ions	Growth O.D	IAA production (μg/ml)
Control	2.45	126.60
Ni ($NiCl_2$, $6H_2O$)	2.05	119.80
Mn ($MnSO_4$, H_2O)	1.89	109.34
Zn ($ZnSO_4$, $7H_2O$)	2.65	161.13
B (H_3BO_3)	2.40	126.60
Cu ($CuSO_4$, $5H_2O$)	2.01	119.09
Fe ($FeSO_4$, $7H_2O$)	2.12	132.36
Critical difference at P = 0.05	0.07	1.83

The bacteria were grown in yeast extract mineral medium with 1% glucose and L-tryptophan (2.0 mg/ml). The metal ions were added at 1.0 μg/ml level individually. The control set contained no metal ions. Other conditions were the same as in Table II.

Optimum concentration of SDS was 1.0 μg/ml (data not given). Changes in the cell wall or membrane by SDS might have increased the availability of tryptophan to the converting enzymes as well as increased the release of IAA from the cell.

Table IV

Effect of nitrogen sources on growth and IAA production of the isolate

Nitrogen sources	Growth O.D.	IAA production (μg/ml)
Control	2.41	126.60
KNO_3	2.86	138.73
NH_4C1	0.55	42.47
$NaNO_3$	2.58	192.96
L-asparagine	2.75	211.09
L-glycine	1.31	66.36
L-glutamine	2.82	132.61
$(NH_4)_2SO_4$	2.82	128.61
Critical difference at P = 0.05	0.03	3.84

The bacteria were grown in yeast extract glucose medium supplemented with L-tryptophan. The nitrogen sources were added at 0.2% level individually. The control set was without any nitrogen sources. Other conditions were the same as in Table III.

Table V

Effect of cell wall affecting agents on growth and IAA production by the Rhizobium *sp.*

Agents	Growth O.D.	IAA production (μg/ml)
Control	2.45	126.50
SDS (1.0 μg/ml)	2.62	150.98
Na-salt of EDTA (5.0 μg/ml)	2.67	134.66
CPC (0.5 μg/ml)	2.28	114.26
Triton × 100 (0.005% v/v)	1.50	54.69
Penicillin (50 IU/ml)	2.55	136.71
Critical difference at P = 0.05	0.04	3.04

The bacteria were grown in yeast extract glucose medium supplemented with L-tryptophan. The control set contained no cell wall affecting agents. SDS = Sodium dodecyl sulfate, CPC = Cetyl pyridinium chloride. Other conditions were the same as in Table III.

Table VI

Increase in growth and IAA production by the affect of L-tryptophan in the presence of most effective supplements

Supplements	Growth		IAA production	
	O.D.	Increase over control	μg/ml	Increase over control
Control	2.45	–	126.60	–
+$ZnSO_4$, $7H_2O$ (0.5 μg/ml)	2.65	8.2	162.00	28.0
+$ZnSO_4$ and + L-asparagine (0.2%)	3.10	26.5	183.60	45.0
+$ZnSO_4$ and + L-asparagine + SDS (1.0 μg/ml)	3.35	36.7	215.22	70.0

Control set contained yeast extract glucose (1%) medium containing L-tryptophan (2.0 mg/ml). Other conditions were the same as in Table I.

To get maximum production of IAA from this *Rhizobium* in culture, the supplements, which individually increased the IAA production to the most, were added to the medium. The *Rhizobium* which initially produced 126.6 μg/ml of IAA in tryptophan supplemented yeast extract glucose broth was induced to produce 215.2 μg/ml of IAA, an increase of about 70% over control (Table VI).

The root nodules of *A. vaginalis* contained moderate amount of IAA (data not shown). The high amount of IAA produced by this *Rhizobium* sp. might be responsible for the nodular IAA. Evensen and Blevins [23] also reported that when they inoculated two different strains of *Rhizobium* sp. to Limabean plants separately an accumulation of GA in the nodules was observed. Hunter [24] reported that inoculation of soybean with mutants resulted in enhanced IAA content in the nodule and altered nodule morphology. In this present investigation, except the cell wall affecting chemicals, all the supplements which increased the IAA production in culture might be available for the bacteria within the nodule for the increased IAA production. By such alteration in supply of metabolites within the nodules, the host plant might induce the symbiont to produce more IAA for its own benefit. There are also reports that IAA produced within root nodules are transported to other plant parts for metabolism [25]. Thus the nodule of this plant may also serve as a rich source of phytohormones for other plant parts when required.

From the above discussion it can be concluded that apart from nitrogen fixation, legume-rhizobia symbiosis should be evaluated afresh in the light of nodular

phytohormones, because the phytohormone production, its supply and metabolism are also important aspects in the symbiosis along with nitrogen fixation, which was initially thought to be the only function.

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MICROBIAL STUDY OF FARMHOUSE EWE CHEESE DURING STORAGE IN OLIVE OIL

R. GÓMEZ*, MONTSERRAT VIOQUE, EMILIA SANCHEZ, CARMEN MATA, L. TEJADA
J. FERNÁNDEZ-SALGUERO

Department of Food Technology, University of Córdoba, Campus de Rabanales, Edificio C-1, Anexo, Ctra N IV, km 396A, E-14014 Córdoba, Spain

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The effect of storing farmhouse ewe cheese in oil and *in vacuo* over long periods of time on physicochemical properties (water activity and pH) and the microbiota of the cheese was investigated. The storage conditions were found to scarcely influence the sample pH. Also, the initial water activity (a_w = 0.961) and its value after 9 months of storage (0.927) were both very similar to those for naturally ripened cheese. The incidence of pathogenic microbial groups was found to decrease with storage time (counts fell below 1 log CFU/g). The flora that effects proteolytic changes in cheese consisted of lactic microorganisms (*viz.* lactococci and lactobacilli), in addition, after 6 months of storage, of enterococci. The last are responsible for the formation of large amounts of soluble nitrogen (SN), non-protein nitrogen (NPN) and aminoacid nitrogen (NH_2–N), which provide this type of cheese with very special sensory features while preventing dehydration and thus lengthening its shelf life.

Keywords: storage of farmhouse ewe cheese, microbial flora

One of the most widely used long-term preservation procedures for a number of farmhouse: ewe cheeses involves storing them in oil. Olive oil avoids dehydration of the cheese while providing it with highly appreciated special features and expanding its shelf life.

Los Pedroches cheese, a traditional ewe cheese variety, is made by using an ancestral procedure in Los Pedroches Valley (a geographical area in the province of Córdoba; southern Spain) between December and May. The cheese is made from uncooked hard paste that is obtained exclusively from raw Merino ewe milk – which

* Corresponding author

restricts consumption to a period of only 60 days after ripening. The milk is supplied with no lactic culture and is usually clotted with vegetable coagulant [1].

Some Spanish, Portuguese, French and Italian ewe cheese varieties are made by using aqueous extracts of dried wild thistle flowers of various species of the genus *Cynara* L, as vegetable coagulant. Thus, *Cynara cardunculus* is usually employed in the making of Portuguese Serra and Serpa cheeses [2, 3], and the Spanish Los Pedroches, La Serena and Torta del Casar cheeses, from ewe milk, as well as Los Ibores cheese; from goat milk, and Flor de Guía cheese, from a mixture of ewe and cow milk [4].

The microbial characteristics of Spanish and Portuguese ewe milk cheeses are widely documented. While the microbiology of Los Pedroches cheese during ripening has been studied by some authors [5, 6], changes in its microflora during storage in olive oil have scarcely been examined. The purpose of the present work was thus to investigate changes in the major microbial groups of ripened ewe cheese preserved by immersion in olive oil.

Materials and methods

Samples

Three batches of four cheeses each were made from unpasteurized raw Merino ewe milk, using a traditional method at a farmhouse in the Los Pedroches region. The milk batches were clotted with an aqueous extract of *Cynara cardunculus* L. obtained from an amount of 70 g of dried flowers that was macerated in 1 L of water for 24 h. The extract was added at a rate such that clotting would take 80–90 min [7]. The milk clotting temperature was 30±1 °C. Following pressing, the cheeses were surface-rubbed with salt and transferred to the laboratory for storage in a controlled ripening chamber at 9±1 °C at a relative humidity (RH) of 80±2%. One cheese from each batch was used for physicochemical and microbiological analyses before the others were stored in oil and packed *in vacuo*. The cheeses were analysed at 90, 180 and 270 days of storage. All samples were analysed in duplicate.

Storage procedure

Cheeses were immersed in olive oil after 3 months of ripening. Following surface brushing, the cheeses were pricked in order to facilitate penetration of the oil and placed in Krehalon MLF-4 280 × 400 mm retractile bags that were filled with 200 g of virgin olive oil of acidity not greater than 0.4° (*viz* 91.6 g of fat, of which 12.3, 72

and 7.3 g was of the saturated, monounsaturated and polyunsaturated type, respectively). Following sealing with NO.2-401 16 mm staples, the bags were vacuum-packaged on a Cryovac 6.570B machine (C.O. Cryovac Division, Woburn, MA) equipped with a vacuum pump and a hot water tunnel for retracting plastic material.

Physico-chemical determinations

Water activity was determined at 20 °C by using a CX-1 dew-point hygrometer from Decagon Devices (Pullman, Washington, DC). pH values were measured potentiometrically by means of a Beckman 3500 digital pH-meter applied to 1:1 w/v aqueous homogenates.

Microbiological analyses

Samples for this type of analysis were prepared following the procedure of Beerens and Luquet [8], for semi-hard paste: an amount of 10 g of cheese was homogenized with 90 ml of a sterile 10% w/v sodium citrate solution, previously warmed to 45 °C, in a Colworth Stomacher 400 apparatus (Seward Medical, London). Serial decimal dilutions of the homogenates in a sterile solution of 0.1% peptone water were plated on the specific media required for the different microbial groups to be examined.

The above-described media and conditions were used to obtain the following counts:

(a) Mesophilic aerobic bacteria [9] were determined with Plate Count Agar (PCA, Oxoid CM325), following incubation at 30 °C for 72 h

(b) Enterobacteria [10] were analysed with Violet Red Bile Glucose Agar (VRBG, Oxoid CM485) after incubation at 37 °C for 24 h

(c) Total coliforms [9] were enumerated in Violet Red Bile Agar medium (VRBA, Oxoid CM107) after 24 h at 37 °C

(d) Faecal coliforms were tested by using the more probable number (MPN) procedure as implemented by De Man [11], and also for gas production in Brilliant Green Bile Lactose Broth (BGB, Oxoid CM31) following incubation at 44 °C for 24 and 48 h. Those tubes that tested positive for gas production were subjected to the indole test.

(e) Staphylococci were estimated on Baird-Parker Agar (BP, Oxoid CM275) plates incubated at 37 °C for 48 h. Coagulase and thermonuclease tests were carried out by using coagulase plasma EDTA (Merck, 1.13305) and DNase (Oxoid CM321), respectively.

(f) Micrococci were counted in Mannitol Salt Agar (MSA, Oxoid CM85) after incubation at 37 °C for 72 h. Colonies isolated from the MSA plates were tested for morphology, the Gram reaction and catalase.

(g) Lactobacilli were enumerated in MRS Agar medium (Oxoid, CM361) after 24 h at 37 °C, under anaerobic conditions. Colonies isolated from the MRS agar plates were tested for the Gram reaction, morphology and catalase. Gas production from glucose was observed in Elliker broth tubes. Hydrolysis of arginine was tested, using Nessler's reagent, in Elliker (ADSA-Micro-2-288) containing 0.1% of glucose and 0.3% of arginine but no ammonium citrate, after 3–7 days at 30 °C.

(h) Lactococci were determined on M17 Agar (Oxoid CM785) according to Terzaghi and Sandine [12], following incubation at 30 °C for 48 h

(i) Enterococci were enumerated in Kanamycin Aesculin Azide Agar (Oxoid CM591) at 37 °C for 24–48 h.

(j) Listeria were determined with previous enrichment in sterile peptone water (37 °C/48 h) and selective enrichment on PALCAM Listeria selective agar (Merck 1175/112122, Darmstadt, Germany) incubated at 37 °C/24 h as described in detail [13].

(k) Clostridium perfringens, on tryptone sulphite neomicin agar (TSN, Oxoid CM246) incubated at 46 °C for 24 h as described in [14].

(l) Yeasts and *moulds* were grown on Potato Dextrose Agar (PDA, Oxoid CM139) acidified with 10% lactic acid (Merck) at 25 °C for 96 h.

All counts are given as log CFU/g, where CFU denotes "colony forming units".

Results and discussion

Table I shows the variation of water activity (a_w), pH and the microbial counts obtained during the oil storage process. After 3 months of ripening in the chamber (day 0), the a_w values for the cheeses were similar; the mean, 0.961, is close to the 0.951 and 0.959 obtained by Carmona et al. [15] in a study of the effects of the addition of starters and the ripening conditions, respectively, on this type of cheese. As can be seen from the Table, a_w decreased moderately with increasing preservation time (to 0.927 after 9 months of storage in oil and *in vacuo*). Between the third and sixth month, such a decrease was negligible; also, this a_w value is similar to the 0.926 reported by Sanjuán [7] for naturally ripened cheeses. Carmona and Gómez [16] obtained water activities from 0.910 to 0.940 at 90 days of ripening with no additional preservation for cheeses stored at 14 °C at 80% RH; these values are similar to ours at 9 months of storage.

pH changes were similar for all cheeses, with differences of only 0,05 units between the initial and final values. Similar values were reported by other authors [7,

17] at 90 days of natural and controlled ripening, respectively. Similarly, a pH of 5.10 was reported by González et al. [18] for La Serena ewe cheese and one of 5.16 by Ruiz et al. [19] for heterogeneous samples of Torta del Casar cheese; both at 60 days of ripening.

In relation to the microbial counts obtained during the oil storage process, all groups exhibited high counts at 3 months of ripening (day 0), consistent with previously reported data [5, 6, 20, 21]. The counts of lactic flora, lactobacilli and lactococci increased with increasing time of storage in oil and under vacuum up to the 90th day, after which they decreased gradually throughout the process. This is consistent with previous findings [22] for Manchego cheese preserved in olive oil. In addition to lactic microbes, enterococci make up the prevailing flora at the late stages of preservation, partly as a result of their broad growth temperature range and of their high tolerance to dysgenetic factors such as the presence of salt and acid. In addition, enterococci remain viable for longer periods than do lactic acid bacteria in the presence of fat.

Table I

Water activity (a_w), pH (mean ± s.d.) and Log microbial counts+ of the cheeses during the storage period in olive oil

a_w, pH/Microbial groups	Storage time (days)			
	0	90	180	270
a_w	0.961±0.005	0.960±0.006	0.931±0.012	0.927±0.006
pH	5.20±0.11	5.17±0.12	5.23±0.17	5.15±0.15
Mesophilic aerobic bacteria	9.10	>9.81	8.10	7.35
Enterobacteriaceae	3.02	<1.23	<1	<1
Total coliforms	2.80	1.70	<1	<1
Faecal coliforms*	2.77	>1.23	<1	<1
Micrococci	8.14	>9.07	8.18	6.76
Staphylococci	7.53	6.97	5.42	4.18
Lactobacilli	7.84	>9.05	8.02	7.14
Lactococci	9.38	9.41.	8.26	7.97
Enterococci	7.97	8.07	8.01	8.20
Yeasts and moulds	1.39	<1	<1	<1

+ Average values for three cheesemaking batches

* MPN/g

Similarly, the counts of mesophilic aerobic bacteria increased over the first 90 days of preservation; the increase was reflected in the counts of lactic flora (> 9.05 log CFU/g for lactobacilli and 9.41 log CFU/g for lactococci) and also in those of micrococci (> 9.07 log CFU/g, which was somewhat higher than the value reported by Sánchez et al. [6]). In this respect, Sánchez et al. [23] reported significantly higher ($P < 0.001$) mesophilic aerobe counts in curds obtained by using vegetable rennet from both *C. cardunculus* and *C. humilis*, than in those based on animal rennet; the differences persisted throughout ripening. In a study on the microbiological quality of cardoons of the genus *Cynara* used in traditional cheesemaking procedures, [24] demonstrated increased counts of total bacterial and enterobacteria in aqueous extracts from wild thistles of the species *C. cardunculus* and *C. humilis*. Such high counts suggest that the addition of cardoon extracts introduces additional microbial contamination in milk. A high enterobacterial content is known to be a major hurdle to the marketing of cheese [25] because coliforms are not only indicators of faecal contamination but also agents of lactose fermentation, which results in early swelling of cheese.

The counts for the other microbial groups studied (*viz.* enterobacteria, coliforms, *E. coli,* and yeasts and moulds) decreased gradually to below 1 log CFU/g after 3 months of storage. Such a marked decrease was probably a result, among others, of the inhibitory effect of lactobacilli and lactococci, which were present in high counts throughout the process. The presence of lactic acid bacteria in competition with the indicators of faecal contamination had previously been noted [26, 27]. These microbes have frequently been used to inhibit growth of enterobacteria, which are poorly resistant to acids.

However, it is worth noting the presence of staphylococci at the end of the process. In Manchego cheese clotted with animal rennet and vacuum-packaged, Nuñez et al. [28] found staphylococcus counts in the region of 2 log CFU/g at 6 months of ripening; this value is well below the 4.18 log CFU/g found at 9 months in this work; however, growth in this microbial group is known to be boosted by the presence of vegetable coagulant instead of the standard animal rennet as a result of the increased time the former takes to clot the milk [29]. No coagulase (+) staphylococci, which produce enterotoxin, were detected at any stage of the study. In addition, no *Listeria* were detected in any of the samples (25 g) at different stages of the study and also no evidence of *Clostridium* was found in any of the samples.

As can be seen from Table I, the highest counts among fermentation flora microbes were exhibited by lactococci (9.38 log CFU/g versus 7.84 and 7.97 log CFU/g for lactobacilli and enterococci, respectively). This situation changed as the preservation time increased. Thus, at 3 months, lactobacilli exhibited increased counts; beyond that point, however, lactococcus and lactobacillus counts – particularly the

latter – decreased gradually until the end of the study. Homofermentative lactobacilli are known to cooperate with lactococci in lactose degradation, their incidence decreasing to a variable extent relative to lactococci as a result.

Finally, yeast and moulds were not detected in any sample after 3 months of preservation. The fact that lactose in the ripened cheese had by that time been metabolized to lactic acid by lactic flora – so it was unavailable to the alterative rnicrobes present –, imposed a constraint on these groups, which can either oxidize lactate or use protein degradation products. In addition, the essentially anaerobic conditions inside the cheese, together with the preservation procedure used in this work, restricted any activity to the cheese surface, mostly by oxidative yeasts.

The incidence of pathogenic bacteria at the end of the oil and vacuum preservation process was markedly decreased (with counts below 1 log CFU/g); also, lactobacilli and lactococcus counts increased at 3 months of preservation but decreased near the end of the process. Consequently, microbial proteolytic activity was due to lactic acid flora at the early stages of storage and largely to enterococci after 6 months. The formation of large amounts of SN, NPN and NH_2–N, the increased content in the non-casein fraction and the presence high-molecular weight polypeptides formed by the action of these microbes on nitrogen compounds, provide this cheese with special, highly appreciated features, Also, it avoids desiccation, thereby lengthening its shelf life.

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SOME EVIDENCES FOR THE INVOLVEMENT OF PLASMID IN DIURON HERBICIDE DEGRADATION

B. A. EL-DEEB[1], A. M. ALI[2], K. A. ALI[1]

[1]Department of Botany, Faculty of Science, 82524, Sohag, Egypt, [2]National Institute of Research, Doki, Cairo, Egypt

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Pseudomonas sp. strain Bk8 was isolated from field soil contaminated with different urea-herbicides. This strain is a plasmid (pBkB)-harbouring organism capable of complete degradation of diuron herbicide. Plasmid-cured strain Bk8M was obtained by treatment of *Pseudomonas* sp. Bk8 with Mitomycin C. This cured strain is capable of only partial degradation of diuron side chain and accumulated a phenolic compound in the medium during growing on diuron as a sole source of carbon and energy. Conjugation experiment was carried out using Bk8M as a recipient and Bk8 as a donor of pBk8 plasmid. The transconjugant was able to degrade a diuron without accumulation of phenolic compound. It was proposed that plasmid pBk8 is self-transmissible and involved in the degradation of diuron aromatic ring but it is not connected with the transformation of diuron into diuron phenol compound.

Keywords: diuron herbicide degradation, plasmid of *Pseudomonas* sp.

Diuron 3-(3,4-dichlorophenyl)1,1-dimethylurea is a selective pre- and post-emergence herbicide that is used for weed control in a wide range of crops and is an example of a substituted urea herbicide. The environmental fate of diuron is of great concern in many areas around the world, where it has been detected in ground water [1]. The biological activity of soil microorganisms is proposed to be the major factor for biodegradation of diuron in soil [2–5]. Various soil bacteria and Fungi transform the urea-herbicide into arylamine, CO_2, and NH_3, using it as a sole source of carbon and nitrogen [6]. Tillmannas et al. [7] described oxidative dealkylation of diuron by the Fungus *Cunninghamella echinulata.* Previous works on the biodegradation pathway of urea-derivatives have shown that the base of decomposition is N-demethylation, hydrolysis followed by oxidation of aromatic ring [8–10].

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Bacterial plasmids may play a significant role in the evolution and dissemination of many genes required for the metabolism of herbicide 2,4-D [11–13], EPTC [14], MCPA [15], Carbofuran [16–18], Bromxynil [19–20], S-atriazin [21] 2,4-Dand MCPP [22]. Most of the works were focussed on the strains that are capable of degrading or transforming the urea-herbicide [8, 23–25]. No detailed studies characterizing possible plasmid involvement in modifying the diuron aromatic ring have been presented.

In this paper we give evidence that in *Pseudomonas* sp. Bk8, the degradation of aromatic ring of diuron is plasmid depended.

Table I

Bacterial strains and plasmids used in this study

Organisms and strains	Plasmid and comments	Phenotype
Pseudomonas, Bk8	Wild-type diuron degrader; Bk8	Dp^+ Strs Rifs
Pseudomonas, Bk8M	Cured derivative of Bk8	Dp^- Strr Rifr
Pseudomonas, Bk8(pBk8)	Transconjugates between Bk8 and Bk8M	Dp^+ Strr Rifr

Dp^+ = ability to degrade the diuron aromatic ring
Dp^- = inability to degrade the diuron aromatic ring
Rifs = Rifampin sensitive
Rifr = Rifampin resistant
Strs = Streptomycin sensitive
Strr = Streptomycin resistant

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used and their plasmids and relevant phenotypes are shown in Table I.

Spontaneous streptomycin-resistant (str^r) mutants were isolated by plating nutrient broth-grown cells on nutrient agar supplemented with 100 μg/ml Rifampin (Rif^r) resistant mutants were isolated by UV induction as described by Miller [26].

Chemicals

Diuro{3-(3,4-dichlorophenyl)1,1-dimethylurea} was purchased from Sigma Co., St. Louis Mo., Agar, Trypton, and yeast extract (YE) were obtained from Difco Laboratories, Mich. Minimal salt medium [27] was used in the present investigation as follows g/l: $(NH_4)SO_4$, 0.5g, $MgSO_4.7H_2O$, 0.2g, $Ca(NO_3)$, 0.05g, Na_2HPO_4, 2.44g and $KHPO_4$ 1.52g.

Culture conditions

Pseudomonas sp. Bk8 and its mutant Bk8M were cultured in basal medium with 2% glucose at 30 °C on Gyrator shaker 150 rpm. For phenolic compounds' accumulation experiments, cells were washed twice in minimal medium and resuspended in 10 ml of minimal medium with 3 mg/ml diuron. Cells were then incubated on a shaker at 100 rpm at 30 °C for 120 h.

Analytical methods

The concentrations of 3,4-dichlorophenol and phenol in the cultures were determined by the modification of 4-aminoantipyrine assay [28]. The absorption maxima were determined for each compound and standard curves were drawn on the basis of spectrophotometric reading at the absorption maxima. Absorption maxima were 507 and 505 nm for the phenolic derivatives of 3,4-dichlorophenol and phenol, respectively. To monitor the accumulation of the phenolic derivatives in the culture medium, 0.1 to 0.3 ml of culture supernatant was used in the 4-aminoantipyrine assay. Reagents were made fresh before each assay. The concentration of diuron was determined as described previously [1].

Plasmid isolation

Plasmid DNA was isolated by a procedure based on the method of Kado and Liu [29].

Plasmid curing

Plasmid curing experiments were performed according to a procedure essentially described by Carlton and Brown [30]. In the present experiments, series of tubes containing LB with a concentration of Mitomycin C, ranging from 0.0001–20

pg/ml were inoculated with loopful of cell from a 48 hour LB culture of *Pseudomonas* sp. Bk8. After incubation by low shaking for 48 hour, 0.01 ml of cell culture from tubes showing only a slight increase in turbidity due to cell growth was diluted in phosphate buffer. Dilutions of these cultures were plated on nutrient agar medium. Single colony isolates were picked, restreaked on nutrient agar and examined for loss of detectable plasmid DNA. These isolates were examined for phenotypic similarity to the plasmid-bearing strain. In addition, growth of strain Bk8 in LB broth medium with no diuron was used as a curing procedure.

Table II

Antibiotic resistance of Pseudomonas sp. Bk8 and its mutant Bk8M

Antibiotic concentration	Bk8	Bk8M
Clindamycin 50 μg/ml	+	+
Tobramycin 50 μg/ml	+	+
Tetracycline 50 μg/ml	–	–
Amikacin 50 μg/ml	+	+
Gentamicin 50 μg/ml	+	+
Norfloxacin 50 μg/ml	+	+
Cephalexin 50 μg/ml	+	+
Fucidin 50 μg/ml	+	+
Streptomycin 50 μg/ml	+	+
Kanamycin 50 μg/ml	+	+
Ampicillin 50 μg/ml	–	–

Bacterial conjugation

In order to link the diuron phenol (Dp^+)-degradation phenotype to the pBK8 plasmid, plasmid was transferred to Bk8M mutant (Dp^-, St^r Rif^r) as a recipient and Bk8 (pBK8) as a donor strain. Filter mating was performed by using a 1:10 ratio of donor cells to recipient cells. Filter was incubated on LB plates for 24 h at 30 °C. Filters were then transferred to 100 ml minimal medium with 50 μg/ml Rifampin and 10 μg/ml 3,4-dichlorophenol as a sole source of carbon in 500 ml Erlenmeyer flasks. The cells were incubated with shaking at 30° C for 48 h, and 0.1 ml fractions were plated onto nutrient

agar plates containing 50 μg/ml Rifampin. Colonies were selected and screened for plasmid presence, appropriate catabolic, heavy metals and antibiotic phenotype.

Resistance to antibiotics

Susceptibility of the organisms to different antibiotics was determined by the agar diffusion technique (Kirby-Bouer disk method, Barry and Thornsberry [31], using different antibiotics disks (Table II).

Resistance to heavy metals

The resistance to heavy metals was determined by agar dilution method of Washington and Sutter [32]. The strains were subjected to the following concentrations of metallic salts: Nickel chloride, 0.5 to 3 mM; Cobalt chloride, 0.5 to 5 mM; Cadmium chloride, 0.5 to 3 mM; Zinc chloride, 0.5 to 4 mM. The metallic salt solutions were sterilized by membrane filtration (0.022 μm) and added to the solid medium. The minimal inhibitor concentration (MIC) was determined by testing twofold serial dilutions of the same metallic salts.

Results

Curing of Pseudomonas sp. pK8 plasmid. Plasmids can be lost from the host cells spontaneously at varying frequencies. Treatment of plasmid-containing cells with compounds such as Mitomycin C, and sodium dodecyl sulfate frequently increase the rate of appearance of plasmid-free cells [30]. In the current study, simply growing the Bk8 strain in YE medium in absence of diuron for 24 generation resulted in approximately 40% loss of the ability to completely degrade the diuron herbicide. The frequency of Bk8M mutants was increased to about 25% with the plasmid-curing agent Ethidium bromide (10 μg/ml).

These cured (Dp^-) strains did, however, retain the ability to grow on diuron as a sole source of carbon but accumulated the phenolic compounds in the medium (Fig. 2). These colonies were not auxotrophic and did not revert at detectable frequency to Dp^+ Diuron (phenol degradation). One of the Dp^- strain was screened for plasmid DNA, but none could be detected either in cesium chloride gradients or on agarose gels (Fig. 1). This Dp^- strain was assumed to be cured of pBK8 and was designated as *Pseudomonas* sp. strain Bk8M.

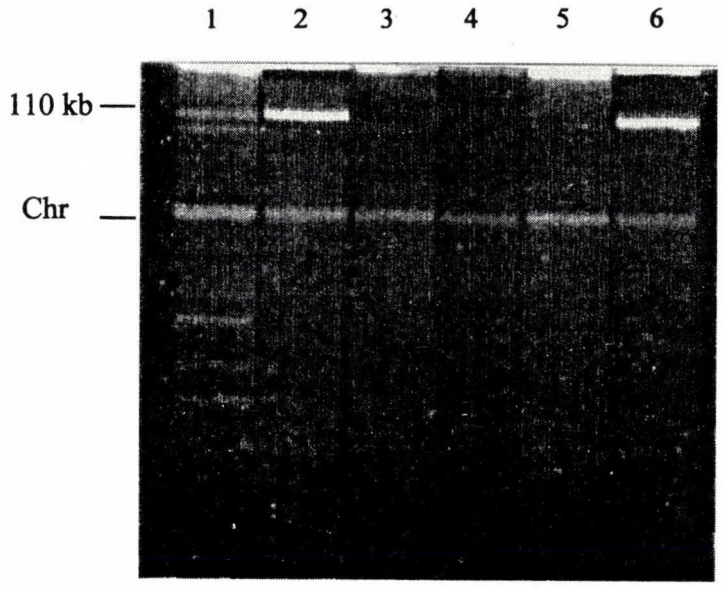

Fig. 1. Plasmid profiles of donor, recipient and transconjugant strains. Lanes; *Salmonella typh.* Plasmids as standard; 1, BK8; 2, cured Bk8M, 3–5, and transconjugant; 6.

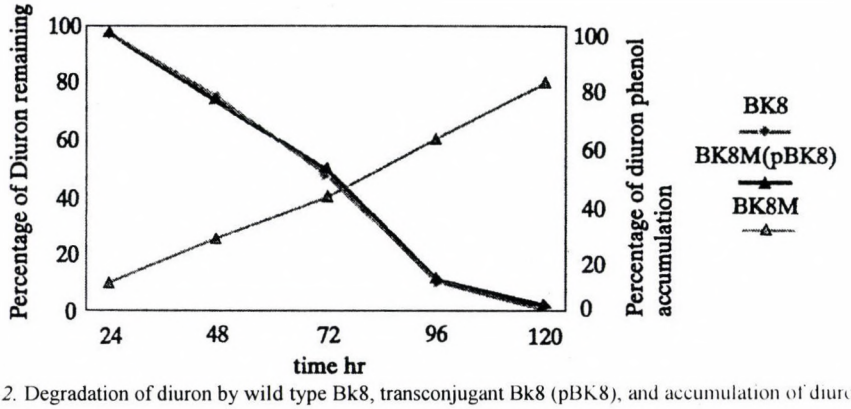

Fig. 2. Degradation of diuron by wild type Bk8, transconjugant Bk8 (pBK8), and accumulation of diuron phenol with cured strain Bk8M

Plasmid transfer by conjugation. Conjugation experiments were performed to transfer the pBK8 of the *Pseudomonas* sp. Bk8 to recipient plasmid-cured strain Bk8M in an attempt to obtain evidence for the role of plasmid in diuron aromatic ring modification. Our preliminary study showed that plasmid-cured strain Bk8 was able to grow on diuron as a sole source of carbon with accumulated phenolic compound in the medium [33].The ability of wild type strain Bk8 to utilize the phenolic compound was, therefore, the logical choice as a selection strategy for obtaining transconjugant strains containing the plasmid. Colonies that arose from the selection plates of the conjugation experiments were found to be Dp^+, streptomycin, Rifampin, and cadmium resistant. They had acquired the large plasmid pBK8 in mating processes (Fig. 1). When grown in minimal medium supplemented with diuron as a sole source of carbon, the transconjugant utilize the herbicide as efficiently as the wild type strain Bk8 (Fig. 2). The frequency of plasmid transfer from Bk8 to its mutant Bk8M ranged from 0.9×10^{-7} to 1.4×10^{-6} per initial donor cell. Moreover, the ability of transconjugants to grow on 3,4-dichlorophenol, phenol, aniline and catechol (Table III) may add further proof for the role of plasmid in diuron phenol catabolism.

Table III

Growth of Pseudomonas *sp. Bk8 strain and its derivatives on different aromatic compounds as a sole source of carbon and energy*

Growth substrate	Bk8	Bk8M	Bk8M (pBk8)
Diuron	+	+	+
Anilin	+	–	+
Phenol	+	–	+
3,4-dichlorophenol	+	–	+

Plasmid profiles. Pseudomonas sp. strain Bk8 was screened for the presence of plasmid DNA by the method of Kado and Liu [29] and a single band was detected after agarose gel electrophoresis. The size of the plasmid was 110 Kb as judged by migration on agarose gel against plasmid of known molecular weight (Fig. 1). This plasmid was designated pBk8.

Testing for additional plasmid-encoded functions. In an attempt to identify additional functions encoded by BK8 plasmid, the wild type strain Bk8 and its mutant were tested for their resistance to different heavy metals and antibiotics. The wild type

strain Bk8 and its mutant Bk8M were resistant to the same concentrations of the tested antibiotics (Table III).

Heavy metal resistance. Overnight culture of the wild type strain BK8 and the cured strain Bk8M were diluted and spread on agar containing the metals concentrations described above (material and methods). The MIC (minimum inhibitor concentration) of metals was studied and summarized in (Table IV). The results show that the MIC of wild type strain is 1 mM, Cd; 3 mM Zn; 1.5 mM, Co and 1 mM, Ni. The cured strain Bk8M lost its resistance to Cd but retained the resistance to Zn, Co and Ni (Table IV).

Plasmid-mediated diuron catabolism. Pseudomonas sp. strain Bk8 was originally isolated from a field soil exhibiting enhanced degradation of the herbicide diuron. It was capable to completely degrade the herbicide diuron at concentration 3 mg/ml without accumulation of diuron phenol or any other metabolite detectable by ultraviolet absorption between 200–600 nm [1, 12, under publication]. Moreover, the isolate Bk8 was able to use the diuron aliphatic side chain as a sole source of carbon.

Table IV

The minimum inhibitor concentration (MIC) of cadmium (Cd), zinc (Zn), cobalt (Co) and nickel (Ni) for Pseudomonas *sp. Bk8 and its derivatives*

Heavy metals	Bk8	Bk8M	Bk8M (Bk8)
Cd	1 mM	–	1 mM
Zn	3 mM	3 mM	3 mM
Ni	1 mM	1 mM	1 mM
Co	1.5 mM	1.5 mM	1.5 mM

The involvement of naturally occurring plasmids in the degradation of many aliphatic and aromatic compounds was described and analyzed in the genus *Pseudomonas* [15, 35–40].

Pseudomonas sp. Bk8 contained a large plasmid designated as pPBK8 with a molecular size of about 110 kb. In order to determine whether this plasmid is required by strain Bk8 for growth on diuron, Bk8 strain was cured of plasmid by growth in LB containing 10 μg/ml Mitomycin C for 48 h. The results presented here suggested that plasmid-encoded genes are mediated in subsequent steps. Moreover, the enzymes required for transformation of diuron into phenolic compound by Bk8 strain were not

encoded on plasmid pBk8, since cured strain Bk8M had the ability to transform diuron and accumulated the phenolic compounds in the medium (Fig. 2).

The biodegradation activity is inhibited by heavy metals and their presence might decrease biodegradation of aromatic compounds in polluted sites [41, 42]. Recently, there is a great interest in bacterial strains that degrade aromatic compounds and tolerate toxic metals [43, 44]. The present results revealed that the plasmid pBk8 are involved in cadmium resistance.

In summary, the finding reported here provide the evidence that a plasmid PBk8 is responsible for both diuron aromatic ring modification and cadmium resistance. Moreover, the Dp^+ (diuron aromatic ring cleavage) marker was always cotransferred with Cd^+ when selecting on cadmium or phenol.

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3RD ANNUAL MEETING OF THE HUNGARIAN STD SOCIETY AND THE 4TH ALPE–ADRIA–DANUBE STD WORKSHOP

12–14 NOVEMBER 1998

BUDAPEST, HUNGARY

ABSTRACTS OF PAPERS AND POSTERS

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HIV INFECTION, AIDS

M. A. WAUGH

Present world trends in STDs and AIDS. Their importance in dermato-venereology

Department of Sexual Health, The General Infirmary at Leeds, UK

There is a dichotomy in the field of Sexually Transmitted Infections between developed countries in North America, Europe and Australasia and the rest of the World. Most of the World, including some recently developing countries in Europe, has high levels of STDs and HIV infection, often causing terrible burdens on the health of the population and the finances of that country.

Dermato-Venereologists should be aware of preventive measures, the effect of genital ulcer disease on STDs, the rise of HIV and clinical methods, such as the syndromic method, used to combat STDs.

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A. STROOBANT

Trends in HIV seroprevalence among patients with sexually transmitted diseases in 17 European Sentinel Networks, 1990–1996

Scientific Institute of Public Health – Louis Pasteur Institute of Hygiene and Epidemiology, Section Epidemiology, Brussel, Belgium

Overall, more than 150,000 STD episodes were registered in this study. In the European network as a whole, the prevalence decreased significantly among IDUs (51% in 1990 to 18% in 1996, Odds Ratio for annual change (OR)=0.87) and among homo/bisexual men (21% in 1990 to 15% in 1996, OR=0.93). In both groups, trends did not differ statistically between networks. Among non-IDU heterosexuals the prevalence changed slightly (0.7% in 1990 and 0.8% in 1996.) However, a significant increase was observed among women (OR=1.14). In addition, trends differed significantly per network: a significant increase occurred in the Belgian and Portuguese networks, while changes are not significant in other networks.

I. DÖMÖK

Review of HIV/AIDS policy, programmes and situation in Hungary

National Centre for Epidemiology, Budapest, Hungary

In Hungary the first HIV positive persons were detected in 1985 and the first AIDS case at the end of 1986. Since that time altogether 729 HIV positive (548 male, 66 female, 115 anonymous) persons have been notified, of whom 297 (270 male and 27 female) already presented the characteristic features of AIDS and 194 died. Thus the cumulative AIDS incidence rate by July 1998 proved to be 29 per million population, which is 11-times less than the reported cumulative global rate. 73% of AIDS cases belonged to the risk group of men having sex with men and 97% of patients were over 12 years of age. Estimated rate of people living with HIV/AIDS was 490 per million (5000) which represented one-tenth of global rate estimated by UNAIDS in 1998.

Factors contributing to this relatively good epidemiological situation were as follows: (a) In the early period of pandemic Hungary was a semiclosed community "safeguarded by the iron curtain" limiting the importation of infection. (b) The spreading of infection began when the diagnostic methods were already available and laboratory facilities were created very early in networks of blood banks, of public

health and of venereological services. (c) Primary prevention activities were started in that early period by the governmental and NG organizations. (d) HIV/AIDS has been regarded primarily as a crisis in public health that had important relations to human rights and not as a crisis in human rights that had some epidemiological relations. (e) Iv. drug abuse remained relatively limited, so far.

K. NAGY

Molecular virology for the better understanding of the progression of AIDS

Department of AIDS and Human Retroviruses, National Institute of Dermatology and Venereology, Budapest, Hungary

More than a decade after AIDS epidemy has reached Hungary the still low rate (0.27/100,000) is due to the early intense biomedical research as well as to the effective education.

To analyse virology of HIV infections, replication capacity and phenotype of the isolates have been characterised to provide proper basis for the clinical diagnosis, treatment and also for follow-up of the spread of HIV/AIDS. Sequencing of the env V3-loop encoding regions of HIV provirus was carried out by gene amplification techniques, helping the determination of the pathogenesis of HIV strains. Nucleotide alignment comparison and representational peptide analysis revealed that the dominant subtype of HIV inducing the majority of the infections in Hungary is the HIV-1 B.

Additional factors contributing to the progression of HIV disease have also been investigated. DNA sequences of HHV-8 from Kaposi's sarcoma lesions and also from lymphocytes of HIV infected and/or AIDS patients were demonstrated in 93% of the cases. Antibodies to HHV-8 indicating the possibility for the subsequent development of Kaposi's sarcoma were detected in 22.2% of HIV infected persons.

Among the genetic factors providing a certain resistance to HIV infection the mutations and DNA polymorphism of the HIV coreceptor CCR5 gene was studied. More than 40% of the long-term nonprogressors, but only 16% of HIV progressors proved to be heterozygote for the CCR5 gene, compared to 23% occurred in the general population.

These molecular virological approaches help us further understanding the geographical distribution and functional significance of the disease diversity in relation to transmission, pathogenesis, progression and the effective therapy of HIV/AIDS.

F. HLADIK[1,2], G. LENTZ[1], A. MCELROY[1], M. J. MCELRATH[1]

Dendritic cell/T cell interactions support co-receptor independent HIV-1 transmission in the human genital tract

[1]Program in Infectious Diseases, Fred Hutchinson Cancer Research Center, Seattle, USA,
[2]Department of Dermatology, Division of Immunology and Infectious Skin Diseases,
University of Vienna, Vienna, Austria

Background. Worldwide, HIV-1 is predominantly transmitted heterosexually. Although mechanisms of transmission have been inferred from model systems in maquaques and the human skin, target cells for HIV-1 in the human genital tract and their role in selection of transmitted viral strains remain undefined.

Methods. Using a novel cell isolation procedure, we analyzed lymphocytes, macrophages and dendritic cells from the human female genital mucosa for expression of HIV-1 co-receptors and infectivity with CCR5- and CXCR4-tropic HIV-1 strains.

Results. Whereas the majority of lymphocytes and macrophages expressed CCR5, none or only few dendritic cells were positive for this receptor by flow cytometry and RT-PCR. In contrast, all three cell types expressed CXCR4. Correspondingly, infection with CCR5-tropic HIV-1_{BA-L} was less efficient for sorted mucosal dendritic cells than for T lymphocytes. Moreover, mucosal T cells were infected with both CCR5- and CXCR4-tropic HIV-1 strains. However, viral replication was blocked in T cells after completion of reverse transcription. Conjugation of T cells with dendritic cells could overcome this post-entry block of replication in T cells as well as the coreceptor restriction on mucosal dendritic cells for both CCR5- and CXCR4-tropic HIV-1 strains.

Conclusions. In the human female genital tract, T cells and macrophages are the primary targets for HIV-1. Interaction of resident dendritic cells with T cells, however, is crucial for local viral amplification and systemic spread. Our findings also demonstrate that mucosal mononuclear cells infiltrating the human genital mucosa are permissive for both M- and T-tropic HIV-1 variants.

D. FORSEA, M. MĂRDĂRESCU, S. PETREA, C. POPESCU, S. TIPLICA

AIDS-children in Romania muco-cutaneous particularities

[1]"N.Gh. Lupu" Colentina Hospital, Bucharest, Romania

Muco-cutaneous lesions have an important place in the pathology of HIV infection.

In children the cutaneous lesions tend to be significantly different from those seen in adults. At the same time, they are more severe and less responsive to treatment. Romania has a particular epidemiological context of HIV infection in children.

More than half of the European children with AIDS were recorded in Romania (4779 cases at 30/06/98). High prevalence areas in our country are Constanta – 1268 cases (26.5%), Bucharest – 350 cases (7.3%), Giurgiu – 332 cases (6.9%) and Galati – 256 cases (5.3%). A peculiar feature of AIDS in Romania is that 89.6% of the recorded cases (5280 at 31/03/98) were represented by children.

We performed a cross-sectional survey of skin diseases in patients with AIDS, admitted in the Department of Infectious Diseases of the "N. Gh. Lupu" Hospital. Our aim was to measure the point prevalence of various skin diseases found in children with AIDS and to identify those which are highly suggestive for immunosuppression.

All children were independently examined by two trained dermatologists and the diagnosis was established by consensus. We examined 82 children with AIDS (44 boys, 32 girls), aged 1–9 years, 76 of the 82 (92%) examined children had cutaneous manifestations, most of them having two or three different types of lesions simultaneously. All of them had thin, dry, transparent teguments covered by furfuraceous scales, hyperliniarity of palms and soles; thin and brittle hair shafts.

Some skin lesions can be considered as markers of AIDS, their dissemination, severity and resistance to treatment testifying for a severe immunocompromised patient and predicting a poor prognosis. Except for severe infections or malignant tumors, the cutaneous lesions by themselves do not shadow the vital prognosis of children with AIDS, but treating these lesions will significantly improve the quality of life of these patients.

W. WENINGER[1], T. PARTANEN[2], S. BREITENEDER-GELEFF[3], C. MAYER[1], H. KOWALSKI[3], M. MILDNER[1], J. PAMMER[3], M. STÜRZL[4], D. KERJASCHKI[3], K. ALITALO[2], E. TSCHACHLER[1]

Expression of VEGFR-3 and podoplanin suggests a lymphatic endothelial cell origin of Kaposi's sarcoma tumor cells

[1]DIAID, Department of Dermatology, [2]Institute of Clinical Pathology, University of Vienna, Austria, [3]Department of Pathology, University of Helsinki, Finland, [4]GSF-Forschungszentrum, Institute for Molecular Virology, Oberschleißheim, Germany

Despite intensive research over the past decade the exact lineage relationship of Kaposi's sarcoma (KS) tumor cells has not yet been settled. In the present study we investigated the expression of 2 markers for lymphatic endothelial cells, i.e. vascular endothelial growth factor receptor-3 (VEGFR-3) and podoplanin, in AIDS and classic KS. Both markers were strongly expressed by cells lining irregular vascular spaces in early KS lesions and by tumor cells in advanced KS. Double-staining experiments by confocal laser microscopy established that VEGFR-3 positive and podoplanin positive cell populations were identical and uniformly expressed CD31. By contrast, these cells were negative for CD45, CD68 and PAL-E excluding their hemopoietic and blood vessel endothelial cell nature. No expression of VEGFR-3 and podoplanin was detected in any of 4 KS-derived spindle cell cultures and in 1 KS-derived autonomously growing cell line (KS-Y1). Our findings that KS tumor cells express two lymphatic EC markers in situ strongly suggest that they are related to or even derived from the lymphatic EC lineage. Lack of these antigens on cultured cells derived from KS lesions indicates that they might not represent tumor cells which grow in tissue culture but rather other cell types present in KS lesions.

M. HENRY, A. UTHMAN, E. TSCHACHLER

Detection of HHV-8 in peripheral blood cell populations in Kaposi's sarcoma patients

Division of Immunology, Allergy and Infectious Diseases. Department of Dermatology Vienna Medical School, Vienna, Austria

Human herpes virus-8 (HHV) has been detected in all forms of Kaposi's sarcoma (KS) as well as in multicentric Castelman's disease and pleural effusion lymphoma (PEL). Although its presence has been reported in B cells, monocytes,

semen mononuclear cells, endothelial cells, macrophages and spindle cells, the target cells of this new herpes virus are still unknown.

To investigate the distribution of HHV-8 in different blood cell populations of patients with and without KS, we used conventional PCR technology. Peripheral blood mononuclear cells (PBMC) of eleven blood samples from 9 patients (4 KS/HIV-, 4 KS/HIV+, and 3 HIV+ without KS) were treated with immunomagnetic beads for serial isolation of four cell populations: CD2+/CD19+/CD14+ and the remaining depleted population CD2–/CD19–/CD14–. By conventional PCR, PBMC were positive for HHV-8 in 1 of 3 KS/HIV- patients, 2 of 3 KS/HIV+ and none of the HIV+ patients without KS. With regard to subpopulations of PBMC, HHV-8 sequences were detected only in CD2–/CD19–/CD14– cells of HIV patients. In contrast, in 2 HIV+ patients HHV-8 was present in all 4 subpopulations of PBMC. In the one HIV+ patient who did not show HHV-8 DNA in the total PBMC fraction, viral DNA was detected in the CD2+ as well as in the CD2–/CD19–/CD14– population. In one of the HIV- patients without KS, the subpopulation of CD19+ cells was the only one which tested positive for HHV-8 DNA.

In summary we found that HHV-8 sequences can be detected more frequently, in PBMC subpopulations of HIV-1 infected than HIV-1 noninfected KS patients. Since in patients with PBMC positive for HHV-8 there is no single subpopulation of cells which alone is positive for this virus, our data suggest that various cell types might harbor HHV-8.

A. PETTER[1], M. GUGER[1], A. CIRESA[2], K. HEIM[2], M. SARCLETTI[1], R. ZANGERLE[1], H. PFISTER[3], P. FRITSCH[1], R. HÖPFL[1]

Specific serum ELISA IgG, IgM, and IgA reactivity to human papillomavirus (HPV) types-6, -11, -16, -18 and -31 VLPs in HIV-infected women

[1]Department of Dermatology and Venereology, [2]Department of Gynecology and Obstetrics, University of Innsbruck, Austria, [3]Department of Virology, University of Köln, Germany

Objective. Immunosuppressed, e.g. HIV-infected persons are about five times as likely as HIV negative controls for developing HPV-related disease such as cervical intraepithelial neoplasia (CIN) and anal squamous intraepithelial lesions (ASIL). A serological assay detecting type specific HPV antibodies would provide additional information to HPV-DNA diagnostic methods, since it could evaluate prior HPV exposure and define persons at risk for HPV-related disease. However humoral

immune response to HPV has only been studied in immunocompetent patients and immunosuppressed men so far. In our observational study, we analyzed the type (HPV types-6, -11, -16, -18, -31) specific serologic response to intact HPV L1 virus like particles (VLPs) in HIV-positive women, compared with a HIV-negative control group.

Methods. Serum samples of 83 HIV-positive women and 102 HIV-negative controls were analyzed by ELISA for specific IgG-, IgA-, IgM-antibodies recognizing HPV types-6, -11, -16, -18 and -31 L1 VLPs. Symptomatic women were followed over time (median 35 months). HPV-DNA was detected by PCR and a dot blot screening assay.

Results. HPV-related lesions (e.g. Condylomata acuminata or CIN) were present in 30 (36%) of 83 HIV-positive women. Progression to invasive cervical cancer has occurred in one patient. Positive IgG reactivity to HPV-6/-11 and -16/18/-31 was seen in 19%/31% and 49%/30%/24%, respectively. In 102 HIV seronegative control patients the percentages were 13%/13% and 14%/7%/9%. IgM responses were low, IgA responses comparable to IgG. The cumulative (and/or) seroprevalence of high risk HPV (-16, -18, -31) was 58% in HIV infected women compared to 19% in HIV negative control patients ($p<0.0000$). The IgG/IgM/IgA seroreactivity was constant over time irrespective of CD4 counts.

Conclusions. The present data suggest a marked increased prevalence of high risk HPV in HIV-positive women, compared to HIV-negative control individuals. This may reflect the higher risk of this population for developing cervical lesions.

K. NAGY, B. KEMÉNY, A. HORVÁTH

Seroprevalence and nucleic acid polymorphism of HHV-8 in AIDS associated Kaposi's sarcoma

National Institute of Dermatology and Venereology, Budapest, Hungary

The involvement of Kaposi's sarcoma associated herpesvirus or human herpesvirus (HHV-) 8 in the etiopathogenesis of Kaposi's sarcoma (KS) has not yet been fully established. We identified and confirmed the presence of HHV-8 DNA in our patients with AIDS associated, iatrogenic and classical KS, and based on our previous studies (NAGY K. et al., Cancer Control 1997) we proposed that HHV-8 may be present in the healthy population as latent persistent infection.

It is remarkable that KS has been developed in less than 10 per cent of the AIDS patients in our region, but HHV-8 DNA was identified in >95 per cent of KS/AIDS

cases, and on the other hand KS is diagnosed in 22 per cent of all malignancies occurring after organ transplantation. These differences in the pathogenesis observed may suggest the circulation of a strain of HHV-8 different from those analyzed in other geographical regions such as the US or Africa.

Therefore we analyzed the presence and DNA sequence variability of ORF26 and K1 ORF regions of HHV-8 in cutaneous and visceral forms of histologically confirmed KS lesions and also in lymphocytes from 27 AIDS associated, iatrogenic and classical KS cases. Moreover the seroprevalence of HHV-8 antibodies in the KS risk groups and in the healthy population of the region was determined.

PCR amplified ORF26 and K1 ORF regions were cloned and sequence variability was analyzed by SSCP. Presence and prevalence of HHV-8 antibodies in sera of 868 individuals including 600 healthy subjects was determined on BCBL-1 cells by IF and also with WB of PMA-stimulated cell lysates and concentrated HHV-8 virions.

An overall of ~95 per cent homology of K1 ORF to known sequences was found, however more variability was detected in the 5' segment (115–463 bp) of K1 ORF, especially in the regions of 125–132 and 293–304 bps. Antibodies to HHV-8 were detected in 22.2 per cent of HIV-1 infected non KS individuals, in 1 per cent of their HIV negative non-KS partners and in >80 per cent of HIV negative KS patients. Among the healthy individuals 2.1 per cent showed the presence of HHV-8 antibodies with titres of 1:100–1:6000.

The significance of our findings include i) the possibility, that the presence of HHV-8 antibodies may be one of the prediction markers of the subsequent progression of KS in some of these individuals, ii) healthy blood donors may latently harbour the transmissible HHV-8, which may affect controlling the security of blood transfusions, iii) differences in the pathogenesis in population groups in our region may be attributable to various additional factors other than HHV-8 strain variability.

A. GEUSAU[1], I. SIMONITSCH[2]

Primary HIV-infection

[1]Department of Dermatology, Div. Immunology, Allergy and Infectious Diseases (DIAID),
[2]Department of Pathology, University of Vienna, Medical School, Austria

A 24-year-old homosexual man, who recently had started a new relationship, developed constitutional symptoms, fever, lymphadenopathia and a maculopapular rash. Laboratory findings showed slightly elevated liver enzymes and markers of

inflammation, thrombopenia (71 G/l, normal 140–440 G/l), and leucopenia (3.0 G/l, normal 4.3–10.1 G/l). An HIV-antibody test (ELISA and Westernblot) was negative, as were serological tests for syphilis. The suspected diagnosis of a primary HIV-infection could be confirmed by a highly elevated serum p24 antigen as well as by the detection of plasma HIV-1 ribonucleic acid (RNA) (6.3×10^5 copies/ml). The patient's CD4 count was decreased (400/μl), the ratio of CD4/CD8 lymphocytes was reversed (0.66). Antiretroviral treatment was initiated as a triple combination therapy (Ritonavir 2×400 mg, Didanosid 2×200 mg, Stavudine 2×40 mg) in combination with a cytostatic agent (Hydroxycarbamid 2×500 mg) and the patient became asymptomatic within a week. Seroconversion to HIV could be demonstrated within two weeks after onset of the symptoms. A histopathological investigation of a skin lesion showed a superficial lymphohistiocytic perivascular dermatitis. Vacuolar changes in the basal layer cells with a few necrotic keratinocytes were classified as an interface dermatitis. Immunohistological examination revealed a marked predominance of CD8+ T cells with coexpression of Granzyme B7 (GB7) and TIA-1, pointing to a highly activated, cytolytic status of these T cells. Expression of the p24 Ag was noted in individual cells of the infiltrate, presumably macrophages and/or Langerhans cells. Fifty to 80% of the patients infected with the HI-virus suffer from an acute mononucleosis like illness, being the skin and oral mucosa the most frequently involved sites. The combination of a maculopapular, asymptomatic rash, predominantly distributed on the upper trunk and on the face, and oral and/or genital ulcers, associated with constitutional complaints should be considered suggestive of a primary HIV-infection. As in our patient, who developed candida-esophagitis, severe transient immunosuppression may even lead to opportunistic infections during the acute phase of seroconversion.

EPIDEMIOLOGY AND CLINICAL ASPECTS OF SYPHILIS

N. TZANKOV, G. SPIROV, N. KIRIAKOVA, G. PECHLIVANOV, R. DENTSHEVA

Analysis of morbidity of syphilis in Bulgaria 1990–1997

Sofia, Bulgaria

The aim of the study is to analyse morbidity of syphilis for 1990–1997 period as well as to estimate the effectiveness of treatment and to summarize causative factors for its increasing. A significant increase of the number of syphilis patients has been

registered during that period – from 378 in 1990 and 542 in 1991 to 2298 in 1996 and 2138 in 1997. An important characteristic of morbidity is the prevalence of contagious forms – the frequency is 94.7% out of all syphilis patients. The number of the earliest stage patients among men is 3 times higher than that among women. The increase of number of lues congenita patients is another important factor. It was 0.26% in 1990 and 1.4% in 1997. The treatment applied is standardized for the country and is considered to be highly effective. Due to the high number of syphilis patients the future efforts should be concentrated on decreasing syphilis morbidity and establishing earlier diagnosis.

M. POTOČNIK[1], M. R. BUTINA[1], A. GODIČ[1], I. BARTENJEV[1], S. LEVIČNIK-STEZINAR[2]

Syphilis in pregnancy in Slovenia

[1]Department of Dermatovenereology, University Medical Centre, Ljubljana, Slovenia,
[2]Blood Transfusion Centre of Slovenia, Ljubjana, Slovenia

Untreated primary or secondary syphilis during pregnancy affects virtually 100 per cent of fetuses; 50 per cent of such pregnancies resulting in premature delivery or perinatal death. Untreated early latent syphilis during pregnancy results in a 40 per cent rate of prematurity or perinatal death. Ten per cent of infants born to mothers with untreated late syphilis shows signs of congenital infection, and the perinatal death rate is increased approximately tenfold. Whereas syphilis is rarely sexually transmissible longer than 2 years after acquisition, women with untreated syphilis apparently may remain infectious for their fetuses for many years, although the proportion of affected fetuses and the severity of fetal disease decrease with longer duration of untreated maternal infection. In Slovenia pregnant women are screened for syphilis in the first and in the third trimester of pregnancy.

Syphilis in Slovenia started to decline toward the end of the sixties and followed such a persistent decline, thus in 1991 5 (3 females) cases, in the years 1992 and 1993 only two cases of early syphilis were registered in the entire country, meaning an incidence of 0.10 per 100,000 inhabitants. Surprising were the data for 1994 when 36 (11 females) cases of early syphilis were registered in the country, meaning an incidence of 1.82 per 100,000 inhabitants. This trend continued in 1995 with 38 (9 females) cases, but in 1996 28 (12 females) cases and in 1997 25 (5 females) cases were registered. In the period 1991–1997 6 pregnant women with early and 11 pregnant women with late syphilis were detected and treated. All mothers and babies were followed up clinically and serologically. Because of the encouraging fact, that the

last case of juvenile congenital syphilis was registered in 1986, the abolition of screening was discussed. The current epidemiological situation of STDs, and especially syphilis, requires consequent screening of pregnant women.

M. BERECZ, V. VÁRKONYI, A. HORVÁTH

Current trends in syphilis epidemiology in Hungary according to the epidemiological data of 1997

National Institute of Dermatology and Venereology, Budapest, Hungary

The number of detected syphilis cases in 1997 in Hungary was 38.35% higher than in 1996. Three cases of connatal lues have been reported, and one newborn had severe symptoms at the time of delivery. 85.47% of all cases was classified as early infectious syphilis. 38.61% was diagnosed in the symptomatic period, 46.86% was detected as syphilis latens recens. The syphilis was transferred from regions neighbouring the borders of the country (East and South Hungary, Middle-Transdanube). Occurrence of syphilis in Hungary is significantly lower than in the surrounding countries, due to the well-organized and functioning dermatological-venereological disease prevention network. A rise in the occurrence of syphilis cases can be assumed.

S. LEVIČNIK-STEZINAR[1], M. POTOČNIK[2]

Surveillance and prevention of transfusion-transmitted syphilis

[1]Blood Transfusion Centre of Slovenia, Ljubljana and [2]Department of Dermato-Venerology, University Clinical Centre, Ljubljana, Slovenia

Treponema pallidum is one of the many agents that can be transmitted by transfusing blood or cellular components. Measures for the prevention of any possible transmissions of infections from blood donors to blood recipients were introduced in the 1950s. According to the principles of modern transfusion therapy, all blood is tested for the presence of anti-*Treponema pallidum* antibodies and every reactive dose of blood is set aside and destroyed. The TPHA and EIA methods of testing are used and FTA is performed for confirmatory testing. Blood donors with reactive test results are notified and at the same time newly discovered cases are reported to the Department of Dermato-Venerology, University Medical Centre, Ljubljana. We determine whether the

person is already registered among the seropositive patients and, or if he/she has ever been treated. We invite the newly discovered cases for an interview and a detailed anamnesis is made, a clinical examination, retesting is done and if necessary retesting using another method. Treatment is prescribed if necessary. An epidemiological questionnaire is completed for each person where answers to questions such as who was the source of infection, the time of infection, other persons with whom the person has had sexual contact are given. In the Blood Transfusion Centre of Slovenia 30 new cases among blood donors from the Ljubljana region were discovered between 1991–97, 10 persons (33.3%) among the newly discovered cases were being treated, 10 (33.33%) persons had been previously treated and were no longer registered and 5 (16.6%) persons were under progressive treatment. One person had a false reactive result for unknown reasons, one person had a cross-reactivity with *Borrelia burgdorferi* and 3 persons (10.0%) did not respond when invited to the interview. During the last decades, Slovenia as well as Europe, have no data about the transmission of syphilis by blood transfusions, which is probably the result of the strict implementation of preventive measures.

B. L. SCHMIDT, M. EDJLALIPOUR, A. LUGÈR

The avidity index in syphilis serology

Ludwig Botzmann Institute for Dermato-Venerological Serodiagnosis, Hospital of the City of Vienna-Lainz, Vienna, Austria

Objectives. The binding strength of antibodies to multivalent antigens, called avidity, is generally low in primary, acute infections and increases in response to the maturation of the immune system. Goal of the present investigations was to find out whether or not there are differences in the specific IgG-avidity in patients with early and late syphilitic infections and if treatment does alter avidity.

Methods. Quantification of specific IgG antibodies was done with a sandwich ELISA-test using a lysate of treponemes as antigen. In addition, antigen-antibody-complexes were dissociated by applying high molar urea solutions after the incubation with serum dilutions. Antibodies still bound to the solid phase were determined by the same ELISA protocol. The antibody index was calculated as the ratio of ELISA units in the untreated well to that in the urea treated well × 100.

Results. The avidity index in primary and early latent syphilis (n=14) and secondary syphilis (n=7) was 34.6±5.9% and 49.2±9.6% respectively. Two years after adequate treatment (n=42), mean values and standard deviation of the avidity index of

patients was 75.1±3.9%. Follow-up studies of individual patients with primary or secondary syphilis revealed a profound increase in the avidity index confirming the data given above.

Conclusions. Measurements of the avidity of *Treponema pallidum* specific IgG immune globulines yielded a marked difference between sera from patients with early active disease and sera from patients two years after treatment.

F. GRUBER[1], A. JOJIĆ[2], V. PEHARDA[1], L. ČABRIJAN[1], M. SAFTIĆ[1]

Treatment of syphilis with azithromycin

[1]Department of Dermatology, Clinical Hospital Centre, and [2]Department of Social Medicine, Medical School, Rijeka, Croatia

Objective. The study was undertaken in order to assess the efficacy and tolerability of the long-acting azalide azithromycin in the treatment of venereal syphilis.

Methods. Fourteen patients with early syphilis (ten males and four females) were included in this open, noncomparative study. The diagnosis was achieved by clinical grounds, dark field examination and by serology. The patients were treated with 1.0 g azithromycin the first days and then 500 mg for 8 days.

Results. At the baseline 5 patients had primary seropositive syphilis, 1 primary seronegative syphilis and 8 patients had secondary syphilis (4 manifest and 4 latent). In patients with primary syphilis bacteriologic eradication was obtained within 24 h, while the chancres healed in 4–8 days.

The exanthema faded in secondary syphilis in 7–9 days.

Complete resolution of serologic tests (VDRL and TPHA) occurred in 4 patients within 3 month and in 9 patients within 6 months. Four patients presented the Jarisch-Herxheimer reaction at the beginning of treatment. No deviation in haematological and biochemical blood tests had been observed. Two other patients had mild gastrointestinal troubles.

Conclusion. Based on the results of this study it may be concluded that oral azithromycin is effective in the treatment of early syphilis. In cases of hypersensitivity to penicillin, azithromycin can be recommended in the therapy of syphilis.

EPIDEMIOLOGY AND CLINICAL ASPECTS OF GONORRHOEA

A. STARY, B. GÖTZ, L. TEODOROWICZ, M. KERSCHBAUMER, E. MESSERITSCH, A. BILINA

Diagnosis of *Neisseria gonorrhoeae* in men and women by using amplification methods

Outpatients Centre for Infectious Venero-Dermatological Diseases, Vienna, Austria

Objective. Aim of the study was to evaluate the performance of the COBAS Amplicor and the LCX assay for the detection of *Neisseria gonorrhoeae* in genital samples of both men and women. In addition, the outcome of testing genital samples was compared with that of first void urine (FVU) in men and vulval swabs in women with gonococcal cultures of the endocervical canal, vulval region, and urethra.

Methods. Urethral and urine samples were collected from 280 women attending the outpatients centre in Vienna because of a suspected genital tract infection, partner control, contact tracing, and health check up were also performed. All samples were tested by the ligase chain reaction (LCR) and by polymerase chain reaction (COBAS Amplicor). Culture of urethral, vulval, and cervical samples was performed on selective (Thayer-Martin) and non-selective (New-York) agar. For discrepant analysis the 16S RNA-amplification was included in the evaluation.

Results. Of the 27 (11.6%) infected men, all were detected in both sample types by LCR and 26 and 24 were detected by the COBAS Amplicor, resulting in a sensitivity of 100% for LCR and 96.3% and 88.9% for urethral and urine specimens for PCR. In women, the sensitivity of LCR was higher for all sample types (FVU 100%, vulva 95.2%, cervix 90.5%) when compared with PCR (FVU 71.4%, vulva 76.2%, cervix 85.7%). Culture detected all infected men and 20 of the 21 (95.2%) infected women.

Conclusions. Gonococcal culture can still be recommended as the gold standard in case of sufficient transport and culture conditions. The performance characteristics of LCR were superior to COBAS Amplicor and positive PCR results have to be confirmed by 16S-RNA amplification. In addition to FVU, vulval smears may substitute invasive sampling for gonococcal diagnosis.

A. SZÉLL

Determination of the genital and extragenital gonorrhoea infections by diagnostic studies

National Institute of Dermato-Venereology, Budapest, Hungary

The most important microbiologic, diagnostic studies are presented in the following lecture. The author reports the modern diagnostic methods and results, which were acquired in the present Hungarian practice and points out the faults of microscopic diagnosis.

The nucleic acid amplification method used for the determination of gonorrhoea infection in genital and extragenital tracts will be presented.

These experiences which are acquired in the foreign and the sexually transmitted diseases center in the Department Dermato-Venerology of Semmelweis University of Medicine will be reported, too.

200 LCR gonorrhoea studies (from the genital and extragenital samples) were done.

Three groups were involved in these studies:

1. Patients and their partners who are already infected with gonorrhoea
2. Homosexual and HIV positive group
3. A group showing up in the STD center for other reasons

T. TISZA[1], V. VÁRKONYI[2], A. SZÉLL[2], A. HORVÁTH[2]

Comparison of single dose azithromycin and penicillin/spectinomycin for the treatment of non-complicated acute genital gonococcal infection

[1]University Clinic of Dermatology and Venereology, Semmelweis Medical University, Budapest and [2]National Institute for Dermato-Venereology, Budapest, Hungary

Objective. To compare the efficacy of 1 g single dose p.o. Azithromycin and procaine penicillin G 3 (in females 2×3) million units IM + 1g probenecid p.o. or spectinomycin 2 (in females 4) g IM for the treatment of non-complicated acute genital gonococcal infection.

Methods. 143 patients (mean age 29.15±8.76 (17–66) years) entered the open, randomized study, 120 were evaluable. Platinum loop specimens for Gram stain, culture and antimicrobial susceptibility testing were collected from the urethra of men presenting with signs and symptoms of acute gonorrhoea and from the cervix and/or

urethra of their female sexual partners. Men were enrolled based on the positive Gram stained smear, while women based on positive culture result after giving informed consent to the study.

Evaluable patients were randomized in the following treatment groups:

A/1. single dose procaine penicillin 3 million units IM + 1 g probenecid p.o., for women repeated one day later (31 patients)

A/2. single dose spectinomycin 2 g for men, 4 g for women IM (29 patients)

B. single dose azithromycin 1 g p.o. (60 patients)

Tests-of-cure were performed in men 24 hours and 7 days, in women 48 hours and 7 days after treatment with smear, culture and DNA hybridization.

Results. The efficacy rate for procaine penicillin treatment was 80% (25/31), for spectinomycin 100% (29/29), for azithromycin 98% (59/60). The difference between the efficacy of procaine penicillin and azithromycin therapy is statistically significant.

Conclusions. Single dose 1 g azithromycin treatment might be a reasonable therapeutic alternative in acute non-complicated genital gonococcal infection.

CHANGES IN SEXUAL BEHAVIOUR AND ITS IMPACT ON THE EPIDEMIOLOGY OF STD'S

B. BUDA

Changing trends in homosexual and heterosexual promiscuity: recent surveys and experiences

Institute of Behavioural Sciences, Semmelweis University of Medicine, Budapest, Hungary

In several countries surveys have been conducted about sexual behaviour and these surveys show significant changes in comparison to the patterns of sexual behaviour found in the 70s and 80s. Especially changes in the prevalence and behavioral manifestations of homosexuality are striking, but also promiscuity has diminished in both sexual orientation. These findings are obviously connected with the AIDS epidemic and with the effort to promote safe sex practices. Thus the findings show perspectives in sex education and health promotion. At the same time, however, they have an impact also on the theory of sexual orientations. In the literature there is an ongoing discussion between the biological (essentialist, materialist) and

constructivist explanations, and the recent findings (among them the data reflecting a high prevalence of bisexuality among homosexuals who claim themselves exclusively homosexuals) corroborate the constructivist side.

J. PÁNOVICS[1], V. VÁRKONYI[4], A. SZÉLL[4], T. TISZA[2], I. SZILLER[3]

Rate and significance of partner infections at patients in chronic prostatovesiculitis

[1]Department of Urology, [2]Department of Dermato-Venereology, [3]Department of Gynecology, Semmelweis University of Medicine, Budapest and [4]National Institute of Dermato-Venereology, Budapest, Hungary

Objectives. Between 01.06.96 and 30.05.97 the authors carried out examinations in partners of patients suffering from chronic lower urinary tract infections.

Methods. In this period 96 patient's sexual partners were examined. The bacteriologic laboratory findings of patients with prostatovesiculitis were compared with those of their sexual partners.

Results. By examining prostatic expression, sperms and urine after prostatic massage of 96 patients with chronic prostatovesiculitis 122 pathogens were diagnosed. In 50 sexual partner cases (52%) 62 pathogens were identified.

Conclusion. Examination of sexual partners of patients with chronic prostatovesiculitis is highly suggested at the phase of setting up the diagnose when the complaints occur. After recovery the patient's partner control is essential to prevent fast relapse.

THE IMPACT OF STD'S ON REPRODUCTIVE CAPACITY AND CARCINOGENESIS. VERTICAL TRANSMISSION

F. PAULIN

STD infections and human reproduction

II. Department of Obstetrics and Gynecology, Semmelweis University School of Medicine, Budapest, Hungary

In Hungary the number of deliveries continuously decreased in the last decade. The infertility and the relative high incidence of prematurity have a great importance.

Acute pelvic inflammatory disease (PID) affects women in their reproductive years and is often a complication of a sexually transmitted disease (STD), particularly *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Infertility, ectopic pregnancy, and chronic lower abdominal pain are common long-term sequelae to acute PID.

Mycoplasma hominis and *Ureaplasma urealyticum* can be isolated with considerable frequency from the human urogenital tract and can cause pyelonephritis and prematurity in addition to the above-mentioned diseases. Genital *Chlamydia trachomatis* (CT) infections are the most common cause of tubal infertility.

Teenagers are at higher risk for sexually transmitted diseases (STDs) than any other age group, for a variety of reasons.

To overcome these problems the gynecologists and obstetricians should have appropriate knowledge, modern diagnostic possibilities and a complex strategy for the screening and the early and effective therapy. We need more teenager, STD and infertility clinics and high level pregnancy care. For early diagnosis and effective care of tubal infertility the modern endoscopical procedures should be applied.

To screen the whole population is impossible. Not all STDs are readily cured by antibiotics, and because many adolescents are asymptomatic, many STDs initially go undetected and untreated. There is a need for innovative psychosocial and behavioral prevention programs to be developed and implemented to reach all adolescents.

P. BŐSZE

Genetics of cervical intraepithelial neoplasia – clinical implications

Clinic of Cancer Genetics and Prevention and Research Center, Budapest, Hungary

Cervical carcinoma like other cancers is a multistep, multigenetic disease apparently initiated by human papillomaviruses (HPVs) in most if not all instances. Infection with certain HPV types is intimately linked with the development of cervical intraepithelial neoplasia (CIN) and invasive cervical cancer. HPV-DNA can be detected over 90% of CIN with PCR (polymerase chain reaction)-based technology and in approximately 70% of invasive cervical carcinomas. More than 70 types of HPV have been identified, and only some of them have oncogenic potential. The most common oncogenic HPVs include HPV-16, HPV-18 and HPV-33. Women with normal cervices infected by oncogenic HPV have an increased risk of developing CIN.

HPVs infect epidermal cell, get into the basal layer at the early stage of infection and replicate and viral capsids are produced in maturing epithelium. Only epidermal cells are permissive for viral replication. Within the cell the virus directly binds to the mitotic spindle (episomal) resulting in polyploidy of the infected cell (productive viral infection). Then the HPV-DNA integrates into the chromosomes of the epithelial cells, i.e. into the host genome and induces other mechanisms such as activation of host genes (ras) etc., that results, through events mainly related to E1 and E2 open reading frames, in production of oncoproteins and aneuploidy. Experimental data suggest that the E6 and E7 oncoproteins encoded by the integrated HPV-16 or HPV-18 are capable to immortalize cells in cell cultures. These cells, however, are not tumorigenic unless ras oncogene is introduced.

CIN is a histologic description of cell phenotypes with not much indication to the biologic behaviour of the lesion. DNA measurement may be the key in understanding the biology of CIN. Consequently, there is no point in comparing DNA pattern with the grade of CIN but with the outcome of the lesion. Indeed, Fu et al. demonstrated that all CIN that progressed to invasive carcinoma had an aneuploid DNA pattern. It appears that all CIN, irrespective of histologic grade, that show aneuploidy are premalignant lesions.

A. NEUER[1], S. DIETERLE[1], P. GIRALDO[2,3], S. SPANDORFER[2], S. S. WITKIN[2]

Immunology of heat shock proteins and genital tract infections – consequences for embryo development

[1]Division of Reproductive Endocrinology and Infertility, University of Witten/Herdecke, Dortmund, Germany, [2]Division of Immunology and Infectious Diseases, Cornell University Medical College, New York, USA, [3]Department of Gynecology and Obstetrics, University of Campinas, Sao Paulo, Brazil

Heat shock proteins are highly conserved proteins present in organisms ranging from bacteria to men. They are both dominant microbial immunogens and among the first proteins produced during mammalian embryo development. Since bacterial and human heat shock proteins share a high degree of amino acid sequence homology, it has been suggested that sensitization to bacterial heat shock proteins during an infection may result in autoimmunity to human heat shock proteins.

Tubal factor infertility is very frequent among couples seeking in vitro fertilization (IVF) and thus it is possible that these patients have been previously sensitized to bacterial heat shock proteins as a consequence of an asymptomatic upper genital tract infection. Due to strict clinical monitoring and precisely timed fertilization these patients represent an ideal study group to investigate the effect of prior sensitization to heat shock proteins on pre-implantation embryo development and implantation failure. Immune sensitization at the level of the cervix to the 60kD heat shock protein (hsp60) has been shown to be associated with implantation failure in some IVF patients. Similarly, the highest prevalence of circulating hsp60 antibodies among IVF patients can be found in sera of women whose embryos failed to develop in vitro. To more directly assess whether humoral immunity to hsp60 influence in vitro embryo development we established a mouse embryo culture model. Monoclonal antibody to mammalian hps60 markedly impaired mouse embryo development in vitro. The presented data suggest that immune sensitization to human hsp60, possibly developed as a consequence of infection, may adversely affect pregnancy outcome in some patients.

G. GROSS

Do we need antiviral agents for genital herpes simplex virus and human papillomavirus infections?

Department of Dermatology and Venereology, University of Rostock, Germany

Herpes virus specific antiviral drugs have turned out to be of extreme value for the treatment of Herpes simplex virus (HSV) strain 1 and strain 2 infections both of genital and extragenital sites. This is especially true for immunosuppressed patients as well as for neonates acquiring HSV from the mother during birth. Antiviral therapy for genital human papillomavirus (HPV) infection would be highly appreciated since none of the available therapies effectively clears genital warts without recurrence. Furthermore a HPV specific antiviral may have the capacity to eradicate active HPV infections, to prevent long-term sequelae and possibly also to interfere with the development of HPV associated malignant tumors. So far only interferons and cytokine inductors like imiquimod, which have certain antiviral activities, are available for therapy. Recently some progress has been made towards antiviral HPV therapy by studies on cidofovir, an antiviral with activities against a broad spectrum of herpesviruses and against HPV.

I. SZILLER[1], S. S. WITKIN[2], K. SEMBERY[1], Z. BARDÓCZY[1], Z. PAPP[1]

Chlamydia trachomatis heat shock protein antibodies and ectopic pregnancy

[1]Department of Obstetrics and Gynecology, Semmelweis University Medical School, Budapest, Hungary, [2]Department of Obstetrics and Gynecology, The New York Hospital-Cornell Medical Center, New York, USA

Clinical and histopathological correlations of immunoreactivity to *Chlamydia trachomatis* and to epitopes of the *C. trachomatis* 60 kDa heat shock protein (hsp60) among women with ectopic pregnancy were evaluated in a case-control study. Serological responses to 13 synthetic peptides corresponding to major epitopes of the chlamydial hsp60 were determined in 69 women treated for ectopic pregnancy and 69 women with uncomplicated pregnancy in utero. Plasma cell salpingitis was detected in 29 (43%) of the ectopic patients. Its presence correlated with antibodies to two hsp60 epitopes, encompassing amino acids 260–271 and 411–422 ($p=0.02$). Antibodies to these two epitopes, along with five other epitopes, also correlated with peritubal

adhesion formation in ectopic pregnant patients (p=0.01). Antibodies to epitopes 260–271 and 188–199 also correlated with a history of pelvic inflammatory disease (p=0.05). Patients with ectopic pregnancy were also more likely to have anti-chlamydial immunoglobulin G (p=0.005) than their intrauterine pregnant controls. Women positive for both *C. trachomatis* and hsp60 epitope antibodies had an increased prevalence of salpingitis, pelvic adhesions or history of PID (p=0.05). In contrast, patients who were positive for only *C. trachomatis* antibodies or only hsp60 epitope antibodies did not differ from antibody-negative patients in each of these categories. Immunity to hsp60, therefore, might subvert homeostatic mechanisms into pro-inflammatory pathological events with adverse clinical consequences.

L. TÖRÖK[1], I. SZŐKE[2], E. DÓSA[2], J. DEÁK[2], S. SCULTÉTY[1]

The prevalence of STD pathogens in male patients with infertility

[1]A. Szent-Györgyi Medical University, [2]Department of Clinical Microbiology, Szeged, Hungary

The STD pathogens may affect male reproductive function in different ways. Possible consequences are impairment of spermatogenesis, induction of auto-immune mechanism, spermatodysfunction, and inflammatory occlusion of the ejaculatory duct. In high concentrations, bacteria and mycoplasmas may decrease the motility of spermatozoa. From January 1997 till July 1998 semen samples of 87 male patients with infertility were tested by microbiological methods (aerobic and anaerobic bacteria, yeasts, mycoplasmas, ureaplasmas, *Chlamydia trachomatis* and *Trichomonas vaginalis*). The following microorganisms were found in the samples of the patients: Candida spp. 4%, *Mycoplasma hominis* 3%, *Ureaplasma urealyticum* 9%, *Chlamydia trachomatis* 1%, *Neisseria gonorrhoeae* and *Trichomonas vaginalis* were not found. Aerobic bacteria were found more frequently (40%) and anaerobic bacterial growth of $\geq 10^6$ CFU/ml was seen only in 28%. Our results showed that among the examined cases the STD pathogens occurred only in low percentage, while aerobic and anaerobic bacteria were isolated much more frequently.

J. MARIN[1], L. BREGANT[2]

Epstein-Barr virus infection in pregnancy – its possible relationship with perinatal misfortunes

[1]Institute of Microbiology and Immunology, Medical Faculty of Ljubljana,
[2]Department of Gynaecology, Clinical Center, Ljubljana

Epstein-Barr virus (EBV) persists after primoinfection in the human host. Over 90% of adult population carries EBV, which is mainly acquired during childhood or adolescence. The clinical picture usually is infectious mononucleosis. Complications are rare but manifold. Susceptibility to EBV infection in pregnant women could be evaluated by screening for EBV antibodies. Normally very small percentage of pregnant women has no detectable EBV antibodies and very rarely they acquire EBV during pregnancy. It is assumed that primary EBV infection during pregnancy is rare. But in normal pregnant women reactivation of EBV frequently occurs. Cellular immune response is apparently suppressed, but high titer of EBV specific antibodies may compensate the reactivated virus. Yet a significant number of cases of deaths and of perinatal misfortunes are still of unknown origin. As has been shown by epidemiological and virological tests EBV seems to become one of the possible agent accounting for some very serious multiple abnormalities and infant's death.

The authors describe the case of possible congenital EBV infection occurring in in term born girl with repeating hemolytic anemia, thrombocytopenia and with a fatal issue. EBV infection was proved by laboratory tests. As the presence of EBV DNA was demonstrated in cervix of 33% of women attending the STD clinic it seems that STD family has grown wider and new members like EBV will probably join.

J. SÖLTZ-SZÖTS, C. HELLER-VITOUCH, R. MALY

An evaluation after five years of routinely HPV-testing

L. Boltzmann-Institute for the Study of Dermato-venerological Infectious Diseases, Vienna, Austria

Objective. Up to now, approximately 100 types of Human Papilloma Viruses (HPV) are known and there is overwhelming evidence that certain types play a major role in the origin of neoplasias in the anogenital tract.

Methods. Since 1994 male and female patients attending our STD outpatients clinic presenting perigenital warts and pathological Papanicolaou (PAP) smears are

routinely investigated. We take smears from the surface of the condylomata and from the cervix in order to detect HPV by using the Digene Hybrid Capture System. High risk HPV types (16, 18, 31, 33, 35, 45, 51, 52, 56) and those of low risk (6, 11, 42, 43, 44) are differentiated.

Results. Between 1994 and 1998 1174 patients (928 females and 246 males) were investigated. HPV could be detected in 601 cases, i.e. 51.19%. In the whole group we found 403 HPV low risk (67.05%) and 373 HPV high risk infections (62.06%) In 145 (24.12%) patients both virus types could be detected. Out of 246 male patients 124 cases were HPV positive (50.41%), only 16.12% of them showing high risk positive results. 85.94% (214) of the 532 female patients presenting condylomata acuminata were positive for HPV low risk. But as 36% of them were coinfected with HPV high risk it results in a total of 113 HPV high risk infections (45.38%). 346 female patients with PAP IIID and IV were in 54.91% HPV positive (190), 98.42% of them being infected with HPV high risk.

Conclusions. Our investigations confirm that there is a connection between genital neoplasia and the infection with HPV high risk types. The high percentage of coinfection of condylomata acuminata with HPV low risk and high risk underlines the demand for a thorough screening of these cases. As the presence of HPV high risk means a high probability for the development of cervical neoplasia, even negative PAP smears should be repeated at shorter intervals.

L. GERGELY

Human papilloma viruses and cervical cancer

Debrecen, Hungary

Carcinoma of the uterine cervix is one of the most prevalent malignant disease of women. Oncogenic human papilloma viruses (HPV type-16, -18, -31, -33, -35, -45, -58) are thought to be the most important risk factors in the development of cervical cancer. The genome of papillomaviruses contains the following regions: early (E) regions coding viral regulatory proteins and oncoproteins, late (L) open reading frames (ORFs) coding structural proteins and a noncoding long-control region (LCR). The products of the E6 and E7 ORFs functionally inactivate two important cellular tumour suppressors, the p53 and the pRB proteins, causing transformation and immortalization.

The long-control region and the E2 protein of HPV are the most important viral factors regulating the transcription of the viral oncogenes E6 and E7. Sequence

variations within these genomic regions may have an impact on the oncogenic potential of the virus.

The most frequent HPV type in cervical neoplasias is HPV16. HPV DNA is present in an episomal form in asymptomatic infections and benign lesions, while it is frequently integrated into the host cell genome in malignant lesions. The integrated state and higher HPV16 positivity of pelvic lymph nodes could indicate higher frequency of disease recurrence.

C. HELLER-VITOUCH, R. MALY, J. SÖLTZ-SZÖTS

Perigenital condylomata acuminata as a marker for cervical intraepithelial neoplasia

L. Boltzmann-Institute for the Study of Dermato-Venerological Infectious Diseases, Vienna, Austria

Objective. Aim of the presented study was to demonstrate whether perigenital condylomata acuminata in female patients are a marker for simultaneously existing cervical intraepithelial neoplasia.

Methods. Female patients attending our STD outpatients clinic presenting perigenital warts were investigated. We took smears from the surface of the condylomata and from the cervix in order to detect Human Papilloma Virus (HPV) by using the Digene Hybrid Capture System. High risk HPV types (-16, -18, -31, -33, -35, -45, -51, -52, -56) and those of low risk (6, 11, 42, 43, 44) were differentiated. Furthermore, Papanicolaou (PAP) smears were taken. In case of pathological PAP results patients were sent to the gynecological department.

Results. 37 patients were recruited for the study. HPV could be detected in 32 of the condylomata smears (86.5%). In 33 cases (89.2%) HPV was found in cervical epithelia. If perigenital warts were positive for HPV, cervix was also positive in 90.6%. Four patients showed positive results on cervix only. High risk HPV types were detected on cervix of 19 patients (51.3%), five of them with pathological PAP smears (PAP IIID and E). One more patient with PAP IIID was high risk HPV positive perigenitally only.

Conclusions. In 16.2% of our patients suffering from condylomata acuminata we observed pathological PAP smears. HPV infection of the cervix could be detected in 89.2%. In each case of perigenital warts patients should be investigated thoroughly and in short intervals with regard to an early diagnosis of intraepithelial inflammation or neoplasia.

M. SKERLEV[1], J. LIPOZENČIĆ[1], M. GRCE[2], A. BASTA-JUZBAŠIĆ[1], K. PAVELIĆ[2]

Human papillomavirus DNA types in men with condylomata acuminata

[1]Department of Dermatology and Venereology of University Hospital Šalata, Medical School, Zagreb University, [2]Institute "Rudjer Boškovic", Zagreb, Croatia

Objectives. Human papillomavirus (HPV) genital infections are one of the most frequent diagnosis in the STD Outpatient Clinic of the Department of Dermatology an Venereology of the Zagreb University Medical School. The association between certain HPV and cervical intraepithelial neoplasia is well documented among Croatian women. However, similar studies among male patients have not been performed so frequently. Thus, the aim of this study is to determine the frequency of certain HPV DNA types in men with clinically visible lesions consistent with HPV genital infection (condylomata acuminata).

Methods. Ninety male patients aged 3 to 68 years attending the STD Clinic of the Department from 1995 to 1997 were included. HPV DNA detection and typing of biopsy specimens taken from clinically visible lesions of the skin and mucous membranes on the anogenital location were performed using polymerase chain reaction (PCR). Type-specific primers for low-risk HPV 6/11 and high risk HPV 16, 18, 31 and 33 were used either in the single (HPV 18) or multiplex PCR.

Results. HPV DNA types 6/11 were found in 72 out of 90 patients. However, high-risk HPV DNA was found in 18 (20%) patients (HPV type 16 in 10 patients, HPV type 18 in 6 patients and HPV 31 and 33 in one patient). High-risk HPV DNA types were significantly more often located on the distal part of the skin and mucous membranes of the external genitalia.

Conclusions. HPV DNA types are not always absolutely consistent with clinical type of HPV genital lesion, i.e. HPV DNA 16 and 18 can also be isolated from condylomata acuminata (and not only plana). Thus, we consider HPV detecting and typing of male genital HPV lesions as an important part of the diagnostic procedure, treatment and follow-up.

P. SENJI, L. ŽELE-STARČEVIĆ, S. KALENIĆ, J. LIPOZENČIĆ, M. SKERLEV

Impact of specimen quality on isolation of *Ureaplasma urealyticum* from the endocervix

Clinical Hospital Center Zagreb, Croatia

Ureaplasma urealyticum is a sexually transmitted, opportunistic bacterium causing different diseases as cervicitis, urethritis, orchiepididymitis, pelvic inflammatory disease, postpartal and postabortal febrility, arthritis in immunocompromised patients, urinary calculosis, male infertility or repeated abortion. It is generally accepted that in case of infection the number of cfu of *Ureaplasma urealyticum* in specimen must be $\geq 10^4$; lower numbers are present in colonisation. To distinguish between infection and colonisation, quantification is mandatory. However, we presumed that the specimen quality, measured by the number of endocervical cells on the agar surface may have an important influence on the detected number of cfu. Therefore, we compared the detected number of *Ureaplasma urealyticum* expressed in cfu with the semiquantitatively determined number of endocervical cells in 1576 specimens. According to our results; there is a direct influence of specimen quality on the quantitative determination of *Ureaplasma urealyticum* in endocervical specimens.

MISCELLANEOUS

P. K. KOHL

Current issues of sexually transmitted infections

Department of Dermatology and Venereology, Neukölln Academic Hospital, Berlin, Germany

Sexually transmitted infections (STI) (sexually transmitted diseases including HIV infection) represent one of the most complex problems in modern medicine. The global problem STI is influenced by a number of factors: different infecting agents with different host relationships and susceptibility to therapy, age group, social status, sexual orientation and sexual behaviour of infected persons in different time frames, absence or presence of national prevention programs and therapy guidelines, different medical specialities dealing with STIs and last but not least reliability of registry data.

Pregnant women, sex workers, recruits, STD clinic attenders and prisoners are often studied populations. Only recently screening data of general populations became available. STIs may be subdivided into curable and non-curable STI. Curable STI are of bacterial (*N. gonorrhoeae*, *C. trachomatis*, *T. pallidum*, *T. vaginalis*) while non-curable STI are of viral origin (HIV, HSV, HPV, Hepatitis).

N. gonorrhoeae and *C. trachomatis* can now easily screened by modern amplification techniques even by self-obtained swabs and home obtained urine specimens. 11.5% of female and 27.2% of male STD clinic attenders in Baltimore/USA were positive for *N. gonorrhoeae* and 10.8% and 13.8% respectively for *C. trachomatis* by COBAS Amplicor system. 1.5% of sexually active female adolescentes were positive for *C. trachomatis* in Antwerp/Belgium in a population based study. In contrast in adolescentes in the USA the prevalence of *C. trachomatis* was 13.3% among non-hispanic blacks, 10.7% among mexican americans, and 5% among non-hispanic whites. For syphilis the incidence rate is 1.4/100,000 in Germany, although 85% of infections are not reported. In the Russian Federation, Ukraine, Belorussia and Moldavia the incidence of syphilis has risen since 1992 to and unprecedented value of 150–100 cases/100.000 population. 3% of HIV patients have syphilis in Seville/Spain. Commonly North America, Europe and Australia are regarded as "developed" countries, but in certain regions and subgroupes of the population on these continents the situation may not differ from "developing" countries.

I. GYÖRGY, J. GY. MEZEY, E. FODOR

Occurrence of *Streptococcus agalactiae* in our clinical cultures

Buda Children's Hospital and Polyclinic, Budapest, Hungary

Streptococcus agalactiae (Lancefield Group B Streptococcus, Str. B) is an opportunistic pathogen, which may cause serious infections in newborn babies and in any person with a compromised immune system at any age as well. It can also be isolated from the throat, vagina, urethra, rectum and faeces of primarily healthy subjects.

As data in literature reveal, its prevalence among pregnant women in different parts of the world ranges from 5 to 25%. During birth about 50% of the newborn babies of infected mothers become colonized. Transmission is also possible during sexual intercourse.

Objective. To determine the occurrence of Str. B in our patients. In the period between 1st January 1996 and 31st December 1997 10,594 various clinical cultures

were examined in our microbiological laboratory. For their analysis the Lányi method was used, and for the detection of Str. B the CAMP test and the Lancefield precipitin test. The antibiotic susceptibility was determined by disc diffusion method.

The occurrence in genital swabs was 9.3% (120/1293), in urine 0.7% (15/2107), in throat 0.8% (30/3967), in nose 1.2% (2/171), and in paranasal sinuses 22% (5/23).

In vitro antibiotic resistance: about 3% of the strains proved to be resistant to β-lactams and macrolid antibiotics, and 5% to clindamycin.

L. TÖRÖK

Differential diagnosis of genital erosions and ulcers

County Hospital, Kecskemét, Hungary

The wide expansion of HIV infection and occurrence of many new cases of syphilis in Hungary increases the importance of differential diagnosis of genital ulcers and erosions. The author presents the most important types of genital erosions and ulcers (both of STD and non-STD origin) and provides guidelines for their differentiation.

F. ROZGONYI, ZS. CSUKÁS, ZS. BEREK, CS. JENEY, K. KAMOTSAY, K. GLATZ

Antibiotic susceptibility of most frequent Gram-positive bacteria with special reference to topically usable drugs

Institute of Microbiology, Semmelweis University of Medicine, Budapest, Hungary

Sensitivity of 920 *Staphylococcus aureus*, 375 *Staphylococcus epidermidis*, 100 *Staphylococcus haemolyticus*, 50 *Staphylococcus hominis*, 60 serogroup-D *Streptococcus*, 30 *Streptococcus agalactiae* and 26 *Streptococcus pyogenes* strains was tested to a variety of antimicrobial agents with the disk diffusion method on Mueller-Hinton agar supplemented with bovine blood in cases of streptococci.

Staphylococci were sensitive to penicillin (9–18%), oxacillin (67–77%), erythromycin (25–56%), clindamycin (38–65%), mupirocine (76–82%), sulfonamide (3–20%), tetracycline (42–53%), chloramphenicol (83–87%), tobramycin (43–69%) and gentamicin (49–70%).

S. aureus was the most sensitive, while *S. hominis* was the most resistant species. All of the 100 *Staphylococcus* strains tested with the microdilution method proved to be sensitive to ebrimycine.

Strains of serogroup-A, -B and -D *Streptococcus* were sensitive to penicillin (100, 84%, and 24% respectively), erythromycin (57%, 93%, 43%), clindamycin (77%, 85%, 33%), mupirocine (100%, 60%, 43%), sulphonamide (67%, 12%, 9%), tetracycline (15%, 19%, 28%) and chloramphenicol (75%, 83%, 76%). All of the 50 streptococcal strains tested with the microdilution method proved to be sensitive to ebrimycine.

In conclusion, an adequate antimicrobial treatment of any Gram-positive superficial infection requires an antibiotic sensitivity test regardless of the causative agent or drug to be used because a bacterial isolate sensitive to all antimicrobials in question seems to be extremely rare.

STATE-OF-ART LECTURES

I. STRAUB

Epidemiological aspects of viral hepatitis

National Center for Epidemiology "B. Johan", Budapest, Hungary

The author gives a general outline about the epidemiological characteristics of different viral hepatitis and summarizes the global and national epidemiological situation.

Currently the epidemiological situation of different viral hepatitis is favourable in Hungary. There is a significant HAV dominance and the level of HAV incidence is very close to the European average. On the basis of HBV prevalence rate Hungary belongs to the territories of low endemicity. A national complex prevention program of viral hepatitis was worked out and introduced at the beginning of the 1990s.

Recent changes in political/social situation have resulted in new public health problems in Hungary. The pauperization especially contributes to increase the enteric infectious diseases (HAV), but in the meantime the poverty, deterioration in hygienic standards, failures in the hospital hygiene, increasing tendency in risk behaviours (i.v. drug abusing, prostitution, etc.) contribute to spreading of HBV/HCV infections, too.

D. PETZOLDT

Sexually transmitted diseases in the new German law for the protection against infective diseases

University Clinic for Dermatology, Heidelberg, Germany

A special law on sexually transmitted diseases is the legal basis for measures attempting to control the most important sexually transmitted disease in Germany. This law was passed in 1953 and therefore is outdated in various respects today.

According to the legislator, in the future there will be no new special law concerning sexually transmitted diseases. Instead, measures on sexually transmitted diseases will be included in a law controlling all important infectious diseases. A bill has been drafted and has been viewed and commented by public institutions and interest groups. This law most likely will be passed in 1999.

The epidemiological control of STDs will be managed by three means:

1. The Robert-Koch-Institute, Berlin, with its central role in the reporting system of infectious diseases, will carry out sentinel investigations if required. These investigations are performed in cooperation with selected institutions involved in the field of health care and disease control. Such measures are taken for those transmissible diseases, which are suspected to pose a major threat to public health and when individual case reports are no longer feasible.

2. A permanent reporting system will be installed. *Treponema pallidum* or HI-virus detected by microbiological resp. biological or serological methods are to be reported. The report has to be anonymous and may only include gender, month and year of birth, the first three figures of the postal code of residence, the probable route of infection and the country, where the infection occurred.

Under the new law cases of gonorrhoea, ulcus molle or lymphogranulomatosis inguinalis are no longer reportable nor is there an obligation for contact tracing or name registration of persons who evade treatment.

3. Institutions of public health (Gesundheitsämter) will provide medical advice, examination and treatment for STD patients. Treatment is free of charge in case the patient is considered at high risk of transmitting the disease and when the patient is unable to personally cover the cost of treatment.

The pros and cons concerning the new legal regulations are discussed. Efforts made by the German Dermatological Society and the German STD-Society in structuring the legal text are reviewed.

POSTERS

A. STARY, B. GÖTZ, E. SCHUH, M. KERSCHBAUMER

Chlamydial diagnosis in women using vulval samples: a comparison of different amplification methods and sample types

Outpatients Centre for Infectious Venero-Dermatological Diseases, Vienna, Austria

Objective. In addition to cervical samples, urine has shown to be an alternative sample type for chlamydia diagnosis in women. To evaluate whether vulval smears may serve as an alternative non-invasive specimen for chlamydial diagnosis, the performance of all commercially available amplification tests (LCX Chlamydia trachomatis assay, COBAS Amplicor, Gen-Probe Transcription-mediated Amplification) with vulval specimens as well as with urine, and cervical samples was compared with culture on endocervical and vulval samples.

Methods. The study was performed in 875 female patients attending the Outpatients' Centre mainly because of a suspected genital tract infection, partner control, contact tracing, and health check up. Swabs from the vulval region were obtained in addition to first void urine (FVU) and samples from the endocervix. All specimens were tested by at least two amplification methods and compared with culture on vulval and cervical samples. For discrepant analysis, amplification of the MOMP-gene and DFA was included in the evaluation.

Results. The sensitivity (Table) and specificity for all sampling sites and amplification methods was high, calculated on infected women as the gold standard established by positive culture or 2 different test procedures.

Test method	Vulva	FVU	Cervix
LCR (77/875)	89.6% (69)	90.9% (70)	92.2% (71)
TMA (25/308)	92.0% (23)	76.0% (19)	88.0% (22)
PCR (25/398)	88.0% (22)	80.0% (21)	88.0% (22)
Culture (77/875)	14.3% (11)	n.d.	59.7% (46)

Conclusion. The results demonstrate that all amplification methods performed with a high sensitivity with vulval smears (89.8%), comparable with urine (86.6%) and

cervical specimens (90.6%). The data indicate that vulval smears can be used as alternative noninvasive specimens useful for chlamydial screening programs.

A. SKAZA MALIGOJ

A research project for assessment of cost-effectiveness screening for *Chlamydia trachomatis* in asymptomatic adolescent males in the Celje region, Slovenia

Institute of Public Health Celje, Slovenia

Objectives. A small number of information is available about the prevalence of *Chlamydia trachomatis* in male population in Slovenia. Therefore the objectives for our research work are to determine the prevalence of chlamydial urethritis and to access the cost-effectiveness of screening for *Chlamydia trachomatis* in sexually active adolescent males in the Celje Region.

Methods. Military recruits attending their routine examinations, before entering military service were examined. The first-void urine collected during routine examinations has been suggested as a sampling technique. Chlamydial antigens were detected in urine samples, using PCR. Only sexually active recruits were included to avoid systematic error. Supposing we want to determine the prevalence of chlamydial urethritis with 1% absolute accuracy and with 95% confidential interval, the hypothesis being the 4% prevalence (based on some previous research in the region), the screening should include 1277 sexually active male adolescents. According to the recorded prevalence of chlamydial urethritis in our cohort a cost effectiveness model was made comparing the direct costs of early diagnosed and adequately treated infection with the costs of the advanced infection and its consequences especially for a female partner. The direct costs were based on the following calculations and estimations: the cost of collecting the urine sample, the cost of laboratory test and the cost of treatment of the infected male and his female partner. The indirect costs include the costs arising from the chlamydial urethritis in a male and their female partner, in case the infection is not adequately treated and it thus results in an advanced infection in women. It has been estimated that the infection is transmitted to female partners during sexual intercourse in 60% of cases. Based on the available data it has been estimated that *Chlamydia trachomatis* causes cervicitis and pelvic inflammation in 20-30% of women. Neonatal infection with *Chlamydia trachomatis* in infants is caused during the passage through birth canal of an infected mother in approximately 2/3 of infants. 50–70% of infants

born by infected mothers develop chlamydial conjunctivitis and 10–20% of these infants develop chlamydial pneumonia.

The data were statistically analysed with the *t*-test and the test of logistic regression.

G. SIMON, A. SZÉLL, V. VÁRKONYI, T. TISZA, A. HORVÁTH

Comparison of E test^R and Fungitest^R in susceptibility testing of yeast strains isolated from STD patients

National Institute for Dermato-Venereology, Budapest, Hungary

Fungitest^R (Sanofi Diagnostics Pasteur, France) is one of the novel antifungal susceptibility tests. The broth dilution method is based on NCCLS proposed standards. It is useful for the testing of amphotericin B (AMB), flucytosine (5FC), miconazole, ketoconazole (KET), itraconazole (ITR) and fluconazole (FLU). The concentration of all antimycotics correlates with the accepted breakpoints and yeast strains can be interpreted as susceptible, intermediate or resistant. E test^R (AB Biodisk, Sweden) is an agar diffusion method for testing of AMB, 5FC, KET, ITR and FLU. E test strips contain continuous concentration gradient of antimycotics and allow to determine the minimal inhibitory concentration (MIC).

Antifungal susceptibilities of 88 yeast strains isolated from homogenous population of patients of STD Outpatients Clinic of National Institute for Dermato-Venereology, Budapest, were evaluated by Fungitest^R and E test^R. Only KET, ITR and FLU were tested because other drugs could not be considered in the therapy of vulvovaginal candidiosis. The number of tested strains were: *Candida (C.) albicans* 45, *C. glabrata* 15, *C. kefyr* 9, *C. krusei* 8, *C. parapsilosis* 5, *C. tropicalis* 5, *C. lusitaniae* 1.

In the determination of susceptible and resistant strains the two methods showed 100% agreement. Among strains with intermediate susceptibility against FLU demonstrated by Fungitest^R, 4 were resistant and 3 susceptible by E test^R.

Our results suggest that all two methods are convenient for routine testing of antimycotic susceptibility. Fungitest^R seems to be easier-to-use while E test^R allows the individual testing of antimycotics and MIC determination.

A. SKAZA MALIGOJ

Voluntary HIV testing in the Celje region, Slovenia

Institute of Public Health Celje, Slovenija

Objectives. To enable everyone to get an anonymous, voluntary and free HIV-test and to offer everybody free medical advice.

Methods. In the region of 300,000 inhabitants there is one testing point located at the Institute of Public Health in Celje. Anyone who wants can get a free and anonymous test every day between 8 and 12 a.m. The antibody tests and the diagnosis are carried out by ELISA test of the 3rd generation which additionally detects IgG and IgM antibodies against HIV1 and HIV2.

Before the actual test a conversation takes place with the person who wants the test so that we can find out their awareness of the illness and the circumstances in which the person might have been infected. According to the time, which has passed since the infection, control tests are recommended within a year after the infection. The patient is given information about the infection and the development of the illness and is issued with condoms. If the patients want they can tell details about how the illness was transmitted and the time of the assumed infection. In addition the patient's sex is recorded.

The test results are handed to the patient in person within two days after the blood sample has been taken.

Results. Since 1987 when the tests started, a total number of 357 people took the test – 205 men and 152 women. The average age of the tested patients is 22.5. The reasons for taking the voluntary test are unprotected sexual intercourse and fear of infection, intravenous drug use. So far all test results have been negative.

Conclusions. The voluntary anonymous tests and information available on HIV are considered to be absolutely necessary, although the epidemic in our country is still in its beginning stage. More and more people, especially couples who are starting their sexual life, are deciding to take the test.

M. FÖLDES, A. DOBOZY

Treatment of urogenital *Chlamydia trachomatis* infections with doxycyclin or azithromycin. A comparison of drug effectiveness

Department of Dermatology, Albert Szent-Györgyi Medical University, Szeged, Hungary

Sixty patients with proven urogenital *Chlamydia trachomatis* infection were treated using either Azitromycin (Sumamed) or Doxycyclin. The mean age was 28 that ranged between 14–42. None of the patients received anti-Chlamydia treatment at least two weeks prior to our study. 29 individuals, 6 males and 23 females, were treated with 1 gr Azithromycin given in one dose. This treatment was effective in 33% of male patients (n=2) and 69.5% of female patients (n=3). 31 individuals, 11 males and 21 females were treated with Doxycyclin, 2×100 mg/day, for 12 days. In this group the therapy was successful in 27% (n=3) of male and 70% (n=15) of female patients. The treatment was well tolerated in both groups, no serious side-effect was noted.

B. NYÉKY, GY. SZŐNYI, GY. GARAMVÖLGYI

Bacterial background of habitual abortion

Schöpf-Mérei Ágost Hospital and Mothercare Center, Budapest, Hungary

150 women complaining habitual abortion after one or more interruptions in their gynecological history asked for a well-termined baby. The taxonomical factor analysis with 28 variants showed that the chronic endometritis was the first causal factor. Several examinations (karyotypes, spermatograms, family pedigrees, psychological investigations, bacteriological examinations of the cervix) were performed before starting of the next pregnancy. On the beginning of this pregnancy the patients have been treated with antiaerobic and antianaerobic medication.

After uncomplained pregnancies at term normal babies were born in 96 cases (64%).

J. ŘEZÁČOVÁ[1], J. MAŠATA[1], M. PŘIBYLOVÁ[2], M. DRAŽDÁKOVÁ[2]

Chlamydia trachomatis – its possible effect on male infertility

[1]Department of Gynecology and Obstetrics, [2]Department of Dermatology, Faculty of Medicine, Charles University, Prague, Czech Republic

Objectives. There is still a discord on the effect of *Chlamydia trachomatis* on male fertility. It causes urethritis, epididymitis, prostatitis and orchitis, however, infection is asymptomatic in 20–80% of cases and therefore frequently not properly treated. The aim of this study was to look for presence of *Chlamydia trachomatis* and antichlamydial antibodies in ejaculate and antichlamydial antibodies in blood serum of male partners of infertile couples and to compare the obtained results with their spermiogrammes.

Methods. Specific antichlamydial IgG and IgA (ELISA) were studied in 49 males. Presence of *Chlamydia trachomatis* (using LSR) and antichlamydial IgG and IgA was looked for in their ejaculates. Spermiogramm was obtained using WHO criteria in the masturbation ejaculate.

Results. 26 out of 49 examined males were normospermic, 7 asthenospermic and 16 oligospermic. Serum antichlamydial IgG was found in 6 patients, serum antichlamydial IgA in 1 out of this 6. In one out of 5 males with serum IgG indicating a passed infection, antichlamydial IgA were found in ejaculate (indicating a chronic infection). In all these patients oligospermia according to WHO criteria was obtained. *Chlamydia trachomatis* were found in no one of patients included in this study.

Conclusion. It was well established that *Chlamydia trachomatis* is one of the causes of female infertility. Role of this infection in male infertility is still under discussion. Our observations suggest that microbiological examination of the ejaculate is not enough to reveal chronic Chlamydial infection in males. Not diagnosed and therefore not treated infection might be a cause of male infertility.

D. GOLUBOVSKI[1], M. POTOČNIK[2], S. PETEK[2]

Pathogenic role of *Ureaplasma urealyticum* in patients with nongonococcal urethritis

[1]Medical Faculty, Institute of Microbiology and Immunology Ljubljana, Slovenia,
[2]Department of Dermatovenereology, University Medical Centre Ljubljana, Slovenia

We studied the frequency of *Ureaplasma urealyticum* in patients with nongonococcal urethritis. We examined clinical samples of adult patients attending the STD Outpatient clinics of the Department of Dermatology in Ljubljana.

We have tested urethral swabs of 188 men and urethral and cervical swabs of 60 women. Samples were collected and transported in transport medium (International Microbio, France). For detection of *Ureaplasma urealyticum* we used two methods: metabolic growth in liquid media and growth on solid media A_7 (International Microbio, France).

We have isolated *Ureaplasma urealyticum* in urethral swabs of men in 32 (17%) cases, and in urethral swabs of women in 21 (35%). In cervical swabs of women we isolated *Ureaplasma urealyticum* in 7 (16%) cases.

Our results show an important role of the isolation of *Ureaplasma urealyticum* in the detection of the etiologic agent of nongonococcal urethritis.

T. NYÁRI[1], J. DEÁK[2], GY. MÉSZÁROS[3], CS. NYÁRI[4]

A cost-effectiveness analysis of screening and treatment for *Chlamydia trachomatis* infection in asymptomatic women

[1]Department of Medical Informatics, [2]Department of Clinical Microbiology, [3]Department of Obstetrics and Gynaecology, Albert Szent-Györgyi Medical University, [4]Department of Economics of Attila József University, Szeged, Hungary

Chlamydial infections of the genital tract do not invariably cause symptoms that would prompt a person to seek medical aid. However, identification and treatment of infected persons is important not only for the individuals but also to prevent the spread of *C. trachomatis* in the society. Screening asymptomatic women for chlamydial infection is the cornerstone of effort to reduce the burden of the disease, since chlamydial cervicitis is not associated with specific complaints. Since the medical costs rise, a new attention to quality and cost of care has become apparent. The key to the prevention of chlamydial infections and their sequelae is screening using a high

performance diagnostic test. In general, the age-based screening provided the greatest cost savings. However, universal screening is desirable in some situations (e.g.: high prevalence). In most cases, screening done by using any criteria and a highly sensitive diagnostic assay should be part of any STD prevention and control program or health plan.

The frequencies of PID and male STDs are poorly documented in Hungary, and the annual cost of chlamydial infection and its sequelae was not yet estimated. In order to determine the prevalence and some of the risk factors of genital *C. trachomatis* infections, we have performed a study among pregnant women attending health centres in different regions of Hungary and a cost-effectiveness analysis of screening of chlamydial infection and its sequelae has been carried out. The prevalence of sequelae is derived from the literature in our analysis of cost-effectiveness. Financial resources are insufficient to offer *C. trachomatis* testing to all women in Hungary (prevalence is 5.9%). Therefore, selection criteria for testing should be constructed for a specific setting that includes young age between 15–19 years. The results of our analysis revealed that the cost of the sequelae of untreated *C. trachomatis* infections is slightly less without the cost of infertility treatment than the costs of using the ELISA method for detecting of *C. trachomatis* of the age group between 15–19 years, which displayed a 11.4% rate of infection.

V. KOVALJEV, P. GREGOROVIĆ-KESOVIJA, V. MIĆOVIĆ

Gardnerella vaginalis **in pregnancy**

Department of Microbiology, Institute of Public Health, Rijeka, Croatia

The purpose of the study is to establish the prevalence of *Gardnerella vaginalis* in pregnant women, importance and relation between microbiological findings and clinical pathology.

The total of 533 pregnant women from the Clinic of Gynecology and Obstetrics in Rijeka was examined during the two years period. Swabs from posterior vaginal fornix are examined aerobically and anaerobically (Gas Pak Anaerobic system) at the Department of Microbiology.

G. vaginalis was isolated from 299 (56.09%) of samples. In pure culture, from 107 (35.79%) samples and in mixed culture from 192 (64.21%) samples, but as predominant isolation. *G. vaginalis* is the most frequent isolation in pregnant women with following diagnoses: abortus imminens, incompetentia cervicis uteri and partus praetemporarius imminens, 253 (84.62%). Out of that number the majority (46.82%) is

connected to abortus imminens. During the first six months of pregnancy, *G. vaginalis* is most frequent, 203 (67.89%).

The second part of the study shows the results of pregnancy outcome. Out of 334 pregnant women that delivered at the clinic, *G. vaginalis* was isolated in 188 (56.29%). Correlation between *G. vaginalis* isolation and birth weight was established. The number of children with birth weight between 1000 and 1499 grams is significantly higher in the group of mothers with *G. vaginalis*. Out of 51 spontaneous abortions, in 33 (64.7%) *G. vaginalis* is isolated. Histopathologic diagnosis such as fibrosis stromae villorum chorialium, chorioamnionitis acuta suppurativa and deciduitis acuta suppurativa are significantly connected to the presence of *G. vaginalis*.

Pregnancy is a state of higher risk for sensibility toward infections with *G. vaginalis*. Influence and correlation between *G. vaginalis* isolation and birth weight is established. Correlation between *G. vaginalis* isolation and histopathologic finding in placenta is also confirmed. Microbiologic screening and therapy in early pregnancy may prevent spontaneous abortion and premature delivery.

M. R. BUTINA[1], A. KANSKY[1], M. POTOČNIK[1], I. BARTENJEV[1], T. AVŠIĆ[2]

Epidemiology of genital herpes infections in Slovenia

[1]Department of Dermatovenereology, University Medical Centre Ljubjana, [2]Institute for Microbiology and Immunology, Medical Faculty, University of Ljubljana, Slovenia

Objective. Genital herpes affects millions of people worldwide, it is probably the most common genital ulcerous disease in the world. Disease is still mainly a result of HSV type 2 infection for which seroprevalence rates as high as 60–90% have been reported in several developing countries. In developed countries it is estimated that approximately 20% of the general population may be seropositive.

In a pilot study we performed in Slovenia in 1996 the seroprevalence was 42%, which is relatively high considering data from the literature. Therefore we decided to perform a more extensive study of genital herpes prevalence in Slovenia and also to investigate the association between presence of antibodies to HSV type 2 and sexual lifestyle.

Methods. Over 200 patients participated to date. They are divided in two groups: in the first group are those, who come to our STD outpatient clinic with a strong suspicion of sexually transmitted infection and in the second group are patients without any history of sexually transmitted disease. Serums of all patients were tested for the presence of the IgG class antibodies against herpes simplex virus (HSV) type 1

(Behring Enzygnost anti HSV IgG) and HSV type 2 (Virotech ELISA Testkit Herpes simplex 2). Seropositivity on the HSV type 2 is considered to be the indicator of genital herpes infection in Slovenia, the association between the presence of HSV type 2 IgG antibodies and gender, age and history of sexually transmitted infection are being determined.

Results. Data are still in process of statistical analyses, the results will be presented at the 4th Alpe-Adria- Danube STD Workshop.

J. DEÁK[1], I. CSEH[2], J. SZÖLLŐSI[3], T. PULAY[4], L. KORNYA[5], M. BAK[6], T. NYÁRI[7], E. WESZELOVSZKY[1], I. JAKAB[8], E. NAGY[1]

Detection of human papillomaviruses with nucleic acid hybridisation

Albert Szent-Györgyi Medical University, [1]Department of Clinical Microbiology, [3]Department of Obstetrics and Gynaecology, [7]Department Medical Informatics, Szeged, [2]Haynal Imre University of Health, Department of Obstetrics and Gynaecology, National Institute of Oncology, [4]Department of Gynaecology, [6]Department of Cytopathology, [5]Péterfy Sándor Hospital, Department of Obstetrics and Gynaecology, [8]Biogal Teva Pharma, Budapest, Hungary

Objective. The Human papillomaviruses (HPV) are regarded as the etiological agents of cervical carcinoma. A multicentre study was organized to determine the prevalence of HPV in the fertile female population in Hungary.

Methods. Simultaneously with the clinical sample collection, a questionnaire interview was performed to acquire data on the life style, sexual practice, etc. Between 1 April and 1 July 1997, 1200 women were examined colposcopically and cervix samples were collected for cytology and the detection of HPV DNA (Digene Hybrid Capture™ System).

Results. 17.4% of the samples were HPV-infected. 3.9% of the patients had acquired low risk, and 10.2% high risk HPV types. 3.4% of the women were simultaneously infected with both low risk and high risk HPV types. The correlation of the results of colposcopy and the HPV hybrid capture assay was 7.5%, whereas that of the results of cytology and the HPV hybrid capture assay was 73%. Statistically significant differences in HPV prevalence were detected as functions of age ($p<0.001$), age at first sexual intercourse ($p<0.01$), the number of sexual partners ($p<0.001$), a changing in sexual partner in the last 3 months ($p<0.001$), marital status ($p<0.001$), parity ($p<0.001$) or smoking ($p<0.001$). No statistically significant differences in HPV prevalence were detected with regard to ethnic group, education, socioeconomic status, dietary habits, working with chemical or radioactive agents, use of oral contraceptives,

age at first birth, previous performance of induced abortion, or the occurrence of spontaneous abortion.

Conclusions. Parallel performance of cytology and HPV hybrid capture assay increases the certainity of the carcinoma prevention diagnostics and serves a quality control of the cytological diagnosis.

V. S. SAKALO, S. SHARMAZAN, L. KALUZHNAYA, Y. KUSHNIRUK, A. SACALO

Treatment of human papilloma virus (HPV) infection with interferon alfa-2b

Institute Urology and Nephrology, Acad. Med. Sci. of Ukraine, Policlinic N1 of Health and Rehabilitation Department under the Cabinet of Ministers of Ukraine, Ukraine

Objectives. HPV infection is often combined with *Trichomonas vaginalis*, gonococcus, *Chlamydia trachomatis* and mycoplasma infection. Goal of this study was to attempt treatment with intraurethral instillation of interferon alfa-2b.

Methods. From the 47 men referred to the urology clinic with visible manifestations of HPV infections or with history with known HPV, 11 were found to have cytological evidence of disease. The treatment of patients begins with antibacterial therapy for eliminations of concomitant microflora. After the initial stage of treatment we conduct a cytological test of urethral swab on HPV presence. The swab was fixed by 95% alcohol and stained by Papanicolaou. The presence of koilocytes in swab is pathognomonic cytological indication of cellular damage by HPV. The visible condyloms were eradicated using a Nd-Yag Laser. In the presence of HPV infection Interferon alfa-2b was used for treatment. For men this medicine was instilled into the urethra. Each patient received 25 million U of Interferon alfa-2b in 3–5 ml of sterile physiological solution. Interferon with holed in the urethra by the patient during 1 hour. The course of treatment was 1 time per week during 6 weeks. Patients were investigated for possible side-effects.

Results. Eleven of 17 patients had urethra cytology positive for HPV infection with no evidence of visible disease. All patients were followed for an average of 12.8 months. Nine of 11 remained disease free the follow-up period. Two persons with recurrence were successfully retreated. The blood testing, semen analysis in all patients was unchanged after the treatment. The patients did not complain of fever, nausea, or dysuria throughout the study.

Conclusions. Associated bacterial-viral infection demands complex treatment with antibiotics and local treatment. We did not observe complications.

C. HELLER-VITOUCH[1], R. MALY[2], E. TSCHACHLER[2], J. SÖLTZ-SZÖTS[1]

Evaluation of a signal amplified hybridization assay for the detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

[1]L. Boltzmann-Institute for the Study of Dermato-Venerological Infectious Diseases, Vienna, Austria,
[2]Department for Immunodermatology and Infectious Diseases, University of Vienna, Vienna, Austria

Objective. A signal amplified hybridization microplate assay for the chemoluminescent detection of *Chlamydia trachomatis*- (CT) and *Neisseria gonorrhoeae*- (GC) DNA in cervical specimens (Digene CT/GC test, hybrid Capture II) was evaluated in comparison with the DNA amplifying Ligase Chain Reaction (LCR) and the Hybridization Assay PACE 2 as well as with gonococcal culture in order to investigate specificity and sensitivity of the new test system.

Methods. We obtained three endocervical swab specimens from female patients attending our STD outpatients clinic, whereby sample collecting was randomized. The samples were tested by the CT/GC test using Hybrid Capture II technology. Furthermore, we performed the LCR and the PACE 2 in order to obtain an enlarged gold standard, defined by the positivity of two independent chlamydia assays. Another endocervical swab for gonococcal culture on Thayer Martin medium was taken.

Results. 241 patients were recruited for the study, 22 of them (9.1%) were positive for CT in at least two of the tests. Digene CT/GC detected 21 of the true positives, which means a sensitivity of 95.5%. Two more samples were positive in LCR only, which might be caused by contamination. No sample was detected false positive, the specificity is 100%. Three patients were positive for GC in culture as well as in Digene CT/GC.

Conclusions. The Digene CT/GC test is easy to handle. It is highly sensitive and specific for the detection of CT and GC. The lack of prove of antibiotic resistance and the comparatively high costs have to be taken into account, when using this test instead of culture for the detection of GC.

Y. KUSHNIRUK, V. SAKALO, S. SHARMAZAN

Reproductive disorders in men with genito-urinary infection

Department of Health and Rehabilitation under the Cabinet of Ministers of Ukraine, Institute of Urology and Nephrology, Academy of Medical Sciences of Ukraine, Ukraine

Objectives. The aim of the work is to study the character of reproductive disorders in men with different genitourinary infections and effectiveness of etiopathogenic treatment with comparison to traditional therapy.

Methods. We performed clinical-laboratory examinations for 268 men aged from 21 to 45 years who complained of infertility in their marriage and of inflammatory diseases of the genitourinary tract. Patients were divided into 2 groups. 73 (27.2%) persons with STD were included into the basic group. 152 (56.8%) men with bacterial infection and 43 (16.0%) with prostatodynia make up the control groups. Urological, laboratory spermatological and microbiological methods were used. Patients with reproductive disorders were complexly treated including etiological, antibioticotherapy, transrectale lazerophototherapy, vitamins, aminoacids and donators of sulfydryl group. The results of this treatment were compared with the traditional therapy.

Results. Clinico-microbiological examination of the basic group there were found different associations of STD microorganisms, including: *Chlamydia trachomatis* in 14.1%, *Ureaplasma urealyticum* in 7.3% and *Trichomonas vaginalis* in 5.8%. We identified Gram-positive and Gram-negative bacteria in patients of the control group. Chronic prostatitis, vesiculitis and epididymitis in different combinations were diagnosed in 94.5% of patients with STD and 67.8% of men with bacterial infection.

Spermatological changes were characterised by teratospermia, leukocyturia, spermaglutinatia, changes of pH, decrease of concentration of fructose and citric acids.

Complex etiopathogenetic treatment based on the nature of spermatological disorders gave positive results in 86.5% and 61.8% in the group of traditional therapy.

Conclusions. Chlamydiosis and ureaplasmosis make negative effect to men reproductive function. Spermatological changes should be kept in mind when treating the patients.

V. VÁRKONYI[1], T. TISZA[2], A. HORVÁTH[1,2], J. MINÁROVITS[3], Á. GYURIS[3]

Evaluation of the immunstatus and viral load of asymptomatic HIV infected patients

[1]National Institute of Dermato-Venereology (NIDV), [2]University Clinic of Dermato-Venereology, Semmelweis University of Medicine, [3]National Institute of Hygiene Research Laboratories, Budapest, Hungary

Objective. To evaluate the immunstatus (CD4+, CD8+cell count, CD4/CD8 ratio) and the viral load of newly verified and regularly followed-up HIV infected patients.

Methods. 34 HIV infected patients were evaluated (mean age at the date of HIV verification: 36.8 years (22–74), 32 men – 26 homosexual, 4 bisexual, 2 heterosexual, 2 women). Plasma viral load was determined with NUCLISENS (ORGANON) in all patients. Viral load was measured in 41.2% of patients at the date of HIV verification or within 10 months thereafter. Patients were classified in 4 groups according to their CD4+ cell count, and were further classified based on plasma viral load.

Results. Group I: CD4+ cell count >500/μl. I/1: viral load<400 molecules/ml: 7 patients (4 of them are known to be infected for 2–4 years. I/4: viral load 10,001–30,000/ml: 1 patient (at the date of verification). I/5: viral load > 30,000/ml: 5 patients (mean duration of infection in 4 patients 4.25 years – 2.8 years).

Group II: CD4+ cell count: 351–500/μl. II/1: viral load <400/ml: 3 patients (in 1 patient at the date of certification, in 2 patients 3 and 8 years after verification, respectively). II/3:viral load 3001–10,000/ml: 1 patient (7 years after verification). II/4: viral load 10,001–30,000/ml: 2 patients (1 patient at the date of verification, 1 patient 4 years after) II/5: viral load >30,000 (4 patients (newly verified). Group III: CD4+ cell count 201–350/μl: III/1: viral load <400/ml: 2 patients (1 patient is newly, another is known to be positive for 2 years). III/3: viral load 3001–10,000/ml: 1 newly patient. III/4: viral load 10,001–30,000/ml: 2 patients (infected for 2- and 3 years, respectively). III/5: viral load: >30,000/ml: 4 patients (1 newly, 3 were known to be infected for 3, 5, 7 years respectively). Group IV: CD4+ cell count 200/ul: IV/5: viral load 30,000/ml: 2 patients (known to be infected for 2 years).

Conclusions. To evaluate the current status of HIV infected patients both CD4+ cell count and viral load should be monitored regularly as they do not necessarily correlate.

P. GREGOROVIĆ-KESOVIJA, V. KOVALJEV, V. MIĆOVIĆ, A. JONJIĆ

Chlamydia trachomatis as the cause of nongonococcal urethritis (NGU) in men

Department of Microbiology, Institute of Public Health, Rijeka, Croatia

Chlamydia trachomatis is currently recognized as one of the leading causes of sexually transmitted diseases (STD). Unfortunately, many infected individuals remain asymptomatic allowing the organism to go undetected and untreated. In men the most common manifestation of chlamydial infections is NGU.

678 urethral swabs of sexually active outpatient males who suffered from NGU were examined during a five years period (1993–1997). The patients aged between 17 and 55 years. For an accurate diagnosis the specimen must contain enough epithelial cells from the infected site. Therefore, one hour prior to sampling the patients had to refrain from urinating.

The specimens were processed and test results were interpreted according to standardised Direct Immunofluorescent technique using monoclonal antibodies to *C. trachomatis* (Orion-Chlamyset).

C. trachomatis has been detected in 175 out of 678 urethral swabs (25.81%). The number of positive specimens has successionally increased in the five-year period of observation. In 1993 and 1997, there were 24 and *44 C. trachomatis* positive patients, respectively.

C. trachomatis has been detected in urethral swabs of different age groups. The youngest patient was eighteen and the oldest was fifty-three years old (medium age 29 years).

The results of our study confirmed *C. trachomatis* as a very important etiological agent in NGU. Due to the fact that the number of adolescents enrolled in this study was low, the risk of STD spreading as well as the consequences of undetected infections are markedly enlarged.

E. ANTONINI, M. A. GONZALEZ INCHAURRAGA, B. ULESSI, G. TREVISAN

Genital herpes simplex infection: the prevalence of HSV-1 and HSV-2

Institute of Dermatology, University of Trieste, Italy

The prevalence of genital herpes has increased worldwide during the past twenty years. Genital herpes is now the most common cause of genital ulcerations in patients attending sexually transmitted disease clinics. Both HSV-1 and HSV-2 cause primary genital herpes and about 80% is HSV-2: they produce an identical clinical picture, but there is evidence that both symptomatic and asymptomatic recurrences are less frequent after HSV-1 genital infection and it has been shown that most people infected with HSV-2 are asymptomatic or have atypical symptoms. The number of recurrences is 3 or 4 per year, but 15% of patients have 8 or more recurrences per year. We report an evaluation of HSV type specific serology in the cases of recurrent genital ulceration over the last six month period in our Institute.

Methods. We have studied twenty-two patients, (5:F–17:M), average age 46.7 years (24–73), affected by genital recurrences of herpes, without a history of herpes labialis and with HSV positive viral culture. Type specific serology was performed using a monoclonal antibody blocking assay.

Results. Seven patients were found to have antibody to HSV-1 only, twelve to HSV-2 only and three to both serotypes. In this selected group the seroprevalence of HSV-1 was of 31.8% and of HSV-2 of 54.5%.

Conclusions. These data suggest an increased frequency of cases of RGH due to HSV-1, and are comparable to several recent English studies, where the proportion of cases due to HSV-1 and HSV-2 seems to show an increase of HSV type 1.

A. STARY[1], S. MAYERHOFER[2], B. GÖTZ[1], E-M. VINZELJ-HORVATH[2]

Diagnosis of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* on genital and extragenital sample types in males and females

[1]Outpatients Centre for Infectious Venero-Dermatological Diseases, Vienna, Austria, [2]Outpatient Clinic for the Diagnosis and Treatment of Sexually Transmitted Diseases, Public Health Department, Vienna, Austria

Objective. In the present study we have compared the performance of LCR for the detection of *C. trachomatis* and *N. gonorrhoeae* in both men and women, by testing pharyngeal, rectal and urine specimens in addition to cervical and vulval samples in women and urethral samples in men.

Methods. The study was performed in a high risk selective group of 696 patients (236 women and 460 men) attending a private outpatients center of STD diagnosis and the STD Center of the Public Health Office in Vienna.

Samples from the pharyngeal, anorectal, and vulval region were collected for LCR and gonococcal culture. In addition, urethral specimens in men and cervical specimens in women were collected for chlamydial and gonococcal culture and for LCR. From most of the patients also urine was available and was tested by LCR for both microorganisms. Discrepant analysis was performed by MOMP-LCR and PILIN-LCR.

Results. The results of chlamydia and gonococcal testing confirm the high sensitivity of LCR for genital samples. In contrast to women, where 51.7% and 75% of rectal specimens were chlamydia and gonococcal positive, respectively, only one man had a positive chlamydia outcome for pharyngeal and none for rectal specimens. Positive results for extragenital gonorrhoea were observed more often than for chlamydia.

Conclusions. Both pharyngeal and rectal chlamydial and gonococcal infections were seldomly observed but occur more often in women than in men. A careful interpretation of LCR results especially for rectal specimens has to be recommended to exclude contamination for women and background amplification for both men and women.

I. HORVÁTH[1], A. HORVÁTH[2], G. VALLUS[3], Z. PROHÁSZKA[2], B. DLUSTUS[3], Á. BARANYAI[3], L. ROMICS[2], G. FÜST[2], I. KARÁDI[2]

From the lues screening to the anticholesterol antibody studies of healthy subjects and patients with peripheral atherosclerotic disease

[1]National Institute of Dermato-Venerology, [2]3rd Department of Internal Medicine of Semmelweis University of Medicine, [3]Department of Vascular Surgery, Central Military Hospital, Budapest, Hungary

In the lues screening a significant number of the biological aspecific positive (BAP) seroreactivity used to be demonstrated. Previously the reactivity was supposed due to the anticardiolipin antibody. The involvement of anticholesterol antibody (ACHA) into the BAP reactivity was proved by us.

The protective role of ACHA against atherosclerosis was demonstrated in animal models. This antibody was found in the sera of almost all healthy persons but no data on the occurrence in disease were reported until now. In our study we measured ACHA levels in sera of patients with peripheral atherosclerosis and healthy controls. IgG ACHA levels were demonstrated in 86 patients (58 male, 28 female, age: 58±10 SD ys) with peripheral atherosclerotic disease and postoperative restenosis. A group of blood donor volunteers (n 218, 107 male, 111 female, age 56±10 SD ys) served as healthy controls. ELISA was developed in our laboratory to detect with high sensitivity the antibodies against native cholesterol. Cholesterol crystals were dissolved in alcohol for coating. Sera were diluted 1 to 800 and a rabbit anti-human IgG was used as conjugate. Antibody levels measured in the patients (12.65±3.4 SEM AU (arbitrary units)/ml) were significantly lower with the Mann-Whitney test ($p=0.0001$) than in the healthy controls (30.1±3.5 SEM AU/ml). ACHA levels in healthy subjects correlated with blood cholesterol levels ($p=0.038$, r -0.19), and no correlation with triglycerid levels was found. According to the animal experiments and our present findings ACHA play an important role in the protection against atherosclerotic process. Further studies aiming to understand the mechanism of the protection are in progress in our laboratory.

G. PECHLIVANOV, N. TZANKOV

Oleoma penis

Sofia, Bulgaria

We present patients mostly from gipsy-s origin and risk social behaviour (prisoners and beggars) having implanted different types of foreign bodies in the preputium. The most typical cases are with injected fatty substances (oleoma penis) and implantation of plastic pellets. We have connected the social personal motivation of the patients and prognosis.

J. MAŠATA, J. REZÁCOVÁ, I. SODJA

The role of sero-diagnostics in management of *Chlamydia* infection

Prague, Czech Republic

Objectives. Chlamydia trachomatis is the most often sexually transferable bacterial agent in industrial countries. In recent decades, the percentage of Chlamydia infection has been growing constantly. The similar situation applies to the Czech Republic, where the prevalence of this disease is rather high. One of the most serious consequences of the infection of genital tract in females is tubal occlusion. Chlamydia induces production of type, subspecies and genus specific antibodies. The presence of IgG antibodies is one of the markers of infection. The objective of our study was to assess the possibility of serum examination, namely as a screening test of tubal damage and diagnostic of chronic infection and compare two diagnostic methods.

Methods. 506 women were included in the study, who were examined in the Ist Department of Gynecology-Obstetrics from September 1993 to July 1997. The women were divided in two groups, sterile females and other ones. For all females two smears of cervix were taken to detect antigen by direct immunofluorescence (DIF, DFA, Orion Chlamyset) and by immunoenzymatic method (ELISA-Wellcozyme set, Murex). For the blood sample (taken from cubital vein), the levels of IgA, IgG antibodies were determined and for antigen-positive women also the level of IgM antibodies (ELISA-BAG set). The group of sterile women was further divided into a subgroup of patients for whom tubal occlusion was proved. For 210 serum-positive women additional serological testing of species was performed by a specific test, micro-immunoluorescence test (MIF) SeroFTA, MRL, Labsystem Savyon). The group was

divided, again, into two groups: sterile females and other ones. For statistical analysis chi square was applied.

Results. The percentage of IgG positive women does not show significant difference between the group of sterile women and the group of other females (62% and 54%) In the group of sterile women where tubal infertility factor was proved by laparoscopy, 92% of females had IgG anti-Chlamydia antibodies in blood serum (statistically significant). If we perform MIF testing, for the group of non-infertile females the percentage of IgG positive cases as a result of previous *C. pneumonia* infection is higher.

Conclusion. Infection by *C. trachomatis* is the most frequent cause of tubal occlusion and determination of the level of IgG antibodies is a suitable test for its diagnosis. Elisa method is more sensitive than MIF, but shows immunoglobulins that are common to all *Chlamydia*.

ERRATUM

K. H. CHAN[1], H. L. CHEN[2], R. X. LUO[3], M. PEIRIS[3], P. WOO[3], W. H. SETO[1], M. H. NG[3]

Serological diagnosis of NPC by recombinant Epstein-Barr virus replication activator fusion protein specific ELISA

[1]Department of Microbiology, Queen Mary Hospital, [2]The University of Hong Kong, Hong Kong and [3]Department of Pharmacology and Molecular Science, School of Medicine, John Hopkins University, Baltimore, Maryland, USA

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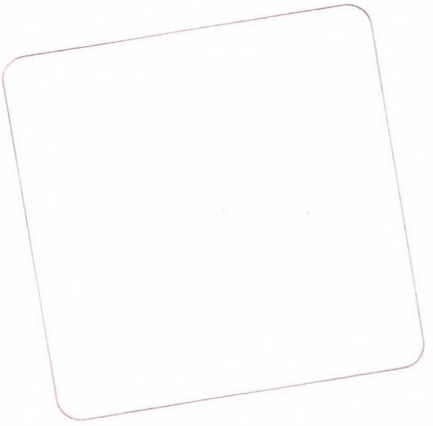

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BIOLOGICAL PHENOTYPE AND CORECEPTOR USAGE OF HUMAN IMMUNODEFICIENCY VIRUS*

(A SHORT REVIEW)

EVA MARIA FENYÖ

Microbiology and Tumorbiology Center, Karolinska Institute, 171 77 Stockholm, Sweden

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Introduction

The AIDS epidemic is well into its second decade and up to date has claimed 13.9 million lives. Human immunodeficiency virus (HIV) is the causative agent of AIDS and according to the December 1998 estimates of UNAIDS (United Nations AIDS Programme) 32.4 million people are living with HIV/AIDS worldwide. The main routes of HIV transmission are sexual, from mother to child or by intravenous drug use. At the beginning of the epidemics blood and blood products played an important role, but testing of blood donors and control of blood products have successively minimized the risk of becoming infected by this route.

HIV is a retrovirus, a member of the lentivirus subfamily. Lentiviruses occur in several animal species and cause slow – often fatal – diseases affecting various organ systems depending on the species and the age of the animal at the time of infection (reviewed in [1]). In HIV-infected humans, gradual depletion of CD4+ T cells results in immunodeficiency after several years and leads to the clinical entity of acquired immunodeficiency syndrome (AIDS). The rate of CD4+ cell depletion is highly variable in different individuals, from stable CD4 counts in long-term nonprogressors

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(>10 years) to a decline of 15×10^6 cells/liter/month leading to AIDS in a few years. Also, infection by HIV type 1 (HIV-1) as known in Central Africa, Europe and the Americas, leads to immunodeficiency much faster than infection with HIV type 2 (HIV-2) in West Africa [2]. Still today we do not know what exactly determines the disease progression rate in individual patients. Conceivably, virus-host cell interactions have a decisive role in this process. Here we shall focus on the biological phenotype of the virus which has been demonstrated to vary according to the severity of HIV infection, thus providing a marker for viral virulence.

Biological variation of HIV-1

The original observation some 14 years ago that the rate of HIV-1 replication and the amounts of virus obtained in primary isolation cultures vary according to the severity of HIV-1 infection in the patient suggested that we might be looking at viral determinants of the pathogenic process. This prompted us to further investigate HIV-1 biological phenotype, such as replication rate, cytopathology and cell tropism in tissue culture, in primary cells and established cell lines. Based on biological phenotype, such as replication rate, HIV-1 isolates could be divided into two major groups [3–5]. In one group virus could be isolated within days from peripheral blood mononuclear cells (PBMC) of HIV-1 infected immunodeficient patients, and was able to induce syncytia not only in PBMC but also in cell lines. Hence the designation, rapid/high or syncytium inducing (SI). The other group of viruses was characterized by a prolonged time to isolation (2–3 weeks), slow replication rate in PBMC, absence of or marginal cytopathology (small syncytia or cell killing) and inability to infect established T-lymphoid and monocytoid cell lines. Most primary HIV-1 infections occur with this latter type of virus, called slow/low or non-syncytium inducing (NSI). Those individuals that do become infected with rapid/high or SI virus lose CD4 cells at a faster rate than slow/low or NSI virus infected individuals [6]. Changes in viral phenotype may also occur within the same infected individual undergoing clinical progression, and have been shown to involve switch from NSI to SI [7, 8]. Using syncytium induction in MT-2 cells to test the phenotype of sequential isolates derived from a cohort of 53 HIV-1 infected homosexual men over a period of 5–8 years, we found that no change in NSI phenotype was associated with a better prognosis (Table I) [9]. Taken together, the data from our group and from several others indicate that HIV-1 biological phenotype is a marker for viral virulence. Recently, these phenotypic traits could be translated into molecular terms, such as coreceptor usage, opening new doors in our understanding of HIV-1 pathogenesis.

Table I

HIV-1 biological phenotype and CD4+ cell decline

MT-2 tropism	No. of patients	CD4+ counts × 10^6 cells/liter	
		End of study	Baseline
Neg/Neg	26	327	489
Neg/Pos	20	104	397
Pos/Pos	6	57	308

Table II

Classification of HIV-1 biological phenotypes

Chemokine receptor usage	New classification	Previous terminology based on	
		cytopathology in MT-2 cells	replication rate in PBMC
CXCR4	X4	SI	rapid/high
CCR5	R5	NSI	slow/low
/CCR3/CCR2b	R3/R2b		
CXCR4 and CCR5	R5X4	SI	rapid/high
and/or CCR3	R3R5X4 or R3X4		

It has long been recognized that HIV-1 uses the CD4 receptor for cell entry [10, 11]. It has also been known that CD4 alone is not enough, since transfection of CD4 into non-human cells did not allow viral entry and infection of cells [12, 13]. By functional cDNA cloning an HIV-1 entry cofactor has recently been identified as a member of the seven transmembrane G-protein coupled receptor family [14]. Discovery of the first cofactor, shown to function as coreceptor for HIV-1 isolates able to infect established cell lines, was soon followed by several others [15–17]. The two phenotypically different groups of HIV-1, while both using CD4, could be distinguished by their coreceptor usage, inasmuch rapid/high or SI viruses were shown to use CXCR4, and slow/low or NSI viruses CCRS [18, 19]. This major pattern was straightforward and allowed the establishment of a new nomenclature (Table II) [20].

What are these cell surface structures that HIV learned to use as keys to enter cells? Recent development in the field of immunology has disclosed, in addition to

traditional cytokines (like interferons and interleukines) the existence of chemoattractant substances – called chemokines – that serve as mediators in cell-to-cell signalling (reviewed in [21]). Chemokines belong to a superfamily and show similarities in their primary structure, characteristically a conservation of 4 cysteines in a molecule composed of 65–95 amino acids. Depending on whether the first two cysteines are intercalated by an amino acid or not, chemokines fall into two major groups, CXC or CC chemokines. From the point of view of HIV-1, SDF-1 (stromal cell-derived factor-1) [22, 23] is the prototype for CXC chemokines and RANTES (regulated on activation normal T cell expressed and secreted), MIP-lα and MIP-1β^2 (32 represent CC chemokines [24]). While SDF-1 is produced in many different tissues and apparently has a house keeping function, RANTES, MIP-lα and MIP-1β are involved in inflammatory processes [25]. Chemokines exert their effect on cells by binding to specific receptor molecules followed by intracellular signalling. Chemokine receptors belong to the family of seven transmembrane G-protein coupled receptors, members of which have been identified as coreceptors for HIV-1. There are at least two consequences of this coincidence: i) chemokines may inhibit HIV-1 replication [24] by preferentially binding to the same receptor(s), ii) receptor availability may select for certain viral variants.

Phenotypic differences correlate with distinct coreceptor usage

To test chemokine receptor usage by primary HIV-1 isolates, human cell lines, such as the U87 glioma and the HOS osteosarcoma, were engineered to stably express CD4 and coreceptors [15, 18, 26]. Following HIV-1 infection, the U87.CD4 cell lines expressing CCR1, CCR2, CCR3, CCR5 or CXCR4 were scored for syncytia and p24 antigen production at day 3–7. GHOST(3) cells expressing the chemokine receptors CCR3, CCR5 or CXCR4 or the orphan receptors Bonzo or BOB, contain the green fluorescence protein (GFP) driven by the HIV-2 long terminal repeat. HIV-infected GHOST(3) cells express GFP and the fluorescence can be observed in a UV microscope and quantitated by flow cytometry. Using the U87.CD4 cell system we could show that HIV-1 isolates of different biological phenotype are distinguished by their ability to use the chemokine receptor CXCR4 for entry into target cells [18, 19]. Slow/low viruses formed syncytia on CCR5-expressing cells only (RS viruses), while rapid/high viruses used CXCR4 either alone or in combination with CCR5 (X4 or RSX4 dual tropic, respectively). Syncytium induction by an R5 and R5X4 virus is illustrated in Figure 1. It has to be pointed out that CXCR4-using viruses could often use several receptors, not only CCR5 but CCR3 and some even CCR2, suggesting that

a broader cell tropism might enable the virus to infect many different cell types and this may conceivably contribute to the increased virulence of these viruses. Moreover, we found that all HIV-1 isolates are syncytium inducing – provided the target cells carry the receptor required for infection by the particular virus. The receptor not only has to be present on the target cell surface but it has to be expressed in a high enough concentration to trigger syncytium formation. We know today that low CCR5 expression on PBMC allowed slow replication but rarely syncytia formation by viruses using this receptor (the so-called slow/low or NSI viruses), whereas high concentrations of CXCR4 in the same cultures allowed fast replication and extensive syncytia formation of viruses using CXCR4 (rapid/high or SI).

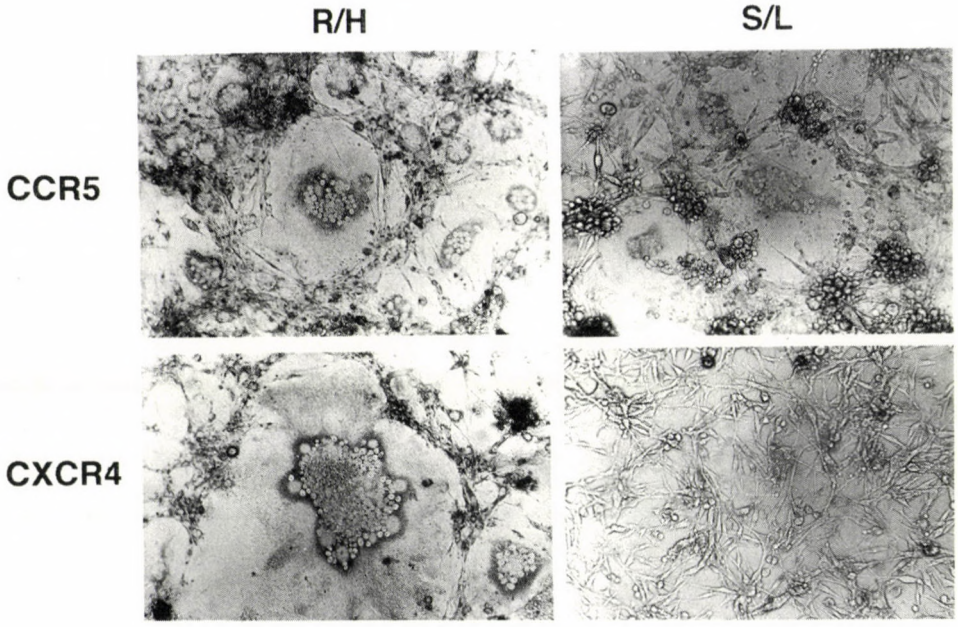

Fig. 1. Syncytium induction by an R5 and R5X4 virus

Sequential isolates from patients with progressive HIV-1 disease may differ in coreceptor usage

Changes in HIV-1 phenotype during clinical progression – often measured as the capacity to induce syncytia in MT-2 cells – have been observed by several groups over the years. Early work has shown that the change involved a phenotypic switch from NSI to SI, as a rule, and lead to the suggestion that SI virus is more virulent than NSI virus [7, 27]. Since we know today that the ability of HIV-1 to infect and induce syncytia in MT-2 cells is dependent on usage of the CXCR4 coreceptor, the phenomenon of viral phenotypic evolution could be revisited in terms of evolution of coreceptor usage. Our results have shown that both in adults and children who acquired HIV-1 infection from their mother, an R5 virus is present early in infection [19].

Clinical progression and decline in CD4 counts is often accompanied by either a switch from R5 to X4 or by a broadening of coreceptor usage yielding multitropic viruses (R5X4 or R3R5X4). In parallel with evolution of coreceptor usage, there is a change in the virus sensitivity to CC chemokines-mediated inhibition [28]. Replication of R5 viruses, but not those using CXCR4 (X4 or multitropic viruses), can be inhibited by RANTES, MIP-lα and often by MIP-1β as well [18]. Evolution to resistance by CC chemokine mediated inhibition occurs in about half of the AIDS patients, while R5 viruses with apparently preserved sensitivity to inhibition by CC chemokines can be recovered from the other half [29]. The impact of this change on the pathogenic process is not well understood. It is an attractive idea that the microenvironment, including cytokines, chemokines and available target cells, in the different organs of an infected individual, selects for virus with different biological properties.

Biological variation appears to be an universal property of HIV-1 isolates across genetic subtypes

Extensive genetic variation of HIV has been well documented (for latest update see ref. [30]). HIV-1 and HIV-2 show an overall difference of 50% in nucleotide sequences. A common measure of differences between viruses and groups of viruses is the divergence between env genes, the most variable of the HIV structural genes. Envelope homology between HIV-1 and HIV-2 is less than 50%. HIV-1 itself can be divided into three distantly related groups; the Major (M) and the Outlier (O) groups, and the newly identified N group of viruses [31]. The M group, which is by far the most widespread, is further subdivided into distinct "clades" or "envelope sequence subtypes", differing by approximately 30–35% at the nucleotide and amino acid

sequence level. The clades are phylogenetic groupings that probably represent a founder effect, that is, the historical beginnings of HIV-1 epidemics in different groups of people around the world. Clade determination is a convenient means of tracking the spread of the virus. *env* clade B, for example, was initially identified in viral isolates from Europe and North America. It has now spread to many other parts of the world. Clade C is the most frequently encountered world wide, mounting to 48% of all HIV-1 infections, while clade A represents 23.5% (UNAIDS 1998 estimates). Emergence of recombinant viruses composed of sequences from different clades in different parts of the virus have been described. For example, an A/B recombinant (A in *gag* and B in *env*) is held responsible for the HIV-1 epidemics in the Kaliningrad area of Russia [32]. The immediate question which arises is what impact has HIV genetic variation on the biology of the virus? Do different clades differ also in virulence? in transmissibility? Is the emergence of recombinant viruses a continuous threat to produce viruses with increased virulence and thereby accelerate the epidemics? Initial studies carried out within the framework of WHO Network involving replication and syncytium induction in PBMC and cell lines, showed no major differences in biological properties of subtypes A–E [33, 34]. More recent work not only confirmed that biological variation is a universal property of HIV-1 isolates across genetic subtypes [35] but showed that coreceptor usage varies with severity of HIV-1 infection. Like in subtype B infections, CXCR4-using viruses were frequently recovered from AIDS patients infected with subtype A, D and E, while individuals in earlier stages of HIV-1 infection yielded predominantly R5 viruses. However, subtype C appears to be at variance with this general pattern, in that isolates are R5, regardless of the severity of HIV-1 infection in the patient (Table III) [36]. Since this is true for subtype C isolates obtained in Sweden (15 isolates), Ethiopia (0/9) and South Africa (1/9) it cannot simply be the result of a founder effect in a certain geographic area. The results suggest that subtype-dependent differences in frequency of usage of certain coreceptors may exist. This opens up the possibility that genetic subtypes, subtype C in particular, may differ in important biological properties such as virulence, tissue tropism and transmissibility.

The promiscuous relative: HIV-2

HIV-2 is less pathogenic than HIV-1 and therefore lends itself for studies on coreceptor usage in relation to pathogenesis. Like HIV-1, a majority (10 out of 11) of HIV-2 isolates use CCR5. Among AIDS patients two out of seven isolates obtained used CXCR4 and showed syncytium induction. Similarities between HIV-1 and HIV-2 end here, however, because unlike HIV-1, most HIV-2 isolates use CCR1, CCR2,

CCR3, BOB and/or perhaps Bonzo as well [37]. Interestingly, nearly all HIV-2 isolates replicate in MT-2 cells, although they do not induce syncytia. In addition to CXCR4, MT-2 cells express BOB mRNA and HIV-2 infection most probably occurs by the orphan receptor BOB. The results indicate that while HIV-1 often evolve to multitropism in the course of the pathogenic process and multitropism is most often associated with CXCR4 usage, multitropism is a general property of HIV-2 isolates. Consequently, multitropism *per se* cannot be held responsible for differences between HIV-1 and HIV-2 pathogenesis.

Table III

Coreceptor usage of HIV-1 genetic subtypes A–E and severity of infection

Subtype	AIDS	No. of isolates	Coreceptor usage	
			CCR5	CXCR4
A	–	12	10	2
	+	8	4	4
B	–	13	13	0
	+	7	1	6
C	–	23	23	0
	+	13	13	0
D	–	5	3	2
	+	9	3	6
E	–	7	6	1
	+	2	0	2

To further investigate the *in vivo* relevance of multitropism, we infected PBMC from individuals homozygous for the deletion in the ccr5 gene (deletion of 32 nucleotides, the so-called Δ32 mutation [38, 39], with 11 HIV-2 and 10 HIV-1 isolates. Taking extracellular antigen production as evidence for productive infection, only cultures infected with viruses using CXCR4 showed signs of virus replication. Testing the antigen negative cultures by PCR revealed that HIV-2 DNA was present in most cases, whereas HIV-1 infected cultures were negative. In HIV-2 infected cultures that were initially antigen negative but PCR positive, virus replication could in some cases be detected several weeks later. Although preliminary, these results suggest that even if the main coreceptors are CCR5 and CXCR4, in the absence of CCR5 the multitropic HIV-2 may utilize, albeit less efficiently, other receptors for cell entry.

Conclusions

Viral biological phenotype is a key player in HIV-1 pathogenesis. Initially, biological phenotype has been defined by replication rate and cytopathology in primary cells and cell lines, but can today be translated into molecular terms, such as usage of different coreceptors at cell entry. CXCR4 usage determines the biological phenotype for viruses of A, B, D and E genetic subtypes and is often associated with AIDS. The fact that the change in virus biological phenotype may occur in the same individual over time and is associated with progressive disease has suggested that CXCR4 using viruses are more virulent. It is tempting to speculate that increased virulence following entry by the CXCR4 receptor is due to differences in receptor-mediated signalling. It remains to be seen whether signalling – if it occurs at all following HIV attachment through the CXCR4 receptor may cause a more severe perturbance of immune functions than signalling through the CCR5 receptor, explaining the differences between viruses using different receptors.

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THE INFLUENCE OF CHROMIUM COMPOUNDS ON YEAST PHYSIOLOGY*

(A REVIEW)

P. RASPOR[1], M. BATIČ[1], POLONA JAMNIK[1], DJ. JOSIĆ[2], RADMILA MILAČIČ[3], MAJA PAŠ[1], MARJETA RECEK[3], VESNA REŽIĆ-DEREANI[3], MIHAELA SKRT[1]

[1]Food Science and Technology Department, Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia, [2]Octapharma, Vienna, Austria, [3]Institut Jožef Stefan, Ljubljana, Slovenia

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Introduction

Metal ions and yeasts metabolism was reviewed quite a few times in the last decades by eminent researchers [1–4]. In spite of this excellent work in the past it is clear that metal ions nutrition in yeasts is still open for further research. After specifying requirements and functions of macroelements in yeasts [4, 5] the era of trace

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elements [6] started and in the recent years one can see many publications considering the problem of trace elements, which seems to be really needed for efficient metabolism. It seems that new analytical approaches [7] busted new wave of research (i.e. NMR, EPR, Raman spectroscopy) into the field of ionic nutrition, which was already in the focus some years ago [6, 8, 9].

The problem of ionic nutrition is also strongly oriented to the human diet. Regarding this topic there are profound reviews available [10, 11] and some of them specially emphasize chromium [12, 13, 15], since it became important through revealing its function in glucose tolerance factor (GTF). Consequently, all the results from studies of ionic nutrition in microbes are oriented towards metabolism of higher organisms and finally to human diet [16, 17]. It is evident that nutrition of human is becoming one of the major fields of research [18] with laying stress on understanding metabolism [19], defining standards of mineral intake [20] and creating functional foods [21, 22].

In the environmental field one can see significance of metal ions in two directions: the pollution control and mining technology. The removal or uptake of metal is concerned with the increasing value of some metals on one hand, and with awareness for ecological effect of toxic metals released into the environment on the other [3, 23–26]. In the area of environmental care the conventional methods for removing metals from polluted streams or ore processing solutions can include traditional chemical and physical processes which can be ineffective or extremely expensive, especially when metals are present in low concentrations from 1 to 100 μg/mL [27, 28]. In addition, the history of the waste (source and its pretreatments) seems to play an important role for selecting proper microbial biomass for biosorption with a goal of bioremediation. In some cases it was proved that although the yeast biomass was capable of removing more than 99% chromium from electroplating wastewater, it was only capable of removing 50–60% of chromium from tannery wastewater [29].

Regarding environment we should be very careful since everything what was released into soil, water or air from technological activities comes back to food chains and consequently – directly or indirectly – to human diet [30]. The link to environmental biochemistry of chromium is evident [31] what is crucial for tracing chromium flux in environmental niches and understanding oxidation-reduction dynamics in chemical [32, 33] as well as in biological world [34].

Moreover, successful ionic nutrition is related to the metal uptake, which is understood as biosorption and bioaccumulation and is important for all living creatures. Selective advantages are confirmed for species which are capable of uptake and retaining ions (from metal or transition elements) available in limited amounts in

surroundings. During the last decades the trace elements such as Mn, Fe, Co, Ni, Zn, V and Cr, were found to be essential or at least beneficial for normal metabolism in all organisms [35].

The metal uptake starts with fast biosorption on the cell wall and then continues with slower bioaccumulation. Bioaccumulation mechanisms consist of metal ion translocation through the cell membrane into the cell and its deposition in adequate store compartment in living cell. In principle, all the steps mentioned are metabolically dependent, what is not the case in metabolically independent passive processes, which are based on different mechanisms for metal sequestering onto the cell surface. Biosorption is strongly affected by combination of other metal ions in cell surroundings [36]. This uptake is metabolically independent but depends on the chemical make-up of the cell wall of particular species. Enormous diversity among yeasts has been found by various approaches of studying yeast cell envelope [37]. Consequently, the metal type, its ionic form in particular solution, and structure of on active binding site responsible for sequestering the metals are factors which should be included into the predicted model when influence on biosorption is concerned. It depends on the degree of affinity between different types of metal species or its ionic forms and the target site at the cell wall molecular structure [38]. Additionally, competition of other ions should not be ignored since there are some observations showing competition effects (i.e. in cadmium/iron system) [39].

Bioaccumulation is generally defined as active mode of metal uptake by living cells [3] and depends on its metabolic activity. Bioaccumulation process can be significantly affected by the presence of metal ions, which are accumulated. Additionally, the tolerance of yeast to the environmental concentration of metal ion is crucial for conducting bioaccumulation. Consequently, yeast ability to control the intracellular concentration of the ion in the range, which is not toxic for metabolic processes, have to be maintained for efficient bioaccumulation.

When a selection of suitable microorganism for metal bioaccumulation is in consideration, the yeasts showed to be advantageous among the microbial candidates, as they have been known to serve human needs for a long time. They can be exploited in metal removing processes from industrial wastes [40], as well as for human and animal nutrition since they belong to microbes which are traditionally recognized as safe [41]. However, the status of safe yeast is dominantly adopted for *Saccharomyces cerevisiae,* which is the common species present in food and feed production as well as in human and animal diet.

Nutrition has been very important in the last years in human and animal diet [11]. Mineral nutrition is one of the key issues in functional foods [21]. As an example of food mineral nutrition chromium can be selected. The chromium function as the key

element in glucose tolerance factor has been studied from different perspectives since 1957 [42]. In respect to chromium metabolism and function there are still many nonclarified situations, which additionally stimulate research in bioaccumulation. Majority of publications claims that it is an essential element for glucose and fat metabolism in mammals [12, 14, 16, 43–45]. There are not many informations about chromium tolerance, ionic nutrition [46], transport [2], biosorption [47, 48] and bioaccumulation in yeasts [49–51]. The natural chromium content in *Saccharomyces cerevisiae* is less than 0.07 μg/g dry mass [49], when its availability depends on natural substrates for growth. It is claimed that its structural function is the stabilization of the tertiary structure of proteins or nucleic acids on one hand and the metabolic function in glucose metabolism in mammals [12, 14] and yeasts [52–54] on the other hand.

Chromium as an ion of choice for yeast metabolism

Chromium is included on the list of essential trace elements for humans and some animals [45] and expresses at least beneficial effect in other living species tested including yeasts [52]. Depending on its form it can be toxic and even cancerogenic [55, 56].

Chromium belongs to the first series of transition metals. Its position in subgroup VI B of the periodic system is surrounded by three elements with a known biological function, like vanadium, manganese, and molybdenum. Chromium can occur in every oxidation state from 2– to 6+, but the most commonly found are 0, 3+, and 6+ [34]. The 2+ state does not exist in the biological system [12, 14, 44]. Tetravalent and pentavalent chromium oxidation states are rare and unstable, like divalent chromium. However, the electron precession resonance (EPR) spectroscopy has shown stabilization of the pentavalent complexes, formed via the reduction of hexavalent chromium with various biological thiols prior to DNA binding [57]. The hexavalent form is almost always linked to oxygen, and forms chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) ions, which are strong oxidants. The hexavalent chromium compounds demonstrate acidic properties and their colors range from yellow to orange [12, 14, 57]. This ionic complex has negative charge what makes it very suitable for transport by sulfate transporters into the cell. These ions are easily reduced to Cr(III) in acidic environment. Additionally, the presence of reductants in the surrounding has been proved to be of extreme importance. In this regard, the case of iron(II) can serve as demonstration of appropriate reductant efficiency [58]. In some yeasts and various fungi, though not *Saccharomyces cerevisiae*, special metal-siderophore interaction occurs (*Rhodotorula* – rhodotorulic acid siderophores) [59–61], which has the function

to promote bioaccumulation of particular ions. Originally, siderophores are chelating agents excreted by many microorganisms to facilitate the uptake of ferric ions [61]. However, there is some evidence that chromium(III) can also be inserted into siderophores. Resulting Cr(III)-siderophore complexes are kinetically inert (i.e. ligand exchange is slow) [60].

The trivalent state of chromium is thermodynamically the most stable and is commonly found in the living systems. Trivalent chromium has a strong tendency to form co-ordination compounds, complexes and chelates. Its co-ordination number is 6, and it can bind ligands to form hexaco-ordinate or octahedral complexes. Consequently, this ionic complex has positive charge what makes it less suitable for transport into the cell. Free chromic ions do not exist in aqueous solutions. They are always co-ordinated, either with water or with other ligands in the solution. Metal-ligand bonds may involve oxygen, nitrogen or sulfur [12, 14, 44, 57, 62].

At neutral or basic pH, Cr-H_2O bonds are modified and Cr-OH^- bonds are created, leading to the formation of giant macromolecules (polymerization of hydrated Cr – known as olation) that precipitate and are thus biologically inert. Oxalate ions and other strong ligands can prevent and reverse olation, while weaker ligands like pyrophosphate, methionine, serine, glycine, leucine, lysine and proline prevent the reaction at physiological pH, what enables functioning of chromium in cell metabolism [12, 14, 57].

Following the message of Mertz [12], the most important oxidation state of chromium in biological systems is 3+ and is therefore not likely to participate in oxidation/reduction reactions. Furthermore, chromium(III) occurs mostly in octahedral configuration with coordination number 6 and its relative inertness of the chromium-ligand bond makes it suitable for structural function, like the stabilization of tertiary structures in proteins or nucleic acids.

The yeast tolerance to metal ions is key parameter in the conditions when yeasts are grown in media loaded with heavy metals. The variation in tolerance or resistance among yeast species can be the result of different cell wall compositions [35]. There is neither much data about the microbial requirements for chromium nor available data for chromium biosorption and bioaccumulation. Frequently it is regarded as a toxic element to which some microorganisms have evolved resistance mechanisms [64].

Most common sites of chromium binding in yeasts are in the cell walls and cell membrane structure. In yeast cell the compounds such as pyrophosphate, polysaccharides, proteins, amino acids (Table I) are potential donors of ligand donor groups [12, 27, 35, 38, 65]. They act as ligand donor groups through oxygen, nitrogen and sulfur [66].

Table I

Amino acids with metal-binding groups in protein [adapted from 38, 60, 63]

Amino acid	Side chain	Character	Metals-binding
Alanine	$-CH_3$	Neutral	/
Arginine	$-CH_2CH_2CH_2NHC(NH_2^+)(NH_2)$	Basic	*
Asparagine	$-CH_2CONH_2$	Neutral	Ca
Aspartic acid	$-CH_2COOH$	Acidic	Ca, Zn, Fe, M(II), M(III)
Cysteine	$-CH_2SH$	Neutral	Zn, Cu, Fe, Ni, Mo
Glutamic acid	$-CH_2CH_2COOH$	Acidic	Ca, Zn, Fe, M(II)
Glutamine	$-CH_2CH_2CONH_2$	Neutral	Fe
Glycine	-H	Nonpolar Hydrophobic	Cr
Histidine	$-CH_2C_3H_3N(NH^+)$	Neutral Basic	Zn, Cu, Mn, Fe, Ni *
Isoleucine	$-CH(CH_3)CH_2CH_3$	Nonpolar Hydrophobic	/
Leucine	$-CH_2CH(CH_3)(CH_3)$	Nonpolar Hydophobic	Cr
Lysine	$-CH_2CH_2CH_2CH_2NH_3^+$	Basic	*, Cr
Methionine	$-CH_2CH_2SCH_3$	Nonpolar Hydrophobic	Cu, Fe, Cr
Phenylalanine	$-CH_2C_6H_6$	Nonpolar Hydrophobic	/
Proline	$-CH_2CH_2CH_2NH_2^+$	Nonpolar Hydrophobic	Cr
Serine	$-CH_2OH$	Neutral	Ca, Cr, M(I)?
Threonine	-CH(OH]CH_3	Neutral	Ca, M(I)?
Tryptophan	$-CH_2C{=}CH(NH)C_6H_4$	Nonpolar Hydrophobic	/
Tyrosine	$-CH_2C_6H_4OH$	Neutral	Zn, Fe, Mn, M(III), Ca
Valine	$-CH(CH_3)(CH_3)$	Nonpolar Hydrophobic	/
γ-carboxyglutamic acid	$-CH_2CH(COOH)_2$		Ca
β-hydroxyaspartic acid	-CH(COOH)(OH)		Ca

* Anion binding

Additionally, it was found in higher organisms that chromium can act as a substitute for magnesium (Mg(II)) in protein (enzyme) sites [38]. It is well known that many ions can interfere with each other [34]. In the case of chromium the action of nickel (Ni(II)) can be compared with chromium action because their co-ordination chemistry tends to be the same, both prefer octahedral co-ordination, and aluminium

(Al(III)) which, like chromium, has a slow rate of ligand exchange and molybdenum (Mo(III)) with a range of octahedral complexes, which resemble compounds of Cr(III) [38].

Regarding chromium as an ion of choice for yeast metabolism, from the mentioned information one could expect that there might be some important activities still to be elucidated.

Chromium accumulation in yeast biomass

Already in 1978 it was noticed that the yeast *S. carlbergensis* was able to uptake chromium from the medium with (^{51}Cr-labelled) chromium chloride. The 5% (w/v) glucose supplement increased the uptake of chromium into the yeast cell. Further glucose feeding to the growth media during cultivation did not improve chromium chloride uptake [67]. Their comments that diffusion is the only transport system into the cell have not been discussed since then. They based their conclusion on repeating glucose feeding. Much later it was reported that chromium(III) has to be organically bounded to be able to pass through the cell membrane [32]. Final explanation is still needed. For chromium(VI) it is claimed to use sulfate and phosphate transport route. Sulfate transporter has a function to translocate chromium(VI) as chromate (CrO_4^{2-}) through the membrane into the cell because sulfate space conformation is very similar to chromate [68, 69]. Since first isolation of cDNA encoding High affinity sulfate transporter [70] there were published also other genes involved in transporting sulfate as well as chromate [71].

The complex structure of yeast envelope [37] protects vital processes in the cell. Chromium ion has to pass different barriers, the cell wall and membrane from the environment to the cell interior [72]. In the translocation process of metal ions into the yeast cell values for pH, temperature, metal's biological availability, etc. should be considered as the most important environmental parameters. When the intracellular metal ion concentration rises to the critical level, it can interfere with vital processes resulting in cell death in the mechanism of metal ion translocation [2, 73].

The metal ions uptake is essentially a biphasic process consisting of a metabolism-independent and a metabolism-dependent step. The initial biosorption step for metal ions is rapid [74, 75], typically only a few minutes in duration [76], and is independent of temperature [77]. In the case of chromium it was found a correlation in yeast *S. cerevisiae* between pH and chromium(III) uptake at different starting pH values. The studied pH range from 2 to 6 showed chromium uptake up to 1.54 μmol/g dry biomass which was conducted at 28 °C for 4 hours in gently mixing conditions

(200 rpm) [78]. Under the applied experimental conditions the cell surface deposition capacity for chromium (CSDC-Cr) of the yeast cells increased with the pH. The initial binding step is thought to involve the microbial cell surface structure (e.g. cell wall components – polysaccharides). This agreed with the observation for other metal ions in the same pH range [79]. Complex growth media reduce bioavailability of the chromium(III) ion for yeast metabolism. pH value around 6 additionally enhances the problem of olation mentioned earlier. To avoid the problem of chromium(III) olation in complex aqueous media the pH 4 was found to be suitable for yeast cultivation [80].

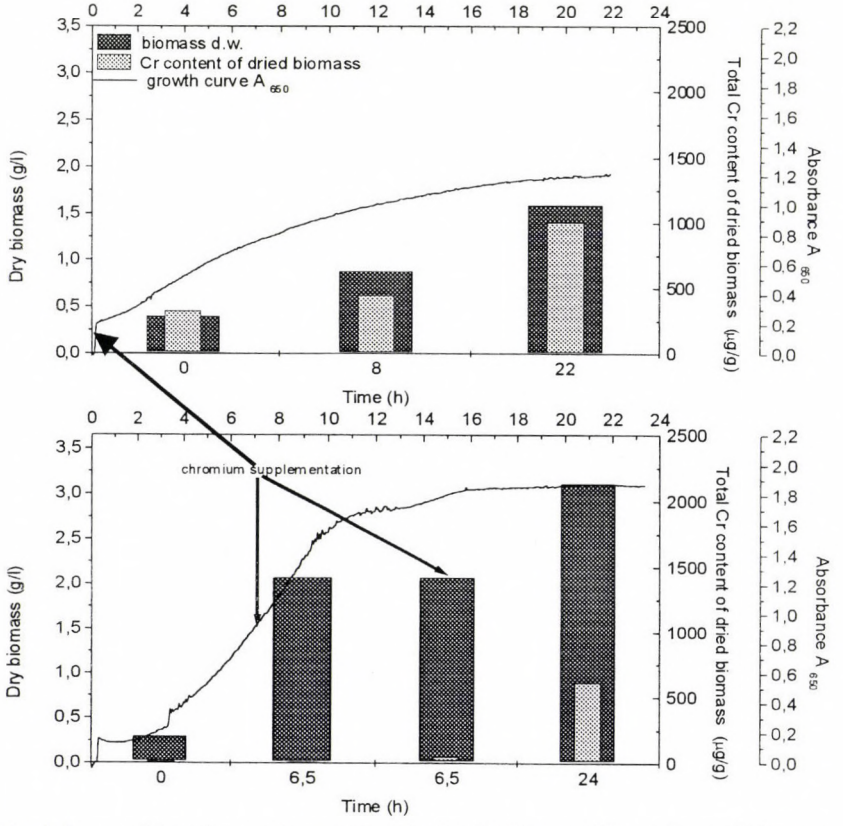

Fig. 1. Impact of chromium supplementation (5 mM Cr(III))in two different phases of bioprocess (at the beginning and in the exponential phase) on its uptake in aerobic cultivation of *Candida intermedia* ZIM 156 (82)

In the absence of the metabolic activity non-viable biomass can still sequester metal ions from surroundings by biosorption [74, 77]. Chromium biosorption capacity of non-viable yeast biomass (heat inactivated) compared to viable counterpart from the same source was 2.5-fold higher [81] what is in favour of technological application in environmental technology (i.e. removing chromium from water streams) when low concentration of metal ion is to be eliminated.

When metal/biomass ratio was below 0.1 μmol/g dry mass Brady and Duncan [75] claimed that metal ion uptake to biomass was dependent on biosorption. *S. cerevisiae,* when glucose was added to the system where biomass and chromium were in the suspension, reached the ratio 0.577 μmol of chromium per g dry yeast biomass, what indicates synchronous biosorption and bioaccumulation. Consequently, in viable yeast biomass chromium uptake was enhanced up to 5-fold [80, 81].

Studying chromium uptake in yeast, biosorption is not of primary importance over bioaccumulation, because chromium enriched biomass by cultivation was found more suitable for food and feed consumption.

If one takes into account mechanism for metal bioaccumulation [9], then active transport indicated is in response to electrochemical proton gradients, generated by membrane-bound H^+-ATPases, across the cytoplasmic and vacuolar membranes. Experimental results suggested the involvement of metabolic activity in chromium(III) ion bioaccumulation. Namely, concentrations of chromium ions higher than 2.40 mM stimulated a more intensive extrusion of H^+ ions from yeasts and glucose even enhanced the proton export out of the cell [80]. Additionally, it was found that some heavy metals can release other cations from the cell (i.e. K(I), Mg(II)). This was observed by exposure of yeast cells to copper [79] but is no data for chromium available.

However, there is no other information whether known metal uptake systems have the capacity to transport chromium(III) ions or whether specific chromium ion transport into the cell even exists. Appropriate timing for chromium supplementation into growth media at different yeast growth phases was proved to be important for chromium bioaccumulation. The results showed that yeasts growth at higher chromium concentration added (10 mM) at the beginning of the bioprocess was more inhibited but chromium(III) uptake into yeast biomass was higher.

Addition of chromium in the middle of the exponential growth phase had no notably effect on yeasts' growth but resulted in approximately 30% lower chromium uptake. Cultivation of yeast *C. intermedia* ZIM 156 in 10 mM chromium(III) doubled (2400 μg/g dry mass) its uptake in comparison to 5 mM concentration in the medium [82]. Similar experiment with *C. utilis* showed growth inhibition at lower concentration [50].

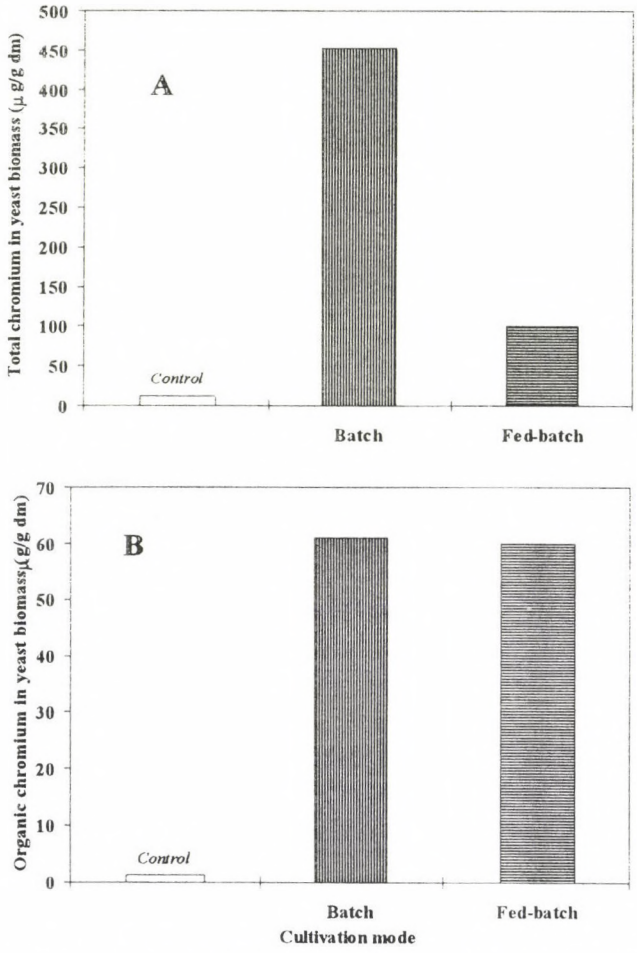

Fig. 2. Concentration of chromium in biomass of *Candida intermedia* ZIM 156 in batch and combined batch/fed-batch culture in medium supplemented with chromium(III) (100 μM $KCr(SO_4)_2 \cdot 12H_2O$) after 20 hours cultivation (Total chromium (A) and organically bounded chromium (B)) [51]

Yeast growth and chromium bioaccumulation in biomass was studied in batch, fed-batch and continuous culture. Regarding chromium effects on biomass production, the batch cultivation mode was found to be better than combined batch/fed-batch. According to Kosman [83] this can be attributed to biosorption capacity of yeast biomass and better uptake of chromium in the beginning of the exponential growth

phase. Such behaviour favors batch over combined batch/fed-batch operating mode in bioaccumulation of chromium(III) [78].

Consequently, total chromium accumulation in batch cultivation was 4.5-fold higher compared to combined batch/fed-batch cultivation mode with yeast *C. intermedia* ZIM 156 [78]. On the contrary, combined batch/fed-batch cultivation mode was found to be beneficial over batch biomass production because of high concentration of organically bounded chromium in yeast biomass and technologically suitable specific growth rate (μ_{max}=0.26 h^{-1}).

The high efficiency of this bioconversion can be seen through total vs. organically bounded chromium ratio in yeast biomass of *C. intermedia* ZIM 156 (Fig. 2) [51]. The organically bounded chromium is considered to be the fraction, which can be extracted from total yeast biomass with NH_4OH [84].

When combined batch/fed-batch cultivation mode is analyzed, yeast *C. intermedia* ZIM 156 was found to be capable of chromium bioconversion into organically bounded chromium fraction as high as 60%. Combined batch/fed-batch cultivation mode showed a 9-fold better chromium ratio in spite of a 22% lower total chromium concentration in yeast biomass.

On the other hand *C. intermedia* ZIM 156 cultivated in batch culture was able to bioconvert 12%, when compared to yeast *S. cerevisiae* ZIM 198 cultivated in the same conditions which was able to convert 23% of chromium which came into the cell into organically bounded fraction. The fraction of organically bounded chromium (org. Cr/tot. Cr) remained constant (6%) in yeast biomass when supplementation with chromium(III) cross 1.44 mM. In bioprocess with 0.96 mM chromium(III) *Saccharomyces cerevisiae* was metabolically capable to incorporate 3.9-fold higher portion of chromium into organically bounded fraction what had a consequence in chromium concentration factor (CCF) in yeast biomass which attained 236-fold higher value than in yeast biomass from control media (without chromium(III) supplementation).

The highest intracellular chromium binding capacity was found in *S. cerevisiae* for organic fractions in the molecular weight range from 10,000 to 100,000 and more [78, 80]. Under normal conditions (no addition of chromium to growth media) chromium distribution was observed in a range of molecular weights between 10,000 and 3000 (Fig. 3). Many cytoplasmic biomolecules have the ability to bind metal ions. The diversity of intracellular organelles provides a wide range of potential binding sites [38, 63, 85].

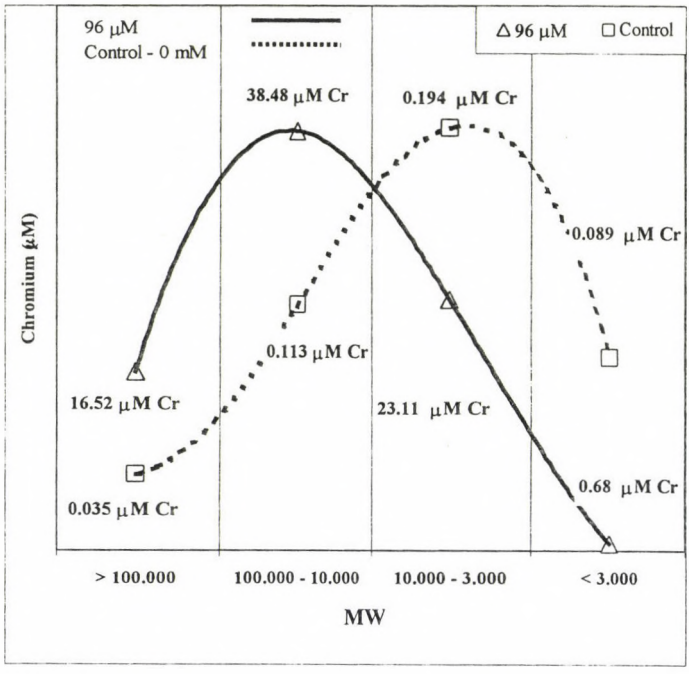

Fig. 3. Chromium organically bounded incorporation factor of different molecular weight fractions of yeast *S. cerevisiae* grown in continuous culture with 28 μM of glucose at dilution rate 0.08 h^{-1} in medium loaded with 96 μM of chromium [80]

Regarding chasing chromium distribution among biomolecules similar results were obtained in biomass of yeast *C. intermedia* isolated as intracellular low-molecular-weight chromium-binding biomolecules [86]. Determination of chromium in cell fractions of biomass collected during cultivation in chromium enriched medium revealed that it was present in all collected chromatographic peaks, however, chromium was unevenly distributed. The comparison of low molecular weight fractions in chromium enriched biomass with control sample showed remarkable difference in chromium distribution and its loading. Fractions with highest chromium concentrations were found in molecular weight range from 1500 to 7000. Two distinguishable peaks in this range contained the highest amount of chromium [86].

Binding sites of chromium, compartmentalization, localization in the cell and finally incorporation processes into yeast biomass are rather complex processes and many details remain to be elucidated. On practical scale we gained some experience to push yeast to accumulate chromium. There are many patents claiming efficiency of

their bioprocessing strategy for yeast biomass enrichment with chromium [87–91] and just few publications [49, 50, 92], what confirm strong economical interest for this product in the future. This is not unusual since yeast biomass enriched with chromium is a source of GTF molecule (glucose tolerance factor) [42, 52, 93–96]. GTF molecule is actually a Cr-binding molecule, which influences glucose metabolism in humans. Some authors claim that GTF activity is correlated with the amount of chromium accumulated in yeast [97] but so far this observation is not clearly proved. Moreover, GTF function in yeast metabolism seems to be important, since there are experiments showing that GTF enhances glucose consumption also in yeast metabolism when added to the growth medium [98, 99].

However, its structure remains to be discovered in spite of many attempts in the past [54, 100–106]. The challenge of GTF is still not completely elucidated with its function in glucose and lipid metabolism [107, 108], in particularly to actual active substance in its molecule [54, 100–104] and with regards to its mechanism [106, 109–111].

Due to the importance of GTF product there have been some attempts to make GTF biosubstitutes as already mentioned [112].

Table II

Chromium concentration in yeast biomass [113, 114]

Yeast	Chromium concentration (μg/g dry mass)
S. cerevisiae	0.7–4.4
C. lipolytica	1.6–3.0
C. boidinii	1.1–5.1
H. platypodis	3.2
P. kluyveri	3.0
Y. lipolytica	0.22
S. starkeyi-henricii	0.19

The natural source of chromium (i.e. enriched yeast biomass) was proved to be more beneficial to glucose metabolism than Cr-picolinate [115, 116] or other Cr-compounds [117–120]. Even more, some publications claim that Cr-picolinate can be cancerogenic [121, 122], what is not the case with chromium enriched yeast biomass [123]. Following current results in the literature and our own results it is obvious that the majority of research is focussed on yeast in aerobic conditions. Anaerobic

environment, in spite that it is widely extended in technology, is not comprehensively approached in the past. The presence of chromium in yeast biomass originated from anaerobic bioprocesses was also studied in traditional process of must fermentation and it was found that chromium had beneficial effect in *S. cerevisiae*. However, stronger production of the reduction compounds were found, consequently [124].

Effect of chromium on yeasts' viability

Chromium can have different effects on yeast metabolism. It can be beneficial if the concentrations are in physiological range. Deficiency symptom from the lack of chromium is not known for yeast, but toxic effects from the excess of it can be recognized clearly with growth inhibition or with analyzing particular biomolecules in the cell (i.e. DNA, RNA, proteins).

Since chromium and also some other metal ions are both essential and potentially toxic, their intracellular concentrations are the subject of precise homeostatic regulation [6].

As chromium is important for humans there were some attempts in the past to isolate yeast with high tolerance to it [125–129].

The problem of metal ions' toxicity and/or resistance is discussed in the literature [19, 57].

The adequate methodology for microbial bioassays for the detection of metal toxicity is important [130–132], since different approaches can give different results, which are not comparable at all. If one wants to optimize growth medium, the tolerance to particular metal ion for selected *yeast* species has to be determined [64]. The survival of living cells depends on their ability to react to alterations in the environment and to appropriately respond to the newly emerged conditions [133]. Knowledge of stress and stress responses is crucial to understand how single-cell and multicellular organisms adapt to changing environmental and physiological conditions [134, 135]. The molecular mechanisms induced upon the exposure of cells to such adverse conditions are commonly designated as stress responses. The aim of stress response mechanisms is to protect cells against the potentially detrimental effects of stress challenges and to repair any molecular damage, and to adjust the metabolism and other cellular processes to the new status [133]. These stress responses involve aspects of sensing, signal transduction, transcriptional and post-translational control, protein-targeting to organelles, bioaccumulation of protectants, and activity of repair functions [136].

Redox metals play a major part in the generation of reactive intermediates (free radicals, other reactive intermediates) in the cell [19, 133] which are potentially the most dangerous for the cell.

To avoid the problems of stress and consequently toxicity of ions in cell surroundings it is crucial to know yeast tolerance limit and spectrum of ions dangerous for the cell. In case of chromium the screening procedure on agar plates based on concentration gradients was developed (fast and reliable), which can serve for rapid assessment of yeast tolerance to potentially toxic metal ions [128, 137, 138].

These screening tests showed great inter- and intra-generic differences among 23 yeast genera and 49 species. Yeasts from genera *Saccharomyces, Candida, Hansenula* and *Kluyveromyces* represented more than 50% of strains tested. The tolerance to chromium(III) was found to be normally distributed and 6% of screened population could tolerate up to 6.9 mM Cr(III) concentration in the medium. Majority (approx. 40%) of yeast strains tolerated up to 5.8 mM and just 4% showed tolerance below 1.5 mM Cr(III) [138].

The mathematical model was established with an aim to characterize yeast viability and mortality.

On the other hand, if more precise data are needed, an advanced procedure may be introduced for precise measurement of yeast viability or mortality. It is more time consuming, but it gives more concise information on yeast characteristics [140]. Viability was defined with four parameters: population condition, cell viability in the buffer and cell viability in the buffer supplemented with the effector and specific cell viability. Mortality was defined with three parameters: cell mortality in the buffer, cell mortality in the buffer supplemented with the effector and specific cell mortality. Parameters were calculated on the bases of total cell count per milliliter and number of viable cells per milliliter in each experimental step.

This model was tested for five chromium(VI) compounds on seven selected yeasts (Fig. 5). The study elucidated that physiological status of yeast population from particular species is not comparable in spite of equal treatment during cultivation.

Population condition demonstrated this status with values in the range from 2.78 to 48.58% in theoretical scale of 100%. With this model one is able to draw conclusion of chromium to yeast viability/mortality as can be seen in Figure 4.

Six chromium(III) compounds were assessed by the same model (Fig. 6). Studies of chromium(III) compounds revealed deviations, which could be attributed to different chemistry of chromium(III) and chromium(VI) compounds in aqueous solution [73, 140].

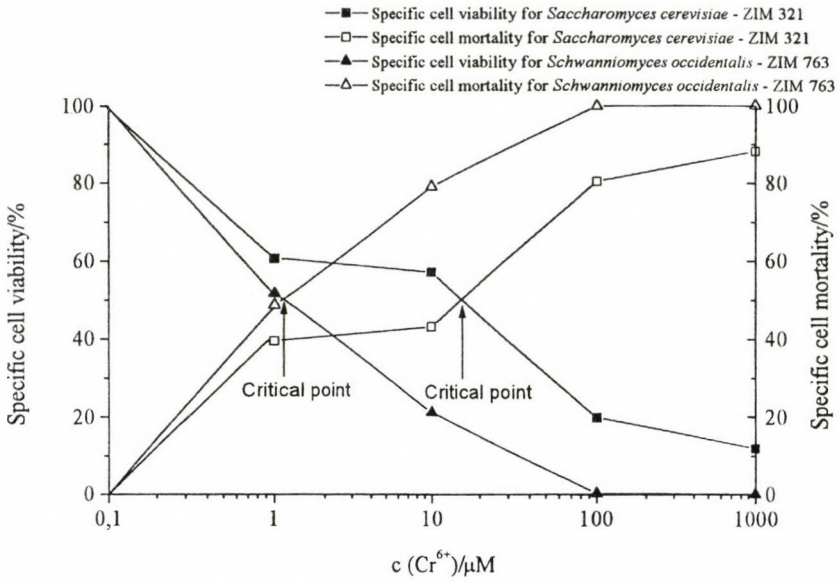

Fig. 4. Model for graphical determination of specific cell viability and specific cell mortality at different concentration of Cr(VI) for yeasts *Saccharomyces cerevisiae* ZIM 321 and *Schwanniomyces occidentalis* ZIM 763 [140]

The aim of these experiments was also to compare toxicity of chromium(III) to chromium(VI) compounds, which are known to be more toxic. The results showed that different species of yeast can exhibit noticeable differences in their ability to tolerate higher concentrations of chromium(III) and chromium(VI).

$CrCl_3 \times 6H_2O$ and $Cr(NO_3)_3 \times 9H_2O$ exhibited the highest toxicity in the case of *Saccharomyces cerevisiae* ZIM 753 and *Candida intermedia* ZIM 156, while very low concentrations of all Cr(III) compounds except $KCr(C_2O_4)_2 \times 3H_2O$ were toxic to *Saccharomyces diastaticus* ZIM 152. However, all tested yeasts showed the greatest survival in the case of $KCr(C_2O_4)_2 \times 3H_2O$ [141].

We found *S. cerevisiae*, *S. paradoxsus and C. intermedia* to be the most tolerant where an average specific cell viability for all Cr(VI) compounds at the highest Cr(VI) concentration of 1000 μM was in the range from 13–25% and 0–2% in the case of less tolerant yeasts *S. bayanus*, *S. pastorianus, S. diastaticus* and *S. occidentalis*. On average the most toxic compound for all yeasts at all Cr(VI) conc. was $KCrClO_3$ and the least toxic compound was K_2CrO_4 [139, 141].

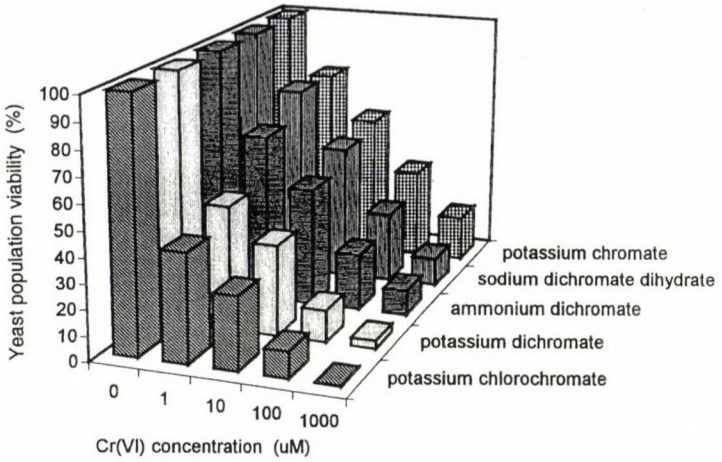

Fig. 5. Yeast population viability calculated from the data for yeasts included into the study (*S. cerevisiae*, *S. paradoxus, C. intermedia, S. bayanus, S. pastorianus, S. diastaticus, S. occidentalis*) presented for particular Cr(VI) compounds [139]

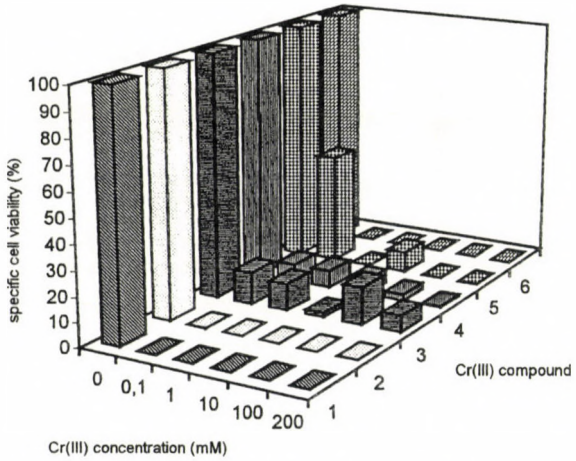

Fig. 6. Effect of higher concentrations of Cr(III) compounds: 1 = $CrCl_3 \times 6H_2O$, 2 = $Cr(NO_3)_3 \times 9H_2O$, 3 = $CrK(SO_4)_2 \times 12H_2O$, 4 = $KCr(C_2O_4)_2 \times 3H_2O$, 5 = $Cr(CH_3COO)_3$, 6 = Cr-citrate on specific survival of yeast *Candida intermedia* ZIM 156 [141]

This might be related to the involved mechanism of detoxification [3, 142], which is based on intracellular quarantine in particular compartment or on blocking transport into the cell.

The most common essential amino acid used in the detoxification of many metals is cysteine, which is found in some yeasts as a dominant residue in the thioneins, or as a component of the γ-glutamyl peptides which may bind metals. Another possibility is that these proteins might be involved in pumping out of the cell. However, there are some indications in this respect for some metabolically important metals like Zn(II) [35], but is no data for chromium so far.

Detoxification mechanisms are studied by some researchers lately and they showed that vacuole is involved in this action in normal metabolism in the cell detoxification. When vacuolar-lacking mutants of *Saccharomyces cerevisiae* were exposed to chromate, they displayed higher sensitivity. However, chromium is bioaccumulated into the cytosolic compartments and not to vacuole also in wild strains [143].

One approach for studying toxicity and tolerance of yeast to chromium(VI) or chromium(III) can be established analytically with a goal to determine intracellular components, which turn on or disappear during prolonged exposure to chromium. During the cultivation of *S. cerevisiae* at dilution rate (D) 0.08 h^{-1} chromium ions exert a harmful effect on yeast cell growth, cell protein and RNA concentrations [144], what was also the case in batch growth [145]. The toxic effect of chromium ions was found as 21% reduction of biomass, 9% reduction of protein and 22% reduction of total cell RNA during continuous cultivation. In batch cultivation, the effect of chromium ions was recognized in growth rate reduction and extension of lag phase [80].

Analyzing distribution of chromium in the cell showed that fractions with molecular weight higher than 100,000 support the mechanism of detoxification at high concentration levels of intracellular chromium in *S. cerevisiae*. Determination of chromium in this cytosolic biomolecules with high molecular weights supports the idea that fast binding of toxic intracellular chromium ion is possible. This is the generally accepted mechanism of action of other heavy metals [146].

Among cell biosynthetic products, metallothionein, the specially designed proteinous molecules for attracting metals (especially toxic ions) are discovered. One group of metallothionein-like molecules found in yeast *S. cerevisiae* is the most studied low molecular weight-cysteine-rich protein for Cu binding [147]. Lately, Ballatori claimed glutathione mercaptides as transport forms of metal ions in the yeast cells [148].

Effect of chromium on yeast genome

Toxicity of chromium is discussed elsewhere [55, 149, 150] since it is important in industry and in life processes. However, interference and all positive and/or negative actions of chromium compounds within the yeast cell and its metabolism is focussed on genome since cumulative mutation can effect future development and expression of genetic informations in DNA structure.

According to early observation, chromium (VI) is areactive towards DNA under physiological conditions *in vitro* [69]. However, *in vivo* the reduction of chromium(VI) by cellular reductants in cell culture (the microsomal cytochrome P-450 system, mitochondrial electron transport chain complexes, aldehyde oxidase, hydrogen peroxide, scorbate and thiols) to the reactive intermediates, including Cr(VI) thioester, Cr(V), Cr(IV) species and free radicals (hydroxyl and thiol radicals), is thought to be an important step in the mechanism of Cr(VI) induced DNA damage [69, 151–155].

There are many publications dealing with DNA lesions induced by chromium(VI) *in vivo* as well as chromium(III) compounds *in vitro,* but they seldom use yeast as a model organism. One can find publications on Cr-DNA adducts, DNA-DNA cross links (DDC) [156, 157], DNA-proteins cross links (DPC) [158] and DNA brakes- single and double [68, 56, 154, 159–161], proving direct reactivity of chromium with DNA. Some of them are dealing with an interference of chromium(III) compounds *in vitro* with enzymes in DNA processing (i.e. polymerase) [152, 162], or with chromium bounded to DNA on the way to alter cleavage side for restriction endonucleases [163]. Many publications deal with free radicals in gene toxicity. Combination of hydrogen peroxide and chromium(III) and chromium(VI) showed DNA damage mediated with Fenton-like reaction [164–166]. Further interesting observation that metal ion-dependent hydrogen peroxide-induced DNA damage is more sequence specific than metal specific opened new dimension of the problem [167].

Few publications claim interference with particular system included in detoxification in the cell. Alcedo and co-workers found chromium(VI) altering metal-inducible expression, but not basal expression of the metallothionein gene. They used this as a proof for suggesting that chromium(VI) has priority to inducible genes [168].

Regarding mutagenesis in yeast there were many publications dealing with the problem of chromium twenty years ago. The majority of them were oriented just to detect mutagenic effect of chromium in different preparations [117, 120]. However, there are some publications, which gave suitable start for further research in the field, like induction of petit mutants by calcium chromate. Its toxic and mutagenic effects on the mitochondria of *S. cerevisiae* were showed [169, 170]. In the same organism,

induction of reverse mutation and mitotic gene conversion by some metal compounds was demonstrated [171].

In the last decade, rapid estimation of chromosomal damage in yeast (i.e. radiation-induced) has been evaluated by pulsed field gel electrophoresis [172–174]. The effects of chromium(VI) compound $K_2Cr_2O_7$ can be visualized in the same manner [175]. PFGE together with densitometry offers opportunity to follow DSB (Double-Stranded Brakes), which were detected as smear of DNA on the gel after yeasts were exposed to chromium(VI). They found that in *in vivo* conditions chromium(VI) compound $K_2Cr_2O_7$ induced double-stranded brakes of chromosomal DNA in all *S. cerevisiae* tested.

Further studies revealed that recombination repair (gene Rad51) reduced gene toxicity of chromium compounds in exponential growth phase for all applied concentrations up to 20 mM of chromium compounds. Excision repair of DNA damage (gene RAD1) was found in the stationary growth phase as crucial for reducing gene toxicity of $K_2Cr_2O_7$.

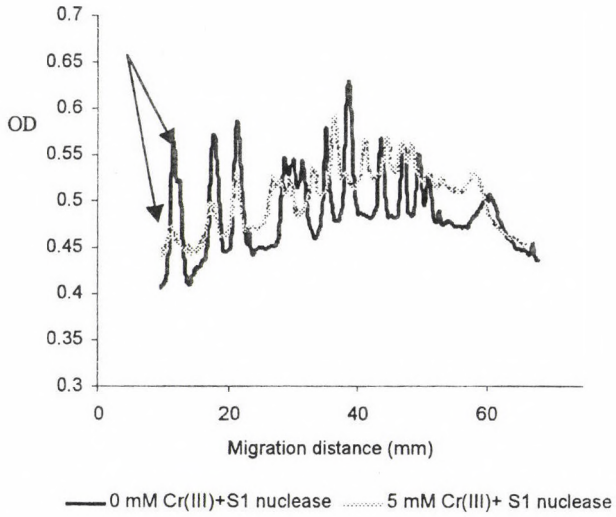

Fig. 7. The effects of chromium(III) compound $K_2Cr_2O_7$ on chromosomal DNA in yeast *S. cerevisiae* ZIM 465 *in vitro* (S1-detected through S1 nuclease activity-cutting SSB on DNA; OD–Optical density) [176]

Additionally, the cells were exposed to chromium(VI) in exponential growth phase and they evidently have excision repair mechanism at high concentration range.

Since lethality is correlated to chromium concentration, the study of cell response showed gene RAD18 responsible for mutation repair. This gene is active in exponential growth phase, but not in stationary phase [176]. As a consequence of $K_2Cr_2O_7$ treatment, chromosomal aberrations were found in *S. cerevisiae in vivo*, specifically on one chromosome (Figure 7). Proposed chromium induced damage was confirmed by survival test with rad51 mutant, since rad51 mutant has no mechanism of repair as is common in wild strain, consequently their survival was strongly reduced. Single-stranded brakes (SSB) were tested in *in vitro* conditions with chromium(III). Nuclease S1 showed to be appropriate tool for detection of spots at chromosomal DNA in *S.cerevisiae* where this DNA damage could occurred. Results suggested that chromium(III) induces structural changes in DNA structure and consequently SSB [176]. Today it is stated that chromium-induced DNA damage can be repaired after mutagenesis [177, 178], since repair mechanism are not damaged by chromium oxidation.

Cells have evolved a number of capabilities to repair macromolecules that are damaged and to inactivate reactive intermediates. Yeast cells contain enzymatic as well as non-enzymatic defense mechanisms against the harmful effects of reactive deviates. The genes encoding components of this defense apparatus, such as those involved in glutathione or thioredoxin biosynthesis, display increased expression after stress challenge. Glutathione and thioredoxin are normally involved in cellular redox-reactions, in particular those involved in the establishment and maintenance of the tertiary structure of proteins [133].

Conclusions

Chromium is an element in yeast metabolism, but when accumulated in excess, becomes toxic. The form and concentration of chromium in the macro- and microenvironment of the yeasts are important. Chromium(VI) with its negative charge enters cell more easily than chromium(III), which is positively charged in physiological conditions. However, all the transport mechanisms are still not clearly defined. Since toxicity is connected with chromium concentration, its manifestation in metabolism is proportional with amount, which can enter the cell and interfere with biological functions. DNA was showed to be damaged by chromium(VI) *in vivo* and chromium(III) *in vitro*. Single-stranded and double-stranded brakes are induced by chromium. During cultivation in batch, fed-batch and continuous processes reduction of RNA, proteins and biomass yield are present. However, there are yeasts, which tolerate higher concentrations of chromium and are more suitable for bioprocessing.

Their fast screening can be conducted by the gradient agar plate method, followed by cell viability/ mortality method, which select adequate yeast. Yeast biomass showed to be able to uptake high amount of chromium by biosorption and bioaccumulation. The ratio between total and organically bound chromium was good. However, the function of chromium in glucose tolerance factor and role of chromium in yeast metabolism remains in focus of current research. The question if metallothionein-like complex, which is able to bind chromium in yeast remains to be opened for further research.

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HIGH PRESSURE PROCESSING OF FOODS FOR MICROBIOLOGICAL SAFETY AND QUALITY*

(A SHORT REVIEW)

M. LINTON[1], MARGARET F. PATTERSON[1,2]

[1]Department of Agriculture for Northern Ireland and [2]Department of Food Science, Food Science Division, The Queen's University of Belfast, Newforge Lane, Belfast BT9 5PX, Northern Ireland, UK

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Abstract

Consumers are demanding foods that are "natural", of good nutritional and sensory quality, free from chemical preservatives, microbiologically safe and with extended shelf-life. High pressure processing can, potentially, meet these criteria. Recent advances in equipment design now allow foods to be processed up to 900 MegaPascals (130,000 psi). However, further work is required to more fully understand the factors that can affect the response of microorganisms, including pathogens, to pressure so that treatments can be optimised and microbiological safety can be assured. This paper describes how the pressure resistance of microorganisms can vary depending on factors such as species, strain, stage of growth and food composition. Strategies for overcoming the problem of pressure resistance will be discussed, for example the use of pressure cycling and the combination of pressure with mild heat. The current commercial uses of high pressure to preserve foods will be reported and potential applications will also be discussed.

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Introduction

High pressure processing of foods is not a new concept. As far back as 1899 Hite [1] was investigating the use of high pressure for the preservation of milk. Recent advances in high pressure technology and the search for alternatives to the traditional heat processing of foods has led to a renewed interest in high pressure for extending the shelf-life and ensuring the microbiological safety of foods.

At present the products which are produced by high pressure processing are mainly high acid fruit products such as juices, jams, jellies, sauces and purees [2]. In Japan, for example, semi-continuous high pressure systems with a capacity of up to 6,000 l/h are used for the bulk treatment of citrus juices. Yeasts and moulds, which are the main cause of spoilage in these products, can be inactivated using relatively low pressures and shelf-life can be extended without adversely affecting colour and flavour and with only a slight decrease in vitamin C content [3]. Other pressure processed foods commercially available include fish and shellfish, ham, rice products and guacamole [4, 5]. As the understanding of the effects of high pressure on foods increases and the conditions required for microbial inactivation are determined, the range and types of food processed by high pressure should increase.

Units of pressure

The SI unit of pressure is the Pascal. 1 Pascal (Pa) = 1 Newton/metre^2

100 MPa ~ 1000 atmospheres ~ 1 kbar ~ 15,000 psi. Typically the pressures used in the treatment of foods are 100–1000 MPa.

Principles of action

There are two general scientific principles of direct relevance to the use of high pressures in food processing: (a) high pressure stimulates reactions that result in a volume decrease but retards reactions that involve a volume increase (Le Chatelier principle) and (b) pressure is transmitted in a uniform and instantaneous manner throughout the whole biological sample, whether the sample is in direct contact with the pressure medium or hermetically sealed in a flexible package that transmits pressure (the isostatic rule) [6]. Thus, the breaking of ionic bonds is enhanced by high pressure as this leads to a volume decrease due to electrostriction of water in the proximity of the ions. Hydrogen bonds tend to be stabilised by high pressure since their

formation involves a volume decrease. The effects of high pressure on hydrophobic interactions depend on the intensity of pressure applied. Hydrophobic interactions are disrupted at pressures below 100 MPa. However, above 100 MPa (as in most food applications) hydrophobic interactions tend to be stabilised due to a volume decrease. Covalent bonds are generally unaffected by pressure [7]. Pressure can therefore disrupt the three dimensional structure of larger molecules or cell structures (proteins, including enzymes, lipids, cell membranes, etc.) but have no effect on small covalently bonded molecules such as vitamins, flavour components and some pigments. The disruption of the various structures leads to the inactivation of microorganisms and enzymes and can induce textural changes in foods.

Factors affecting inactivation of bacteria by high pressure

High pressure inactivation of microorganisms is not clearly understood but normally involves a perturbation of the cell membrane. This has been shown in pressure treated cells by an increase in extracellular ATP [5] and by increased uptake of ethidium bromide and propidium iodide [8]. Further evidence of the importance of the membrane in pressure inactivation can be seen in the dependence of pressure resistance on the fluidity of the cell membrane. For example, *Lactobacillus plantarum* cells which were grown at sub-optimal temperature showed an increase in unsaturated fatty acid content and were more resistant to pressure [9].

Other mechanisms of action which may be responsible for microbial inactivation include the denaturation of key enzymes and the disruption of ribosomes. DNA is largely unaffected by high pressure but enzymes involved in replication and transcription may be inactivated [10].

A number of factors determine the resistance of a microorganism to pressure. Simple first order reactions (straight line relationships) have been reported for the inactivation of some vegetative bacteria by pressure [11]. However, other studies have shown that deviations from the first order might occur. In many cases the inactivation curves show exponential decay although the shape of the curves may change with treatment temperature. For example, first order kinetics were reported when *Escherichia coli* was treated at 250 MPa at 40 or 50 °C over a period of 20 min. However at temperatures below 30 °C the curve shape changed to that of exponential decay (second order kinetics) [12]. It was suggested that the "tail" indicated that a small fraction of the population appeared to be less sensitive when the temperature was below 30 °C. These observations may be explained by an altered membrane composition of the organisms as a consequence of a liquid-gel transformation in the

membrane near 30 °C. Eze [13] reported such phase transitions in *E. coli* membranes. Similar exponential decay curves have been found when *Yersinia enterocolitica*, *E. coli* O157:H7, *Listeria monoctyogenes*, *Salmonella typhimurium* and *S. enteritidis* are pressurised at 20 °C in phosphate buffered saline (PBS) at pH 7.0 [14]. Metrick, Hoover and Farkas [15] also reported tailing effects for *S. typhimurium* and *S. senftenberg* pressurised from 238 MPa to 340 MPa at 23 °C. In these studies, when the resistant tail population was isolated, grown and again exposed to pressure, there was no significant difference in the pressure resistance between it and the original culture. It has been suggested that the tailing phenomenon may be due to heterogeneity in the population, such as age, clumping, genetic variation or experimental conditions [16].

Gram-positive bacteria are generally more pressure resistant than Gram-negatives, while spores are most resistant and can survive pressures greater than 1000 MPa. However, there are some exceptions. Patterson et al. [14] found that *E. coli* O157:H7 could be relatively resistant to high pressure. Resistance varies between species and between different strains of the same species. For example Benito et al. [8] showed that strains of *E. coli* O157:H7 exhibited differences in resistance to high pressure and Patterson et al. [14] found a significant difference in pressure resistance between strains of both *E. coli* O157:H7 and *L. monocytogenes*. The reason for this variation between strains is not known but for some bacteria there is a correlation between pressure resistance and thermotolerance. Growth phase also has an effect on the pressure resistance, with bacteria in the log phase being more sensitive to high pressure than those in the stationary, dormant or death phase [10]. The composition of the pressurising medium also determines pressure resistance. Proteins, carbohydrates and lipids all confer resistance as does a reduced A_w. It is therefore important that process conditions are evaluated in real foods and not extrapolated from data obtained in other substrates.

The conditions during pressure treatment can have a significant effect on resistance. The temperature during pressurisation can have a marked effect on the inactivation of cells. In one study the destruction of microorganisms in 2 mM sodium phosphate buffer, pH 7.0 was investigated [17]. Pressures ranging from 100 to 400 MPa were applied for 20 min. at either –20 °C or +20 °C. In most cases the microbial inactivation of vegetative organisms, including *S. bareilly, V. parahaemolyticus* and *St. aureus*, was greater at –20 °C than at +20 °C. Similar trends were observed in another investigation where minced beef was inoculated with *Citrobacter freundii* (used as an indicator organism for *Salmonella* spp.) and subjected to high pressure processing for 20 min at a variety of temperatures [18]. A reduction of at least 10^5 was obtained with 280 MPa at 20 °C, or 230 MPa at 4 °C or 150 MPa at 50 °C.

The combination of pressure with relatively mild heat may be a useful strategy for dealing with pressure resistant vegetative pathogens [19] and spores [20]. Simple models have been developed which predict the pressure and temperature conditions required to achieve a given level of inactivation in named foods. These conditions can then be used to determine the optimum treatment, in terms of sensory quality, suitable for each product [19].

The pH of the substrate also has an important effect on the pressure resistance and most microorganisms are more susceptible to high pressure when the pH of the suspending medium is sub-optimal. For example, it has been shown that the pressure resistance of *E. coli* O157:H7 in orange juice is dependent on the pH of the juice, the degree of inactivation increasing as pH decreases [21]. Survival of *E. coli* O157:H7 in orange juice during storage is also dependent on pH. When *E. coli* O157:H7 was stored in orange juice at 3 °C, following a mild pressure treatment (400 MPa for 1 min at 10 °C) the survival times at low pH decreased relative to an untreated control (Table I).

Table I

Time taken during storage at 3 °C for the numbers of *E. coli* O157:H7 (NCTC 12079) in orange juice to reduce by 5 log cycles

pH	Storage time (days) to achieve a 5 log inactivation	
	Control	400 MPa /1 min /10 °C
3.4	13	3
3.6	16	6
3.9	>25	8

These results indicate that the damage caused to *E. coli* O157:H7 during sublethal pressure treatment, made it more susceptible to the combination of low pH and low temperature encountered during refrigerated storage in orange juice. This effect should also be taken into consideration when enumerating bacteria after pressure treatment as selective media may not allow growth of pressure-injured cells which may subsequently multiply under suitable conditions.

Sublethal pressure treatment also reduces the thermotolerance of *E. coli* O157:H7. When *E. coli* O157:H7 was inoculated into skimmed milk and pressure

treated at 200 MPa for 30 min at 10 °C the D_{10} values obtained were significantly lower than those obtained in untreated controls (Table II).

This sensitisation to heat should be considered when conditions are being determined for combination treatments, such as high pressure treatment followed by heating.

Table II

D values (min) obtained when *E. coli* O157:H7 (NCTC 12079) was suspended in skimmed milk and pressure treated prior to heat treatment at 55, 58 and 60 °C

Temperature (°C)	D values (min)	
	Control	200 MPa /30min /10 °C
55	16.38	10.50
58	8.10	4.88
60	2.44	1.51

Future development of high pressure processed foods

Although high pressure offers opportunities for food processing there are a number of restrictions, which should be considered. The resistance of bacterial spores to high pressure may mean that high pressure will have to be used in tandem with heat or other processes to give the required level of inactivation in susceptible foods. Pressure combined with heat, and pressure followed by heat (or vice versa) have been suggested for the inactivation of spores. Pressure cycling, using alternating high and low pressure treatments has also been proposed as a method for increasing spore inactivation [20]. Spore germination can be induced using relatively low pressures and the germinated spores may then be inactivated by moderately high pressure [13].

Pressure-treated fruit juices also present a problem in that the high pressure treatments used do not inactivate polyphenol oxidase and consequently these products require chilling during storage to prevent enzymatic browning.

The range of commercially available pressure-processed products is small at present but there are opportunities for further development and the production of a

wide range of pressure-treated products. High pressure affects the functionality of large protein and carbohydrate molecules, an attribute that may allow the optimisation of food manufacturing processes and the production of novel foods. For example, high pressure can be used to improve the functional properties of food and food ingredients such as the texture and emulsifying, whipping and dough forming properties [9]. Applications of high pressure include the production of high strength acid set gels, the restructuring of meat and fish, tenderization of meat, reduced ripening time for cheeses, tempering of chocolate and pressure shift freezing and thawing.

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ABSTRACTS OF PAPERS AND POSTERS

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PLENARY SESSION

M. DEGRÉ

Cytomegalovirus interaction with blood and endothelial cells

Institute of Medical Microbiology, University of Oslo, Rikshospitalet, Oslo, Norway

The mononuclear cells of the haemopoietic system are probably an important reservoir of latent human cytomegalovirus (HCMV) and also play a major role in the pathogenesis of infections. HCMV DNA was detected in CD34+ cells (both HLA-DR– and +), from seropositive donors. Colony formation and single cell growth of progenital cells from bone marrow (BM), stimulated with growth stimulating factors, were inhibited by HCMV, while progenitor cells from cord blood (CB) were not affected. Differences in the cell cycling activity might be at least part of the explanation. The mechanism(s) of inhibitory effect were examined in haematopoietic cell lines. HCMV inhibited the proliferation of KG-1, MO7 and U937 cell lines comparable to that of the primary haematopoietic cells. Uptake of virus was shown by the presence of pp65 lower matrix protein, but no replication was found and no transcription of the immediate early proteins was found. Transcription of the cells with pp71 tegument gene, but not with pp65 and pp150 or IE genes caused inhibition of the cell proliferation. Inoculation of cells with dense bodies, containing matrix proteins but no nucleic acid, also caused inhibition. HCMV infection of MO7 cells induced apoptosis in approximately 70% of the cells, indicating that the HCMV induced inhibition of the growth of haematopoietic cells is at least in part due to apoptosis. Matrix proteins may play an important part in this effect while IE and E proteins may have a minor role. Endothelial cells seem to play an important part in the pathogenesis of HCMV infections. HCMV have a strong tropism for vascular endothelium and microvascular inflammation, occlusion and ischemic injury are stages in the development of viral disease. HCMV productively infect endothelial cells, but cells from different organs have different susceptibility. Microvascular cells from intestinal endothelial cells (HIMEC) are more susceptible to HCMV infection than endothelial cells from umbilical vein (HUVEC) Also the IL-1β-induced cellular adhesion molecules, E-selectin, VCAM-1 and ICAM-1, differed between HIMEC and HUV#VEC. This may explain the development of gastrointestinal HCMV disease in humans.

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É. M. FENYŐ

HIV biological phenotype and chemokine receptor usage

Microbiology and Tumorbiology Center, Karolinska Institute, Stockholm, Sweden

Human immunodeficiency virus type 1 (HIV-1) biological phenotype, such as replication rate and cytopathology in culture, has long been recognized as marker for viral virulence. The ability to translate these phenotypic traits into molecular terms brought HIV biological variation into focus during the past three years. It has been recognized that HIV-1 uses members of the seven transmembrane domain chemokine receptor family as co-receptors to CD4 for membrane fusion and entry into cells. The two most well-defined HIV-1 co-receptors are CXCR4 and CCR5, members of the CXC (α) and CC (β) chemokine receptor subfamilies, respectively. Primary HIV-1 isolates previously termed rapid/high or syncytium inducing (SI) which readily infect activated PBMC and $CD4^+$ T cell lines are defined by their use of the CXC-chemokine receptor CXCR4. Slow/low or non-syncytium inducing (NSI) viruses, which preferentially infect activated PBMC are defined by their use of members of the CC-chemokine receptor family, principally CCR-5. This allowed the introduction of a new terminology: CXCR4 using viruses have been termed X4 and CCR5-using viruses R5. In addition to CXCR4, some rapid/high viruses use CCR5, CCR3 or CCR2B and thus have a broader host range than slow/low viruses. The fact that the change in virus biological phenotype may occur in the same individual over time and is associated with progressive disease has suggested that CXCR4 using viruses are more virulent. We asked the question whether the pattern established with HIV-1 isolates of genetic subtype B holds true for subtypes A, C, D and E. Of the 40 patients with non-AIDS 34 yielded CCR5-using (R5) virus (85% vs 80% for subtype B). None of the viruses, including HIV-1 subtype B, were able to use any other chemokine receptor in this group. Conversely, many of the subtype A, B, D and E viruses derived from AIDS patients used CXCR4 (18 out of 40, 45%). Viruses using CXCR4 were in half of the cases able to use other receptors as well, most frequently CCR5 or CCR3, and were thus dual-tropic or multitropic. Interestingly, HIV-1 of subtype C differed from this general pattern in that CXCR4 usage was very infrequent. In fact, all of nine Ethiopian or four Swedish AIDS patients with subtype C virus infection yielded virus that used CCR5 only. Furthermore, we compared receptor usage of HIV-1 with the less pathogenic HIV-2. Similarly to HIV-1, some of the HIV-2 infected AIDS patients yielded viruses using CXCR4, instead of or in addition to CCR5. In contrast to HIV-1, the ability to use several coreceptors, particularly CCR1, CCR2 and CCR3 in combination with CCR5 and/or CXCR4, characterized 10 out of 11 HIV-2 isolates,

regardless of the severity of infection. The results indicate that multitropism *per se* cannot explain the differences between HIV-1 and HIV-2 pathogenesis.

P. RASPOR

The influence of chromium compounds on yeast physiology

Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

Successful, ionic nutrition is related to the metals' biosorption and bioaccumulation, which are important and essential for all living creatures. Selective advantage is confirmed for those species which are capable of accumulating and retaining those components which are present in limited, trace amounts in surroundings. Uptake of metal ions from the environment is mediated by biosorption and bioaccumulation mechanisms. The uptake starts with fast metal biosorption on the cell wall and then continues with slower bioaccumulation. Bio accumulation is generally defined as active mode of action by living cells. Tolerance of particular organism to particular metals ion may reflect the ability of an organism to survive in an environment with a high concentration of metals, or to store it. This can be due to an ability to control the intracellular concentrations of the ion in the range, which is not toxic to its metabolic processes. Effect of chromium on yeasts growth and the problem on metal ions toxicity and/or resistance to it is discussed through a new model for evaluation. For first screening the agar diffusion test is shown to be essential followed by advanced procedure for precise measurement of yeast viability or mortality. Values for pH, temperature, a metal's biological availability, etc. are considered as one of the most important environmental parameters in the mechanism of metal ion translocation. The metal ion's uptake is essentially a biphasic process consisting of a metabolism-independent and metabolism-dependent step. The initial biosorption step for metal ions is rapid. In the case of chromium we found at different starting pH values in yeast *S. cerevisiae* correlation between pH and chromium (III) accumulation in the pH range from 2 to 6. Under the experimental conditions applied, the cell surface deposition capacity for chromium increased with the pH. During cultivation in batch operation mode, the high inhibitory effect of supplemented chromium (III) in the media on yeast growth was observed during prolonged lag phase and consequently reduction of biomass yield. The toxic effect of chromium ions was found during continuous cultivation in biomass. During the cultivation of *C. intermedia* in combined batch/fed-batch mode, favourable chromium ratio in biomass was achieved, as well as higher yeast cell capacity in transformation of accumulated chromium into organic fraction.

On the contrary, higher biomass accumulation in batch cultivation mode supported a higher total chromium accumulation capacity. The high concentration of chromium in the environment always causes a reduction of RNA and protein concentration on the cellular level. On the macro scale, chromium causes a reduction of growth rate and biomass production, and an extension of lag phase. In *S. cerevisiae* organic compounds with a molecular weight from 100,000 to 10,000 showed the highest intracellular chromium binding capacity. The diversity of intracellular organelles and biomolecules provides a wide range of potential binding sites. When a higher concentration of chromium was present in the environment, a 47 times higher amount was found in the yeast biomass. The distribution was found in favour of organically bound chromium. Speciation of organically bound chromium by a molecular sieve showed that the organic substances with a molecular weight of between 100,000 and 10,000 expressed a high binding capacity to a chromium ion. Similar results were obtained in yeast biomass *C. intermedia* isolated as intracellular low-molecular-weight chromium-binding polymers.

A. L. DEMAIN

Importance of amino acids in regulating microbial secondary metabolism

M.I.T. Cambridge, MA, USA

Amino acids have major positive or negative effects on the production of secondary metabolites. Methionine stimulates cephalosporin C production by *Cephalosporium acremonium* by acting as an inducer of several cephalosporin synthases; it also supplies the sulfur atom of the antibiotic. Lysine stimulation of cephamycin C production in *Streptomyces clavuligerus* is due to induction of lysine-□-aminotransferase, the first enzyme involved in bacterial conversion of lysine to the precursor, α-aminoadipate. Valine stimulates tyrosin biosynthesis in *Streptomyces fradiae* by inducing valine dehydrogenase, the first enzyme leading from valine to the small acid precursors of the macrolide ring. Other examples include stimulation of nikkomycin synthesis by branched amino acids, tryptophan induction of ergot alkaloid synthesis, phenylalanine stimulation of benzodiazapene alkaloid formation and leucine induction of bacitracin synthetase. On the negative side, lysine inhibits penicillin production by *Penicillium chrysogenum* due to inhibition of homocitrate synthase, the first enzyme leading to formation of the α-aminoadipate precursor in fungi. Also methionine inhibits biosynthesis of many antibiotics to which it contributes a methyl

group, e.g., anthramycin, thienamycin, esperamicin and rapamycin. Recent experiments indicate that this is due to methionine repression of the methylating enzyme system. Leucine interferes with *Monascus* red pigment formation by a mechanism thought to involve enhanced decay of pigment synthase(s).

C. ELMERICH

Diazotrophs associated with cereal crops

Unit de Physiologie Cellulaire, Institut Pasteur, Paris, France

The biological process responsible for the reduction of molecular nitrogen to ammonia, catalyzed by the nitrogenase enzyme, is referred to as biological nitrogen fixation. Cereal crops are the most important sources of food. In particular rice is the major food for more than a third of the world's population. It is now recognized that plant growth promoting rhizobacteria (PGPR) play an important role in agriculture. Attempts to enumerate and to identify the nitrogen-fixing bacterial species associated with cereal crops and other grasses have revealed a large diversity and complexity. In general, nitrogen-fixing root-associated diazotrophs are soil bacteria able to colonize the root surface. Some, including *Azospirillum*, invade the superficial layers of the cortex. Obligate endophytes that inhabit the vascular system of the plant have been described. *Azospirillum* enhances the plant growth primarily by colonization of the root surface that results in increasing the proliferation of the root hairs and of the root system of the host plant. This effect has been tentatively attributed to the production of auxins like compounds such as indole-3-acetic acid (IAA) by the bacterium. Surface colonization of wheat roots by *Azospirillum* involves the polar flagellum and the production of surface polysaccharides. Bacteria on the root surface are ovoid in shape, resembling differentiated cyst-like cells. Data on the genetic determinants involved in the colonization process will be presented.

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BACTERIAL PATHOGENS AND PATHOGENESIS OF MEDICAL AND VETERINARY IMPORTANCE

H. U. BERTSCHINGER[1], E. MEIJERINK[2], P. VÖGELI[2]

Prevention of infectious diseases of animals through breeding for genetic resistance

[1]Institute of Veterinary Bacteriology, University of Zürich, [2]Institute of Animal Science, Swiss Federal Institute of Technology, Zürich, Switzerland

Most informations on genetic resistance to infectious diseases have so far been obtained from laboratory mice and from poultry. The impact on animal production has been minimal, whereas vegetable farming and horticulture have derived considerable advantage from the development of genetically resistant plant breeds. Research into microbial virulence factors has in many cases revealed an absolute need for adhesion to highly specific receptor structures on host tissues. Genetic polymorphisms may cause variation in these receptor structures. Examples of economical relevance have been found in the field of porcine enteric *Escherichia coli* infections. In case of fimbrial type F4, many pigs have no enterocyte brush border receptors for enterotoxigenic *E. coli* strains with this adhesin. The receptor status is inherited in a simple Mendelian fashion, presence of receptor being dominant over its absence. The receptor phenotype can only be identified by means of a microscopic enterocyte adhesion test, i.e. after death of the pig. The second common fimbrial type, F18, is associated with porcine *E. coli* strains producing Shiga-like toxin 2e and/or enterotoxin(s). The F18 receptor is also inherited as a dominant trait. Our group has found that it is coded for by a gene on chromosome 6, which is perfectly linked to – and probably identical with – the gene for fucosyltransferase 1 (*FUT1*). The genotype at *FUT1* can be determined by PCR starting with DNA extracted from a blood or tissue sample. This allows the selection for breeding stock of homozygously resistant pigs, a method, which is already practiced in Swiss pedigree herds.

H. KARCH, W. BRUNDER, H. SCHMIDT

New discoveries in molecular biology of enterohaemorrhagic *Escherichia coli* O157

Institute for Hygiene and Microbiology, University of Würzburg, Germany

Since 1982, enterohaemorrhagic *Escherichia coli* (EHEC) have been identified as a cause of diarrhoea and haemorrhagic colitis. The most serious complication of the infection is the haemolytic-uraemic syndrome (HUS) that develops in 5 to 10% of children with diarrhoea. Shiga toxins (Stx) are the most important virulence factors of EHEC known at present. After reaching the bloodstream, the toxins cause damage of endothelial cells but also of tubular cells in the kidneys and these results in renal failure. In EHEC O157 isolates from patients we were able to identify seven different combinations of *stx* genes that occurred with different frequency. The genes encoding Stx are located in the genomes of prophages that are integrated in EHEC chromosomes. In addition, EHEC O157 strains possess a chromosomally located pathogenicity island termed LEE that contains numerous pathogenicity genes including the *eae* gene encoding intimin. Moreover, EHEC O157 strains harbor a 93-kb plasmid where are located the genes encoding the EHEC-hemolysin (EHEC-HlyA), a serin protease (EspP) that cleaves factor V and a protein called ToxB that shows homology with the toxin of *Clostridium difficile*. The EHEC O157 strains exist in two variants, namely non-sorbitol-fermenting O157:H7 and sorbitol-fermenting O157:H– strains that are evolutionary older. Our results obtained up to now demonstrate marked differences in epidemiology of the infection caused by the respective EHEC O157 variants. EHEC O157:H7 strains occur worldwide whereas sorbitol-fermenting strains have been found in Germany and recently also in the Czech Republic. While the EHEC O157:H7 strains occur mainly during warm months, the sorbitol-fermenting strains are more frequent during cold season of the year. In addition, differences exist with regard to the resistance to heavy metals, the plasmid structure, and the reservoir. We postulate the hypothesis that sorbitol-fermenting O157:H– strains occur only in human intestine, whereas non-sorbitol-fermenting O157:H7 strains got adapted to other hosts, such as cattle, which enables more rapid spread of the strains.

M. AWAD-MASALMEH

Virulence factors of VTEC bacteria isolated from animals, meat and meat products

II. Medical Clinic for Ruminants and Swine's, Veterinary University, Vienna, Austria

VTEC strains of the serotype O157 (n=15) and of other serotypes (n=70) were tested for harbouring genes encoding for the virulence factors *hly*, *pCVD419*, *eaeA*, *astA*, *katP*, *espP*, *etpD*, *ileX* and *colD157*. Gene detection was performed by PCR. Furthermore, the adhesion properties and sensitivity of all VTEC strains were investigated on HeLa-cells-monolayer and in Etest (AB BIODISK, Sweden). Genes encoding for *astA*, *katP* and *colD157* virulence factors were not detected in any strain of the serotype O157. VTEC of this serotype isolated from humans, meatproducts and intestinal contents from ruminants showed a very similar gene-profile (*hlyA*, *astA*, *eaeA*, *espP*, *etpD* and *ileX*). Strains of the serotype O157 showed a weak and not reproducible adherence to HeLa-cells-monolayer and all of them were sensitive to enrofloxacin, ampicillin and amoxacillin *in vitro*. The gene encoding *katP* virulence factor was not found in any strains of the isolated non-O157-VTEC serotypes. The non-O157-VTEC isolates from meat products showed genes encoding for *hlyA* in 40%, *eaeA* in 20%, *astA* in 40%, *etpD* and *ileX* in 10% each. Similar results were also observed in cases of VTEC isolates from fecal samples of ruminants. They possessed the genes for *hlyA*, *eaeA*, *espP*, *colD157* and *ileX* in 40, 20, 16, 14, 6 and 4%, respectively. These isolated VTEC strains showed a broad spectrum of gene composition. 10% of them have attached to HeLa-cells-monolayer and were resistant to tetracycline (40%) and ampicillin (20%).

I. TÓTH[1], ZS. RUZSICS[2], V. KARCAGI[3], B. NAGY[1]

Rifampicin – resistance associated mutation in *fliC* flagellar gene of *E. coli* O157:H7

[1]Veterinary Medical Research Institute, Hungarian Academy of Sciences, [2]"B. Johan" National Centre for Epidemiology, [3]Center for Public Health, Budapest, Hungary

E. coli O157:H7 prototype strains 7785 and EDL933 and their spontaneous rifampicin resistant (Rif^r) mutants with reduced motility and non-motile (NM) phenotype were compared by PCR using a primer-pair specific for the variable region and the C terminus of the *fliC* flagellin gene. DNA was amplified from the wild type *E.*

coli O157:H7 strains and from K-12 strains, but no amplicon was detected from the corresponding Rif^r mutants analysed by agarose electrophoresis. In Southern blot analysis no polymorphism could be seen among these wild type and mutant *E. coli* O157 strains when genomic DNA samples of 7785, EDL933 their Rif mutants and an *E. coli* O157:NM were digested with either *BamHI* or *HinfI* and were probed with the *E. coli* K-12-specific amplicon. *fliC*-specific amplicon hybridised to a different sized *BamHI* fragment in *E. coli* K-12 and O157 strains, but to the same sized *HinfI* fragment as in *E. coli* K-12.

L. E. HÖLZLE, M. M. WITTENBRINK

Molecular characterisation of chlamydiae from swine

Institute of Veterinary Bacteriology, University of Zürich, Switzerland

In our report lung and intestine of 49 pigs with respiratory diseases and endocervical swabs from 205 sows with reproductive disorders were investigated for *Chlamydia* infection by polymerase chain reaction. Samples from 49 healthy slaughter pigs and endocervical swabs from 30 fertile sows served as controls. PCR primers targeted DNA sequences flanking almost the entire *Chlamydia ompl* gene and sequences flanking a 590-bp fragment of the *Chlamydia omp2* gene. PCR amplicons of the expected size were generated from 49.0% of pigs with respiratory disease and 60.0% of sows with reproductive disorders. Corresponding values for the respiratory healthy controls were significantly lower (24.5%; $p<0.05$). No PCR amplicons were obtained from endocervical swabs of fertile sows. By DNA hybridization of PCR amplicons a high prevalence of mixed infections with *C. psittaci* serotype 1 and *C. trachomatis* in the porcine lung and intestine was found and further confirmed by restriction fragment length polymorphism analysis and nucleotide analysis of the *ompl*-gene-PCR amplicons. 81.3% of the PCR amplicons from endocervical swabs were identified as *C. psittaci* serotype 1, indicating an association of the known genitopathogenic *C. psittaci* serotype 1 with reproductive disorders in sows. Nucleotide sequence analysis of *ompl* gene amplicons identified as porcine *C. trachomatis* shared maximum 82.7% homology with the reference strain S45.

C. L. TURNBOUGH JR.

Gene regulation by reiterative transcription and transcriptional start site switching

Department of Microbiology, University of Alabama, Birmingham, Alabama, USA

Recent studies of gene regulation from this lab have elucidated a number of *E. coli* control mechanisms that employ two unusual reactions catalyzed by RNA polymerase: reiterative transcription and transcriptional start site switching. Reiterative transcription is the repetitive addition of a nucleotide (UMP in this case) caused by slippage between a homopolymeric stretch of nascent transcript and a stretch of (≥3) complementary nucleotides in the DNA template. Transcriptional start site switching is the regulated selection of alternative transcript start sites at a particular promoter. One or both of these reactions have been shown to play key roles in pyrimidine-mediated regulation of a number of operons involved in pyrimidine nucleotide biosynthesis and salvage, including the *pyrBI*, *pyrC*, *carAB*, *codBA*, and *upp* operons. Details of these regulatory mechanisms will be described, and the use of these reactions in global gene regulation will be discussed.

O. BENEDEK, J. KNURR, B. VINSON, C. L. TURNBOUGH JR.

Identification of peptide ligands that bind *Bacillus subtilis* spores

Department of Microbiology, University of Alabama, Birmingham, Alabama, USA

Using a phage display screening system based on a combinatorial library of random 7-amino acid peptides individually displayed on the surface of the filamentous coliphage M13, we identified peptide ligands that bind tightly to the surface of *Bacillus subtilis* spores. The tight-binding phages were selected from the library by several rounds of biopanning, after which individual phage were isolated, amplified, and their genomic DNA extracted. The peptide-encoding region of the genome was sequenced to determine the amino acid sequence of the tight-binding peptide. These peptides were chemically synthesized and tested for use in spore capture and identification. The tight-binding peptides identified contain a consensus sequence found near the amino terminus of the SpsC protein, which apparently binds to the surface of the *B. subtilis* spore and is involved in spore coat polysaccharide synthesis. Presumably our peptides mimic the coat-binding region of SpsC. Several tight-binding peptides were

fluorescently labelled and shown to be capable of species-specific identification of spores by fluorescence activated cell sorting (FACS). One of these peptides was also attached to a water-insoluble polymer, coated on a glass slide, and shown to specifically capture *B. subtilis* spores in aqueous suspension. Fluorescently-labelled monoclonal antibodies against M13 phage are currently being used to detect the *B. subtilis* tight-binder phage on the surface of the spore to improve FACS analysis.

B. KOCSIS, I. KUSTOS, Z. PÉTERFI

Isolation and characterisation of a *Shigella sonnei* absolute rough mutant

Department of Medical Microbiology and Immunology, University Medical School, Pécs, Hungary

Shigella sonnei phase I (Kis) strain was isolated in our institute many years ago. This strain was mutagenized by alkylating agents. Great number of rough mutants were isolated and characterized by phagetyping with R-specific phages, hydrophobicity and antibiotic sensitivity tests. The mutants were divided into groups. One representative of each group was cultivated, their lipopolysaccharide (LPS) was extracted and analyzed by polyacrylamide-electrophoresis and gaschromatography. We realized that one of these strains is an absolute rough mutant: its LPS contains only lipid A and KDO. We compared our absolute rough mutant with the original *S. sonnei* phase I and *Salmonella minnesota* R595 absolute rough strain. We investigated the whole protein profiles of bacterial lysates and outer membrane proteins extracted from our strains by capillary electrophoresis. The serological cross-reactions were analyzed by indirect haemagglutination, ELISA test and immunoblotting method. We plan to test our absolute rough mutant in biological experiments, too.

Z. TIGYI, T. PÁL

Relationship between the sensitivity to *Shigella sonnei* colicin type 7 and the presence of the invasion plasmid in enteroinvasive *Escherichia coli*

Department of Medical Microbiology and Immunology, University Medical School, Pécs, Hungary

Horak has shown that the sensitivity to the type 7 colicin of *Shigella sonnei* in *Escherichia coli* strains of the "EIEC serogroups" correlated with their virulence, i.e.

the positivity in the Serény test. The virulence of these strains is the result of the co-ordinated expression of genes located both on the chromosome and on the invasion plasmid. In the current study the role of the invasion plasmid in the sensitivity to colicin type 7 was investigated. First, from a virulent, colicin sensitive O143 EIEC strain 21 independent colonies having lost the invasion plasmid were selected. All the derivatives became resistant to the colicin. Next, investigating the "opposite" situation, 114 independent colicin resistant clones were isolated from the virulent patent strain. As judged by the capacity to bind Congo Red, after the colicin selection 90.4% of the clones lost the invasion plasmid functionally or physically, while a similar passage without a colicin selection resulted in a 20% loss only. After mobilizing the invasion plasmid into a colicin resistant *E. coli* K-12 strain the transconjugant became sensitive to colicin type 7. Taken together, all these data suggest a positive correlation between the sensitivity to colicin type 7 and the presence of the invasion plasmid of EIEC strains. The fact, however, that *S. flexneri* strains carrying similar virulence plasmid are resistant to this colicin suggests the additive role of factors coded by chromosomal genes, too.

I. KUSTOS[1], V. TÓTH[1,2], F. KILÁR[3], B. KOCSIS[1], L. EMŐDY[1]

Investigations of outer membrane components of *Proteus penneri* strains by electrophoretic methods

[1]Department of Medical Microbiology and Immunology, [2]Department of Stomatology, [3]Central Research Laboratory, University Medical School, Pécs, Hungary

The electrophoretic profiles of lipopolysaccharides (LPS), outer membrane proteins (OMP), and the whole protein content of bacterial lysates of thirteen wild, and two mutant *Proteus penneri* strains were determined. The whole protein profiles and the OMPs were determined by "dynamic sieving" capillary electrophoresis (CE). This is a modern, fast and quantitative method, which provides the opportunity for determination of molecular weight, and the estimation of relative percentage of different proteins. CE requires very small sample amounts, it can be automated, and provides results within a few minutes.

The whole protein profiles of the *P. penneri* strains – although contained several peaks – were dominated by the OMPs. Therefore we prepared the OMPs according to the modified method of Osborn and Munson. The OMP profiles of the *P. penneri* strains were dominated by two major proteins of 39 and 43 kDa. The ratio of these two

proteins were 3:2 in 12 strains, while in two mutant strains (357/1 and 357/S) their ratio was 1:1. One isolate (H1209) had more characteristic peaks in its pattern.

The LPSs of *P. penneri* strains were determined by SDS-PAGE. 12 of the 15 strains showed S (smooth) from by silver staining, while one wild strain and the two mutant strains (357/1, 357/S, H1209) showed R (rough) form in the gels.

The organization of the outer membrane of Gram-negative bacteria requires specific interactions between OMPs and LPS, and these interactions could not occur properly with defective LPS. In these mutants the levels of major outer membrane proteins are decreased. In our study the three *P. penneri* strains synthesising rough form of LPS showed alterations in the OMP, and in the whole protein profiles as well, by electrophoretic methods. These outer membrane components create the surface of Gram-negative bacteria, thus they play an important role in the interactions with the host cells, and in the pathogenicity. Furthermore, they are responsible for the permeability properties of the outer membrane, therefore their alterations can influence the antibiotic sensitivity, and the therapeutic opportunities.

W. HRYNIEWICZ, A. SKOCZYNSKA

Characteristics of major clinical pathogens responsible for meningitis in Poland

National Reference Centre for Bacterial Meningitis, Sera and Vaccines Central Research Laboratory, Warsaw, Poland

Bacterial meningitis remains major cause of morbidity and mortality world-wide, especially in children. Beyond the perinatal period, there are three major causative agents of bacterial sepsis and meningitis in industrialised countries: *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae.* During 1997–98 the National Reference Centre for Bacterial Meningitis collected 220 strains isolated from cerebrospinal fluid from patients with meningitis. Among them the most common was *N. meningitidis* (n=90, 40.9%), followed by *H. influenzae* (n=58, 26.4%) and *S. pneumoniae* (n=46, 20.9%). Most of *N. meningitidis* and *H. influenzae* strains were isolated from children below the age of five. *S. pneumoniae* was mainly isolated from adult patients. Out of meningococcal strains 88.9% belonged to group B. Most of them were highly sensitive to penicillin, however nine (10%) of them showed decreased susceptibility to penicillin with MIC higher than 0.06 mg/l. All *H. influenzae* belonged to serotype b and were susceptible to 3rd generation cephalosporins and chloramphenicol. Five strains (8.6%) produced β-lactamases. Two isolates were

resistant and 10 exhibited intermediate susceptibility to cotrimoxazole. Eight isolates (13.8%) exhibited intermediate susceptibility to rifampin. Broad distribution of serotypes was found among pneumococcal strains of which the most common were serotypes 3, 8 and 22F. Penicillin nonsusceptible strains constituted 13% of all pneumococcal isolates (4 resistant and 2 intermediate susceptible strains). Three of resistant strains belonged to 23F serotype. Among pneumococci 22% were resistant to chloramphenicol and 8.8% to cotrimoxazole. Molecular techniques such as PCR based methods and PFGE have been also developed for epidemiological typing as well as for identification.

E. NAGY[1], I. SZŐKE[1], L. TÖRÖK[2]

Role of anaerobic bacteria in chronic prostatitis and male infertility

[1]Department of Clinical Microbiology, [2]Department of Urology, Albert Szent-Györgyi Medical University, Szeged, Hungary

The accurate diagnosis of chronic prostatitis syndromes poses a major challenge to physicians and clinical microbiologists. About half of all men suffer from symptoms of prostatitis during some part of their life. In acute bacterial prostatitis, Gram-negative bacteria are the most common pathogens. The roles of Gram-positive bacteria in chronic bacterial prostatitis and of *Chlamydia trachomatis* in "non-bacterial" prostatitis are debated. Empirical antibiotic therapy is often used for the treatment of chronic prostatitis.

During this study, the urethral discharges and the prostatic fluids after prostate massage of 80 patients with therapy-resistant chronic prostatitis were cultured in parallel in aerobic and anaerobic environments. Thirty-seven patients exhibited infertility problems, with a 100% decrease in the motility of their sperms. The samples of all patients were screened for the presence of *C. trachomatis*, *M. hominis* and *U. ureolyticum*. Thirty of the 80 patients gave negative culturing results for all pathogens screened, but 43% of the patients harbored $>10^5$ CFU/ml anaerobic bacteria alone or together with aerobic bacteria. The most frequently isolated anaerobic bacteria were *B. ureolyticus*, *Prevotella* spp., *Porphyromonas* spp. and *Peptostreptococcus*. *In vitro* tests after incubation for 2 to 18 hours demonstrated the negative effects of the anaerobic bacteria isolated from these patients on the motility of healthy sperms. In patients with massive anaerobic infection, long-term antibiotic therapy active against anaerobic bacteria led to elimination of the complaints, and the symptoms of chronic prostatitis. The use of molecular diagnostic methods will be discussed.

J. SZENTANDRÁSSY[1], M. NÉMEDI[2], E. TÓTH[2], D. SZABÓ[3], ZS. FILETÓTH[4], CS. JENEY[3], F. ROZGONYI[3]

SHV-5 extended spectrum β-lactamase producing *Klebsiella pneumoniae* (ESBL-KP) in a perinatal intensive care unit (PICU) of a Hungarian hospital

[1]Microbiological Laboratory, [2]Perinatal Intensive Care Unit, "Géza Hetényi" County Hospital, Szolnok, [3]Institute of Microbiology, "Semmelweis" University Medical School, [4]Clinical Epidemiology Unit, National Institute of Traumatology, Budapest, Hungary

A cluster of cases caused by ESBL-KP was described in the PICU of the County Hospital of Szolnok (Hungary) with 15 primary cases. Although the occurrence of ESBL-KP was described previously in Hungary. The appearance of ESBL-KP in the PIC of this hospital is genuine because the ESBL-detecting E-test was introduced for all ceftazidime-resistant or intermediate resistant *Enterobacteriaceae* isolates four months before the first recognized case and the screening frequency of premature babies remained the same. This correlates with the worldwide accepted opinion that all ceftazidime-resistant or intermediate resistant isolates of *K. pneumoniae* are suspected for ESBL production. Between June and October 1998 out of 132 newborns admitted to the PICU a total of 14 cases were found to be colonized or clinically infected and in one case contamination could occur. To determine whether the cluster on the PICU was due to ESBL-KP, all strains of this species isolated during a four-month period were identified in an ATB system, examined for antibiotic resistance, phenotype and β-lactamase production. Representative strains were further characterized by plasmid restriction profiling with *Eco*RI and *Hind*III, arbitrarily primed (AP)-PCR analysis with ERIC-2 primer, and ESBL-typing. The gene sequence responsible for the ESBL production was confirmed by PCR method in all ESBL-producing *K. pneumoniae* isolates and in one strain of *S. marcescens*. All strains produced SHV-5 extended-spectrum β-lactamase. Among 15 *K. pneumoniae* strains, 14 *K. pneumoniae* strains were identical.

K. LATKÓCZY, F. ROZGONYI

A comparative study on factors influencing the susceptibility of bacteria to trimethoprim/sulfamethoxazole combination

Institute of Microbiology, "Semmelweis" University Medical School, Budapest, Hungary

The aim of this study was to examine that the relatively high incidence of resistance to the combination trimethoprim/sulfamethoxazole in strains of the family *Enterobacteriaceae* reported from different Hungarian laboratories could be due to the resistance of bacteria or to some technical errors. Susceptibility to trimethoprim/sulfamethoxazole of 100 strains belonging to the family *Enterobacteriaceae* was parallelly determined on agar plates prescribed by the HUMAN Ltd, Budapest, Hungary, and on Mueller-Hinton agar plates (bioMérieux) recommended by the NCCLS using both OXOID and HUMAN discs. Minimal inhibitory concentrations of trimethoprim/sulfamethoxazole for the strains were measured by the E-test on both media and the results were compared to each other and to those obtained with the disc diffusion method. There was no significant difference between the results obtained with the HUMAN and OXOID discs as well as the E-test. In contrast, 77% of the strains showed sensitivity on bioMérieux Mueller-Hinton agar, while only 70% exhibited sensitivity on the agar prescribed by the HUMAN in the Resistest sheet. In conclusion, the results of the sensitivity tests to trimethoprim/sulfamethoxazole combination may considerably depend on the composition and quality of media used in different laboratories. The correct laboratory measurements greatly influence the choice of antibiotics and the cost of the treatment. Omission of trimethoprim/sulfamethoxazole combination attributable to a misdetermination of resistance seems to be unfounded in many cases.

K. KAMOTSAY, I. DUNAY, ZS. CSUKÁS, K. LATKÓCZY, K. GLATZ, ZS. BEREK, F. ROZGONYI

Distribution and antibiotic susceptibility of blood culture isolates

Institute of Microbiology, "Semmelweis" University Medical School, Budapest, Hungary

Between 1 January and 31 December 1998, 426 microbes were isolated from 2587 blood culture bottles. 65% of the bacteria belonged to the Gram-positive group and 35% of them belonged to the Gram-negative group. 0.02% of the positive results

were *Candida* spp. The most frequent microorganisms were *Staphylococcus aureus* (29%), followed by coagulase-negative *Staphylococcus* spp. (20%), *Enterobacter* spp. (13%), group-D *Streptococcus* (7%) and *Escherichia coli* (7%). The most potent drugs against *S. aureus* were vancomycin (100%), teicoplanin (92%), chloramphenicol (92%), netilmicin (74%), ciprofloxacin (62%), oxacillin (48%), and against coagulase-negative *Staphylococcus* spp. vancomycin (100%), teicoplanin (83%), chloramphenicol (90%), netilmicin (67%), ciprofloxacin (90%), oxacillin (60%). Strains of group-D *Streptococcus* were susceptible to vancomycin (100%), teicoplanin (80%), imipenem (87.5%), piperacillin (76.5%) and amoxicillin/ampicillin (67%). In case of *Enterobacter* spp. the most effective drugs were imipenem (94%), and ciprofloxacin (77%). Strains of *E. coli* were susceptible to imipenem (100%), cefuroxime (74%), ceftriaxone (88%), ceftazidime (88%), cefotaxim (66%). The most potent drugs against *Pseudomonas* spp. were netilmicin (73%), amikacin (65%) and ciprofloxacin (55%). In conclusion, bloodstream infections of inpatients seem to be caused by multiple resistant bacterial strains.

V. TALESKI[1], T. L. HADFIELD[2], S. STOJKOVSKI[1]

Human brucellosis in the Republic of Macedonia and current diagnostic possibilities

[1]Department of Microbiology, Institute of Preventive Medical Care, Military Health Institution Centre, Skopje, Republic of Macedonia, [2]Armed Forces Institute of Pathology (AFIP), Washington DC, USA

Brucellosis is a typical zoonosis caused by bacteria of genus *Brucella* that belongs to risk group III of laboratory hazards and present a potential biological weapon.

The epidemic and epizootic of brucellosis in the Republic of Macedonia started in 1980. A total of 7337 cases of human brucellosis were documented until the end of 1998, with average morbidity of 18.3/100,000 (highest rate of 44.2/100,000 in 1992). The disease is markedly seasonal with lowest morbidity in December and highest in May and June. According to the questionnaires 34% of patients were infected by direct contact, 23% by alimentary way and 43% buy both, including aerosols. The patients were male in 65%, female in 35%, citizens in 20% and peasants in 80%. The highest percentage of disease in animals was reported in 1992, when out of 1,163,000 examined herds of sheep and goats (source of infection), 6890 (0.6%) were infected. *Brucella melitensis* biotype-2 was recognized as the etiological agent.

The diagnosis of human brucellosis is mainly based on the clinical features of the disease and the classical serological tests: Rose Bengal – Slide Agglutination Test, Wright – Serum Agglutination Test in Tubes, Coombs – Antihuman Globulin Test, CFTf Complement Fixation Test and 2-Mercaptoethanol Test. Our two comparative studies pointed out the significant superior sensitivity and specificity of competition ELISA (c-ELISA – CVL, New Haw, Addlestone, UK) and ELISA (NOVUM-Diagnostica) than the classical serologic tests, especial in diagnosis of chronic brucellosis. The bacteriological isolation and identification is not implemented, since no adequate laboratories exist in Republic of Macedonia. Primary isolation and identification of *Brucella* spp. is difficult, since all species are slow growing and fastidious. Molecular diagnostic techniques, based on PCR, allow to overcome bacterial isolation and identification, by *in vitro* amplification of the DNA-target, and therefore has the potential to result in a quicker (one day only) and more reliable diagnosis of *Brucella* infection. The first PCR results of our collaborative study with AFIP, in diagnosis of human brucellosis by PCR from blood, were four positive out of ten. Mononuclear cells were separated by Becton Dickinson's VACUTAINER CPT Cell Preparation Tubes with sodium citrate. PCR reactions were designed around the insertion sequence IS711 and upstream genes for the *Brucella* species. The testing was done on a Perkin Elmer DNA sequence detector model 7700. Thermal cycling master mix consisted of 200 μM dNTP's, UNG, 50 μM Mg^{++}, Taq Gold, forward primer and reverse primer and probe. Thermal cycling conditions were 10 minutes at 94 °C, followed by 40 cycles of 94 °C for 20 seconds and 60 °C for 60 seconds (2 step PCR).

A. K. T. KIRSCHNER[1], A. FARNLEITNER[2], A. HERZIG[3], G. KAVKA[2], S. KÖLBL[4], B. VELIMIROV[1], T. ZECHMEISTER[5]

Determination of the potential toxical risk of botulism neurotoxin C1, responsible for the outbreak of avian botulism, and the relation to ecological variables of flat saltwater pools in the Austro-Hungarian National Park Neusiedler See – Seewinkel (Fertő Hanság)

[1]Institut für Medizinische Biologie, Universität Wien, [2]Institut für Wassergute des BAW, Wien, [3]Biologische Station Neusiedler See, Illmitz, [4]Institut für Virusseuchenbekämpfung, [5]Institut für Allgemeine Biochemie, Vienna Biocenter, Wien, Austria

Botulism neurotoxin, serotype C1 causes animal botulism. This toxin is produced under anaerobic conditions by *Clostridium botulinum* strains, infected with tox+ phages containing the toxigenic gene. Type C1 is worldwide common in avian species, causing massive outbreaks in wild waterfowl. Especially in the fiat salt water

pools and surrounding marshlands of the Austro-Hungarian national park Neusiedler See–Seewinkel (Fertő Hanság), avian botulism has been diagnosed for several times since 1982, often leading to the collapse of several endangered bird populations.

In our planned interdisciplinary study we want to elucidate the connections between the potential occurrence of Botulism Neurotoxin C1 production and the ecological variables in selected areas of the national park. The ecological variables include chemophysical data (e.g. temperature, pH, O_2, nutrients, substrate availability) and microbiological data (e.g. bacterial numbers, biomass and production) in both the sediment and the water column of the flat saltwater pools. In addition to field investigations, microcosm experiments will be conducted, simulating different ecological conditions (e.g. substrate enrichment, different chemophysical treatments of the sediments, addition of toxigenic cells) in order to find out the key factors being responsible for successful toxin production.

The toxin shall be routinely detected via mouse-bioassay. Additionally, *in vitro* techniques (enzymatic and immunological) as alternatives to the mouse-bioassay shall be tested. Extracted and purified DNA of the environmental samples will be analysed for different types of tox+ phages by PCR, DGGE and sequencing.

The results of this study shall provide a basis for future monitoring and management programs, which should dam massive outbreaks of the disease in the national park, and which should also be applicable as a model for other bird-protecting areas worldwide.

I. RYCHLIK[1], L. CARDOVA[1], A. SVESTKOVA[1], G. MARTIN[2], U. METHNER[2]

Interactions of stationary cultures of *Salmonella typhimurium* F98 and its defined mutants

[1]Veterinary Research Institute, Brno, Czech Republic, [2]Federal Institute for Health Protection of Consumers and Veterinary Medicine, Jena, Germany

Currently used live attenuated vaccine strains of *Salmonella typhimurium* in poultry are less microbiologically competitive and do not protect very young chicken against infection till protective immunity 10 to 14 days post-immunization is raised. Therefore we screened more then 3000 Tn mutants of *Salmonella typhimurium* to identify genes involved in microbiological competition which occurs soon after reaching stationary phase of growth. Mutants identified belonged to i) general regulators (*oxr*A, *oxr*G, *crp*, *cya*, *arc*A), ii) respiration (*nuo*G, *cyd*A, *unc*H), and iii) chemotaxis (*fli*D, *fli*M). Further mutations occurred in *omp*C, *omp*D, *tdc*C, *aro*A and

*aro*D. All the mutants were characterized by their biochemical activities, flow cytometry and their ability to restore the wild type phenotype by the addition of homoserine lactone and related amino acids. Finally, defined mutations (*ompC*, *asd*) and *lux*AB fusions (*rpo*S, *sdi*A, *gyr*A) in selected genes were created to assess the events in *Salmonella* cells entering stationary phase of growth. Most of the interactions can be explained by differences in nutrient uptake and utilization, however cell-to-cell signalling cannot be excluded. All the findings can be utilised in new vaccine development.

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U. METHNER, A. BERNDT, G. STEINBACH

Combination of vaccination and competitive exclusion to prevent *Salmonella* infection in chickens

Federal Institute for Health Protection of Consumers and Veterinary Medicine, Jena, Germany

Vaccination and competitive exclusion (CE) represent accepted prophylactic measures to control *Salmonella* infections in chickens. To use the advantages of both CE technique and vaccination with live *Salmonella* vaccines the combination of these methods was studied.

It has been the purpose of our experiments to study competitive exclusion, inhibitory and immunological effects after combined use of CE and vaccination with both attenuated live *Salmonella* vaccine or non-attenuated *Salmonella* wild-type strains against *Salmonella* infection in chickens of different ages.

SPF chickens were pretreated using combined or unique administration of CE and vaccination with live *Salmonella typhimurium* strains on days 1 and 2 of life and challenged with a *S. typhimurium* strain on days 3, 15 or 40 of life. The caecal colonization of both the vaccine and the challenge strain and the antibody response after infection were examined to evaluate the protective effects of the different combinations.

The exclusion effect of the CE culture against *Salmonella* infection could be seen in very young chicks and was still considerable on day 40 of life of the birds. The combined administration of competitive exclusion and immunization resulted in a considerable additional protective effect above the level of the respective exclusive application of these prophylactic measures. Administration of the *Salmonella* vaccine strain prior to or simultaneously with the CE culture produced the best protective effect

because such combinations ensure an adequate persistence of the vaccine strain as prerequisite for the expression of colonization inhibition effects and a strong immune response. The full exploitation of this potential using attenuated live *Salmonella* vaccines will require the presence of high inhibitory and immunogenic properties of the vaccine strain after attenuation of a selected parent strain.

Z. SZÉNÁSI[1], I. VERÉB[1], T. ENDO[2], K. YAGITA[2], E. NAGY[1]

Detection of *Legionella* spp. in water samples from cooling towers and in clinical samples by the polymerase chain reaction (PCR)

[1]Department of Clinical Microbiology, "Albert Szent-Györgyi" University Medical School, Szeged, Hungary, [2]Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan

Legionellosis may occur when viable legionellae in aerosols are inhaled into the lower respiratory tract, occasionally causing pneumonia. Surveying of legionellae in the environment (such as lakes, streams, water supplies and cooling towers) and in clinical samples is needed to prevent and control legionellosis. Since culture techniques have limitations (long incubation period, recovery rate of less than 100%), we introduced PCR methods with primers specific for the genus *Legionella* (5S rRNA gene) and *L. pneumophila* (*mip* gene). Using seeded water samples, and a clinical isolate from BAL, we found that PCR of the 108-bp fragment of the Legionellae 5S rRNA gene was capable to detect legionellae in simulated specimen containing more than 10 cfu/ml. The advantage of PCR is that it may give results in one day, in contrast to conventional culture methods. This allows the introduction of an immediate antimicrobial therapy. However, by the introduction of PCR method, the conventional culture methods cannot be omitted. First, the sensitivity of the PCR, in contrast to conventional culture methods, is difficult to improve by increasing the sample volume because of the possible presence of inhibitors in the sample. Second, the method is a presence/absence test and can give, at most, semi-quantitative results. The same sample should be retested with culture methods to obtain quantitative data and strains for epidemiologic typing. In conclusion, the PCR and culture methods may complement each other in the monitoring of water samples and in clinical diagnosis.

D. SZAKÁL, T. PÁL

The effect of selective enrichment media on the growth of enteroinvasive *Escherichia coli*

Department of Medical Microbiology and Immunology, University Medical School, Pécs, Hungary

Due to the lack of fully specific biochemical markers the recognition of enteroinvasive *Escherichia coli* (EIEC) among the members of the fecal flora is often difficult. Even with the introduction of *Shigella*/EIEC-specific DNA probes colonies (usually 6–10) are randomly selected for the assay. In order to enhance the "hit-ratio" of such a selection procedure we investigated the growth supporting effect of different media on, EIEC, and compared it to the corresponding effect on *Shigella*, *Salmonella* and fecal *E. coli* strains. Diluted cultures of 79 EIEC, 42 *Shigella*, 22 *Salmonella* and 53 faecal *E. coli* strains were spread on DC, Hynes-modified DC, Salmonella-Shigella Agar (SS), and XLD, and – serving as a non-selective control – on a Tryptic Soy agar plates. The number of colonies found on the selective media were expressed as the % of the one found on non-selective TSA plates. The strains were also inoculated into liquid Selenite F, and Gram-negative broth. After 8–12 hours of incubation, the changes in the cell counts were compared to the respective 0 time values. *Salmonella* strains grew best on all solid media, while fecal *E. coli* the least. The growth of EIEC and *Shigella* were remarkably identical. All species grew best on SS while the two DCs were the most inhibitory. However, when their respective effect on EIEC was compared to that on normal *E. coli*, XLD and the two DCs were comparatively selective, while SS plates showed very limited selectivity. Selenite broth and Gram-negative broth did not provide any selective advantage for EIEC over normal *E. coli*. Based on this observations we recommend highly selective plates, like DC or XLD, to be included in the diagnostic panel when searching for EIEC.

R. AUCKENTHALER

Diagnosis of mycobacteria: new developments

Laboratoire Central de Bacteriologie, Hopital Cantonal Universitaire de Geneve, Geneve, Switzerland

Tuberculosis (TB) has increased in certain parts of the world due to war, socioeconomic insufficiency, HIV and lack of TB surveillance. Today one-third of the world's population is infected with *M. tuberculosis* and according WHO there are 8–10

million new TB cases each year. Multidrug resistant strains (MDR) are emerging and laboratories are challenged to provide rapid identification and antimicrobial susceptibility testing for effective treatment. Various methods have been recently developed in the view to increase the sensitivity and to speed up diagnosis: i) Direct examination including cytocentrifugation, auramine staining and amplification of *M. tuberculosis* complex in direct specimens. ii) Culture including solid and liquid media using the semi-automated methods BACTEC 460 TB or automated BACTEC 9240, BACTEC 960 with Mycobacteria growth indicator tube MGIT (Becton Dickinson), MB/BacT (Organon Teknika), ESP automated test systems (Accumed). iii) Detection of positive cultures by probes or amplification. The PCR products can be automatically detected (Cobas Amplicor) or analyzed by sequencing. iv) Susceptibility tests using the automated culture systems or other technologies such as detection of resistant genes to rifampicin and isoniazid by PCR. Today, microbiology laboratories should be able to ensure that results concerning acid-fast bacilli in specimens are available promptly (ideally, within 24 hours of specimen collection), TB control programs should have access to adequate mycobacteriology laboratory services. Reports of isolation and identification of *M. tuberculosis* should be available within 10–14 days, and reports of drug-susceptibility tests should be available within 15–30 days of specimen collection (Centers for Disease Control and Prevention, MMWR 1995°; 44°: RR-11).

B. SZABÓ[1], C. MISZTI[1], L. MAJOROS[1], L. MÓDIS[2], A. BERTA[2]

Isolation, identification and eradication of *Agrobacterium radiobacter* from human endophthalmitis

[1]Department of Microbiology, [2]Department of Ophthalmology, University Medical School, Debrecen, Hungary

A. radiobacter is a plant pathogen but occasionally may infect humans as well. Shortly after removal of an intraocular foreign body from the vitreous space of a 41-year-old male a serious endophthalmitis had developed. *A. radiobacter* was cultured from the vitreous fluid. The diagnosis was based on growth characteristics, microscopic morphology and results of 21 biochemical reactions. The *A. radiobacter* was sensitive to the majority of antibiotics, only the trimethroprim-sulphametoxazol proved to be ineffective *in vitro*. Pars plana type vitrectomy, local amikacin and ceftazidime as well as postoperative systemic amikacin and ceftazidime administration resulted in healing and clearing of the eyeball. This is the first report of human eye infection caused by this plant pathogen introduced by a foreign body.

G. SZMOLLÉNY[1], I. TÓTH[1], K. RÁSKY[1], F. PÉTERFY[2], U. DINJUS[3], F. V. VAN ZIJDERVELDT[4], B. NAGY[1]

Modified monoclonal antibody enzyme-linked immunosorbent assays for detection of specific antibodies to *Salmonella enteritidis* in poultry

[1]Veterinary Medical Research Institute, Hungarian Academy of Sciences, [2]Diagnosticum Ltd., Budapest, Hungary, [3]BGVV Institute of Bacterial Animal Diseases and Zoonoses, Jena, Germany, [4]DLO-Institute for Animal Science and Health, Lelystad, The Netherlands

In order to facilitate the detection of *S. enteritidis* infections in poultry flocks as part of the Hungarian *Salmonella* reduction campaign, monoclonal antibody based enzyme-linked immunosorbent assays (ELISA systems) have been developed and tested for detection of antibodies against *S. enteritidis* in poultry (*Gallus domesticus*). Western-Blot studies indicated the specificity of the monoclonal antibody (Mab) against *S. enteritidis* flagellin (9G3) and direct competition ELISA assays revealed that this Mab was directed against a different epitope as compared to the Mab developed by Van Zijderveldt et al. (1992) earlier. By the use of this new Mab and by modifications of the earlier systems of blocking ELISA and double-antibody sandwich (DAS) blocking ELISA, it was possible to differentiate antibody response of young hens vaccinated with a *S. enteritidis* vaccine from an antibody response of *S. gallinarum/pullorum* infection. Results indicated that the DAS-blocking ELISA was adequately specific and sensitive in detection of *S. enteritidis* infection at different ages similarly to the ELISA system of Van Zijderveldt.

Á. DÁN[1,2], I. FERENCNÉ[1], P. DE SANTIS[3], M. TENK[1,2], B. HARRACH[1], L. STIPKOVITS[1]

Investigations on the presence of *Mycoplasma mycoides* ssp. *mycoides* small colony type in Hungarian cattle herds by PCR

[1]Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary, [2]Central Veterinary Institute, Budapest, Hungary, [3]Instituto Zooprofilattico Sperimentale dell' Abruzzo e del Molise "G. Caporale", Teramo, Italy

Mycoplasma mycoides subspecies *mycoides* small colony type (MmmSC) is the etiological agent of contagious bovine pleuropneumonia (CBPP), a disease causing considerable economic losses. Using PCR method, we have investigated 1003 cattle originated from 110 farms located in 20 different districts of Hungary in order to attempt the detection of possible MmmSC infection with this very sensitive method.

Lung tissues (953 samples) from the slaughtered cattles and nasal swabs (50 samples) were collected for DNA extraction. Examinations of the samples were performed with three different PCR assays described previously. Four hundred eighty samples were examined using *M. mycoides* cluster specific primers, 325 samples by MmmSc species specific primers and 198 samples were tested with MmmSC species specific primers in nested PCR. All the samples were negative regardless of the primers applied. Testing the sensitivity with DNA extracted from lung tissue of an artificially infected cow (used as positive control in the study) the nested PCR proved to be 100 times more sensitive than the one step PCRs. The same tissues and swabs were tested also by culturing and using selective media and were found to be negative for MmmSC. Our results strengthen the statement that CBPP is absent in Hungary.

R. ROSENGARTEN, C. CITTI

Host-pathogen interactions in *Mycoplasma* pathogenesis: immune evasion and exploitation of host cells

Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine, Vienna, Austria

The genus *Mycoplasma* belongs to a group of prokaryotes which are the smallest and simplest self-replicating organisms, of which many species are known to be etiologic agents of disease in man and animals. They are characterized by i) their lack of a cell wall emphasizing membrane surface proteins as the key components for a variety of functions involving host interactions and immune avoidance; ii) their small genome with a limited coding capacity that makes them "minimal cells" which are dependent on the supply of nutrients from their in-host environments; and iii) the apparent paucity of recognizable components that regulate gene expression in response to environmental changes. All of these features have recently been confirmed by the complete genome sequence of two mycoplasma species, namely the human pathogens *Mycoplasma genitalium* and *Mycoplasma pneumoniae*. From an evolutionary point of view this enormous reduction of genetic information that precludes several conventional metabolic pathways must have led to the obligate parasitic mode of life of these organisms. The parasitic lifestyle forced the mycoplasmas to maintain a high number of genes devoted to attachment and to exploit diverse mechanisms of genetic and phenotypic variation as a strategy for survival and adaptation to the microenvironmental changes encountered in the host, including those through adaptive immunity. In fact, the molecular characterization of genetic mechanisms directed towards evasion of the host immune system is one of the "hot" subjects of current

mycoplasma research. Several recent studies in this area indicate that high-frequency, reversible mutations affecting both the structure (size) and the expression of abundant membrane surface proteins may be widespread among different pathogenic mycoplasma species, which underscores their importance as a means of governing key functional aspects of these organisms. Although the functional consequences of this mutation-based structural variation, phase variation or antigenic variation are still poorly understood, the impact of these variations imposed by multiple types of mutations associated with individual genes may be considerable and may contribute in many ways to the survival, propagation, and virulence potential of a pathogenic mycoplasma species. Recent interesting findings that some species of pathogenic mycoplasmas are capable of active invasion into non-phagocytic host cells will open new ways for defining the role of genetic variation in mycoplasma-host cell interactions and will provide new insights into the molecular events of mycoplasma pathogenesis.

G. STANEK

Lyme borreliosis in Europe

Institute of Hygiene, Medical Faculty, University of Vienna, Vienna, Austria

No other tick-borne disease has sparked and held such an interest for physicians, scientists, the pharmaceutical industry and the public over the last 10 years as much as Lyme borreliosis (or Lyme disease). Is there a reason for this? Is the clinical presentation so destructive that Lyme borreliosis makes so many people afraid of it? Is the disease so frequently seen that the fear is justified? What is the secret of this spirochetal bacterial disease named Lyme borreliosis?

There may be many answers. One may be that besides the main clinical pictures that have been defined there are others which are poorly defined. Then, it is still a matter of controversy whether borrelia can be cleared completely from the body by antibiotic therapy. Further, there is controversy about the effect of high concentrations of specific antibodies in healthy persons. Lastly, there is evidence for induction of autoimmune processes by borrelia infection.

Lyme borreliosis occurs throughout Europe. The tick attack rate in humans ranges between 4% and 50% of the population. About 2% of persons bitten by ticks will develop clinical disease. It presents most frequently with a characteristic rash, the skin disorder erythema (chronicum) migrans (ECM). When comparing results of old

and new epidemiological studies it is obvious that the relative number of ECM cases has increased and that of neuroborreliosis and acrodermatitis has decreased.

What has also been observed is that the annual incidence rate is highest in countries of Central Europe such as Slovenia, Austria and Southern Germany (over 100 cases per 100,000 population). Southern Sweden has also a remarkably high incidence rate (69 per 100,000) whilst Ireland and the UK record incidence rates of 0.3 to 0.6 per 100,000.

There are now several countries in Europe where reporting of Lyme borreliosis is mandatory. However, reporting of Lyme borreliosis alone would not guarantee that more reliable figures will be obtained. The drawbacks in establishing incidence and prevalence data are mostly due to missing facilities for long-term observation of patients and are also to serodiagnostic criteria, which vary between laboratories.

F. ROZGONYI, Á. GHIDÁN

The effect of iodine polyvinylpyrrolidone (Betadine®) on multiple antibiotic resistant bacteria and *Candida albicans*

Institute of Microbiology, Semmelweis University Medical School, Budapest, Hungary

The bactericidal and fungicidal effects of Betadine at different concentrations were examined on 10^5 and 10^9 microorganisms/ml using a plate count method and a biophotometric measurement. Betadine was bactericidal and fungicidal even in 100-fold dilution, i.e. 0.1% concentration on methicillin-resistant *Staphylococcus aureus,* vancomycin-resistant *Enterococcus faecalis,* extended spectrum beta-lactamase producing *Klebsiella pneumoniae* and fluconazole-resistant *Candida albicans* strains. 500-fold and 1000-fold dilutions suspended the bactericidal and fungicidal effects, however, a bacteriostatic and fungostatic effects still existed for a longer period of time. The results indicate that using Betadine at proper concentrations to disinfect skin and mucosal surfaces prevents colonisation of multiple-resistant bacteria and *Candida.* Betadine at lower concentrations may delay the multiplication of such microorganisms consequently the infections may not take place.

R. NEMCOVÁ, A. BOMBA, S. GANCARCIKOVÁ, R. HERICH, P. GUBA

Effect of administration of lactobacilli and fructooligosaccharides on the fecal microflora in weaning piglets

Research Institute of Veterinary Medicine, Kosice, Slovak Republic

The influence of administration of *Lactobacillus casei* alone and a mixture of *Lactobacillus casei* and fructooligosaccharide (FOS) on faecal bacteria counts in the weanling pigs was investigated. In faeces of experimental animals receiving the mixture of *Lactobacillus casei* and FOS, significantly higher *Lactobacillus* ($p<0.01$), *Bifidobacterium* ($p<0.05$), total anaerobes ($p<0.05$), and total aerobes ($p<0.05$) counts have been found as compared to the control and significantly higher anaerobes ($p<0.05$), total aerobes ($p<0.05$), *Bifidobacterium* ($p<0.05$), and *Lactobacillus* ($p<0.05$) counts compared to *Lactobacillus casei* group. Compared to the control, significant decrease in *Clostridium* ($p<0.05$), *Enterobacteria* ($p<0.01$) counts was observed as well as an insignificant decrease in *Coliform* counts by 1 log. *Enterococcus* counts were significantly reduced ($p<0.001$) compared to both control group and *Lactobacillus casei* receiving group. In faeces of experimental animals receiving *Lactobacillus casei,* significant decrease in *Clostridium* ($p<0.05$) and *Enterobacteria* ($p<0.05$) counts as compared to the control was recorded. *Coliform* counts were by 0.5 log lower compared to control. This difference, however, was insignificant similarly like with *Coliform* in previous experimental group due to the great variance of values in individual groups. *Lactobacillus, Enterococcus* and total anaerobes counts were identical in both groups. An insignificant increase in total aerobes in favour of experimental group was recorded and vice versa, there was an insignificant decrease in *Bifidobacterium* as compared to the control group. The results obtained point to a synergic effect of the combination of *Lactobacillus casei* and fructooligosaccharide on numbers of bacterial populations observed in the faeces of the weanling pigs. The combination of probiotics and non-digestible carbohydrates may be a way of stabilization and/or potentiation of the effect of probiotics. Such potentiated probiotics indicate a realistic way of using biological preparations in the prevention of gastrointestinal diseases in weaned pigs.

E. SZABÓ, A. SKEDSMO, E. PÁTRI, L. EMŐDY, T. PÁL

The occurrence of curly fimbriae on enterotoxigenic *Escherichia coli*

Department of Medical Microbiology and Immunology, University Medical School, Pécs, Hungary

A total of 109 enterotoxigenic *Escherichia coli* (ETEC) strains of different geographical origin were investigated for the expression of curli type fimbriae. Strains were grown in micro-cultures of colonization factor broth at 30 and 37 °C. The presence of curli was detected by a polyclonal antibody raised against the curli (SEF17) of a *Salmonella enteritidis* strain. Of the 109 strains 56 gave a positive reaction at 30 °C arbitrarily defined as when reaching the 50% of the optical density obtained with the homologous *Salmonella* strain. The average OD of the positive isolates was: 128.62 ± 87.13% of the positive control, while the corresponding value for the negative isolates was 16.12 ± 11.40%. The expression of curli was more pronounced in all of the 56 positive isolates, and the $OD_{30\ °C}$ / $OD_{37\ °C}$ ratio exceeded 2 in all but three of them (average: 19.47 ± 25.83). Morphologically, the curli of ETEC was indistinguishable from that of *Salmonella*. It mediated the binding of the Congo Red dye at 30 °C, and – in most strains – its presence was associated with the aggregative nature of the isolate. Based on these findings it is concluded that curli is a frequent component of the ETEC cell structure. Similarly to animal pathogen *E. coli*, and unlike in *S. enteritidis* it is better expressed at ambient temperatures.

GY. SCHNEIDER, D. SZAKÁL, T. PÁL

Detection of enterohaemorrhagic *Escherichia coli* serotype O157 with colony blot technique

Department of Medical Microbiology and Immunology, University Medical School, Pécs, Hungary

With the help of a polyclonal antisera raised against boiled bacteria colonies of Enterohaemorrhagic *Escherichia coli* serogroup O157 in fecal samples were detected. Contaminated stool samples were evenly spread on to Tryptic Soy Agar plates and were covered with nitrocellulose membranes. After overnight incubation and pretreatment to eliminate the internal enzyme activity of the samples, colony prints of the pathogens were visualized by the O157-specific serum followed by an Alkaline Phosphatase-labelled conjugate. By this method as low as 10^5 pathogen germs in the

presence of 10^8 c.f.u. background flora pro gramm feces could safely be detected. The major advantage of this technique is that it could also detect the increasing number of sorbitol positive O157 isolates, left unnoticed by the classical diagnostic approaches.

GY. SCHNEIDER[1], S. FUCHS[2], M. KERÉNYI[1], I. MÜHLDORFER[2], J. HACKER[2], L. EMŐDY[1]

The effect of *rec*A and *leu*X loci on the virulence of enterohaemorrhagic *Escherichia coli*

[1]Department of Medical Microbiology, University Medical School, Pécs, Hungary,
[2]Institut für Molekulare Infektionsbiologie, Universität Würzburg, Würzburg, Germany

In this report the impact of the *rec*A and *leu*X loci on the virulence of the enterohaemorrhagic *Escherichia coli* (EHEC) strain 86–24 was evaluated. Comparative virulence assays were performed with the wild-type isolate, its *rec*A and *leu*X mutants, and derivatives trans-complemented with the cloned *rec*A or *leu*X genes.

In vitro assays revealed that Shiga toxin 2 production was significantly reduced in the mutant strain, and trans-complementation with the *rec*A, gene reconstituted the full toxic capacity. Intravenous lethality and lung toxicity assays in mice showed a strongly reduced virulence for the *rec*A mutant, while the trans-complemented derivative exhibited a degree of virulence comparable to that of the wild-type isolate. There was, however, no difference between the wild-type strain and the *rec*A mutant in the capacity to colonize the mouse intestine after oral administration of the bacteria.

No difference could be detected in Shiga toxin production and parenteral mouse virulence of the wild-type strain and its *leu*X mutant. At the same time this mutant showed a reduced capacity to colonize the mouse intestine.

The original aim with production of *rec*A mutants was to construct genetically stable, recombination deficient bacterial derivatives for prospective vaccine studies. Our results indicate that this mutation may also be utilized as a tool of attenuation.

Z. PÉTERFI, B. KOCSIS

Optimization of ELISA test used for detection of serological cross-reaction between lipopolysaccharides

Department of Medical Microbiology and Immunology, University Medical School, Pécs, Hungary

Enzyme-linked immunosorbent assay (ELISA) is probably the most frequently used method for estimation of antibodies. In case of lipopolysaccharide (LPS) antigens their poor coating to microplate is a problem. Takahashi et al. described a good method for coating LPS antigens using poly-L-lysine for precoating. Another problem of the ELISA method is the nonspecific binding of antibodies to the plastic wells. To reduce this disadvantageous phenomenon, blocking agent, such as bovine serum albumin, casein is commonly used. We have to choose the blocking agent carefully because unfortunately LPS can bind proteins aspecifically. This process can inhibit LPS-specific antibody activity and diminish the sensitivity of ELISA test. In this poster we present an ELISA for LPS in which normal goat serum is used for blocking instead of bovine serum albumin and casein. Goat serum gave statistically significantly lower OD value for negative control and statistically significantly higher OD values for positive control. Our study on *Shigella sonnei, Escherichia coli* and *Proteus morganii* lipopolysaccharides demonstrates that this type of ELISA using goat serum for blocking is the best method not only for detection of LPS and anti-LPS antibody reaction, but in cross-reaction study, too.

CS. MISKOLCI[1], I. LABÁDI[2], T. KURIHARA[3], N. MOTOHASHI[4], J. MOLNÁR[1]

G-C rich regions of plasmid DNA can be the target in antiplasmid effect of phenothiazines

[1]Institute of Microbiology, "Albert Szent-Györgyi" University Medical School, Szeged, Hungary, [2]Department of Inorganic and Analytical Chemistry, Faculty of Science, Josai University, Sakadoshi, Saitama, [3]Meiji College of Pharmacy, Tanashi-si, Tokyo, Japan

Phenothiazines have been shown to inhibit the growth of *E. coli* bacteria and promote the elimination of plasmids. It has been proposed that some of these effects may be correlated to the binding of the phenotiazine to DNA by intercalation. Heterocyclic compounds such as caffeine and indole intercalate weakly with DNA and therefore may promote the stacking of phenotiazines. To test this hypothesis, we have studied the plasmid elimination effect of the phenotiazine promethazine in the presence

of xanthine analogs caffeine, guanosine monophosphate and indole which themselves have no effect on the growth of *E. coli*.

The order of suppression of plasmid elimination produced by promethazine combined with different agents reflected the polarizing power of each agent. There might be a competition between the phenotiazine and GMP for intercalation sites of the DNA, by the binding of the phenotiazine to the G-C rich region or by molecular stacking of promethazine into the G-C rich region of plasmid DNA promoted by the xanthine analogs.

M. HERPAY, É. CZIRÓK, I. GADÓ, H. MILCH

Laboratory strategy in diagnosis of Shiga toxin producing *Escherichia coli* in Hungary

"B. Johan" National Center for Epidemiology, Budapest, Hungary

Altogether 29 Shiga toxin-producing *Escherichia coli* (STEC) were isolated and characterized according to their serotypes, virulence markers, and association with human illness. For screening faecal samples were examined for free Shiga toxins (Stx(s)), additionally they were cultivated on sorbitol MacConkey (SMAC), cefixime and tellurite (CT)-SMAC and chromogenic plating media (BBL CHROMagar O157). Comparison of *E. coli* O157 detection was made between direct plating, immunomagnetic separation (IMS) and plating. Premier EHEC enzyme immunoassay was applied for the detection of Shiga toxins (Stx(s)). Specimens positive by Premier EHEC test and negative for *E. coli* O157 were serotyped. Our O157 STEC positive human faecal samples originated from bloody diarrhoea (13), nonbloody diarrhoea (2) and HUS (1). In 13 cases free Stx were detected. Two mixed cultures and 5 isolates (O157-2, O18ab-2, O19-1) of these cases were Stx-positive, too. In two cases STEC strains (O157-1, O76-1) could be isolated, however Stx(s) could not be detected in their faecal samples. Mixed cultures of laters were also Stx(s) positive. All faecal samples from patients with diarrhoea should be screened for the most frequent serogroup O157, or if this is not possible, at least those from patients with bloody diarrhoea. CT-SMAC and CHROMagar are selective for O157 STEC. Premier EHEC test is valuable as a routine method for the detection of non-O157 Stx-producing *E. coli*. Successful Stx diagnosis can be expected from different kinds of simultaneously performed methods.

K. TÖRŐ[1], ZS. CSUKÁS[2], L. TÓTH[1], F. ROZGONYI[2]

Combined effects of pre/postnatal risk factors and microbiological agents in sudden infant death

[1]Institute of Forensic Medicine, [2]Institute of Microbiology, "Semmelweis" University Medical School, Budapest, Hungary

Objectives. Many pre- and postnatal factors have an important role in the pathomechanism of sudden infant death. We investigated whether the combined action of pre/postnatal effects and bacterial colonization in throat further increase the risk for SIDS.

Methods. Questionnaire-based screening for SIDS risk factors based on family interview and taking samples for microbiological examination was performed among healthy symptom-free infants. The outcome was compared to the history data of SIDS cases. This case-control study included 17 SIDS and 74 controls and was analysed by conditional logistic regression.

Results. The outcome of our survey showed that infants living in crowded housing, with low birth weight or twin, and exposed to drugs, smoking or narcotics during gestation, having young unmarried mother/are at risk for SIDS and microbiological agents. The information feedback to the concerned family doctors and health visitors will be the basis for an increased attention paid to the future care of these infants.

Conclusion. Identification of SIDS risk factors in healthy infants could be highly important in the prevention of sudden infant death.

ZS. CSUKÁS[1], F. ROZGONYI[1], K. TÖRŐ[2], I. JANKOVICS[3]

Microbiological study among SIDS victims

[1]Institute of Microbiology, [2]Institute of Forensic Medicine, Semmelweis University Medical School, [3]"Béla Johan" National Center for Epidemiology, Budapest, Hungary

The potential role of microbiological agents was investigated in 15 cases of Sudden Infant Death Syndrome and in 15 non-SIDS cases in Budapest between September 1996 and April 1999. Autopsy, histological examination and producing *Staphylococcus aureus*, Enterobacteriaceae and *Candida albicans* strains in large number and by the detection of Parainfluenza Type 2 virus antigen. *Staphylococcus aureus* microbiological tests were performed on samples of blood, cerebrospinal fluid,

pharyngeal samples and lung tissue from infants under six months died suddenly without previous diseases. The multifactorial pathomechanism of SIDS was suggested by the isolation of toxin proved the predominant bacteria in the SIDS cases. Nasopharyngeal microbial flora and *Staphylococcus aureus* carrying state of 100 age matched healthy infants were tested during the same period. *Staphylococcus aureus* was isolated from 53% of SIDS cases and 37% from healthy infants (OR=1.986). The enterotoxin and TSST-1 toxin producing activity of *Staphylococcus aureus* showed a characteristic difference. The toxigenic *Staphylococcus aureus* was detected in 46% of SIDS cases and 16% of healthy infants (OR=4.5). The distribution of toxigenic and non-toxigenic isolates was 85% in SIDS cases and 43% in healthy infants (OR=7.875).

GY. GUNICS[1], S. FARKAS[1], N. MOTOHASHI[2], A. SHAH[3], M. KAWASE[3], S. SAITO[4], J. MOLNÁR[1]

The modification of antibiotic resistance in some Gram-negative bacteria

[1]Department of Microbiology, "Albert Szent-Györgyi" University Medical School, Szeged, Hungary, [2]Meiji Pharmaceutical University, Kiyose, Tokyo, Japan, [3]Department of Chemistry, Saurashtra University, Rajkot, India, [4]Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama, Japan

The Verapamil as a well-known resistance modifier and its newly synthetized derivatives were tested for synergy with some antibiotics. The compound had no direct antibacterial effect on various *E. coli* strains. The antibacterial effect of Ampicillin was enhanced in the presence of the majority of Verapamil analogues on a laboratory strain. Compound [G1o] and Verapamil were antagonistic with Ampicillin. Compounds [G2, G3, G8, G9, G11, G13, Nifedipine] among eleven Verapamil analogues were synergistic after 24 hours incubation. When Verapamil analogues were tested on clinical isolates of *E. coli* Ampicillin^s, Erythromycin^r, Verapamil had moderate synergistic effect with Ampicillin. Compound [G1] antagonized the antibacterial effect of Ampicillin. Synergistic effect was found with Erythromycin on *E. coli* in the presence of [G4, G5, G6 or G8]. Non of the G-compounds had synergy with Ampicillin or Erythromycin in one polyresistant clinical isolate of *E. coli*. The structure activity relationship of Verapamil analogues and their synergy with Ampicillin and Erythromycin will be discussed.

K. ANTMANN[1], P. ANDERLIK[2], Á. GHIDÁN[2], F. ROZGONYI[2]

Characterisation of *Staphylococcus aureus* strains isolated from air and patients during a hospital surveillance

[1]Institute of Public Health, [2]Institute of Microbiology, "Semmelweis" University Medical School, Budapest, Hungary

In a previous bacteriological investigation in Törökbálint Pulmonary Hospital 37 *Staphylococcus aureus* strains have been isolated from the air sample of the wards and from the nasopharyngeal swabs of patients nursed in these wards. Primary characterisation of the strains by clumping factor reaction, lipase, lecitinase tests and antibiotic sensitivity patterns indicated two clusters of the strains. Further studies on minimal inhibiting concentration (MIC) of methicillin for the strains showed 16 strains to be methicillin resistant. The degree of virulence and the presence of the above-mentioned properties strongly indicate a common origin of methicillin resistant strains isolated from both the ward air and the patients being nursed in these wards. The results indicate a possibility for air-mediated nosocomial methicillin resistant *Staphylococcus aureus* infection.

Á. GHIDÁN[1], CS. JENEY[1], K. CSISZÁR[2], F. ROZGONYI[1]

Detection of vancomycin resistance in *Enterococcus faecalis* using the PCR method

[1]Institute of Microbiology, "Semmelweis" University Medical School, Budapest, [2]Public Health Station of Nógrád County, Salgótarján, Hungary

Enterococci are members of the normal flora of the intestinal tract, however, they may occasionally play a role in nosocomial infections. Recently, the number of nosocomial infections caused by vancomycin-resistant *enterococci* (VRE) has considerably increased in both the USA and Western Europe. In the USA, less than 1% of such infections were caused by VRE in the year 1989, while they increased to 7.9% in 1993. Noble et al. proved a conjugative transfer of vancomycin-resistance from VRE to *Staphylococcus aureus* by *in vitro* methods, which species plays also a significant role in nosocomial infections. The genes responsible for vancomycin-resistance (*vanA*, *vanB*, *vanC*) can be shown precisely and reproducibly by the PCR method. A VRE strain was isolated in Salgótarján and the present investigation aimed to show the

carriage of any of vancomycin-resistance genes in this strain using a PCR and a Multiplex PCR technique, respectively. It was possible to show the presence of *vanA* gene in the strain responsible for both the vancomycin and teicoplanin-resistance. Until now in Hungary only a few VRE nosocomial infections were described, however the result of this study indicates that an increase in the frequency of such infections should be accounted in the near future.

M. ZUTIC[1], Z. LEPSANOVIC[2], D. KRNJAIC[3], R. ASANIN[3]

Antimicrobial susceptibility and plasmid profiles of *Actinobacillus pleuropneumoniae* strains isolated from swine

[1]Veterinary Institute, [2]Institute of Microbiology VMA, [3]Department of Microbiology, Faculty of Veterinary Medicine, Belgrade, Yugoslavia

Eight strains of *Actinobacillus pleuropneumoniae* were isolated from the lungs of pigs (weighing 25–35 kg) in the outbreak of pleuropneumonia in Yugoslavia in the spring of 1998. The isolated strains were subjected to antibiotic sensitivity testing and plasmid profiling. The susceptibility of the isolates to antimicrobial agents was determined by the agar disc diffusion method. Seven of the eight isolated strains were sensitive to all antimicrobial agents. Only one strain was resistant to ampicillin, amoxacillin and tetracycline. In order to determine the genetic basis of this drug-resistant strain we undertook to isolate the plasmid DNA and subsequently subjected it to electrophoresis in agarose gel. That strain possessed plasmids of 3.5:, 6.2:, 10 and 50 megadaltons. As we had isolated only plasmids from the resistant strain *Actinobacillus pleuropneumoniae*, we came to the conclusion that the present resistance to beta-lactams and tetracycline was the consequence of the existence of the specific plasmid profile.

A. WERNICKI, A. PUCHALSKI, R. URBAN-CHMIEL

Antimicrobial susceptibilities and plasmid DNA profiles of *Pasteurella haemolytica* strains

Department of General Prophylaxis and Bird Diseases, Faculty of Veterinary Medicine, University of Agriculture, Lublin, Poland

Pasteurella haemolytica is an important agent causing shipping fever or pneumonic pasteurellosis in cattle. In most population of agents antimicrobial resistance is often found, so the purpose of this study was to examine and compare antibiotic-resistance and plasmid profiles *P. haemolytica* strains isolated from cattle with shipping fever or pneumonic pasteurellosis. We report on plasmid profiles characterization and antibiotic resistance of *P. haemolytica* strains from several sites in Poland. The pathogens were isolated from nasal swabs and trachea (live animals) or lungs (died animals). Bacteria were identified by using standard criteria:

– Growth on 5% sheep blood agar plates

– Antimicrobial susceptibilities were determined using a disk-diffusion assay on Mueller Hinton agar plates with 5% sheep blood.

– The DNA plasmid preparations were made using a modification of the lysis procedure according to Sambrook et al. (1989). Plasmid DNA preparations from 18-hour cultures in BHI were electrophoresed in horizontal agarose gels in TBE buffer. Gels were stained with ethidium bromide and photographed over UV light.

Multiple antimicrobial resistance was observed. Besides there is no correlation of plasmid content and antimicrobial resistance.

The presence and absence of plasmids were observed of isolates that have multiple resistance to antimicrobials.

J. PÁSZTI[1], I. GADÓ[1], V. G. LÁSZLÓ[2], B. NAGY[3], J. KIRÁLY[1], P. KOPPÁNY[1], L. ORBÁN[1], H. MILCH[1]

Occurrence and spread of multiple resistant *Salmonella enterica* sv. *Typhimurium* strains in Hungary

[1]"B. Johan" National Center for Epidemiology, [2]National Food Investigation Institute, [3]Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary

The number of publications reporting the occurrence of strains resistant to one, two, three or more antibiotics increased from the middle of the nineties. The majority

of the reports on the rising tendency of multiple resistance concerned mainly the strains of animal origin. It was observed that most of these strains belonged to DT104. The Hungarian National Salmonella Reference Laboratory collected the data on the sensitivity to antibiotics and the distribution of phage types (PT) of *S. typhimurium* of human origin, since 1960. According to our data multiple resistance occurred more frequently from 1996 onwards, the increase continued in 1997 and it reached 55% in 1998. The distribution of phage types was also examined using the method of Felix-Callow. On the basis of statistical analysis a change was found in the distribution of phage types. Parallel with the higher incidence of multiple resistance the ratio of PTs 2 and 2c increased. There were no consequent changes in the level of PT 35 and PT 4 and Nt (not typable) decreased. Phage types of 45 strains were compared using Felix-Callow's and Anderson's methods. All of the strains of PT 2 and 2c belonged to DT 104, famous in the literature.

R. KISS[1], M. FÜZI[2], CS. BOGNÁR[3], M. FEDERIGHI[4], C. MAGRAS[4]

Detection of viable but non-culturable microorganisms. Detection of *Campylobacter* by the use of acridine orange

[1]"József Fodor" National Centre of Public Health, [2]Publich Health Laboratory Service, [3]"Béla Johan" National Centre of Epidemiology, Budapest, Hungary, [4]Veterinary University, Nantes, France

The public health network performs *Campylobacter* surveillance in case of outbreaks or sporadic cases of enteric diseases since the eighties. The number of campylobacteriosis cases shows a continuous increase – as we reported several times – but the examination of the causative, *Campylobacter* contaminated food and environment is performed very rarely. Diseases were caused by contaminated milk, egg, poultry, pork meat, salad and water in Hungary.

As a routine, control of foods does not prescribe the involvement of *Campylobacter*. In the course of our investigations – in accordance with the international literature data – we found high rate of positivity when examining poultry, meat, Danube-water using pre-enrichment, enrichment, filtration and active migration method. From heat-treated and frozen foods in several cases the cultivation of stressed microorganisms did not succeed. As the cultivation of *Campylobacter* is a demanding method we compared three methods:

1. CAT (Oxoid) supplemented Preston enrichment and CCDA (ISO 102720 standard),

2. methods prescribed by the 3640/24-1989 *Campylobacter jejuni* Hungarian standard,
3. examinations using acridine orange.

Surface samples were taken by the use of TIMI cleanness sampler (Labsystem) from poultry meat and the environment of processing. Microscope slides were stained by acridine orange.

In the smear of the corkscrew-shape or twisted forms of *Campylobacter* were seen. Higher positivity was reached by the use of ISO 10272 and CAT. By the rapid method of staining also those laboratories get information on *Campylobacter* contamination, which do not possess facilities for cultivation. Laboratories performing *Campylobacter* cultivation, however, may receive rapid information indicating positivity, which fact may be verified by cultivation.

By the use of CAT, we isolated *C. jejuni, C. coli, C. lari, C. upsaliensis* species according to ISO 10272 standard.

R. KISS[1], E. NAGY PAPP[2], ZS. FARAGÓ[2]

Experiences by the use of rapid methods for hygienic and epidemiological connections in the course of food microbiological supervision and epidemiological surveillance of *Salmonella*

[1]"József Fodor" National Centre of Public Health, [2]Public Health Laboratory Service, Budapest, Hungary

Nowadays in our country salmonellae are the most important foodborne pathogens. The detected and registered enteric diseases in Hungary in 1998 fell into the following categories: Salmonellosis (18,108), Shigellosis (752), *E. coli* enteritis (272), Campylobacteriosis (7,941), Yersiniosis (144), Enteritis infectiosa (15,909). We do not know in how many cases physicians were not contacted when the symptoms were observed and in the 15,909 enteritis cases isolations of the pathogenic agent did not succeed.

In our present work we report that during routine supervisions, examinations of the background of diseases and/or observation of hygienic deficiencies, when applying methods of relevant standards, in some cases the detection of the causative agent, could not be performed from environmental samples. By the use of immunomagnetic separation IMS (DYNAL) and ELISA (LOCATE) methods, respectively, the detection of the concerned microorganisms was successful in the environment of the production of the food.

Isolation of *Salmonella bareilly* on contrast to classical methods gave 100% sensitivity in a child's home outbreak. Detection of *Salmonella enteritidis* by the use of valid standard was not successful from environmental samples taken in the place of preparation of the foods in a school outbreak. Using IMS method, a hygienic sample taken from a washed colander, proved to be positive, thus it was obvious that hygienic deficiencies occurred in the kitchen. The efficacy of the disinfectant cleaning has to be improved.

Sensitive and rapid methods like IMS and ELISA can help to clear up the mistakes of the hygiene for the quality assurance, the performance of the improving steps and the implementation of good manufacturing practice.

G. VÖRÖS, L. BAJNOK

Effect of *Bacillus toyoi* (ToyoCerin®) on the performance, mortality and aerob caecal flora of young rabbits kept in a large-scale farm

PO-RA-VET Research and Development Ltd., Gödöllő, Hungary

An eleven week long trial with ToyoCerin^{R} (50, 100, 200 ppm in feed) with control group was carried out at a rabbit farm of 800 does. Does involved in this experiment started to consume ToyoCerin treated feed from 5 days before delivering. Litters of 70 does were equalized to 9 after kindling and the same feed including 50, 100, 200 ppm ToyoCerin was consumed by suckling and weaned rabbits. Every ToyoCerin dose did not significantly improve the weight gain of weaned rabbit caged either individually or in small groups. Regarding to the whole postweaning period feed conversion ratio of ToyoCerin treated groups were not significantly better than that of the control group. Mortality of suckling rabbits of ToyoCerin treated groups were far more lower than that of the control group. Especially losses of 100 ppm ToyoCerin treated group was very low (5.9%). Mortality of weaned and treated rabbits caused by enteric diseases was also lower immediately after weaning than mortality of the control group. *Bacillus toyoi* depending on the ToyoCerin dose and treating time composed 28–70% of aerob flora of caeca of ToyoCerin treated rabbits. Comparing the effect of different dose 100 ppm ToyoCerin achieved the best performance and mortality figures in this trial so we recommend to use this dose in practice.

Ž. CVETNIĆ[1], M. OCEPEK[2], B. KRT[2], H. KOVAČIĆ[1], K. BRLEK[3], J. TRSTENJAK[3]

Use of different tests in diagnosis of *Mycobacterium paratuberculosis* in infected cattle

[1]Croatian Veterinary Institute, Zagreb, Croatia, [2]School of Veterinary Medicine, Ljubljana, Slovenia, [3]Farm of Dairy Cows "Varazdinka", Krzovljangrad, Croatia

During a regular annual tuberculinisation on the farm of dairy cows of Frisian breed 12.1% of unspecific reactions to tuberculin was established. Some of the cows had chronic watery diarrhoea and were markedly skinny, thus such clinical symptoms indicated paratuberculosis. The blood samples of 205 cattle on the farm were serologically analysed using the following methods: enzyme-linked immunosorbent assay – ELISA (IDEXX), complement fixation (CF) test and agar gel immunodiffusion – AGID test (Bioveta), *Mycobacterium paratuberculosis* gamma interferon test kit – γ-IFN (IDEXX). The repeated allergy test (P.P.D. – Johnin-Etlik) was also carried out as well as the DNA-test (IDEXX) from the dung of cattle reacting positively or suspectedly with one of the tests mentioned. Applying some of the tests mentioned above, positive reactions were established in 39 (19%) out of 205 cattle examined. When using ELISA positive reactions were established in 20 (9.7%) of cattle, with CF test 3 (1.5%) cows were positive and 9 (4.4%) of them suspected, with AGID test 4 (1.95%) of them were positive and with γ-IFN test reactions were established in 23 (11.3%) cattle. When using the allergy test in 22 (10.7%) cattle the swelling of skin wrinkle larger than 2 mm was established and by DNA probe positive reaction was established in 10 (25.6%) cattle out of 39 samples analysed. Combining several tests (ELISA, γ-IFN, DNA-probe) and using faecal culture reliable diagnostics of paratuberculosis in cattle can be obtained.

K. J. HÁBER[1], M. TAKÁCS[2], A. NAGY[1], A. SZENDRŐI[2], P. DEZSŐ[1], GY. BERENCSI[2], J. MINÁROVITS[1]

Cloning and sequencing of mycobacteriophage DNAs

[1]Microbiological Research Group, [2]Department of Virology, "Béla Johan" National Center for Epidemiology, Budapest, Hungary

Two phages – differing from each other in their morphologies and DNAs – were separated by linear gradient equilibrium centrifugation from mycobacteriohage D29 in our laboratory. Phage D29A (of higher density) has isometric heads and long,

noncontractile tails; phage D29F also has long, noncontractile tails but its heads are elongated. Comparing these phages to the sequence of mycobacteriophage D29 published in 1998 we found that phage D29A DNA shows some similarity to D29 DNA: (i) both DNAs have only one EcoRI cleavage site (ii) non of these DNAs are cleaved by SacI endonuclease. On the contrary, D29F DNA has several cleavage sites of EcoRI and that of SacI enzyme. For the purpose of sequencing we began to clone D29A and D29F DNA fragments into pUC18 plasmid. A clone containing a ~1.2 kb KpnI-EcoRI fragment- and clones harbouring ~1.3-, 1.2- and 0.4 kb KpnI–EcoRI fragments were isolated from D29A and D29F DNA, respectively. Cleavage sites of SmaI-, SacI-, BamHI- and PstI enzymes were determined on the ~5.8kb EcoRI-EcoRI fragment of D29F-DNA inserted into the vector earlier. On the basis of this map subclones were also constructed. We also present a search of the GenBank database carried out with sequences determined from these clones.

Z. SHERKO, E. GRABOCKA

The microbial quality of some kinds of sausages on Tirana market

Institute of Public Health, Environment and Health Department, Food Microbiological Laboratory, Tirana, Albania

The aim of the present study is to perform an assessment of the microbial qualities of some kinds of sausages on the market and to identify dangerous products. The assortments of sausages' samples were analytically examined for the following microbes aerobe mesofile microorganisms, Coliforms, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, *Clostridium perfringens*. 7% of the examinated sausages contained Coliforms and *E. coli*, which are indicators of feacal contamination, so are not consumed. The data related to the presence of *Clostridium perfringens* indicate that their storage is not good. *Salmonella* and *Staphylococcus aureus* are not present in examined sausages' samples.

The transition from a centralized state-controlled system towards a free market resulted in drastic changes in the food production and distribution system of Albania. The lack of incentives in the former system, gradually installed a chronicle insufficiency of availability for food products. The adopted initial policy for filling this gap, was an unconditional support of all free initiatives within the food system. This policy created within a short time the expected abundance of food products on the market, but it associated with the creation of considerable problems on food safety and the consequent impairment of public health.

E. A. M. BORAS[1], R. KISS[2], G. KOVÁCS[1], K. MÁRIALIGETI[1]

Isolation and characterisation of *Listeria* spp. from food and environmental sources

[1]Department of Microbiology, L. Eötvös University, [2]"József Fodor" National Centre for Public Health, Budapest, Hungary

The results of our examinations showed that *Listeria* spp. however not frequent, can be isolated from environmental samples like food, sewage, sewage sludge cake, soil, and rotting leaf material. Clear results were obtained by using a two stage enrichment technique and three "selective" media. PALCAM, Oxford, and Blood agars were highly effective and differential.

Isolates were identified by the use of BBL Crystal Gram-positive technique as *Listeria monocytogenes* except two (RIA, RIC) which were delineated as *L. grayi* and *L. murrayi*. Serotyping was also done for all 35 isolates of *Listeria*. The serotypes of food isolates consisted of (six) 1/2a, (four) 3a and (one) 4b strains, whereas strains from sewage, sewage sludge cake consisted of (four) 1/2a, (ten) 3a, (three) 4ab and (one) 4b serotype. Samples from rotting leaf material, and soil contained (four) 3a, (one) 4e and (one) 4c type strain.

Comparison of the BBL Crystal identification results with 16S rDNA sequence based identification showed a good correlation except two ARDRA groups. Group A strain (identified as *L. monocytogenes* by BBL Crystal test) proved to be *L. ivanovii* and group H, RIA strain (BBL identified as *Listeria grayi*) could be determined by sequencing as *L. innocua*.

The results of our survey suggest that there is a considerable reservoir of *Listeria monocytogenes* in human and animal populations. The organism exists either as commensal in the gut or as a causative agent of subclinical or clinical infections, occurring from time to time following ingestion. We have shown that *Listeria monocytogenes* is widely distributed in sewage, food and soil, and rotting leaf material. It is interesting to note that sewage sludge cake (used as an agricultural fertiliser) was found to harbour few listerias. We have to call attention to the use of CAMP test, as not using it can lead to improper identification with BBL Crystal.

I. STEINHAUSEROVA, K. FOJTIKOVA

Identification of *Campylobacter* spp. isolated from animals by using polymerase chain reaction

Department of Meat Hygiene and Technology, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic

Campylobacter spp. is one of the most frequent causes of food-born diseases in humans in the last years. *C. jejuni* and *C. coli* are the most important species in the genus *Campylobacter*. In several cases *C. lari* and *C. upsaliensis* were recorded as the cause of human enteritis.

In the present study, rectal swabs from ill pigs with enteritis were examined. The purpose of this work was to find a simple and reliable method suitable for identification of *C. jejuni* and *C. coli*. Cultivation and biochemical testing were done by means of standard isolation and identification techniques. Then, samples were tested by using Polymerase Chain Reaction. *C. jejuni* and *C. coli* were identified together on the basis of amplification in the genome region coding for flagellin genes flaA and flaB and then analysed by Restriction Fragment Length Polymorphism.

A. H. KANJO

Our experience with Crystal identification system E/NF

Microbiology Laboratory, Árpád Hospital, Budapest, Hungary

Since more than two years we have been using several traditional and other commercial non-automated systems in our laboratory for the identification of isolated bacteria.

Some of the isolates were identified with Crystal Enteric/Non-Fermenter ID Kit (Crystal E/NF) system which is based on 30 enzymatic and biochemical substrates divided over three rows of 10 wells. The BBL CRYSTAL Identification (ID) System associated accessories include light box or panel viewer, color reaction chart, report form and software package.

The aim of this work is to evaluate the accuracy of this system for the identification of different groups of bacteria isolated from several clinical specimens.

Altogether 700 clinical isolates of fermentative and nonfermentative bacteria including the family Enterobacteriaceae, and genera *Stenotrophomonas, Pasteurella*

and members of the most common nosocomial pathogens *Pseudomonas* and *Acinetobacter* spp., mostly isolated from Intensive Care Units (ICUs) and surgical wards, were investigated.

147 *Acinetobacter baumanii* strains were tested. 117 strains were identified with no unusual reaction. The identification score was above 99%. *Escherichia coli* strains (145) were correctly identified with this system, too, 78 were identified with no unusual reaction. Mostly melibiose substrate gave unusual results (for 49 strains). 93 *Enterobacter cloacea* strains were identified. For 84 strains the identification score was above 98%. Glycine and inositol substrates were evaluated as giving mostly unusual reactions.

It can also be concluded that as the percentage of positive wells in a panel increases, the ratio of borderline reactions similarly increases. This in turn influences the value of test score.

A. A. MATOUG[1], K. S. GHENGHESH[1], R. KISS[2]

Characterisation of aeromonads isolated from different sources in Hungary and Libya

[1]Faculty of Medicine, Tripoli, Libya, [2]"József Fodor" National Center of Public Health, National Institute of Food Hygiene and Nutrition, Budapest, Hungary

In humans *Aeromonas* species are known to cause food-borne and water-borne infections, like gastroenteritis, septicaemia. Recently a number of food-poisoning outbreaks have been associated with these organisms. It is believed that some members of the genus *Aeromonas* are more virulent (e.g. *Aeromonas hydrophila* and *Aeromonas sobria*) than others (e.g. *Aeromonas caviae*). Therefore, exact identification of aeromonads isolated from different sources has a prime importance in understanding the epidemiology of these organisms and their role in diseases they cause in man.

In our work pathogens were searched in faeces, food, haemocultures, environmental, and wound samples. For the isolation of *Aeromonas* blood agar and GSP (SIGMA) agar were used. From 87 *Aeromonas* strains 27 were isolated in Hungary and 60 strains in Libya. The strains originated from water (44.4%), food (14.8%) fish, raw milk, chicken sausage, salad, and clinical specimens (40.8%) faecal samples, haemoculture, wound. The identification of the strains was performed by BBL Crystal Rapid Stool/Enteric system.

13% of the Hungarian isolates were *Aeromonas hydrophila*, 48% *Aeromonas sobria*, 9% *Aeromonas caviae* and 30% of the isolates had atypical reactions. From the

Aeromonas sobria strains, 64% were isolated from water, 9% from food, and 27% from clinical specimens.

Of the Libyan strains 40% were *Aeromonas hydrophila*, 35% *Aeromonas caviae*, 18% *Aeromonas sobria* and 7% were atypical *Aeromonas* species. Of the 24 *Aeromonas hydrophila* from Libya 54% were from water, 25% from food and 21% from clinical specimens.

In conclusion, water appears to be a major source of the pathogenic *Aeromonas* in Hungary and Libya. In some outbreaks when enteropathogens were not isolated *Aeromonas* caused enteritis. Furthermore, the presence of atypical *Aeromonas* species among the strains studied warrants the use of more sophisticated techniques (in our work Biolog GN microplate system was used) for the speciation of these organisms.

M. MATSUOKA[1], K. ENDOU[1], H. KOBAYASHI[1], M. INOUE[2], Y. NAKAJIMA[1]

A plasmid that encodes three genes for resistance to macrolide antibiotics in *Staphylococcus aureus*

[1]Division of Microbiology, Hokkaido College of Pharmacy, Otaru, Hokkaido,
[2]Department of Microbiology, Kitasato University, School of Medicine, Kitasato, Sagamihara, Kanagawa, Japan

We had demonstrated that plasmid pMS97 – obtained from a 1971 clinically isolated *Staphylococcus aureus* strain MS8968 resistant to macrolide (Mac) antibiotics – carried an *msrA* gene and uncharacterized *erm* genes, respectively. The *msrA* encodes a cytoplasmic membrane protein that mediates the so-called "active Mac-efflux" (designated hereafter as *msrSA'*) and *erm* encodes a methyltransferase by which a specific adenine residue of 23S rRNA is modified: methylation prevents Mac antibiotics from binding to the 50S ribosomal subunit.

Interestingly, we found, in addition, an *mph*-like gene (*mphBM*) present together with *msrSA'* and *erm* on pMS97. By a BLASTP analysis, the gene *mphBM* product has 49% identity and 67% similarity to the amino acid sequence of MPH(2')II encoded by *mphB* from *Escherichia coli*. The order of genes was 5'-*msrSA'*-*mphBM*-3', with a 342-base pair spacer sequence. Although we have not yet determined where *erm* gene is located on pMS97, the gene seems to be downstream from *mphBM*. This finding suggests a warning concerning the imprudent use of antibiotics.

MEDICAL, VETERINARY AND PLANT VIROLOGY

S. BELÁK

Molecular epizootiology of pestiviruses

Section for Research and Development, Department of Virology, The National Veterinary Institute, Uppsala, Sweden

The genetic variability and the molecular epizootiology of pestiviruses were studied by our group and the observations are summarized in this article. The *Pestivirus* genus in the *Flaviviridae* family comprises four genotypes, namely classical swine fever virus (CSFV), bovine viral diarrhea virus (BVDV) types I and II and Border disease virus (BDV), major pathogens all over the world. The current nomenclature and classification based on host-species, should be revised, because crossing of host-species was observed in case of several genotypes. The aim of the present study was to obtain data for molecular characterization and epidemiology of pestiviruses. The analysis of the 3' untranslated region (3'-UTR) of the viral genome provided a clear separation of the four genotypes. Inter-genotypic recombination was not observed, but BDV and BVDV I showed great intra-genotypic variability. Various deletions and insertions were observed in the 3'-UTR, further demonstrating that the 3'-UTR was less conserved than the 5'-UTR. In studies on the genetic variability of CSFV, a large collection of viruses obtained from 20 countries over a period of a half-century was analysed by RT-PCR and direct sequencing. When parts of the E2 and the NS5B (the putative polymerase gene) coding regions were analysed, two main phylogenetic groups were separated, indicating that the virus apparently evolved in two ancestor nodes. Investigating the genetic variability of CSFV in the rather restricted geographic area of Central Europe, comparative sequence analysis of the 5'UTR, the E2/NS2 and NS2 gene regions was performed. The viruses were separated into subgroups that largely coincided with their regions of origin. Another study showed that a simple restriction endonuclease cleavage assay of the 5'NC PCR products was useful to discriminate vaccine virus strains and recent field virus variants in Europe. The collected sequence data allow the rapid classification of newly emerging pestiviruses. For example, a pestivirus termed Frijters, which was spreading in swine populations in Europe, was identified as not of the CSFV genotype, but rather as a variant of an ovine pestivirus. This information prevented the unnecessary slaughter of large numbers of pigs. By studying the genetic variability of BVDV, two new subgroups of type I virus, distinct from subgroups Ia and Ib, were detected when

viruses from Southern Africa were analysed. Additionally, pathogenicity markers in cytopathogenic BVDV strains were identified and different types of genetic rearrangements were described within the NS2-NS3 gene region. *In vivo* studies were performed with two genetically distinct isolates of African BVD viruses. In New Zealand, the presence of BVDV I in cattle and BDV in sheep was proved, while BVDV II was found in fetal calf serum samples of USA origin. By studying a number of relatively small farms in Sweden, a strict, herd-specific genetic clustering of BVDV was observed. The molecular characterization of ovine pestiviruses at the 5'-UTR and the N^{pro} region revealed that sheep may naturally be infected not only with BDV, but also with BVDV types I and II. This indicates the inadequacy of the current host-species based nomenclature and classification of pestiviruses. In summary, the genetic studies provided novel data for characterization and classification of pestiviruses. The sequences were deposited in the GenBank and the obtained findings contributed to the establishment of molecular epizootiology. By means of rapid sequence analysis, the viruses are promptly identified during the new outbreaks. Molecular epizootiology provides powerful novel means to control the diseases caused by pestiviruses.

K. SZARKA, GY. VERESS, J. KÓNYA, L. GERGELY

p53Arg codon 72 homozygous genotype – a genetic risk factor in human papillomavirus associated cervical cancer?

Department of Microbiology, University Medical School of Debrecen, Debrecen, Hungary

A recent study suggested that the p53Arg (at residue 72) homozygous genotype could be a potential genetic risk factor in cervical cancer among white women. To confirm this result we examined the proportion of p53 genotypes in a larger number of patients with cervical cancer (n=82) and in patients with squamous intraepithelial lesions (SIL) (n=86) compared to a control population (n=87).

We used allele-specific primers to amplify the p53Arg and p53Pro sequences and examined the proportion of genotypes in the study populations using χ^2-test.

The distributions of p53Arg homozygous, heterozygous and p53Pro homozygous genotypes were 63%, 27% and 10%, respectively, in cervical cancer patients, 53%, 36% and 8% in individuals with SIL, and 60%, 36% and 4% in control population. Using chi-square test, no significant difference was found between genotype frequencies in the study groups.

Thus, the p53Arg homozygous genotype does not seem to increase the risk of cervical cancer in Hungarian women.

O. ZORMAN-ROJS[1], B. FILIPIC[2], M. CERNE[1]

An attempt to isolate field strains of a very virulent Infectious Bursal Disease Virus on chicken embryonal fibroblasts

[1]Institute for Health Care of Poultry, Veterinary Faculty, [2]Institute of Microbiology and Immunology, Ljubljana, Slovenia

Infectious bursal disease (IBD) is an acute virus induced infection characterized by the destruction of lymphocytes in the bursa of Fabricius and other lymphoid organs. The pathogenesis of viral infection may be induced by several mechanisms, including immune response such as antibody production and cell-mediated reactions.

Seven field isolates of IBD virus (IBDV) obtained from field outbreaks of IBD in Slovenia from 1993 to 1995 were examined. The disease was confirmed by pathological examinations and in some cases also by challenge test on SPF chickens.

For the isolation on tissue culture bursal homogenates were used. Isolates were serially passaged on chicken embryonal fibroblasts. The first appearance of cytopathic effect (CPE) was found after the sixth passage. The time required to see the CPE was approximately six days. With the passages this was shorted to 3 to 4 days. The strength of the isolates was determined by TCID50. To confirm the virus, neutralisation test on chicken embryonal fibroblasts was done. As a control the Winterfield strain of IBDV and antiserum against it was used. The cross-neutralisation data showed that field isolates belong to the IBDV type 1.

J. RAJČÁNI, I. VOJVODOVÁ, I. ORAVCOVÁ, M. KÚDELOVÁ, J. KOŠOVSKÝ, J. MATIS

Characterisation of strain HSZP of herpes simplex virus type 1 (HSV 1)

Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia

The HSZP strain of HSV1 isolated from labial herpes was excessively passaged and adapted to chick embryo cells. The virus formed large plaques due to formation of widespread giant cells, but was not pathogenic for adult mice after peripheral inoculation. These properties are related to two specific mutations in the UL27 (gB) gene. One mutation alters the aa Arg at position 858 of the gB polypeptide to *His*, rendering less flexible a short β-sheet between 2 longer α-helices in its cytoplasmic domain. Another mutation at aa 108 (Asn to *Ser*) is located in the gB^{HSZP} ectodomain, namely in the epitope responsible for formation of antibodies, which participate in NK-

cell action (ADCC reaction). The gK (UL53) gene showed a single mutation (aa 198 is *Arg* and not Gln) unrelated to the *syn* phenotype. Thus, our sequencing results confirm that HSZP virus has a syn gB (*syn*3) mutation rather than at syn gK (*syn*1) mutation. The failure of HSZP to induce early (virion) **h**ost **s**hutoff is due to a bulk of mutations in the UL41 (**vhs**) gene, causing aa changes at positions 18 (*His* instead of Arg) in the conservative region I of *vhs* polypeptide as well as in its box A (aa 384, 384, 386, 392 and 452) and the close neighborhood (aa 374 is *Arg* instead of Leu). The latter mutations are in accord with the finding that deletion of the last 107 aa from the *vhs* polypeptide or insertion of heterologous sequences at position 374 can abolish the shutoff function. Alterations in gC of HSZP are manifested by lower affinity of this virus to lectins possibly due to their truncated glycosylation. The gC polypeptide has at least 5 mutations as related to the gC UL44 sequence of strain Glasgow 17, from which two are HSZP-specific (at aa 139 and 147), while another three are common for gC^{HSZP} and gC^{KOS}. The former two mutations (at both positions we found *Trp* instead of Arg) are situated in the epitope (locusII), which is responsible for binding of gC to the glycosoaminoglycan receptor(s). Nevertheless, the immunogenic properties of gC^{HSZP} were not essentially altered. The experimental efficacy of the strain HSZP based candidate vaccine showed that the vaccine not only protects and cross-protects against lethal virus challenge, but may also reduce the extent of latency and virus reactivation in the rabbit eye model.

D. SALAMON[1], M. TAKÁCS[2], S. MYÖHANEN[3], Z. MARCSEK[4], GY. BERENCSI[2], J. MINAROVITS[5]

Methylation analysis of the latent origin of DNA replication (*ori*P) in Epstein–Barr virus genomes

[1]2nd Department of Pathology, Semmelweis Medical University, [2]Division of Virology, National Center for Epidemiology, Budapest, Hungary, [3]Department of Biochemistry and Biotechnology, University of Kuopio, Kuopio, Finland, [4]National Center for Hygiene, [5]Microbiological Research Group, National Center for Epidemiology, Budapest, Hungary

Latent episomal genomes of Epstein–Barr virus represent a suitable model system for replication and methylation of chromosomal DNA in mammals. We analyzed the methylation patterns of CpG dinucleotides in oriP of Epstein–Barr virus using automated fluorescent genomic sequencing of bisulfite-modified DNA samples. The minimal origin of DNA replication (which contains a dyad symmetry sequence) was unmethylated in 9 human cell lines carrying latent Epstein–Barr virus genomes. 5' from this region we observed focal points of *de novo* DNA methylation at nonrandom

positions in the majority of Burkitt's lymphoma cell lines studied while the viral genomes in lymphoblastoid cell lines were completely unmethylated. Clustering of highly methylated cytosines suggests that *de novo* methylation can spread *in vivo* in cis.

D. VÖDRÖS[1,3], R. THORSSTENSSON[2], G. BIBERFELD[2], E. M. FENYŐ[1]

Co-receptor usage of sequential simian immunodeficiency virus (SIVsm) isolates from cynomolgus monkeys with progressive disease

[1]Microbiology and Tumorbiology Center, Karolinska Institutet, [2]Swedish Institute for Infectious Disease Control, Stockholm, Sweden, [3]Microbiological Research Group, National Center for Epidemiology, Budapest, Hungary

Sequential virus isolates from seven cynomolgus monkeys experimentally infected with SIVsm (sooty mangabey origin) were studied for co-receptor usage in human osteosarcoma cell lines, GHOST(3), engineered to stably express CD4 and each of the receptors CCR3, CCR5, CXCR4, Bonzo and BOB. The cell lines also contained the green fluorescent protein (GFP) gene driven by the HIV-2 LTR. Activation of GFP expression following infection with SIVsm was observed in fluorescent microscope and quantitated by flow cytometry. Antigen production was measured by HIV-2 antigen ELISA.

Details of the progressive immunodeficiency and the antigenic changes of the virus isolates has been described earlier (Zhang et al. Virology, 197:609, 1993). In the present work, we infected the GHOST(3) cell lines with different SIVsm inoculum viruses and two or three reisolates each from seven monkeys. All virus isolates used CCR5 and BOB, and with the exception of one also used Bonzo as co-receptor for cell entry. CCR5 usage was very efficient and stable in all of the monkeys studied. While uniform at the beginning, Bonzo and BOB usage of later reisolates varied in efficiency. Also, CXCR4 usage was evident with early reisolates, but disappeared gradually over time.

The results show multiple changes in virus populations of monkeys undergoing progressive immunodeficiency.

L. PALKOVICS[1], N. KARAMOVA[2], D. PRIBÉK[3], E. BALÁZS[1]

Changes on the 5' noncoding region of plum pox virus in connection with symptom development

[1]Agricultural Biotechnology Center, Gödöllő, Hungary, [2]Kazan State University, Kazan, Tatarstan, [3]Plant Protection Institute, Budapest, Hungary

Plum pox virus is a member of the Potyviridae, the largest family of plant viruses. The genome of PPV is a messenger-polarity RNA molecule of 9786 nucleotides (nt) in length (PPV-SK68 Acc.No.: M92280) with a VPg protein at the 5' end and a poly(A) tail at the 3' end. The genomic RNA has a single open reading frame which is translated into a large polyprotein.

The role of the 5' noncoding region (NCR) in virus replication and translation initiation has been analysed in detail, but limited information is available on the contribution of this region to disease symptom development. We have identified two nucleotide changes in the 5' NCR which induce clearly different and considerably milder symptoms than those induced by the wild-type PPV-SK68 on *Nicotiana benthamiana*. Both of nt position (G_{94} and C_{117}) are important in symptom expression, and both mutation alone had quite similar effects on symptom phenotype. The amount of viral RNA in the systemic infected leaves was not modified by the mutations. The only difference between the parental and the mutated strains could be detected at the coat protein level. These results suggest that the reduced protein levels can be responsible for the mild symptomes.

C. ROS, S. BELÁK

Bovine herpesvirus types 1 and 5, caprine, cervine and rangiferine herpesviruses types 1: studies of genetic relationship and improved molecular methods for detection and identification

Department of Virology, The National Veterinary Institute, Uppsala, Sweden

The glycoprotein B and D genes have been partially sequenced from five ruminant alphaherpesviruses, bovine herpesvirus 1 (BHV-1), bovine herpesvirus 5 (BHV-5), caprine herpesvirus 1 (CapHV-1), cervine herpesvirus 1 (CerHV-1) and rangiferine herpesvirus 1 (RanHV-1). The nucleotide sequence alignments revealed a highly conserved gB gene, with homologies ranging between 87.2% to 99.6%, and a more variable gD gene, with homologies ranging between 71.3% to 98.9%. The

phylogenetic analysis of the gB and gD nucleotide and deduced amino acid sequences revealed that BHV-5 is the most closely related virus to the BHV-1.1/BHV-1.2 cluster and CapHV-1 is the most distant. The phylogenetic data showed a close relationship of all the studied viruses with suid herpesvirus 1.

Based on the sequence data from the gB gene, a nested PCR combined with restriction enzyme analysis (REA) of the PCR products has been developed for the simultaneous detection and identification of the studied viruses. Nested primers have been selected from highly conserved sequence stretches in order to amplify a region of 294-bp in all the five viruses, and a subsequent REA of the PCR products allowed the specific identification. A mimic molecule has been constructed to serve as internal standard of the amplification efficiency. The practical diagnostic applicability of the assay has been evaluated on clinical samples consisting of semen and organ specimens from experimentally infected animals.

K. NAGY, B. KEMÉNY, A. HORVÁTH

CCR5 Δ32 deletion affects course of disease in HHV-8 infected asymptomatic HIV patients

National Institute of Dermato-Venerology, Budapest, Hungary

HHV-8 has a role in the pathogenesis of Kaposi's sarcoma (KS). It is more frequent in HIV infection, and its presence may predict AIDS associated KS. Δ32 mutation of CCR5 chemokine receptor gene results in partial resistance to macrophage tropic HIV-1 infection *in vitro*, and a slower disease progression *in vivo*. Interaction of Δ32 mutation of CCR5 gene and prevalence and role of HHV-8 infection in the course of HIV disease was analysed in asymptomatic HIV positive patients.

Methods: HHV-8 antibodies to LANA were detected by IF, and verified by ORF26 PCR. CCR5 genotype was determined in 192 cases by PCR using purified DNA from PBMCs of 93 asymptomatic HIV carriers (including long-term non-progressors /LTNP/), 20 HIV negative sexual contacts and 79 healthy individuals.

Results and conclusion: i) a Δ32 allele frequency of 0.086 was found in HIV positive patients which was significantly lower than that of in the healthy group: 0.132, ii) Δ32 was most frequent in the LTNP group: 0.200, iii) HHV-8 prevalence in HIV infected group was 25%, similar to that of Western Europe and US, iv) in the HIV positive group with HHV-8 coinfection Δ32 heterozygotism was 16.7%, while without HHV-8 infection, this was 29.4%. This suggests that people with CCR5 Δ32 deletion has an immune system more resistant to HHV-8 infection.

I. MIHÁLY[1], L. TELEGDY[2], E. IBRÁNYI[3], A. LUKÁCS[1], L. RÓKUSZ[4], É. BÁNKUTI[1], J. DÓCZY[1]

Features of hepatitis C infection in personnel involved directly in health care

[1]Virology Laboratory, [2]Internal Department III, [3]Internal Department IV, St. László Hospital for Infectious Diseases, [4]Internal Department, Hospital of Defence Forces, Budapest, Hungary

Serum samples of 477 hospital workers (HWs) were tested with hepatitis C virus (HCV) EIA. HCV-RNA were measured of the HCV seropositive samples (Amplicor). The genotypes were determined by a line-hybridisation assay (Innogenetics) or by a serotyping ELISA (Murex). A total of 15 (3.1%) of the HWs proved to be HCV antibody positive. The seroprevalence was 0.7%, 4.5%, 9.2% and 9.5% in the age groups 21–30, 31–40, 41–50 and above 50 years of age, respectively. Eleven workers had HCV-RNA in their sera (73.3%). Genotype distribution of HCVs was as follows: six were 1b, two were 1, one was 3a, two were 4 and three were not typeable. Six of the workers developed chronic C hepatitis, out of them three nurses were infected by needlestick injury. The treatment was interferon-alfa alone or with ribavirin.

Conclusions: 1. The risk of HCV infection increases with age but it is lower than that of hepatitis B infection. 2. Needlestick injury is associated with an increased risk for acquiring HCV infection. 3. The carrier state of HCV and the titre of HCV genome-copies is changing during the years, independently of the therapy. 4. The HCV genotype distribution in HWs differs significantly from that of the donor population in Hungary. 5. None of the chronic "C" hepatitis HWs responded with complete and/or sustained remission to the treatment with interferon-alfa. 6. The HWs must be alert to the danger of occupational infections and pay vigorous attention to strict infection control procedures.

J. BURGYÁN[1], H. SZUTORISZ[1,2], GY. BISZTRAY[2]

The replication and 3'-end repair of tobacco necrosis virus RNA have different structural and sequence requirements

[1]Agricultural Biotechnology Center, Gödöllő, [2]Department of Genetics and Plant Breeding, University of Horticulture and Food Industry, Budapest, Hungary

Tobacco necrosis virus (TNV) as the majority of plant viruses have single-stranded RNA genomes which are frequently exposed to the action of cellular nucleases during their life cycle. Therefore, protective (active or passive) mechanisms that maintain the integrity of the 3'-terminal sequences would be very advantageous for viral RNAs. We showed that the deletion of up to 5 nt from the 3' end of TNV genome is repaired *in planta*, similarly to turnip crinkle and cymbidium ringspot viruses in the Tombuviridae family. In addition, we demonstrated that only one unit of 10 nt long repeated sequences located at the 3' terminus of TNV is required for the genome replication. However, the RNA lacking one unit of 10 nt repeats was not repaired, because the 8 nt stem in the hairpin structure formed by the repeated sequences is essential for the repair. Using mutant RNA transcripts we also showed that the structure and not repeated sequence itself are required for 3'-end repair of the TNV RNA. This result shows that the replication and repair of the viral RNA are different activities of the same enzyme (replicase). Alternatively, the 3'-end repair of viral RNAs is a result of cellular enzyme playing a role analogous to that of cellular telomerases.

A. BÁCSI,[1] J. ARANYOSI[2], Z. BECK[1], J. SZABÓ[1], K. SZARKA[1], F. D. TÓTH[1]

Syncytiotrophoblast cells are permissive to the complete replicative cycle of human cytomegalovirus by contact with placental macrophage

[1]Institute of Microbiology, [2]Department of Obstetrics and Gynecology, University Medical School, Debrecen, Hungary

Maternofetal transmission of human cytomegalovirus (HCMV) is the most common cause of congenital viral infection. How HCMV crosses the syncytiotrophoblast (ST), the barrier between maternal blood and fetal tissue in the villous placenta is unknown. Although syncytiotrophoblasts can be infected by human cytomegalovirus (HCMV), ST cells do not support the complete viral reproductive cycle or HCMV replication may occur in less than 3% of ST cells. On the basis of these observations we investigated whether placental macrophages might enhance

activation of HCMV carried in ST cells and infected ST cells would be capable of transmitting virus to neighbouring macrophages. For this purpose, we studied HCMV replication in ST cells grown alone or cocultured with uninfected placental macrophages. Our results demonstrated that HCMV gene expression in ST cells was markedly upregulated by coculture with macrophages, resulting in release of substantial amounts of infectious virus from primarily infected cells. After having become permissive for viral replication, ST cells delivered HCMV to the cocultured macrophages as evidenced by detection of virus-specific antigens in these cells. The stimulatory effect of coculture on HCMV gene expression in ST cells was mediated by marked interleukin 8 and transforming growth factor β1 release from macrophages, an effect caused by contact between the different placental cells. Our findings indicate an interactive role for the ST layer and placental macrophages in the dissemination of HCMV among placental tissue. Eventually, these interactions may contribute to the transmission of HCMV from mother to the fetus.

J. SZABÓ[1], Z. BECK[1], É. CSOMÁN[1], X. LIU[2], I. ANDIRKÓ[1], J. KISS[1], A. BÁCSI[1], P. EBBESEN[2], F. D. TÓTH[1]

Interactions between human immuno-deficiency virus type 1 (HIV-1) and human T-cell leukaemia-lymphoma virus type I (HTLV-I) in macrophages cultured *in vitro*: implications for *in vivo* pathogenesis

[1]Institute of Microbiology, University Medical School, Debrecen, Hungary, [2]Department of Virus and Cancer, The Danish Cancer Society, Aarhus, Denmark

Coinfection with HIV-1 and HTLV-I is associated with a high frequency of tropical spastic paraparesis (TSP) or an increased risk of development of AIDS. Since *in vitro* dual infection of CD4+T-lymphocytes with HIV-1 and HTLV-I was reported to result in a moderate enhancement of replication of both viruses, we investigated the interactions between HIV-1 and HTLV-I in cultures of monocyte-derived macrophages. For study, the lymphotropic IIIB and macrophagetropic Ada-M strains of HIV-1 were used. The HTLV-I was prepared from the supernatants of the virus producing MT-2 cell line.

We found that coinfection of macrophages with T-lymphotropic HIV-1 and HTLV-I significantly enhanced HIV-1 replication, whereas double infection of the cells with macrophagetropic HIV-1 and HTLV-I resulted in marked upregulation of HTLV-I production. Stimulatory interactions between HIV-1 and HTLV-I were mediated by their trans-acting proteins.

Results of study on nuclear translocation of proviral DNA showed that the tax gene product of HTLV-I was able to facilitate the nuclear import of the reverse transcribed HIV-1IIIB DNA. In contrast, the HIV-1 Tat protein did not increase the intranuclear trafficking of HTLV-I DNA, which suggests an other mechanism for HTLV-I enhancement by the tat gene product. In conclusion, this study provides possible mechanisms whereby coinfection of an individual with HIV-1 and HTLV-I may influence the clinical outcome of double infection.

GY. SZITTYA, J. BURGYÁN

Identification of host range determinant gene(s) in tombusviruses

Agricultural Biotechnology Center, Gödöllő, Hungary

The infection of plants by viruses requires a series of compatible interactions between viral and host factors. Many times, closely related viruses are not able to infect systemically the same plant. To study the host specificity and other properties of tombusviruses we used the closely related tomato bushy stunt virus pepper isolate (TBSV-P) and cymbidium ringspot virus (CymRSV) in our experiments. We observed that *Capsicum annum* and *Datura stramonium* plants can be systemically infected with TBSV-P, but not with CymRSV. We also observed that this incompatibility between CymRSV and *Datura stramonium* is due to a very fast hypersensitive like response. In addition, it is also demonstrated by press blot analysis that CymRSV is able to replicate in the primary infected *Datura stramonium* cells, but the plant quickly confines the virus by a hypersensitive like response so the sacrifice of a small area of tissue limits further spread of the virus, thereby protecting the whole plant from systemic invasion and disease. To test the role of factors contributing to host specificity we have analyzed the systemic infection abilities of hybrid viral RNAs constructed by exchanging the genes between TBSV-P and CymRSV. Infection of *Capsicum annum* and *Datura stramonium* plants with CymRSV, TBSV-P and hybrid viral genomes revealed that the capsid protein (41K) or the RNA, which encodes the capsid protein, has a crucial role in host specificity.

I. KISS[1], S. KECSKEMÉTI[1], J. TANYI[1], S. B. KLINGEBORN[2], S. BELÁK[2]

Studies on the invasion and distribution of feline coronaviruses in naturally and experimentally infected cats

[1]Department of Virology, Veterinary Institute of Debrecen, Hungary, [2]National Veterinary Institute, Uppsala, Sweden

Two RT-PCR assays were developed for the amplification of the highly conservative nucleocapsid (N) and ORF7b genomic regions, respectively. The two assays were applied to study the shedding and distribution of the virus in naturally and experimentally infected cats. The infection remained subclinical, but the two PCR assays revealed that the majoriy of the animals shed the virus via feces throughout the experiment and the virus could be detected in the blood and in the large intestines. In addition, in a few cases sequences corresponding to the N region were amplified from the cortex, dura mater, pancreas, lungs, third eyelid, and the heart muscle. Interestingly, the ORF7b region was only detected in the pancreas and in the heart muscle. These data correlate with previously reported *in vitro* observations, indicating that the ORF7b region can be lost during virus replication.

The single strand conformational polymorphism (SSCP) analysis of the PCR products revealed that FCoV has quasispecies nature during *in vivo* replication. In the large intestines, both genomic regions showed continuous changes during the course of infection. In the other organs, the two examined regions were highly conserved, as it was revealed by SSCP and nucleotide sequencing.

CS. JENEY, O. DOBAY, B. BANIZS, K. GLATZ, É. ÁDÁM, I. NÁSZ

In the absence of epsilon-cop the human adenovirus type 5 regurgitates into the extracellular space

Institute of Microbiology, Semmelweis Medical University, Budapest, Hungary

The inhibition of the adenoviral entry process by weak base chloroquine after preincubation by the drug is a well-known phenomenon. The proposed mechanism of inhibition is the pH elevation in the endosomal system preventing the virus to enter into the cytoplasm. We studied the kinetics of the inhibition of entry by chloroquine parallel to the kinetics of accumulation of the pH indicator fluorescent dye acridine orange in the endosomal system. It was found that the pH change takes place well before the inhibition of entry suggesting that the pH change and the inhibition of entry are not

directly associated. The ε-cop protein involved in the transfer of material in the endosomal system has an endosomal pH dependent binding to the endosomal membrane but the pH change do not trigger the dissociation of this protein. We investigated whether this protein could be involved in the adenoviral entry process. Radioiodinated adenovirus particles were detected to be regurgitated to the extracellular space in response to chloroquine treatment having the same behaviour what HRPO shows in the absence of functional ε-cop. Additionally 1d1F cells, a CHO cell line which express a thermosensitive ε-cop mutant, cannot be infected readily by human adenovirus type at nonpermissive temperature and the radioiodinated virus regurgitate into the medium and on the contrary at permissive temperature the infection takes place and no regurgitation was detected.

R. IZADPANAH[1,4], M. BENKŐ[2], Á. DÁN[2,3], K. URSU[2,3], M. RUSVAI[1], B. HARRACH[2]

Genetic analysis of the fiber gene and the early region 4 of bovine adenovirus 2

[1]Department of Microbiology and Infectious Diseases, University of Veterinary Sciences, [2]Veterinary Research Institute, Hungarian Academy of Sciences, [3]Central Veterinary Institute, Budapest, Hungary, [4]RAZI Vaccine and Serum Institute, Teheran, Iran

Bovine adenovirus 2 (BAdV-2) is a nonenveloped double-stranded DNA virus which is a mild pathogen causing economic losses in cattle industry occasionally. Physical mapping of the BAdV-2 genome has been accomplished and many parts of it including two-third of the fiber gene and the right end of the genome, have been sequenced. Now, we report on the sequencing and analysis of the region which is completing the fiber gene (knob) and the E4 region. We have used the *Sal*I-*Eco*RI fragment located at 63–90.5 map units in transposon insertion based DNA sequencing. Based on the new sequence data, the size of the fiber gene could be specified to be 560 amino acid long of which the knob region is 176 amino acids. Our sequencing also revealed a new ORF in the E4 region. Homology comparison with human adenovirus 2 indicated that this region contains a gene homologous to the gene of the 17 kD protein. The reanalysis of the E4 region previously sequenced by Y. Haj-Ahmad's group shows slight differences in the ORFs compared to the published data. It is interesting that according to our results in BAdV-2, the ORF closest to the right ITR has a similarity to the dUTPase found in eukaryotic organisms, as well as in pox viruses and on the opposite end of the genome of fowl adenovirus 1 and 9 (published as "type 8").

M. H. ISMAIL[1], A. A. ABOU-ZEID[2]

Studies on alfalfa mosaic virus of alfalfa in Egypt

[1]Botany Department, Faculty of Science, Cairo University, [2]Virology and Mycoplasma Department, Agricultural Research Center, Cairo, Egypt

Alfalfa mosaic virus (AMV) was detected on alfalfa plants, cultivated at different locations of Egyptian farms. The virus was transmitted by sap to 13 species, readily aphid and seed-borne in a limited number of hosts. The virus was inactivated after 10 minutes at 56–58 °C, 72–80 hours at room temperature. The dilution end point is between 10 and $1:8\times10^{-3}$. The virus was detected by ELISA and by immunosorbent electron microscopy. The latter offers the advantage of particle morphology, which appeared bacilli from of similar size to the other AMV isolates. The virus did not react with antisera of different virus groups, naturally infecting alfalfa plants.

M. H. ISMAIL[1], M. S. AHMED[2]

Properties of sweet potato feathery mottle virus (SPMFV)

[1]Botany Department, Faculty of Science, [2]Botany Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt

An isolate of SPFMV was isolated from leaves of *Ipomoea batatas* cv. "Mabrouka" plants exhibiting mosaic symptoms, malformation, chlorotic spotting, vein mottling and twisting of leaf margin. The virus was graft and sap transmissible, and nonpersistently transmitted by *Myzus persicae* and *Aphis gossypii*. In *Ipomoea setosa* sap, the viral properties were: thermal inactivation point at 60–65 °C, dilution end point between 10^{-3} and 10^{-4}. It remained infectious in the sap for less than 12 hours. Fifty-one plant species from families were surveyed for susceptibility but only five *Ipomoea* species were infected. Antisera, produced to the purified virus had a titer of 1:1024 in the microprecipitin test. In dip preparation, the virus particles were usually flexuous filaments of approximately 830–850 nm long. Serological studies, using dot-ELISA on nitrocellulose membrane (Dot-blot or immunoblot) revealed that the causative agent was SPFMV solely. Dot-blot was more effective in detecting the SPFMV in symptomatic than asymptomatic leaves of sweetpotato plants. Ultrastructural examination of infected tissue of *Ipomoea batatas* leaves revealed that the virus

induced cylindrical cytoplasmic pinwheel inclusions. This SPFMV strain, most probably, belong to the common (C) strain of SPFMV.

Z. DIVÉKI, D. SZILASSY, K. SALÁNKI, E. BALÁZS

Monitoring the movement of RNA viruses in plant tissue: the role of tomato aspermy cucumovirus (TAV; Bromoviridae) coat protein in cell-to-cell movement

Agricultural Biotechnology Center, Gödöllő, Hungary

Both cell-to-cell and long-distance movements are crucial functions in the pathogenesis of plant viruses. In cell-to-cell movement, the viruses use the plasmodesmata connecting neighboring cells. This movement is primarily facilitated by different virus-encoded movement proteins, although several other gene products (e.g. coat proteins) and host factors are involved. Precise monitoring of the spread of virus infection in plant tissues is necessary for studying the function of different genes and noncoding sequences, which could participate in this process. The jellyfish green fluorescent protein (GFP) is recently one of the most popular reporter gene used in molecular biology. The use of this reporter gene is also opened a new horizont in plant virology. In contrast with *in situ* hybridization, this method does not require the disruption of the plant material. Therefore, it facilitates studying the kinetics of virus movement directly in the intact plant tissue. TAV has a tripartite RNA genome; the coat protein (CP) is located on the RNA3 segment. Using GFP reporter gene, we constructed different fusion proteins with the TAV CP. Plants were coinoculated using *in vitro* transcripts of cucumber mosaic cucumovirus (CMV, *Bromoviridae*) RNA1 and RNA2 cDNA clones and the TAV RNA3 constructs. Virus movement was monitored by epifluorescent microscopy *in vivo*. Our results confirmed that the CP is essential for the cell-to-cell movement, and CP partly retained the ability to promote virus movement in fusion protein form in the examined host-virus systems.

MEDICAL, VETERINARY AND PLANT VIROLOGY AND PARASITOLOGY

D. SZABÓ[1], D. SHARPLES[2], GY. HAJÓS[3], J. MOLNÁR[1]

Interactions of ellipticines with nucleic acids and reverse transcriptase

[1]Institute of Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary,
[2]School of Pharmacy and Pharmaceutical Sciences, The University of Manchester, England,
[3]Central Research Institute of Chemistry, Hungarian Academy of Sciences, Budapest, Hungary

Inhibition of retrovirus replication can be achieved by several ways e.g. the inhibition of one of the key enzymes or the complex formation with nucleic acids can result in a cessation of virus replication. In model experiments the effect of some ellipticines were tested on the reverse transcriptase of Moloney leukemia virus. It was found that H 3001 and RZ 541 had nearly the same inhibitory effect on the reverse transcriptase possibly due to the similar structure.

When the interactions of the two compounds were tested with DNA, polyA-polyU and tRNA, the intercalation was shown as one of the most important binding mechanism of nucleic acids. Weak interaction was formed between ellipticines and polyA-polyU and tRNA. The later interaction is not specific for the native form of tRNA, because the binding was the same in the presence or absence of magnesium ion.

N. Y. A. SPIVAK, N. O. TIMOSHOK, V. ZOTSENKO, O. N. MIKHAILENKO

Inductors of interferon – a new class of preparation with antiviral properties

Zobolothy Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kiev, Ukraine

Interferons (IFN) are regarded as the class of cytokines and are the family of proteins that possess antiviral, immunomodulating and anti-tumor activity. It permits to refer them as polyfunctional bioregulators of wide spectra of action. Of many years experience of IFN preparations' use in clinical practice permitted to determine its effectiveness for prophylaxis and treating of viral, bacterial and some of oncological diseases.

At the same time there is the other alternative way of obtaining all positive IFN effects. The idea of such approach brings to action the own interferon-productive

system: using interferon inducers. At present this method has got the name "endogenic interferongenesis". The possibility of use of such method has become obvious.

It is necessary to mention the fact that IFN inducers also induce tumor necrosis factor synthesis and interleukins, which play an important role in immunoreactiveness' regulation.

Purposeful screening among the wide spectrum of compounds of natural origin as well as synthetic ones (natural and synthetical RNA two chains, polycations, lectines, polysaccharides, fluorenons, acridanones, phenylmidazothyasoles, gosypoles analogues and others) revealed some preparations with high chemical-therapeutical index, permitted to consider them for use in medical practice.

I. STILLER[1], ZS. NEER[1], A. PÁL[2], B. TARÓDI[1], R. PUSZTAI[1]

Prevalence of antibodies to human herpesvirus-6 among women of childbearing age

[1]Department of Microbiology, [2]Department of Obsterics and Gynecology, Albert Szent-Györgyi Medical University, Szeged, Hungary

The human herpesvirus-6 (HHV-6) can cross the placenta during viraemia in pregnant women causing congenital infection. Since there are no data on the prevalence of antibodies to HHV-6 in the Hungarian women, the incidence of HHV-6 infection in pregnancy cannot be predicted.

The prevalence of HHV-6 specific IgG was tested in 180 women of childbearing age.

The study group was comprised of 60 women with normal pregnancy, 60 pregnant women with fetus suspected having congenital virus infection and 60 healthy blood donors. Plasma/serum samples were examined by immunofluorescence assay. Antibody titers ≥10 were considered positive. Fluorescence of infected cells was scored from 0 (no fluorescence) to +++ (very strong fluorescence) .

Ninety-eight percent of blood donors and 97% of the 120 pregnant women were positive for IgG to HHV-6. The rate of seropositivity in case of normal and diseased pregnancies was the same. The distribution of the ++ anti-HHV-6 activities was similar in the three groups, but 57% of the plasmas from women with diseased pregnancy showed very strong fluorescence.

Because of the high seroprevalence of HHV-6 IgG antibodies among women of childbearing age, primary infection followed by the infection of the fetus is expected to be very low. However, there is uncertainty about the role of possible reactivation of the

latent virus when the immunosurveillance decreases during pregnancy. Furthermore, antibodies to HHV-6 may not prevent infection with a new variant of HHV-6.

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E. UJHELYI[1], E. KORCHMA[2], V. TARJÁN[1], J. SZABÓ[1], P. VÁGÓ[2]

HIV, hepatitis B and C epidemiology in a stomatology department in Hungary

[1]National Blood Transfusion Service, [2]Institute of Stomatology, Budapest, Hungary

Twenty-seven care staff members from the Preserve Dental Surgery of Semmelweis Medical Surgery (female: 20, male: 7, medical doctors: 18) and 92 care staff members from Stomatology Institute (female: 73, male: 19, medical doctors: 38) were investigated for HIV antibody, hepatitis B antigen (HBsAg), hepatitis B antibody (aHBS), and hepatitis C antibody, to analyse the HIV and hepatitis epidemiology status in one of group at highest risk for hepatitis B.

No HIV and HCV positive cases have been found among the 119 care staff members.

No HBsAg positive cases were found in the Dental Surgery, but 2 (2%) HBsAg positive cases were checked and confirmed in the Stomatology Institute.

To analyse the aHBS results in the two groups of dental care staff members 4 groups were divided. 1. aHBS negative, 2. aHBS positive (the antibody titer was very low, the staff members must have been vaccinated against hepatitis B some years ago), 3. aHBS positive (the antibody titer is relatively high, the staff members have been vaccinated 1–2 years ago, or they are HBV infected), 4. aHBS positive (the aHBS titer is extremely high, the staff members were HBV infected some years ago). According to the serological findings of hepatitis B infection, in the case of acute infection the HBsAg can be detected between 8–24 weeks after infection, in case of chronic infection after the 8th weeks continuously, the aHBS antibody in case of acute infection can be detected after 26th weeks of HBV infection. There are some weeks, (around 24–26th weeks after infection) when no antigen or antibody can be detected. That time the aHBC detection can help the diagnosis.

The following results were got:

1. In the Dental Surgery 4 staff members (15%) had no aHBS antibody, 4 staff members (15%) had low titer aHBS antibody, 3 staff members (11%) had medium titer

aHBS antibody, 16 staff members (59%) had extremely high titer aHBS antibody (10 of them were MD).

2. In the Stomatology Institute: 37 staff members (41%) had no aHBS antibody, 13 staff members (14%) had low titer aHBS antibody, 11 staff members (12%) had medium titer aHBS antibody, 30 staff members (33%) had extremely high titer aHBS antibody (13 of them were MD).

The aHBC investigation have been performed at the groups with low and medium level of aHBS antibody, to analyse whether these staff members were HBV infected or vaccinated.

0/7 aHBC positive cases have been found in the Dental Surgery, 4/24 aHBC positive cases (1 from low, 3 from the group of medium titer of aHBS antibody) have been found in the Stomatology Institute. They are thus HBV infected, not vaccinated persons. At the high-risk groups of hepatitis B infection – at the dental medical care staff – the ratio of HBV infection seems to be very high. The hepatitis vaccination is surely recommended.

I. MUCSI[1], J. MOLNÁR[1], N. MOTOHASHI[2]

Combined effects of benzo(α)phenothiazines and acyclovir against herpes simplex virus in cell culture

[1]Department of Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary,
[2]Department of Medical Chemistry, Meiji College of Pharmacy, Tokyo, Japan

The combined antiviral effects of some benzo[*a*]phenothiazine derivatives and acycloguanosine (acyclovir, ACV) were studied on multiplication of herpes simplex virus type 2 (HSV-2) in Vero cells. Previously it was demonstrated that some phenothiazine and benzo[*a*]phenothiazine derivatives exhibited antiviral activity against HSV-2. Several anti-herpesvirus drugs are available but ACV is currently one of the most effective agent for the treatment of herpes simplex virus infections. Resistance to ACV may emerge particularly in severely immunocompromised patients. However, the combination of antiviral agents should have additive or synergic activity and should delay the development of drug resistance. In the present studies the simultaneous application of benzo[*a*]phenothiazine derivatives and ACV during the serial passages of an plaque-purified ACV sensitive HSV-2 strain resulted in decrease or disappearance of the infective virus population. The antiviral effect of ACV on a wild strain of HSV-2 was enhanced in the presence of 5-oxo-5H-benzo[*a*]phenothiazine in yield reduction test. A mathematical formula was used to

interpret the drug interaction and the combination exhibited synergy. The results suggested that a special combination of some antiviral drugs with benzo[*a*]phenothiazines can increase the antiviral action probably due to reduction of the mutagenic rate in the virus population.

M. MEZEI[1], K. BALOG[1], M. TAKÁCS[2], Á. GYURIS[1], J. SEGESDI[1], Á. BAKOS[1], D. VÖDRÖS[1], D. BÁNHEGYI[3], GY. BERENCSI[2], J. MINÁROVITS[1]

Genetic subtypes of HIV-1 in Hungary

[1]Microbiological Research Group, [2]Department of Virology, National Center for Epidemiology, [3]Szt. László Hospital, Budapest, Hungary

HIV-1 shows remarkable genetic variation, which is the result of a high mutation rate, rapid viral turnover and recombination of viral genomes. Genetic variation is most pronounced in the viral envelope gene. Phylogenetic analyses of diverse envelope sequences have shown that HIV-1 can be classified into multiple genetic clades or subtypes. Thus, ten genetic subtypes named A through J in group M (major) and a group of other subtype (group O, outlying) have been identified. Amino acid sequence variation within a subtype generally ranges from 5 to 20% , and between subtypes from 25 to 35%. All genetic subtypes have been found in Central African countries, whereas subtype B predominates in Europe and the USA.

HIV-1 genetic subtypes were determined in 20 adults from Hungary. Peripheral blood mononuclear cells (PBMCs) of HIV seropositive individuals and AIDS patients of the St. László Hospital, Budapest were cocultivated with PBMCs of HIV seronegative blood donors and PCR amplified *env* regions of HIV-1 proviral DNAs were analysed by heteroduplex mobility assay (HMA) and genomic sequencing. HMA results revealed that HIV-1 gp120 sequences from most patients were of subtype B. DNA sequencing confirmed the HMA results.

This study shows that subtype B is the predominant HIV-1 clade at present in Hungary.

V. ŠUBIKOVÁ[1], J. ŠUBÍK[2]

Soil-borne viruses of sugar beet detected in Slovakia

[1]Institute of Experimental Phytopathology and Entomology, Ivanka pri Dunaji, [2]Department of Microbiology and Virology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovak Republic

Rhizomania, a devastating disease of sugar beet, *Beta vulgaris* L., caused by beet necrotic yellow vein virus (BNYVV), a member of the furovirus group has been detected in Slovakia since 1990. Furoviruses infecting sugar beet are vectored by the soil-borne fungus *Polymyxa betae* Keskin, a member of the *Plasmodiophoromycetes*. Our recent surveys point to a serious infestation resulting in a significant decrease of sugar beet yields and its sugar content. Visualization of fungal vector *P. betae* involved in this disease was also described. Out of 61 field samples of sugar beets, tested by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), for the presence of BNYVV in their roots, 24 samples gave positive results. Our virus isolates of beet necrotic yellow vein virus belong to the A-type of BNYVV with quadripartite genome. In 5 samples also a mixture infection of BNYVV and beet soil-borne furovirus (BSBV) was detected. Moreover, a natural infection of sugar beet by tobacco necrosis necrovirus (TNV-D), vectored by fungus *Olpidium brassicae* was detected in at least seven localities of Slovakia using DAS-ELISA. TNV-D was detected in mixed infections with BNYVV. The occurrence of tobacco mosaic tobamovirus (TMV) in mixed infections with BNYVV in rhizomania infected sugar beet roots in four localities of Slovakia was also detected. Rhizomania is caused by BNYVV, but there may be complicated interactions between BNYVV and BSBV and the vector, *Polymyxa betae*, as well as other viruses, which should be paid more attention to.

V. TARJÁN, E. UJHELYI

HIV, hepatitis B and C status of Hungarian drug addicts

HIV Confirmatory Laboratory, National Blood Bank Service, Budapest, Hungary

Aim. To collect information about the HIV, HBV, HCV and CMV status of the Hungarian i.v. and non i.v. drug users.

Patients. Saliva collection devices together with a questionnaire were sent to every known Hungarian drug user treating institution. The saliva samples and from time to time blood samples were taken from the drug users and sent to the laboratory.

Methods were modified for saliva samples, and the following tests were used:

antiHIV-1/-2: Detect HIV^{MC} (v.2) (BioChem Immunosystems Inc.)

antiHCV: Abbott HCV EIA 3.0.

HbsAg: Abbott Auszyme^{R} MC Dynamic

antiHBs: Abbott AUSAB^{R} EIA

antiCMV: Abbott CMV Total Ab EIA

Results. Data from our former study of i.v. drug users shows that when 145 saliva and 180 blood samples were examined, HIV Ab positive patients were not found. 0/145 respectively 5/180 HbsAg positive, and 22/145 respectively 28/180 HCV positive samples were found.

Conclusions:

– saliva sample is a useful material for virologic examinations,
– until 03. 99. we did not find a HIV positive patient among drug users,
– the percentage of HCV seroconverters was the same if we had saliva or blood samples (16%),
– the occurrence of HbsAg positivity was lower (2.7%) than it was expected.

J. DEÁK[1], E. NAGY[1], E. SZÖLLŐSI[2], GY. MÉSZÁROS[2], K. BOHUS[2], T. NYÁRI[3]

Follow-up study for determination of human papillomaviruses

[1]Department of Clinical Microbiology, [2]Department of Obstetrics and Gynecology,
[3]Department of Medical Informatics, Albert Szent-Györgyi Medical University, Szeged, Hungary

The human papillomaviruses (HPV) are regarded to be associated with cervical carcinoma. A follow-up study was organized to determine the prevalence of HPV in a population at low or high risk of HPV in cervical samples (n=45). Two samples were collected at an interval of 8–18 months. Simultaneously with the HPV determination, cervix samples were collected for cytology and blood samples for the determination of serum antibody titers against sexually transmissible viruses and *Chlamydia trachomatis* from the HPV-tested women. The average reported prevalence of HPV in Hungarian asymptomatic women is 17.4%. 45.5% of initially HPV-positive symptomatic women were also positive in the second examination. In the first screening the HPV positivity was 43.4%. 6.8% of the patients had acquired low-risk, and 34.1% high-risk types of HPV. 4.6% of the women were infected with both low- and high-risk types. CMV IgM

and IgG antibodies were present in 0.0% and 73.3% of the patients, HSV 1 IgM and IgG in 8.9% and 80.0%, HSV 2 IgM and IgG in 0.0% and 77.8%, EBV IgM and IgG in 2.2% and 97.8%. There were no HCV-positives among the patients. *C. trachomatis* IgM and IgG were detectable in 60.0% and 51.1%. The present level of HPV positivity was only 45.5% in initially HPV-positive women. The HPV infection may be transient. The correlation of HPV positivity and sexually transmissible virus antibodies was relatively high. The follow-up of cytologically or molecular diagnostically HPV-positive women is very important concerning the prevention of cervical carcinoma.

E. BARABÁS-HAJDÚ[1], Z. PÁVAI[2], N. OPRIS[3], Á. GÁLL[3]

In vitro study on the *Giardia lamblia* cysts' behaviour at different pHs of the environment

[1]Department of Microbiology and Parasitology, [2]Department of Anatomy, [3]Students, University of Pharmacy, Tirgu-Mureş, Romania

Giardia lamblia infection occurs in humans by ingestion of the infectious cysts present in contaminated water or food. Excystation begins in the stomach due to its acidic pH. The released trophozoites colonize the upper part of the small intestine where they attach to the apical edge of the epithelial cells and multiply by binary fission. Some of the trophozoites encyst, producing a rigid cyst wall that protects them after being excreted with the feces. We try to reproduce *in vitro* the steps of the life cycle of this primitive eukaryote starting with the excystation process. In our study *Giardia lamblia* cysts obtained from persons with symptomatic giardiasis, after being purified by using the sucrose-gradient technique and concentrated by centrifugation at 600 g/min for 15 minutes, are counted in a haemocytometer and 10^5 cysts are exposed to different pH-s of the environment, using 1 ml of HCl sol. 1N, pHs 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5; 5.0; 5.5 and 6.0. After incubation in cell culturing chambers at 37 °C for 5, 10, 15, 20, 25, 30, 35, 40, 45 minutes and 1 h, samples are taken and carefully examined under the microscope, magnified by 400. In spite of all our efforts excystation was obtained only at pH 3.5 where excystation rate was very low (2%). We continue our study to obtain enough trophozoites to be able to cultivate these important microorganisms.

I. KUCERA, S. RADULOVIC, S. VELIMIROVIC, N. BULAJIC, M. TATIC, V. BAJOVIC

Finding of intestinal protozoa cysts in stool using three methods

Institute of Microbiology, Authority for Preventive Medicine, Military Medical Academy, Faculty of Biology, Belgrade, Yugoslavia

This paper presents quantitative and qualitative comparison of findings of cysts of intestinal protozoa by comparative using of three methods: formalin-ether concentration by Ritchie (FAC), 33% Zinc Sulfate concentration (FZS) and gradient centrifugation in ficoll (FGC). In 28 (65,12%) of 43 examined samples the cysts of 5 species of intestinal protozoa have been found: *E. coli* (FAC 15; FZS 14; FGC 9), *E. nana* (9; 9; 8), *G. lamblia* (5; 5; 4), *B. hominis* (2; 3; 4) and *J. bütschlii* (2; 1; 1). Analysing the number of positive findings, mean number of the cysts per high power field, appearance of the cysts and the removal efficiency of other fecal contents, it has been concluded that FAC is the most suitable for concentration of most cysts of intestinal protozoa and for the use in routine diagnosis, because it provides the highest number of cysts. Hypertonic solution of $ZnSO_4$ deforms the cysts, which was seen in most smears. FGC method provides the best removing of fecal contents, but the number of cysts is the smallest per high power field; however, it appears particularly suitable for concentration of *Blastocystis hominis* cysts, which retain their viability, because inoculated into nutritive media they have been developed to typical vacuolar forms.

GY. TÖMÖRY[1], ZS. SZÉNÁSI[1], J. SZABÓ[2], M. JESZENSZKY[3], ZS. OZSVÁR[4], E. VESZELOVSZKY[1], E. NAGY[1]

Analysis of the serological results of *Toxoplasma* screening of pregnant women in Szeged

[1]Department of Clinical Microbiology, [2]Department of Obstetrics and Gynaecology, [3]Department of Infectious Diseases, Municipal Hospital of Szeged, Szeged, [4]Department of Infectology, Szent György Hospital, Székesfehérvár, Hungary

Toxoplasma gondii infection of the fetus can only be discovered or prevented by the appropriate serological screening and subsequent treatment of the mother and her offspring.

First in Hungary a general screening program was started in the Szeged region for the early detection of congenital toxoplasmosis in pregnant women. Between 1987

and 1998 all pregnant women in this region (a total of 26877 persons) were routinely screened by modern serological and molecular biological methods (CFT, anti-P30 ELISA IgA, IgM and IgG determination, PCR amplification).

The women were first screened within the first 16 weeks of gestation for toxoplasma antibodies by complement fixation test (CFT). Seronegative cases were retested for seroconversion every second month. Patients with CFT titers ≥1:128 were retested for anti-P30 immunoglobulin A (IgA), IgM and IgG antibodies by ELISA in order to distinguish the acute and chronic phase of the infection. Appropriate treatment was immediately started both in the mothers suspicious of "acute" toxoplasmosis and in their newborns. The urine samples of the newborns were examined by PCR detected B1 gene of *Toxoplasma gondii.* No cases of congenital toxoplasmosis has been detected among the infants and children so far. Thus, we consider the program highly successful for congenital toxoplasmosis screening. A detailed analysis of serological data of the 2357 pregnant women screened in 1998 is also presented.

T. LASKAY, H. LAUFS, N. JAHNKE, W. SOLBACH

Leishmania prevent apoptosis of neutrophil granulocytes

Institute for Medical Microbiology and Hygiene, Medical University of Lübeck, Lübeck, Germany

The protozoa of the genus *Leishmania* are intracellular parasites responsible for diseases of major relevance in tropical and subtropical areas of the world. In mammals, these parasites are strictly intracellular and replicate in the phagolysosome of macrophages. Extracellular parasites are rapidly killed by complement mediated lysis. Therefore, for the survival of parasites in mammals, it is essential to be taken up by phagocytic cells to avoid hostile serum factors. We have recently demonstrated that the accumulation of monocytes/macrophages at the site of infection takes at least 48 hours. The leukocytes that are recruited rapidly to the subcutaneous site of infection are neutrophil granulocytes. Therefore, in the absence of macrophages, granulocytes could provide and intracellular site for the parasites in the first hours/days of infection. In the present study we investigated the potential role of granulocytes in the uptake of *Leishmania. In vivo* we could demonstrate intracellular parasites in granulocytes in the early phase of experimental infection in mice. *In vitro* analysis of human granulocytes has revealed that neutrophil granulocytes are able to take up both opsonized and non-opsonized *Leishmania.* We found that the apoptotic death of neutrophil granulocytes is

significantly delayed after co-incubation with nonopsonized *Leishmania*. These data suggest that granulocytes may provide an intracellular environment for the survival of *Leishmania* within the first days of infection.

GENERAL AND MICROBIAL IMMUNOLOGY

É. RAJNAVÖLGYI[1], A. HORVÁTH[1], N. NAGY[2], Á. SIMON[1], I. K. FALK[2], I. ERNBERG[2], É. KLEIN[2]

The role of Cd4+ T-lymphocytes in viral infections

[1]Department of Immunology, L. Eötvös University, Göd, Hungary, [2]Karolinska Institute, Microbiology and Tumorbiology Center, Stockholm, Sweden

Major histocompatibility complex (MHC) class II-restricted CD4+ T-lymphocytes are major regulators of immune responses. Besides delivering cognate help for antigen-specific B-lymphocytes they are able to condition dendritic cells for efficient antigen presentation and support the generation, differentiation and survival of CD8+ cytolytic T-lymphocytes (CTL). CD4+ T-cells may exert cytotoxic activity mediated by the Fas/FasL or by the tumor necrosis factor (TNF) receptor/TNF interaction. Their other effector functions are related to the secretion of a wide array of cytokines. These mechanisms are involved in the down regulation of the virus-specific immune response when the pathogen is eliminated and also in the maintenance of sustained antibody and CTL memory.

In our studies the protective role of CD4+ T-lymphocytes, directed against a subdominant influenza haemagglutinin (HA) epitope not affected by antigenic drift, was demonstrated in BALB/c mice. Repeated injections with linear or branched synthetic peptides, comprising the CD4+ epitope and an inscribed in B-cell determinant, resulted in the activation of peptide-specific CD4+ T-cells as well as in the production of peptide-specific antibodies. Peptide preimmunization elicited a subtype cross-reactive immune response and conferred enhanced protection against lethal oral reinfection. Our results demonstrate that activation of functionally relevant CD4+ T-lymphocytes can generate efficient anti-viral memory.

In the human system promiscuous, MHC class II-restricted CD4+ T-cell epitopes were identified in a repetitive region of the Epstein–Barr virus (EBV) nuclear antigen-6 (EBNA6). The majority of EBV carriers produce antibodies to this region, which encompasses multiple overlapping core regions with binding capacity to a group of related HLA-DR molecules. Peptide-specific CD4+ T-cells, isolated from EBV seropositive individuals, could also recognize B-lymphocytes, which expressed EBNA6 provided they were MHC-matched. Opposing the strict MHC class I-restricted recognition of EBV-infected cells by CD8+ T-lymphocytes these results suggest that memory CD4+ T cells are focused to such regions of latent viral antigens which can be

recognized and maintain a sustained CTL and antibody memory in many individuals. These CD4+ T-cells may also be important for the development of CD8+ CTL which recognize latently infected EBNA6-expressing B-cells which have the capacity to proliferate and are controlled by the cellular immune response.

J. ONGRÁDI[1,2], A. AHMAD[2], A. HORVÁTH[1], J. MENEZES[2]

Induction of key cytokines in PBMC by human herpesvirus 7

[1]National Institute of Dermato-Venerology, Budapest, Hungary, [2]Department of Medical Microbiology and Immunology, University of Montreal, Montreal, Canada

Altered cytokine profile induced by viruses may contribute to the pathomechanism of acute and chronic infections. Human herpesvirus-7 (HHV-7) isolated recently is one of the causative agents of exanthem subitum and pityriasis rosea, and its latent infections are frequently activated in immunosuppressed patients. Pathomechanism of neither HHV-7 nor these clinical entities has been revealed yet. Therefore, the production of some key immunomodulatory cytokines by peripheral blood mononuclear cells (PBMC) was studied upon primary and secondary infections *in vitro*. After combined treatments with live or inactivated viral preparations and/or mitogens (PHA, LPS, anti-CD3 monoclonal antibodies) some of the cytokines (IL-1β, -2, -4, -6, 10, IFN-γ, TNF-α) were quantified by sandwich ELISA kits, and TGF-β was bioassayed on mink lung epithelial cells. It was established that individual cytokines were produced at maximal output at different time periods after infection; and their levels depended on primary or secondary HHV-7 infections. Inactivated viral preparations also induced cytokine release. Production of IL-2 and IFN-γ after mitogenic stimulation was augmented in primary, but was diminished in secondary infections. Release of TNF-α was parallel to that of IL-1β, but the combined effects of HHV-7 with mitogens increased the level of IL-1β. Induction of IL-4 and IL-6 was not affected, that of TGF-β was augmented by HHV-7. HHV-7 induced IL-10 production, which latter is known to inhibit cytokine release from helper T cells and consequently might play a role in those inflammatory skin diseases mentioned above. In contrast to the closely related HHV-6 found in severe immunocompromised conditions, the effect of HHV-7 on the cytokine balance seems to be mild. HHV-7 induced skin disorders and immunosuppression show a tendency of spontaneous recovery.

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K. MEGYERI, Y. MÁNDI, I. ROSZTÓCZY

Induction of cytokine production by different *Staphylococcus* strains

Department of Microbiology, Szent-Györgyi Albert Medical University, Szeged, Hungary

The aim of our studies was to compare the cytokine inducing activities of different *Staphylococcus aureus, S. epidermidis* and *S. saprophyticus* strains. Our results demonstrated that *S. aureus* strains induced a transient interferon (IFN) production with highest yield between 12 and 20 h after induction. The IFN activity ranged from 300–2500 IU/ml. Serologic characterization of *S. aureus*-induced IFN indicated that alpha-IFN was the predominant type in these preparations. *S. epidermidis* strains induced low-titre (10–20 IU/ml) IFN production. No IFN was detected in supernatants of human mononuclear cells (MNC) stimulated with *S. saprophyticus.* Thus, the higher IFN-inducing activity of *S. aureus* may be characteristic of this species. Our RT-PCR assay demonstrated *S. aureus*-specific increase in IFNα mRNA. Transcription of IFNα gene was transient reaching a maximum between 4 and 8 h after induction and decreasing to baseline level at 24 h. A very weak expression was detectable in MNC stimulated with *S. epidermidis* at 4 h postinduction. We were unable to identify *S. saprophyticus*-specific increase in IFNα mRNA. Restriction analysis with Hinc II endonuclease which is specific for IFNα2 suggests the presence of this subtype in *S. aureus*-induced MNC. IL-6 production was also measured. The *S. aureus, S. epidermidis* and *S. saprophyticus* species stimulated high-titre IL-6 production. Only slight differences were observed in the IL-6 inducing activities of the *Staphylococcus* strains tested. A multiprobe RNase protection assay was used to assess gamma-IFN, IL-1 alpha, IL-1 beta, IL-1 Ra, IL-6, IL-10, IL-12 p35 and p40 mRNA levels. A high increase in IL-1 alpha, IL-1 beta, IL-1 Ra, IL-6 and IL-12 p40 transcription was detected in MNC stimulated with *Staphylococci* for 8 h, and all of the tested *Staphylococcus* strains proved to be very efficient in mediating induction of these genes. Production of IL-1, IL-6, IL-12 and IFN-alpha might play an important role in the pathogenic mechanisms of diseases caused by coagulase-positive as well as coagulase-negative *Staphylococcus* strains.

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Y. MÁNDI[1], GY. FARKAS[2], T. TAKÁCS[3]

Relevance of procalcitonin in the prediction of sepsis and infected pancreas necrosis

[1]Department of Microbiology, [2]Department of Surgery, [3]1st Department of Medicine, Albert Szent-Györgyi Medical University, Szeged, Hungary

Procalcitonin (PCT) is a diagnostic parameter of bacterial infections. PCT is only induced with systemic reactions of the host to the infection. Infected pancreatic necrosis is an absolute indication for surgical intervention, therefore an early and accurate laboratory diagnosis is necessary to confirm the infection. The aim of this study was to analyse the clinical value of PCT for the prediction of infected necrosis and sepsis in comparison with IL-6 and sICAM-1.

A total of 25 patients were investigated; 10 patients with sterile necrosis, 10 with infected necrosis, 5 with sepsis with different origin. The concentrations of PCT in the patients' sera were measured by immunoluminometric assay (BRAHMS PCT Lumitest), the IL-6 concentrations by bioassay, applying the B-9 cell line, and sICAM-1 by ELISA (R&D). PCT was found in relatively high concentrations (25±11.5 ng/ml) only in patients with sepsis and infected necrosis. Positive values (>1 ng/ml) preceded positive bacterial results from either blood or surgical samples. In contrast, IL-6 and sICAM-1 were overproduced in both types (infected and sterile) of necrosis (150±50 pg/ml and 750±125 ng/ml, respectively), and their levels remained elevated for several days even after surgical elimination of the infected focus. We could not detect PCT in patient's leukocytes by immunoblotting.

Elevation of serum IL-6 and sICAM-1 level is characteristic in systemic inflammatory response syndrome either of infectious or noninfectious origin. On the contrary, the PCT level is an accurate and available parameter for early diagnosis of sepsis, and for the discrimination of infected pancreas necrosis, and a helpful marker for surgical intervention. Further studies are necessary to identify the PCT-producing cells.

J. SZABÓ[1], Z. PROHÁSZKA[2], F. D. TÓTH[1], Á. GYURIS[3], J. SEGESDI[3], D. BÁNHEGYI[4], E. UJHELYI[5], J. MINÁROVITS[3], G. FÜST[2]

Strong correlation between the complement-mediated antibody-dependent enhancement of HIV-1 infection and plasma viral load

[1]Institute of Microbiology, University Medical School, Debrecen, Hungary, [2]3rd Department of Medicine, Semmelweis Medical University, and Research Group of Metabolism, Genetics and Immunology, Hungarian Academy of Sciences, Budapest, Hungary, [3]Research Group of Microbiology, National Center of Epidemiology, Budapest, Hungary, [4]Deparment of Immunology, St. László Hospital, Budapest, Hungary, [5]National Blood Transfusion Center, Budapest, Hungary

Previously we have demonstrated that complement-mediated antibody-dependent enhancement (C-ADE) of HIV-1 infection correlates with accelerated immunosuppression and disease progression in HIV-1 infected individuals. In the present work the relationship between C-ADE and plasma HIV-1 RNA concentrations was studied. C-ADE was measured by the method established in our laboratories previously. The results were expressed by an enhancement/neutralization index (E/N I). HIV-1 RNA levels were determined with the Amplicor monitor (Roche), and the NASBA (Organon Teknika) kits. Three studies were performed. a) C-ADE and HIV-1 RNA concentrations were determined in serum and plasma aliquots taken at the same time from 98 HIV patients. We found a highly significant ($p < 0.0001$) positive correlation between E/N I values reflecting the extent of HIV-1 infection enhancement and plasma HIV-1 RNA levels. Both E/N I and HIV-1 RNA levels negatively correlated to the CD4+ cell counts. b) In four seroconversion patients (Boston Biomedica) C-ADE was first detected just before to, or concomitantly with, seroconversion. c) Changes of HIV-1 RNA concentration and C-ADE during a 17-month follow-up period were determined in 18 HIV infected patients. Both E/N I values and HIV-1, RNA levels significantly ($p < 0.001$) increased. The strong association between the extent of the complement-mediated antibody-dependent enhancement of HIV-1 infection and the plasma viral load in HIV patients indicates that C-ADE correlates with HIV replication *in vivo* and potentially contributes to the progression of HIV disease.

P. SOÓS[1], H-J. SCHUBERT[2], K-R. DEPNER[2]

Immunological studies on porcine leukocytes during classical swine fever infection

[1]Department for Microbiology and Infectious Diseases, University of Veterinary Science, Budapest, Hungary, [2]Hannover Veterinary School, Hanover, Germany

The classical swine fever is an infectious disease caused by Pestivirus (Flaviviridae). The course and the epizootical properties of this infection described in Ohio (1833) have changed in the last decades. While the epizootics showed the typical, classical form of the disease in the first part of this century, nowadays it can appear in different forms. These forms manifest themselves in chronic or fetopathogenic courses.

Ten weaner pigs were infected with CSF virus strain in this experiment, and blood samples were taken for flow cytometric examination, beside the clinical, pathological and virological observation. During the infection one pig became convalescent, chronic form developed in two pigs, and the others died in the classical acute form.

It was investigated in this study what kinds of changes can be observed in the lymphocyte subsets during a CSF infection. The questions were as follows:

a, Which leukocyte subsets are infected with CSF virus, at what time?

b, Are these subsets permanently virus positive or just temporarily?

c, Are there any differences among the subsets?

Firstly the virus antigen could be detected in the monocytes. Some percentages of these cells proved to be virus positive on day 10 p.i. The kinetics of the infection was very steep, because on day 13 p.i., when the first lymphocytes became virus positive, the virus antigen could be found in 70–90% of the monocytes. This high percentage of positive monocytes remained until death.

Looking at the CD6+ cells, it could be established that the rate of these cells decreased until day 6 of the infection, but afterwards it began to increase and reached different values, depending on the pigs, it could be 75% as well. The CD6+ cells were less virus positive than CD6- ones at every pig. In case of the swine with chronic form both the CD6+ and the CD6- cells were less infected than those of the other pigs.

Similar difference could be observed at the CD4, CD8 subsets, because both the CD4+CD8+ and the CD8+ cells were obviously more expressed virus positive than the CD4+ and the CD4-CD8-subsets, but essentially all subsets could be infected. The rate of CD8+ cells increased during the disease, while the number of double-positive cells decreased, and at the CD4+ cells was altering.

During the experience no virus antigen could be detected in the leukocytes of the pig, which became convalescent, though the changes in the leukocyte subsets were very similar to those pigs, where the virus antigen could be detected.

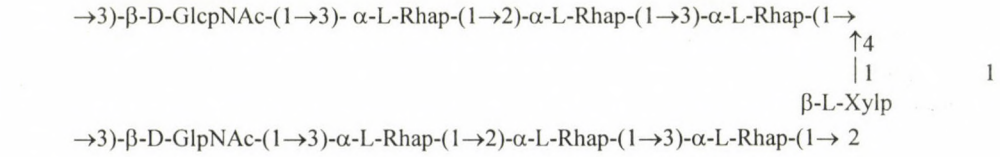
The examinations showed that essentially every leukocyte subset can be infected with the CSF virus, but in very different amounts.

L. D. VARBANETS, N. V. MOSKALENKO

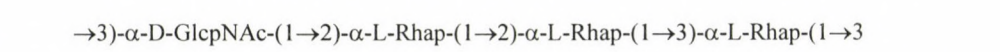
Interferon inducing activity of *Ralstonia solanacearum* lipopolysaccharides

Institute of Microbiology and Virology, National Academy of Sciences, Kiev, Ukraine

Ralstonia solanacearum is a most destructive bacterial pathogen affecting plants in more than thirty-three genera. These bacteria are transmitted via food crops (tomato, potato, sweet pepper and others) to the warm-blooded macroorganisms affecting their immune system. So far as lipopolysaccharides (LPS) of Gram-negative bacteria are known as classical immunomodulators, interferon inducing activity of *R. solanacearum* ICMP 5712 and 7859 LPS and its structural components were studied. O-specific polysaccharide (O-PS) of *R. solanacearum* ICMP 5712 contained two types of structurally different oligosaccharide repeating units: branched pentasaccharide (70%) and linear tetrasaccharide (30%) with the following structures:



while O-PS of *R. solanacearum* ICMP 7859 was characterized the structure 3:



As the main structural components of the core-oligosaccharide (CO) heptose, 2-keto-3-deoxy-mannooctonic acid, rhamnose and glucose were observed. The chemical identification of lipid A indicate that $C_{12:0}$ (2.5%), $C_{14:0}$ (22.0%), 3-OH-$C_{14:0}$ (35.9%), $C_{16:0}$ (5.9%), 3-OH-$C_{16:0}$ (5.9%), $C_{18:0}$ (1.6%), 3-OH-$C_{18:0}$ (7.1%) were the predominant fatty acids.

The studies indicate that *R. solanacearum* LPS effectively induces γ-interferon (IN) production (530 $U/_{50/ml}$) comparable with activity of classical immunomodulator – *Escherichia coli* LPS ("Sigma"). The high levels of γ-IN production were observed in response to lipid A (1070 $U/_{50/ml}$) and CO stimulation of mice peritoneal macrophages. The O-PS have been shown to be low in interferon inducing activity (320 $U/_{50/ml}$). In order to estimate the chemical groups which are responsible for interon inducing activity, coordination compounds of the *R. solanacearum* ICMP 7859 LPS and lipid A with germanium were obtained. LPS preserved interferon-inducing activity, while lipid A modification have led to complete loss of activity. The results of IR-spectroscopy of modified preparations and interferon inducing activity of a number of synthesized compounds give the possibility to suppose the following: phosphate at C4' GlcN II can be responsible for interferon inducing activity, while carboxylic groups of CO or O-specific polysaccharides are responsible for that in native LPS molecule.

J. TÓVÁRI[1], I. NÉMETH[1], S. H. M. JEURISSEN[2], J. M. SHARMA[3], T. F. DAVISON[4], CS. N. DRÉN[1]

Comparative assay of chicken and geese immunoglobulins and lymphocytes

[1]Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary, [2]Institute for Animal Science and Health, The Netherlands, [3]University of Minnesota, College of Veterinary Medicine, USA, [4]Institute for Animal Health, Compton, UK

Of the economically important avian species, only the domestic chicken s immune system is well studied. The immune system of other important birds, with the exception of duck, an *Anseriform* bird, is poorly understood. The same is true for the domestic goose (*Anser anser domesticus*), an economically important species in Hungary. Without basic research, species-specific immunological, reagents cannot be produced, which are essential in disease diagnosis and control. Goose IgG, IgM and IgA enriched immunoglobulin fractions were isolated by the combination of ammonium sulphate precipitation (9–14% v/v concentration), DEAE-Sephacel and Sepharose CL-6B chromatography. Goose IgG were eluted from Sepharose CL-6B column at the position of the chicken IgG. Using rabbit anti-goose immune sera two precipitation lines were visualized by two-dimensional immunelectrophoresis which are tentatively called IgG1 and IgG2. Using the rabbit anti-goose IgG sera several immunogen proteins of *Mycoplasma* spp. were identified by Western-blot analysis. The IgG fraction of rabbit anti-goose IgG was conjugated with FITC, which is routinely

used for the detection of antibodies, developed in geese against various infectious agents. Mouse monoclonal antibody (mAb) developed against chicken IgM recognized goose bursa cells by immunohistochemistry but not by continuous-flow cytofluorimetry (FACS). Mouse anti-chicken CD44 mAb recognized goose thymus cells and the cortico-medullary epithelial cells in the goose Bursa of Fabricius. Mouse mAbs developed against goose thymus-derived cells recognized in various combination goose T-, B-, and, some of the mAbs, chicken B-cells, both by immunohistochemistry and FACS analysis. These results support the notion that species-specific reagents are needed for studying the structure and function of species belonging to *Anseriform* order.

B. FILIPIC[1], O. ZORMAN-ROJS[2]

Biological activity of interferons in the sera of commercial poultry flocks vaccinated under the standard immunoprophylactic programmes

[1]Institute of Microbiology and Immunology, Medical Faculty, [2]Institute for Health Care of Poultry, Veterinary Faculty, Ljubljana, Slovenia

Commercial poultry flocks receive a number of vaccines to protect them from the environmental exposure to pathogens. Immunisation is the principal method used to control several virus infections. Immune response may be influenced by several host mechanisms, such as antibody production and cell-mediated reactions including interferons. The present experiments were aimed to test the biological activity of α-interferon in the sera of commercial poultry flocks in comparison to the antibody response. The sera were obtained from broiler flocks and from broiler chicks. Broiler breeders were vaccinated with the live vaccines against: Marek disease, Newcastle disease, infectious bursal disease, reovirus, infectious bronchitis, avian pox, avian encephalomyelitis and revaccinated with inactivated vaccine against Newcastle disease, reovirus, infectious bursal disease and infectious bronchitis. Broilers were vaccinated with live vaccines against infectious bursal disease and Newcastle disease. As a negative control, the sera from unvaccinated chickens were used. To determine the biological activity of interferon, sera were examined on the chicken embrional fibroblasts with VSV as a challenge virus in comparison to the chicken IFN standard (50 I.U./ml). In broilers' sera an average titer of 600 I.U./ml was found, while in the sera from broiler breeders, the average titer was higher, around 784 I.U./ml. The titer

found in the sera of nonvaccinated chickens was less than 50 I.U./ml. The possible biological role of serum interferons in connection to the immune status of the chickens will be discussed.

B. FILIPIC[1], S. TOTH[2], S. KOREN[1]

Modification of Eagle's medium to cultivate the adherent cells in suspension

[1]Institute of Microbiology and Immunology, Medical Faculty of Ljubljana, Slovenia,
[2]Institute of Biotechnology, Attila József University of Science, Szeged, Hungary

To cultivate the monolayer (adherent) cells in suspension, different techniques as well as different rather complex media were developed and more or less successfully used till recently. The presented experiments were aimed to develop a simple modification of Eagle's medium, that will make possible the cultivation of the normally monolayer-growing cells in suspension conditions. Throughout the experiments the following normaly adherent-growing cells were tested: (a) cell lines: HeLa, WiREF (Wistar rat embrional fibroblasts), MDBK (Bovine kidney), PLA (Adult pig kidney); (b) primary cell culture: CF (Chicken embrional fibroblasts). All of them normally grow in a monolayer in Eagle's medium supplemented with 5–10% of FCS (Fetal Calf Serum). To cultivate the monolayer cells in suspension, the Eagle's medium was changed as follows: glucose, phosphates and glutamine content was increased: glucose to 3000 mg/l, $NaH_2PO_4 \times 2H_2O$ to 1500 mg/l and glutamine to 3000 mg/l. The content of $CaCl_2$ was decreased to 40 mg/l. To enhance the cell growth to the medium 3.125 ml of insuline and 110 mg/l of sodium pyruvate was added. Instead of FCS, 15% of Tryptose-phosphate-broth: peptone mixture was used. The obtained results show that HeLa, WiREF, MDBK, PLA and CF can be successfully adapted for the cultivation in suspension. The use of the proposed medium and various cultivation conditions for the more efficient multiplication of VSV on HeLa, WiREF, MDBK, PLA and CF and porcine interferon-β induction on PLA cells will be shown.

J. IVÁN[1], N. NAGY[2], I. KACSKOVICS[3]

Examination of B cell development in the bursa Fabricii after *in-ovo* injection of an infectious bursal disease immune complex vaccine

[1]Veterinary Research Institute, Hungarian Academy of Sciences, [2]Department of Human Morphology and Developmental Biology, Semmelweis University Medical School, [3]Department of Physiology and Biochemistry, University of Veterinary Science, Budapest, Hungary

The functional activity of the bursal B-lymphocytes was studied in a critical period in which gene conversion occurs, a known feature providing immune repertoire in chicken. To evaluate the influence of a newly developed immune complex vaccine against infectious bursal disease (IBD) on this stage 18-day-old SPF chicken embryos were vaccinated and the changes of chB1 gene expression were studied from one-day-old chickens up to 48 days of age. ChB1 lectin has been recently described as a marker of bursal B-cells undergoing gene conversion. Northern blot analysis showed a dramatic decline in the expression levels 7 days after hatching. Detectable gene expression returned around day 35 with individual differences. Immunohistochemical studies detected vigorous B-cell depletion from the bursal follicles at day 7 after hatching and also showed follicles' regeneration. These results coincide with the Northern blot suggesting a reversible character of this damage. Based on our study this immune complex vaccine temporarily interrupted the normal development of the bursa Fabricii inducing a strong depletion of the bursal B-cells but after about a four-week regeneration phase the follicles were repopulated by functionally active B-cells.

É. BARABÁS, K. NAGY, V. VÁRKONYI, R. GONZALEZ, A. HORVÁTH

Disregulated mRNA expression of type 1 (IFNγ) but not type 2 (IL-10, IL-4) cytokines in asymptomatic phase of HIV infection

National Institute of Dermato-Venerology, Budapest, Hungary

The spontaneous expression of mRNA of IFNγ (type 1), and IL-10, IL-4 (type 2) cytokines in asymptomatic phase of HIV infection were analyzed and correlation between mRNA levels and disease progression was studied. In separated PBMCs of 1. HIV infected individuals with <200 CD4+ cells/μl; 2. 300–400 CD4+ cells/μl; 3. >500 CD4+ cells/μl; 4. their HIV-negative homo/bisexual partners; 5. uninfected atopic patients; 6. healthy male controls relative expression of mRNA for IFNγ and IL-10, IL-4 with respect to β-actin were determined by semi-quantitative RT-PCR analysis.

Spontaneous IFNγ mRNA in all of HIV infected groups (independently of CD4+ cell count) was significantly elevated compared to that of noninfected groups ($p<0.015$, Student's *t*-test). There was no significant difference in IFNγ mRNA expression between HIV-infected groups as well as between noninfected groups. Transcriptional level of IL-10 mRNA was moderated in all groups studied and there were no significant differences. IL-4, however, showed significantly increased expression in atopic group ($p<0.01$). Based on increased IFNγ mRNA expression these results suggest an elevated antiviral activity in immune system in asymptomatic phase of HIV infection. Elevation in type 2 cytokines mRNA levels was not observed in cross-sectional study. However, type 2 cytokines may have a significant role in the progression of disease, which could be analyzed by longitudinal study.

W. SOLBACH, T. LASKAY

Role of innate and adaptive immunity for resistance to Leishmania infection

Institute for Medical Microbiology and Hygiene, University Luebeck, Luebeck, Germany

Cutaneous infection of mice with *Leishmania major* (*L. major*) is a well-established experimental model of chronic disease caused by an intracellular parasite. In this infection model, most strains of mice develop a Th1-dominated immune response, which is associated with healing. Conversely, some strains succumb to the infection. In these susceptible animals the immune response is dominated by Th2 cells. The adaptive development of the respective Th responses is governed by innate components of the immune system. They include the number and function of (NK) cells, macrophages and granulocytes and the resulting cytokine and chemokine milieu at the site of infection. On the site of the parasite the species and dose of the infecting inoculum influence T cell development. Among the lymphokines, production of IL-4 in the first 24 hours of infection is required for development of susceptibility. Lack of IL-4 at this stage confers resistance, most likely through permitting the action of IFN-γ provided by NK cells. NK cell recruitment itself is under the tight control of the expression of activating chemokines like IP-10, MCP-1 and lymphotactin.

AGRICULTURAL AND FOOD MICROBIOLOGY

M. F. PATTERSON[1,2], M. LINTON[1], J. M. J. MCCLEMENTS[1]

High pressure processing of foods for microbiological safety and quality

[1]Food Science Division, Department of Agriculture for Northern Ireland, [2]Department of Food Science, The Queen's University of Belfast, Belfast, Northern Ireland, UK

Consumers are demanding foods that are "natural", of good nutritional and sensory quality, free from chemical preservatives, microbiologically safe and with extended shelf-life. High pressure processing can, potentially, meet these criteria. Recent advances in equipment design now allow foods to be processed up to 900 MegaPascals (130,000 psi). However, further work is required to more fully understand the factors that can affect the response of microorganisms, including pathogens, to pressure so that treatments can be optimised and microbiological safety can be assured. This presentation will describe how the pressure resistance of microorganisms can vary depending on factors such as species, strain, stage of growth and food composition. Strategies for overcoming the problem of pressure resistance will be discussed, for example the use of pressure cycling and the combination of pressure with mild heat. The current commercial uses of high pressure to preserve foods will be reported and potential applications will also be discussed.

S. I. A. EL-SAID[2], M. M. ZAKI[1], N. G. EL-GAMAL[2]

In vitro and *in vivo* biological control for soil-borne fungi infected geranium plants (*Pelargonium graveolens* L.)

[1]Agricultural Microbiology Department, Ain Shams University, Cairo, [2]Plant Pathology Department N.R.C., Dokki, Giza, Egypt

Geranium is one of the most important medicinal plants grown in Egypt. This plant is usually severely affected by wilt and root diseases. The present work was conducted as a trial for the biological control of these diseases to reduce the deleterious effects of fungicides.

In vitro test was performed to detect the antagonistic activity of 500 bacterial, 147 fungal and 112 actinomycete isolates to antagonize these pathogens. Results

revealed that only a small fraction of these isolates were active antagonists with varying degrees.

In vitro studies under greenhouse conditions showed the activity of *Trichoderma harzianum*, *Aspergillus terreus*, *Bacillus subtilis*, *B. lognisporum* and two streptomycete isolates in antagonizing the pathogens and reducing the disease incidence.

Nowadays, biological control is becoming a necessary component for safe and effective plant disease management, especially with the increasing knowledge about the harmful effects of chemical pesticides on the environment (Harman and Taylor 1992, Mehrotra et al. 1993).

J. FARKAS, É. ANDRÁSSY, K. POLYÁK-FEHÉR, L. MÉSZÁROS

Improvement of microbiological safety of vacuum-cooked meals by gamma irradiation

Department of Refrigeration and Livestock Products' Technology, University of Horticulture and Food Industry, Budapest, Hungary

Due to their good quality and convenience, production and trade of vacuum-packaged then cooked-chilled (sous-vide) meals are increasing in countries with well-developed cold chain. However, because of their minimal processing, the microbiological safety of sous-vide foods is vulnerable to contamination of their ingredients with spores of psychrotrophic pathogenic spore-formers such as non-proteolytic clostridia and *Bacillus cereus,* especially in case of temperature-abuse. Using a psychrotrophic strain of *Bacillus cereus* as test organism, inoculated packs of prepared meals such as cooked beef in tomato paste and smoked-cured pork with stewed beans were treated with combinations of pasteurizing heat treatments and gamma irradiation with 4 or 5 kGy. Before and after the treatments and periodically during storage at 10 °C, total aerobic and total anaerobic viable cell counts and, selectively, the viable cell counts of *Bacillus cereus* and sulphite-reducing clostridia have been determined. The effect of the treatment-order (first irradiation and subsequent heating, or, first heating and subsequent irradiation) was also studied. Sensory testing of uninoculated samples proved that the combination-preserved meals were of acceptable quality. The microbiological investigations showed that the medium-dose irradiation of prepared meals prior to their quality-friendly "sous-vide" cooking sensitized the surviving bacterial spores against the heat treatment and increased thereby considerably their microbiological safety and keeping quality.

N. BOIKO[1], Z. FÁBRY[2], V. LYTVYN[3], M. MIHÁLY[4]

Prophylactic effectiveness of a new bacterial biopreparation, "Monosporine-PK", against acute gastrointestinal infections of some agricultural animals and poultry

[1]Department of Plant Physiology and Biotechnology, [2]Department of Biochemistry and Pharmacology, Uzhhorod State University, Uzhhorod, [3]Department of Epizootiology, National Agriculture University, Kiev, [4]Transcarpathian Epidemiological Control Center, Uzhhorod, Ukraine

It has been found that from the diseased calves and piglets, enterobacteria were regularly excreted, ranked by genera according to their excretion frequency as follows: *Escherichia* (45%); *Klebsiella* (16.5%); *Citrobacter* (12.5%); *Proteus* (9%); *Shigella* (6%); *Serratia* (4.5%); *Yersinia* (4%); *Hafnia* (1%); *Enterobacter* (0.5%). Certain peculiarities of the diseased chickens' intestinal microflora had been found reflected by prevalence of *Escherichia*, *Salmonella*, *Proteus* genera enterobacterias. The biopreparation "Monosporine-PK" manufactured in two medical forms according to the Ukrainian Technical Specification ТУ У 46.15.275 - 97 in quantity of three million doses. It was applied to prevent acute gastric disturbance of the above etiology of the new born diseased animals, in compliance with the State Testing methods and Biopreparation Use Instruction approved by Ministry of Agriculture and Food Production of Ukraine. "Monosporine-PK"'s effectiveness was compared with that of "Bacterin-SL", "Lactosporine", "Bifidumbacterine". "Monosporine-PK" and "Bifidumbacterine" were observed to reveal the highest protective activity. Impact of the eubiotics applied upon the gastrointestinal microflora of animal and poultry, was studied as well as histological studies of the organs were carried out and haematological indices of blood were studied. Correlative dependence between the weight increase of the newly born calves and piglets and chickens and the thyroid gland's functional state as revealed is worth to most peculiar attention. Using the immuno-ferment analysis, we showed that during pathological processes (bacterial diarrhea, dyspepsia), thyroxin (T_4) triiodothyronine (T_3) and thyrotrophin (TTG) contents in the blood of the experimental animals changed ambiguously. Depending on seriousness of the disease, we observed decrease of T_3 and T_4 contents with increase of TTG and increase of T_3 and T_4 contents with TTG decrease. These result in protein synthesis recession, dominancy of catabolism over anabolism. Preliminary use of "Monosporine-PK" may ensure normalization of the thyroid gland's functional indices.

Cs. Mohácsi-Farkas[1], G. Kiskó[1], J. Farkas[2], T. Sáray[2]

Investigations on application of antimicrobial agents from plants for food preservation

[1]Department of Microbiology and Biotechnology, [2]Department of Refrigeration and Livestock Products' Technology, University of Horticulture and Food Industry, Budapest, Hungary

In the frame of an INCO-COPERNICUS EU-RTD cooperation project, the feasibility in laboratory scale of application of various essential oils produced from spices and herbs by the Department of Food Technology, Kaunas University of Technology, Kaunas, Lithuania, has been assessed as biopreservatives. Antimicrobial effects of volatiles from dill seed, thyme or oregano on the microflora of shredded cabbage and carrot cubes aerobically packaged in polyethylene pouches and stored at various refrigeration temperatures were tested. Antimicrobial activity of essential oils of thyme, dill weed and oregano added to pizza cream, mayonnaise and tomato juice, respectively, was also studied. Both the useful antimicrobial and the unwanted plant-physiological or sensory effects were strongly influenced by the concentration and the mode of application (vapour phase, spray or dipping solution) of essential oils. The very intensive flavour of essential oils limits their use as general food biopreservatives and the assessment of their utility in food preservation requires an item-by-item approach.

K. Czelleng[1], Z. Szépréti[2], Z. Klement[1]

Research aspects of the green pepper pathogen *Pseudomonas viridiflava*'s virulence factors

[1]Plant Protection Institute of the Hungarian Academy of Sciences, [2]University of Horticulture and Food Industry, Budapest, Hungary

Recently we have started to study the phytopathogenic bacteria and their compatible interaction with plants in question.

In our research work we are focusing on the molecular background and the working mechanism of the plant-microbe interactions in case of phytopathogenic *Pseudomonands*.

It will be demonstrated that there are at least two different bacteria which cause sort rot in green pepper. They are distinguishable because they cause different type of

symptoms. Actually *Pseudomonas viridiflava* produces some green-brown lesions while *Erwinia carotovora* causes some "water clean" kind of lesions.

On the other hand, we would like to explain how we approach the research of the virulence genes. In this case we are using two of recombinant DNA technics: one of them is the "Transposon mutagenesis" and the other one is the "*In vivo* Expression Technology".

We are using some sub-possibilities both of the above-mentioned technics which multiply the chance to find some virulense genes. To obtain good results first we had to create a transposon and some plasmid constructions, called: pTnGC; pIvi4; pIvi5. We can use all the three constructions for promoter tagging which occur by the same reporter system they have.

Their fusioned reporter system contains a promoterless *gfp* (Green Fluorescent Protein) and a promoterless *cat* (Chloramphenicol Acetyl Transferase). Using of these constructions we can screen recombinant bacteria on Petri dishes and *in plants* as well.

V. RAZAVILAR, S. SHEKARFOROUSH

Factorial growth of *Clostridium perfringens* as affected by temperature, salt, pH, acid type and storage time

Department of Food Hygiene, Faculty of Veterinary Medicine, Tehran, Iran

This study was initiated to evaluate the fate of *Clostridium perfringens* type A spores in a model broth system affected by various growth factors. The effects of temperature (42, 35, 25 and 15 °C), salt concentration (0.25, 2 and 4%), pH (7.2, 6.5, 5.9 and 5.3), kind of acid (hydrochloric and acetic acid), and storage time (up to 44 days), with inoculum levels of 10^2 to 10^4 spores in Brain Heart Infusion broth under aerobic condition was evaluated. Growth measurement was conducted using the log probability percentage (log P%) of one *C. perfringens* type A spore growth in the BHI broth model. The log P (%) of *C. perfringens* was affected significantly by temperature, salt, pH, acid and storage time. The interactive effect of salt × pH, temperature × pH, salt × temperature and salt × storage time was also significant ($0.0001<P<0.02$). The log P% of bacteria increased by increasing pH, temperature and storage time and decreased by increasing concentration of salt ($p<0.001$). Acetic acid showed more inhibitory effect than hydrochloric acid. Among the levels of factors used in this study, temperature (25 and 15 °C) showed the highest and salt (4%) showed the lowest bacteriostatic effect. This study was made in aerobic condition (normal

atmosphere in food storage), but the results may bc different (less inhibitory effects) in anaerobic condition.

K. KÖVES-PÉCHY[1], B. BIRÓ[1], I. VÖRÖS[1], T. TAKÁCS[1], R. J. STRASSER[2]

Enhanced activity of inoculated associative and obligate nitrogen-fixers (*Azospirillum* and *Rhizobium* sp.) by AM fungi

[1]Research Institute for Soil Science and Agricultural Chemistry, Hungarian Academy of Sciences, Budapest, Hungary, [2]Laboratory of Bioenergetics, University of Geneva, Jussy-Geneva, Switzerland

The effects of single or tripartite co-inoculations of some nitrogen-fixers (*Azospirillum*-S, *Rhizobium*-R) and the indigenious arbuscular mycorrhizal fungi (M) were examined on the growth and development of alfalfa (*Medicago sativa* L.) in two soils originating from different environmental conditions.

Original (*C=normal rhizosphere*), sterilised (γ-irradiated, *G=no microbes*), or sterile but bacterial-reinoculated (*GB=no AMF*) soil-treatments were used for inoculating the host by the micro-symbionts separately or as combinations in pot experiments. Beside the mass production, nutrient contents, nodulation activity, MPN counts of associative diazotrophs and the AMF colonisation measurements, the physiological stage of the hosts was also examined by a new chlorophyll fluorescence rise (*OJIP*) test.

All micro-symbionts alone (*S, R, M*) or combined (*RS, RM, RSM*) increased the physiological parameters (the plant fitness) of the hosts in the sterile, γ-irradiated /*G*/ soils, suggesting the synergistic effect of these beneficial microbes in optimal cases. Competence of the indigenous microflora on the other hand had a great influence depending on the origin of the soil-types. Inoculation proved to be more effective on the old arable soil (with intensive agricultural usage), in comparison to the more species-rich and diverse soil from a natural woodland environment. AMF inoculation enhanced the abundance and activity of the nitrogen-fixers, and increased the stress resistance of the alfalfa (stress-buffer effect). Chlorofill fluorescence measurement proved to be an adaptable technique for the early *in situ* selection of candidate inocula-strains in case of these beneficial microbes.

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CED REES, CER DODD, P.J. HILL, W. M. WAITES

Development and use of techniques for studying microorganisms *in situ* in natural and man-made environments

Division of Food Science, Sutton Bonington Campus, University of Nottingham, Loughborough, Leics., UK

Most studies of microorganisms have been performed by growth of single organisms in suspension in liquid media in laboratory conditions. However, in both man-made and natural environments, microorganisms grow in mixed culture and are often not planktonic but sessile. There is now a large amount of evidence which shows that microorganisms growing attached to surfaces form biofilms, where, compared with planktonic cells, cells are more resistant to a number of external forces including drying, antibiotics, chemical disinfectants, bacterial viruses and even heat. In order to study such organisms, techniques which allow their numbers and their physiology and biochemistry to be examined *in situ* without perturbation need to be developed.

Our studies have used the green fluorescent protein from *Aequorea victoria* and the *lux* gene system from *Photorhabdus luminescens* as marker and reporter genes, as well as 16S rRNA-directed oligonucleotide and antibody-linked probes to differentiate between different species and between their surface components. Using such methods, coupled to molecular biology and image analysis techniques, we have demonstrated that, compared with the flagellated wild type, a mutant of *Listeria monocytogenes*, which does not produce flagella, shows a much reduced ability to attach to stainless steel surfaces. We have also been able to detect the sites of growth of *Salmonella in situ* within whole eukaryotic organisms including plants and mice.

These and other examples of such innovative techniques will be discussed in order to demonstrate that the methods are now available which will allow mixed cultures of bacteria to be studied *in situ* in both natural and man-made environments.

GY. OROS

Acquired tolerance to benomyl modifies developmental stage response of *Botrytis cinerea* Pers. to various chemicals

Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary

There were investigated differences in responses of hyphal growth, conidium formation and germination process of benomyl sensitive (BS) and benomyl tolerant

(BR) *Botrytis cinerea* strains originated from grape to natural compounds (55 metal salts and 13 antibiotics) and synthetic chemicals (28 organo-metal complexes and 7 organic ligands, 21 azole, 7 dimethylmorpholine, 9 halogenated, 12 nitro-, 10 benzimidazole, 4 carboxamide derivatives and 11 other fungicides) *in vitro*. In general, the conidiogenesis was about twice more sensitive than hyphal growth, however the acquired benomyl tolerance affected the growth at higher degree than the conidiogenesis (~20% and ~5% increase in sensitivity, respectively, as related to wild type). The similarity in responses of these two events was high ($p^2 \geq 0.9$), and the reaction of benomyl tolerant strain was more uniform than that of the wild type (71 and 50%, respectively).

The compounds on the base of their growth inhibitory effects could be clustered into five groups (A-E) and the most actives are denominated:

The compounds of group A and B exhibited low or medium activity against BS strain and were active against BR one. They formed two distinct clusters and significant exponential relationship was revealed ($r>0.95$) in both case. Majority of inorganic salts and sodium dimethyldithiocarbamate (DDC), sodium ethylene*bis*dithiocarbamate, 8-hydroxyquinoline, *cis*-tridemorph and dodine as well as phenylcarbamate fungicides belong to these two groups. The compounds of group C (benzimidazole derivatives, copper dodinium sulfate and cymoxanil) were eminently active against BS strain and weakly against BR strain. The compounds of goup D did not differ in activity against the two *B. cinerea* strains. The antibiotics, the most active metal salts (Cr, Cd, Ce, Hg) and the metal-organic compounds, and most of organic xenobiotics belongs to this group. The group E comprised compounds inactive against *B. cinerea* (Na, K, Rb, Mg, Ca, most of dyes, carboxanilide fungicides).

Some metal salts (Ge, Cs, Bi, U) and fungicides (andoprim, fenpropimorph, hymexazole, kasugamycin), which slightly retarded the hyphal growth and did not affect the conidium formation of BS strain, exhibited significant activity against conidiogenesis of BR strain.

The most active xenobiotics surpassed the activity of antibiotics and metal salts except Cr, Cd, Ag and Hg that, together with benomyl and diethofencarb (against BS and BR, respectively) were among the most effective compounds inhibiting all events. Optimum for conidium germination was between pH 5–6 for both strains, and the initiation of germ tube formation was inhibited over pH 7.0 ($R^2=0.85$). The most active compounds against initiation of germination and germ tuba elongation were carboxine, DDC, dichlone, dodine, and iprobenfos; against embranchment of hyphae and parasexual process metconazole, myclobutanil, *cis*-tridemorph, triadimenole methyl ether, nodine and vanadium The developmental stage tolerance of *B. cinerea* was as

follows: initiation of conidium germination > germ tuba elongation > embranchment > apical growth of hyphae > parasexual process > conidiogenesis.

The increased sensitivity of benomyl tolerant strain to metal salt indicates that the appearance of acquired tolerance to benomyl leads to increasing of sensitivity of *B. cinerea* to natural factors of selection.

J. BECZNER[1], I. VIDÁCS[1], J. TELEGDI[2], GY. KANYÓ-PRINCES[3]

Bacterial attachment and growth on surfaces in the poultry processing industry

[1]Microbiological and Postharvest Biology Department, Central Food Research Institute, [2]Corrosion Chemistry Department, Central Research Institute for Chemistry, Hungarian Academy of Sciences, Budapest, [3]Bábolna Poultry Processing Plant, Győr, Hungary

Adhesion of microbes to different metals and their propagation on the surfaces is well known. The biofilm consists of extracellular microbial products, mainly polysaccharides, and embedded microbes and other materials. The biofilm induced a number of difficulties, i.e. energy loss in industrial cooling water systems, microbiological corrosion, contamination with pathogen and spoilage microbes in the food industry. In the medical practice biofilm formation on catheters, implantatums, pacemakers and other materials in the body may cause health problems. Within the biofilm the physico-chemical environment, the distribution of nutrition change – considering the survival – might be useful for the microbes. From practical point of view, the most important change is the increase of bacterial resistance toward disinfectants and other factors. The culturing of microbes from the biofilm is difficult and non-reliable. Investigating the poultry processing line the places, where the biofilm formation is the most probable, were detected. The most critical places were selected and investigated during processing and after cleaning using traditional methods (total microbial count, *Enterobacteriaceae* and *Streptococcus faecalis*). In addition to the microbiological investigation the adhesion and propagation of microbes were proved by microscopic investigations, too (epifluorescence microscopy, atomic force microscopy).

The investigations are part of the OTKA project No. T 020792.

R. BOŽANIĆ, I. ROGELJ[2], L. J. TRATNIK[1]

The influence of inulin addition on sensory and microbiological quality of fermented beverages from cow's and goat's milks

[1]Faculty of Food Technology and Biotechnology University of Zagreb, Zagreb, Croatia, [2]Zootechnical Department, Biotechnical Faculty, University of Ljubljana, Domzale, Slovenia

Prebiotics are dietary additives that encourage the selective growth of beneficial organisms in the intestinal tract. Most prebiotics that have been studied up to date are nondigestible oligosaccharides. The most common of these are fructooligosaccharides composed of β-fructan units; in this group inulin and its enzymatic hydrolysate are included. Beneficial properties of prebiotics are of interest in the production of fermented milk beverages. The type of milk is very important for the fermented milk production as well. Although goat's milk, according to published information, is used for therapeutic purpose, the production of fermented goat's milk is not significantly investigated until now. In this work the fermentation of cow's and goat's milk with and without inulin addition is investigated. Each type of milk was divided in two parts and in one part inulin was added (1.5%). Milk was fermented with ABT4 culture, containing *Streptococcus thermophilus, Lactobacillus acidophilus* and *Bifidobacterium* sp. Fermented samples were stored in refrigerator (5 °C) for 28 days. On 1st, 7th, 14th, 21st and 28th day pH was measured and microbiological and sensory analysis were performed. The fermentation of cow's milk was about 1 hour shorter (5^{27}) than goat's milk fermentation (6^{25}). At the end of fermentation Str : Bif : Lb ratio in all samples was similar 40: 33: 27, respectively. Viable count of *Streptococcus* was the highest, log N= around 10^9/ml, and remained unchanged during 4 weeks of storage. During the fermentation lactobacilli grew poorly and their survival during storage was inferior. After first week of storage viable count of lactobacilli in all samples was lower than log $N=10^6$. Sensory taste of fermented products was excellent. Samples with inulin possessed better consistency and higher viscosity. The inulin addition showed no influence on fermented cow's milk taste, while the specific flavour of goat was less expressed. Sineresis in goat's fermented milk samples with inulin was negligible compared with samples without inulin. This observation has shown great effect of inulin on stability of coagulum. The same observation was noticed at the 28th day of storage.

Z. NAÁR[1], M. KECSKÉS[2]

Co-existence of different species of *Trichoderma* genus in various soil types

[1]Department of Botany, Eszterházy Károly College, Eger, [2]Agricultural-, Environmental Microbiology and Soil Biotechnology Ph.D. Subprogram, Gödöllő University of Agricultural Sciences, Budapest, Hungary

Trichoderma species have very similar environmental requirements, however they can establish and maintain at the same time and in the virtually same habitat of soil. This co-existence of different *Trichoderma* species in various soil types of Hungary was studied (granted by OTKA F025924) to reveal physico-chemical parameters rendering their co-occurrence. The species composition of *Trichoderma* genus was recorded in 24 soil samples collected from A-horizons of various soil types at different parts of Hungary. The decreasing frequency order of total 13 species was as follows: *T. harzianum*, *T. virens*, *T. viride*, *T. atroviride*, *T. tomentosum*, *T. minutisporum*, *T. spirale*, *T. hamatum*, *T. koningii*, *T. longipilis*, *T. polysporum*, *T. strictipilis*, *T. strigosum*. Five out of 24 soil samples contained only one *Trichoderma* species, thus their co-occurrence seemed to be a common phenomenon. The number of co-existing species ranged between 1–5, which allowed further analyses. Co-existence matrix was constructed. The list of species in decreasing order of number of co-existing other species was as follows: with 11 species: *T. viride*, with 10 species: *T. harzianum*, with 9 species: *T. tomentosum*, *T. virens*, with 6 species: *T. atroviride*, *T. spirale*, with 4 species: *T. hamatum*, *T. koningii*, *T. longipilis*, *T. minutisporum*, *T. strictipilis*, with 3 species: *T. polysporum*, with 2 species: *T. strigosum*. 26 physico-chemical soil parameters and colonizing ability by *Trichoderma* were determined for each soil sample and used for constructing mathematical models to reveal a group of factors influencing the co-existence of *Trichoderma* fungi.

E. JAKUCS

Thelephoraceae-mycorrhizae in Hungarian *Populus*-forests

Department of Plant Anatomy, Loránd Eötvös University, Budapest, Hungary

Besides *Boletaceae* and *Russulaceae*, *Thelephoraceae* are the most widespread ectomycorrhizal fungi in needle and deciduous forests playing an outstanding role in water and mineral supply of trees. However, up to now only few ectomycorrhizae of

the family has been described comprehensively. *Thelephoraceae*-ectomycorrhizae isolated from soil samples taken in different *Populus alba* forests of the Hungarian Plain has been characterized using the morphological and anatomical methods, introduced by AGERER, including SEM, PhC and Nomarski microscopy and histochemical investigations. Three unknown ectomycorrhizae, belonging to the genus *Tomentella*, has been described from *Populus*-roots. Identification of the species was carried out by comparing morphology of cystidia and ITS-DNA-sequences of ectomycorrhizae and fruitbodies.

E. B. GÓRSKA[1], S. RUSSEL[1], J. LABETOWICZ[2]

The occurrence of mesophilic, cellulolytic bacilli in typical lessive soil under differentiated mineral and organic fertilization conditions

[1]Department of Agricultural Microbiology, [2]Department of Agricultural Chemistry, Warsaw Agricultural University, Warsaw, Poland

In the present paper the effect of organic and mineral fertilization and term of soil samples collection on occurrence of cellulolytic bacilli in typical lessive soil from Łyczyn was studied. The investigated soil samples were collected five times during vegetation season from experimental pots of long-term, static fertilization experiment at Łyczyn. The number (MPN) of mesophilic, cellulolytic bacilli was determined by dilution method using mineral liquid medium amended with strips of filter paper as a sole carbon source. Before inoculation of growth medium the proper dilutions of soil suspensions were pasteurized at temp. 80 °C for 15 minutes. The cultures were incubated at temp. 28 °C for two weeks. The presence of investigated bacteria were checked macro- and microscopically. During macroscopic analysis the change of colour of filter paper strips, its looseness and the presence of slime was considered. The results were calculated statistically using two-way analysis of variance method. It was shown that occurrence of mesophilic cellulolytic bacilli in investigated soil samples depended on term of soil samples collection, climatic conditions and organic and mineral fertilization. The highest number spore-forming, cellulolytic bacteria was observed in July in soil samples collected from limed pots fertilized with manure and full mineral fertilization (NPK). From meteorological dates appeared that July was the most wet and warm month of the year. The lowest value MPN of the investigated bacteria was observed in June.

K. RIGÓ[1], J. TÉREN[2], J. VARGA[1]

Ochratoxin contamination and decomposition caused by *Aspergillus* species

[1]Department of Microbiology, Attila József University, Szeged, [2]Animal Health and Food Control Station, Szeged, Hungary

Ochratoxin A (OA) is a nephropathic mycotoxin which was discovered in 1965 as a secondary metabolite of *Aspergillus ochraceus* strains. In the subsequent years several other *Aspergillus* and *Penicillium* species were described as producers of this toxin. We examined the OA production of *Aspergillus* strains isolated from green coffee beans and other plant products by using an immunochemical technique. Besides *A. ochraceus*, black *Aspergilli* and an *A. fumigatus* isolate have also been found to produce OA, indicating that *A. ochraceus* is not the only source of OA contamination of plant products. Elimination of OA from agricultural products including poultry feed, cereals and green coffee beans can be accomplished by chemical and biological methods. During our studies, several *Aspergillus* species were examined for their ability to degrade OA. *A. fumigatus* and *A. niger* strains could eliminate OA effectively from culture media. *A. niger* is one of the few fungal species which has received the GRAS (generally regarded as safe) status due to its low toxigenicity. An atoxigenic *A. niger* strain could degrade OA both in liquid and solid media within 5 days, and the less toxic degradation product, ochratoxin A was also eliminated in the next two days. Further studies are in progress to examine the ability of this strain to degrade OA in agricultural products. A fast microwave-assisted method has also been developed to measure the ergosterol content of contaminated plant products. The amount of ergosterol was found to correlate well with the amount of fungal biomass and mycotoxin production, consequently this method could be used for predictive purposes.

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E. B. GÓRSKA[1], S. RUSSEL[1], B. TUDEK[2]

Effect of growth conditions on cellulolytic activity of *Bacillus circulans*

[1]Department of Agricultural Microbiology, Warsaw Agricultural University, [2]Department of Molecular Biology, Institute of Biochemistry and Biophysics, Warsaw, Poland

Cellulolytic strain of *Bacillus circulans* was isolated from samples of typical lessive soil collected from long-term, static fertilization experiment at Łyczyn. The

isolated strain of bacteria was cultured in mineral medium at temp. 28 °C. The culture was incubated stationary and on rotary shaker. The culture medium was amended with carboxymethylcellulose, filter paper strips or Avicel cellulose. The ability of isolated strain of bacteria to degrade of above-mentioned substrates was measured on the base of production of cellulolytic enzymes e.g. cellobiase, CMC-ase and FP-ase. The activity of cellulolytic enzymes was determined by Ghose and Mandels method, measuring the level of reducing sugars in the reaction mixture. The characteristic of investigated strain was done on the base of morphology and biochemical properties. The ratio of % GC in DNA was also determined. The isolated strain has been classified as *Bacillus circulans*. It was found that complex of cellulolytic enzymes produced by *B. circulans* does not contain cellobiase. Intensity of cellulolytic enzymes produced by *B. circulans* depended on aeration and carbon source in the culture medium. Significantly higher cellulolytic activity was found in supernatants obtained from shaken cultures than from stationary ones. The highest activity of CMC-ase was found in medium with CMC as carbon source. In shaked cultures the production of FP-ase was practically the same in growth media amended with CMC and Avicel cellulose as a sole carbon source. Optimum activity of both investigated cellulolytic enzymes was found at pH 7.0 and temp. 50 °C.

H. E. A. F. BAYOUMI HAMUDA[1], A. KHALIF[2], L. KÖDÖBÖCZ[1], M. KECSKÉS[1]

Effect of dicyandiamide, nitrapyrin, 2,4-D and thiourea on the growth and symbiosis of *Vicia faba – Rhizobium leguminosarum* bv. *viciae*

[1]Agricultural-, Environmental Microbiology and Soil Biotechnology Ph.D. Subprogram, Gödöllő University of Agricultural Sciences, Budapest, [2]Environmental Microbiology M.Sc. Subgroup, Gödöllő University of Agricultural Sciences, Budapest, Hungary

Decreasing the time of nitrification and increasing the time of nitrogen availability for plant absorption should increase the efficiency of soil fertility as well as the useful biological N_2-fixation. Inhibiting nitrification offers the availability of nitrogen in the reduced form and thus may prove to be a useful tool in maximizing soil bioproductivity and minimizing water pollution with oxidized nitrogen forms. The effects of nitrification inhibitors – Dicyandiamide (1-cyanoguanidine), Nitrapyran (2-chloro-6-(trichloromethyl)-pyridine), 2,4-D (2,4-Dichlorophenoxyacetic acid) and Thiourea at 0, 0.1, 1, 10, and 100 mg l^{-1} on the growth and the respiratory activities of the *Rhizobium leguminosarum* bv. *viciae* strains (E1012, HB-3841*str*$^+$, Lóbab Z, and Bükköny 75/4) were studied *in vitro* (in yeast extract mannitol broth medium using microfermentor and

Warburg's respirometer methods). Plant seedlings inoculated with *Rhizobium* strains in acidic (pH 4.7) Gödöllő brown forest soil with low humus (1.22%) content treated with Dicyandiamide, Nitrapyran, 2,4-D and Thiourea at 0, 0.1, 1, 10, and 100 mg kg^{-1} soil was also studied *in vivo*. The results of *in vivo* experiments supported the *in vitro* observations, in which Dicyandiamide had relatively low effect on the growth and respiratory activities of microsymbionts and symbiotic parameters (plant growth and dry weight, nodulation potential and N_2-fixation). However, Thiourea was the most toxic inhibitor, but 2,4-D and Nitrapyrin had a moderate effect in both ecosystems. The results showed that the symbiotic parameters of the plant seedlings inoculated with Lóbab Z, and Bükköny 75/4 strains were higher than those inoculated with E1012 or HB-3841*str*$^+$ strains in all treatments.

J. STARÝ, A. LUKEŠOVÁ, A. NOVÁKOVÁ, V. KRIŠTUFEK

Interaction between oribatid mites (Acari: Oribatida) and soil microflora

Institute of Soil Biology, Academy of Sciences of the Czech Republic, Ceské Budejovice, Czech Republic

Seasonal occurrence of soil actinomycetes, algae and fungi on the surface and in the gut of nine species of oribatid were studied. Model oribatid species were sampled from three experimental plots during 1997. Altogether 25 species of soil algae, 35 species of soil fungi as well as 94 strains of actinomycetes were found. Number of isolated soil microorganisms is dePoribatid mite species. Composition and number of microfloral species isolated from one oribatid species vary distinctly during the season. The biggest number of soil algae species was found in summer, on the other hand number of soil fungi species were very low during summer with maximum in spring and autumn. Food preference was studied using all nine oribatid species and material of all species of soil microflora found on the surface and in the gut of each oribatid mite species. Distinct interspecific differences were found in preference in all groups of studied soil microflora. Some preferred species of algae and fungi were completely destroyed by digestion, some ones were passed trough oribatid gut without any damage. Some species of soil algae and fungi were assigned as important food source for studied oribatid mites; other ones are spread in soil on the body surface or in the oribatid mite gut.

T. SZILI-KOVÁCS[1], D. KLEIN[2]

Enforced N-immobilisation to accelerate secondary succession in sandy grassland

[1]Research Institute for Soil Science and Agricultural Chemistry, Hungarian Academy of Sciences, Budapest, Hungary, [2]Department of Microbiology, University of Colorado, Fort Collins, USA

It is a presumption of many ecologists that the vegetation recovery of disturbed lands highly depends on the soil N-availability. High available-N inhibits expansion of natural vegetation while favourable for early-seral r-strategists.

Experiments were designed to test the applicability of nitrogen immobilization as means of accelerating the recovery of endemic open sand grassland (Festucetum vaginatae danubiale) on old-field in the Great Hungarian Plain. Sucrose and oak sawdust as carbon sources were selected for field application to increase microbial N-immobilization. The quantity of C-sources applied in the field was determined according to the previous lab experiment. The sawdust was broadcasted only once in early spring while sucrose 4-times during the vegetation season.

Three experimental sites were located along an elevation gradient parallel with an aridity and productivity gradient. Soil organic matter, microbial biomass-C, cellulose decomposition and inorganic-N were measured during the vegetation season.

Soil organic matter, microbial biomass and decomposition rate changed from site to site in accordance with the elevation gradient. We found significant increase in microbial biomass-C and decrease in soil available-N in two sites. The third site having the highest organic-C did not show significant differences due to the carbon source addition. Cellulose decomposition rate was significantly lower in treated plots only in one site.

The plant community did not show any alteration due to the treatment in the first year.

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T. PICEK[1], R. TYKVA[2], H. ŠANTRUČKOVÁ[1], M. SIMEK[1], B. PAVLU[2]

Glucose decomposition in soil after change of aeration status

[1]Institute of Soil Biology AS CR and Faculty of Biological Sciences, University of South Bohemia, Ceske Budejovice, [2]Institute of Organic Chemistry and Biochemistry AS CR, Prague, Czech Republic

Soil was preincubated in oxic and/or anoxic conditions, respectively, for 10 days. Then ^{14}C-labeled glucose was added, aeration status was changed from oxic to anoxic and *vice versa* and soil was incubated for next 3 days. Soils incubated in permanently oxic and in permanently anoxic conditions were used as controls. Oxic incubation was performed in continuous flow system in a flow of CO_2-free air and anoxic incubation was carried out in static conditions in O_2-free N_2. Glucose consumption, 0.5 M K_2SO_4 extractable C (C_{EXTR}; $^{14}C_{EXTR}$), C in microbial cells (C_{MIC}; $^{14}C_{MIC}$), ^{14}C incorporated into polymeric compounds ($^{14}C_{POLY}$), CO_2 and $^{14}CO_2$ were measured 0, 8, 16, 24, 48 and 72 hours after glucose addition. If soil was incubated in **oxic conditions** all glucose was consumed within 72 hours and no effect of oxic and/or anoxic preincubation on ^{14}C distribution was observed. Thus, at the end of experiment about 25% of ^{14}C consumed were found in $^{14}CO_2$, 1.5% in $^{14}C_{EXTR}$, 16% in $^{14}C_{MIC}$ and about 56% in $^{14}C_{POLY}$ fractions. However, mineralization of soil organic C and its assimilation into microbial biomass were enhanced when anoxic conditions were changed to oxic as compared to permanently oxic incubation. If soil was incubated in **anoxic conditions**, glucose consumption was strongly inhibited by oxic preincubation, as only 35.8% of added glucose were consumed within 72 hours; 51.4% of consumed glucose C were evolved as $^{14}CO_2$, 4.5% were found in $^{14}C_{EXTR}$, 23.2% in $^{14}C_{MIC}$ and 20.9% in $^{14}C_{POLY}$ fractions. If soil was incubated in permanently anoxic conditions, all glucose was consumed within 72 hours; 30% of consumed glucose C were found in $^{14}CO_2$, 27.5% in $^{14}C_{EXTR}$, 8.4% in $^{14}C_{MIC}$ and 34.1% in $^{14}C_{POLY}$ fractions. No significant effect of pre-incubation on mineralization and assimilation of soil organic C in anoxic conditions was observed.

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L. MARILLEY[1], U. A. HARTWIG[2], M. ARAGNO[1]

Influence of an elevated atmospheric CO_2 content on rhizobacterial populations beneath *Lolium perenne* and *Trifolium repens*

[1]Laboratory of Microbiology, University of Neuchâtel, [2]Institute of Plant Sciences, Swiss Federal Institute of Technology, Zürich, Switzerland

Due to human activities, the atmospheric CO_2 concentration is increasing. However, the possible consequence on soil ecosystem is far from understood. The major influence is indirect because CO_2 concentration in soils is at least 10-fold greater than in atmosphere. Consequently, the influence on soil microorganisms is thought to occur by the intermediate of plant roots.

The rhizospheres of clover and ryegrass were divided into three fractions: the bulk soil, the rhizospheric soil and the rhizoplane-endorhizosphere. Bacterial community structure was assessed after isolation of DNA, PCR amplification and construction of cloned 16S rDNA libraries. The cloned 16S rDNA were then partially sequenced and analysed by a phylogenetic approach.

Our data show a very high bacterial diversity in soil, which is dominated by clones related to yet-uncultivated microorganisms. The phylogenetic diversity dramatically decreases in the root environment, leading to a dominance of pseudomonads and rhizobia in the clover rhizosphere and to a dominance of pseudomonads in the ryegrass rhizosphere. The selective effect of plant roots is increased under elevated CO_2. Under these conditions, rhizobia are dominant in the clover rhizosphere and the dominance of pseudomonads is increased in the ryegrass rhizosphere. Carbon source utilization analysis at the community-level confirms the CO_2-induced changes in the structure of the bacterial community. This work provides evidence for CO_2-induced changes in the structure of the rhizosphere bacterial populations, suggesting a possible alteration of the plant-growth-promoting-rhizobacterial effect and a root-mediated adjustment of bacterial populations to CO_2-induced increase in plant nitrogen requirement.

L. ZSOMBIK, G. J. KÖVICS

Preliminary data for overwintering of *Diaporthe helianthi* (anam.: *Phomopsis helianthi*) causing brown spot (stem cancer) of sunflowers in Eastern Hungary

Department of Plant Protection, Debrecen Agricultural University, Debrecen, Hungary

Almost two decades passed since a destructive sunflower pathogen *Diaporthe helianthi* Muntanola-Cvetkovic, Mihaljcevic and Petrov (anamorph: *Phomopsis helianthi* Muntanola-Cvetkovic, Mihaljcevic and Petrov) was observed (Mihaljcevic et al., 1980) and described in Yugoslavia (Muntanola-Cvetkovic et al., 1981). First record of brown spot (stem cancer) was published by Németh et al. (1981) in Hungary. Since then this fungus became the most serious problem of the sunflower growers to manage. Early experiences showed that the fungus can remain viable and infective only on infected stem pieces that overwintered above the soil surface, no pathogen was reisolated from stem pieces buried in the ground 5, 15, or 30 cm deep (Vörös et al., 1983). Both *Phomopsis* conidiomata (pycnidia) and *Diaporthe* ascomata (perithecia) can overwinter on stem fragments of sunflowers depending on ecological influences. *In vitro* only beta-conidia were found (Franic-Mihajlovic et al., 1994) however sterile alpha-conidia can also occur *in vivo*. The role of ascospores as the main source of primary inoculum is argued. Ascospores first appear mainly in June (Jinga et al., 1987). Ascospore traps were described for monitoring *Diaporthe helianthi* epidemics (Delos et al., 1995).

In a fungicide application experiment we observed an early epidemic of *Diaporthe-Phomopsis* disease with leaf necrotic symptoms on 12 June 1998. Later on a heavy epidemic developed on stems causing serious losses in yield in spite of fungicide applications. In the case of early infection it seems more reasonable to apply fungicides preventively at an early growth stage (BBCH 16–18) than in stage of inflorescence emergence (BBCH 51–55).

Aims of our present experiments were to identify the overwintered forms of fungus and primary sources of inocula, which can contribute to an early epidemic situation. Stem debris was collected from five sunflower plots of trans-Tisza region (Eastern Hungary) in early March 1999. Conidiomata and spores of 100 samples were examined by light microscopy. Thirty conidiomata and conidia were studied of each sample for identification.

All pycnidia produced beta-conidia except Debrecen/01 sample, which yielded alpha-conidia beside beta-ones. We also observed pycnidia and conidia of *Phoma macdonaldi*, the causing agent of black stem at 1/3 of samples.

Infested stem residues were put in wet chambers to stimulate an early ascomata and ascospores production. After 10-day incubation period formation of perithecia and ascospores have started. Another part of samples serve for weekly monitoring of ascomata/ascocpores production *in vivo* which is in progress by ascospore traps.

TH. MÜLLER[1], E.-M. OTT[2]

Occurrence and possible ecological function of enterococci on forage grass

[1]Institute of Microbial Ecology and Soil Biology, Centre for Agricultural Landscape and Land Use Research Müncheberg, Paulinenaue, [2]Institute of Ecologically Compatible Animal Husbandry, University of Rostock, Rostock, Germany

Enterococci are frequently found in grassland independent of utilisation for pasture farming. Their main reservoir is the litter layer. They colonise the rhizosphere as well as the above ground parts of grass, where they live in a truly epiphytic relationship with the plants, i.e. they are able to reproduce there. As saprophytic bacteria they are part of a naturally-occurring biological control of pathogens. Bacteriocin production in this group is very common, and enterocins are known to show activity against a broad spectrum of accompanying bacteria. This could possibly be of particular importance with respect to the elevated numbers of microorganisms in extensively used grassland for suppression of species potentially pathogenic to animals or plants. Plant samples were taken from five meadows in a fen land area in NE-Germany in the course of a growing season. Enterococci could be detected at all investigation sites and on each sampling occasion in numbers of 10^1–10^4 CFU/g of grass. From each site 30 isolates per sampling occasion were identified (whole-cell protein pattern on SDS-PAGE and restriction analysis of PCR-amplified 16S rDNA) and tested for their ability to produce bacteriocins. About 20% of the isolates were antagonistically active against other Gram-positive species. This activity was also detected when the enterococcal strains grew on "phylloplane agar", a medium which simulates the low level nutrient conditions found on the leaf surface, in a temperature range between 4 and 37 °C.

I. J. HOLB

Influence of acidity in structure development of *Monilinia fructigena*

Plant Protection Department, Agricultural University of Debrecen, Debrecen, Hungary

In some fruit growing areas, *Monilinia fructigena* (Aderh. & Ruhl.) Honey is one of the main pathogens causing fruit rot. Together with *Monilinia laxa* (Aderh. & Ruhl.) Honey and *Monilinia fructicola* (Winter) Honey forms the group of brown rot fungi. Plant pathologists have studied the brown rot fungi very intensively to decrease fruit loss, and these investigations suggested that overwintered mummified fruits played an important role in the pathogen life cycle as a main source for primary inoculum, but the amount of primary inoculum depends on the quality of mummies. Many factors (internal quality of the fruit, abiotic conditions) influence the mummification process after infection. During this mummification process (i.e. hardening of the fruit tissue), stroma formation develops inside the fruit, which serves as overwintering structure. For rational control is important to know which conditions are favourable for the development of stroma inside the fruit and how this process reacts to changes in the environment. Although there is a lot known about this subject, for example many factors (nutrition, temperature, light, age of the culture) have already been examined but study about the influence of acidity on stroma formation in *Monilinia fructigena* has not been described yet, although it is one of the main factors of the internal quality of the fruit and probably plays an important role in the development of stroma formation. Therefore physiological study were made. The aim was to evaluate the effect of different pH (acidity) ranges in the disease progress of *Monilinia fructigena* on agar plates (*in vitro*) and in apple fruits (*in vivo*). In our experiment, two isolates of *Monilinia fructigena* were used (JAP.2316 /Japanese/ and HU.D2 /Hungarian/) which represented the characteristic features of the two main *Monilinia fructigena* groups. For the agar experiment buffered PDA (Oxoid) media were prepared with a range of the initial pH from 2.5 to 6.5 (pH 2.5, 3.5, 4.5, 5.5 and 6.5). The dishes were inoculated with 5 mm plug of each *Monilinia fructigena* isolate. Incubation took place at 23 °C in darkness. The cultures were allowed to develop for three days and then daily the diameter of the colony in mm were measured until the colonies filled the dishes. When the stromata were considered mature, the stromatal plates were harvested. For fruit experiment cv. James Grieve apple fruits 2 weeks before fruit harvested were used in five course of time. Inoculation technique was the same as it is described in agar experiment. During stromata development in fruits, the pH changes of the fruit were detected according to the disease progress (after 7, 14, 28 and 35 days of artificial inoculation). In the plates the most intensive mycelial growth

was at pH 4.5 and pH 3.5, resp. in Japanese and Hungarian isolate. After having appeared stroma formation on cultures, the pH of the cultures was finally stabilized by the fungus between 4.6–5.4 (without control) 20 and 50 days after incubation, resp. in Japanese and Hungarian isolate. Both isolates formed the highest amount of stromata at pH 5.5. The Japanese isolate produced about twices or three times more stromata per Petri dishes depending on the initial pH. Higher pH (> pH 4.0) was also more favourable for stroma formation in the fruit experiment. Presented pH changes, weight of the stromata and fruit assessment showed that a higher pH (pH 4.0–5.5) was more favourable for stromata development than lower pH (pH 3.0–3.2) of healthy fruits. Consequently, low acid content fruits of apple cultivars or matured fruits are likely to be favourable for mummification of *Monilinia fructigena*.

R. I. GVOZDYAK, L. V. KABASHNA, L. A. PASICHYK, E. A. MAKARCHUK

Interaction between endophytic bacteria of wheat seeds and pathogens

Zabolotny Institute of Microbiology and Virology, Kiev, Ukraine

Endophytic microflora of four varieties of wheat: Kharkivska 37, Khersonska 86, Albatros, Lutescens was investigated. Special attention was given to the sterilization of seeds and removal of epiphytic microflora. Absence of epiphytic microflora on sterilized seeds was checked by washing them in a broth, with the subsequent incubation. Absence of growth indicated the sterility of seeds. From free epiphyte seeds endophytic bacteria were isolated by plating their pestled mass on a nutrient agar and placement of the externally steriled seeds on the surface of nutrient medium. Depending on the variety the quantity of wheat seeds containing the internal bacteria was different, but this did not exceed 60%. Among isolated bacteria phytopathogens were not revealed. Certain endophytic isolates belong to *Pantoea agglomerans* and the genus *Bacillus*. Spraying of wheat seeds by these endophytes completely suppressed their overlaying by micromycetes, in the control overlaying of seeds by micromycetes was 70–100%. Endophytic bacteria of wheat seeds were not antagonistic to *Pseudomonas syringae* pv. *atrofaciens*, the dangerous disease agent of wheat. At the same time endophytes affect a pathogen aggressiveness. By artificial infection of plants at the time of vegetation with the mixture of cells of pathogenic and endophytic bacteria aggressiveness of *Pseudomonas syringae* pv. *atrofaciens* was significantly lowered. At some ratio of pathogen and endophyt, pathogen lost ability to cause diseases in plants.

Á. OLÁH-ZSUPOSNÉ[1], J. KÁTAI[2], M. BESSENYEI[2]

The effect of amelioration on the quantity of soil bacteria and microbial activity of soil

[1]Research Institute, Karcag, [2]Department of Soil Science and Microbiology, Faculty of Agriculture, Debrecen Agricultural University, Debrecen, Hungary

About 80% of soils used for cultivation are exposed to physical, chemical and biological loading. Furthermore, more than 51% of Hungarian soils have unfavourable properties, which require improvement. In order to prevent soil pollution and to improve soils having unfavourable properties, it is very important to do microbiological research in this field.

Field trials were conducted on meadow chernozem soil in the Research Institute Karcag, in1997. The spot experimental site was set up with 7 different plant cultures and by applying two different limes – limestone powder and lime ooze, originating from a sugar factory – this procedure was repeated 4 times. This paper deals with the effect of soil improving materials on the microbiological processes in a meadow chernozen soil. In the course of laboratory analyses, the total number of bacteria, the amount of aerobic N_2-fixing, aerobic nitrifying and cellulose decomposing bacteria, as well as some important soil enzymes' activities – phosphatase, catalase, urease, and invertase – were determined from the soil of two plant cultures – maize and sugar beet. Soil samples were taken 3 times during the growing season.

In soils on which sugar beet was cultivated the favourable effect of limestone effected the quantity of total bacterium number and nitrifying bacteria. The advantageous effect of lime ooze was measured in case of aerobic N_2-fixing and cellulose decomposing bacteria.

In the soil samples taken from maize fields the effect of lime ooze was more favourable on the quantity change of soil bacteria than the limestone – except for total bacteria count.

On the basis of the average result of similar treatments performed on both plant cultures, the effect of lime ooze is more favourable on the life-activity of the soil bacteria, than the lime powder.

Both of the two soil improving materials proved to be very effective on soil enzymes, and CO_2 production increased in a similar level.

In conclusion, it can be stated that the effect of two soil improving materials – lime ooze and limestone powder were favourable on the life activity of soil bacteria, their number increased as compared with the control treatment.

The number of aerobic N_2-fixing and nitrifying bacteria increased in the case of lime ooze treatment. The activities of soil enzymes and the production of CO_2 were influenced positively by both two limings.

Á. OLÁH-ZSUPOSNÉ[1], J. KÁTAI[2], M. BESSENYEI[2]

The effect of cultivation on the amount of soil bacteria and some enzyme activity

[1]Research Institute, Karcag, [2]Department of Soil Science and Microbiology, Faculty of Agriculture, Debrecen Agricultural University, Debrecen, Hungary

The most common/universal antropogenic effect on cultivated soil is soil cultivation and soil utilisation. This is the reason why it is very important to research the microbiological consequences of the different cultivation methods.

Soil cultivation and utilisation field trials were set up and conducted on the experimental site of the Karcag Research Institute on meadow chernozem soil, in 1997. In the experimental field two types of soil cultivation methods were compared – traditional ploughing and conservation tillage – with 7 different plant cultures, as well as according to the size of small and large plots, repeated 4 times.

The effect of cultivation methods on the quantity changes of some soil bacteria – total number of bacteria; aerobic N_2-fixing; nitrifying; aerobic decomposing bacteria – as well as the activities of some soil enzymes – catalase, phosphatase; urease; invertase – and CO_2 production were examined and evaluated. During the growing season, soil samples were taken three times, in spring, summer and autumn. According to the results originating from the spring soil sampling, the bacterium number increased 7–42% in the case of conservation tillage.

In autumn the opposite was experienced. In traditional ploughing treatments the number of soil bacteria increased except in case of nitrifying. The differences between the two cultivation methods were between 19–62% depending on the different physiological groups of bacteria. It is probable that by the effect of long-term-rainy weather, the soil conditions for aerobic soil bacteria were decreased in the cultivated layer of conservation tillage soil.

As for the results related to enzymes activities, it can be stated that both in spring and autumn the treatment of conservation tillage increased the activities of different soil enzymes from 2–25%. The most favourable effect can be experienced in the case of catalase. CO_2 production increased by 6.6% in spring and, by 20% in autumn, in case of conservation tillage.

In conclusion it can be stated that the number of bacteria examined – total number of bacteria; aerobic N_2-fixing; aerobic cellulose decomposing bacterium – was higher in the treatments using traditional cultivation – except for nitrification The activities of soil enzymes – phosphatase; urease; catalase sacharase – as well as CO_2 production increased as a result of conservation tillage.

B. A. EL-DEEB

Isolation and characterization of bacterial strain which is able to utilise a molluscicide bayluscide as a sole source of carbon and nitrogen and evidence for the involvement of plasmid in bayluscide degradation

Botany Department, Faculty of Science, South Valley University, Sohag, Egypt

Egypt is considered to be the home of Schistosomiasis and also one of the most severe endemic areas. It has been the site of numerous control efforts. One of these efforts is the application of molluscicide bayluscide *Pseudomonas* sp strain Bal 1 was isolated from a field contaminated with molluscicide bayluscide. This strain was able to utilize a bayluscide as a sole source of carbon and nitrogen. The degradation of bayluscide by Bal 1 strain is mediated by pBE1 (58 Kb) and pBE2 (110 Kb) plasmids. The loss of these plasmids resulted an irreversibly mutant unable to degrade bayluscide. The transfer of these plasmids from wild type strain Bal 1 to Bal 1M mutant, restored completely its capability to degrade the molluscicide. It was proposed that pBE1 and pBE2 are conjugated plasmids and involved in the bayluscide degradation.

A. E. EL-KARAMITY

Response of some lentil cultivars to inoculation and spraying with molybdenum

Agronomy Department, Faculty of Agriculture, Minia University, Egypt

A field experiment was performed during two successive seasons of 1993/94 and 1994/95 at the experimental farm, Faculty of Agriculture, Minia University to study the effects of inoculation with *Rhizobium leguminosarum* and three concentrations of molybdenum (0, 10 and 20 ppm) as foliar spray on nodulation,

growth, yield and its components of three lentil cultivars (Giza 9, Giza 370 and Precoz).

The obtained results of each season and the combined analysis of the two seasons revealed that the inoculated plants significantly surpassed uninoculated ones in number and dry weight of nodules/plant, dry weight/plant, number of branches, pods and seeds/plant, 100-seed weight, seed and straw yield/feddan. Plant height was not affected by inoculation. All above-mentioned traits were significant and gradually increased with increasing molybdenum concentration from 0 to 20 ppm. It is worthy to note that seed yield and its components were not significantly different by Mo application at rates of 10 and 20 ppm.

Nodulation, growth, yield and its components' characteristics except number of seeds/pod were significantly different among the tested cultivars. In general, Giza 370 cultivar recorded the highest values for the previous characters except 100-seed weight followed by Giza 9, whereas the lowest values were achieved by Precoz cultivar. The heaviest 100-seed weight was estimated in Precoz cultivar, while the lightest 100-seed weight was recorded in Giza 9. Number of seeds/pod was not affected by any of the tested factors. Either inoculation or Mo application delayed flowering of lentils. The earliest flowering cultivar was Precoz followed by Giza 370, while the latest one was Giza 9.

Inoculation × Mo concentrations interaction had significant effect on number and dry weight of nodules, dry weight/plant, number of branches and pods/plant and 100-seed weight. Inoculation × cultivars interaction had significant effect on number and dry weight of nodules, number of branches and pods/plant and straw yield/feddan, while number of nodules/plant, dry weight/plant, plant height, number of branches and pods/plant and seed yield/feddan were significantly affected by the interaction between Mo concentrations and cultivars. Inoculation × Mo concentrations × cultivars had a significant effect on dry weight/plant, number of branches and pods/plant in each season.

H. E. A. F. BAYOUMI HAMUDA[1], H. ABDORHIM[2], I. JEVCSÁK[1], H. GODWAR[2], M. KECSKÉS[1]

Ecotoxicological tests of Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+} on the growth and symbiosis of *Vicia faba – Rhizobium leguminosarum* bv. *viciae*

[1]Agricultural-, Environmental Microbiology and Soil Biotechnology Ph.D. Subprogram,
[2]Environmental Microbiology M.Sc. Subgroup, Gödöllő University of Agricultural Sciences, Budapest, Hungary

In soil ecotoxicological studies, the toxicity of heavy metals is usually investigated by mixing metal salts at different concentrations into the soil. In such tests, no attention is paid to the possible effect of the anionic partner of the investigated metal, which may itself has an adverse effect on soil microbiota. Investigations were carried out using four different techniques (paper disk, hole, streak and microfermentor) to study the effects of different polluted materials (Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+}) applied to cultural medium (*in vitro*) and to soil (*in vivo*) in five concentrations (10, 20, 40, 80, and 160 mg l^{-1} medium or mg kg^{-1} soil) on growth of *Rhizobium leguminosarum* bv. *viciae* and the symbiotic interaction with *Vicia faba* L. plant. The results showed that there were no differences among the four techniques used. Therefore, the results of microfermentor method are summarized here. Strain of Lóbab Z proved to have the highest tolerance among the strains to the tested chemicals ions followed by Bükköny 75/4, HB-3841*str*$^{+}$, and E1012. The ions of Pb^{2+} were the least toxic and Ni^{2+} and Cu^{2+} were the most toxic ions *in vitro* followed by Cd^{2+}. An agroecosystem symbiotic model of mesocosm experiments was also conducted in greenhouse using sterile acidic (pH 4.7) brown forest soil of Gödöllő to study the effects of ions *in vitro* on the growth of horse bean seedlings and its symbioses with *Rhizobium leguminosarum* bv. *viciae* (E1012, HB-3841*str*$^{+}$, Lóbab Z, and Bükköny 75/4) strains. Results indicated that Ni^{2+} and Cu^{2+} ions highly inhibited plant growth, nodulation and N_2-fixation at 40–80 mg $^{-1}$ soil in the applied dose. The plants inoculated with Lóbab Z, and Bükköny 75/4 were more tolerant to the polluted ions than others. These inhibitions were reduced when the soil was treated with Cu^{2+}-containing fungicide (Cobox) before the plantation and rhizobial inoculation by three weeks. Also, the results proved that the chloride forms compared to sulfate forms of metals had more ecotoxicological effects both *in vitro* and *in vivo*.

B. BIRÓ[1], L. KÖDÖBÖCZ[2], M. KECSKÉS[2], P. L. EBERBACH[3]

Comparative *in vitro* study for the herbicide sensitivity of various authentic and Hungarian *Rhizobium* strains belonging to different species

[1]Research Institute for Soil Science and Agricultural Chemistry, Hungarian Academy of Sciences, Budapest, [2]Agricultural-, Environmental Microbiology and Soil Biotechnology Ph.D. Subprogram, Gödöllő University of Agricultural Sciences, Budapest, Hungary, [3]University of Melbourne, Melbourne, Australia

Compatibility of the legume cultivations by various xenobiotics is the crucial point of developing the sustainable agricultural practices. Among the agrochemicals, herbicide applications are the most frequent treatments throughout the World. The effect of the most abundant herbicides (of the *Rhizobium*-legume systems) with various active ingredients, such as 2,4-D, trifluraline, MCPA or N-phosphono-methyl-glycine has been examined in *in vitro* conditions. Four concentrations (0.1, 1, 10 and 100 μg l^{-1}) of these chemicals were used in a micro-fermentor (or in a bio-photometer). The cell number of the various strains was measured after 15 hours of incubation (or permanently in every 20 minutes), starting from the 10^6 CFU ml^{-1} in liquid YEM media. Six authentic *Rhizobium* strains of the Australian Type Culture Collection belonging to various species, such as *R. leguminosarum* bv. *trifolii* (NA-25, NA-14, NA-71) bv. *viciae* (NA-503) bv. *phaseoli* (NA-575), *R. meliloti* (NA-355) was examined, and also some other Hungarian isolates from *Coronilla varia*, *Lupinus albus*, *Trifolium repens*, *Medicago sativa* and *Glycine max*, so as to compare the inter- and intra-specific variations of the sensitivity. Among the herbicides investigated, the glyphosate with N-phosphono-methyl-glycine content proved to be the less harmful for the *in vitro* growth of rhizobia. After 14 hours of incubation the cell number was reduced by 0–31% only at the 10 μg l^{-1} concentration comparing to the untreated control. Due to the more frequent use of glyphosate in the clover-grass mixtures abroad, the authentic *R. leguminosarum* bv. *trifolii* strains were found to be less sensitive comparing to the home inoculum strains for clover. Importance of the effecting periods of pesticides in the adaptation and the natural selection of tolerant lines, is therefore concluded. Sensitivity on the other hand proved to be positively correlated rather with the single isolates and the origin, than the species and hosts of the fast- and slow-growing *Rhizobium* (*Bradyrhizobium*) sp.

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W. MERBACH[1], S. RUPPEL[2]

Influence of different N compounds on the dinitrogen fixation of *Azospirillum* and *Pantoea*

[1]Institute of Soil Science and Plant Nutrition, University of Halle/Wittenberg, [2]Institute of Vegetable and Ornamental Plant Science, Grossbeeren, Germany

The dinitrogen fixation activity of *Azospirillum* sp. and *Pantoea agglomerans* strains was determined by ^{15}N incorporation after incubation with ^{15}N labeled air and/or by acetylene reduction. These bacterial strains were able to fix N_2 both in pure culture and in association with wheat plants in hydroponics. Nitrogenase activity of *Azospirillum* sp. in pure culture was more rapidly inhibited by the addition of ammonium than nitrate. The N_2 fixation of *P. agglomerans* decreased only by ammonium addition, but was stimulated by nitrate. Nitrogen fixation in association with wheat plants remained unaffected by both N compounds. However, nitrogen derived from the atmosphere (Ndfa) contributed only very slightly to the overall nitrogen nutrition of the plants.

ZS. MAYER[1,2], T. MÁTRAI[1], ZS. KÓKAI[1], I. SALAMON[1]

Aspergillus invertase (AI) test for the rapid detection of feed spoilage

[1]Research Institute for Animal Breeding and Nutrition, [2]Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary

From hygienical point of wiev *Aspergillus* spp., more exactly the members of the *A. flavus* group (*A. flavus, A. parasiticus, A. oryzae, A. nomius*) are the most important moulds:

a) They start earliest to deteriorate feed at a low a_w,

b) generate moisture for further moulds growing at higher a_w,

c) produce important mycotoxines, like aflatoxines and ochratoxines.

The principle of the *Aspergillus* invertase (AI) test is based on three research experiences:

1. A technique has been developed, where mycelia of primarily *Aspergillus*, but also of *Penicillium* and *Poecilomyces* spp. could be grown rapidly in a 1–4 mm deep liquid medium at 37 °C.

2. Fungi common in feed can grow on Czapek-Dox medium, whose substrate is sucrose, must be able to produce invertase necessarily.

3. Very small mycalial growth, invisible for naked eye can be detected through invertase activity in the submerse mycelium growing system by detecting the apparition of reducing sugar.

Procedure, aspecific interactions are described, sensitivity and practical use discussed.

Zs. Mayer[1,2], T. Mátrai[1], Zs. Kókai[1], I. Salamon[1]

Growth kinetics of common freed moulds monitored in constant a_w moisture chamber

[1]Research Institute for Animal Breeding and Nutrition, [2]Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary

A simple model for constant a_w moisture chambers is proposed, based on the standard relative humidity over saturated aequous solutions of NH_4NO_3 (0.62), NaCl (0.75), KCl (0.84) and KH_2PO_4 (0.95). The chambers of two litre could be closed air-tight, the atmosphere got to an equilibrium at the 8th hours. Two gms of sterilized ground feed samples of particle size less then 0.2 mm or whole grains were placed on aluminium trays inoculated and incubated at 25 °C for 26 days.

Samples were taken and mould counts were determined according to ISO 7954.

Inoculated by *A. parasiticus* at a level of log 6.5, at a_w=0.95, mould count started to grow rapidly, by approx. 0.5 log/day, attaining 8–9 log at the 19th day. At a_w=0.84, mould count started to decrease till the 6th day, by 0.15 log/day, then started to grow by 0.2 log/day and attained the same magnitude at the 19th day. At a_w=0.75, slight decrease until the 6th day could be also observed, then mould count increased to the initial level. At a_w=0.62, initial mould count stagnated.

A. flavus and *A. parasiticus* and a *Penicillium* strain combined inoculations at a_w=0.95 and a_w=0.84 showed the similar propagation kinetics, except the decreases during the first 6 days. At higher a_w values *Penicillium* dominated rapidly while at lower a_w its propagation started after the 6th day only.

K. POSTA[1], G. BAKONYI[2], I. KISS[2], M. FÁBIÁN[2], P. NAGY

Density dependent regulation of arbuscular mycorrhizal fungi by fungivorous collembolan

[1]Department of Microbiology, [2]Department of Zoology and Ecology, Gödöllő University of Agricultural Sciences, Gödöllő, Hungary

Arbuscular mycorrhizal (AM) fungi increase the plant nutrient uptake under different circumstances. Enhanced phosphorus uptake by AM plants compared to that of non-AM plants is one of the best known among related phenomena. Soil fauna can affect AM fungi directly by grazing. This influence could result in positive or negative effects on plant nutrient uptake.

One reason of these results may be the density dependence of this interaction. A field experiment was set up to test whether collembolan density can have a direct impact on the number of spore, hyphal length and colonization of *Glomus mosseae* on maize plant.

In addition, collembolan influence on the number of fungi and *Trichoderma* spp. was studied to see whether fungivorous collembolan predominantly graze on AM hyphae or other fungal hyphae.

A clear and strong density dependence was found regarding the number of spore and colonization of AM. Collembolans in low density enhanced the number of spore and colonization of mycorrhizal fungi, while in a greater numbers decreased these parameters. No such correlation was observed with respect to hyphal length. *Trichoderma* spp. density showed an opposite trend. Lowest values were found at moderate collembolan density.

M. JUHÁSZ-ROMÁN[1], E. SIMON[2], Á. TÓTH[3]

Special fermented milks with low protein- and phenylalanine content for phenylketonuric persons

[1]Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, [2]Corporation of Nestlé Hungária, Szerencs, [3]Department of Chemistry and Biochemistry, University of Horticulture and Food Industry, Budapest, Hungary

For phenylketonunoric persons is very important a dietetic life-style.

Normal kefir cultura and intestinal Lactobacilli were used to produce the therapeutic kefir and probiotics from low protein- and phenylalanin- based milk.

The next chemical and microbiological parameters were examined in details:
– acidity,
– aroma compounds,
– viable cell count of Lactic acid bacteria,
– antibiotic sensitivity of intestinal Lactobacilli,
– phenylalanin content.

The acidity was higher and the aroma profile was poor in case of probiotics than in normal kefir therefore we supplemented these products with fruit juices.

(This supplementation seems to be the best with heated ananas for the phenylchetunoric children.)

E. SZIGETI[1], O. REICHART[2], J. FARKAS[3], B. BECKER[4], W. H. HOLZAPFEL[4]

Modelling growth of *Lactococcus lactis, Listeria monocytogenes* and *Bacillus cereus* as a function of pH and temperature measured by turbidimetry

[1]Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, [2]Department of Food Hygiene, University of Veterinary Science, [3]Department of Refrigeration and Livestock Products' Technology, University of Horticulture and Food Industry, Budapest, Hungary, [4]Institute of Hygiene and Toxicology, Federal Research Centre for Nutrition, Karlsruhe, Germany

Combined effects of pH and temperature on growth of *Lactococcus lactis* BFE 920, *Listeria monocytogenes* SLCC 2540 serotype 3b and *Bacillus cereus* CCM 2010 were studied by turbidimetric measurements in two semi-synthetic media using a Bioscreen C equipment (Labsystem Oy, Finland). Detection times (DT) belonging to the first significant changes in optical density were determined. Linear correlation could be established between DT and logarithm of colony forming units (cfu) determined by conventional spread-plate technique.

In the range of 2–8 $\log_{10}$ cfu the determination coefficients (R^2) of the linear regressions were higher than 0.98 at each strain-medium combination.

In order to describe the relationship between the detection time (DT) and the environmental factors involving the initial cell number, a mathematical model was constructed:

$$\log_{10}DT = a + b_1 \times \log_{10}N_o + b_2 \times (pH\text{-}pH_{opt})^2 + b_3 \times (T\text{-}T_{opt})^2,$$

where

N_o: initial cell count (cfu/ml)

a, b_1, b_2, b_3: constants depending on media and microbes

pH: pH of the medium

pH_{opt}: pH optimum of the growth obtained from the model

T: temperature of the medium (°C)

T_{opt}: temperature optimum of the growth obtained from the model (°C).

From the results of the mathematical modelling the following conclusions can be drawn:

– The predictive model applied for the experimental data describes the DT values with close correlation as a function of the initial cell number, pH and temperature. The determination coefficients lasted from 0.976 to 0.996.

– There is no significant difference between the determination coefficients of the fitting related to the two media.

– The optimum values of pH obtained from the model for both media were nearly identical in the case of the same microorganism, while the temperature optimum were differing with 1–4 °C.

– The a, b_1, b_2, coefficients belonging to the two media at the same microorganism do not differ significantly at 95% level, which means that the effects of the initial cell number and the pH on the growth do not vary significantly in the media.

The b_3 coefficients proved to be significantly (95% level) differing at every microorganism, so the temperature effect of the two media on the growth is not identical. This conclusion is in coincidence with the varying temperature-optimum values corresponding to the two media at the same microorganism.

L. KREDICS[1], ZS. ANTAL[2], L. MANCZINGER[1]

Influence of water potential and temperature on growth, enzyme secretion and *in vitro* enzyme activities of *Trichoderma harzianum*

[1]Department of Microbiology, Attila József University, Szeged, [2]Research Group for Microbiology, Hungarian Academy of Sciences and Attila József University, Szeged, Hungary

Influence of water potential and temperature on linear mycelial growth, secretion and *in vitro* activities of β-glucosidase, cellobiohydrolase, β-xylosidase, exochitinase and chymotrypsin enzymes of *Trichoderma harzianum* was studied. Nearly linear correlation was found between water activity and colony growth rate at 25 °C and at 10 °C with higher growth rates at higher temperature and water potential. Secretion of the enzymes depended on the water potential of the liquid media and not on the type of the salt (NaCl or KCl) used as osmoticum. Different water potential values were optimal for the secretion of the different enzymes. *In vitro* enzyme activities were significantly affected by water potential and temperature. All enzyme

activities were lower at lower temperatures. Significant activities were measured for most of the enzymes even at the water potential value of –14.80 MPa, which is below the limit of mycelial growth (–11.54 MPa). These results suggest the possibility of using mutants with improved xerotolerance for biocontrol purposes in soils with lower water potential.

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M. L. KECSKÉS[1], I. SCHRÖDER[2], B. C. AHOHUENDO[3], M. KUHLMANN[4], W. DRÖGE-LASER[4], Z. BOZSÓ[1], G. P. OTT[1], Z. KLEMENT[1], K. RUDOLPH[2]

A candidate factor in resistance mechanisms induced by phytopathogenic pseudomonads and xanthomonads: lipopolysaccharide

[1]Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary,[2] Plant Protection Institute, Georg-August University of Göttingen, [3]National University of Benin, Cotonou, Benin, [4]Botanical Institute, Georg-August University of Göttingen, Göttingen, Germany

It has been long known that pretreatment of leaves by heat killed or avirulent (mutant) phytopathogenic bacteria causes "locally induced resistance" and gives protection against a second challenge inoculation with virulent bacteria. However, not much was known about the possible signals that evolved on behalf of the plant side or the nature of the inducing principle.

We used purified substances of lipopolysaccharide (LPS) extracted from the outer cell-membrane of some *Xanthomonas campestris* pvs. (*Xc*) to test the effect of these bacterial fractions in tobacco leaves by both classical and molecular biological methods.

With our repeated experiments we could confirm our earlier visual observation that the normally appearing hypersensitive response (HR) in tobacco could be prevented when 0.2–0.5 mg/ml of LPS extracts were applied prior to *Xc* or *Pseudomonas syringae* pv. *syringae* 61 (10^7–10^8 cfu/ml) bacterial inoculation. The nonappearance of HR was markedly influenced by the LPS concentration, the physiological state of the leaf/plant and the incubation temperature throughout the experiment.

Total RNA extracts of leaves were pretreated with LPS, living or heat killed bacteria. In order to investigate possible accumulation of specific mRNA of the treated plant tissues extracts were subjected to agarose gelelectrophoresis followed by Northern-blot and hybridization with radioactive (^{32}P) cDNA probes coding for PR1a

(pathogenesis-related protein 1a), PAL (phenylalanin ammonia lyase) and sesquiterpene-cyclase (4-epi-aristolochene synthase, EAS4).

We concluded that due to the LPS pretreatment, beside our visual observation of nonappearance of HR (induced resistance), specific accumulation of *PR1a*, *PAL*, *EAS4* mRNA could be detected. These results indicate a possible role of LPS or its subcomponents in the recognition processes of both pathogenesis and of induced resistance mechanism.

H. DOMJÁN-KOVÁCS[1], K. RÁSKY[2], A. FÁBIÁN[1]

Chemiluminescence enzyme immunoassay, a screening method for selective detection of *E. coli* O157:H7 from food

[1]National Food Investigation Institute, [2]Diagnosticum Ltd, Budapest, Hungary

Verocytotoxin producing *E. coli* (VTEC) is now recognized as a major cause of haemorrhagic colitis and haemolytic-uremic syndrome. Although great variety of VTEC serogroups have been implicated in human disease, *E. coli* O157:H7 is the most prevalent strain. Since these pathogens may be present in food and environmental samples in only small numbers, sensitive methods are needed for their detection. This study was done for the evaluation of a Chemiluminescence Enzyme Immunoassay developed for the detection of *E. coli* O157:H7. For this aim different *E. coli* O157 serotypes were used. The sensitivity and specificity of the kit was determined from the decimal dilutions of the 24-hour broth cultures of the test strains. According to this trial the sensitivity of the kit is 10^3–10^4 cell/cm^3, and it is specific for *E. coli* O157. Further on 25 g ground beef samples were prepared and inoculated with *E. coli* O157:H7 with different CFU g^{-1}. The samples were incubated in 225 ml mEC + n at 42 °C for 4 hours and the assays were performed. According to the results with the CLIA test 10^1-10^2 *E. coli* O157 g^{-1} can be detected from the sample. So this kit seems to be suitable for screening the samples before selective cultivation of *E. coli* O157: H7.

M. MAREKOVÁ[1], A. LAUKOVÁ[2], I. F. NES[3]

Study of bacteriocin-like activity produced by environmental enterococcal strains

[1]Institute of Medical Chemistry and Biochemistry, MF UPJS, [2]Institute of Animal Physiology, SAS, Kosice, Slovakia, [3]Laboratory of Microbial Gene Technology, NLH, As, Norway

Bacteriocins are antimicrobial proteins, protein aggregates or protein complexes produced by several bacteria with antagonistic activities against other, more or less closely related, bacterial strains. In the majority bacteriocin-like producing strains isolated from humans, food or other sources were described.

Fifteen strains of *Enterococcus* sp., which were isolated from wastewater, were obtained as bacteria producing bacteriocin-like substances. As indicator of antimicrobial activity 22 strains were used. All examined enterococci produced inhibitory agents, which showed a wide range of inhibition against Gram-positive and Gram-negative indicator organisms from different sources. Clear zones of inhibition (diameter 2–21 mm) were observed. Most bacteriocin-like substances produced by the strains of enterococci were stable and no decrease in activity were detected after 3 month at 10 °C, by freezing (–20 °C) and long-term storage at 4 °C and –20 °C. The maximum activity was produced by overnight cultures. The inhibitory activity was not reduced by heat treatment at 50 °C, 80 °C and 100 °C for 30 min. Some from studied enterococcal strains contained a plasmid DNA, but the evidence for plasmid-associated bacteriocin production needs additional experiments.

F. DELAŠ[1], S. DURAKOVIĆ[1], I. DELAŠ[2], B. RADIĆ[3]

Relationship between biomass synthesis and ochratoxin A (OTA) production in mould *Aspergillus ochraceus* NRRL 3174 grown on a soil substrate in pure and mixed culture

[1]Faculty of Food Technology and Biotechnology, [2]School of Medicine, [3]Institute for Medical Research and Occupational Health, Zagreb, Croatia

In this study, the growth of ochratoxicogenic mould *Aspergillus ochraceus* NRRL 3174 on a solid substrate (corn grains) was investigated, as well as the parameters which influence ochratoxin A (OTA) synthesis. The mould was incubated as pure culture, as well as mixed culture together with moulds *Trichotecium roseum* ZMPBF 1226 and *Fusarium* sp. ZMPBF 1215, which were found not to produce OTA.

The biomass yield was measured by chitin method, while OTA concentrations were determined fluorodensitometrically. The growth monitoring was performed through 5 weeks during the cultivation in stationary phase, at incubation temperatures 15 °C, 20 °C, 25 °C and 30 °C, with 38% water content in the substrate. Inoculation was performed with 10^6 spores g^{-1} substrate. The highest level of OTA production was reached after three weeks of cultivation at 20 °C and was found to be 790 μg g^{-1} and 290 μg g^{-1} dry mycelium for pure and mixed culture, respectively. During the cultivation at 25 °C, under otherwise same conditions, OTA synthesis was reduced about 45% (355 μg g^{-1}) in pure, and 28% (210 μg g^{-1}) in mixed culture. At the same time, higher incubation temperature caused increased biomass production for 40% (12.40 mg g^{-1}) in pure and 20% (12.90 mg g^{-1}) in mixed culture. These results show the ability of mixed culture to inhibit OTA production, especially at lower temperatures (15 °C and 20 °C), where moulds *Trichotecium roseum* ZMPBF 1226 and *Fusarium* sp. ZMPBF 1215 prevail.

L. A. PASICHNYK, R. I. GVOZDYAK, S. F. KHODOS

Bacterial microflora of seeds and growing wheat plants

Zabolotny Institute of Microbiology and Virology, Kiev, Ukraine

Bacterial microflora healthy seeds and growing wheat plants of different varieties were investigated. Epiphytic bacteria were isolated by washing of seeds, leaves and ears by sterile water with subsequent plating of dilution on nutritious media. In 4–5 days the colonies were picked up and analysed.

It is established that main microflora of wheat seeds is presented by *Pantoea agglomerans* (50–90% depending on the varieties). Total quantity of bacteria on a surface of seeds also depends on the varieties. Absolutely another picture is observed on the growing wheat plants. In a phase of plantlet *P. agglomerans* they are absent on the plants, and in a phase of booting they are detected very seldom. But in process of seeds' maturing their quantity is increasing, and gaining maximum in a milk-dough stage. It is necessary to note that with maturing of seeds the total quantity of bacteria on leaves and ears is increasing. By morphological, physiological and biochemical properties bacteria isolated from healthy wheat plants were identified as *Pseudomonas* spp., *Erwinia* spp. and *Bacillus* spp. The main epiphytic bacteria of the genus *Pseudomonas* belong to saprophytic bacteria *P. fluorescens*. Besides, we isolated the agent *P. syringae* pv. *atrofaciens*, which does not differ in biological properties from *P.*

s. pv. *atrofaciens*, isolated from the affected tissue of wheat. Epiphytic strains of *P. s.* pv. *atrofaciens* at the artificial infection are pathogenic for wheat.

Thus epiphytic microflora of seeds and growing wheat plants includes *P. agglomerans*, *P. fluorescens*, *P. s.* pv. *atrofaciens*, and also bacteria of the genera *Erwinia* and *Bacillus*.

T. BINTSIS[1], R. DAVIES[1], E. LITOPOULOU-TZANETAKI[2], R. K. ROBINSON[1]

Microbiology of Feta cheese brine

[1]The University of Reading, Department of Food Science and Technology, Whiteknights, Reading, UK,
[2]Laboratory of Dairy Technology, Faculty of Agriculture, Aristotle University of Thessaloniki, Thessaloniki, Greece

The primary and secondary micro-floras of two batches of Feta cheese brine from two different factories in Northern Greece were investigated. The microbiological analysis included total count, coliforms, enterococci, *Staphylococcus* spp., yeasts, lactic acid bacteria, salt-tolerant bacteria, *Listeria* spp. and *Yersinia enterocolitica*. Chemical analyses (pH, NaCl, free fatty acids, ash, protein and lactose content) were also carried out; representative samples of brine were taken in duplicate at 0, 10, 20, 30, and 60 days from manufacture. The brines had low pH values (4.8–4.0) and high salt contents (5.7–8.0%) which along with nutrients leaching from the cheese, made them a special medium with a specific micro-flora. The total counts were 1.4×10^7–3.1×10^8 cfu/ml, the counts for lactobacilli and streptococci/lactococci were 1.5×10^6–1.8×10^8 cfu/ml and 2×10^6–1×10^8 cfu/ml, respectively, and yeasts were found to be 1.1×10^5–1.9×10^6. Coagulase-negative *Staphylococcus* spp. declined from 2.8×10^4 to 30 cfu/ml over the 60 days, while coliforms were present in only one sample and decreased during maturation; enterococci were found only in one sample at low numbers (1.9×10^3 cfu/ml). The high numbers of lactobacilli, streptococci/lactococci and yeasts suggest that these microbial groups play an important role during the maturation stage of Feta cheese, but the survival of pathogenic bacteria could be a problem. Consequently, a novel decontamination system using UV/furocoumarins was applied to simulated cheese brines in order to determine the effect of the system on the natural micro-flora and on selected pathogenic bacteria capable of surviving in cheese brines.

I. JEVCSÁK[1], B. BIRÓ[2], H. E. A. F. BAYOUMI HAMUDA[1], M. KECSKÉS[1]

PGPR effect and herbicide sensitivity of some pseudomonads depending on their origin and plant hosts

[1]Agricultural-, Environmental Microbiology and Soil Biotechnology Ph.D. Subprogram, Gödöllő University of Agricultural Sciences, Budapest, [2]Research Institute for Soil Science and Agricultural Chemistry, Hungarian Academy of Sciences, Budapest, Hungary

The *in vitro* growth of some home-isolated PGPR pseudomonads were examined in liquid nutrient broth supplemented with glyphosate (N-phosphono-methyl-glycine) herbicide. Four concentrations (0.1, 1, 10 and 100 μg l^{-1}) were used in a micro-fermentor, where the cell number of the various strains was investigated after 14 hours of incubation starting from the 10^6 CFU ml^{-1} in liquid YEM media. Different isolates of the fluorescens-putida type *Pseudomonas* sp. were collected from various sites and hosts, such as crownvetch (*Coronilla varia*), sugarbeet (*Beta vulgaris*), potato (*Solanum tuberosum*), lupine (*Lupinus albus*), river Tisza etc, so as to select candidate isolates for inocula-production. Plant growth promoting (PGPR) effect of the various strains was also calculated from the *in vitro* siderophore production. Glyphosate herbicide was found to have only a slight influence of the *in vitro* growth rate of the tested pseudomonads. No significant differences developed among the effect of various rates, except the 100 μg l^{-1} dose in some cases. There was no correlation between the siderophore production or the herbicide sensitivity and also between the host-plants or the origin (rhizospere or water) of the strains. Testing some physiological characteristics, such as the antagonistic ability against the soil-borne plant pathogens or the sensitivity to abiotic stress factors (pH, salinity, drought, etc.) proved to be especially important.

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I. VIDÁCS[1], G. KISKÓ[2]

DSC measurements of sporeforming bacterium *Bacillus cereus* T

[1]Department of Microbiology, Central Food Research Institute, [2]University of Horticulture and Food Industry, Budapest, Hungary

In our study we have tried to detect the changes occurring in *Bacillus cereus* T spores due to irradiation with the help of the DSC instrument. Suspension of 10^{10} spore/cm^3 was irradiated with 3kGy with a ^{60}Co source. The DSC results were

compared with results obtained with the measurement of untreated control spores. The heat resistance of the irradiated spores has decreased due to the treatment, which is shown by the shifting of the endothermic processes to a lower temperature range.

The other aim of the experiment was to determine if the curve obtained by an isotherm DSC measurement could be interpreted as a growth curve, and if so under what circumstances.

The suspension of 10^5 spore/cm^3 *Bacillus cereus* T spores was placed into the DSC cell. The cultivation was carried out at 37 °C and the heat-current changes were monitored continuously. Parallel to this measurement the growth curve of the microbe was also determined with traditional plating method under the same circumstances. Based on the incubation time we have compared the two curves and we have found the following: the detection limit of the DSC instrument is above 10^6 microbe/cm^3, correlation between the measured heat-current changes and the real growth can be found between 10^7–10^9 microbe/cm^3. This means that only at very high concentration of the microbes can the growth parameters (growth rate and lag-phase) determined and calculated from the DSC isotherm measurements.

S. FARKAS[1], GY. GUNICS[2], A. HEGEDÜS[3], M. KECSKÉS[1]

Susceptibility of *Rhizobium* and *E. coli* strains to different antibiotics

[1]Agricultural-, Environmental Microbiology and Soil Biotechnology Ph.D. Subprogram, Gödöllő University of Agricultural Sciences, Budapest, [2]Albert Szent-Györgyi Medical University, Faculty of Health Sciences, [3]Teachers Training College Gyula Juhász, Szeged, Hungary

Antibiotics' susceptibility of two *Sinorhizobium meliloti*, five *Rhizobium leguminosarum* bv., three *Bradyrhizobium* sp. and six *Escherichia coli* strains was tested with RESISTEST, BBL and E-test disk-diffusion, and Checkerboard methods. The interaction of Ampicillin and Gentamicin with Imipramine was studied on *S. meliloti*, *R. leguminosarum* bv. *phaseoli* and *E. coli* K12 RP4 strains. The *S. meliloti*, *E. coli* K12 drd$^+$ and *E. coli* K12 RP4 strains were resistant against more than ten antibiotics. The other *Rhizobium* and *E. coli* strains were resistant against five to ten antibiotics. Synergistic effect was found with Ampicillin and Gentamicin on *E. coli* K12 RP4 and *S. meliloti* strains in the presence of Imipramine. The additive effect of Ampicillin and Gentamicin was tested with Imipramine on *R. leguminosarum* bv. *phaseoli* strain.

A. HEGEDŰS[1], H. E. A. F. BAYOUMI HAMUDA[2], M. KECSKÉS[2]

Enhanced growth of gerbera plants in the presence of *Trichoderma* spp. inoculates

[1]"Gyula Juhász" Teachers Training College, Szeged, [2]Agricultural-, Environmental Microbiology and Soil Biotechnology Ph.D. Subprogram, Gödöllő University of Agricultural Sciences, Budapest, Hungary

The plant-microbe interaction would be indirect when the microbial population produces biologically active substances, which affect plant growth. These might be positive when these substances promote growth.

We studied the effect of *Trichoderma viride* on nine of *Gerbera* c.v. (Macho, Zigone, Snowball, Rebecca, Fame, Robijn, Parade, Pascal, and Fanny) growth. The Gerbera plants were taken from tissue culture and were grown – under greenhouse climate – in sterilized peat pots before the treatments of plant roots with *Trichoderma viride* solution. The roots were immersed into the fungi solution for ten minutes. The plants were watered with the nutrient solution every day. The content of the solution was 30–22–20 (N–P–K) 1.5 g/litre, magnesium sulphate 2 g/litre and calcium nitrate 2 g/litre: the young plants were grown for eight weeks then they were planted into sterilized peat soil in 10 litres plastic containers. We measured chlorophyll and carbohydrate content of the leaves, yield and flower quality of plants.

Differences among the growth of treated and control plants are significant.

M. A. ABDEL-SATER

Antagonistic interactions between fungal pathogens and leaf surface fungi of onion

Botany Department, Faculty of Science, Assiut University, Assiut, Egypt

Twenty species belonging to 13 genera were identified from leaf surfaces of onion plant on glucose-cellulose-Czapek's agar at 28 °C. The microbial numbers in the phyllosphere of the plant tested was found regularly and display a decrease throughout the growth period on the two isolation media. While the count of phylloplane fungi increased with the increase of plant age till February and decreased till the end of the experimental period. The most prevalent fungi were *Alternaria alternata*, *Aspergillus niger*, *A. sydowii*, *A. versicolor*, *Cladosporium herbarum*, *Cochliobolus lunatus*,

Pleospora herbarum, *Setosphaeria rostrata* and *Ulocladium botrytis* on the two type of media:

The prevalent phyllosphere and phylloplane fungi of onion (*Allium cepa*) were screened for their antagonistic activity against the pathogenic fungus *Pleospora herbarum* in plate cultures. The culture filtrates of *A. niger*, *A. sydowii*, *E. nidulans*, *S. chartarum* and *C. lunatus* caused high inhibition of *P. herbarum*, followed by *S. rostrata*, *A. alternata*, *C. herbarum*. While filtrate of *U. botrytis* was less effective in reducting the growth of the tested fungus, thus under the influence of such fungal antagonist the growth of the fungus is controlled. Volatiles produced from the most fungal cultures have no significant inhibition on the growth of the fungal pathogen tested. Among the fungal isolates screened for their ability to produce pectinase enzyme, about 87% of the isolates could produce this enzyme with variable degrees of pectin hydrolysis.

J. MÁTÉ[1], I. RIMÓCZI[2], I. LENTI[1]

Mycorrhizal fungus relationships in the oak forests of the Bátorliget primordial marsh

[1]Agricultural College Department, Gödöllő University of Agricultural Sciences, Nyíregyháza,
[2]University of Horticulture and Food Industry, Budapest, Hungary

We have recorded 577 basidial fungus species on the territory of the Bátorliget primordial marsh in 1995–1996. This is five times as many as we have known to be in Bátorliget on the basis of Ubrizsy's work (1953).

The 124 mycorrhizal fungi account for nearly one-third of the recorded species, and the 24 genera concern a rather wide range of macrofungi. From the usually pine-mycorrhizal fungi those with greater plasticity in relation to the tree-partner are to be found under the Quercus robur L. (*Xerocomus badius* (FR.) KÜHN. ex GILB.).

The number of mycorrhizal fungus species collected on the territory of the Convallario-Quercetum tiliosum phytocoenosis amounts to 102 and they belong to 27 genera. The *Amanita*, *Boletus*, *Crtinarius*, *Hebeloma*, *Inocybe*, *Lactarius*, *Russula*, *Tricholoma* and *Xerocomus* genera represent the greatest number.

The number of mycorrhizal fungus species found under the Quercus robur amounts to 91 and they are represented by 21 genera. The *Russula* genus constitutes the mycorrhizal relationship with the greatest number of species (19) followed by the *Amanita* (9) and the *Boletus* (2) genera.

According to Einhellinger (1987) the Russula faginae ROMAGN., the name of which already expresses its linkage to the beech, is a typically Carici-Fagetum species. On the Bátorliget primordial marsh we have found the *Russula faginae* fungus under the Quercus robur. Similarly we have recorded the *Hygroporus chyrodon* (BATSCH et FR.) FR. species here as well, which is the fungus of Fagetalia.

A. L. KANSOH, Y. M. H. ABU-AYANA, F. F. ABDEL-MOHSEN

Evaluation of encapsulated copper salts or copper complexes by various types of vinyl polymers as fungicides

Microbial Chemistry Department, National Research Centre, Dokki, Cairo, Egypt

The biological activity of Cu-salts or Cu-complexes encapsulated by different kinds of polymers were studied against various types of fungal strains. The dose and rate of leaching of Cu-ions were significantly controlled by the type of polymer film used and their solubility in the medium. The different kinds of used polymers improved the tenacity of the fungicides on the leaf surfaces and also improved the dispersion of Cu-salts suspension. The results provided laboratory support for the concept that the polymers containing chemically bound biocides were useful for controlling microorganisms' growth. The effective concentrations of the biocides were 0.1–0.2 mg/ml. In field application, encapsulation appears to be a feasible route to obtain both economic and environmental advantages that can be used in rainy and windy places. The kinetics of Cu-uptake by fungal strains were studied to determine the difference in their behaviour. The uptake strategy was examined by TEM. In addition, the histological studies on in cucumber leaves showed a good entrance with high efficiency. The acute and subacute toxicity of these compounds were also studied.

L. DURAKOVIĆ, Z. PETROVIĆ, F. DELAŠ, M. GLANCER, S. DURAKOVIĆ

Dehydroacetic acid and the newly synthesised Schiff base to control ochratoxin A accumulation

Faculty of Food Technology and Biotechnology, Zagreb, Croatia

The potential for inhibition of ochratoxin A accumulation by ochratoxigenic fungus *Aspergillus ochraceus* NRRL 3174 was investigated using dehydroacetic acid (DHA) and the newly synthesized Schiff base 3-/2-Aminophenylimino(p-toluoyl)-4-

hydroxy-6-(p-tolyl)-2H-pyran-2-one in yeast extract-sucrose (YES) medium at pH 5.5. YES medium was treated with various amounts of DHA and Schiff base after inoculation with *A. ochraceus*. Experiments were carried out in a stationary culture at temperatures of 20 °C and 28 °C during 28 days. Mycelial dry weights were determined gravimetrically, and concentration of ochratoxin A was measured fluorodensitometrically using a Camag TLC Scanner. DHA concentrations of 1.0 μmol L^{-1} and 10.0 μmol L^{-1}, respectively, stimulated mould growth and ochratoxin A accumulation, but concentrations higher than 50.0 μmol L^{-1} produced an inhibitory effect. In the presence of low Schiff base concentration, mould growth was decreased by 80% and toxin concentrations by 70% or completely.

CS. HAJDÚ

Involvement of mono- and polysporic cultures of wild *Pleurotus* spp. in the breading work

Korona Spawn Plant and Research Laboratory, Demjén, Department of Plant Physiology, University of Horticulture and Food Industry, Budapest, Hungary

The oyster mushrooms (*Pleurotus* sp.) are cultivated in the largest quantity in the world after *Agaricus bisporus*. The cultivation of these mushrooms has been increased in some areas of the world, so their importance is undoubted.

In the breeding work in the Korona Spawn Plant and Research Laboratory, wild *P. ostreatus* and *P. pulmonarius* tribes with wide geographic distribution and significant genetic variability are used in breeding programs. The aim is to create hybrids, which can fruit at higher temperature, thus suitable for solving the problem of summer cultivation.

This paper details the process of making tissue, mono- and multispore cultures from wild specimens, the observation of these cultures' development and morphological features in laboratory conditions under optimal and extreme circumstances.

J. GEML

Utilisation of wild isolates of *Agaricus* spp. in mushroom breeding programs

Korona Spawn Plant and Research Laboratory, Demjén, Hungary

The mushroom produced in the greatest amount today is *Agaricus bisporus*. The importance of using wild varieties of this species in breeding new commercial strains has been realized by several researchers in the last decade. These wild types can be used to improve the commercial strains' growing and marketing characteristics, including better consistency, longer shelf life, flavour, resistance to pests and diseases. The objective of commercial mushroom breeding is to bring together these desired characteristics from two or more different individuals or strains. In addition to this, breeding programs include the creation and selection of desired traits.

This paper introduces the most important breeding methods used for *Agaricus* in the Korona Spawn Plant and Research Laboratory, including the preparation of tissue, mono- and multispore cultures from wild isolates, the method and criteria of selection in the laboratory and during grow-out trials.

V. GRISHKO

Changes in bacterial numbers of microbial cenosis in soils contaminated with fluorides

Krivorozhsky Botanical Garden NAS of Ukraine, Ukraine

Qualitative and quantitative characterizations of soil microbocenose are often used for diagnostics of biological processes taking place in soils contaminated with heavy meatals, nitrogen compounds, organics. But, effects of fluorine containing effluents of industrial factories upon bacteria of microbial cenosis in soils are poorly investigated. The study of bacteria numbers grown on meat-peptone agar (MPA) and sporulating bacteria showed considerable changes in their amounts at soils with various levels of fluorine contamination. Thus, when fluorine contents were increased from 1.1 to 5.2; 86.2, 120.2 and 169.4 mg/kg in top soil layer (0–10 cm), numbers of MPA-drown bacteria were decreased accordingly by 6.1, 2.5, 64.5 and 86.2%. Examination of samples from deeper soil layers (15–30 cm) resulted in fluorine content exceeding that in the control soil samples. Increased fluorine content in soil layer of 15–30 cm

reduced numbers of microorganisms grown on MPA by 3.2 times. Increased amounts of sporulating bacteria indicated, in our opinion, forming of less favourable conditions in soil contaminated with fluorides. In control soils it had been found a tendency to increasing of sporulating bacteria in lower soil horizonts. Investigations showed that in soils contaminated with fluorine significant changes in bacterial cenosis took place.

O. SYSHCHIKOVA, V. GRISHKO

The study of dominant bacterial species in soils polluted with coke-chemical industry effluents

Krivorozhsky Botanical Garden, NAS of Ukraine, Ukraine

Coal processing at the coke-chemical factories leads to soil accumulation of large toxicant amounts (phenoles, sulphocyanates, pyridines, cyanides, sulphur, etc.). Thus, 1m2 soil at coke-chemical factory area can accumulate per day: phenoles, 1.1 g; cyanides, 0.02 g; pyridine, 0.2 g and sulphur, 25 g; also, it can contain of sulphocyanates, 240–4200 mg/kg. That is why finding of microorganism strains able to destruct and utilize of these toxicants has an important significance for soil improvement. The study of changes in bacterial cenosis in contaminated soil was a first stage of our work. With that aim we identified any ammonificating microorganism strains, which often appeared in control and strong-contaminated soils. In the control soil dominant species were from families of *Bacillaceae*, *Bacteroidaceae* and *Micrococcaceae*. *Bacillaceae* family was represented with species of *Bacillus* genus; *Micrococcaceae* and *Bacteroidaceae* – with *Bacteroides*, *Butyrivibrio* and *Micrococcus* genera. Analysis of obtained data showed that 50% of species were from *Bacillus* genus, 25% of them *Bacteroides* genus. Essential changes were observed in taxonomical structure of bacterial cenosis in polluted soils. In strong-contaminated soils under 70% of species were not found in control soil. From nine identified species *B. megaterium*, *B. subtilis*, *B. pumilus*, *B. alvei*, *B. pulvifaciens*, *B. lentus*, *B. circulans* and *M. varians* was dominant.

PATHOGENESIS, TAXONOMY, MOLECULAR BIOLOGY AND PHYSIOLOGY OF YEASTS AND FUNGI

H. PRILLINGER, W. SCHWEIGKOFLER, K. LOPANDIC

Systematics of Asco- and Basidiomycota based on cell wall sugars, 18S rDNA sequences and urease activity

Institut für Angewandte Mikrobiologie, Universität für Bodenkultur, Wien, Austria

Differing from the morphological systematics, where commonly bipartite systems (e.g. Basidiomycota: Hetero- and Homobasidiomycetes; Ascomycota: Hemi- and Euascomycetes) prevail, tripartite systems dominate, when molecular characteristics like the qualitative and quantitative monosaccharide pattern of purified cell walls or partial or complete sequences of ribosomal DNAs are used. Based on cell wall sugars and complete 18S ribosomal DNA sequences the Basidiomycota are divided into three classes: the Urediniomycetes, the Ustilaginomycetes and the Hymenomycetes. Similarly three classes are found in the Ascomycota: the Hemiascomycetes, the Protomycetes and the Euascomycetes. Within the Ascomycota cell wall sugars can only be used to show that the Protomycetes are a sister group of the Euascomycetes. The presence of urease activity and the ultrastructure of septal pores are additional characters, which suggest a sister group relationship between the Protomycetes and the Euascomycetes. The Hemiascomycetes occupy a basal position to this sister group. Morphological and ultrastructural data of *Mixia osmundae* (Nishida et al:, Can J Bot 73 (suppl. 1): S660–S666), cell wall sugars of *Taphrina vestergrenii* (Prillinger et al. Z Mykol 56: 219–250, 1990) and 5S ribosomal DNA data from Gottschalk & Blanz (Z Mykol 51: 205–243, 1985) and Walker (System Appl Microbiol 6: 48–53, 1985) suggest the Protomycetes to be ancestral to the Euascomycetes and the Urediniomycetes of the Basidiomycota. Although yeasts predominate within the Hemiascomycetes, yeasts or yeast stages occur within all classes of the Ascomycota and the Basidiomycota. Within the Basidiomycota a polyphyletic origin of the smut fungi, the non-gilled Hymenomycetes, the gilled mushrooms and the "Gasteromycetes" become obvious. Within the Ascomycota the "Plectomycetes" as indicated by the Erysiphales, the bitunicate Ascomycota ("Loculoascomycetes", black yeasts) as indicated by the Chaetothyriales, and the Ophiostomatales appear to be heterogenous. Based on sequence information of the ribosomal DNA there is no need for the artificial group of the Deuteromycetes.

I. MIKLÓS[1], M. SIPICZKY[1,2]

Characterisation of a *Schizosaccharomyces pombe* mutant strain, defective in cytokinesis

[1]Department of Genetics, Lajos Kossuth University, [2]Institute of Human Genetics, Medical University, Debrecen, Hungary

Cytokinesis is the last landmark event of the cell cycle. Our understanding of the control of cytokinesis is limited. Study of genetically tractable systems such as the fission yeast *S. pombe* are useful to address this problem. So here we report on the characterization of a temperature sensitive *S. pombe* mutant strain, defective in cell separation.

Mutation in the gene *spl1*$^+$ confers a pleiotropic phenotype to the cells. For example they have a changed morphology with bent cell shape, failure in cytokinesis, sensitivity to caffeine and $CaCl_2$. The gene product might interact with other cytokinesis proteins, as the morphology of the double mutants spl1-1 cdc (cell division cycle) 8–27, spl1-1 cdc 3–6 suggest. Upon transforming the spll-1 mutant with a wild-type genomic library, a new pro-t RNA gene was found as a suppressor. DNA cloning and further molecular investigation of the gene *spl1-1* are under way.

E. ZILAHI[1], M. SIPICZKI[1.2]

Investigation of the *sep*15 gene in *Schizosaccharomyces pombe*

[1]Institute of Human Genetics, Medical University, [2]Department of Genetics, Lajos Kossuth University, Debrecen, Hungary

S-phase, mitosis and cytokinesis are the landmarks of the eukaryotic cell cycle. *Schizosaccharomyces pombe* is an eukaryotic haploid organism. Fission yeast cells grow by elongation at their ends and divide by binary fission after forming a centrally placed septum.

We have isolated and identified a number of cytokinesis mutants called sep mutants. Sep mutants are able to form septa, yet they are unable to complete cytokinesis. Since the daughter cells do not separate, the mutants form mycelia. We are particularly interested in *sep*15 mutant, which is sterile in addition to displaying sep phenotype. Using classical genetic methods it was established that the inheritance of sep phenotype and sterility are recessive features encoded by different genes.

We have tried to find out functional interactions creating double mutants between *sep*15 and other cdc (cell division cycle) mutants.

In addition to classical genetic methods we have cloned the *sep*15 gene, which is essential. Determining of the DNA sequence of *sep*15 gene, additional molecular investigations are required to reveal what process the gene is involved in. The complete characterization of *sep*15 gene may give further understandings of the regulation of cytokinesis.

A. BOZSIK [1,2], Á. GRALLERT[1], M. SIPICZKI[1,2]

The cytological and genetical study of the dimorphic yeast *Schizosaccharomyces japonicus*

[1]Department of Genetics, Lajos Kossuth University, [2]Institute of Human Genetics, Medical University, Debrecen, Hungary

The majority of microscopic fungi including several pathogenic species is able to proliferate either in unicellular or filamentous form. This dimorphism promotes their adaptation to the changes of environmental conditions. The unicellular yeast morphology is more advantageous in fluid media while the filamentous mycelial form benefits solid substrates. In case of pathogenic dimorphic species it is the filamentous form that is generally more destroying, as it is easier for it to invade into the host tissues. The species *Schizosaccharomyces japonicus* possesses both morphology and can convert these shapes according to the changes of environmental conditions serves as a good model organism in studying metamorphosis. We thus revealed that starving provokes the transformation of unicellular yeast cells into filaments in solid media; this metamorphosis is maintained by the food gradient and can take place only in specific temperature interval. This event involves several drastic changes. The polarity of growth alters: yeast cells grow bipolarly, filamentous hyphae grow unipolarly. Yeast cells divide exactly in the middle while mycelial cells form a smaller apical and a larger distal daughter cell. Hyphae grow much faster than yeast cells due to the large vacuoles at the non-growth pole of the cell. The situation of interphase actin is bipolarly in yeast cells and unipolarly in hyphae. Even the organization of microtubules is different in mycelial hyphae. Yeast cells grow only on the surface of the solid substrate, hyphae, however, invade into the medium. The two morphologies can convert into each other reversibly. The changes of shapes depend on the level of cAMP: metamorphosis can be prevented by raising cAMP level. We isolated several

morphological and auxotrophic mutants from the wild type of the strain. With the help of these mutants we started genetic analysis. We intend to generate a cloning shuttle-vector.

T. KRAMARENKO, H. KARP, A. IVASK, T. ALAMÄE

Glucose phosphorylating enzymes and sugar repression in methylotrophic yeast *Hansenula polymorpha*

Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia

Glucose phosphorylating enzymes are suggested to be implicated in glucose repression and glucose signalling in a number of organisms: yeasts, plants and humans. In *Saccharomyces cerevisiae* only one (hexokinase PII) of the three glucose phosphorylating enzymes is most probably involved in glucose repression. Hexokinase might generate a repressing regulatory metabolite from glucose, but other possibilities such as protein-protein interactions between hexokinase and other regulatory proteins are also possible.

Methylotrophic yeast *Hansenula polymorpha* is suggested to be a reliable alternative model for the study of glucose repression mechanisms in yeasts since it has a perfect glucose-repressed metabolic system (enzymes and organelles implicated in methanol utilization) that can be used as a model. In addition, we have characterized another well-defined glucose-repressed system in *H. polymorpha*-maltase that is implicated in utilization of disaccharides. Regulation of synthesis of maltase has widely been used for the study of glucose repression mechanisms in different yeasts. Therefore, it will be possible to compare data obtained by using *H. polymorpha* model with data concerning other yeasts.

We have described the spectrum and kinetic properties of glucose phosphorylating enzymes in a methylotrophic yeast *Hansenula polymorpha*. By using mutagenesis, selection and genetic crosses we have isolated strains of *H. polymorpha* that carry different combinations of glucose phosphorylating enzymes. These isolates were used to study glucose and fructose repression of maltase and methanol-oxidizing enzymes.

R. COSTENOBLE[1], T. BRANDBERG[1], L. ADLER[2], C. NIKLASSON[1], G. LIDÉN[1]

Expression of the bacterial *mtlD* gene in a glycerol-3-phosphate dehydrogenase-deficient mutant of *Saccharomyces cerevisiae*

[1]Department of Chemical Reaction Engineering, Chalmers University of Technology, [2]Department of Cell and Molecular Biology, Microbiology Group, Göteborg University, Göteborg, Sweden

Baker's yeast (*Saccharomyces cerevisiae*) can be used for the production of other chemicals than ethanol, its primary industrial product. These products can be substances, which the yeast produces naturally, or substances, which it can produce only after genetic engineering.

Yeast cells frequently use biochemical reduction reactions in their metabolism. The various enzymes performing this type of reactions often need a specific substance, the NAD^+/NADH co-factor, to perform properly. One of these enzymes is glycerol-3-phosphate dehydrogenase (GPD), which is involved in the synthesis of glycerol. In earlier research, it was shown that a mutant lacking this enzyme stops to grow under anaerobic conditions. Since these cells could not produce glycerol anymore, they could not regenerate NADH, which builds up under anaerobic conditions. This led to growth cessation because the NADH-excess strongly inhibits other enzymes.

The objective of the presented project is to use this anaerobically induced NADH-excess to drive bioreductions yielding other products than glycerol. If this were possible, NAD^+ would be regenerated and anaerobic growth could (re-)occur. This would make the GPD-deficient mutant a basis for a powerful, anaerobic bioreduction system. The first bioreduction investigated, is the production of mannitol, an artificial sweetener.

A GPD-deficient mutant of *S. cerevisiae* was transformed with the *mtlD* gene, which encodes bacterial mannitol-1-phosphate dehydrogenase. This enzyme catalyzes an NADH-dependent reduction of fructose-6-phosphate to mannitol-1-phosphate, which after dephosphorylation forms mannitol. The transformed GPD-deficient mutant produced mannitol under anaerobic conditions and only small amounts under aerobic conditions. Extracellular mannitol concentrations of 0.5 g/l were reached.

A. J. CLUTTERBUCK

Aspergillus chromosomes, maps and sex

Division of Molecular Genetics, Institute of Biomedical and Life Sciences,
University of Glasgow, Glasgow, Scotland

In the early 1950s G. Pontecorvo chose the homothallic ascomycete fungus *Aspergillus nidulans* as a model genetic organism for investigating the relationship between recombination and gene function. Homothallicity means that any strain can be crossed with any other, and has resulted in a large collection of mutant strains in Glasgow and elsewhere, all derived from a single wild type. Another consequence of homothallism is the possibility of building a self-consistent genetic map. There is now also a cosmid contig map, but unfortunately the two do not always agree. Almost certainly there are mistakes in both, but the genetic map has the advantage that each new linkage reinforces the pre-existing map; moreover, a framework for each chromosome is provided by mitotic recombination. While molecular genetics, based on cloning and gene replacement does not need to know the location of any gene, telomere-associated variegation and gene clusters pose two position-related puzzles, well exemplified in this fungus. Homothallicity in *A. nidulans* is itself something of a puzzle. Firstly, it is evident that two strains participating in sexual reproduction take up distinct male and female roles, e.g. in the donation of mitochondria. Secondly, the complex dance of nuclei in the formation of thousands of asci in one fruiting body must surely require mutual recognition by the partners involved. We now have circumstantial evidence that there may be a mating type switch mechanism, reminiscent of that in yeast, which occurs only in the sexual phase of this fungus.

J. ŠUBÍK

Mutational analysis of the regulatory protein Pdr3p involved in multiple drug resistance in fungi

Department of Microbiology and Virology, Faculty of Natural Sciences, Comenius University,
Bratislava, Slovak Republic

Multiple or pleiotropic drug resistance refers to genetic alterations giving to a complex spectrum of tolerance to cytotoxic compounds having different structure and intracellular targets. It can arise due to the overexpression of energy-dependent efflux pumps belonging to either ABC (ATP-Binding Cassette) or MFS (Major Facilitator

Superfamily) transport families. In the yeast *Saccharomyces cerevisiae*, the expression of transporter coding genes involved in multiple drug resistance is under the control of two networks of regulators specified by the *YAP* and *PDR* genes. The *PDR3* gene encoding transcriptional activator of the Zn_2Cys_6 family has been subjected to mutational analysis. *In vitro* mutagenesis of this gene was used to induce multidrug resistance phenotype and the resulting gain-of-function mutants were isolated and characterized. Thirteen amino acid substitutions in Pdr3p were identified that resulted in overexpression of two ABC drug-efflux pumps, Pdr5p and Snq2p, and rendered yeast cells simultaneously resistant to different drugs. These substitutions were clustered in two protein segments corresponding to the new central regulatory domain and the carboxy-terminal activation domain which may play an important role in protein-protein or protein-DNA interactions during transcription activation by Pdr3p. The results may contribute to the better understanding of the multidrug resistance phenomenon in general, but more particularly in the closely related pathogenic species *Candida albicans*.

J. ZALA

Antifungal drug susceptibility/resistance in the medical mycology

Mycological Department, "B. Johan" National Center for Epidemiology, Budapest, Hungary

In the 1990s antifungal drug resistance became a very important problem in the treatment of systemic mycotic infections. As, because of the increasing number of immunocompromised patients, the number and the severity of these infections show a rising trend, a successful therapy should be essential.

Earlier the Amphotericin B was the unique tool against the life threatening systemic mycoses, but due to its strong undesirable side-effects the research turned for finding new antifungal agents. The introduction of the new generation of azoles (triazoles) led to better results, but a quite new problem – the resistance – developed. Triazoles (fluconazole, itraconazole) inhibiting the biosynthesis of ergosterol the major and essential membrane compound of the fungal cells influence the function of many enzymes. Because of the direct membrane damaging effect of amphotericin B the decreased susceptibility is very rare. The fungal cell could protect itself against the indirect enzyme based effect of azoles by a lot of ways, resulting to many type of azole resistant strains. Several factors can lead to the presence of a resistant strain in a patient: Intrinsic resistance of the colonising strain, replacement of the sensitive endogenous strain with a more resistant *Candida albicans* or other *Candida* species,

genetic alteration and transient gene expression, changes in the structure and composition of the cell membrane, alteration in the cell type and in the enzyme production, etc. Since these processes are very complicated and the data between the *in vitro* and clinical findings are controversial, a very effective co-operation among laboratories and hospital wards is necessary.

L. MAJOROS, C. MISZTI, B. SZABÓ

Isolation and identification of *Candida albicans* and non-albicans *Candida* species from humans by traditional and new methods

Department of Microbiology, University Medical School of Debrecen, Debrecen, Hungary

Our laboratory isolated 553 *Candida* species from 28419 specimens in 1998. After evaluation of germ tube tests we tried to identify *Candida* species using CHROM agar culture method and by 32 biochemical reactions (ID 32 panel). The most frequent isolate was *C. albicans* (62.2%), whereas the non-albicans *Candida* species were found less frequently (*C. glabrata* 12.8%, *C. tropicalis* 4.5%, *C. krusei* 3.6%). The frequency of other non-albicans *Candida* species was below 1%.

The antimycotic sensitivity of our isolates was tested by determination of MIC values and by E-test. Results of MIC test showed that Amphotericin B and 5-flucytosine were the most effective drugs.

In order to analyse the karyotype of recurrent *Candida* infections we used pulse-field electrophoresis. The identical chromosome pattern served as a proof in identification the same *Candida* strain in a recurrent infection. The pulse-field method was also suitable for exact determination of *Candida* species in cases when traditional methods gave questionable results.

R. DEÁK[1], A. MARÁZ[1], H. AARTS[2]

Discrimination of *Candida glabrata* and *C. guilliermondii* clinical isolates by molecular typing

[1]Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary, [2]State University for Quality Control of Agricultural Products (RIKILT-DLO), Wageningen, The Netherlands

Among the species belonging to the *Candida* genus several are capable of causing mycosis, named candidiasis. They are present on the skin and mucous membranes as the members of the normal flora and are also frequently isolated from natural and man-made habitats as saprophytic species. Last year we reported results of our molecular genotyping study on *Candida glabrata* clinical isolates, which showed two distinct clusters of strains by RFLP analysis of rDNA gene sequences and RAPD-PCR fingerprinting methods. These results suggested the presence of a distinct species inside *C. glabrata* or consequent misidentification of strains belonging to other closely related species. Physiological and biochemical typing of the strains, which separated those from the other group including the type strain, made it probable that they belong to *C. guilliermondii*. Further molecular analysis by ribotyping and AFLP fingerprinting analysis confirmed this suggestion. These results indicate that simplified short tests, used for routine identification of clinical yeast isolates, can cause consequent misidentification, which can be avoided by using molecular typing methods.

M. S. YOUSSEF

Mycopathological studies of *Tinea* diseases in Sohag Governorate, Egypt

Botany Department, Faculty of Science, South Valley University, Sohag, Egypt

Eighty cases of dermatomycoses were recorded in patients from Sohag Dermatology hospitals during November 1997 – April 1998. Cases of tinea versicolor, tinea capitis, tinea corporis, tinea cruris, tinea pedis and tinea manuum were diagnosed. Tinea versicolor, tinea capitis and tinea corporis were the most dominant dermatophytic diseases in Sohag Governorate. Generally, males were more susceptible to dermatomycoses than females (77.5% versus 22.5% of examined cases). All dermatophytic specimens were examined directly by microscope and the results were positive in 66 cases out of 80 (82.5%). Nine fungal species belonging to 5 genera were identified and collected from 80 cases of ringworm examined. Dermatophytes

identified from examined cases of human tinea diseases were *Microsporum canis, M. gypseum, Trichophyton mentagrophytes, T. rubrum* and *T. violaceum*. The isolated closely related fungi to dermatophytes were *Chrysosporium keratinophilum, C. tropicum, Malassezia furfur* and *Candida albicans. Malassezia furfur* and *Trichophyton mentagrophytes* were the main causative fungi of tinea versicolor. However, only the first fungus could be detected by direct microscopic examination of the specimens. *Microsporum canis* and *Trichophyton violaceum* were the most prevalent dermatophytes causing tinea capitis and tinea corporis, while *T. mentagrophytes* and *T. rubrum* were the most common dermatophytes causing tinea cruris, tinea pedis and tinea manuum. *Candida albicans* a closely related fungus to dermatophytes was considered to be the main causative pathogen of tinea cruris.

P. ROMANO

Selection of starter cultures for winemaking

Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali, Universitá degli Studi della Basilicata, Potenza, Italy

Collections of *Saccharomyces* wine strains from natural fermentations have demonstrated a strong strain diversity and the different strain specific patterns can be presumed as typical for each fermentation. In recent years data have been accumulated which indicate that the characteristics of the yeast strains used in wine-making play an important part in wine quality. Therefore, the use of the same strain for fermenting different types may be nonappropriate, due to a potential uniformity of aromatic characteristics in the final products. In order to typify each product for the varietal and geographic characteristics, it becomes useful to isolate natural autochthonous strains, which, in addition to the desirable technological characteristics, can exhibit a metabolic profile corresponding to each wine.

Bearing this in mind, we have developed a methodological approach for selecting strains, which are more appropriate to the standard individual characteristics of each wine. This approach is based on the characterisation of indigenous wine *S. cerevisiae* strains for technological traits and the results for each strain are correlated to quality and individual determinants of the wine. Successively, the selected cultures should be tested for the genetic segregation of technological traits in order to identify strains completely homozygous for the characteristics considered.

In this report thirty strains of *S. cerevisiae*, isolated from different Aglianico grape musts, were tested for the production of secondary compounds. A considerable

phenotypic variation was found, confirming the environmental role of natural yeasts and the importance of the main and desirable traits, that the selected culture must possess to preserve the individual characteristics of that specific product.

Only 4 strains, exhibiting a metabolic profile corresponding to the individual Aglianico wine characteristics, were selected and underwent to genetic analysis. All the strains were homozygous for the homothallism gene, whereas most single spore cultures, tested for the production of secondary compounds, were heterozygous from one to more metabolic characteristics.

The application of this strain selection approach allows obtaining a final product characterized by the desired aromatic profile, consistent with flavour-determinants, which are typical of each wine.

A. MARÁZ, A. POMÁZI

Molecular genotyping as a tool in the study of population dynamics of yeasts during wine fermentation

Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary

Contribution and role of different species of yeasts to the quality and character of wine have not yet been elucidated. Selective pressure of the environmental factors, which affect growth, fermentation and metabolite production of yeasts, are under a continuous change during wine fermentation. Successive changes of different species and of the strains belonging to a given species can be monitored and studied effectively by the application of molecular genotyping methods as plasmid profile analysis, electrophoretic karyotyping, RFLP analysis of rDNA or mitochondrial DNA and PCR amplification of random DNA sequences.

Successive changes of the wine fermenting *Saccharomyces cerevisiae* killer populations were monitored by electrophoretic profile analysis of viral RNAs and also by the molecular fingerprinting of their nuclear genotypes. Genetic determinants for the toxin production were found to be dsRNA plasmids in every killer strain, differing in the size of M dsRNA molecules. Killer toxins of activity at pH 4 were associated with different M dsRNA molecules, but killer strains from the same fermentation always harboured M dsRNA of the same size.

Electrophoretic karyotyping showed high degree of heterogeneity when killer yeast strains from different fermentations were compared, while more similarity was found when strains from the same fermentation were analysed. Both similarity and

dissimilarity in the PFGE karyograms of the isolates were reflected in the cluster analysis of the RAPD-PCR profiles but more detailed distinction of strains, which belonged to the same PFGE group, was achieved by the latter method.

We found less diverse *Saccharomyces cerevisiae* yeast populations when non-killer yeast isolates were subjected to molecular genotyping. Selection and dominance of unique strains were observed during increase of the ethanol content in wine.

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I. SÁRVÁRI HORVÁTH, M. J. TAHERZADEH, C. NIKLASSON, G. LIDÉN

Physiological effects of furfural on anaerobic continuous cultivation of *Saccharomyces cerevisiae*

Department of Chemical Reaction Engineering, Chalmers University of Technology, Göteborg, Sweden

A large source of potentially cheap substrates to be used for fermentative production of ethanol by yeast are residues from agriculture and forestry. A common problem in the utilization of these substrates is the presence of inhibitory substances, particularly if acid hydrolysis is used for obtaining the monosaccharides. Major inhibitors in dilute-acid hydrolyzates from different kinds of wood have been identified to be furfural and hydroxymethyl-furfural (HMF).

Earlier work has shown that furfural and HMF can be converted by yeast to less inhibitory compounds, primarily their corresponding alcohols (furfuryl alcohol and hydroxymethyl-furfuryl alcohol). In addition to furfuryl alcohol, another metabolite was detected. The structure of this compound has been shown to be an addition product of furfural and pyruvate.

In the present work the physiological response of *Saccharomyces cerevisiae* CBS 8066 to different concentrations of furfural in anaerobic, continuous cultivations was examined. The growth and product formation of *S. cerevisiae* was investigated at three different dilution rates in the presence of various furfural concentrations in the feed. In addition the transient metabolic response was followed by pulse experiments in which furfural was injected into steady-state continuous cultures.

The specific uptake rate of furfural (q_f) determined in the pulse addition experiments, depended on the dilution rate. The maximum values of q_f, obtained were in agreement with previously determined values in batch culture experiments. Furthermore, the maximum specific uptake rate of furfural was clearly higher than the highest obtainable steady-state q_f value in chemostat before wash out occurred.

Evidently, cell growth stops at lower q_f, than the maximum q_f possible, which results in wash-out of the culture. The exact mechanism of the growth inhibition still needs further studies.

M. S. YOUSSEF

Antidermatophytic activity of some medicinal plant essential oils and aqueous extracts against isolated human skin pathogenic fungi

Botany Department, Faculty of Science, South Valley University, Sohag, Egypt

Antidermatophytic activity of both essential oils and aqueous extracts of 16 different medicinal plants was estimated against seven species of dermatophytes isolated from human skin mycotic diseases in Sohag Governorate, Egypt. These pathogenic fungi were *Microsporum canis, M. gypseum, Trichophyton mentagrophytes, T. rubrum, T. violaceum, Chrysosporium keratinophilum* and *C. tropicum*. The data clearly elucidated that both of essential oils and aqueous extracts of Ceylon cinnamon and greater galangal had a wide-spectrum antidermatophytic activity, whereas the two tested extracts of sweet flag had wide- and mediate-spectrum antidermatophytic activity, respectively. The essential oils of wild tea, garlic and thyme had mediate-spectrum activity, whereas their aqueous extracts possessed wide-spectrum moderate antidermatophytic activity. On the other hand, only the aqueous extract of cabbage seeds proved to have a mediate-spectrum inhibitory effect, whilst the two tested extracts of cardmom and the essential oils of eucalyptus and glinus in addition to the aqueous extract of radish were of limited-spectrum antidermatophytic activity.

T. PAPP, Á. NAGY, ZS. PALÁGYI, M. VASTAG, CS. VÁGVÖLGYI

Genetic studies on sexual processes of *Gilbertella persicaria*

Department of Microbiology, Attila József University, Szeged, Hungary

Gilbertella persicaria (Eddy) Hesseltine is an agriculturally important postharvest pathogen. Similarly to the majority of the organisms belonging in the Zygomycetes, this species is heterothallic. The sexual cycle of most of these fungi is rather long, which makes genetic studies difficult. In contrast, after the mating of the partner strains and zygospore formation, *G. persicaria* requires a short period of

dormancy (5–6 days) for germination of the zygospore, and it might therefore be used as an excellent experimental object in genetic studies.

Little is known about the genetic background of the inheritance of the Zygomycetes. Previous studies with another species (*Mucor hiemalis*) suggested non-Mendelian distribution of the alleles among the offsprings. The present work involved a study of the presence of the two mating types (+/–) among the descendants and also the distribution of RAPD (random amplified polymorphic DNA) markers in the meiotic products derived from isolated zygospores. The results suggested that the inheritance of both the mating types and the mating type-specific RAPD markers is non-Mendelian; more than 90% of the progenies were found to be of (–) mating type. The distribution of other RAPD markers and the appearance of new (recombinant) RAPD patterns were also analysed in detail.

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Á. JUHÁSZ[1], ZS. HAMARI[2], B. TÓTH[1], L. FERENCZY[1,2], F. KEVEI[1]

Interpretation of mtDNA recombination events among vegetative incompatible *Aspergillus japonicus* strains

[1]Department of Microbiology, Attila József University, [2]Microbiological Research Group, Hungarian Academy of Sciences, Szeged, Hungary

The mitochondrial genomes of strains of *A. japonicus* belonging to the imperfect black *Aspergilli* display highly variable RFLP patterns. Transmissions of mitochondria were carried out between oligomycin resistant mutant (oliR) strain as standard donor and sensitive recipient ones with different mtDNA RFLP patterns by using protoplast fusion technique. All intraspecific mitochondrial transfer experiments resulted in oliR progeny with recombinant and/or unchanged donor mtDNAs. In our previous work physical and functional maps of a recombinant' and its parent's mtDNAs were developed for interpretation of recombination processes. The results elucidated that the recombination process was based on movement of introns. The recombinant progenies inherit basically the resistant donor mtDNAs, which are modified by introns of recipient mtDNA. Four introns were determined, which are involved in formation of recombinant characters. These introns were used as DNA probes in hybridisation experiments to determine intronic relations among different recombinant progenies and their parents. In our recent work another recombinant and its parents were also investigated to find novel mobile introns, which may play a role in

recombination events. Physical and functional maps of parental and recombinant mtDNAs were constructed and compared. Regions involved in recombination processes were studied at sequence level.

ZS. ANTAL[1], L. MANCZINGER[2], L. KREDICS[2], L. FERENCZY[1,2]

Investigation of the mitochondrial DNA organisation of *Trichoderma harzianum* strain

[1]Microbiological Research Group, Hungarian Academy of Sciences, Szeged,
[2]Department of Microbiology, Attila József University, Szeged, Hungary

Trichoderma isolates are known as potent biocontrol agents. Some isolates were successfully used against phytopathogenic fungi such as *Pythium debaryanum*, *Rhizoctonia solani* and *Sclerotium rolfsii*. Mitochondrial DNA organisation is less studied in *Trichoderma* species. For this reason, mitochondria were isolated from a mycoparasitic *Trichoderma harzianum* strain and nucleic acids were purified by lysing the mitochondria with the aim of constructing a detailed physical and functional map of the mtDNA in this filamentous fungus. The mtDNAs were found to be circular, 32 kb in size. A physical map was constructed through the analysis of double-digestion patterns obtained by using three restriction enzymes, *Eco*RI, *Eco*RV and *Cfo*I. Heterologous gene probes were used for the construction of a functional map, which derived from *Aspergillus nidulans* (L-rRNS, S-rRNS, cobA, ATP-ase subunit 9), or *A. carbonarius* mitochondrial DNA (NADH dehydrogenase subunit 5). All gene probes hybridized to the mtDNA of *T. harzianum*. The gene order was similar to that observed in *A. nidulans*.

R. TÓTH, I. PFEIFFER, J. KUCSERA

A comparative analysis of the mitochondrial genomes of *Saccharomyces dairensis* strains CBS 421 and CBS 4309

Department of Microbiology, József Attila University, Szeged, Hungary

Saccharomyces dairensis is a member of the *sensu lato* group of the *Saccharomyces* genus, but its exact taxonomic position is debated. The type strain of the species, CBS 421, was isolated by Naganishi, in 1917. The CBS 4309 strain was first described as a separate species, *Saccharomyces castellii*, by Capriotti, in 1967.

Later taxonomic studies listed both strains as *S. dairensis*, since distinction was unreliable with conventional methods.

Electrophoretic karyotyping and nuclear DNA hybridization have also been carried out within the *Saccharomyces* genus. In cases of other species, a study of the RFLP patterns of the mitochondrial genomes proved to be a useful tool for determining taxonomic position.

In our study, we compared the RFLP patterns of the type strain of *S. dairensis* (CBS 421) and the CBS 4309 strain with the use of various restriction endonucleases. We measured the size of the mitochondrial genomes, and carried out DNA-DNA hybridization, using heterologous gene probes from *Aspergillus nidulans*, and *Candida parapsilosis*, to determine the location of individual genes.

Examination of mitochondrial mutants is another practical approach in the study of mitochondrial genome organization. Some yeasts remain viable with impaired respiratory functions, and form smaller, 'petite', colonies on solid medium, versus the normal, 'grand' phenotype. Petite phenotype may also result from nuclear mutations.

We successfully isolated petite mutants of both strains using ethidium-bromide induction. According to literature, ethidium-bromide treatment produces mutations mainly on the mtDNA. Deciding whether the isolated petite mutants are cytoplasmic or nuclear is one of our research aims.

A. GÁCSER, I. PFEIFFER, J. KUCSERA

Intraspecific polymorphisms among isolates of *Cryptococcus hungaricus*

Department of Microbiology, Attila József University, Szeged, Hungary

In our previous studies six *Cryptococcus hungaricus* strains derived from various geographic area proved to be polymorphic both in their nuclear and mitochondrial genome organisation. Thermo-sensitivity and ability of carotenoid pigment production are their common features. The morphological and physiological properties of six strains were investigated. Carbohydrate-assimilation was examined and five types of C-sources were found to be useful for differentiation. In the study of their nuclear organisation the ITS region of ribosomal DNA were amplified and double-digested. RFLP patterns of ITS sequences showed that only two strains were identical and the others showed striking differences. Physical and functional maps of the mitochondrial DNA of the six different strains were constructed. Results of the mtDNA organisation correlate with those of nuclear ITS organisation. On the basis of molecular characteristics one of the strains extremely differs from the others, not only

in its restriction map but in its native appearance on agarose gel. The native mtDNA sample is separated to eight bands with different sizes. The study of organisation of mitochondrial genome of this particular strain is in progress.

I. FAUST, B. JAKAB, J. HIDASY, M. PESTI

Parasexual recombination of *Candida albicans* morphological mutants

Department of Genetics and Microbiology, Janus Pannonius University, Pécs, Hungary

A wild-type strain of *Candida albicans* (ATCC 10261) was used to obtain double auxotrophic mutants by nitrosoguanidine (NTG) treatment. Absence of complementation in hybrids for lysine auxotrophy of different origins proved that the three double auxotrophs possessed an allelic mutation for this selective marker. Repeated NTG treatment resulted in numerous colony morphological mutants. Colonies of various mutants were multiplied with the application of a single cell descendent method. In this way four stable segregants were selected, characterized and used for complementation analysis by parasexual recombination. Examination of the hybrids of these colony morphological mutants of *C. albicans* is under way.

B. TÓTH[1], ZS. HAMARI[2], ZS. BEER[1], F. KEVEI[1]

Detailed physical and functional maps of mitochondrial DNAs of an *Aspergillus niger* and an *A. tubingensis* strain and their interspecific recombinants

[1]Department of Microbiology, Attila József University, [2]Microbiological Research Group, Hungarian Academy of Sciences, Szeged, Hungary

Mitochondrial DNA (mtDNA) polymorphisms are frequently observed among imperfect black *Aspergilli*. Strains of the *Aspergillus niger* species aggregate can be divided into three groups based on their nuclear and mitochondrial DNA RFLPs. MtDNA RFLP types 1, 2 and 3 correspond to ribosomal DNA types I, II and III. These groups equal three different species, *A. niger*, *A. tubingensis* and *A. brasiliensis*. MtDNA types 1 and 2 consist of several subgroups (1a-1e, and 2a-2f, respectively). Successful mitochondrial transfers were carried out between incompatible strains possessing different mtDNA patterns by selecting for the oligomycin resistance carried by the mtDNA of the donor parent. Intraspecific transfers resulted in a single type of

recombinant RFLP profile, while interspecific transfers resulted in recombinant progenies with highly variable mtDNA RFLP patterns. The 1a → 2b interspecific transfer gave most variable recombinant mtDNA patterns. Sizes of the observed recombinant mtDNAs also varied, some of them were smaller than the parental mtDNAs, while others harboured larger mtDNAs than the parental strains.

Physical and functional maps of the parental strains and intraspecific recombinants were constructed, and the homologous fragments, which exhibited size differences between the parental strains and the recombinants, were cloned and sequenced in order to identify the region where the recombination event took place.

ZS. NEER, I. PFEIFFER, J. KUCSERA

Examination of killer phenotype attributed to dsRNA viruses in *Cryptococcus hungaricus*

Department of Microbiology, József Attila University, Szeged, Hungary

The most researched, and thus best known, species within the *Cryptococcus* genus is *C. neoformans*, due to its pathogenicity. Our research was conducted on *Cryptococcus hungaricus,* a less known species of the genus. The species was first described by János Zsolt, under the name *Dioszegia hungarica.* Later (1970) Pfaff and Fell observed the same vegetative multiplication, that was thought to be unique to *D. hungarica*, in other *Cryptococcaceae* species, and therefore considered the *Dioszegia* genus redundant.

Double-stranded RNA viruses have been isolated from the CBS 6569 strain of *C. hungaricus*, and this strain shows killer phenotype. In our study we tried to prove that the killer attribute is attached to virus particles by deleting the virus particles from the cells, using cycloheximide and acridine-orange treatments, and then examining whether the obtained strains still exhibit the killer phenotype.

We tested the mechanism, through which the toxin protein is acting, by fluorescent staining. Using various media and incubation temperatures the activity of the toxin. Furthermore, we examined the stability of the protein by exposing it to pH ranges differing from the optimum, prior to incubation. Our aim is to establish the effective mechanism and spectrum of the toxin, as well as trying to find sequence homology with the genomes of viruses found in ascosporous and related basidiosporous fungi.

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T. PAPP[1], CS. FEKETE[2], M. VASTAG[1], Á. NAGY[1], CS. VÁGVÖLGYI[1]

Presence of double-stranded RNA molecules and virus-like particles in *Rhizopus* strains

[1]Department of Microbiology, Attila József University, Szeged, [2]Institute of Plant Sciences, Agricultural Biotechnology Center, Gödöllő, Hungary

Double-stranded ribonucleic acid (dsRNA) molecules have been found in a wide variety of phylogenetically-diverse fungi. However, the screening of fungal species belonging in the Zygomycetes from this aspect has been neglected. dsRNA molecules have so far been observed only in three genera: *Entomophaga*, *Mucor* and *Mortierella*.

In the present study, 26 strains representing 5 *Rhizopus* species were examined for the incidence of double-stranded RNA elements. These genetic elements were found to be present in 5 strains: 1 *R. oryzae*, 1 *R. microsporus* and 3 *R. stolonifer* strains. Electrophoretic separation of the nucleic acids revealed 5 different RNA patterns, with 1 to 5 discrete dsRNA bands. The molecular weights of these dsRNA bands ranged from 2.7 to 9.1 kbp. The presence of virus-like particles was also investigated by electronmicroscopy; all 5 strains were found to harbour virus-like particles. This is the first description of a mycovirus in the *Rhizopus* genus.

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T. NAGY, K. KISS, J. ZALA

Theoretical comparative study of yeast identification with some test kits

Department of Mycology, "Béla Johan" National Center for Epidemiology, Budapest, Hungary

The fungal infections are increasing and there is a demand of correct identification of the species. The selection of strains of yeast species on Chromagar (BBL) is very useful, however, identification of yeast species with it is not verified.

Funid program developed in our department for identification was used to evaluate usefulness of test kits for yeast identification.

There have been several problems both in the scientific literature and with the test kits for yeast identification (Auxacolor, Mycotube, API20CAUX, API32ID).

Our preliminary results have shown good correspondence among Auxacolor, Mycotube, API 32ID and further comparisons have been intended to be done. There are some advantages of test kits:

- Standard composition,
- Standard method.

The main problems with test kits are:

- The names of some yeast species are incorrect.
- Some yeast species no longer exist.
- The available codes are insufficient and/or unreliable for correct identification (c.f. kit documentation).
- The spectrum of clinically important yeast species seems to be wider than that, which the test kits can distinguish.
- The results between two certain test kits are almost incomparable because of the different fungal spectra and the names of species.

Proposals:

- The accepted changes in scientific literature should be involved in test kits.
- The species that are most important for clinically important yeast identification should be declared.
- The names of the yeast species should be standardized.

K. KISS, J. ZALA, T. NAGY

Comparison of *in vitro* antifungal susceptibility tests

Department of Mycology, "Béla Johan" National Center for Epidemiology, Budapest, Hungary

Fungal infections have increased significantly over the past years. Besides, the frequency of nosocomial fungaemia due to unusual yeasts has increased dramatically. Resistance of yeast human pathogens to antifungal agents used in hospitals has been rarely reported during 70s and 80s. It is only during the last decade that antifungal drug resistance has been recognised as a significant problem. Therefore the *in vitro* antifungal susceptibility tests have important role in choosing the acceptable treatment and recognizing the drug resistance.

In our study three methods for antifungal susceptibility test were studied. The E-test for Amphotericin B (on semi-synthetic agar medium), Itraconazole (on Casitone-agar medium), Fluconazole (Casitone-agar) were compared with Fungitest. The disc diffusion test for Ketoconazole and Miconazole (both semi-synthetic agar mediums) and 5-Fluoro-citozine (semi-synthetic agar) were compared with Fungitest. Sixty-one

Candida strains were examined for all three tests. The results with E-test and Fungitest with Amphotericin B were the same (93%). The E-test for Itraconazole was according to Fungitest 80%. The results were the same with Ketoconazole, Miconazole, 5-Fluorocitozine in 80%, 87% and 93%, respectively. The azoles especially Fluconazole were the least corresponding. The different mechanism of action (e.g. Fluconazole has fungistatic effect while Amphotericin B has fungicid effect) may be a reason for that.

V. G. ARZUMANIAN

New synthetic media for cultivation of lipophilic yeast *Malassezia* spp.

Mechnikov Research Institute for Vaccines and Sera, Russian Academy of Medical Sciences, Moscow, Russia

The lipophilic yeast *Malassezia* spp. (the recent name of genera – *Pityrosporum*) is the only human saprophyte yeast. Their role in some skin pathologies – pityriasis, seborrheic and atopic dermatitis, psoriasis were intensively studied. Some metabolites of *Malassezia* can be strong allergens. Cultivation of *Malassezia* on usual lipid-containing media is not difficult but some components of these media are allergens themselves. In this case synthetic media would be more useful. Since 1976 (Nazzaro-Porro) we do not know other publications concerning the growth of *Malassezia* on synthetic media but we could not use these recipes. So the aim of the present research was to construct a synthetic media for *Malassezia* spp. Growth capacity of the *Malassezia* spp. (*Pityrosporum* spp.) on the synthetic nutritional media has been studied. Isolates of *Malassezia* have been obtained from healthy human skin and identified by the method of Guillot and Gueho (1996). The modified Dixon's medium was taken as a prototype for preparing the synthetic media. New media contained asparagine, Tween 40 and emulsifier as carbon and energy sources. The isolates of *Malassezia sympodialis* have demonstrated relatively fast growth on such media, in contrast to *M. globosa*. The duration of exponential growth phase was 1–3 days depending on the isolate.

Z. GAZDAG[1], N. FARKAS[1], ZS. FEKETE[1], N. HARTVIG[1], M. NYITRAI[2], J. BELÁGYI[3], M. PESTI[1]

Chromium(III) and (VI)-induced plasma membrane processes in fission yeast

[1]Department of Genetics and Microbiology, Janus Pannonius University, [2]Department of Biophysics, [3]Central Research Laboratory, University Medical School, Pécs, Hungary

Auxotrophic and heterothallic strains of *Schizosaccharomyces pombe* were used to obtain chromium(VI)-sensitive and resistant mutants. The effects of the Cr(VI) anions on the plasma membrane were studied *in vivo* by applying electron paramagnetic resonance spectroscopy. 5-doxylstearic acid (5-SASL) and 3-doxylbutyric acid (HO-185) spin probes were used to label the membrane. The order parameter (S) was calculated at different temperatures (0–25 °C). Addition of 225 μM $K_2Cr_2O_7$ significantly decreased the phase transition temperatures of the 5-SASL-membrane of the wild-type strain CRW-6 and the sensitive mutant CRS-6.51, but slightly increased the phase transition temperature of the resistant mutant CRR-6.66, as revealed by the HO-185 label. The general cellular oxidant dihydrorhodamine 123 (DHR) was applied to characterize the Cr(VI)-induced oxidative changes. The results suggested a strong, membrane-localised oxidation of DHR induced by Cr(VI). The Cr(III)- induced membrane alteration caused the loss of OD_{260} materials from the cells.

J. VARGHA, É. KEVEI, K. RIGÓ, B. TÓTH

Phylogenetic analysis of the toxigenic *Aspergillus ochraceus* species

Department of Microbiology, Attila József University, Szeged, Hungary

The genetic variability of *Aspergillus ochraceus* was examined using genotypic methods. Based on the *Hae*III-*Bgl*II generated mitochondrial DNA restriction profiles, most isolates could be classified into two distinct groups. These two groups could also be distinguished by the random amplified polymorphic DNA technique, and by using telomeric or IGS-specific sequences as primers to amplify fungal DNA in the PCR reactions. None of the isolates exhibiting type 2 mtDNA profiles produce ochratoxins. Some strains (e.g. *A. ochraceus* ICMP 939) displayed strain-specific mitochondrial DNA patterns, and their amplified DNA profiles were also different from all other *A. ochraceus* strains examined. Phylogenetic analysis of sequences of the intergenic transcribed spacer region of some of the strains resulted in a dendrogram of the same

topology as that based on mitochondrial DNA and amplified DNA data. Based on these results, *A. ochraceus* ICMP 939 possibly represents a new ochratoxigenic species within *Aspergillus* section *Circumdati*.

D. DLAUCHY, J. TORNAI-LEHOCZKI, G. PÉTER

Identification of foodborne yeasts on the basis of 18S rDNA

National Collection of Agricultural and Industrial Microorganisms, University of Horticulture and Food Science, Budapest, Hungary

Yeast contribution to the production of alcoholic beverages and bread is widely known, however, their role as spoilage agents is often neglected and underestimated. The importance of yeast in the spoilage of foods is increasing. Process and quality control both require the reliable and rapid identification of foodborne yeasts. The yeasts are traditionally characterized and identified by 60 to 90 morphological, physiological and biochemical tests. The reliability of these criteria in yeast identification is often questioned. The determination of 60–90 characteristics is hardly feasible and time-consuming task for routine identification in the industrial laboratories and culture collections. The aim of our study was to develop a simple and rapid method to identify yeast strains on the basis of their 18S rDNA. We used the restriction patterns of 18S rDNA with the neighbouring ITS region, digested with four (*Hae*III, *Msp*I, *Alu*I, *Rsa*I) different four-base cutting enzymes for differentiation and identification. Up to date we examined the restriction patterns of 18S rDNA of 130 frequent yeast species mainly associated with food, wine, beer and soft drinks. For constructing a database of restriction fragment patterns, the gels have been scanned and analysed using the Molecular Analyst Fingerprint 2.0 software (BIORAD). The use of four different enzymes proved to be sufficient in strain identification except only the species of genus *Debaryomyces*.

L. BÁNSZKY, A. MARÁZ

Genotypic characterisation of strains belonging to the *Schizosaccharomyces* genus by RAPD fingerprinting and ribotyping

Department of Microbiology and Biotechnology, University of Horticulture and Food Science, Budapest, Hungary

In this work several strains belonging to the *Schizosaccharomyces* genus were characterized by molecular typing methods such as RAPD-PCR analysis and ribotyping (RFLP of amplified rDNA sequence). These PCR-based molecular typing technics can be useful in the identification and comparison of yeasts species and strains. By the application of the ribotyping method, a rDNA sequence was amplified flanked by the specific primers ITS4 and NS1. The amplified sequence contained the whole nuclear small rDNA, the 5.8S rDNA, two ITS regions and a small part of the nuclear large rDNA genes. The amplified rDNA sequence was digested with different restriction enzymes (*Hae* III, *Msp* I, *Scr*F I, *Sau* 3 A I) of four- and five-base recognition site. RAPD-PCR fingerprinting was carried out with four different 10-base random oligonucleotide primers of 50, 70, and 80% G+C contents. Dendograms based on the RFLP of rDNA sequences and RAPD-PCR showed three distinct clusters of strains with low degree of similarity, which corresponded to the currently accepted three species of the genus *Schizosaccharomyces* as follows: *Schizosaccharomyces pombe*, *Schizosaccharomyces octosporus* and *Schizosaccharomyces japonicus*. Any of the four used restriction enzymes was suitable for differentiation of *Schizosaccharomyces japonicus* and the other *Schizosaccharomyces* strains, while restriction enzymes *Hae* III and *Sau* 3 A I resulted also differences between *Schizosaccharomyces octosporus* and *Schizosaccharomyces pombe* strains. Within each of these species ribotyping did not show any difference, indicating that this method is excellent for identification at the species level. RAPD-PCR analysis showed also the previous three clusters of species, but showed more differences at intraspecies level. We could not find however, any clustering of the formerly delineated varieties of *Schizosaccharomyces pombe* and *Schizosaccharomyces japonicus*.

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T. KLEFFLER, T. DEÁK, A. MARÁZ

Biodiversity of yeast biota in vineyard and wine fermentation

Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary

In the oenological process from grapes to wine, yeasts are of primary importance. Numerous previous investigations show that the composition of yeasts varies in the different vineyards. The diversity of yeasts is generally high on the grapes and the beginning of the fermentation but during the middle and last phase *S. cerevisiae* strains take over the leading role.

The places of our experiments were: Etyek vineyard, preprocessing equipments and the processing factory in Budafok (Hungarovin), which are using active dry *S. cerevisiae* strains as starter cultures.

We identified the strains isolated from the samples at spices level with traditional simplified identification test (SIM) or with the commercially produced API 20C and API 30C diagnostic kits. The following molecular genotyping methods were also used: pulsed field gel electrophoresis and RAPD-PCR.

Our result showed that the number of yeast cells increased by about twenty times on grapes and leaves. We found several yeasts above the dominant *Aureobasidium pullulans*. The number of *S. cerevisiae* isolates was low. In the processing equipments *Kloeckera* and *Torulaspora* species became dominant but the *S. cerevisiae* strains also increased. The biomass of yeast population was growing by two order of magnitude. In the main fermentation of must *S. cerevisiae* took over the leading role.

There are some questions, what the current molecular genotyping experiments have to answer. For example:

– Were there other *S. cerevisiae* strains above the active dry yeasts in the last fermentation phase that came from the grapes and enriched in the equipments?

– Were such yeasts isolated that survived in the equipments and had an important role in the fermentation?

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I. GRECO, P. ROMANO

Molecular biology strategies as biotechnological tools in wine quality

Dipartimento di Biologia, Difesa e Biotechnologie Agro-Forestali, Università degli Studi della Basilicata, Italy

To study the diversity present in a biological system is essential to determine its individual genotype and geographic characteristics. In this context the biological source of the genetic information allows to individuate any genetic traits, without environmental effects, and also to determine the degree of genetic polymorphism. As we are focussing our attention on the conservation of individual characteristics of Aglianico of Vulture, the typical grape variety of Basilicata region, we have chosen to investigate molecular and genetic variability in vine clones of Aglianico of Vulture cultivar and in autochthonous strains of *Saccharomyces cerevisiae*. The development of the polymerase chain reaction (PCR) has created fascinating possibilities in biotechnology, since virtually any target DNA can be amplified from complex samples and the use of RAPDs primers allows to analyse a great part of the genome in a single population. We applied these molecular techniques to assess genetic diversity of vine clones and yeast strains. In this study, thirteen vine clones of Aglianico of Vulture, collected on the basis of morphological characteristics and tested by RAPDs analysis, exhibited certain variability. Fifty strains of *S. cerevisiae*, isolated from different spontaneous fermentation of Aglianico of Vulture, were characterized phenotypically for technological traits, such as resistance to SO_2 and copper, ethanol tolerance, production of some fermentation by-products. Of these, twenty strains, representing the different phenotypes found, were selected and characterized by molecular analysis. A strong polymorphism was found with the expression of biotypes, differing in size and/or number of bands. The preliminary results have demonstrated a high genetic variability in biological natural sources both in vine plant and in yeast strains. The goal of our study is to identify suitable associations between vine clone genotype and yeast strain genotype that, besides imparting desirable organoleptic qualities, can also preserve the individual characteristics of the wine under consideration.

K. SZOMBAT, A. POMÁZI, A. MARÁZ

Epidemiological study of entomopathogenic fungus *Ascophaera apis* by RAPD-PCR fingerprinting

Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary

In the last decade very considerable losses were experienced in colonies of honey bee (*Apis mellifera* L.) due to the disease named "chalkbrood". Little is known, however about epidemiology of the fungus *Ascosphaera apis*, which is the causing agent of chalkbrood.

We used random amplified polymorphic DNA (RAPD) analysis for answering the question: if i) one or more virulent strains are involved in the epidemics located in different apiaries of Hungary or ii) no connection could be found between the outbreaks of chalkbrood.

Fifty-one representative *A. apis* strains, which were isolated from different parts of Hungary, were investigated. Three random primers were proved to be suitable for the PCR amplification reactions. On the base of banding patterns obtained, cluster analysis was carried out with the Molecular Analyst/PC Fingerprinting software (Bio-Rad).

The constructed dendrogram reflected that high variability existed in the Hungarian populations of *A. apis*. Furthermore there was no correlation found either between the RAPD patterns or the geographic origin of the isolates.

Our results suggest that a genetically diverse species was responsible for the chalkbrood disease of honeybee in Hungary.

E. VARGA[1], A. MARÁZ[2], K. CSEDŐ[1]

Effect of microelement enrichment to the vitamin content of yeast

[1]Department of Pharmacognosy, University of Medicine and Pharmacy, Targu Mures, Romania,
[2]University of Horticulture and Food Industry, Department of Microbiology and Biotechnology, Budapest, Hungary

Yeast cells enriched in microelements are frequently used as paramedicinal drugs. Yeasts are able to take up certain microelements and to build into organic molecules, which are more favourable to human and animal organisms.

In the course of our experiments we determined the microelement (Zn, Fe, Cr, Se) content of yeasts after 24 hour fermentation.

We prepared yeasts enriched in zinc-iron-selenium and chromium microelements by fermentation in 2 L volume. From this biomass we determined concentration of microelements with ICP-AES method. The microelement content was as follows: Cr=4 μg/g d.w., Fe=104 μg/g d.w., Se=2159 μg/g d.w. and Zn=1075 μg/g d.w.

Also we determined the B_1 and B_2 vitamin content of the yeast biomass with a microbiological method in viable cells and after thermal inactivation.

We found 50–70% loss of the original vitamin content during inactivation of cells.

We prepared yeast biomass enriched with chromium and selenium microelements in 20 L fermentor. The microelement content of this biomass was as follows: Se=1841 μg/g d.w. and Cr=9 μg/g d.w. We are using this biomass to animal diet after thermal inactivation. Experiments are in progress.

Á. SVEICZER[1], B. GYŐRFFY[1], A. CSIKÁSZ-NAGY[1], J. M. MITCHISON[2], B. NOVÁK[1]

Quantised cycles in the fission yeast wee1-50 cdc15 double mutant

[1]Department of Agricultural Chemical Technology, Technical University of Budapest, Budapest, Hungary, [2]Institute of Cell, Animal and Population Biology, University of Edinburgh, Edinburgh, Great Britain

The fission yeast, *Schizosaccharomyces pombe* is a classical test organism for studying eukaryotic cell cycle, because its cylindrical cells grow exclusively at the tips and divide by binary fission producing two identical progenies. A simple method often used is taking time-lapse films of growing fission yeast cultures in a thermostated photomicroscopic field. We have recently analysed many cell cycle mutants by this method. The most interesting case was the *wee1-50 cdc25Δ* double mutant where the main regulators of mitotic onset (wee1, an inhibitory tyrosine kinase; cdc25, its tyrosine phosphatase pair) are both absent. We have found that the cycle time is quantised in this population and the cycle time of a cell is not determined by its birth length. Those cells having long cycles abort mitosis from an early stage and return back to G2 phase. We have developed a mathematical model in which mik1, the back-up enzyme of wee1 is activated during early mitosis, therefore the mitosis promoting factor (MPF) becomes inactivated, causing an improper exit from mitosis instead of separating the sister chromatids.

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BACTERIAL BIODIVERSITY, GENERAL AND MOLECULAR ECOLOGY AND SYSTEMATICS

F. A. RAINEY

Ionizing radiation: a selective enrichment tool in culturable diversity assessment

Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana, USA

Our limited knowledge of the diversity of members of the domain Bacteria has been demonstrated using the culture-independent approach. Soon more taxa will exist in the form of inserts in a plasmid and as 16S rRNA gene sequences in a database than as pure cultures in culture collections. Investigation of the characteristics and function of such environtaxa is not possible. The microbial diversity of various environments have been studied to different degrees, some extensively, others poorly. Arid and hyper-arid soils are examples of environments about which little is known of the microbial components and their function. With the aim of expanding our knowledge of the culturable diversity of arid soils, and further investigate the link between ionizing radiation and desiccation resistance, we exposed soils to various doses of gamma ionizing radiation, ranging from 0.1 and 3.0 MRad.

Plating of these soils on routine culture media allows us to determine the numbers and the phylogenetic diversity of the survivors obtained at all doses. Using this approach we have been able to add novel species to already existing genera and to discover new genera. This selective enrichment technique has added at least eight additional species to the classical ionizing radiation resistant genus *Deinococcus*, six of these coming from a single soil sample. Many of the survivors belong to the single species genus *Geodermatophilus* and their isolation has expanded this genus, at both the species and strain level. Strains representing novel genera within the cytophaga group and alpha-proteobacteria lineages have also been isolated. The fact that ionizing radiation eliminates fast-growing and potentially inhibitory competitors within the microbial community, allowing the novel taxa to proliferate has made this isolation strategy successful. This study has provided novel strains and species of existing genera, and demonstrated that ionizing radiation resistance may be more prevalent across the domain Bacteria than was previously tho

M. LEBUHN, B. MOGGE, M. SCHLOTER, M. STOFFELS, A. HARTMANN

Improved *in situ* tracking of *Sinorhizobium meliloti* by a multiple staining approach, and effects of inoculation with *S. meliloti* on the rhizoplane microflora of alfalfa

GSF-National Research Center for Environment and Health, Institute of Soil Ecology, Neuherberg, Germany

Alfalfa (*Medicago sativa*) was inoculated with *S. meliloti* (wt 2011: wildtype; L 33: *Luc*$^+$; control: no inoculation) in a greenhouse experiment.

A multiple staining protocol was elaborated for improved *in situ* tracking of *Sinorhizobium meliloti*. It comprised *in situ* hybridization with fluorescently labeled 16S rRNA targeted oligonucleotide probes (FISH) and a new probe (SINO) which is specific for the genus *Sinorhizobium*, fluorescently labeled antibodies specific for *S. meliloti* LPS, and DAPI counterstaining. Signals were visualized using confocal laser scanning microscopy (CLSM) and digital image analysis. Using this approach, we could demonstrate the presence of *S. meliloti* bacteroids *in situ* in nodule cuttings of alfalfa, and of bacteroid-like pleiomorphic *S. meliloti* bacteria at the rhizoplane and in pure culture. In contrast to plant DNA, bacterial DNA was not adequately stained by DAPI on and in plant tissues. DAPI was probably absorbed by excess plant DNA, microbial DNA may not have been sufficient in double-strand supercoiled conformation, and access to DAPI may have been hindered by plant tissue. DAPI is hence of limited use for the staining of plant associated bacteria *in situ*.

Effects of inoculation on the rhizoplane microflora were evaluated by FISH of filtered rhizoplane extracts and DAPI counterstaining. We obtained no significant differences between the inoculated variants. The portion of hybridizable rhizoplane bacteria (HRB) was 3-fold higher in the inoculated variants (37%) as compared to the control (14%). The fractions of the inoculated variants were dominated by bacteria reacting with the probe against α-*Proteobacteria*. *S. meliloti* bacteroids were in majority with 54% of HRB, as compared to 3% in the noninoculated control. They were extracted from the interior of nodules. In contrast, bacteria reacting with the probe against (β-*Proteobacteria* were in majority in the noninoculated control, representing 31% of HRB). Other tested phylogenetic groups were less represented.

E. SZÁLLÁS[1], G. KOVÁCS[2], O. ORAVECZ[1,2], A. FODOR[1], E. STACKEBRANDT[3]

Phylogenetic analysis and symbiotic relations of *Photorhabdus* and *Xenorhabdus*

[1]Department of Genetics, [2]Department of Microbiology, L. Eötvös University, Budapest, Hungary, [3]DSMZ, Braunschweig, Germany

We report the complete sequence of 73 entomopathogenic, *Xenorhabdus* and *Photorhabdus* bacterium strains symbiotically associated with entomopathogenic nematodes, *Steinernema* spp., and *Heterorhabditis* spp., respectively. While the present pattern of symbiotic relations of *Steinernema* and *Xenorhabdus* strains seems to be the result of co-speciation, bacterium strains belonging to the only species (*P. luminescence*) of *Photorhabdus* genus (comprising 11 16S rDNA subclusters) are symbionts of different strains of 6 phylogenetic species of *Heterorhabditis* genus. All but four are obligate symbionts, the rest was isolated from human wounds and cannot be retained as symbiont. *Xenorhabdus* and *Photorhabdus* are different genera and the branching point of their phylogenetic separation can be traced back. Some of the 16S rDNA subclusters (II, IV, VII) comprised of symbionts of a single *Heterorhabditis* species, while others (III) include symbionts of three different species. With some limitation, *H. bacteriaphora*, *H. marelata* and *H. megidis* strains could be utilized each other's symbionts, while the *Photorhabdus* strains of tropical Heterorhabditis strains could be utilized only by the tropical strains. Our data indicate that c-cospeciation has been started in *Heterorhabditis Photorhabdus* relations as well.

CS. DOBOLYI[1], I. KERESZTÉNYI[2]

Studies on availability of microbiological ecotoxicological methods in the biodegradation of hydrocarbons in aqueous phase

[1]Department of Microbiology, Gödöllő University of Agricultural Science, Gödöllő, [2]Hungarian Oil and Gas Co., Research and Development, Százhalombatta, Hungary

Ecotoxicity gains great importance when the environmental hazard value of hydrocarbons that cannot be completely biodegraded is tested, or where the process of biodecay is slow. The availability of different methods based on the activity of microbes or their enzymes was studied for testing the ecotoxicity during biodegradation processes. We investigated the ecotoxicological effects of two crude oil derivates on the reproduction of the bacteria *Azomonas agile* and *Pseudomonas fluorescens*, the

green alga *Raphidocellis subcapitata*, and the bioluminescence of *Vibrio fischeri*. The concentration ranges at which the inhibition of the reproduction of *A. agile* reached the level of 50% were 150–200 mg/L at gas oil and about 1000 mg/L at lubricant fluid, and the 50% inhibition of *P. fluorescens* was found in the same range of the tested chemicals. Both chemicals affected the reproduction of the test alga *R. subcapitata*, the change in reproduction was exactly quantified by the determination of cell counts. Both gas oil and lubricant compounds caused significant inhibition in bioluminescence (EC_{50}: 150 mg/L). The activity of the enzyme bacterial luciferase was rapidly affected by the substances which evolved in process of gas oil biodegradation: the level of 25–30% inhibition was experienced at the start and more than 80% could be measured after a ten-day-long incubation. In progress of biodegradation the value of EC_{50} notably decreased in the case of the gas oil, while it showed a slight decrease in the case of the tested lubricant. The changes in ecotoxicity were proved with the reaction of all applied test organisms during the biodegradation of different hydrocarbons. The ecotoxicological monitoring in this field is proposed to be based on simultaneous determination with several different methods.

E. TÓTH, K. MÁRIALIGETI, L. HAVASI, I. S. MOKHTAR

Comparative studies on the bacteriology of wound myiasis of sheep caused by *Wohlfahrtia magnifica*

Department of Microbiology, L. Eötvös University, Budapest, Hungary

In the last years the bacteriology of traumatic wohlfahrtiosis of sheep was studied in detail (the isolated bacteria were identified by using pheno-, geno and chemotaxonomical methods). Comparing the healthy and myiatic regions of sheep it could be demonstrated in general that: i) the number of pyogenic cocci (e.g. *Staphylococcus* sp.) decreased during the development of the maggot containing lesions, later they totally disappeared from the wound; ii) the number of Gram-positive aerobic cocci (*Micrococcus* sp., *Arthrobacter* sp.) also decreased in the wound during the fly development; iii) bacteria belonging to the family *Enterobacteriaceae* could be detected just from the healthy regions.

In the bacterial communities of different developmental stages of the fly there are also characteristic differences: 1. during pupation bacteria belonging to the genus *Proteus* almost totally disappear; 2. the number of a characteristic but so far unknown microorganism (going to be a new γ-proteobacterium) also decreased during the

metamorphosis of the fly; 3. B*acillus* spp., *Corynebacterium* sp. *Micrococcus* sp., *Arthrobacter* sp. were present in all stages.

Later the biotic interaction among these microorganisms using antibacterial assays as well as some aspects of the significance of these bacteria in the development of *Wohlfahrtia magnifica* were tested. The new group of bacteria together with the *Arthrobacter-Micrococcus* group presumably has an important role in the metamorphosis of the fly as they have strong chitinase activity. It could be also proven that the shifts in the bacterial communities usually were results of collective function of several microbial interactions: a) the authentic *Staphylococcus* strains as well as those originating from the sheep and the test organisms from the family Enterobacteriaceae were sensitive for compounds produced by *P. mirabilis, E. coli, Serratia* sp., *A. johnsonii, B. diminuta, P. Stuartii*; b) almost all tested bacteria were sensitive for compounds produced by *Enterococcus faecalis*; c) the new (presumably symbiontic) bacteria and the *Proteus vulgaris* strains were extremely sensitive for the metabolic compounds of lot of other bacterial strains; d) the low number of aerobic cocci in the wound containing third stage fly larvae can be connected mainly to the recurrently appearing anaerobic conditions and possibly not to the direct function of other bacteria.

A. K. BORSODI[1], A. MICSINAI[1], I. MIKLÓS[2], O. ORAVECZ[1], CS. ROMSICS[1]

Bacterial communities participating in the biodegradation of *Phragmites* rhizomes

[1]Department of Microbiology, [2]Department of Botany, L. Eötvös University, Budapest, Hungary

Microbial activity contributing to the breakdown of organic matter plays a crucial role in the nutrient cycling of wetland ecosystems. In this study investigations were carried out on the microbial communities participating in the biodegradation of reed (*Phragmites australis* /Cav./ Trin et Steudel), which is a dominant species in the littoral vegetation of Hungarian lakes.

Following a reed harvest in winter, reed rhizomes were exposed in net bags floating on the water surface to biodegradation. Seasonal samples were taken. From the four samples bacterial strains have been isolated by taking scrapings of the reed rhizome surfaces and tested using classical microbial methods. The carbon-source utilisation pattern was investigated with BIOLOG plates. The results were subjected to numerical analysis and species level identification was carried out.

The results have shown that the late autumn and winter samples, and the late spring and summer samples were in closer relationship to each other, based on their

bacterial community structure. In the former two samples primarily facultatively chemolitotrophic bacteria (*Xanthobacter, Ancylobacter, Alcaligenes, Hydrogenophaga*) were identified with the potential to establish a closed nitrogen cycle within the bacterial biofilm formed on decaying rhizomes and showing preference towards organic acids and not easily degradable biopolymers (cellulose). In the late spring and summer samples, however, facultatively anaerobic fermentative bacteria (*Aeromonas*) were dominant utilizing primarily simple sugars and easily degradable biopolymers. Bacteria possessing oxidative metabolism (*Pseudomonas, Bacillus*) were characteristic in the colder periods.

The PCA method applied to the carbon-source utilisation patterns of the strains of different seasons gives further evidence of a seasonal dynamic.

G. M. ZDOROVENKO[1], YU. A. KNIREL[2], L. M. YAKOVLEVA[1]

Composition and structure of lipopolysaccharide macromolecule as taxonomic criteria in classification of *Pseudomonas syringae*

[1]D. K. Zabolotny Institute of Microbiology and Virology, National Academy of Sciences of Ukraine, Kiev, Ukraine, [2]N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia

Pseudomonas syringae is a group of widespread phytopathogenic bacteria, which include of about 40 pathovars with not fixed taxonomic rank.

We have isolated and studied the LPSs from *Pseudomonas syringae* strains (total 35), representing different pathovars (total 17). Due to peculiarities of LPS macromolecule architecture, their parts (lipid A, core, O-chain), having different conservative levels, give information on evolutionary aspect. We studied separately the different parts of LPS-macromolecule obtained after it was cleaved by mild acetic acid hydrolysis.

In lipid A fractions of all strains glucosamine, ethanolamine-phosphate and similar fatty acids were detected. The set of oxyacids identified (3-OHC10:0, 2-OHC12:0, 3-OHC12:0) is common for the typical representatives of *Pseudomonas* genus.

Studies on the core oligosaccharide fractions also showed their composition usual for pseudomonads. Rhamnose, glucose, glucosamine, galactosamine, KDO, alanine, phosphate as typical components were detected. Composition of core oligosaccharide was more variable in comparison with that of lipid A. 5 chemotypes of core were detected.

Analysing the monosaccharide composition 7 chemotypes of O-chains were distinguished. And thus, the comparative study showed the divergence in the course of evolution of the LPS-macromolecule composition, in the strains studied, from 1 to 7 chemotypes on the levels: lipid A - core - O-chain.

The O-chains structures were determined on the basis routine of and modern methods, first of all by different variants of N.M.R.-spectroscopy. From these data follows that in all strains studied, independent on their taxonomic rank, the O-chains are built up by a general principle, their backbones are represented by rhamnans: *L*-, *D*- or mixed (*DL*-) with tri- or tetrasaccharide O-repeats, having identical structures in the majority of the strains. This is in a good agreement with inclusion of the host-plant differentiated species into *Pseudomonas syringae* as conditionally presented in Bergey's Manual. Presence and nature of the lateral branch substituents (*D*-fucose, *D*-rhamnose, *N*-acethyl-*D*-glucosamine, *N*-acethyl-3-amino-*D*-fucose) and the mode of binding of those with the main O-chain determined the serogrouping of the strains.

P. SCHUMANN[1], I. GROTH[2], E. STACKEBRANDT[1]

New genera of actinomycetes with menaquinone MK-8(H_4) and lysine- or ornithine-containing peptidoglycan

[1]DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig,
[2]Hans-Knöll-Institut für Naturstoff-Forschung e. V., Jena, Germany

A high number of actinomycetes isolated for the biotechnological screening were studied by chemotaxonomic methods. Peptidoglycan diamino acids and menaquinone patterns turned out to be of highest discriminatory power for coryneform genera. A group of isolates attracted special attention because of the occurrence of lysine or ornithine as diagnostic diamino acid and MK-8(H_4) as major isoprenoid quinone – a combination of chemotaxonomic characteristics unique for actinobacteria up to that time. On the basis of their detailed peptidoglycan structure, the isolates were classified into five groups. Peptidoglycan type A4α was represented by three variations: L-Lys←L-Ala←L-Ala←L-Glu (novel variation, strain DSM 11294), L-Lys←L-Ser←D-Asp (A11.36, strain DSM 11295) and L-Lys←L-Glu (A11.54, strain DSM 12333). Two novel variations L-Orn←$Gly_{(1-2)}$←D-Glu and L-Orn←Gly←D-Asp of peptidoglycan type A4β were detected in strains DSM 12335 and DSM 12362, respectively. These groups were additionally differentiated by cellular fatty acid profiles and polar lipid patterns. Phylogenetic analyses based on 16S rDNA sequence comparison revealed that

the strains with unusual sets of chemotaxonomic characteristics represent five separate lines of descent within the suborder *Micrococcineae*. Consequently, five new genera have been proposed to accommodate these isolates.

G. KOVÁCS, M. NIKOLAUSZ, A. HALBRITTER, I. VILLÁNYI, H. M. RIFAAT, K. MÁRIALIGETI

Bacterial communities of cattail (*Typha angustifolia*) rhizoplane

Department of Microbiology, L. Eötvös University, Budapest, Hungary

The bacterial communities inhabiting the rhizoplane of *Typha angustifolia*, a common macrophyton in floating mat ecosystems was investigated by using classical and molecular methods. At first 250 bacterial strains, derived from root samples, were characterised by investigation of numerous phenotypic properties. Based on results of computer generated cluster analysis selected strains of each phenon were characterized and determined by BIOLOG metabolic fingerprint assay. Strains chosen from each phenon were investigated by 16S rDNA sequence analysis to determine their precise taxonomic position. The results demonstrate that the culturable members of rhizoplane bacterial community are dominated by Gram-positive bacteria, by *Bacillus pumilus* as main coloniser. Other Gram-positive bacteria like *Staphylococcus warneri, Kocuria* spp., *Microbacterium lactis, Arthrobacter agilis* and *Streptomyces* spp., *Micromonospora* spp. appeared to be less frequent. The Gram-negative isolates represent principally *Acinetobacter lwoffi, A. radioresistens, Xanthobacter flavus, Agrobacterium tumefaciens, Rahnella aquatica, Erwinia* sp. and *Pseudomonas fluorescens*.

ARDRA analysis of 16S rDNA-clones obtained from direct DNA-extraction of root mass was carried out in order to detect a broader range of bacterial community. In contrast with the culturable members of the rhizoplane, a significant part of clones analysed has fallen into the group of δ-proteobacteria represented by numerous sulphate-reducers. Other clones were clustered together with *Frankia*, *Clostridium*, *Rhodomicrobium*, *Rubrivivax*, *Janthinobacterium* and *Pseudomonas* spp.

A. VÖLGYI[1], T. FARKAS[2], A. FODOR[1], S. FORST[3]

Phospholipid membrane fluidity and fatty acid profile in natural phase variants, transposon-induced, deletion and complemented phase-variant mutant of the entomopathogenic bacterium *Xenorhabdus nematophilus*

[1]Department of Genetics, L. Eötvös University, Budapest, [2]Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary, [3]University of Wisconsin-Milwaukee, Milwaukee, Wisconsin, USA

Phase variation is one of the most peculiar feature of entomopathogenic nematode symbiont bacteria, *Xenorhabdus* and *Photorhabdus*. In *X. nematophilus*, the shift from the phase 1 cells to phase 2 occurs spontaneously and is of very low reversibility. A several gene active in Phase one switch off, while the lipase switched off in Phase 1 switches on at phase shift. By mutating Phase 1 cells by using a *Tn10* minitransposon, conjugation and selection, several mutants of phase 2 phenotypes were isolated. By analysing one of them, finally the wild type allele of novel gene (*var-1*), capable of complement the transposon tagged and deletion mutant was isolated, cloned and sequences. One of the pleiotropic phenotypes of the secondary (phase 2) cells is more rigid nature of the membranes, which could be detected both by biochemical and biophysical methods. We found that both the Tn10 induced, and the deletion mutant for *var-1* showed the phase 2 phenotype while the complemented ones showed the phase 1 phenotype. We hope we did manage to isolate one of the most important regulatory gene of the gene-cascade system behind the phase variation of *X. nematophilus*.

J. MAKK, É. ÁCS, B. BESZTERI, O. ORAVECZ, K. SZABÓ

Investigation of diatoms associated bacterial communities of the River Danube

Department of Microbiology, L. Eötvös University, Budapest, Hungary

Epilithon microbial communities, which colonize and grow on gravel, pebbles and rocks are important in aquatic environments, responsible for nutrient cycling, degradation of xenobiotics. Among algae the diatoms are found to be dominant groups of the epilithon communities of the River Danube. According to our electron microscopic investigations the biofilms on illuminated surfaces contain many types of cells, including bacteria on surfaces of the diatoms, so one has to realise that the surfaces of them serve habitats for different bacteria.

One of our study sites was at the north-eastern part of Szentendre Island in the River Danube main arm, where gravel samples were collected from the northern water gaining area of bank wall filtered drinking water wells serving Budapest. At other study site artificial substrata were submerged in the river at 50–60 cm water depth at 1669 river km, Alsógöd. These were incubated for 6 days. Both collected samples were plated onto algal media in order to isolate diatoms, and then associated bacteria. The representative strains were selected on the basis of cultural-morphological-features, certain biochemical investigation and ARDRA analysis. Selected groups were identified by the Biolog phenotypic fingerprint method and 16S rDNA sequencing.

Concerning the Gram-negative organisms of our identification work strains belong to genera typically isolated from aquatic environments such as *Pseudomonas, Caulobacter, Aeromonas, Mycoplana, Sinorhizobium, Flavimonas, Flavobacterium, Sphingomonas,* and facultative methylotrophic and/or H_2 autotrophic bacteria such as *Xanthobacter, Variovorax, Ancylobacter, Azospirillum, Methylobacterium.* Among the Gram-positive bacteria *Bacillus, Microbacterium, Streptomyces, Rhodococcus, Nocardia, Aureobacterium, Rathayibacter species* were found.

The bacteria isolated are able to utilise such sugars and amino acids as only carbon sources, which have been proved to be among extracellular products of diatoms. Thus we can suppose that association between diatoms and bacteria is based also upon metabolic interactions.

T. PÁL

Experiences obtained from the microbiological examinations of activated sludge sewage treatment plants of Hungary

BIOTOP Bt. Budapest, Hungary

The author deals with saprobiological problems of the biological sewage treatment plants since 1965. In the latest years in Hungary several biological sewage treatment plants have been built mainly on the base of activated sludge technology.

Beside the technical problems of the construction and operation of these plants a few efforts have been paid so far for the microbiological problems of them. However, according to the experiences gained from the microscopical sludge investigation a poor quality final effluent is very often caused directly by the less than optimum condition of the sludge flocs in the plant. The standard investigating procedure used most often nowadays for checking the course of the treatment process and providing information for adjustment, if necessary, involves mainly chemical and physical analysis giving

little direct information to plant operators about the actual quality of the activated sludge floc in the aeration tank. As a result it is often not possible to indicate the cause of disturbance in the treatment process and, consequently, to initiate corrective action in order to improve plant operation.

Microscopic investigations of activated sludge is a simple analysis which gives information about the form and structure of flocs, the presence of filamentous microorganisms, the number of protozoa, etc.

The author as an iexpert has made several microscopical investigations during the last years in different communal and industrial sewage treatment plants in Hungary.

In this lecture his experiences will be summarized which can contribute to a better insight into the composition and structure of flocs and through this into the functioning of the activated sludge process.

A digitalized video film enregistered by the author will be shown demonstrating the problem of bulking sludge, etc.

M. NIKOLAUSZ, K. MÁRIALIGETI, O. ORAVECZ, CS. ROMSICS

Changes in the composition of eel gill bacterial communities as a function of eel nematode infection

Department of Microbiology, L. Eötvös University, Budapest, Hungary

The cause of the mass destruction of eel in lake Balaton is presumably a result of joint influence of different factors (pesticide poisoning, effect of animal parasite, lack of oxygen, etc.), but it is certain that bacterial infections may play important role as predisposing factors. The most important target point of fish pathogens is the gill, particularly if floating and breathing of the fish is injured by an eel-nematode (*Anguillicola crassus*) parasitism.

In order to get a more detailed insight into the composition of the gill bacterial community of healthy and infected eels a polyphasic approach has been chosen. Homogenized gill tissue samples were used for obtaining pure cultures moreover for direct DNA extraction. Pure cultures were described by some phenotypic characters, grouped by ARDRA (Amplified Ribosomal DNA Restriction Analysis) and identified by partial sequencing of PCR amplified 16S rDNA.

The DNA isolated directly served as a template for amplifying 16S rDNA of the gill bacterial community. A second nested PCR resulted sufficient product for cloning. A clone library was generated by blunt-end cloning and it was screened using ARDRA

method. Representative members of different band pattern groups were sequenced partially. The sequences were aligned and analysed by the ARB phylogenetic software package and compared to rDNA databases for taxonomic purpose.

The most dominant species from pure cultures proved to be *Aeromonas veronii* in healthy eels, but its dominance is restricted by a more diverse community dominated by *Pseudomonas putida* and *Pseudomonas stutzeri* in eel nematode infected, sick eels. *Dietzia maris, Shewanella putrefaciens, Acinetobacter* spp., *Plesiomonas shigelloides, Comamonas* sp. also were found in the gill tissues of infected eels. They are facultative pathogens in mammals but their pathogenicity in case of fish is not always clear and must be examined and proved later.

The clone library can be described with the dominance of *Aeromonas veronii* in healthy eels. Other dominant species are *Arthrobacter oxydans*, *Acinetobacter johnsonii*, *Nocardioides simplex*. The evaluation of data is in progress, but they support the results based on the culturing methods that in healthy eels the *Aeromonas* dominance is changed to a more diverse community where potential fish pathogens may appear, in the nematode infected animals.

H. PAMJAV[1], D. TRIGA[1], E. SZÁLLÁS[1], A. FODOR[1], ZS. BUZÁS[2]

PhastSystem PAGE PCR-RFLP analysis: new molecular technique for identification and phylogenetic analysis of entomopathogenic nematode – symbiotic bacteria, *Photorhabdus* and *Xenorhabdus*

[1]Department of Genetics, L. Eötvös University, Budapest, [2]Agricultural Biotechnology Center, Gödöllő, Hungary

A recently newly developed PhastSystem polyacrylamide Electrophoresis method was adopted to analyse the RFLP pattern of the PCR-amplified spacer region of the 16S - 23S rDNA operon of *Photorhabdus luminescens* and several *Xenorhabdus* spp. Three enzymes (*Alu*I, *Hinf*I, *Mse*I) provided highly reproducible patterns. On the basis of comparative pattern analysis DSMZ types strains of *X. nematophilus, X. bovienii, X. poinarii, X. beddingii* and *P. luminescens* could be identified unambiguously. Natural isolates and laboratory strains of *the Xenorhabdus* species hardly differed from the respective type strain. Symbionts isolated from different *Steinernema* (nematode host) species provide characteristic pattern, indicating that the cospeciation is characteristic for the symbiotic pattern of *Steinernema/Xenorhabdus* relations. Photorhabdus strains belonging to different 16S rDNA subclusters also provided characteristic pattern, but there were significant differences between some

strains belonging to the same 16S rDNA groups. Considering that the PhastSystem PAGE technique proved to be also suitable for molecular identification of the nematode symbionts on the basis of PCR-RFLP analysis of the internal transcribes spacer (ITS) region of the 18S - 5.8S -26S rRNA operon, there is a molecular tool for studying coevolution of the nematode-bacterium symbiotic complexes available.

M. K. KILANI[1], A. H. KANJO[2]

Bacterial load of the air of Budapest

[1]Department of Microbiology, L. Eötvös University, [2]State Public Health Service, Budapest, Hungary

In this study the total colony counts of heterotrophic-aerobic bacteria was investigated and the existence of several kinds of bacteria (at species level) in the air of Budapest at 15 different places was characterized.

This highest mean CFU counts were found at Gergely street and at Kálvin square. Isolated and purified strains proved that Gram positive organisms absolutely dominated. The same is true for catalase positivity. Among cocci, members of the genus *Micrococcuss* dominated, in leading position with *M. luteus.* Among rod shaped bacteria, members of the genus *Bacillus* dominated with *B. brevis*.

Coryneforms and nocardioforms were also present. Members of genera *Microbacterium, Arthrobacter, Brevibacterium, Cellulomonas, Rhodococcus, Clavibacter* and *Gordona* were identified.

E. L. ZDOROVENKO[1,2], YU. A. KNIREL[1], V. V. OVOD[3]

Structures of O-polysaccharide chains of *Pseudomonas syringae* pv. *garcae* LPS

[1]N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia, [2]Taras Shevchenko Kiev University, Kiev, Ukraine, [3]Institute of Medical Technology, University of Tampere, Tampere, Finland

Pseudomonas syringae pv. *garcae* is cause of coffee diseases. Lipopolysaccharides (LPSs) from microbial cells of *P. syringae* pv. *garcae* (three strains) were extracted by saline solution. O-specific polysaccharide fractions (OPSs) were obtained by gel-filtrating the products of LPSs by mild acid hydrolysis. Using ^{1}H- and ^{13}C-NMR spectroscopy, including two-dimensional spectroscopy, sugar and

methylation analyses and Smith degradation the structures of OPSs were elucidated. It was found that in all strains studied they belong to a structural group that is characterized by L-rhamnan backbone with tetrasaccharide O repeats. OPS of one strain had linear structure. Others were branched in different positions with a monosaccharide side chain of 3-acetamido-3-deoxy-D-fucose (D-Fuc3NAc).

All of them included O repeats with structures 1 and 2, which differ in the position of substitution of one of the rhamnose (Rha) residues.

→2)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→
1

→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→
2

This unique type of structural heterogeneity in the main OPS chain has been previously demonstrated for OPSs of some other *P. syringae* pathovars studied which have L-rhamnan main chain with Fuc3NAc as the lateral substituent. Of them, the OPS that of *P. syringae* pv. coriandricola GSPB 2028 (W-43) most closely resembles to the OPSs studied.

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T. HANCZÁR[1], R. CSÁKI[1], L. BODROSSY[1,2], J. COLIN MURRELL[3], K. L. KOVÁCS[1,2]

Hydrogenases in methanotrophic bacteria

[1]Department of Biotechnology, University of Szeged, [2]Institute of Biophysics, Biological Research Centre, Szeged, Hungary, [3]Department of Biological Sciences, University of Warwick, Coventry, UK

The thermotolerant methanotroph *Methylococcus capsulatus* strain Bath was shown to have genes for the small and large subunits of a typical Type I (Ni-Fe) hydrogenase by Southern hybridisation with a heterologous probe from *Thiocapsa roseopersicina*. These genes have been cloned and the hydrogenase structural genes have been sequenced and compared with the sequences of known hydrogenase genes from other organisms. The hydrogenase genes have been used in marker exchange mutagenesis experiment in order to create hydrogenase-minus mutants of *Methylococcus capsulatus*. Hydrogenase activity has also been examined in whole cell assay with *Methylococcus* grown under a variety of different growth conditions. Preliminary biochemical characterisation of the hydrogenase enzyme has also been carried out.

A. GATTINGER, L. ZELLES, J. C. MUNCH

Characterization of methanogenic and methanotrophic microorganisms in agricultural soils by phospholipid analysis

GSF-National Research Center for Environment and Health, Institute of Soil Ecology, Neuherberg, Germany

We report on a method which enables the estimation of methanogenic (anaerobic) and methanotrophic (aerobic) microorganisms in soil ecosystems based on phospholipid analysis. Methanotrophs which are Gram-negative bacteria contain ester-linked monounsaturated fatty acids in their polar membran lipids, whereas the phospholipids of members of the domain *Archaea* including methanogens lack the ester linkage. Their membrane lipids posses ether linkages. This biochemical differentiation can serve as a complement to molecular approaches of the domains *Bacteria* and *Archaea* in soil. Examples for the application of this phospholipid assay in a current research project are also contributed.

M. MUELLER[1], U. BEHRENDT[1], P. LENTZSCH[1], J. KIESEL[2]

Characterisation of bacterial communities from the phyllosphere of crop plants

[1]ZALF e.V., Institute of Microbial Ecology and Soil Biology, [2]Institute of Landscape Modelling 2, Müncheberg, Germany

Whereas the diversity of plants and animals in agroecosystems receives great attention, the diversity of microorganisms is often neglected in the description of the biological diversity in a natural ecosystem. However, several methods have been suggested for genetic and functional analyses of microbial communities in the phyllosphere of crop plants. Moreover, there is an increasing demand for information about the influence of landscape structures, climate factors and land management practices on their composition. The present study was carried out to assess the possibility to use four different biological parameters concerning the phylloplane microorganisms to characterise these microbial communities:

1. Total number of enterobacteria, a frequently occurring and important group on the surface of crop plants; 2. Part of the individual species of *Pantoea agglomerans* within the population of the enterobacteria;

3. Intraspecific genetic diversity of this species determined by PCR fingerprint using repetitive DNA sequences;

4. Enzyme activities (α- and ß-glucosidase, acid and alkaline phosphatase) regarded as a term for the general metabolic activity of the whole microbial community.

Spatial and temporal variations of these biological parameters were presented along a transect in North-Eastern Germany about 200 kilometres in length with 40 sampling places in various distances. The study area comprises, as a result of its glacial origin in Young Pleistocene, a wide variety and a complex arrangement of land forms and landscape elements. We demonstrated the distribution of the biological parameters depending on several types of Young Pleistocene landscapes on regions of different rainfalls and on the kind of crop plants.

A. FŰZI[1], ZS. LANGÓ[1], L. G.-TÓTH[2], I. MUSKÓ[2]

Bacteriological data of the molluscs and the planktonic crustaceans of Lake Balaton

[1]Department of Microbiology, L. Eötvös University, Budapest, [2]Balaton Limnological Research Institute, Tihany, Hungary

The molluscs and the planktonic crustaceans are filter-feeding organisms and some of them are considered to be effective bacterial feeders. The aim of our work was to identify the bacterial communities of the hindgut content of three molluscs (*Anodonta* sp., *Unio* sp., *Dreicena* sp.), the gut content of two planktonic crustaceans (*Corophium* sp. and *Daphnia* sp.) and homogenized body mass of *Bosmia* sp. and *Eudiaptomus* sp. Up to the present we have not got any information on bacterial communities of these animals living in different aquatic habitats, and especially the Lake Balaton. We collected specimens of molluscs and planktonic crustaceans at two sampling sites (eastern part - Tihany and western part - Keszthely) of Lake Balaton. Seventy-two representative strains were studied in detail by their morphological, physiological and biochemical characters. Based on the phenetic data a dendrogram was generated by using the S_M coefficient and UPGMA algorithm with the help of the SPSS for Windows 6.0 software. Nine characteristic clusters were obtained. Representative strains of phena were identified by the Biolog metabolic fingerprint system, too. The dominant clusters contain the following species: *Acinetobacter johnsonii, Pseudomonas diminuta, Aeromonas media, Aeromonas veronii, Klebsiella oxytoca*, *Enterobacter cloacae*, all Gram negative organisms (79% of strains). The

species of family Enterobacteriaceae were characteristic only in the hindgut of the bigger size molluscs (*Anodonta* sp., *Unio* sp.). In the planktonic crustaceans no enterobacteria were detected.

G. DIÓSI, L. G. GAZSÓ, GY. FARKAS

Study of microorganisms and biofilm development in connection with nuclear waste management

National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary

In order to the correct planning of a nuclear waste disposal, the corrosive and migrative effects induced by the potentially developing biofilms on the surface of the container must be considered. Biofilms are defined as matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces. Each biofilm bacterium lives in a customized microniche in a complex microbial community that has primitive homeostasis, a primitive circulatory system, and metabolic cooperativity. Each of these sessile cells reacts to its special environment so that it differs fundamentally from a planktonic cell of the same species. Direct observations have clearly shown that biofilm bacteria predominate, numerically and metabolically, in virtually all nutrient-sufficient ecosystems. Therefore, these sessile organisms predominate in most of the environmental, industrial, and medical problems and processes of interest to microbiologists. Our work was to examine the samples (stone, water, technical water) taken from the uranium mine at the Mecsek Hill (a potential place for high level waste disposal) and also the water samples from the interim storage of the spent fuel at AEKI. Our aim was to isolate the microorganisms from these samples and to study the metabolism of them. We checked the isolated aerobic and anaerobic strains' gas and siderofore productivity and also examined the biofilms consisting of these bacterial strains and developed on the surfaces of stainless steel. We obtained useful information about the cell morphology, cellular metabolism, and the physical architecture of the biofilm matrix.

E. M. OTT[1], TH. MÜLLER[2]

Bacteriocins of plant-associated enterococci

[1]Institute of Ecologically Compatible Animal Husbandry, University of Rostock, Rostock, [2]Institute of Microbial Ecology and Soil Biology, Centre for Agricultural Landscape and Land Use Research Müncheberg, Paulinenaue, Germany

Six bacteriocin-producing strains of plant-associated enterococci (*E. faecalis* P 7644, P 8041, P 8403, *E. faecium* P 8079, *E. mundtii* P 7826, P 8382) obtained in an ecological study on extensive managed meadows were used for comparative studies of their antagonistic substances. Tests were carried out with bacteriocin crude extracts produced by ammonium sulphate precipitation, dialysis and freeze-drying. The antibacterial activity of all extracts was completely destroyed by treatment with proteinase K, pronase E and papain, while α-amylase treatment showed no effect on activity. Pepsin, α-chymotrypsin and trypsin inactivated antagonistic compounds produced by *E. faecium* and *E. mundtii* strains while they reduced activity of those produced by strains of *E. faecalis*. All bacteriocins were pH- and heat-stable except E 8382, which loses activity when boiled for more than 10 min.

To test antimicrobial activity spectra of crude extracts, a total of 109 bacterial strains were used as indicator in the Agar-Diffusion-Assay. The antibacterial substances affected a wide spectrum of lactic acid bacteria, especially enterococci. All enterocins strongly inhibited *L. monocytogenes* and showed activity against clostridia. Additionally, extracts E 7644 and E 8041 (both produced by an *E. faecalis*) caused weak inhibition of a *Bacillus* strain and several strains of *Clavibacter* and *Curtobacterium*. Gram-negative indicators were not affected. Cluster analysis of the results grouped the enterocins corresponding to the species the producing strains belong to. This finding suggests that strains of the same enterococcal species produce bacteriocins of similar characteristics.

K. SZABÓ[1], É. ÁCS[1], É. PÁPISTA[2], K. T. KISS[3], S. BARRETO[1], J. MAKK[1], B. BESZTERI[1]

Phytoplankton and periphyton investigations in Soroksár Danube

[1]Department of Microbiology, L. Eötvös University, Budapest, [2]North-Hungarian Waterworks, Salgótarján, [3]Hungarian Danube Research Station, Hungarian Academy of Sciences, Göd, Hungary

The 58 km long Soroksár side arm of river Danube is regulated with locks. The upper lock is found on left side of the River Danube at Southern part of Budapest (1642

riv.km). This large side arm has recreational, industrial and agricultural water supply function for the region, and it receives half of the treated waste water of Budapest. The water is most polluted on the upper part (58–38 riv.km) of the arm (unsuited for bathing) and less polluted on the lower part (22–0 riv.km) with some beaches.

Reed-periphyton and phytoplankton samples were taken in November 1996, January, April and July 1997, from the upper part of the Soroksár-Danube at Taksony (39 riv.km), and July and November 1998, January and May 1999 from the lower part at Ráckeve (19 riv.km). The aim of this study was to compare the seasonal and vertical distribution of periphytic algae both on old and on green reed-stems, at the upper and lower part of side-arm, concerning the taxonomic composition, abundance and chlorophyll-a content, among others. In addition, we calculated the abundance, chlorophyll-a content and qualitative-quantitative composition of phytoplankton.

The vertical distribution of abundance and chlorophyll-a values were usually the lowest just below the water surface, had a maximum somewhere on the upper part of the stem and more or less evenly decreased toward the bottom. This tendency was the opposite in July along the reed-stem.

The abundance and chlorophyll-a values of periphyton were higher at Ráckeve, while that of phytoplankton at Taksony.

The proportion of green algae was higher at Ráckeve in every season.

Some diatoms showed the same vertical distribution along the reed stems. The relative abundance of *Amphora lybica* Ehr., *Coconeis placentula* Ehr. and *Eunotia arcus* Ehr. increased and that of *Gomphonema minutum* Ag., *G. parvulum* Kütz., *Navicula capitatoradiata* Germain and *Nitzschia dissipata* (Kütz.) Grun. decreased toward the bottom.

According to the chlorophyll-a concentration of phytoplankton, the trophic state of the side-arm at Taksony was mesotrophic in November and July, eutrophic in January and hypertrophic in April. It was hypertrophic at Ráckeve in July and May, oligo-mesotrophic in November and January.

B. Beszteri, É. Ács, J. Makk, G. Kovács

Application of molecular methods in diatom taxonomy

Department of Microbiology, L. Eötvös University, Budapest, Hungary

Molecular biological methods are becoming widespread tools also in phycology recently. Until now the most commonly used technique is sequence analysis of 18S rDNA, most frequently with the aim of determining relationships of large taxonomical

units (divisions, classes, orders) of algae and other eukaryotes. The purpose of our work is to use this tool in diatom taxonomy on a lower taxonomic scale.

Diatom taxonomy is a good example of morphology-based taxonomy because several morphological characters found on the finely structured silica valves serve as key differential characters. Generally electron microscopic techniques are used in these examinations, and the number of species described and identified by small differences in valve structure is increasing fast. Much less investigations can be found on non-morphology based (e.g. biochemical, molecular biological, ecological) differences between and within diatom species. We are trying to use 18S rDNA and ITS sequence comparisons to determine genetic differences between some "morpho" species.

As the first step, cultivation and purification experiments were made. For purification of the cultures both streaking and single-cell isolation methods and their combinations were used. For sequence determinations the widely used methods of PCR amplification and direct sequencing of the product were used. As the first step of our study we determined sequences of some well growing diatom species (e.g. *Melosira varians*, *Amphora montana*, *Gomphonema parvulum*, *Navicula minima*, *Navicula subminuscula*, *Phaeodactylum tricornutum*,) and compared them to those already available. The phylogenetic tree constructed does not fit exactly with the tree based on phenotypic characters.

M. LEBUHN[1,2], W. ACHOUAK[2], O. BERGE[1], M. SCHLOTER[1], A. HARTMANN[1], T. HEULIN[2]

Polyphasic taxonomy of *Ochrobactrum* spp. isolates from environmental samples

[1]DSV-DEVM, Laboratoire d'Ecologie Microbienne de la Rhizosphére, CNRS-CEA, CEA Cadarche, St. Paul Léz Durance, France, [2]GSF-National Research Center for Environment and Health, Institute of Soil Ecology, Neuherberg, Germany

The genus *Ochrobactrum* consists of 2 species, *O. anthropi* and *O. intermedium*, and belongs to the rRNA superfamily IV of the α-2-*Proteobacteria*. Most *Ochrobactrum* isolates are from human clinical specimens. 16S rDNA homology among these both *Ochrobactrum* species and to their closest relatives, *Brucella* sp., is higher than 98%.

Monoclonal antibodies (mABs) were raised against an *O. anthropi* soil isolate. They did not cross-react with any described related species. Using one of these antibodies, a large strain collective was immunotrapped from two french soils and from roots of two wheat cultivars that were grown in these soils in phytotrones. The

immunotrapped collective was grouped on rep-PCR profile similarities. Representatives of these rep-groups were examined in a polyphasic taxonomic study on morphological, phenotypical (BIOLOG, API, sensitivity to antibiotics), serological (ELISA), and genotypical (ARDRA and homology of 16S rDNA, DNA/DNA reassociation) characters.

We obtained 13 distinctly different major rep-groups (A–M), partly comprising several subgroups. The results from the different morphological, phenotypical and genotypical approaches accordingly prove that rep-group A–I strains are *O. anthropi*, and rep-group L and M strains *O. intermedium*. Rep-group J and K isolates constitute 2 new *Ochrobactrum* species which will be described as *O. tritici* and *O. grignonense*, respectively. In phylogenetic 16S rDNA trees, however, *Brucella* sequences were interspersed in the *Ochrobactrum* cluster and one *O. anthropi* sequence was found within the *O. tritici* cluster. 16S rDNA homology values within the Ochrobactrum–Brucella group were very high (>96%). A similar situation was reported for the Rhizobium–*Agrobacterium* branch. Various species of α-2-*Proteobacteria* including *Ochrobactrum* have at least two 16S rRNA operons that obviously can be different. The phylogenetic 16S rRNA approach is hence of limited use as decisive means for species definition within the α-2-Proteobacteria.

G. DARABOS

Bacteriological investigations on soil cover of karstic areas

Department of Physical Geography, Loránd Eötvös University, Budapest, Hungary

Some soil-microorganisms living in soil cover of karstic areas promote limestone weathering by produced inorganic or organic acids or ligands and by altered chemical composition of the soil atmosphere and the infiltrating water. The aim of the present investigation was to get more information about microbiological communities in the soil of limestone areas and their role in the processes of karst corrosion. Soil samples were collected on the Aggtelek Karst, in Hungary. From the samples the CFU number of the main groups of microbes was determined. Gram-positive microbes dominated in all samples. Furthermore, all together 250 isolates were collected, from them 10 Streptomyces sp. and 10 other bacterial strains were selected to use in new laboratory model-system regarding the production of $CaCO_3$-aggressive matters. The identification all of the bacterial strains was facilitated by phenotypic methods. The shifting of pH-values and the amount of dissolved $CaCO_3$ effected by the selected microorganisms were measured under different ecological conditions. It was clearly

shown by the different tests that the various ecological factors (pH-value, the amount of acids, etc.) influence the activity of microorganisms that is decisive in the production of aggressive material and at the same time the microbiological products could change the ecological factors. Moreover, the earlier disregarded *Streptomyces* strains, are probably most important in the solution of limestone. These examinations were preliminary experiments to determine the circumstances of the $CaCO_3$-aggressive substance production by microbes and their role in the process of karst corrosion.

A. LAUKOVÁ[1], P. JURIŠ[2], S. CZIKKORVÁ[1], Z. VASILKOVÁ[2], M. MAREKOVÁ[3], I. KRUPICER[2]

Inhibitory effect of enterocins against hygienic important bacteria in the cattle and pig slurry environment

[1]Institute of Animal Physiology, [2]Parasitological Institute, Slovak Academy of Sciences, [3]Institute of Medical Chemistry and Biochemistry, Medical Faculty, University of P. J. Šafárik, Košice, Slovakia

The preferred intensive methods of cattle and pig production in Slovakia are associated with the consequence of large volumes of slurry requiring disposal. This has resulted in pollution problems (pathogenic bacteria and odour) leading to increased pressure on farmers to find satisfactory methods to manage waste. Aerobic and/or anaerobic treatment can be very effective in dealing with a wide range of these problems, but it is often not popular due to the high energy costs implied. Therefore, one possible way to solve this problem could be to use the ability of some bacteria to produce antimicrobial substances. Bacteriocins are proteinaceous compounds with an antagonistic activity generally exerted on closely related bacteria as well as on hygienic important strains. Knowledge of some bacteriocins, especially those produced by lactic acid bacteria has expanded dramatically over the last decade. Therefore, the aim of our study was to report the results associated with the experimental application of our two bacteriocins produced by *Enterococcus faecium* CCM 4231 and *Ent. faecalis* V24 strains for slurry treatment. The slurry used in our experiments was collected from a farm located in Liptovská Teplicka or Spišské Vlachy and from a pig farm in Figa (Slovakia). In all experiments, the slurry was filtered and boiled to kill bacteria. Then both, the experimental and control samples (ES, CS) were inoculated with indicator organisms: *Listeria monocytogenes*, *Yersinia enterocolitica* and *Enterobacter cloacae* (1% inoculum of 10^7 cfu ml^{-1}). Enterocin CCM 4231 was added to ES at the beginning of the experiment in conc. of 3200 AU ml^{-1}. Enterocin-like V24 was added to ES in a

logarithmic growth phase of indicators in conc. 800–1600 AU ml^{-1}. Samples with enterocin CCM 4231 were incubated for 2 weeks at 30–32°C. On the other hand, samples with bacteriocin (bc) V24 were incubated only for 24 h in water incubator at the same temperatures. Stable, suppressing effect of enterocin CCM 4231 on the growth of listerial cells was noted. When bc V24 was added to the growing cells of *L. monocytogenes*, the inhibitory effect was found already 1 h after bc addition. The highest inhibition was noted after 2 h with effectivity up to 24 h. When bc V24 was added to ES with *Ent. cloacae* inoculum, no effect was detected in the moment as well as after 1 h of bc addition. There, the inhibitory effect started after 2 h of bc addition with increased effectivity up to 24 h. *Yersinia enterocolitica* was inhibited already 1 h after bc V24 addition with stable inhibitory activity up to 24 h. Our results indicate the possible use of bacteriocin producing strains and/or their bacteriocins to decrease or eliminate contaminants in slurry.

D. ELHOTTOVÁ[1], J. TŘÍSKA[2]

GC-MS/MS detection of poly-β-hydroxyalkanoates (PHA) as prokaryotic storage compounds

[1]Institute of Soil Biology, [2]Institute of Landscape Ecology AS CR, České Budějovice, Czech Republic

Poly-β-hydroxyalkanoates (PHA) compounds are accumulated under conditions of nutrient unbalance by many bacterial species belonging to diverse taxa. They are considered as important markers of the growth and nutritional status of bacterial community. The PHA contain 3-hydroxyacyl monomer units with 3–14 C atoms. The most abundant PHA compound is poly-β-hydroxybutyrate (4 C) that has been often the only monitored PHA compound in majority of ecological studies. A lack of sensitive methods is a reason that the other PHA monomers are not observed in majority of environmental analyses. The goals of the study were (i) to modify the trace analysis for PHB determination (Elhottová et al., 1997) to complex determination of PHA composition, (ii) screening pure bacterial cultures to show distribution of individual PHA monomers. In principle the method is based on a single-phase extraction of lipids from the sample; release of monomer units (3-hydroxy fatty acids) from PHA by alkaline hydrolysis; monomer derivatization by N-tert-butyl-dimethylsilyl-N-methyltrifluoracetamide (MTBSTFA); and finally detection of derivatized products (MTBSTFA-3hydroxyacids) by GC-MS/MS method. Modification consisted in the final detection step of GC-MS/MS analysis. The analysis was divided to individual detection segments corresponding to retention time and *m/z* value of selected ions of

individual derivatized PHA monomers. The fragment ions $(M-57)^+$ and $(M-15)^+$ were selected. This method allowed detection of all potential PHA monomers in one analysis on the trace level (10^{-15} mol μL^{-1} of injected volume). Screening of 13 bacterial strains showed distribution of following PHA monomers: the most abundant were 3-hydroxyacids with 4 and 5 C (50% of bacterial strains), less abundant were 3-hydroxyacids with 8 and 10 C (31%), 12 C (23%), 9 C and 6 C (15%) and minority group represented 3-hydroxyacids with 7 and 11 C (7%).

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E. BÖSZÖRMÉNYI, K. LENGYEL, H. PAMJAV, E. SZÁLLÁS, A. FODOR

Gnotobiological analysis of *Heterohabditis/Photorhabdus* symbiotic complexes

Department of Genetics, L. Eötvös University, Budapest, Hungary

The RFLP patterns of the PCR amplified internally transcribed spacer (ITS1 - 5–8S rRNA and ITS2) region the 18S–28S rRNA genes, 13 strains of 6 entomopathogenic nematode (EPN) phylogenetic species belonging to the *Heterorhabditis* genus and those of amplified internally spacer region of their bacterial (*Photorhabdus*) symbionts were determined. Species specific patterns were identified reproducibly and the possibilities of cospeciation are discussed. We accomplished a large-scale gnotobiology study by growing nematode strains on each other's symbiont. Both dauerlarvae and axenic J1s were grown on bacteria in Petri dishes (TSY media), the next generation dauers were regained, *Galleria mellonella* larvae were infected, and the bacteria were regained from the next generation of dauers and then determined.

The results are discussed with the aspect of coevolution.

M. LEBUHN[1,2], W. ACHOUAK[2], M. SCHLOTER[1], O. BERGE[2], A. HARTMANN[1], T. HEULIN[2]

Geno- and phenotypic diversity of *Ochrobactrum* sp. isolates from soil and wheat roots

[1]GSF-National Center for Environment and Health, Institute of Soil Ecology, Neuherberg, Germany,
[2]DSV-DEVM, Laboratoire d'Ecologie Microbienne de la Rhizosphére, CNRS-CEA, CEA Cadarche, St. Paul Léz Durance, France

We had carried out a polyphasic taxonomic study on bacterial isolates that were immunotrapped from french soils and from the wheat rhizoplane (see poster "Polyphasic taxonomy of *Ochrobactrum* spp. isolates from environmental samples" by M. Lebuhn, W. Achouak, M. Schloter, H. Meier, O. Berge, A. Hartmann, and T. Heulin). We demonstrated the presence of *Ochrobactrum anthropi*, *O. intermedium*, *O. tritici* sp. nov., and *O. grignonense* sp. nov. in the samples. Here we report on geno- and phenotypic diversity of the immunotrapped *Ochrobactrum* strain collective at different scales of taxonomic resolution, focusing on differences between bulk soil and rhizoplane populations.

Genotypic diversity below the species level were assessed using rep-PCR. Phenotype performances and phenotypic diversity of taxons were compared determining substrate utilization capacity (SUC) and substrate utilization versatility (SUV).

The immunotrapped strains were distributed on 9 major rep-groups. Eight rep-groups and 3 species (*O. anthropi*, *O. intermedium*, *O. grignonense*) were isolated from bulk soil, whereas only 3 rep-groups and 2 species (*O. anthropi*, *O. tritici*) were isolated from the rhizoplane. Six rep-groups of *O. anthropi* were isolated from bulk soil, but only 2 from the rhizoplane. Intra-generic diversity of *Ochrobactrum* as well as intra-species diversity of *O. anthropi* were hence higher in bulk soil than on the wheat rhizoplane.

Our results on SUC and SUV showed that at the intra-species level metabolic capacity and versatility was higher for the bulk soil than for the rhizoplane subpopulations of *O. anthropi*. They also indicated that at the intra-generic level *Ochrobactrum* species from soil had tendentially a higher metabolic capacity and versatility than species from soil.

I. S. MOKHTAR, E. TÓTH, K. MÁRIALIGETI

Changes in the anaerobic bacterial community structure of skin in ewes due to *Wohlfahrtia* myiasis

Department of Microbiology, L. Eötvös University, Budapest, Hungary

Wohlfahrtia magnifica is considered to be one of the most important myiasis causing species of livestock, poultry and humans in several countries in the World. In Hungary up to 40% of animals in sheep flocks may become infected during the summer. Former studies on the microbiology of wound myiasis caused by *Wohlfahrtia magnifica* have clearly demonstrated that alterations in the skin bacterial communities, and the resulting production of the attractant volatiles can be responsible for the initiation of disease.

Healthy skin and myiatic lesion for anaerobic heterotrophic bacterial communities were investigated and compared by numerical taxonomic methods, moreover the volatile and non-volatile fermentation end products of the isolates were investigated. An important shift in the species composition is evident, the amount of strictly anaerobic bacteria usually found in abscesses, etc. is higher in the lesion than in the healthy skin. The strains isolated from the myiatic lesions have a broader potential for volatile acid production than their from healthy skin originating counterparts.

M. N. RESKÓNÉ[1], A. BORSODI[2]

Long-term monitoring of sulphur cycle associated microbial communities in Lake Velencei

[1]Central Danubian Environmental Protection Agency, Székesfehérvár, [2]Department of Microbiology, L. Eötvös University, Budapest Hungary

The Lake Velencei is a shallow lake, which has eutrophicated in a natural way. The most studied period of the lake was in the past one and a half decades. The shallow swampy lake has considerably changed due to the basin recreation. The once reed-covered lake remained undisturbed only on one-third of its area after 1983. As a result of basin recreation the preceding natural habitats have changed or vanished, implicating the rearrangement of the once unique diversity of the lake. Today the lake consists of two, well-separable regions where the reed-covered area with small water surfaces have conserved the sulfuratum characteristics of the lake.

The dry period beginning in the late 80s has triggered off several limnological problems still unsolved. Due to low water level after 1991 the ecological balance of the lake has disequilibrated, a considerable change could have been observed in the water quality (concentration of salts, oxigen-, temperature-, light conditions).

The major part of the organic materials arriving with the water supply is decomposed here, keeping stable the water areas used for tourism.

The dry period starting in the 80s has triggered several limnological questions to solve. The low water level from 1991 caused a shift in the ecological balance of the lake, the water quality has considerably changed (concentration, oxygen, temperature, light conditions), the structure of living companies has rearranged.

Between 1993 and 98 we have studied the microbial sulfur cycle associated with bacteria in connection with sulfate transports in order to interpret the ecological modifications. We have compared and according to chemical caracteristics valued the sulfuratum-like bacteriological results obtained from breeding the bacteria participating in the sulfate circulation in the water and sediment. Tracing the quantitative transformation of microbes present in the sediment structure of the lake we could conclude that during the long period of low water level the frontiers of the sulfuratum-like waters have drafted, the anaerob representatives of the typical companies based on sulfate forms have been present only in the sediment. With the elevation of water level the examined representatives of aerobe and anaerobe groups have reappeared in the water-sediment system. Different regions became characterizable by the ratios compared to each other. Following the quantitative alteration of typical life-communities based on sulfate forms we get a suitable method for a long-term monitoring of the ecological state of the lake.

A. L. KANSOH, A. M-ALI, A. A. EL-GAMMAL

Xylanolytic activities of *Streptomyces* sp.

Microbial Chemistry Department, National Research Centre, Dokki, Cairo, Egypt

Twenty-four different strains of *Streptomyces* sp. isolated from Egyptian soil were tested for their ability to produce extracellular xylanases. Of all these isolates, a *Streptomyces* sp. that had the highest potential for xylanolytic activity was chosen. From various morphological, physiological and antagonistic properties, this isolate was found to belong to *Streptomyces lividans*. Factors affecting xylanase production by this organism in a basal salt medium containing purified sugar-cane-bagasse xylan as a sole carbon source were examined. A noticeable increase in enzyme activity was observed

in the presence of peptone or soyabean meal. However, a slight increase was noticed with ammonium sulfate. Optimum production for xylanase was achieved after five days incubation on a rotary shaker (180 rpm) at 30 °C. The initial pH values were around neutrality. In addition, this organism has high potential for xylanolytic activity when grown on lignocellulosic wastes including corn cobs, wheat bran, peanut shells, sawdust, wheat straw and sugar-cane-bagasse. Partial purification of the enzyme in the culture supernatant was achieved by salting out at 50–80% ammonium sulfate saturation with a purification of 9.03 fold recovery.

M. A. DOW

Validity of Gram reaction of fresh environmental isolates

Department of Microbiology, L. Eötvös University, Budapest, Hungary

During the early days of bacteriology the detection of bacterial cells in tissues was difficult since most of the staining methods used coloured the bacterial cells equally. In 1884 Christian Gram attempted the development of a procedure that would differentially stain "schizomycetes" from tissue cells, which is now known as the Gram staining procedure. This method of staining gained wide application because of its simplicity and quickness in the tentative classification of bacteria into two groups, Gram-positive and Gam-negative. In the age of molecular procedures in bacterial taxonomy one could argue that there is no need anymore for Gram differentiation. We have to realize, however, that most of clinical microbiology laboratories base their diagnostic work on classical phenotypic characterisation, where the "everfirst test" is Gram staining. This situation will not change dramatically in the forthcoming decade.

In our work, different Gram staining methods were compared using authentic strains. KOH test and aminopeptidase were also used as confirmation. The best staining method was chosen, and applied on a broad set of freshly isolated strains. Altogether 216 strains were tested by KOH, aminopeptidase test, and Gram stain. Bacteria were isolated from the gills of eels caught by electric fishing in Lake Balaton. The eels were in part healthy, but in part eel nematode (*Anguillicola crassus*) infected. The bacteria were isolated from gill tissue macerate. All cultures were streaked and repeatedly reisolated to ensure purity and maintained on adequate slant agar. All the strains were subjected to ARDRA grouping, and representative members from each ARDRA group were identified by partial 16S rDNA sequence analysis.

Using the staining procedure on 18 to 24 h cultures, 151 (69.9%) of the strains were found to be Gram-negative, 64 (29%) to be Gram-positive, and only 1 (0.5%) to

be Gram-variable. Comparing the results with the result of sequence analysis, false Gram staining reactions are encountered with genera *Acinetobacter*, *Microbacterium*, *Deinococcus*, *Bacillus* and *Aeromonas*. KOH test gave the best correlation with sequence results. In case of aminopeptidase problems arose with pigmented strains, but in a limited number (2–3%) nonpigmented strains gave also false results.

J. PAPP

Microbiota on leaves of oats grown in lead an zinc mine spoils

Department of Plant Physiology and Microbiology, Babes-Bolyai University, Cluj-Napoca, Romania

Microflora of oats leaves was studied. The oat plants were grown in lead and zinc mine spoils submitted to remediation procedures under laboratory conditions. The colony-forming microorganisms that could be cultivated on malt extract agar used as nutrient medium belongs to six groups: rod-shaped Gram-negative bacteria, nonsporogenous Gram-positive bacteria, endospore-forming Gram-positive bacteria, Gram-positive cocci, yeasts and filamentous fungi. Gram-negative bacteria were the most frequently found microorganisms in the phyllosphere of oats, followed by Gram-positive cocci and filamentous fungi. The nutrient medium made it possible to record a more frequent occurrence of aerobic rod-shaped Gram-positive bacteria and Gram-positive cocci than that expectable based on literature data.

M. VARGHA, G. SZABÓ, K. MÁRIALIGETI

Investigation of the decomposition of atrazine in a bank-wall filtered well model system

Department of Microbiology, L. Eötvös University, Budapest, Hungary

Atrazine is one of the most abundant herbicides in the world. Due to its widespread agricultural usage, it can be detected in soil, subsurface and surface water-flows. Its constant concentration is 1–2 ppb in Danube river. The drinking water of many cities in Hungary, including Budapest, is obtained from the Danube through bank-wall filtered wells. In well water, not even trace amount of atrazine is detectable, therefore it is presumed that the compound is degraded in the gravelbed. However, the investigation of biotransformation and other filtering processes is difficult on site. In

this study, a laboratory model system containing natural sediment sample was constructed in order to examine biodegradation. Validity of the model system was tested by chemical and hygienic measurements, the change of parameters having importance in drinking water quality was followed throughout the system. The results (e.g. significant decrease of nitrite and ammonium ion concentration) show high correlation with the data from previous on site study. Microbial communities of the gravelbed sample were investigated by the characterization and partial identification of microorganisms isolated from the model. Dominant Gram negative strains belong to *Pseudomonas* genus (mainly RNA group I), and include several facultative H_2 autotrophic strains. Gram-positive strains are mainly coryneforms or *Bacillus* species. In general, species composition is similar to natural river sediment communities. In conclusion, the model represents well the gravelbed in every respect.

Atrazine degradation was examined in laboratory and field experiments as well. However, our study can unite the advantages of both methods, since circumstances can be standardized, but the complexity of communities and their metabolism approaches that of natural systems. Microorganism capable of atrazine mineralization were only recently isolated, and are usually *Pseudomonas* spp. In the model, biodegradation of supplied atrazine was detected after induction. Major metabolite was hydroxi-atrazine. Four strains isolated from the sediment sample were found capable of atrazine degradation. Metabolism of the most efficient atrazine degrading strain (identified as *Tsukamurella paurometabolum*) was investigated in details, and it presumably differs from previously known pathways.

H. M. RIFAAT, K. MÁRIALIGETI, G. KOVÁCS

Comparative analysis of actinomycete communities of cattail and papyrus rhizoplane

Department of Microbiology, L. Eötvös University, Budapest, Hungary

Wetlands play an especially important role in diverting plant material towards fossilisation. These habitats act as sinks for the atmospheric carbon, since mineralization of organic materials is inhibited by the effects of the specific local N and S cycles, the anaerobic environment, by low pH value, etc. Therefore it is not surprising that microbial activity was detected to be highest in the rhizosphere of wetland plants.

Cattail samples from a floating mat in the Soroksár Arm of the River Danube and papyrus samples from a floating mat in the River Nile were collected. The "root-

hair" regions of the samples were cut off, washed with aseptically prepared solutions and plated. Using the plate-count technique with three different media suited for the cultivation of actinomycetes, in the case of cattail, an average of 10^3 CFU/g was detected whereas papyrus germ count values were 10^4 CFU/g. All actinomycete colonies were isolated, subjected to purification and differential diagnostic analysis (phenotypical test and partial 16 S rDNA sequencing). Samples taken from the cattail rhizoplane of a floating mat community at Soroksár arm contained approximately equal "amount" of monosporic and polysporic "actinomycetes", whereas monosporic actinomycetes were mostly absent from the papyrus samples. In the former case the dominant actinomycetes were *Str. anulatus*, *Str. albidoflavus, Str. rochei, Micromonospora chalcea* and *M. carbonacea*. In the papyrus rhizoplane *Streptomyces anulatus* and *M. carbonacea* dominated.

The ecological tolerance abilities of the members of dominant groups revealed during laboratory investigation indicate that these bacteria might be active in the rhizosphere and can be present there in their vegetative forms.

B. IMZILN

Incidence of mesophilic *Aeromonas* within aquatic environments in Marrakech, Morocco

Department of Biology, Microbiology Laboratory, Faculty of Sciences Semlalia, Cadi Ayyad University, Marrakech, Morocco

Under arid climate regions, water shortage is the main problem of the population of these areas. Generally, in Marrakech, hurries did not exceed 250 mm per year, and drinking water is generally obtained from fresh waters (river waters, lakes, etc). During the past twenty years, mesophilic aeromonads have been frequently recognized as responsible for several diseases, both in humans and in animals. Indeed, aquatic environments were frequently considered as the major sources of infection by these bacteria. Furthermore, *Aeromonas* bacteria have been recognized able to grow fast in temperate area and when conditions are favourable. The incidence of mesophilic aeromonads in river waters, and sewage outfalls during 12 months in Marrakech, Morocco was determined. Water samples were collected from 8 sites in different geographic areas of the town. Samples of water were collected from fixed sites at three rivers (Oukaimeden, Ourika and Tensift). Samples from open sewers were taken from two representative sites in the spreading field of the town. Bacteriological analysis were done from samples, and *Aeromonas* isolates were identified by standard

biochemical tests and tested for their ability to produce some virulence factors. Densities of *Aeromonas* spp. varied from 10^4 cfu/100 ml to 5.6×10^6 cfu/100 ml. Higher concentrations were recorded in raw wastewater's and the lowest in waters from Oukaimeden river. The proportion of *Aeromonas sobria* to other species increased considerably (especially in warm periods). Among the tested strains, the majority 98.4 and 96% of *Aeromonas sobria* and *Aeromonas hydrophila*, respectively produced haemolysin. Among the *Aeromonas caviae* strains 53% were found to be haemolytic. Survival study demonstrated that *Aeromonas* strains were able to grow in river waters and quickly in river waters supplemented with sewage or organic matter. In conclusion, we think that in warm regions, as Marrakech, the presence of mesophilic aeromonads with great concentrations in several aquatic ecosystems may be a serious risk of infection and consequently will be a reason behind the restriction of moult-uses of these waters. Further investigations on the fate and vulnerability of mesophilic aeromonads in aquatic environments, where the risk of direct infection to humans is high, are required.

A. HALBRITTER[1], V. KRISTUFEK[2]

Milestones of microbial ecology and the key personalities behind

[1]Department of Microbiology, L. Eötvös University, Budapest, Hungary, [2]Institute of Soil Biology, Czech Academy of Science, Ceske Budejovice, Czech Republic

Microbial ecology emerged as an energetic and dynamic branch of science only in the early 1960s, and as such new and fast developing sciences looks into the future. The history of microbial ecology is therefore not widely known even among microbial ecologists, although their subject's position among other branches, the research topics, and fundamentals cannot be fully understood, only in historical context. 'Ecologists' think only in macro-organisms, and the studies in the history of microbiology concentrated on fields like medical microbiology or microbial genetics. But many of the concepts and paradigms of ecology were born in the field of microbiology, enough to mention that even the term 'ecology' in its current use was first defined by a protistologist, Ernst Häeckel.

As microbial ecology is getting more and more role in university education, we tried to look up material for this topic and build up a short introduction for students of biology. As a skeleton of the history of microbial ecology we have chosen some main ecologically important topics, namely the *existence, role, physiology* and *communities* of microbes and tried to follow the development of the knowledge on these problems.

Among the hundreds of scientists who deserve salute we included only those, whose name can be connected to a main step of the elucidation of the problems. Some of them were not microbial ecologist *sensu stricto* but all they (even the earliest pioneers) shared an ecological approach to the problems.

G. FURGANI, D. TRIGA, H. PAMJAV, E. SZÁLLÁS, A. FODOR

Gnotobiological analysis of *Steinernema/Xenorhabdus* symbiotic complexes

Department of Genetics, L. Eötvös University, Budapest, Hungary

The RFLP patterns of the PCR amplified internally transcribed spacer (ITS1 - 5-8S rRNA and ITS2) region the 18S - 28S rRNA genes 18 strains of 12 entomopathogenic nematode (EPN) species belonging to the *Steinernema* genus and those of amplified internally spacer region of their bacterial (*Xenorhabdus*) symbionts were determined. Species specific patterns were identified reproducibly and the cospeciation is proved. We accomplished a large-scale gnotobiology study by growing nematode strains on each other's symbiont. Both dauerlarvae and axenic J1s were grown on bacteria in Petri dishes (TSY media), the next generation dauers were regained, *Galleria mellonella* larvae were infected, and the bacteria were regained from the next generation of dauers and then determined.

The results are discussed with the aspect of coevolution.

A. MICSINAI, A. HORVÁTH, A. K. BORSODI

The bacterial communities inhabiting the rhizomes of healthy and degrading reed stands

Department of Microbiology, L. Eötvös University, Budapest, Hungary

Rhizome samples from healthy and degrading reed (*Phragmites australis* /Cav./ Trin et Steudel) stands were taken in November 1998. Endophytic and outer surface attached bacteria were isolated using the serial dilution and plating method. The obtained 246 bacterial strains were subjected to phenotypic testing, numerical analysis and BIOLOG identification.

The results have shown that there is a marked difference in the bacterial community structure of the healthy and degrading reed rhizomes. The outer surfaces of

healthy rhizomes are characterized by *Aeromonas media*, *Enterobacter* sp. and fluorescent pigment producing *Pseudomonas* sp. *Aeromonas media* and *Pseudomonas* sp. can also be found on the inner surfaces together with a group of unidentified Gram positive, facultatively fermentative bacteria.

Rhizomes from degrading reed stands, however, contained *Aeromonas veronii/sobria*, *Erwinia* sp., a group of unidentified, facultatively slow fermenting bacteria and another group of unidentified, pigmented bacteria exhibiting cell cycle. Bacteria isolated from the inner surfaces of the degrading reed rhizomes belonged mainly to the latter group.

The differences detected in the community structure might explain some aspects of the reed decline. The presence of fluorescent pseudomonads in the healthy stands might contribute to the resistance of the plants. Their disappearance in the degrading reed and the occurrence of several facultatively fermentative bacteria can either be an indication or a consequence of the premature rotting of rhizomes.

PHYSIOLOGICAL AND MOLECULAR BASIS OF METABOLITE OVERPRODUCTION

L. KARAFFA, K. VÁCZY, E. SÁNDOR, I. PÓCSI, A. SZENTIRMAI

Intracellular peroxide concentrations correlate with cyanide-resistant alternative respiration in *Acremonium chrysogenum*

Department of Microbiology and Biotechnology, Lajos Kossuth University, Debrecen, Hungary

Besides the cytochrome pathway, the filamentous fungus *A. chrysogenum* also exhibits a cyanide-resistant, salicylhidroxamic acid (SHAM) sensitive, non-phosphorylating alternative respiration. It has been reported that upon addition of inhibitors of the cytochrome pathway high rates of H_2O_2 production in mitochondria could be observed. On the other hand, inhibition of the cytochrome pathway normally results in enhanced cyanide-resistant respiration. A correlation between alternative respiration and the presence of peroxides in the culture medium has already been demonstrated in higher plants like *Petunia hybrida* and in the yeast *Hansenula anomala*. In this work we will show that enhancement of the intracellular peroxide concentration, either resulted from the direct addition of H_2O_2 or from a facilitated production due to the inhibition of the enzyme catalase by salicylic acid, coincides with an increased cyanide-resistant alternative respiration in *A. chrysogenum* and has a severe impact on the basic growth parameters (biomass production, substrate consumption, growth yield) of cells.

L. KARAFFA, E. SÁNDOR, A. SZENTIRMAI

Chemostat – a powerful tool in basic research

Department of Microbiology and Biotechnology, Kossuth Lajos University, Debrecen, Hungary

Growth and product formation in batch culture is a process that always terminates after some finite and usually relatively short time interval. Altering the batch process by continuously supplying fresh nutrient medium to a well-stirred culture and simultaneously withdrawing the broth containing cells and products results in a culture called chemostat, in which growth can be maintained for prolonged periods. Furthermore, a steady-state can be maintained, thus the cell concentration, specific growth rate and culture environment (e.g. nutrient and product concentrations) do not

change with time. As a consequence, chemostat culture provides a unique tool for investigating the response of microorganisms to their environment and for elucidating the relations between an organism and its environment.

In this lecture we will focus on the changes in the morphology and differentiation of the fungus *Acremonium chrysogenum*, that can be characterized by dimorphism. In batch culture a progressing fragmentation and also a significant vacuolation process can be observed resulting in wide, swollen cells at the stationary phase in contrast with the filamentous morphology of the exponential phase. It was established that the fragmentation process is due to changes in specific growth rate during cultivation. On the other hand, vacuolation depends on the sugar consumption rate rather than the growth rate. The importance of glucose-limited chemostat cultures in differentiating between the two phenomene is discussed.

E. SÁNDOR, L. KARAFFA, A. SZENTIRMAI

Acridine orange a sensitive indicator of metabolic activity

Department of Microbiology and Biotechnology, Lajos Kossuth University, Debrecen, Hungary

The fluorescent dye Acridine Orange (AO; 3,6-bis[dimethylamino]acridine) penetrates through membranes in a neutral form and is accumulated (trapped) as a protonated form inside acidic compartments (e.g. vacuoles). At low concentrations, AO fluoresces at 530 nm, which appears green. In compartments with AO concentrations above appr. 500 μm, a metachromatic shift occurs and the fluorescence emission shifts to 660 nm, giving an orange-red colour. Several attempts were made in order to elucidate the physiological significance of this change in colour.

In the present work we will demonstrate that Acridine Orange is suitable for the assessment of the metabolic activity of filamentous fungi. The quantitative and visible method is based on the reversible red-green shift in the colour of the dye, quantified by image analysis and was tested with *Acremonium chrysogenum* and *Aspergillus nidulans*. It was established that under circumstances leading to high substrate uptake and a subsequently high biomass production and specific growth rate, cells exhibit an overhelmingly green colour, while under carbon starvation leading to low biomass production and specific growth rates, they look orange-red. To our knowledge, this is the first application of a fluorescent dye for the estimation of the metabolic activity of whole mycelia and the first – although indirect – evidence regarding the connection between the Acridine Orange staining and the physiological state of cell.

C. P. KUBICEK, J. STRAUSS

Carbon catabolite repression in *Aspergillus nidulans*

Institut für Biochemische Technologie, Sektion Mikrobielle Biochemie, TU Wien, Vienna, Austria

For a vast array of microbes, carbon response mechanisms ensure that catabolism of glucose results in severe repression of genes encoding enzymes for the degradation of alternative carbon sources. Among eukaryotic microorganisms, carbon catabolite repression has mainly been studied in the yeast *Saccharomyces cerevisiae* and to a lesser extent in filamentous ascomycetes such as *Aspergillus nidulans*. In the latter organism, carbon catabolite repression participates in the regulation of the expression of genes required for the utilization of carbon sources such as starch, cellulose, hemicellulose or pectin, and is thus of industrial relevance. Although there is much more known on the respective events in *S. cerevisiae*, carbon catabolite repression in *A. nidulans* appears to differ in the latter in a number of important aspects, such as structure and function of the DNA-binding protein (Mig1p vs. CreA) and signalling to it, thus necessitating studies with this organism. This review will focus on our recent work on the mechanism of functional activation of CreA by glucose, and on genes/proteins involved in this process.

Z. NAGY[1], A. SZENTIRMAI[1], S. BIRÓ[2]

Purification and properties of β-galactosidase from *Penicillium chrysogenum*

[1]Department of Microbiology and Biotechnology, Kossuth University, Debrecen, Hungary,
[2]Department of Human Genetics, University Medical School, Debrecen, Hungary

β-galactosidase is a well-known enzyme that hydrolyzes lactose. It is widely distributed in nature and many studies have been reported on the physiology and regulation of the enzyme from different sources, including bacteria, yeasts, moulds, plants and animals.

In *P. chrysogenum* the synthesis of the enzyme is induced by lactose and repressed by glucose. We studied the mechanism of induction/repression of the enzyme and found an interesting correlation with cAMP level in this species.

We also describe a procedure for the rapid purification and some molecular properties of an intracellular β-galactosidase of *P. chrysogenum*.

When *P. chrysogenum* is grown on lactose as carbon source, it synthesizes one active form of β-galactosidase. The enzyme was purified by ammonium sulphate

precipitation and substrate affinity chromatography. Its homogeneity was confirmed by SDS/PAGE. β-galactosidase of *P. chrysogenum* is a stable enzyme with an optimum pH value of 4.0 and an optimum temperature of 30 °C.

S. BIRÓ[1], ZS. BIRKÓ[1], A. SÜMEGI[1], A. VINNAI[1], G. WEZEL[3], F. SZESZÁK[1], S. VITÁLIS[1], P. SZABÓ[2], Z. KELE[2], T. JANÁKY[2]

Characterisation of the gene for factor C, an extracellular signal protein involved in morphological differentiation of *Streptomyces griseus*

[1]Department of Human Genetics, University Medical School of Debrecen, Hungary,
[2]Department of Medicinal Chemistry, Albert Szent-Györgyi Medical University, Szeged, Hungary,
[3]Department of Biochemistry, Leiden University, Gorlaeus Laboratory, Leiden, The Netherlands

The gene encoding factor C (*facC*), an extracellular signal protein of cellular differentiation was cloned from *Streptomyces griseus* 45H and the complete nucleotide sequence was determined. The deduced amino acid sequence was also confirmed by high performance liquid chromatography/electrospray ionization – mass spectrometry analysis. The full length protein consists of 324 amino acids with a molecular mass of 34 523 Da. The mature extracellular 286 amino acid protein (M_r 31 038) probably was produced by cleaving off a 38 amino acid secretion signal sequence. Southern hybridization revealed the presence of the gene for factor C (*facC*) in several other *Streptomyces* strains but searching of the databases failed to identify a protein with significant homology to factor C. The effect of the expression of factor C from low and high copy number vectors in different *S. griseus* strains on cytodifferentiation and antibiotic production and its possible role will be discussed.

G. H. KELEMEN, M. J. BUTTNER

Transcriptional switching during morphogenesis in *Streptomyces coelicolor* A3(2)

John Innes Centre, Norwich, UK

Two alternative sigma factors, encoded by *whiG* and *sigF*, play important roles in the transformation of the multigenomic aerial mycelium into chains of unigenomic spores in *Streptomyces coelicolor*. The initiation of sporulation is dependent on *whiG*, while *sigF* is involved in spore maturation after sporulation septa have been laid down.

Transcription studies performed on both these genes in wild type and in several *whi* mutants showed *whiG* transcription to be independent of known sporulation genes, while *sigF* transcription is dependent on all six known early sporulation (*whi*) genes. However, *sigF* is not directly dependent on *whiG*, suggesting an involvement of other sigma factors in sporulation.

In a search for suitable candidates, a new group of genes homologous to *sigF* has been identified and three of them have been sequenced. The genes encode alternative sigma factors that belong to the group of sporulation specific and stress response sigma factors of *Bacillus subtilis*. Several of the new sigma factor genes are potentially part of polycistronic operons and they are preceded by sequences coding for putative anti-sigma factors. The biological functions of these new sigma factors and their activation are currently being investigated and their potential involvement in differentiation will be discussed.

A. PENYIGE[1], GY. BARABÁS[2]

The involvement of GTP-binding proteins in triggering morphological differentiation and antibiotic production in *Streptomyces griseus*

[1]Institute of Medical Biology, Albert Szent-Györgyi Medical University, Szeged,
[2]Institute of Humangenetics, University Medical School Debrecen, Hungary

Previously we have shown that Streptomyces strains possess GTP-binding proteins (GBPs). Stimulation of GBPs lead to restoration of sporulation and antibiotic production in a bald mutant of *S. griseus*. In a search to identify possible signal transduction pathways triggered by stimulated GBPs, we have found two possible targets in the bald mutant.

(i) The activity and substrate specificity of the ADP-ribosyltransferase (ADPRTase) enzyme was altered as a consequence of stimulation of GBPs. ADPRTase is responsible for ADP-ribosylation – a posttranslational modification – of proteins. Previously this enzyme was shown to be involved in the regulation of sporulation in *S. griseus*.

(ii) Stimulation of GBPs induces an immediate depolarization of the membrane potential in the bald mutant. This electric signal might regulate voltage-gated ion channels in the cellular membrane of cells. We have found that the flux of Ca^{2+} ions was affected by the state of membrane potential. It is well known that the intracellular Ca^{2+} concentration is an important factor affecting the differentiation of *Streptomyces*

species Therefore we suggest that modulation of the membrane potential serves as a regulatory signal during the morphological differentiation process.

Interestingly, we have also found that A-factor potentiates the effect of GBPs.

R. MARITS[1,2], D. FLEGO[2], A. ERIKSSON[2], V. KŐIV[1,2], M-B. KARLSSON[2], E. T. PALVA[3]

A two-component regulatory system *peh*R*peh*S, controls endopolygalacturonase production and virulence in the plant pathogen *Erwinia carotovora* subsp. *carotovora*

[1]Institute of Molecular and Cell Biology, University of Tartu, Tartu, Estonia, [2]Department of Plant Biology, Uppsala Genetic Center, Swedish University of Agricultural Sciences, Uppsala, Sweden, [3]Department of Biosciences, Division of Genetics, University of Helsinki, Helsinki, Finland

Bacteria have evolved several mechanisms to sense environmental changes and to regulate its own gene expression accordingly. The pathogenicity of the major plant pathogenic enterobacterium *Erwinia carotovora* subsp. *carotovora* (*E. c.* subsp. *carotovora*) is correlated with its ability to produce and secrete several extracellular enzymes that can attack components of the plant cell wall. The synthesis of the corresponding extracellular enzymes is regulated by complex regulatory network involving both global and enzyme-specific factors and is also responsive to several environmental stimuli. Virulence in the *E. c.* subsp. *carotovora* has been shown to be globally regulated by a small diffusible signal molecule called the autoinducer. In addition we have isolated, sequenced and characterized the two-component regulatory system (*pehR pehS*) from *E. c.* subsp. *carotovora,* which is involved in specific positive regulation of PehA (endopolygalacturonase) production. The mutations in either *pehR* or *pehS*, genes caused reduced virulence capability on the tobacco seedlings. The *pehR* (regulatory component) and *pehS* (sensor component) genes are similarly organised in an operon like *phoP-phoQ* regulatory systems of *Escherichia coli* and *Salmonella typhimurium*. The amino acid sequence of PehRS and PhoPQ are highly conserved except the regions, which may correspond to the periplasmic loop. Functional similarity of *pehR pehS* and *phoP phoQ* has been shown by genetic complementation.

E. SÁNDOR[1], A. JUHÁSZ[1], L. KARAFFA[1], G. C. PAUL[2], I. PÓCSI[1], C.R. THOMAS[2], A. SZENTIRMAI[1]

Assessment of metabolic activity of filamentous fungi using acridine orange

[1]Deparment of Microbiology and Biotechnology, Lajos Kossuth University, Debrecen, Hungary,
[2]School of Chemical Engineering, The University of Birmingham, Birmingham, UK

Acridine Orange (AO; 3,6 bis [dimethylamino] acridine) fluoresces at 530 nm which appears green and also at 660 nm, giving a red colour. In this study we tried to establish whether there is a correlation between the physiological activity (respiration, specific growth rate, biomass production) of *Acremonium chrysogenum* and *Aspergillus nidulans* cultures and their colour upon AO staining.

In the exponential phase of batch cultures, mycelia exhibited an overwhelmingly green colour, whereas stationery phase could be characterized with cells stained red. The ratio of areas stained with red and green as a function of cultivation time exactly covered the growth curve of cultures.

In fed-batch cultivation, stationary phase cells supplied with glucose re-initiated growth, enhanced respiration rate and looked green-like upon AO staining. Glucose exhaustion restored all these parameters, but the whole process could be repeated. Addition of a carbon source with different type of catabolism (soybean oil) also coincided with green colour upon AO staining.

Supplying the cultures with different levels of oxygen by modifying the oxygen transfer rates resulted in a positive correlation between Kla values and the ratio of the green colour.

In chemostat cultures, specific growth rate was inversely proportional with the ratio of areas stained with red and green, respectively.

M. VASTAG, G. KRISZTINA, T. PAPP, K. ÁCS, CS. VÁGVÖLGYI

The antifungal activity of lovastatin against *Rhizomucor* strains

Department of Microbiology, Attila József University, Szeged, Hungary

The members of the genus *Rhizomucor* (Zygomycetes) are distinct from *Mucor* by their thermophilic nature. These fungi are of value in both theoretical and applied microbiology. They are good producers of different extracellular enzymes, while in other cases they may be the agents of frequently fatal opportunistic mycotic diseases.

Lovastatin (mevinolin) was discovered as a cholesterol-lowering fungal metabolite in an *Aspergillus terreus* culture. Its action is connected with the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyzes an early, rate-limiting step in the biosynthesis of sterols and isoprenes. In the present study, the antifungal activity of lovastatin against 24 *Rhizomucor* strains representing three species (*R. pusillus, R. miehei* and *R. tauricus*) was investigated. The inhibitory effect of lovastatin on colony radial extension was different under different culturing conditions: it was less effective at lower pH and on complex media. A substantial strain-to-strain variability in the sensitivity was also detected, however, in all the conditions tested, the *R. pusillus* strains revealed greater sensitivity to lovastatin than the investigated *R. miehei* strains.

M. N. DAVYDOVA, F. K. MUKHITOVA

Overproduction of extracellular hydrocarbons by *Desulfovibrio desulfuricans*

Kazan Institute of Biochemistry and Biophysics, Kazan Scientific Centre, Academy of Science of Russia, Kazan, Russia

It is known that sulfate reducing bacteria when growing on organic substrates can produce oxygen-containing and gaseous products. Hydrocarbons are present in small amounts in culture media. It will be stressing for bacteria when growing with lactate at law concentration of CO (up to 5%). The appreciable increase of reduced NADP is observed. Under these conditions the extracellular organic compounds have been found in the growth media. The long-chain hydrocarbons have been synthesized by cell suspensions of *D. desulfuricans* in the presence of lactate or acetate in the atmosphere of 10% CO + 10% H_2. The results of inhibitor analysis have shown that synthesis was not limited by ATP. When the reduced equivalents (NADH or NAD(P)H) were added the yield of the reaction products increased. One can suppose that the excess of reduced equivalents in the cells of *D. desulfuricans* induced the processes directed towards their spilling.

IVANOVICS MEMORIAL SESSION

E. FARKAS

My always respected good friend György Iványovics

The author briefly recalls those difficult decades following the Second World War, when he cooperated in many different ways with Professor Iványovics. He speaks about the help provided to the Virological Department under his leadership, in so many aspects by the mutual professional and moral support. He will always remember the very successful and high-level work of Professor Iványovics in leading the Hungarian Society for Microbiology and as editor-in-chief of Acta Microbiologica Hungarica. The author came to recognize Professor Iványovics as an outstanding organizer and in all respects a totally dedicated human being.

S. KOCH

György and the viruses. Post-war Hungarian virology initiated by György Iványovics

This is a deliberately subjective commemoration of my Master and paternal friend György, with whom I had the privilege of working from 1945 till 1953. He remained my first and sharpest critic and a reliable friend until his death in September 1980. For ever I shall remain obliged to him for all he taught me as a scientific leader, a "boss", and last but not least an experienced mentor in the affairs of life. His deep and comprehensive knowledge of microbiology as a whole, his persistent interest in Science, his indefatigable participation in the everyday laboratory work, and his great sense of humour made him an exemplary personality for all his students, followers and friends. His minor venial weaknesses made him even more human and brought him even closer to those able to appreciate his qualities. As a scientist, he was not only the founder of the post-war Hungarian school of microbiologists, but also a highly reputed personality both in Hungary and abroad, as illustrated by the numerous honours he was awarded.

L. ALFÖLDI

The megacin story

Institute of Genetics, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary

In 1953, Professor Ivánovics was occupied with the isolation of *Bacillus megaterium* strains from natural habitats and their screening for vitamin B_{12} producers. He was puzzled by the behaviour of one strain (No. 216), which often lysed spontaneously. This strain proved to be the first bacteriocin-producing Gram-positive bacterium. He found the phenomenon so interesting that he studied megacin and megacinogeny for the next 25 years, until the end of his active life. Even so, some aspects of megacinogeny remain unresolved at the present time.

I. BEREK

The story of the biochemical and genetic analysis of porphyrin biosynthesis in *Bacillus subtilis*

Department of Food Technology and the Environment, College of Food Industry, Attila József University, Szeged, Hungary

The story of studies of porphyrin biosynthesis in *Bacillus subtilis* started in 1965 with the activities of Professor György Ivánovics and his staff. Numerous porphyrin auxotrophic mutants were isolated from the 168 trp C2 strain of *Bacillus subtilis* by selection with streptomycin. Some of them could be supplemented with ALA, while the majority grew only in the presence of hemin. Among the latter strains, the syntropism test allowed the distinction of two groups differing in phenotype, viz. feeders accumulating ALA and non-feeders accumulating not ALA but other porphyrin intermediates. On the basis of transductional studies, feeders and non-feeders could be divided into two and four groups, respectively. Biochemical investigation revealed that, with one exception, one enzyme of the porphyrin biosynthesis was coordinated to each hem locus. The following genes were identified: hemA – ALA synthetase, hem B – ALA dehydrase, hem C – PBG deaminase, hem D – uroporphyrinogen cosynthetase, hem E – uroporphyrinogen decarboxylase, hem F – coproporphyrinogen oxidase, and hem G – protoporphyrin iron chelatase. Mapping of the hem genes demonstrated that the hem A, hem B, hem C, and hem D loci are located on the left replication arm of the *Bacillus subtilis* chromosome, and the other known hem loci on the right arm.

I. B. HOLLAND

The secretion of *E. coli* haemolysin by the type I, ABC-dependent mechanism

Institut de Génétique et Microbiologie, Université Paris-Sud, Orsay, France

Type I secretion from *E. coli* involves a transenvelope translocator composed of the inner membrane protein HlyB, and the inner membrane protein HlyD, which spans the periplasm to interact with the third protein TolC, an outer membrane pore. Haemolysin, HlyA, a 107 kDa toxin (forming Ca^{2+}/K^{+} channels in target membranes), docks with the translocator using a 46 amino acid secretion signal, at the extreme C-terminus of the HlyA molecule. Secretion is extremely rapid and direct to the external surface, without any periplasmic intermediate. HlyA is apparently unfolded during translocation and the C-terminal secretion signal, which is not removed during transport, is involved in refolding the toxin before it leaves the surface. We have recently obtained evidence that HlyD may have two functions, one to provide a pathway across the bacterial envelope, and also to provide a multimeric folding chamber, providing a chaperone-like function, perhaps in association with TolC. HlyB is the bacterial prototype of an ABC-(ATPase) transporter, a member of the super family of proteins, including Mdr (P-glycoprotein), Mrp and CFTR, which are important in human disease. HlyB provides energy for transport through its highly conserved, cytoplasmic ATPase domain. We are currently analysing the 3-dimensional structure of this domain and this structure and its possible function will be discussed.

I. DÖMÖK

Prospects in eradication of wild poliovirus

National Centre for Epidemiology, Budapest, Hungary

The global eradication of poliomyelitis means the complete eradication of wild and potentially wild polioviruses. The essential activities are as follows. (1) Interruption of the transmission of wild polioviruses by (a) a strong routine immunization programme; (b) national immunization days; and (c) "mopping-up" immunizations. (2) Certification of the polio-free status by AFP and/or alternative surveillance. (3) Laboratory containment of wild polioviruses. (4) Discontinuance of immunization with OPV and containment of vaccine and vaccine-derived strains.

Substantial results have already been achieved in the interruption of transmission and in the process of certification. In the American Region of WHO, the last indigenous polio case occurred in 1991 (Peru); in the Western Pacific Region, no polio case due to wild poliovirus has been detected since 1997; and in the European Region, all the countries (50) have remained free from poliomyelitis, except Turkey in 1998. The South-East Asian and African Regions have remained the major reservoirs. The certification process is under progress in non-endemic countries controlled by the Global Commissions of WHO, with contributions from the Regional Commission and National Committees. The laboratory containment of polioviruses will be essential, since their possible introduction from laboratories into the community has been documented. Strategies to stop vaccinations with OPV depend on the persistence of vaccine-derived strains in the population and on the prevalence of long-term excretors among immunodeficient persons. At any event, after cessation of vaccinations, even vaccine-derived strains and stockpiled OPV should be under maximum laboratory containment.

A. DOBOZY, L. KEMÉNY, R. GYULAI

Human herpesvirus type 8 in angiogenic tumors

Department of Dermatology, Albert Szent-Györgyi Medical University, Szeged, Hungary

The discovery of human herpesvirus 8 (HHV8) in Kaposi's sarcoma (KS) tissue from AIDS patients has opened up new vistas in virology and oncology. HHV8 DNA has been found in all forms of KS, suggesting that it might be involved in the pathogenesis of KS. Additionally, HHV8 has been detected in both malignant and benign lymphoproliferative diseases, such as body cavity-based B-cell lymphomas and multicentric Castleman disease. The association of KS with HHV8 raised the question, whether HHV8 can also be associated with other endothelial cell-derived vascular neoplasms. Benign vascular lesions (hemangiomas, lymphangiomas, pyogenic granulomas and hemangiopericytomas) were found not to contain HHV8 DNA. However, there are contradictory data concerning the presence of HHV8 in angiosarcomas. In contrast with previous reports, we detected HHV8 sequences in patients with angiosarcoma of the face. The seemingly contradictory findings might be due to differences in the samples examined. Additionally, we could detect HHV8 DNA in angiolymphoid hyperplasia with eosinophilia (ALHE). This benign disorder is characterized by multiple soft angiomatous tumors, usually appearing on the face, ear or scalp. The main histological feature of ALHE is the proliferation of atypical

endothelial cells (as seen in KS) accompanied by eosinophilic infiltration in the dermal and subdermal connective tissue. The presence of HHV8 in both benign and malignant proliferations of endothelial cells suggests that the virus alone is not sufficient to produce a specific lesion.

J. FÖLDES

Effects of gyrase-inhibiting fluoro-quinolones on transformation and transfection in cells of *Bacillus subtilis*

Department of Clinical Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary

The quinolones inhibit gyrase in bacterial cells, disturb the equilibrium of the biological processes and at the same time induce RecA protein for repair. Transformation and transfection reactions appear useful to study the mode of action of the quinolones. Transformation and transfection of competent cells of *Bacillus subtilis* 168 I^- were performed with the DNA of the wild strain and the SP50 phage. Ciprofloxacin, Ofloxacin and Pefloxacin were added at subinhibitory concentrations to the systems in different stages of the genetic process. The rate of transformation was calculated and the kinetic curve of transfection was constructed. The quinolones do not inhibit the uptake of DNA and the recombination process in the transformation. The number of transformants correlates with the decrease in growth rate of the bacterial cells. The gyrase inhibitors stop phage synthesis in any stage of transfection and continuously decrease the number of phages produced. The quinolones exhibit quantitative differences in their action on transfection.

J. MOLNÁR, GY. GUNICS, CS. MISKOLCI

Models for reversal of resistance in bacteria and fungi

Department of Clinical Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary

Antibiotic resistant pathogens are emerging in the population. Many of the potent antibiotics and antitumor drugs have lost their effectiveness in the last decades. Antibiotic resistant bacteria are known to be responsible for more than 50% of hospital acquired infections. The multiple resistance means that patients with infections are ill for a longer time and they are at a greater risk of dying. At the same time too few drugs

are developed to replace those which have lost their effectiveness. Therefore new combinations of compounds are needed to overcome drug resistance.

There is an urgent need to analyse the nature of interaction between chemotherapeutics and resistance reversing compounds. In model experiments the extrachromosomal genetic code of prokaryotes and eukaryotes was eliminated from bacteria and yeast by some heterocyclic compounds. Heterocyclics can affect DNA and membrane functions as well. As a consequence, some representative compounds were able to synergyze the effects of a few antibiotics. The resistance reversing effect was dependent on the chemical structure, and stereospecificity of the resistance modifiers. The mechanisms of synergy between chemotherapeutics and resistance modifiers will be discussed.

A. MICZÁK[1,2], K. HÖNER ZU BENTROP[1], D. G. RUSSELL[1]

Lipid catabolism and intracellular survival of mycobacteria

[1]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, USA; [2]Department of Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary

Pathogenic mycobacteria survive and replicate in macrophages. Phagosomes containing these bacteria do not fuse with lysosomes and the vesicles are only mildly acidified. In the search for the mechanisms by which pathogenic mycobacteria survive in the host macrophages, several intracellularly expressed genes have been identified. One of these genes codes for isocitrate lyase (Icl). The *Mycobacterium tuberculosis CSU93 icl* gene was cloned, expressed in *E. coli* and purified. Icl plays a key role in the glyoxylate cycle and is essential as an anapleurotic enzyme for growth on acetate and certain fatty acids as carbon sources. Its production and activity are enhanced under minimal growth conditions when supplemented with acetate or palmitate. In addition to Western–blots and enzyme assays, its expression in *Mycobacterium tuberculosis* under different growth conditions was also monitored by fusing the protein with green fluorescent protein under regulation of its own promoter.

To elucidate the glyoxylate bypass in more detail, we cloned the gene for malate synthase, too. Its expression in *E. coli* results in an active enzyme. In contrast with Icl, it appears to be expressed constitutively.

M. KÁLMÁN[1], E. SZÖLLŐSI[1], A. FÓNAGY[1], M. ZIMÁNYI[1], L. LACZKOVICS[2]

Examination of serum antibodies to *Campylobacter jejuni.* ssp. *jejuni* and GM_1 antibodies in peripheral neuropathy

[1]Institute of Public Health and Medical Officer Service, [2]Department of Experimental Surgery, Albert Szent-Györgyi Medical University, Szeged, Hungary

The Guillain-Barré syndrome (GBS) is the most common cause of peripheral paralysis. Two-thirds of GBS patients develop the syndrome following various infections, the leading cause being campylobacter enteritis. Patients with GBS subsequent to *C. jej.* ssp. *jej.* enteritis frequently exhibit a high antibody titer to GM_1 ganglioside, probably through molecular mimicry between ganglioside and the LPS of *C. jej.* ssp. *jej.* isolated from GBS patients. We examined serum antibodies (IgGAM, IgG and IgM) to *C. jej.* ssp. *jej.* and to GM_1 ganglioside by ELISA. The serum samples were collected from patients with GBS, meningitis, encephalitis or acute flaccid paralysis, from healthy young adults, some working under conditions of risk (poultry-processing factory) and from *C. jej.* ssp. *jej.*-positive persons (with enteritis, but without GBS). Elevated GM_1 antibody titers were detected in 6 of 7 GBS patients demonstrating high IgGAM antibody titers to *C. jej.* ssp. *jej.* The serum of a patient with encephalitis displayed a high IgGAM titer to *C. jej.* ssp. *jej.* and to GM_1 ganglioside, but this patient had received DIPERTE vaccine 5 days earlier. 7% of the healthy adults and 59% of those working under conditions of risk demonstrated a high IgGAM titer to *C. jej.* ssp. *jej.*, but the sera of only a few of those working under conditions of risk exhibited a low antibody titer to GM_1 as well. The sera of *C. jej.* ssp. *jej.*-positive patients without GBS showed a high IgGAM titer, but no antibody to GM_1 ganglioside was detected.

E. NAGY, J. SÓKI, E. FODOR, E. URBÁN, I. SZŐKE

Antimicrobial resistance in anaerobic bacteria

Department of Clinical Microbiology, „Albert Szent-Györgyi" University Medical School, Szeged, Hungary

Human pathogenic anaerobic bacteria were susceptible until a few years ago to antimicrobial agents used in profilaxis and treatment of anaerobic infections. However, during the past years resistance to certain antianaerobic drugs were reported more frequently from different parts of the world. The Etest method (more reliable than the

disc diffusion method) became available for routine laboratories to evaluate the resistance of clinical isolates to the most widely used antibiotics.

Antibiotic resistance due to beta-lactamase activity is observed most frequently among *B. fragilis* group strains, however more and more isolates belonging to *Prevotella*, *Porphyromonas* and *Fusobacterium* were reported to be resistant to different antimicrobial agents. The metallo-beta-lactamase production of *B. fragilis* is responsible for the resistance to carbapenems. The presence of the *cfi*A gene, coding for the production of this enzyme, was detected by PCR among carbapenem susceptible strains in different parts of Europe including Hungary. Decreased permeability and modification of the PBPs was also reported as the cause of resistance to cefoxitin, ureidopenicillins and inhibitor combinations among our isolates. Clindamycin resistance among clinically important anaerobes vary in different parts of the world. Low number of resistant strains among Gram-negative anaerobes to metronidazole were also detected in different countries. Indirect resistance to metronidazole was observed in our isolates if *Enterococcus* strains were co-cultured. The presence of the *nim* gene found in mentronidazole resistant *Bacteroides* strains was detected in enterococci. According to the *in vitro* data, the newest fluoroquinolons, such as trovafloxacin, clinafloxacin moxifloxacin and grepafloxacin have considerable antianaerobic effect.

I. M. VARGA, A. P. BATTEN

Fulminant cerebral listeriosis

K-W Hospital, Kitchener, Ontario, Canada

Listeria monocytogenes (LM) infection in adults usually involves the central nervous system (CNS). In Canada, about 90% of the cases have one or more known predisposing factors, somehow facilitating LM invasion.

We present a fulminant case of cerebral listeriosis which caused the death of the patient before the proper diagnosis was established.

Listerial cerebritis simulating a cerebrovascular accident is known to occur, but is not widely recognized or described. Listerial CNS infections are usually subacute, but this case demonstrates that a fulminant course is also possible. Establishment of the correct diagnosis depends on the bacterial culturing of blood or spinal fluid. In the climate of cost-cutting, these cultures may not even be considered and a false diagnosis may be readily accepted. The blood cultures in the moribund state of this patient were

actually ordered to exclude infection, as no infective signs and symptoms appeared until the last hours of the patient's life.

Only speculations may be made as concerns the numbers of false diagnoses of similar patients, as there may be more of them than are recognized. With appropriate clinical suspicion and bacterial cultures, the lives of these patients may be saved.

G. ÁDÁM[1], M. GORJANACZ[1], I. TÖRÖK[2], B. M. MECHLER[2], I. KISS[1]

The role of the OHO-31 protein, an importin-α homologue, in the oogenesis of *Drosophila melanogaster*

[1]Institute of Genetics, Biological Research Center, Szeged, Hungary, [2]German Cancer Research Center, Heidelberg, Germany

The importin-α (I-α) protein plays a crucial role in the import of proteins into the nucleus as an adaptor, which binds cargo proteins to the importin-β receptor. The I-α homologue gene, *oho31,* was identified by P transposon-mediated mutagenesis in *Drosophila.* In the wild type, OHO31 is mainly expressed in the ovary and the early embryo. The intragenic deletion $oho31^{D14}$ abolishes OHO31 protein production and causes female sterility, while the nuclear protein import remains normal in the somatic tissues. Sterile females show numerous abnormalities in egg chamber development. The eggs are ventralized, the oocytes are smaller than normal with signs of degeneration, material transport from the nurse cells to and maternal mRNA localization in the oocyte are defective, etc. In wild-type egg chambers, OHO31 protein is abundant in stage 10 oocytes, concentrating along the cortical cytoskeletal network, but not in the nucleus. After disrupting the actin cytoskeleton with cytochalasine B, OHO31 enters the nucleus. These results suggest that this I-α homologue has a specific function in egg chamber development, which is distinct from the protein import into the nucleus.

I. FROLOV[2], E. AGAPOV[2], T. HOFFMAN[2], B. PRÁGAI[1], M. LIPPA[2], S. SCHLESINGER[2], C. M. RICE[2]

Selection of RNA replicons capable of persistent non-cytopathic replication in mammalian cells

[1]Department of Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary,
[2]Department of Molecular Microbiology, Washington University Medical School, St. Louis, MO, USA

Using engineered Sindbis virus RNA replicons expressing puromycin acetyltransferase as a dominant selectable marker, we identified mutations allowing persistent, non-cytopathic replication in BHK-21 cells. Two of these adaptive mutations involved single amino acid substitutions in the C-terminal portion of nsP2, the viral helicase-protease. This work also provides a series of alphavirus replicons for non-cytopathic gene expression studies (Agapov et al., PNAS USA 95: 12989-94, 1998) and a general strategy for selecting RNA viral mutants adapted to different cellular environments.

I. MÉCS

Biological effects of interferons

Institute of Biotechnology, Zoltán Bay Foundation for Applied Research, Szeged, Hungary

The antiviral mode of action of interferon was pursued in interferon-pretreated and Semliki forest virus-infected chick cells. The progeny virus was inhibited in a dose-response relation, while several viral intermediates exhibited the following decreasing sequence of sensitivity: parent-like single-stranded (+) RNA, viral RNA polymerase and the replicative form double-stranded viral RNA. The data suggest that the antiviral action of interferon may be related to the integrity of parent-like viral (+) RNA.

Interferon inducers or interferon treatment decrease the inflammatory responses in carrageenin paw edema of mice, in a dose-response manner, which can be suspended with anti-interferon immune sera. Human leukocyte interferon-alpha (Egiferon^R) also inhibits the inflammatory responses in carrageenin-treated mice, the extent of this inhibition being subtype-dependent. The data suggest that, apart from its antiviral action, interferon has several roles in the primary defensory mechanisms, including the inhibition of inflammations.

I. BÉLÁDI

Avian and human interferon studies initiated by György Ivánovics

Department of Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary

One of the interests of Professor György Ivánovics in 1959–60 was the kinetics of inactivation of the Aujeszky virus by ultraviolet (UV) irradiation. Accordingly, we studied whether the formation of small plaques was due to the increased capacity of interferon (IFN) induction by the UV-treated virus. Human adenoviruses were applied as negative control, because at that time adenoviruses (in contrast to other viruses) were considered to be unable to induce IFN. It turned out that adenoviruses, while not multiplying in chick embryo fibroblasts, are effective inducers of IFN. This was the beginning of a long-lasting study on the IFN-inducing ability of adenoviruses, and mainly that of human type 12. On the basis of our intensive IFN work, we joined in with the human leukocyte IFN production programme of the EGIS Pharmaceutical Company. Technology for the production of human leukocyte IFN and of the inducer Sendai virus has been developed on a laboratory scale. The quality and the biological properties of the produced IFN (Egiferon) have been studied. The antigenicity was compared with that of the recombinant IFNs.

INTERACTION OF VIRAL AND BACTERIAL INFECTIONS

M. DEGRÉ

Interaction of viral and bacterial infections

Institute of Microbiology, University of Oslo, Rikshospitalet, Oslo, Norway

Humans and animals are constantly being inoculated with various microorganisms, including potentially pathogenic viruses and bacteria. Some of these microorganisms may interact directly with other microorganisms, and many of these can interact indirectly by exerting a direct or indirect effect on the various factors of the defence mechanism of the host organism. Over the years we have studied such interactions both in the respiratory tract and in the intestinal tract in mouse model systems supplemented with *in vitro* studies. Several viruses, including Sendai virus, reduce the efficiency of some of the first line defence systems in the respiratory tract against superinfection by Haemophilus influenzae, such as the mucocilliary flow and the antibacterial effect of alveolar macrophages. A further effect on the macrophage activities is exerted indirectly by inducing production of interferons, which in turn also influence phagocytosis and bactericidal activities. Several different viruses influence the process of internalisation of invasive bacteria into non-professional phagocytes. Both direct and indirect mechanisms are involved, and the effect is only partially dependent on replication of the viruses. Viral infections may also influence the haematopoiesis and reduce the development and differentiation of monocytes/macrophages. Similar effects can also be observed by some by-products of the viral infections, as interferons and cytokines.

R. P. BERCOFF

The molecular basis of recombination of poliovirus: implications for eradication programmes

Department of Cell and Developmental Biology, University of Rome "La Sapienza", Rome, Italy

The 5'-terminal extra-cistronic region of poliovirus RNA type 1 (Mahoney strain) contains 10 sport open reading frames (ORFs) starting with alternative translation initiation codons. In the course of experiments designed to assess the potential role of these putative "hidden" frames, we came across a double mutation that

completely abrogated the infectivity of full-length cDNA clones. These substitutions did not alter the ability of the IRES to direct internal initiation of translation in bicistronic mRNAs, and did not prevent the synthesis of plus- or minus-strand viral RNAs.

The infectivity of the mutated poliovirus cDNAs could be rescued in *trans* by co-transfecting the target COS-1 cells with an expression vector containing just the 5'-ECR of poliovirus type 2 (Lansing strain). Direct sequence analysis of the viral genome revealed that the infectious viruses recovered by co-transfection were recombinants Lansing/Mahoney, with points of "*crossing-over*" that varied from one experiment to another.

The infectivity of poliovirus cDNA was also restored by co-transfecting short RNA transcripts of the wt 5'-ECR (Lansing). Blocking the 3'-end of the "*helper*" RNA with cordycepin (a 3'-deoxy analog of ATP) abolished the ability of the transcript to rescue infections virus, consistent with the notion that following hybridization of the "*helper*" RNA to homologous sequences in the minus-strand template, the viral RNA polymerase had extended the primer generating a recombinant type 2-type 1 infectious genome by a mechanism that we propose to call of "primer-alignment-and-extension".

V. ENDRÉSZ[1], K. BURIÁN[1], D. VIRÓK[1], Z. KIS[1], K. BODA[2], Z. PROHÁSZKA[3], G. FÜST[3], É. GÖNCZÖL[1]

Human cytomegalovirus and *Chlamydia pneumoniae* specific antibodies in sera of patients with coronary heart disease

[1]Departments of Medical Microbiology, [2]Department of Informatics, Albert Szent-Györgyi Medical University, Szeged, [3]IIIrd Department of Medicine, Semmelweis Medical University, Budapest, Hungary

Associations between a wide variety of infectious agents and atherosclerosis have been described in the literature. Most of the published studies relate to human cytomegalovirus (HCMV) and *Chlamydia pneumoniae (C. pneumoniae)* bacterium. DNA and antigens of these infectious agents have been localized in atherosclerotic lesions using PCR and immunocyto-chemistry. Seroepidemiologic data also support a relationship between HCMV and *C. pneumoniae* infection and the development of coronary atherosclerosis. We have evaluated the serologic evidence of this association in four groups of patients with coronary heart disease in Hungary. Sera of 156 patients with signs of severe, 44 of mild coronary atherosclerosis, 41 with angina but without atherosclerotic coronary alterations and 96 regular blood donors were tested for

antibodies against full HCMV antigens and HCMV immediate early-1 (IE1) antigen in ELISA tests, and against *C. pneumoniae* in ELISA and microimmunofluorescence (MIF) tests. By statistic evaluation of data no significant difference was detectable for the frequency of HCMV seropositivity and HCMV specific antibody level in the study groups. However, *C. pneumoniae* specific antibodies were detected in significantly higher proportion in both groups of patients with coronary heart disease than in control groups by using the MIF test. Among sera with high HCMV-IE1 antibody level the frequency of *C. pneumoniae* MIF positivity was significantly higher than in sera with low anti-HCMV-IE1 antibody level, suggesting an interaction between these two pathogens. These results indicate that *C. pneumoniae* infection might be related to the development of atherosclerosis.

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A. VARGA[1], W. SOTOKOSTKA-KÖHLER[2], W. PRESBER[2], V. VON BAEHR[3], R. VON BAEHR[3], R. LUCIUS[1], D. VOLK[3], J. MOLNÁR[5]

Interaction between protozoan parasites and cancer cells: *Toxoplasma* infection is able to reverse multidrug resistance of mouse lymphoma and human gastric cancer cells *in vitro*

[1]Department of Molecular Parasitology, Biological Institute, [2]Institute of Microbiology, [3]Department of Medical Immunology, Faculty of Medicine (Charité), Humboldt University, Berlin, [4]Society for Interdisciplinary Immunology, Munich, Germany, [5]Department of Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary

Most intracellular parasites weaken their host cell but do not kill it. Our aim was to examine the effect of parasite infection on a multidrug resistant (mdr) tumor cell.

Here we report that an infection of cancer cells with *T. gondii* could reduce the multidrug resistance of the tumor cells against the cytostatic drugs.

Two mouse lymphoma cell lines (Mdr L 5718 and Par 5718) were infected with *Toxoplasma gondii in vitro* and the reduction of efflux pump activity of the cells was measured. The drug accumulation (Rhodamine-123) was increased in the infected mdr cell lines compared with non-infected mdr and parental cells, and no effect was shown by infection in the parental cell line. The mdr-1-gene expression was reduced in the infected cell lines 48 hours after the infection. A co-cultivation of *Toxoplasma gondii* with mdr-cell lines separated by a microfilter from tumor cells was performed, but this co-cultivation did not change the mdr efflux activity.

The effect of *Toxoplasma gondii* infection on the efflux pump activity and mdr-1 gene expression was also examined in the human gastric cancer cells. A sensitization of resistance of gastric cancer cells was also achieved by parasite infection.

This phenomenon is evidence that a reduction of resistance in tumor cells *in vitro* can be achieved by a parasite infection. It is yet unclear whether an active infection or a substance of *T. gondii* is responsible for this phenomenon.

E. M. ELNIFRO, R. J. COOPER, P. E. KLAPPER, A. B. TULLO

Multiplex PCR for the diagnosis of viral and chlamydial conjunctivitis

School of Medicine, University of Manchester, Clinical Virology, CMHT and Royal Eye Hospital, Manchester, UK

Objective. To develop a multiplex PCR for the detection of HSV, adenovirus and *C. trachomatis* in eye swabs and compare its performance to the single PCR, cell culture isolation, Amplicor PCR and immune dot blot test.

Methods. Single PCRs were first optimised and combined systematically to develop the multiplex PCR. The test was evaluated in eye swabs which were treated with a simple lysis buffer as a method of DNA preparation.

Results. The multiplex PCR was found to have detection limits of 400, 300 and 140 copies of adenovirus, HSV and *C. trachomatis* DNA, respectively. In a prospective study of 429 patients with conjunctivitis or keratitis, the test was evaluated against the single PCRs, cell culture isolation for adenovirus and HSV and the Amplicor PCR and immune dot blot test for *C. trachomatis*. A total of 95 patients were positive for one of these organisms (49 for adenovirus, 32 for HSV and 14 for *C. trachomatis*). Of the 49 adenovirus-positive patients, the multiplex PCR was positive in 48, the adenovirus single PCR in 48 and cell culture in 18 and was not tested in 12. Of the 32 patients positive for HSV, 29 were positive by the multiplex PCR, 30 by HSV single PCR and 29 by cell culture. *C. trachomatis* was detected in 12 patients by both the multiplex PCR and the single PCR, in 14 by the Amplicor PCR and in 8 by immune dot blot test which was reported equivocal in another 2 patients.

Conclusion. The newly developed multiplex PCR is sensitive, rapid, cost effective and can replace single PCRs, cell culture and immune dot blot for the diagnosis of viral and chlamydial conjunctivitis.

L. SEGANTI[1], S. PISANI[1], C. GALLINELLI[1], A. LUKIC[2], F. NOBILI[2], G. VETRANO[2], M. IMPERI[3], A. M. DEGENER[3], F. CHIARINI[1]

Viral and bacterial coinfections in cervicitis

[1]Institute of Microbiology, [2]Intitute of Obstetrics and Gynecology, [3]Department of Cell and Developmental Biology, University of Rome, Rome, Italy

Different sexually transmitted viral and bacterial infections in women often occur concomitantly and generally, signs, symptoms as well as routine clinical investigations, allow to formulate only a diagnostic hypothesis. In cervicitis, microorganisms can induce subclinical and latent infections and the detection of etiological agents is almost neglected. In this study, cervical samples of women with past and present symptoms of inflammation were tested for the presence of viruses and bacteria commonly involved in urogenital tract infections. By molecular and routinely diagnostic procedures, we looked for 45 genital human papillomaviruses (HPV), herpes simplex viruses (HSV), adenoviruses, *C. trachomatis*, *M. hominis*, *U. urealyticum*, and *M. genitalum*. A low correlation between colposcopy and HPV genomic DNA detection was observed. Double and triple infections were evidenced in symptomatic women with flogosis and abnormal transformation zones, whereas in women with negative colposcopic findings none or only one agent was discovered. HPV DNA was always present in coinfection with bacteria, adenovirus or HSV. *In vitro* assays carried out to investigate a possible interaction between HPV and HSV, both associated with carcinoma of human cervix, showed in HSV2 infected HeLa cells, a modulation of mRNA transcription of HPV18 constitutively expressed. Results obtained emphasize the pivotal role of the microbiology laboratory in cervical multiple infections' detection.

F. SUPERTI[1], M. G. AMMENDOLIA[1], A. TINARI[1], F. IOSI[1], A. M. DI BIASE[2], G. PETRONE[2], M. P. CONTE[2], L. SEGANTI[2]

Coinfection of enterocyte-like cells by rotavirus and *Yersinia* species

[1]Laboratory of Ultrastructure, ISS, [2]Institute of Microbiology, Università La Sapienza, Rome, Italy

Cell modification induced by viruses whose normal habitat is the intestinal tract can alter the adherence and invasiveness of bacterial pathogens, favouring the establishment of more severe infections. In this study, a mixed infection with rotavirus and either *Y. pseudotuberculosis* or *Y. enterocolitica* was analysed in Caco-2 cells, an

enterocyte-like cell line highly susceptible to these pathogens. Results obtained showed an increase of bacterial adhesion and internalization in rotavirus infected cells. This enhancement was dependent upon the time of rotavirus infection and the maximal increase was observed 5 hours post viral infection. Similar results were obtained with *E. coli* HB101(pRI203) strain, harbouring the inv gene from *Y. pseudotuberculosis,* known to be preinfected with rotavirus resulted in a decreased viral antigen synthesis. Transmission electron microscopy observations confirm these findings. Results obtained suggested that the rotavirus infection may induce alterations at the level of enterocyte-like cell surfaces, which modulate and favour the early interaction of *Y. enterocolitica* or *Y. pseudotuberculosis* with target cells.

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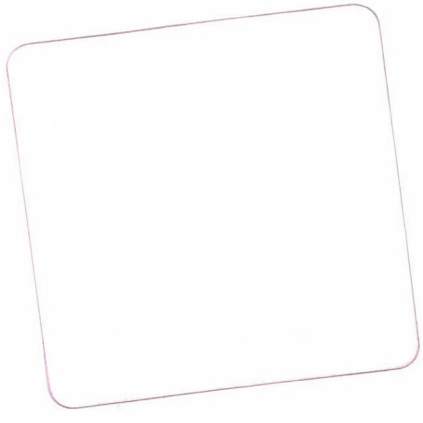

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ANCIENT AND MODERN RETROVIRUSES*

R. A. WEISS

Windeyer Institute of Medical Sciences, University College London 46 Cleveland Street, London W1P 6DB, UK

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Retroviruses are transmitted in two distinct ways: as infectious particles and as 'endogenous' proviral DNA integrated in the germ line of the host. Modern infectious viruses such as HIV-1 and HIV-2 recently infected mankind from chimpanzee and simian hosts, whereas human endogenous retroviral genomes have been present throughout old world primate evolution. Human T-cell leukemia viruses (HTLV-1 and II) have a much older human provenance than HIV, although new zoonoses from simians may also occur. We have recently characterized new retroviruses in pigs and humans. Porcine endogenous retroviral (PERV) genomes are carried in chromosomal DNA but can be activated to produce virions that are infectious for human cells, which has raised concern over human xenotransplantation using pig tissues. Human retrovirus 5 (HRV-5) is detected as an exogenous genome in association with arthritis and systemic lupus erythematosus.

Keywords: transmission of retroviruses, porcine endogenous retroviruses, human retrovirus

The study of retroviruses [1] began early this century when swamp fever in horses was shown by Vallée and Carré in 1904 to be caused by a filterable agent which we now know to be the lentivirus, equine infectious anemia virus. Retroviruses became focused on malignant disease with the discovery of avian leukosis virus in 1908 by Ellerman and Bang, avian sarcoma virus in 1911 by Rous, murine mammary tumor virus in 1936 by Bittner, murine leukaemia virus in 1951 by Gross, and human T-cell leukemia virus in 1980 by Gallo and associates. In the era of AIDS, with the discovery of human immunodeficiency virus type 1 (HIV-1) in 1983 by Barré-Sinoussi, Chermann and Montagnier and HIV-2 in 1986 by Clavel and Montagnier, emphasis in retrovirus research has, of course, switched to immunopathology.

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Aside from disease, retroviruses have provided important insights into and tools for molecular biology [1]. For example, without their study we would not have reverse transcriptase in order to make cDNA, we would have remained ignorant of the role of oncogenes in cancer for a much longer period, and we would not have such useful vectors for gene transfer and gene therapy.

Retroviruses can be lethal pathogens, none more so than HIV-1. UNAIDS estimates that by December 1999, this virus had already infected some 50 million people worldwide and killed 14 million of them. HIV-1 is thoroughly modern virus, having probably arisen as a zoonosis from chimpanzees during the 20th century [2, 3], and once established raced through the human population. Human T cell leukemia virus type I (HTLV-I), in contrast, travelled with African slaves to the New World, and HTLV-II is present in native Americans as well as African pigmies. Yet, more recent zoonoses of variants of HTLV-I from monkeys and apes has probably also occurred [4]. Other retroviruses are ancient and asymptomatic infectious agents exemplified by that most intimate of host-parasite relations, endogenous retroviral genomes integrated into chromosomal DNA. These are vertically transmitted as inherited, Mendelian traits. Some human endogenous retroviruses (HERV) have been in human ancestors for at least 50 million years [5, 6].

The contrasting modes of transmission of HIV and HERV highlight the different rates of evolution between an infectious, high turnover genome, and one embedded as integrated DNA in the germ line of the host. But a common feature is that retroviruses can jump host species, sometimes across wide phylogenetic distances [7].

That is reason enough to treat the porcine endogenous retroviruses (PERV) described below with caution, as they can infect human cells when activated from pig chromosomal DNA as infectious virions.

In our laboratory, we have recently investigated two distinct types of retrovirus of potential but as yet ill-understood impact for human health. The first is a group of endogenous C-type retroviruses in pigs [8] which are important in respect to the use of porcine cells and tissues for human xenotransplantation [9]. The second is a recently discovered but probably ancient virus called human retrovirus 5 (HRV-5) [10]. It is associated with arthritis and systemic lupus erythematosus [11] although it may represent a more widespread infection among healthy people.

Porcine Endogenous Retroviruses (PERV)

The release of PERV particles by pig kidney cell lines has been known for over 25 years [12, 13]. With the growing interest in using pig tissues and organs as a source

for transplantation into humans [9], we thought it was important to determine whether PERV particles might have a human host range.

Initially we showed that PERV released from the PK-15 pig kidney cell line could infect certain human cells in culture [8]. In contrast, PERV released from the mini-pig kidney cell line, MPK, appeared to infect only swine cells. Sequence analysis in *pol* showed that the PERVs of PK-15 and MPK were closely similar [8]. In collaboration with J. Stoye's group at the National Institute of Medical Research, Mill Hill, we detected two, distinct envelope sequences, PERV-A and PERV-B in 293 cells infected by PK-15 virions [14]. The virus released from MPK cells represents a third envelope type, PERV-C, and the differences in host range of PERV-A, -B and -C can be attributed to their envelope glycoproteins recognising distinct cell surface receptors [15].

The PERV genomes are most closely related to GALV and other mammalian C-type (gamma) retroviruses. PERV-A and -B are present as multiple copies in the normal DNA of all breeds of pig examined [14], though PERV-C may be more restricted. Thus it would appear to be a difficult prospect to attempt to breed swine for use in human xenotransplantation that are free of PERV genomes. Provided the Mendelian genomes encoding potentially infectious PERV can be identified, however, their elimination might become feasible when genetic knock-out technology for swine is developed.

The infection of human cells in culture by PERV raises concern that these viruses could also infect transplant recipients, and possibly be transmitted onwards to patients' contacts. The transplantation of porcine tissue to an immunosuppressed human, and the generation of transgenic pigs bearing human genes to prevent hyperacute rejection of xenografts may heighten the risk of pig to human transmission [16]. These anxieties have stimulated a retrospective analysis of patients who have been exposed to swine tissues. To date, published data are available on approximately 180 individuals who have been exposed to living pig tissues in various ways; including 10 diabetes patients transplanted with swine pancreatic islets [17], 2 renal dialysis patients whose circulation was linked for a short time period to swine kidneys extracorporeally [18] and 100 patients in Russia whose blood was perfused through pig spleens [19]. PERV infection was not evident in any peripheral blood samples taken from these patients months or years after their exposure to porcine tissue. However, 23 of the 100 spleen perfused patients appeared to sustain long-term survival of pig cells containing PERV sequences in the human body [19]. We may therefore conclude that although PERVs can be released from healthy porcine tissues in primary culture, such as pig lymphocytes [20] and endothelial cells [21], they are not highly contagious for

humans. The PCR and serological assays developed for these surveys [17–19] will be suitable for monitoring xenotransplant recipients.

Moreover, some anti-HIV drugs targeted to reverse transcription such as Zidovudin inhibit PERV replication. Nevertheless, close surveillance of patients and their contacts will be essential if xenotransplantation is to proceed.

Human Retrovirus 5 and Autoimmune Disease

Speculation that persistent retrovirus infections might play a role in the pathogenesis of autoimmune and chronic inflammatory diseases tends to come in and out of fashion [22]. Particles resembling retroviruses have been seen in tissues from patients with Sjögren's syndrome [23, 24], rheumatoid arthritis [25] and psoriasis [26]. Recently, a human endogenous retroviral genome (HERV-K) closely related to those expressed in human testicular tumors [5] and the normal placenta [27] was also implicated in insulin dependent diabetes mellitus [28]. A different retrovirus has been identified in multiple sclerosis [29], but the significance of these findings remains controversial. This genome is closely related or identical to the recently characterized endogenous genome, HERV-W [30, 31].

In collaboration with P. Venables at the Kennedy Institute of Rheumatology, our laboratory examined Sjögren's syndrome for retroviral particles by concentrating putative virions on sucrose gradients from tissue biopsies or short term cultures of affected salivary glands. We detected weak reverse transcriptase (RT) activity in fractions with the buoyant density typical of retrovirus particles. Using degenerate primers to retroviral *pol* (RT) and *pro* (protease) sequences, we amplified a sequence of 932 base pairs with open reading frames representing a novel retroviral sequence related to B-type and D-type (beta) retroviruses [10]. As HERV-K particles also belong to the beta-retrovirus subfamily, we expected that this new retroviral element would be endogenous. However, when we examined normal human tissues and cell lines by Southern blotting and by PCR amplification using sequences specific to this genome, we found that the retrovirus is not endogenous. We must therefore assume that it is transmitted by human to human infection but we do not know as yet its prevalence and mode of transmission. We have provisionally named this novel retrovirus human retrovirus 5 (HRV-5) as it came to light after HTLV-1 and -2 and HIV-1 and -2 [10].

HRV-5 is genetically most closely related to simian D-type retroviruses, such as Mason-Pfizer monkey virus, which cause immune deficiency, and to rodent intracisternal A-type particles and murine mammary tumour virus. Although we initially detected HRV-5 RNA in particles concentrated from tissues of patients with

Sjögren's syndrome, normal salivary glands and lymphoma [10], further analysis based on PCR detection and amplification of DNA samples rather than RT-PCR of virion RNA failed to support any specific association of HRV-5 with Sjögren's syndrome or lymphoma [32, 33]. HRV-5 proviral DNA appears to be present at extremely low virus load; nested PCR is required for detection in most of the positive samples, giving a 2% frequency in the studies cited above. This may represent the prevalence of HRV-5 infection in the human samples studied. It may, however, be an underestimate due to the threshold of detection for a virus of such low load, since we detected a higher frequency of infection using RT-PCR of sucrose gradients [10].

Table I

Detection of human retrovirus 5 DNA in human tissue samples[a]

Tissue	Number tested	Number positive
Salivary Gland		
Sjögren's syndrome	86	1
Normal	9	0
Synovium		
Rheumatoid arthritis	25	12
Other arthritides	13	8
Normal	13	0
Lymph node		
Lymphoma	78	3
Non-malignant	64	1
Blood		
Rheumatoid arthritis	66	8
Systemic lupus erythematosus	69	11
Normal	103	1

[a]Data from 10, 11, 33

A significantly increased detection rate of HRV-5 DNA is found in patients with arthritis or systemic lupus erythematosis (SLE). Table I lists the frequency of detection in our surveys [11, 33]. The synovium of arthritic joints is a frequent tissue for virus detection, but it is noteworthy that HRV-5 is not specific to rheumatoid arthritis; it is also found in synovial tissue from patients with other arthropathies such as

osteoarthritis [11]. We have recently extended the HRV-5 genome sequence into the *gag* gene. By expressing Gag antigens it should be possible to develop serological assays for HRV-5 for use in more detailed epidemiological and clinical studies.

In summary, we have evidence of human infection by a previously unknown beta-retrovirus, HRV-5, which is distantly related to simian D-type retroviruses. The virus is present in affected tissues at very low frequency, probably less than one provirus among 10,000 cells. Thus far, it is found most frequently in arthritides and SLE, but it would be unwise at this point to claim a causative role. Nonetheless, the discovery of a new human retrovirus merits further study on its mode of transmission and as a potential pathogen.

Conclusion

These two brief accounts of PERV and HRV-5 serve to remind us that novel retroviruses will continue to intrigue medical and veterinary virologists. New assays in clinical and diagnostic virology will allow the detection and characterization of these infections.

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EXAMINATION OF CD11B/C, CD54 AND CD62 EXPRESSION ON CELLS IN TRYPSIN-TRIGGERED EMPHYSEMA MODEL

ERZSÉBET SZOLNOKI[1], E. REICHART[2], SOPHIE MARCHAL[3]

[1]3rd Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Móricz Zs. 22, H–4004 Debrecen, Hungary, [2]Unité de Physiopathologie Respiratoire, U 14 Inserm et Université R. Poincaré Vandoeuvre-Les- Nancy, [3]Laboratoire de Recherches en Oncologie, Centre A. Vautrin, Vandoeuvre-Les-Nancy, France

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We studied the CD11b/c, CD54, CD62 expression on BAL (bronco-alveolar lavage) cells of rats by flow cytometry in a trypsin-triggered emphysema model. We made BAL sampling two and a half hour after trypsin infusion, in early inflammatory phase. Rats were divided into three groups: 1. negative controls, 2. saline-treated, 3. trypsin-infused rats. We found significantly ($p<0.05$) increased number of neutrophil granulocytes in BAL of trypsin-treated group, comparing with controls. By flow cytometry in trypsin-treated group: 1). We found a significantly higher expression of CD54 on BAL macrophages ($p<0.05$) 2). There was a lower, not significant CD11b/c expression on neutrophils and on macrophages in BAL, comparing with other groups. 3). A low, but not significant CD62 expression could be detected on neutrophils and on lymphocytes in BAL. We conclude: 1). Two and a half hour after trypsin infusion, macrophages are strongly activated, and play an important role in the neutrophil transendothelial migration in the early inflammatory phase of this model. 2). Neutrophils are high in number in BAL, but they are hardly activated in this early phase. 3). After trypsin infusion having a lower CD62 expression, lymphocytes seem to be involved as well.

Keywords: CD11b/c, CD54, CD62 expression, trypsin triggered emphysema model, BAL cells

Previously we have shown that emphysema develops in rats and hamsters several weeks after one intravenous perfusion of trypsin (TIV) [1, 3, 5]. Trypsin having no elastolytic activity cannot induce emphysema by itself. The acute phase (two and a half hour after infusion) of the experiment is characterized by an influx of neutrophil granulocytes within the lung microvasculature and terminal airspaces as it was

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demonstrated both histologically and by lung lavages [4], so we considered they may be responsible for delayed emphysema. Doerschuk and other authors stated that circulating neutrophils undergo a generalized response, that increases their margination throughout the lung, after two, four and eight hours of instillation of Streptococcus pneumonia. And only 1–2% of total neutrophils migrated out of the lungs. In order to verify activation of cells – including neutrophils, macrophages during this trypsin-induced acute leucostasis, we studied the cell composition of the liquids of lung lavage of trypsin-injected and control-rats by flow cytometry using monoclonal antibodies.

Materials and methods

Animals

Adult male rats (Wistar strain, IFFA CREDO, Fr) weighing 244 ± 55 g at the start of the experiment were maintained in air filtered and ventilated boxes several days in the husbandry before starting the experiment. They were distributed into three groups: 10 trypsin-perfused rats, 10 saline-treated rats and 17 controls (statistically optimised number: number of rats in each treated group × square root of the number of treated groups).

Anaesthesia

Rats were food-starved during one day before the experiment; water was given *ad libitum* during starvation. They were anaesthetized by an intraperitoneal injection of sodium pentobarbital (38 mg/kg body weight).

Trypsin administration

Pancreatic trypsin (Sigma type III, 10 U BAEE/mg of protein) was dissolved in sterile isotonic saline (2 mg/ml) and renewed each hour for avoiding autolysis. Trypsin or saline solution was perfused into the left jugular vein using a pump for two hours. Trypsin was given at the dose of 4.5 mg/kg/hour under general anaesthesia. Body temperature was kept by warming rats on a heating blanket. After two hours we waited thirty minutes, then performed a lung lavage.

Broncho-alveolar lavage (BAL)

A Carrieri canula was introduced into the trachea. The thorax was opened and the lungs were carefully excised and placed flat on a Petri dish to avoid gravity

differences between the different lobes. Lungs were then washed through the cannula with 30 ml of warmed (37 °C) sterile saline. Six 5 ml syringes were used successively, each being pushed and pulled 5 times, during 15 minutes. A push-pull lasted 30 seconds. The liquids of BAL were pooled and centrifuged at 250 g for 15 min at 4 °C. The pellets were then suspended in phosphate buffer solution (1 ml of PBS) for a quantitative count of neutrophil granulocytes, monocytes and lymphocytes using the Thoma method.

Blood sampling and preparations

Thirty minutes after the end of the perfusion 2 ml blood were sampled carefully of the abdominal vein by puncture on EDTA. Then we separated the cells of the blood by using a 6% dextran solution during 45 minutes at room temperature. The supernatant was centrifuged at 800 *g* for 8 minutes and the pellet resuspended in 1 ml of PBS. We counted neutrophil granulocytes by Thoma method and detected them by staining.

Staining

Trypan blue was used to detect living cells and May-Grunwald for identifying neutrophils in BAL and blood. We made blood smears stained for a differential count. To make a differential count of BAL cells we used cytospin method (200,000 cell/ml) with 200 µl of cell suspension at 170 g for 15 minutes. Blood and BAL smears were stained by Papanicolau method for counting.

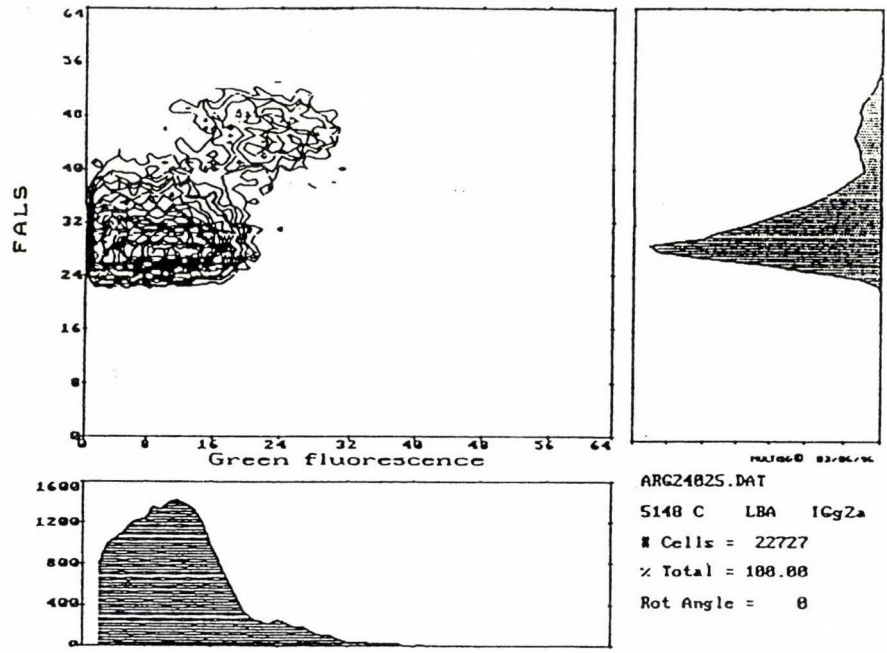
Labelling techniques

For BAL and blood we used: CD11b/c purified mouse anti-rat monoclonal antibody 22081D, 0.5 mg Pharmingen. CD62 L-selectin LECAM-1 purified hamster anti-rat monoclonal antibody 22481D, 0.5 mg Pharmingen. CD54 ICAM-1, purified mouse anti-rat monoclonal antibody 22491D, 0.5 mg, Pharmingen.

Isotype controls: IgG2a, X0943 Dako A/S Denmark and IgG1, X0931 DAKO A/S Denmark.

Method of labelling

It consisted of two successive labellings. First, one antibody was added to the sample at the dose of 1 µg/million of suspended cells for one hour. Then the suspension was washed twice with PBS. A second FITC labelled antibody was added at the dose of 10 µl/100 µl of the cell suspension for half an hour. It was washed twice and centrifuged at 400 *g* for 5 minutes.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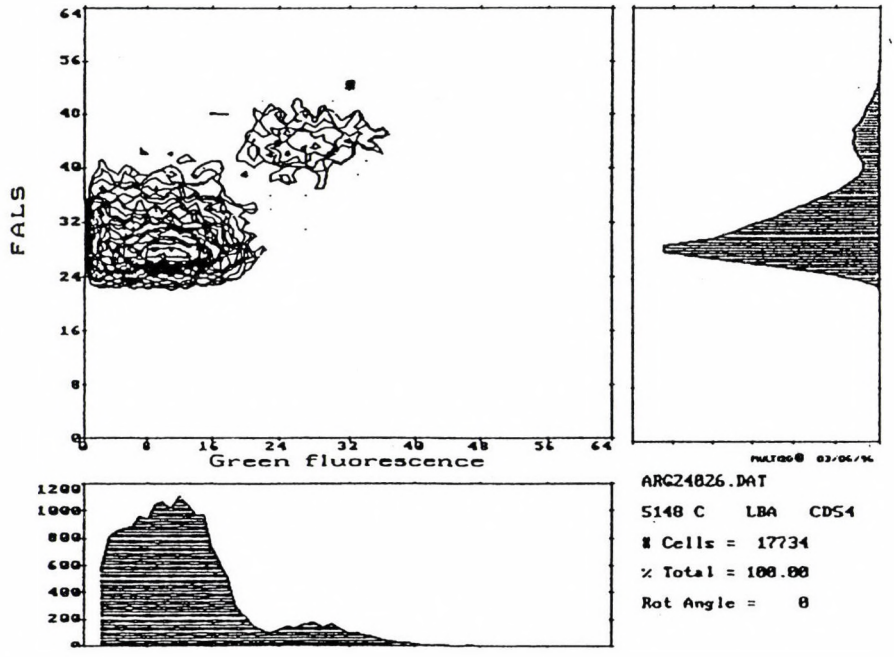
Green fluorescence
ARG24025.DAT
5148 C LBA IGgZa
Cells = 22727
% Total = 100.00
Rot Angle = 0


FALS
Green fluorescence
ARG24026.DAT
5148 C LBA CD54
Cells = 17734
% Total = 100.00
Rot Angle = 0

Flow-cytometry

It was performed cell suspension containing 0.2×10^6/ml by a cell sorter (OrthoCyte by Ortho Diagnostic Systems Roissy Fr). 100 µl of FITC labelled antibody-cell suspension was analysed at 520 nm and 560 nm using an excitation wavelength of 488 nm. Cells were identified according to the cell size and on the basis of nucleus/cytoplasm ratio. Cell subpopulations of BAL and blood were screened on a two axis graph.

Statistics

Blood and BAL values were tested for correlation in each group. We used ANOVA with one factor to evaluate the differences between the groups of trypsin-treated or saline-treated rats and controls, using standard errors of the mean (SEM).

Table I

Cells counts in the BAL (%)

	Neutrophils	Macrophages	Lymphocytes
TIV-rats	41.0± 4.8*	44.6±7.1	13.9±3.4
Controls	22.9±3.7	62.1±6.1	18.9±5.3
Saline-treated	32.9±6.3	47.1±9.9	18.6±4.0

*$p < 0.05$ SEM

Results

Rats were distributed into three groups: 10 trypsin infused rats (TIV), 10 saline-treated rats, and 17 controls. On differential counts we found a significant ($p<0.05$) increase in proportion of neutrophil granulocytes in BAL (Table I) of TIV group, comparing with other groups.

←

Fig. 1. Flow cytometry pattern in controls (CD54)

Figure 1 shows flow cytometry pattern in controls (CD54). Figure 2 shows flow cytometry pattern in TIV group (CD54). The pattern of flow cytometry in BAL are represented in controls and TIV rats. The different cell types are distinguished on the basis of size and nucleus/cytoplasm ratio. The cell fluorescence is compared with an isotype control. The granulocytes of TIV rats appeared more fluorescent and proportioned than that of controls

Figure 3 shows the differences between TIV-rats and controls in labelling BAL cells. TIV rats were characterized by: 1. significantly high expression of CD54 on BAL macrophages(49.8% ± 6.9 SEM $p < 0.05$) 2. Low, but not significant CD11b/c expression on neutrophils (6.8% ± 2) and on macrophages (4.7% ± 1.2) in BAL. 3. Low but not significant CD62 expression detected on neutrophils (9% ± 5.4) and on lymphocytes (0.1% ± 0.04) in BAL.

Discussion

According to our data we deduce that the first step in the acute inflammatory phase is the activation of macrophages by a trypsin infusion in TIV group. It is likely that macrophages were possibly activated by trypsin since they were significantly higher in number in BAL (relatively to the BAL of corresponding controls) and they had a significantly ($p < 0.05$) higher expression of CD54. Nevertheless they have a low CD11b/CD18 expression on their cell surface [6]. The number of neutrophils were significantly higher ($p < 0.05$) in TIV group as well. Apparently they were partially activated having a lower CD62 and CD11b/CD18 expression. It was previously published in the literature that selections mediate a degree of adhesion which is strong enough to induce rolling along the vessel wall [7, 8] and that β1 and β2 integrins are substantial in strong cell adhesion and transendothelial migration [6, 9]. According to our results, macrophages may play an important role in the neutrophil transendothelial migration, in the initial inflammatory phase of this emphysema model [9, 10]. Indeed an increased expression of ICAM-1 on BAL macrophages underlies their role in the early inflammatory process, namely in the extravasation of neutrophils. The intravenously administered trypsin expectively alters the endothelial surface (increased expression of E selectin under effect of TNFα and IL-1) and the chemokines produced

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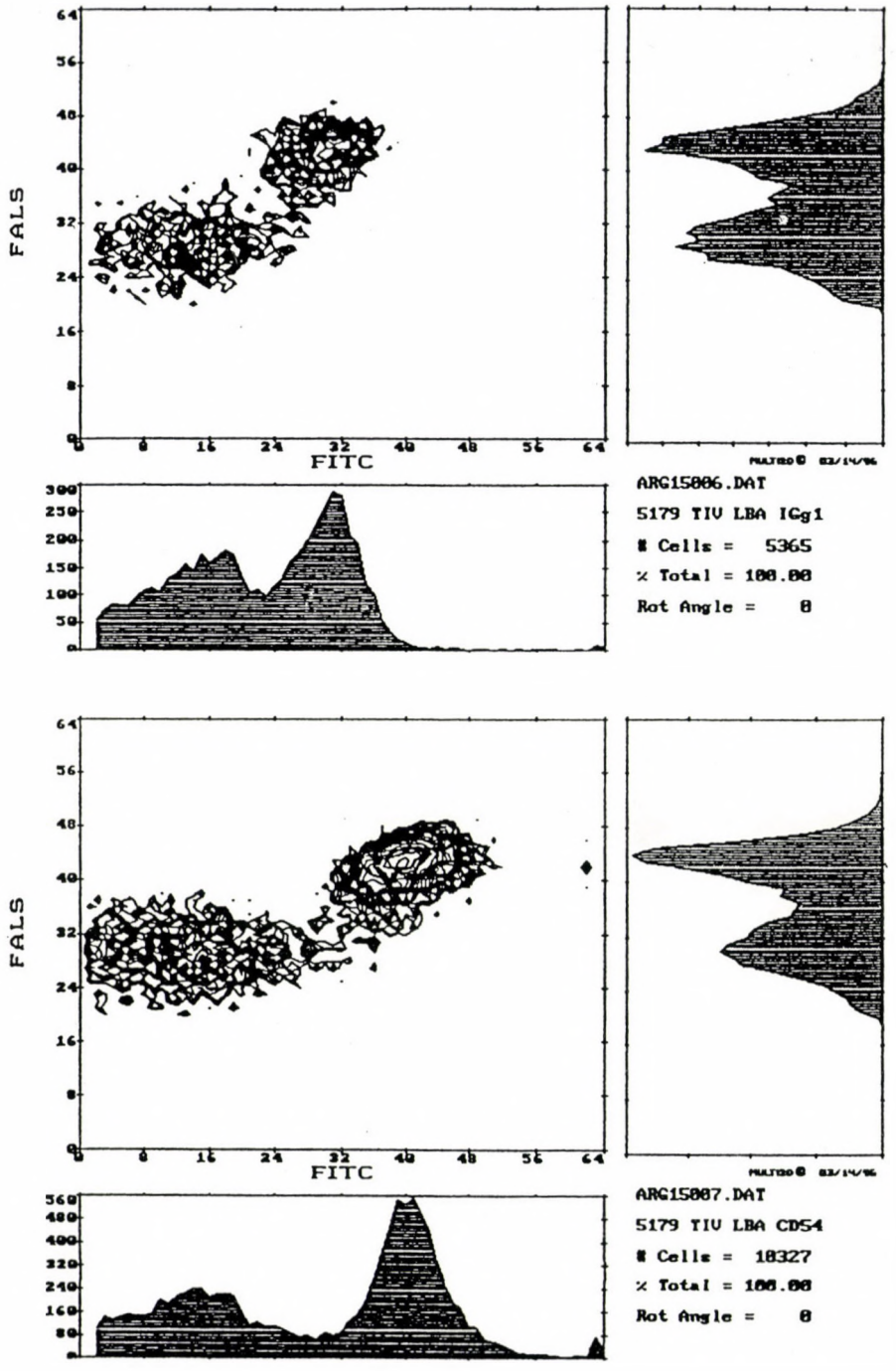
Fig. 2. Flow cytometry pattern in TIV group (CD54)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FITC
ARG15006.DAT
5179 TIV LBA IGg1
Cells = 5365
% Total = 100.00
Rot Angle = 0
FALS
FITC
ARG15007.DAT
5179 TIV LBA CD54
Cells = 10327
% Total = 100.00
Rot Angle = 0

by the endothelium and macrophages (IL-8) can trigger neutrophil chemotaxis and adhesion through activating neutrophil integrins, CD11b/CD18 [11]. So theoretically the expression of CD11b/CD18 on the surface of neutrophils is enough to emigrate, but it can increase under the effect of chemokines – mainly IL-8, being produced locally [9] – and leads to perpetuation of their activated state causing inflammation, release of proteases and production of reactive oxygen metabolites and lung injury-emphysema.

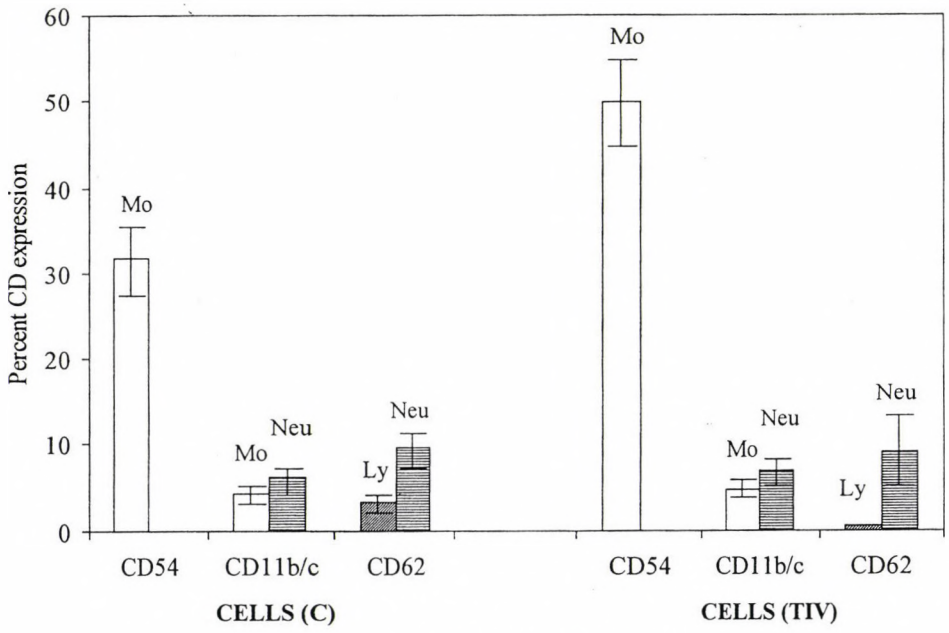

Fig. 3. CD54, CD11b/c, and CD62 expression on BAL cells

A peculiar result was found for lymphocytes. They were significantly lower in number in BAL of TIV group comparing with blood samples. This fact was considered as a pooling effect similarly found by other authors [12–14]. They were in an activated form as well, having a lower CD62 expression in BAL fluids of rats. The activated macrophages also can act on adhesion and activation of lymphocytes since it is known, that early response cytokine TNFα increases the expression of not only ICAM-1 but VCAM-1 as well on vascular endothelium *in vitro* [15]. It would be a subject to verify it in this model.

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RELATIONSHIP BETWEEN THE OCCURRENCE OF ANTI-SSA, ANTI-SSB AUTOANTIBODIES AND HLA CLASS II ALLELES FROM THE ASPECT OF *IN VITRO* INHIBITORY EFFECT OF GLUCOCORTICOSTEROID ON THE ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IN PATIENTS WITH PRIMARY SJÖGREN'S SYNDROME

A. KOVÁCS[1], ÉVA SZEKERES[1], LIVIA BEREK[1], EMŐKE ENDREFFY[2], L. KOVÁCS[3], ÉVA MAKULA[4], J. ELLER[5], GY. POKORNY[3], ILDIKÓ B. PETRI[1]

[1]Blood Transfusion Centre, [2]Department of Pediatrics, [3]First Department of Internal Medicine, [4]Department of Radiology, [5]Department of Medical Informatics, Albert Szent-Györgyi Medical University, P.O. Box 427, H–6701 Szeged, Hungary

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Though at present there is no evidence-based algorithm for the treatment of primary Sjögren's syndrome, it is generally accepted that glucocorticosteroid (GS) therapy must be introduced in cases with severe systemic manifestations. As the side-effects of the GSs are well known, it would be useful to know in advance how the patients will respond to this type of treatment. For this reason we measured the *in vitro* steroid sensitivity of 29 SS patients using inhibition of antibody dependent cellular cytotoxicity (ADCC) test by methylprednisolone compared to that of 28 controls. SS patients proved to be significantly less sensitive to GSs than controls (inhibition of ADCC reaction: 42.4 vs 53.1%; $p < 0.01$). This was especially true in SS patients with anti-SSA and/or SSB autoantibody positivity and with HLA-DR2 and/or -DR3 alleles. Comparing the results of the *in vitro* GS sensitivity and the clinical effectiveness of the previously applied corticosteroid therapy it seems that steroid inhibition of ADCC reaction has a predictive value in determination of *in vivo* sensitivity to GSs. However, in patients with decreased in vitro GS sensitivity a more expressed *in vivo* steroid sensitivity cannot be excluded.

Keywords: HLA class II alleles, ADCC, Sjögren's syndrome

Sjögren's syndrome (SS) is one of the systemic inflammatory autoimmune diseases characterized by lymphocytic infiltration of exocrine glands, first of all the salivary and lacrimal ones. This results in xerostomia and xerophthalmia, which are the obligatory symptoms of the disease. The sicca symptoms can be present alone (primary

SS), but can join other well-defined autoimmune connective tissue diseases (e.g. Rheumatoid arthritis, Systemic lupus erythematosus, etc.). This latter form is called secondary SS. In primary SS, beside the local symptoms, systemic manifestations (articular, lung, vascular, etc.) can also occur.

In SS patients, focal lymphoid infiltrates (predominantly CD4+ T-cells) are present in the lacrimal and salivary glands. Another important feature is the expression of HLA-DR antigens on the glandular epithelial cells [1]. Clinically, primary SS is characterized by hyperactivity of B cells which is manifested in polyclonal hypergammaglobulinaemia, and overproduction of different autoantibodies such as rheumatoid factor, anti-SSA/Ro and anti-SSB/La antibodies. The latter two antibodies are the most characteristic of the disease, and these are directed against small ribonucleoprotein antigens. SS is a multifactorial disease, and in its pathogenesis not only the immunological changes, but also genetic predisposition and environmental factors, such as viruses, are involved [2, 3].

Despite progress in understanding the pathogenesis of SS, at present there is no therapeutical modality that is effective towards the overall disease. For the treatment of dry mouth and eyes, the exocrine substitution and drugs, which can increase the lacrimal and salivary output, are the most important means of alleviating the sicca symptoms [4]. In cases with severe, sometimes life-threatening internal (kidney, lung, vascular, etc.) manifestations, GSs are generally used [5]. Taking into account the well-known short and long-term side-effects of steroid therapy, it would be essential to know in advance, whether a patient will or will not respond to GSs. For this reason we measured antibody-dependent cellular cytotoxicity (ADCC) reaction and *in vitro* methylprednisolone inhibition of this reaction in patients with SS by methods described by Petri et al. [6, 7]. We wanted to know whether these *in vitro* examinations may help to determine the *in vivo* steroid sensitivity of SS patients if GS therapy is required. Another question was if HLA class II genotype, autoantibody positivity and clinical manifestations had any influence on GS sensitivity.

Patients and methods

ADCC activity and *in vitro* methylprednisolone inhibition of the reaction were determined in 29 patients with primary Sjögren's syndrome and in 28 age and sex-matched healthy blood donors as controls. All SS patients met the European Diagnostic Criteria (1993) [8].

Of the 29 patients all but one were female. The mean age of patients was 55 years (range: 32–74 years) and the mean duration of the symptoms was 10.3 years (range: 1–20 years).

In the *ADCC reaction* fresh, human "0" Rh (D) positive red blood cells were used as target cells. Human anti-D serum was adsorbed onto the cells [9] labelled with $^{51}Cr/Na_2\ ^{51}CrO_4$; 7–8 Gbq/mg Cr, Amersham. The effector lymphocytes were isolated on Ficoll Uromiro gradient after treatment of the whole blood with colloidal iron powder (GAF, USA). The effector/target cell ratio was adjusted to 10:1. Methylprednisolone was added to the culture medium for a final concentration of 10 μg/ml. The spontaneous activity was given by the count rates for cultures without anti-D antibody. The total activity was calculated as the radioactivity of labelled red blood cells lysed in distilled water. The cells were incubated at 37 °C in a 5% CO_2 thermostat for 18 hours. The cytotoxicity and steroid inhibition were calculated by the next formulas:

$$\text{cytotoxicity \%} = \frac{\text{test supernatant cpm} - \text{spontaneous cpm}}{\text{incorporated total activity cpm}} \times 100$$

$$\text{steroid inhibition (\%)} = \frac{\text{ADCC with steroid (\%)}}{\text{ADCC without steroid (\%)}} \times 100$$

Grade of sensitivity to GSs was given as a percentage of the inhibition of the ADCC reaction due to the steroid. Steroid sensitivity was defined when inhibition of basic ADCC was >30% [6, 7].

Examination of HLA status and autoantibody profile – statistical analysis

HLA II class antigenes, DRB1, DQA1 and DQB1 alleles were investigated in 28 of the 29 SS patients. Genomic DNA was extracted by a standard phenol-chloroform-proteinase-K method [10]. Polymerase chain reaction (PCR) was carried out: 1. for DRB1 using Amplicor TM PCR Diagnostics KIT (Roche Diagnostics Systems), 2. for DQA1 using Ota's PCR-RFLP method [11], 3. for DQB1 using Mercier's PCR-RFLP method [12]. After polyacrylamide gel (12%) electrophoresis the alleles were determined by comparison of estimated fragment size with the predicted size.

Anti-SSA and anti-SSB autoantibody positivity was determined by enzyme-linked immunosorbent assay (Epignost, Leonding/Linz, Austria).

Statistical analysis was made by Dunnett-2-sided *t*-test, and Spearman's correlation coefficients were calculated as well.

Table I

Main clinical manifestations and laboratory variables in patients with primary Sjögren's syndrome (n=29)

Manifestations and laboratory changes	Occurrence in frequency (%)
Parotid enlargement 10/29	(35%)
Articular 29/29	(100%)
Renal 4/29	(14%)
Vascular Raynaud 13/29	(45%)
Purpura 5/29	(17%)
Vasculitis 2/29	(7%)
Upper airway 27/29	(93%)
Lower airway 10/29	(35%)
Hepatomegaly 23/29	(79%)
Splenomegaly 7/29	(24%)
Lymphadenopathy 12/29	(41%)
Anaemia 14/29	(48%)
Leukopenia 15/29	(52%)
Hypergammaglobulinemia 15/29	(52%)
Antibody positivity	
Antinuclear 20/29	(69%)
IgM Rheumatoid factor 23/29	(79%)
Anti-SSA 19/29	(66%)
Anti-SSB 12/29	(41%)
Both Anti-SSA+SSB positive 11/29	(38%)
Both anti-SSA+SSB negative 9/29	(32%)

Results

The clinical manifestations and laboratory changes are summarized in Table I. Articular involvement (arthritis and arthralgia) occurred in all patients, followed by involvement of the upper airways (93%), anaemia and/or leukopenia (66%), Raynaud's phenomenon (45%), parotid enlargement (35%), lower airway disease (35%), purpura (17%), kidney involvement (14%). Examining the autoantibody profile of the patients, IgM rheumatoid factor positivity occurred in 79%, whereas ANA-positivity and anti-SSA and/or anti-SSB was detected in 69% of the patients.

Analyzing the results of ADCC reaction, there was a tendency for elevated ADCC reaction in SS patients (48.3 ± 15.7%), but the difference did not reach the level of significance comparing to that of controls (41.4 ± 14.1%).

In contrast, the *in vitro* GS inhibition of the ADCC reaction was significantly lower ($p < 0.01$) in SS patients (42.4 ± 15.8%) than that in controls (53.1 ± 13.1%). The range of steroid inhibition varied between 39.9% and 66.2% in the healthy blood donors' group and between 0 and 74% in SS patients. Twenty-three of 29 patients (79.3%) proved to be methylprednisolone sensitive, whereas 6 patients (20.7%) showed a decreased sensitivity to GSs. This rate of sensitivity did not differ from that of the controls. We analyzed the results of *in vitro* methylprednisolone inhibition of ADCC in SS patients on the basis of antibody profile and HLA status. The results are shown in Table II and III. The sensitivity to GSs decreased significantly in patients with anti-SSA and/or anti-SSB antibody positivity as compared to the controls. The difference proved also to be significant between the results of anti-SSB negative SS patients and controls. However, there were not any significant differences when the results of SS patients were compared with or without antibody positivity (Table II).

Table II

Steroid inhibition of ADCC reaction in primary Sjögren's syndrome (SS) patients (n=29) with or without anti-SSA and/or SSB antibody positivity comparing to controls (n=28)

	Degree of inhibition (%)	
Controls:	53.1±13.1	
All SS patients	42.4±15.8*	p<0.01
SS patients with		
anti-SSA+ (n=19)	39.6±16.4*	p<0.005
anti-SSA– (n=10)	47.7±16.0	NS
anti-SSB+ (n=12)	40.1±16.6*	p<0.05
anti-SSB– (n=17)	43.9±15.5*	p<0.05
anti-SSA and SSB+ (n=11)	39.6±17.3*	p<0.02
anti-SSA and SSB– (n=9)	47.9±14.7	NS

+: patients with antibody positivity
–: patients with antibody negativity
*: statistically significant differences as compared to controls
NS: not significant

Table III

Steroid inhibition of ADCC reaction in Sjögren's syndrome (SS) patients (n=28•) with or without HLA DR2 and/or DR3 positivity as comparing to controls (n=28)

	Degree of inhibition (%)	
Controls:	53.1±13.16	
All SS patients	42.4±15.8*	
SS patients with HLA phenotype of		
DR2 positivity (n=15)	37.9±20.8*	p<0.005
DR2 negativity (n=13)	45.8±10.6	NS
DR3 positivity (n=16)	40.3±15.6*	p<0.005
DR3 negativity (n=12)	44.7±17.0	NS
DR2 and DR3 positivity (n=6)	36.6±15.4**	p<0.005
DR2 and DR3 negativity (n=5)	52.6±16.2	NS

**: significant difference as compared to controls and to DR2 and DR3 negative patients
•: HLA phenotyping was carried out in 28 of the 29 SS patients
*: significant difference as compared to controls
NS: not significant

In HLA-DR2 and/or -DR3 positive patients the GS sensitivity was significantly lower than in controls. Though the steroid sensitivity was lower in SS patients with DR2 or DR3 positivity than in DR2 or DR3 negative patients, the differences were not statistically significant. In contrast, in cases with DR2/3 heterozygosity the difference proved to be statistically significant not only in comparison to controls, but also to SS patients carrying neither DR2 nor DR3.

Moreover we also evaluated the influence of combinations of both HLA haplotype (DR2, DR3), anti-SSA and SSB antibody positivity on the values of *in vitro* GS sensitivity (Table IV). We observed a tendency that SS patients with at least one of these alleles plus one of these autoantibodies exhibited lower sensitivity to methylprednisolone than SS patients who possessed none or only one of the above-mentioned immunological and genetic markers. However, the difference proved to be significant only between DR2 plus anti-SSA positive and DR2 plus anti-SSA negative subgroups ($p < 0.05$).

Eight of 29 SS patients needed GS therapy because of systemic manifestations during the course of the disease. Six of the 8 steroid treated patients proved to be GS sensitive according to the *in vitro* examinations. This was in concordance with the clinical improvement after the administration of GSs. In contrast, 2 of the 8 steroid

treated patients showed a decreased methylprednisolone sensitivity on the basis of the ADCC inhibition. When they had to be treated with high dose of GS due to severe liver and lung involvements, they responded, however, well to this therapy.

Table IV

Steroid inhibition of ADCC reaction in Sjögren's syndrome (SS) patients (n=28•) concerning both autoantibody and HLA status and in controls (n=28)

	Degree of inhibition (%)			
SS patients	DR2+	DR2–	DR3+	DR3–
Anti-SSA+	n=7	n=11	n=12	n=6
	32.1±22.2%*	43.5±10.7%	37.6±16.8%	42.2±17.6%
Anti-SSA–	n=6	n=4	n=4	n=6
	44.7±16.6%	52.3±8.4%*	48.5±7.7%	47.2±17.6%
Anti-SSB+	n=3	n=8	n=9	n=2
	34.7±31.6%	38.7±10.7%	36.9±18.0%	51.0±7.7%
Anti-SSB–	n=10	n=7	n=7	n=10
	38.9±17.6%	51.1±8.5%	44.7±11.7%	43.4±18.3%
Anti-SSA+,SSB+	n=4	n=7	n=8	n=2
	34.7±31.6%	40.6±11.3%	35.7±18.9%	51.0±7.1%
Anti-SSA–,SSB–	n=6	n=3	n=3	n=6
	44.7±16.6%	54.3±9.0%	49.3±9.2%	47.2±17.6%
Controls (n=28)	53.1±13.2			
All SS patients (n=28)	42.4±15.8			

+: positivity
–: negativity
•: HLA phenotyping was carried out in 28 of the 29 SS patients
*: significant difference between the two subgroups

Discussion

As the ethiology of SS is unknown, a causal treatment is not available at present. Since there are considerable evidences for its immunological background, therefore immune-regulating drugs may be of therapeutic value. In spite of this fact, there is no evidence-based algorithm for the treatment. While the severe extraglandular manifestations (e.g. lung, kidney, etc.) can be decreased by administration of

immunosuppressive treatment, local symptoms may fundamentally not be altered by this type of therapy [13].

Among the immunoregulatory modalities the GSs are used most frequently. For this reason it would be desirable to know in advance the therapeutic response to this type of drugs in patients with SS. This fact made us to investigate the ADCC reaction and *in vitro* methylprednisolone inhibition of this reaction in our SS patients.

The role of RNP, Sm and SSA-specific antisera from patients with lupus erythematosus in inducing ADCC of target cells coated with nonhistone nuclear antigens was investigated by Norris et al. [14]. In SLE patients with high titer anti-RNP sera, a significantly increased ADCC was seen using monocytes, T-lymphocytes, and low-density lymphocyte effector. Using monocyte effectors, a significantly increased ADCC reaction was detected in patients with anti-RNP, anti-Sm, and anti-SSA autoantibodies. In contrast, neutrophils as effectors were ineffective in any tested nuclear antigen-antibody system.

Petri et al. [7] published a significant correlation between the clinical effect of prednisolone treatment, i.e. the prednisolone sensitivity, and the degree of GS sensitivity based on the percentage of ADCC inhibition in children with nephrotic syndrome.

Since mainly the IgG Fc-receptor bearing lymphocytes take part in the ADCC reaction, it is conceivable that the differences in GS sensitivity may be correlated with a shift in the proportion of different T-lymphocyte subpopulations. Crabtree et al. [15] presented considerable evidence that GSs inhibit T-cell proliferation by blocking of production of T-cell Growth Factor. Parillo and Fauci [16] found that GSs suppressed NK-cell activity in humans.

Anti-SSA autoantibodies have been shown to increase ADCC *in vitro* [14]. As a decreased GS sensitivity was observed in patients with anti-SSA and/or anti-SSB antibody positivity, it is conceivable that at certain genetic background these autoantibodies can influence the GS sensitivity, however, the exact mechanism *in vivo* is unknown. In the literature, only the beneficial effect of corticosteroids on circulating anti-ds-DNA titre has been proven [17].

Methylprednisolone inhibition of the ADCC reaction was significantly decreased in pediatric autoimmune patients suffering from juvenile chronic arthritis or idiopathic thrombocytopenia [18].

Our working group [19] used the test of ADCC to investigate the *in vitro* GS sensitivity in patients with chronic uremia and in patients who underwent renal transplantation. While a positive correlation was detected between the HLA-DR6 positivity and steroid sensitivity, a negative association was found in HLA-B8 carriers with the steroid sensitivity.

In our SS patients, the effector peripheral lymphocytes demonstrated an increased ADCC reaction on the target red blood cells compared to those of controls, however, this was only a tendency. In SS patients and controls the rate of *in vitro* steroid sensitivity/resistance did not differ significantly, using 30% inhibition of ADCC reaction as a borderline. In contrast, the average GS inhibition showed a significant decrease in SS patients as compared to controls. This difference was more expressed in SS patients with HLA-DR2 or -DR3 haplotype than in patients not having these haplotypes. When these two alleles occurred together – in cases of DR2/DR3 heterozygotes – the decrease of GS sensitivity was significant comparing not only to controls, but to SS patients without these two alleles.

We observed a similar correlation between the grade of sensitivity to GSs and anti-SSA and SSB autoantibody positivity. Our results suggest that anti-SSA antibody positivity has a greater impact on GS sensitivity.

Furthermore the tendency of being less sensitive to GSs was more expressed in any cases when both type of predisposing factors (genetic and immunological) occurred together. This is especially true for joint occurrence of HLA-DR2 and anti-SSA positivity. Comparing the results of the *in vitro* steroid sensitivity and the clinical effectiveness of the previously applied GS therapy, it seems that *in vitro* methylprednisolone inhibition of ADCC reaction has a predictive value in determination of *in vivo* steroid sensitivity. However, in patients who exhibited a greatly decreased *in vitro* GS sensitivity, the *in vivo* steroid sensitivity cannot be excluded. For this reason, in severe cases with life-threatening manifestations GSs remain the main factors of therapy [20].

We conclude that sensitivity to GSs in patients with SS is influenced by both genetic haplotype and anti-SSA/anti-SSB seropositivity. Co-existence of these two factors may cause a decreased sensitivity to GSs. *In vitro* ADCC steroid inhibition test of the peripheral lymphocytes, coupled with the analysis of HLA class II allele polymorphism and autoantibody profile, was found to be a useful method for the detection of the grade of GS sensitivity in SS. This method may have a predictive value for making a decision of introducing of chronic GS therapy in other systemic autoimmune diseases, as well.

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THE VALUE OF PYROLYSIS MASS SPECTROMETRY TO INVESTIGATE NOSOCOMIAL OUTBREAKS CAUSED BY *SERRATIA MARCESCENS*

ELISABETH NAGY[1], ZSÓFIA KOCZIÁN[1], Z. PRÁGAI[1], PENELOPE R. SISSON[2], R. FREEMAN[2]

[1]Department of Clinical Microbiology, Albert Szent-Györgyi Medical University, P.O. Box 482, H–6701 Szeged, Hungary, and [2]Public Health Laboratory, General Hospital, Westgate Road, Newcastle upon Tyne, NE4 6BE, England

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Simultaneous outbreaks of *S. marcescens* infection going on in the Neonatal Intensive Care Unit and the Surgical Department of the same hospital were investigated by pyrolysis mass spectrometry (PyMS). The PyMS analysis of the strains clearly demonstrated that the two outbreaks were caused by different strains. The 14 *S. marcescens* isolates from the first outbreak were closely related, with the exception of one environmental isolate, which did not harbour the ESBL plasmid, which was present in all other isolates. However, the phage type of all 14 isolates was the same. Among the 9 *S. marcescens* isolates from the second outbreak, PyMS clearly distinguished 3 that exhibited gentamicin resistance from the remaining 6 gentamicin-susceptible isolates. Phage typing was unhelpful in this case, as none of the isolates were typable. The PyMS typing of nosocomial outbreak strains can reach the level of discrimination approaching that achieved by molecular genetic analysis.

Keywords: Pyrolysis mass spectrometry, *Serratia marcescens*, nosocomial outbreaks

Serratia marcescens is a low-grade pathogen, but may cause infections and has been associated with nosocomial outbreaks in hospital environment [1, 2]. It has been confirmed that most isolates of *S. marcescens* produce chromosomal Class I cephalosporinase, AmpC beta-lactamase, which is inducible. Despite the elevated level of beta-lactamase due to induction by some beta-lactam antibiotics, most *S. marcescens* isolates remain susceptible to third-generation cephalosporins. However, there is evidence that some isolates of this organism may display resistance to these broad spectrum cephalosporins and monobactams in consequence of the parallel stably

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derepressed production of Class I beta-lactamase and alterations in the outer membrane permeability [3]. Plasmids encoding for extended-spectrum beta-lactamases (ESBL) have not been found frequently in this species.

Epidemiological studies of outbreaks caused by *S. marcescens* have been hindered by the lack of readily available and discriminatory typing systems. Traditional typing methods such as biotyping, serotyping, bacteriocin typing [4], antibiogram analysis, phage typing [5] and plasmid typing [6] have not always been able to distinguish separate strains of *S. marcescens*, or have been affected by physiological factors. Various genotyping techniques have recently been used to characterize *S. marcescens*, including ribotyping [7], PCR analysis [8] and pulsed-field gel electrophoresis typing [2]. Pyrolysis mass spectrometry (PyMS) has proved to be a rapid and simple technique for making inter-strain comparisons within a wide range of bacterial species [9]. It has been applied to the study of outbreaks of infection with considerable success [10–12] and proven that can characterize organisms with a level of discrimination approaching that achieved by genetic analysis [12].

Two simultaneous outbreaks of infection due to *S. marcescens* within one hospital afforded an opportunity to assess the usefulness of PyMS in the typing of this pathogen.

Materials and methods

The first outbreak of *S. marcescens* infection was observed in the Neonatal Intensive Care Unit (NICU) during a 4 months period. Twenty patients were involved. Nine of the 20 were colonised only in the nose or the stomach, while 11 additionally had symptoms of sepsis with positive CSF or blood cultures. The *S. marcescens* strains isolated from these 11 patients were available for typing, as were 3 isolates obtained from the environment of the patients during the outbreak. Two of these 3 were isolated from inside of two incubators and the third was from the surface of a tonometer. During the same period another outbreak of *S. marcescens* was observed in the Department of Surgery of the same hospital, in a ward treating patients with acute and chronic pancreatitis. At that time the toilets and bathroom and some of the rooms of the ward were undergoing reconstruction, causing the patients to be housed more densely than usual. No isolates were collected from the environment during this outbreak.

Twenty-four isolates of *S. marcescens*, including 14 from the first outbreak and 9 from the second, were preserved in stab cultures and analysed by PyMS. One epidemiologically-unrelated isolate was obtained from the tracheal aspirate of a patient in the medical ICU. All isolates were demonstrated by the ATB identification system

(BioMerieux, France) to have the same biochemical profile. Details of the isolate designations with the source are shown in Table I.

Table I

Origins of the S. marcescens *isolates*

Isolate	Outbreak	Source	Bacteriocin type	Phage type	Discriminating resistance to antibiotics
a	1	Nose	NA	3/11	$ESBL^+$
b	1	Nose	4, 6, 8	3/11	$ESBL^+$
c	1	Blood culture	4, 7, 10	3/11	$ESBL^+$
d	1	Trachea	4, 6, 8	3/11	$ESBL^+$
e	1	Nose	4, 6, 7, 8	3/11	$ESBL^+$
f	1	Nose	4, 8, 10	3/11	$ESBL^+$
g	1	CSF	4, 6, 8	3/11	$ESBL^+$
h	1	Stomach	4, 6, 8	3/11	$ESBL^+$
i	1	Blood culture	4, 7, 10	3/11	$ESBL^+$
j	1	Environment	1, 2, 10	3/11	$ESBL^-$
k	1	Environment	NA	3/11	$ESBL^+$
l	1	Environment	NA	3/11	$ESBL^+$
m	1	Throat	4, 6, 8	3/11	$ESBL^+$
n	1	Pus	3, 4, 6, 7, 8, 10	3/11	$ESBL^+$
o	S	Tracheal aspirate	NA	NA	
p	2	Ascites	NA	NT	$Genta^R$
q	2	Blood culture	NA	NT	$Genta^S$
r	2	Blood culture	NA	NT	$Genta^S$
s	2	Pancreatic cyst	NA	NT	$Genta^R$
t	2	Pus	NA	NT	$Genta^R$
u	2	Drain	NA	NT	$Genta^S$
v	2	Blood culture	NA	NT	$Genta^S$
w	2	Blood culture	NA	NT	$Genta^S$
x	2	Drain	NA	NT	$Genta^S$

S = sporadic isolate, epidemiologically unrelated to either outbreak
NA = not available
NT = not typable
$ESBL^+$ = isolates with an MIC 32 μg/ml of cefotaxime, ceftriaxone and cefoperazone
$ESBL^-$ = isolates with an MIC 1 μg/ml of cefotaxime, ceftriaxone and cefoperazone

The resistance patterns of all isolates were determined by the disc diffusion method on Muller-Hinton agar. The MICs of selected antibiotics were measured by the Etest (AB Biodisk, Sweden). The presence of Class I beta-lactamase was detected by the disc approximation test, imipenem being used as inducer and ceftazidime as indicator antibiotic [13]. The plasmid encoded ESBL production of the strains was detected by use of the ESBL Etest strip [14]. An MIC ratio of ceftazidime and ceftazidime + clavulanic acid ≥4 was considered indicative for the presence of ESBL. Plasmid DNA was extracted from selected ESBL producing *S. marcescens* isolates from the first outbreak, by using the Wizard™ Minipreps DNA Purification System (Promega, Medison, WI). Plasmid DNA was transferred to the *E. coli* K12 J53-2 *rifR* strain. Purified plasmid DNA of the clinical isolates and their transconjugants was digested with *Eco*RI under conditions recommended by the supplier (Pharmacia Biotech, Uppsala, Sweden) and analysed on 0.9% agarose gel. PCR was performed (GeneAmp System 9600, Perin-Elmer) to amplify a 475 bp sequence, specific for the SHV gene, as described earlier [15]. During the SSCP analysis of the SHV gene endonuclease digestion of the PCR product was carried out with *Pst*I (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions and the single-stranded DNA fragments were compared with those of known reference of SHV-2, SHV-3, SHV-4 and SHV-5 genes separated on 12% polyacrylamide gel with a mini gel apparatus (BioRad) as described previously [15].

Phage typing was carried out according to the method of Pitt et al. [16] and bacteriocin typing was done with the method described by Traub [17].

For PyMS analysis, the isolates were subcultured in duplicate onto a single batch of diagnostic-sensitivity agar (DST; Oxoid), spread to give single colonies and incubated at 37 °C overnight. Colonial material from each subculture was smeared onto triplicate pyrolysis foils (Horizon Instruments Ltd., Heathfield, Sussex, UK) suspended in pyrolysis tubes. The tubes were dried at 80 °C for 5 min and then examined as a single batch on a Horizon Instruments PyMS 200X pyrolysis mass spectrometer at a Curie point of 530 °C. Integrated ion counts at unit mass intervals from 51 to 200 were recorded on floppy disk, together with the pyrolysis sequence number and total iron count for each foil. Multivariant analysis of the PyMS spectral data was performed as documented elsewhere [10].

Results and discussion

The ATB (BioMerieux) ID 32 GN strips proved that all isolates derived from the two outbreaks had the same biotype (phenotypic characteristics). No differences

were observed in the biochemical tests with this kit. Of the 14 *S. marcescens* isolates originating from the first outbreak (NICU) 13 exhibited the same resistance pattern. Resistance to ampicillin, amoxicillin/clavulanic acid, third-generation cephalosporins (such as cefotaxime, ceftriaxone and cefoperazone), tetracycline, gentamicin, tobramycin and netilmicin was observed The isolates displayed intermediate susceptibility to ceftazidime (MIC 8 µl/ml) and susceptibility to imipenem, amikacin and ciprofloxacin. They produced an inducible Class I beta-lactamase together with a plasmid-encoded ESBL (Table I). The MICs of cefotaxime, ceftriaxone and cefoperazone were 32 µg/ml. For these isolates and their *E. coli* transconjugants plasmid analysis revealed a plasmid with a size of approximately 150 kb. The *Eco*RI restriction patterns did not allow a distinction of the plasmids (data not shown). The PCR-SSCP analysis of the ESBL gene revealed the presence of an SHV-2 type enzyme. However, one strain isolated from the environment (j) during the outbreak was resistant only to ampicillin, amoxicillin/clavulanic acid, tetracycline, gentamicin, tobramycin and netilmicin and fully susceptible to the third-generation cephalosporins (MICs 1 µg/ml) imipenem, amikacin and ciprofloxacin. The 9 *S. marcescens* strains isolated during the second outbreak gave a completely different resistance pattern. They were resistant to ampicillin, amoxicillin/clavulanic acid and ofloxacin, and susceptible to third-generation cephalosporins, imipenem, tobramycin, netilmicin and amikacin. They exhibited differences in resistance to gentamicin. Three of them (p, s and t) were resistant to gentamicin with MIC >256 µg/ml, whereas the others were susceptible with MIC 2 µg/ml (Table I).

All isolates from the first outbreak (ESBL-positive and ESBL-negative) belonged in the phage type 3/11, whereas none of those isolated during the second outbreak were typable (Table I). Bacteriocin typing was carried out only on the isolates from the NICU. Considerable differences were observed between the isolates concerning the bacteriocin lysis spectrum (Table I).

PyMS analysis of the dataset using triplicate spectra from duplicate subcultures demonstrated excellent reproducibility (data not shown) and the results were in complete accordance with those obtained when the dataset was analysed with the six replicate PyMS spectra of each isolate being labeled as a distinct PyMS group. The similarity dendogram derived from analysis of the complete dataset is shown in Figure 1. PyMS analysis divided the isolates into two clusters, as indicated. Cluster I comprised all the isolates from outbreak 1, together with the epidemiologically

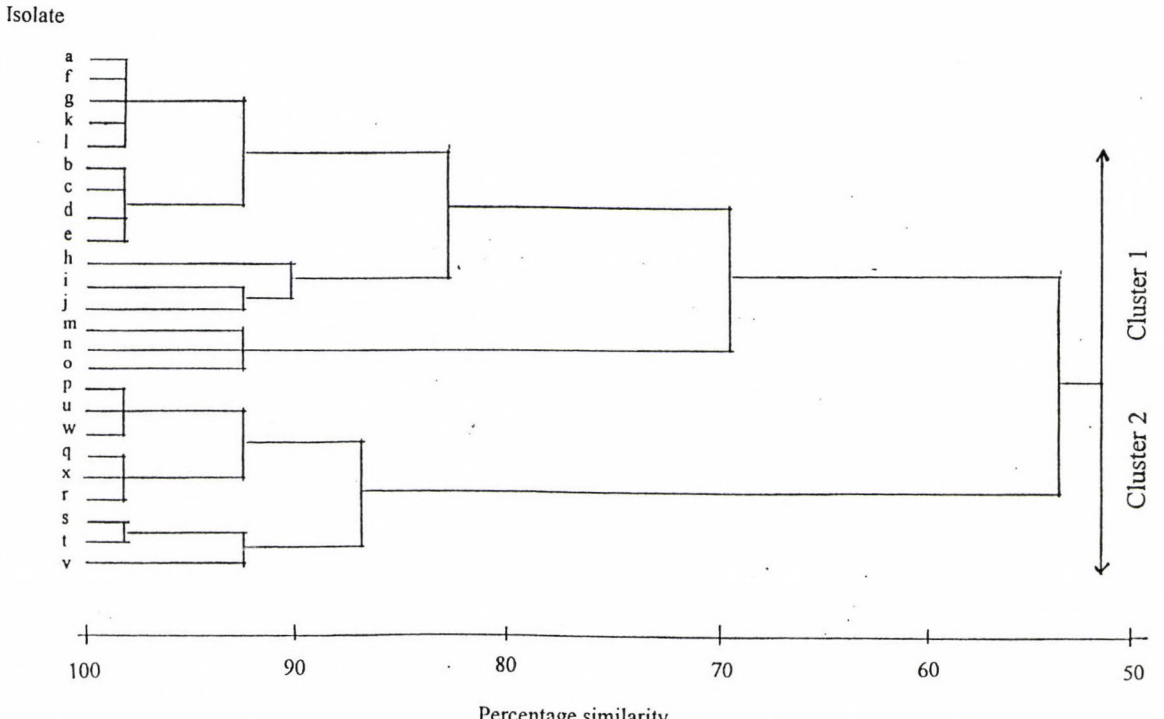

Fig. 1. Dendogram showing the relationships revealed by PyMS analysis between the *S. marcescens* isolates originating from the two outbreaks

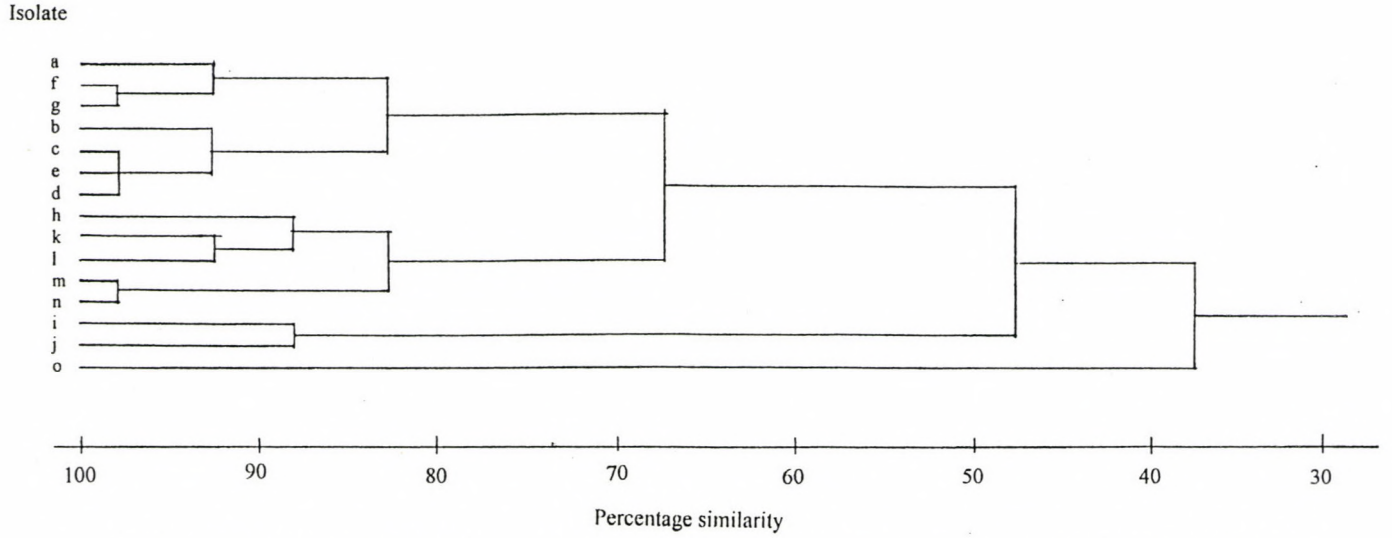

Fig. 2. Dendogram showing the relationships revealed by PyMS analysis between the *S. marcescens* isolates obtained during the outbreak 1

unrelated isolate (o). The second cluster contained only isolates of *S. marcescens* from outbreak 2. Subsequent analyses were performed on the PyMS data from cluster 1 and cluster 2 separately.

The further PyMS analysis of the cluster I dataset is shown in Figure 2. The epidemiologically unrelated isolate of *S. marcescens* (isolate o) is distinguished from the remainder, which continue to cluster closely together. Isolates j and i (one of the environmental isolates, which did not harbour the ESBL plasmid and a blood culture isolate from the first patient involved in the outbreak, respectively) show the least similarity to the other isolates associated with outbreak 1, which may reflect phenotypic variation or absence of the resistance plasmid.

On further PyMS analysis of the dataset for cluster 2, 6 isolates remained closely linked to each other (Figure 3). All these isolates differed from the others by being susceptible to gentamicin (Table I). Isolate p (from ascitic fluid) was distinguished by PyMS, while isolates s and t (from a pancreatic cyst and pus, respectively) were similar to each other, but distinct from the main cluster of isolates from outbreak 2. These 3 strains were resistant to gentamicin.

These two simultaneous outbreaks of *S. marcescens* infections, in two different wards, were managed on the assumption that they were caused by two different strains. This view was based on the differences in the antibiotic patterns of the two sets of isolates and the phage typing results. PyMS analysis clearly confirmed this assumption. During the first outbreak, however, one isolate obtained from the environment gave the same phage typing result, but differed from the other isolates in its antibiotic resistance. It produced only the inducible Class I chromosomal beta-lactamase, while all the other strains exhibited also a plasmid coded SHV-2-type ESBL. PyMS clearly distinguished this environmental isolate, together with another isolate, which originated from the first patient of this outbreak, from the rest of the strains. The fact that *Klebsiella pneumoniae* strains, exhibiting also the SHV-2-type ESBL gene, were isolated from the same patients in the same ward in the same time [18], led us to consider the possibility of the acquisition of this gene by the susceptible *S. marcescens* during the outbreak.

PyMS analysis of the isolates from the second outbreak was even more useful, as it clearly distinguished the 3 isolates exhibiting gentamicin resistance from the 6 gentamicin-susceptible isolates. Other typing methods, such as phage typing were not useful in this case, as all the isolates tested belonged in the nontypable category.

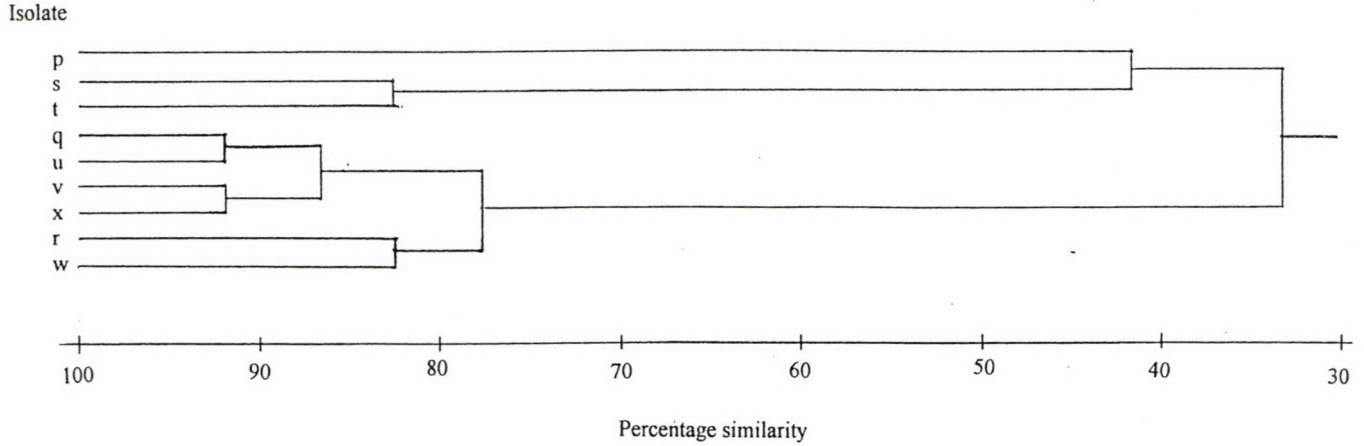

Fig. 3. Dendogram showing the relationships revealed by PyMS analysis between the *S. marcescens* isolates obtained during the outbreak 2

The characterization of putative outbreak strains by PyMS is rapid and results are available on the same day. Where the method is available close to the clinical microbiological laboratory, it may help as an effective outbreak control measure. In our case, it was used as a special additional typing method to prove the relatedness of *S. marcescens* isolates considered to be closely related but differing in some respects (bacteriocin type, antibiotic resistance). It was interesting that in both outbreaks, despite of the close relatedness, PyMS clearly distinguished isolates exhibiting differences only in resistance genes.

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RAPID COMBINED ASSAY FOR *SALMONELLA* DETECTION IN FOOD SAMPLES

I. GADÓ[1], P. MAJOR[2], MARGIT KIRÁLY[1], MARGIT GY. PLÁVECZKY[2]

[1]National Center for Epidemiology "B. Johan", P.O. Box 64, H–1966 Budapest and [2]"J. Fodor" National Center of Public Health, National Institute of Food Hygiene and Nutrition, Gyáli út 3/a, H–1097 Budapest, Hungary

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A rapid method was developed to detect salmonellae in food samples. The method gave a possibility to obtain results after 28 h 30 min. The preenrichment in buffered peptone water lasted for 6 h, the enrichment in Rappaport-Vassiliadis medium was applied for 18 h followed by PCR with INVA1-INVA2 primer pair, adapting Chiu and Ou's method. This procedure was suitable to demonstrate salmonella contamination at min. 10 cfu/25 g sample. Out of 18 samples there was a good agreement between the results of the conventional and rapid methods in case of 17 samples. PCR with SPVC1-SPVC2 primer pair informing about the presence of virulence plasmid was performed in separate tubes, because decreased sensitivity was observed in case of multiplex PCR.

Keywords: *Salmonella* detection, food samples, rapid combined assay, PCR

The incidence of salmonellosis increased considerably in the last decades. It is one of the most commonly reported causes of food-borne diseases in the Western world [1]. In a 3-year surveillance programme on food-borne infection conducted by the WHO in Europe *Salmonella* spp. was identified in 84.5% of the outbreaks, where a causative agent was reported [2]. The majority of the salmonellosis cases were due to the consumption of egg, meat, poultry products and chocolate [3–7].

Demonstration of salmonellas on food samples can be made with conventional procedures: cultivation in non-selective, then in selective media, followed by identification in solid differentiation media and by serological and biochemical methods [8–11]. These procedures are simple, but time-consuming: an unambiguous diagnosis needs 5–6 days [12–13]. Therefore, it was important for the epidemiological practice to elaborate rapid diagnostic methods, which were sufficiently sensitive and

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specific at the same time [14–16]. These methods were developed in four main directions:

1. Methods based on the changes of impedance [4]. These can be favourably applied to screen the negative samples. In case of positive result, however, it is necessary to confirm it with other methods; for this reason they cannot be used as rapid methods.

2. DNA-DNA and RNA-DNA hybridization [17–21]. Their time requirement is relatively long (more than 2 days) and false positive results appear frequently [22].

3. Methods based on enzymatic immunoassay (ELISA) [7, 23–26]. The most prominent is the Salmonella-Tek assay (Organon-Teknika Corp., Durham, N. C.) [7]. This test, however, still requires 48 h to complete [27].

4. Procedures based on PCR. These seem to be the most advantageous in respect of rapidity and specificity. Regarding the sensitivity, the methods are distributed in a wide scale: n. 10^0–10^2 cfu/PCR are given the most frequently as the detection limit. The target can be some genes which are present in all salmonellas [28–35] or in *S. enterica* [36]; it may be a common sequence of the salmonella virulence plasmids [37–38] or some serotype specific genes [39–41].

To qualify a food sample to be negative (i.e. to declare that there is no single cfu in the 25 g sample) none of the PCR methods are sufficiently sensitive in themselves. Therefore, it is necessary to perform cultivation combined with enrichment, which generally increases the duration of the procedure to 24–30 h.

In the present work it was our objective to qualify the food samples on the day following their receival by the combination of enrichment and a suitable PCR method.

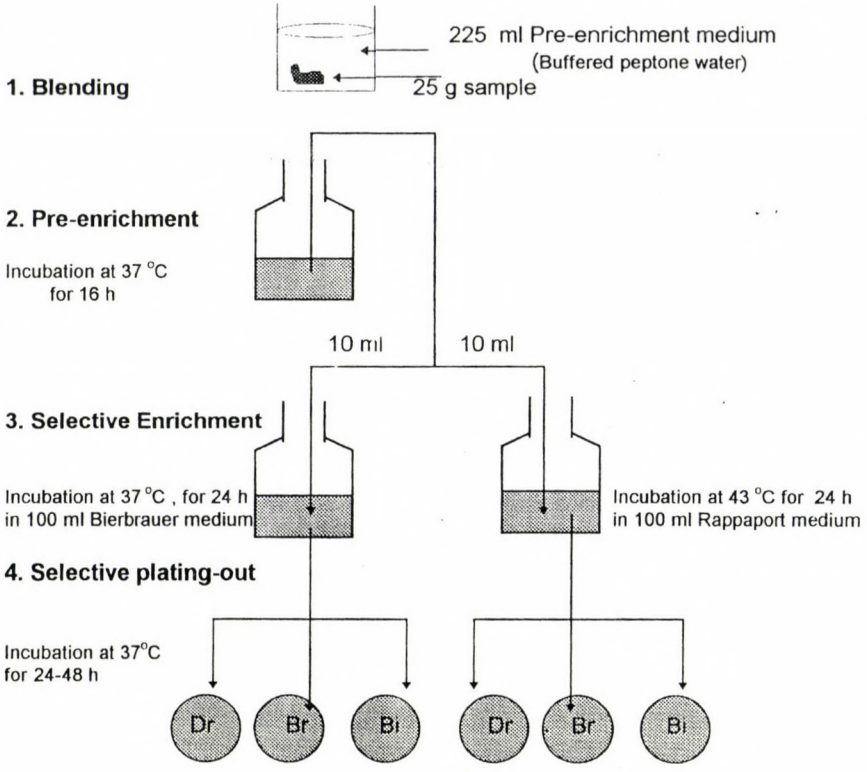
Materials and methods

Food samples

The raw pork, chicken meat and liver samples were got from the National Food Investigation Institute, Budapest; the caraway- and coriander seed and black pepper samples originated from the National Public Health and Medical Officer Service of Budapest, the brawn and sausage samples from the National Public Health and Medical Officer Service of county Pest.

Bacterial strains

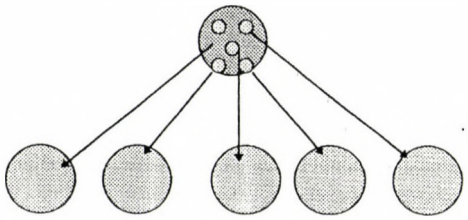
A clinical strain of *S. enterica* sv. *Enteritidis* (further: *S. enteritidis*) 152/96 was used for sensitivity and model experiments.



Dr = Drigalski agar
Br = Phenol red/brilliant green agar
Bi = Bismuth sulphite agar

5. Selection of colonies for confirmation

Incubation on nutrient agar at 37°C for 24-48 h



6. Biochemical and serological confirmation (24-48 h)

Fig. 1. General protocol of the conventional method

The general protocol (Hungarian Standard MS2 3640/8-80) of the conventional procedures is shown in Fig. 1. Their time demand is 5–7 days.

The "rapid method" is a combination of the preenrichment and enrichment steps of the conventional protocol and a PCR method. Its time requirement is 28 h 30 min. The preenrichment was made for 6 h in buffered peptone water (BPW) at 37 °C, then the enrichment was performed for 18 h in Rappaport (R) or Rappaport-Vassiliadis (R-V) media at 43 °C or 37 °C, respectively. One ml culture, after washing twice was resuspended in 100 µl distilled water and boiled for 10 min. The supernatants of the boiled suspensions were used as templates in PCR.

PCR

Chiu and Ou's method [35] was used with minor modifications. Composition of the reaction mixture (50 µl): 5 µl Promega buffer (100 mM Tris.HCl pH=9, 500 mM KCl, 1% Triton X-100), 2 mM MgCl, dNTP-s 300 µM each.

Primers: INVA1, INVA2, SPVC1 and SPVC2, 0.5 µM each, 1.25 U Taq DNA polymerase (Promega), 8 µl template. The sequence of primers and the size of amplicons are presented in Table I. The amplification was performed in thermocycler "Progene" (Techne, Cambridge, UK.) with the programme as follows: initial denaturation at 94 °C for 1 min followed by 40 cycles with 1 cycle consisting of 30 sec 94 °C, 30 sec 56 °C and 2 min 72 °C. After the last cycle the mixture was incubated for 10 min at 72 °C, then mixed with tracking dye.

Table I

Synthetic oligonucleotides used as primers for PCR

Gene	Primer	Sequence	Positions	Size of amlicon (bp)
SpvC	SPVC-1	ACTCCTTGCACAACCAAATGCGGA	505–528	571
	SPVC-2	TCTTCTGCATTTCGCCACCATCA	1052–1075	
invA	INVA-1	ACAGTGCTCGTTTACGACCTGAAT	104–127	244
	INVA-2	AGACGACTGGTACTGATCGATAAT	324–347	

Detection of the amplicons

The PCR products were examined with agarose gel electrophoresis using 1.6% agarose (Sigma type II) in TAE buffer containing 1 μg/ml ethidium bromide. The amplicons were photographed in UV light. As marker pGEM digested with HinfI, RsaI and SinI enzymes (Promega) was used.

Results

First the sensitivity of PCR method is presented with pure *S. enteritidis* cell suspension (Fig. 2.), which was boiled and diluted with tenfold steps; PCR was made from the dilutions using INVA1-INVA2 and SPVC1-SPVC2 primer pairs separately and in common reaction mixture. The concentration of $MgCl_2$ and dNTPs and the number of PCR cycles were increased related to the original method. The size of amplicons corresponded to those of written by Chiu and Ou [35] (244 and 571 bp). The limit of detection of gene *invA* was 10^2 cfu/PCR, which meant a 20 times higher sensitivity compared to the original publication. The demonstration of gene *spvC* required ten times more templates. In case of multiplex method the sensitivity decreased to 1/100 level.

In the next experiment 25 g of salmonella-free, but not sterilized raw pork samples were spiked with 1-10-100 cfu *S. enteritidis* grown in BPW O/N. They were incubated for 6 h in BPW, then in R-V enrichment medium for 18 h at 37 °C. The cells were washed twice, resuspended in 1/10 volume and used after boiling as templates with INVA1-INVA2 primers. 244 bp bands were obtained in case of infection with 10–100 cfu/25 g sample in the presence of 3×10^6 cfu/25 g background flora (Fig. 3).The results were similar plating to selective agar from the washed cell suspensions. Amplifications were also made from the same suspensions with SPVC1-SPVC2 primer pair (Fig. 4). In spite of the fact, that the detection of gene spvC was less sensitive in case of pure cell suspension (Fig. 2), after enrichment the limit of detection was similar in respect of both genes.

By conventional and "rapid" method 18 food samples were examined using INVA1-INVA2 primer pair (Table II). In case of the first 7 samples (331–344) the enrichment was made in R medium at 43 °C. Similar results were obtained by six samples with both procedures, but in case of one sample the "rapid" method resulted in false negative answer, in consequence of the low (10^4 cfu/ml) cell concentration of the washed suspension.

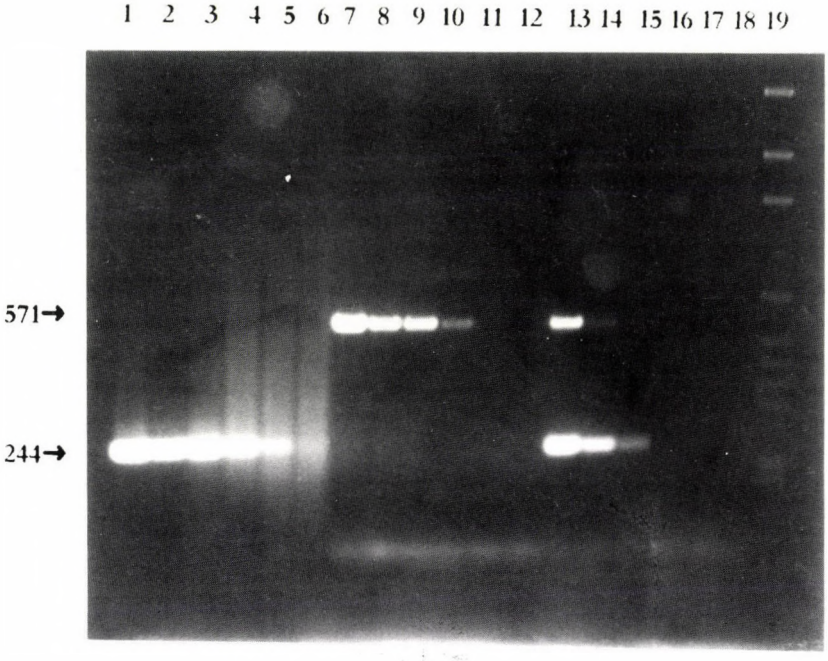


Fig. 2. Sensitivity of single and multiplex PCRs.
Template: supernatant of boiled suspension of *S. enteritidis* 152/96. Concentrations of template (cfu/50 µl): lanes 1, 7, 13: 10^6, lanes 2, 8, 14: 10^5, lanes 3, 9, 15: 10^4, lanes 4, 10, 16: 10^3, lanes 5, 11, 17: 10^2, lanes 6, 12, 18: blanks. Primers: lanes 1–6: INVA1-INVA2, lanes 7–12: SPVC1-SPVC2, lanes 13–18: INVA1-INVA2, and SPVC1-SPVC2. Lane 19: marker (pGEM, Promega). Fragments: 2645, 1605, 1198, 676, 517, 460, 396, 350, 222 bp

In case of the next 5 samples (351–361) the enrichment was performed also in R-V medium at 37 °C, representing milder selection. This resulted in a 100% agreement with the conventional method applying R medium at 43 °C. Therefore, the last 6 samples (371–383) were enriched only by means of the milder selection and neither false negative, nor false positive results were found. The positive results of 4 samples were reproduced also with SPVC primers.

Discussion

The qualification of food samples in respect of salmonella-contamination required a method, which was suitable for the detection of the whole genus or at least

the whole *S. enterica* species; its high sensitivity and quickness made it possible to do the epidemiological measures in due time. Obviously the application of PCR was necessary for these requirements. Among the numerous PCR methods developed for salmonella detection Chiu and Ou's [35] method targeting the genes *invA* and *spvC* seemed to be more promising. Its sensitivity was 2000, and 200 cfu/PCR in respect of *invA* and *spvC*, respectively. One of its targets (*invA*) was present in the whole genus [33, 42–44] and its chromosomal localization provided high stability. Both genes are absent in the non-salmonella spp. resulting high specificity. In our experiments the sensitivity of the detection of the more important, genus specific gene (*invA*) was higher, than it was written originally. The "rapid" method based on this PCR was suitable to demonstrate the salmonella contamination of food samples in case of min. 10 cfu/25 g. This sensitivity does not fulfil the maximum demand (to detect 1 cfu/25 g) similarly to the methods described earlier. In respect of the time requirement our combination means a sufficient compromise which may help the epidemiological practice in Hungary.

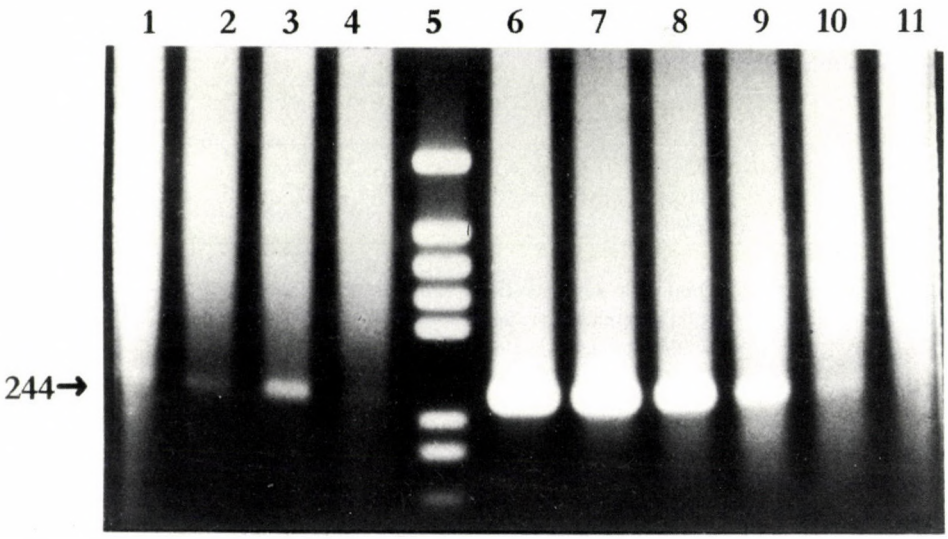


Fig. 3. Sensitivity of the "rapid" method with the amplification of gene *invA*.
Samples of 25 g raw pork were infected with 1 (lane 1), 10 (lane 2) and 100 (lane 3) cfu *S. enteritidis* 152/96 O/N broth culture. Lane 4: uninfected control. Background contamination of the samples: 3×10^6/25 g. Lane 5: marker (pGEM, Promega). Fragments: 676, 517, 460, 396, 350, 222 bp. Lanes 6–10: PCR sensitivity control 1.5×10^5–1.5×10^1 cfu/tube directly. Lane 11: blank

Table II

Salmonella-contamination test of food samples with "rapid" and conventional procedures

Sample		Conventional procedure		Rapid procedure	
N°	Quality	R, 43°C	R-V, 37°C	R, 43°C	R-V, 37°C
331		+		+	
332	raw pork	+		+	
333		+		+	
341		–		–	
342	raw pork	–		–	
343		+		–	
344		–		–	
351	caraway seed	–	–	–	–
352		–	–	–	–
353	coriander	+	+	–	+
354		+	–	–	+
361	raw chicken liver	+	+	+	+
371	black-pepper	–			–
372		–			–
373*	raw chicken meat	+			+
381*	brawn	+			+
382*	brawn	+			+
383*	sausage	+			+

Enrichment was performed in Rappaport (R) or Rappaport - Vassiliadis (R-V) media.
Generally, INVA1-INVA2 primers were applied.
*PCR with SPVC1-SPVC2 primers was also performed with identical results.

The gene *spvC* localizes on the virulence plasmids of numerous important *S. enterica* serovars (*Enteritidis, Typhimurium, Dublin, Choleraesuis*, etc.) [45]. The *spv* operon is necessary for the widespread of salmonella infection from the intestinal tract and for the development of systemic diseases. The simultaneous detection of this gene is not indispensable for the qualification of the samples, it may be advantageous, however, it does not increase the time requirement and the expenses are enhanced in a relatively small degree, but information may be obtained on the pathogenicity of the infective agent. Besides, the gene invA may be absent in some salmonella strains; 2% was found to be *invA*$^-$ [46]. The sensitivity of *spvC* detection from pure cell suspensions was ten times smaller, than that of *invA* in contrast with Chiu and Ou's

results, but the model experiments made with artificially infected samples exhibited the detection limit in a similar scale in case of both genes.

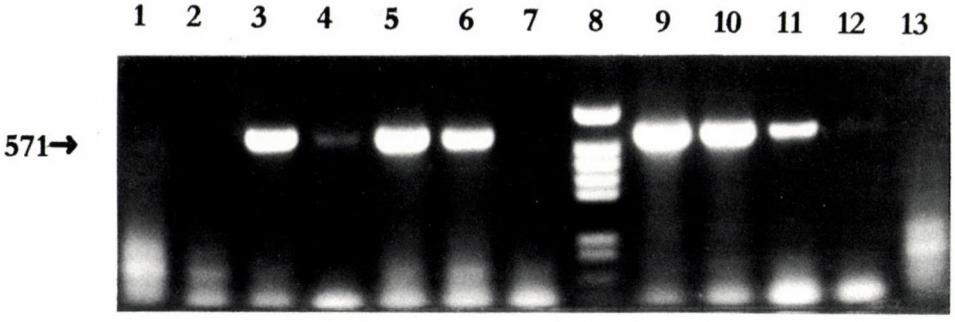


Fig. 4. Sensitivity of the "rapid" method with the amplification of gene *spvC*. Samples of 25 g raw pork were infected with 1 (lanes 1–2), 10 (lanes 3–4) and 100 (lanes 5–6) cfu *S. enteritidis* 152/96 O/N broth culture. Lane 7: uninfected control. Background contamination of the samples: 3×10^6/25 g. Lane 8: marker (pGEM, Promega). Fragments: 676, 517, 460, 396, 350, 222, 179, 122 bp. Lanes 9–12: PCR sensitivity control (8×10^4–8×10^1 cfu/tube, directly. Lane 13: blank

The application of multiplex method is unadvisable considering the decreased sensitivity of *invA* amplification in the presence of both primer pairs. This result is analogous with the inhibited amplification of EHEC genes caused by multiplex PCR [47].

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PROTEUS VIRULENCE: INVOLVEMENT OF THE PORE FORMING α-HEMOLYSIN

(A SHORT REVIEW)

V. TÓTH[1,2], L. EMŐDY[1]

[1]Department of Medical Microbiology and Immunology, University Medical School, Szigeti u. 12, H–7643 Pécs, [2]Department of Stomatology, University Medical School, Dischka Gy. u. 5, H–7621 Pécs, Hungary

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The genus *Proteus* belongs to the tribe of *Proteae* in the family of *Enterobacteriaceae*, and consists of five species: *P. mirabilis*, *P. vulgaris*, *P. morganii*, *P. penneri* and *P. myxofaciens*. They are distinguished from the rest of *Enterobacteriaceae* by their ability to deaminate phenylalanine and tryptophane. They hydrolyze urea and gelatin and fail to ferment lactose, mannose, dulcitol and malonate; and do not form lysine and arginine decarboxylase or β-galactosidase [1]. Colonies produce distinct "burned chocolate" odor and frequently show the characteristics of swarming motility on solid media. *P. mirabilis*, *P. vulgaris* and *P. morganii* are widely recognized human pathogens. They have been isolated from urinary tract infections, wounds, ear, and nosocomial bacteremic infections, often in immuncompromised patients [2–6]. *P. myxofaciens* has no clinical interest to this time. *P. penneri* as *species nova* was nominated by the recommendation of Hickman and co-workers [7]. Formerly it was recognized as *P. vulgaris* biogroup 1 or indole negative *P. vulgaris* [8, 9]. Although it has been less commonly isolated from clinical samples than the other three human pathogenic Proteus species, it has nevertheless been connected with infections of the urinary tract, wounds and has been isolated from the feces of both healthy and diarrheic individuals [10–12]. Potential virulence factors responsible for virulence of *Proteae* are: IgA protease, urease, type3 fimbriae associated with MR/K haemagglutinins of at least two antigenic types, endotoxin, swarming motility and HlyA and/or HpmA type hemolysins [for review see ref. 13].

In the followings we give a survey of accumulated concepts about the position and characteristics of HlyA type α-hemolysins both in general and with emphasis on virulence functions in the tribe of *Proteae*.

Keywords: *Proteus* virulence, α-hemolysin

Position of HlyA type hemolysins among bacterial cytolysins

Several types of bacteria can cause lysis of erythrocytes. The toxins usually damage not only erythrocytes but also other types of eukaryotic cells [14]. A comprehensive name as cytolysin was introduced to them by Bernheimer [15]. Several types of mode of action on target cells have been described. Cytolysis readily performs after enzymatic degradation of cell membrane: *Pseudomonas aeruginosa* [16] and *Clostridium perfringens* [17] have been shown to produce an extracellular phospholipase that can cause hemolysis in this way. β-toxin of *Staphylococcus aureus*, a sphingomyelinase C, can act also enzymatically on erythrocytes [18].

The concept of membrane damage, different from enzymatic digestion, through insertion of alien pore forming proteins into a target lipid bilayer was first advanced in the complement field to explain how terminal C5b-C9 complement components cause cytolysis [19]. Analogous phenomena were found to operate in the field of bacterial toxins [20]. These proteinaceous materials differ in structure and pore forming capability from species to species but are identical in their capability to undergo a transition from a primarily hydrophilic state to an amphiphilic one. The exposure of apolar regions in the molecules permit their spontaneous insertion into the hydrophobic membrane domains to form the walls of aqueous transmembrane pores. They serve as a noncontrolled way of ion exchange between the target cell and the environment. The consecutive water inflow into the cell leads to swelling and blow up of it. Several types of pore forming toxins have been recognized, and they can differ in molecular structure, mode of activation and/or secretion machinery. The prototypes of pore forming hemolysins of Gram positive bacteria are α-hemolysin (α-toxin), a major virulence factor of *Staphylococcus aureus* [21], and streptolysin O of *Streptococcus pyogenes* [22].

Hemolysis caused by *Escherichia coli*, a member of the family *Enterobacteriaceae* on a solid blood agar plate has been described long ago [23]. The long-existing inconsistency that *E. coli* hemolysin either was found to be filterable by some authors [24] and not filterable by others [25] was solved in 1963 by Smith who suggested the existence of two types of hemolysins and distinctly determined them [26]: α-hemolysin was specified as cell free filterable – and β-hemolysin as cell bound one. Hemolysin α (HlyA) of *E. coli* is probably the most thoroughly studied pore forming toxin of Gram negative bacteria and regarded as the prototype of secreted hemolysins in *Enterobacteriaceae*. All later recognized HlyA type hemolysins produced by other enteric bacteria (i.e. *Proteae*) or even more diverse microbes are compared to this toxin of *E. coli*. Another designation – RTX or repeat toxin – of HlyA type toxins came from the tandem repeats of nine amino acids at their carboxy half (see

later in details). The HlyA-RTX toxin family embodies two subgroups by their effect: 1). cytolysins causing total lysis of targeted cells, and 2). leukotoxins which can act specifically either on ruminant [14] or on primate [27] leukocytes.

Hemolysins in the genus *Proteus*

Several attempts have been made to identify hemolysins of this genus. Wenner and Rettger [28] did not find any hemolytic activity for *P. mirabilis* contrary to Taylor [29] and Yacob [30] who reported hemolytic activity in all strains tested. Studying the hemolytic activity of several strains of *Proteae*, after overnight incubation at 37 °C, only some discoloration could be noted around the colonies on blood-agar plate solid media. After incubation for another 24 hours clear-cut zones of hemolysis could be seen. Conclusion was driven by Peerbooms and co-workers [31] that this hemolysin resembled to the β hemolysin of *E. coli* [26, 32]. They found it very unstable, and unlike the secreted α-hemolysin it was strongly cell-associated. Swihart and Welch [33] found that all examined *P. mirabilis* and *P. vulgaris* strains produced a calcium independent (HpmA type) hemolysin. Parallelly it was shown that strains in four members of the genus – *P. mirabilis*, *P. penneri*, *P. vulgaris* and *P. morganii* [34–36] were able to produce a secreted, cell free hemolysin. This latter hemolysin represents a toxin family closely related to the HlyA of *E. coli* [37, 38], and reacts with polyclonal antiserum to *E. coli* α-hemolysin [65].

Considering all the above data actually two types of chromosomally determined hemolysins are distinguished in the tribe of *Proteae*: 1. the calcium dependent secreted α-hemolysin, belonging to the RTX-HlyA toxin family, and 2. the calcium independent HpmA type hemolysin responsible for delayed hemolysis [39].

It is worth mentioning that among a large number of representatives of the closely related *Providencia* genus no one hemolytic strain was found [34].

As HpmA is not the subject of this paper we only mention here that it is cytolytic not only for erythrocytes but also for a wide range of eukaryotic cell types including human renal proximal tubular epithelial cells [40]. HpmA producing *Proteus* strains exhibit a higher virulence than non hemolytic ones in intravenous mouse assays [31, 41], and an obvious toxic effect is involved in their killing capacity [42].

Table I

Exoproteins belonging to the RTX toxin family

Bacterium	Toxin	Designation
Escherichia coli	hemolysin	HlyA
	enterohemolysin	EhxA
Proteus morganii	hemolysin	MmxA
Proteus vulgaris	hemolysin	PvxA
Proteus penneri	hemolysin	PpxA*
Pasteurella haemolytica	leukotoxin	LktA
Actinobacillus pleuropneumoniae	hemolysin	ApxIA
	hemolysin	ApxIIA
	leukotoxin	ApxIIIA
Actinobacillus actinomycetemcomitans	leukotoxin	AaltA
Actinobacillus suis	hemolysin	AshA
Bordetella pertussis	adenylate cyclase-hemolysin	CyaA
Moraxella bovis	hemolysin	MbxA

*proposed designation

HlyA-RTX Toxins of *Proteus* spp. (and of other bacterial taxa)

The very similar molecular composition with repeat units, the specific mode of secretion and activation, and the common organization of genetic information justified that toxic products of bacteria with either close or even remote taxonomic position be placed in the same toxin family (Table I). At the same time a possible phylogenetic relation pointing to a common ancestor organism was suggested. This idea was substantiated by experimental data showing that GC ratios of HlyA determinants in different genera are comparable. As the HlyA operon of *E. coli* contains a GC composition of 40 mol [43], some 10% lower than that of the total genom, this species cannot be the phylogenetic source of this attribute. On the contrary the 40% GC ratio is very similar to that of *Proteus*, making this genus a likely candidate ancestor [44]. HlyA production is rarely ever seen in *P. mirabilis*, the most frequently isolated species of *Proteae* but it is commonly found in *P. morganii* and *P. penneri* suggesting the latter two species being the possible ancestor [45]. Genes coding for virulence factors may be structurally organized in **Pa**thogenicity islands – Pais – on the chromosome [46]. Blum et al. [47] identified two Pais on the chromosome of the uropathogenic *E. coli* strain 536 both harbouring the complete genetic information for synthesis, activation and

secretion of HlyA. These chromosomal regions are frequently subject to reorganization events like deletion and recombination. The genetic instability of these DNA regions [48, 49] coding for virulence gene clusters further supports the plausibility of a relatively frequent gene transfer – including virulence genes – among bacteria living in close proximity in the same niche. As *P. penneri* shows frequent reorganizations affecting the hemolysin operon, and its chromosomal GC ratio is identical to that of the HlyA genes in other bacterial species, it seems to be the most likely source where genetic information of HlyA toxins might have spread from.

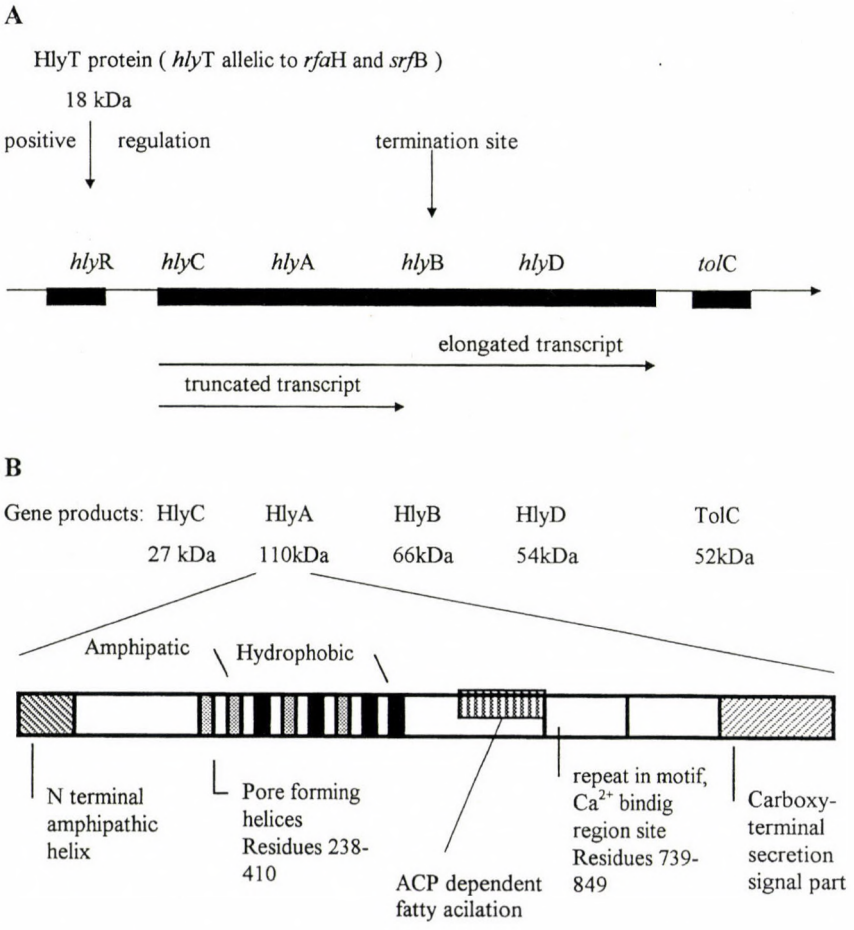

Fig. 1. Genetic determinants of the haemolytic RTX toxins (A) and structure of the HlyA protein (B)

Genetic organization, activation and secretion of HlyA

The *hly*A operon (Fig. 1.A) consists of four genes: *hly*C, *hly*A, *hly*B and *hly*D [50], coding four proteins with molecular weights of 27 kDa, 110 kDa, 66 kDa and 54 kDa, respectively (Fig. 1.B). Transcription starts upstream of *hly*C and proceeds to a rho-independent termination site located between the *hly*A and *hly*B producing a truncated transcript [51]. Bailey and co-workers [52] found that another protein (HlyT) could be an upstream cis acting transcriptional activator of hemolysin synthesis. It regulates positively the transcript initiation and possibly antitermination, generating consequently the elongated hemolysin transcript and the transport proteins HlyB and HlyD, and so it facilitates the synthesis and secretion of the toxin molecule. Gene *hly*T (the gene for HlyT protein is encoded trans on the chromosome a stream to *hly*C and roved to be allelic to *rfa*H and *sfr*B, which regulate transcription of genes encoding synthesis of the sex pilus and the lipopolysaccharide core portion. Mutants in *rfa*H have been found to produce 20–100-fold less HlyA than normal strains. In addition to this Wandersman and Létoffé [53] found that *gal*U can affect hemolysin secretion. Mutations in the *rfa*H or *gal*U loci result in a deep rough core region of the outer membrane, where the TolC content is significantly depleted. This minor outer membrane protein seems to be responsible for the expression of many unlinked and unrelated genes involved in protein secretion of Gram negative bacteria.

The inactive pro-hemolysin is activated by acylation to toxic HlyA by HlyC [54, 55]. This process requires an acylated acyl carrier protein (acyl-ACP) [55, 56].

HlyC proteins can influence the target cell specificity [57] of the activated toxin. RTX leukotoxin (LktA) of *Pasteurella haemolytica* is highly toxic for ruminant leukocytes, but not for erythrocytes. The RTX toxin of *Actinobacillus pleuropneumoniae* (AppA) lyses both leukocytes and erythrocytes.

HlyA is secreted by a HlyB and HlyD directed way, but TolC, an outer membrane protein, regulated by an independent chromosomal locus, is also involved. HlyB is responsible for association with the cytoplasmic membrane [58, 59], it forms six to eight α-helical transmembrane segments [60] with the carboxy terminal region located in the cytoplasm, and containing an ATP binding site. The transmembrane arrangements and the ATP binding motif make HlyB very similar to the members of the ABC superfamily (ATP-Binding Cassette) transport proteins [61–63]. HlyD could be liable for bridge forming between the cytoplasmic and outer membranes of the cell through which activated hemolysin crosses the periplasmic space and is directly extruded to the medium without forming a periplasma intermediate. HlyD is probably situated in the periplasma with its carboxy end and anchored to the cytoplasmic membrane with its amino terminal part [60]. The fact that HlyA has never been found

in the periplasma [64] suggests that it is translocated possibly in a one step process through fusion sites between the cytoplasmic membrane and the outer membrane.

Functional structure of the toxin

The functional structure of α-hemolysin is shown in Fig. 1.B. The amino terminal amphipathic helix is thought to form the pore in eukaryotic cell membranes. Activation takes place on the fatty acid(s) side chain situated between the amphipathic pore forming domain and Ca^{2+} binding site which carries the characteristic RTX motif [65]. The common structural feature of repeats (**R**epeat in **T**o**X**ins) are the tandem repeats of nine amino acids (Leu/Ile/Phe-X-Gly-Gly-X-Gly-Asn/Asp-Asp-X). It is thought to be involved in interaction with the target receptors of cells. The carboxy terminal end of the toxin serves as secretion signal [66].

Mode of action

In mammalian cells hemolysin generates 1–3 nm cation selective membrane pores with consecutive water inflow into the cell and blow up of it [67]. This process could be inhibited by Dextran 4, molecular weight of 4000 kDa, given at 30 mM concentration. This molecule seems to be big enough not to trespass the channels and able to counteract the osmotic pressure of the cytoplasm. It was estimated by Eberspächer et al. [67] that at least 100 HlyA molecules per erythrocyte are needed for lysis to occur within 60 minutes of incubation.

The possible role of HlyA in the pathogenicity

The possible role of hemolytic and non-hemolytic (leukotoxic) RTX toxins as virulence factors has long been ascertained either by human clinical studies [68–72] or by experimental studies applying both *in vitro* and animal models [73, 74].

HlyA exerts a lytic effect on various erythrocyte species, and is toxic for cellular elements of the immune system and a wide range of other cell types including renal tubular cells [75]. By lysing erythrocytes it may liberate iron necessary for bacterial multiplication *in vivo* [76]. The toxic effect on lymphocytes, leukocytes and mononuclear cells interferes with the function of the immune system [77]. In case when subtoxic amounts of HlyA affect these cell types various cytokine responses –

IL-1 β, IL-6, TNF-α – are induced [78], and together with the inflammatory mediators released from endothelial cells and platelets [65, 79] local and generalized inflammatory reactions are induced.

Table II

Virulence of HlyA producing Proteus strains and their isogenic non-hemolytic derivatives*

Virulense assay	Hly+	Hly–
Cytotoxicity ED_{50} (HeLa, HEp-2, Vero)	10^5–$10^{6§}$	$> 10^{8§}$
Intravenous infection with 2.5×10^8 cells	death within 24 h hemoglobinuria	no death no hemoglobinuria
Lung toxicity	hemorrhagic lung edema death within 2–24 h	no death no symptoms
LD_{50} in chicken embryos	$< 10^{2§}$	$> 10^{7§}$

**P. morganii* and *P. penneri*
§bacterial cells

In animal virulence assays this contribution of HlyA to virulence was experimentally documented [80–82]. When hemolytic and non-hemolytic wild type *P. morganii* strains and actinomycin-D treated non hemolytic derivatives were compared in lung toxicity and intraperitoneal virulence assays in mice or in chicken embryo virulence test a marked difference in virulence for the favour of the hemolytic bacteria could be observed. HlyA was supposed to have an acute in vivo toxic effect because of the rapid appearance of fatal hemorrhagic lung edema, and the very short survival time in intraperitoneally infected mice [83].

To establish the role of HlyA in *P. penneri* pathogenicity, the virulence of four types of strains in respect to hemolysin production was compared: 1) wild type strains producing both calcium dependent HlyA and cell bound calcium independent hemolysin; 2) wild type strains producing cell bound hemolysin only; 3) spontaneous non-hemolytic mutants; and 4) transposon mutant *P. penneri* strains with no hemolytic capacity. On HeLa, Hep-2, or Vero cell lines all the wild type strains exhibited >50% lethality when 10^5–10^6 bacterial cells were given to tissue culture plates [84]. The

cytotoxic effect of the strains producing cell-bound hemolysin might be explained by a mechanism similar to the toxic effect elicited by *Escherichia coli* strains producing the so-called contact entero-hemolysin [85]. In case of the spontaneous and transposon induced non-hemolytic mutants 10^8 bacterial cells elicited no detectable changes on the tissue culture cells [84]. When cell free culture supernatants were used only the preparations of strains producing HlyA type filterable hemolysin exhibited cytotoxic activity (Tóth,V.: unpublished data). In virulence titration experiments in mice hemolytic wild type strains exhibited a virulence with 10^7–10^8 range of lethal doses, but the spontaneous and transposon mutants failed to show any effect even when 5×10^8 bacterial cells were applied. Cells of hemolytic *P. penneri* strains, similarly to *P. morganii*, killed the animals within a couple of hours after the infection, indicating an acute toxic effect rather than a real infectious process with *in vivo* bacterial multiplication. None of the non-hemolytic derivates elicited any symptoms, and no death occurred when comparable doses were applied [86]. In the mouse respiratory infection assay *P. penneri* strains with cell bound hemolysin showed decreased or no toxic effect. Animals given the non-hemolytic mutants remained free of symptoms during the 48-hour observation period [86].

The fact that rabbits infected with either HlyA producing *E. coli* or *Proteus* strains develop high titre anti-HlyA antibodies not only indicates the expression of hemolysin genes *in vivo* but also proves that specific lymphocyte clones are triggered. The protective effect of these anti-hemolytic antibodies was evaluated in neutralization assays using the mouse lung toxicity test and allantoic infection of chicken embryos. In both systems neutralization could be observed when *E. coli* or *Proteus* strains were preincubated with immune sera raised either by HlyA producing *E. coli* or *Proteus* strains. Specific anti-HlyA antibodies are produced also in patients suffering from infections caused by bacterial strains producing HlyA toxin [87]. These antibodies are capable of neutralizing the toxic effects of α-hemolytic *E. coli* or *Proteus* strains.

The above *in vitro* and *in vivo* data strongly support the idea that HlyA type hemolysin of *Proteae* is a major virulence factor contributing to the pathogenetic process by exerting a toxic effect on the infected tissue and invading inflammatory cells at the site of infection. It should also be taken into consideration that the complex procedure of bacterial infections can be characterized as the result of concerted action of various virulence factors in a given host organism [88]. α-Haemolysin is only one of these factors but certainly a significant one in the case of *Proteus* infections caused by HlyA producing strains.

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VIRAL CONTAMINANTS OF POLIOMYELITIS VACCINES

J. G. SINKOVICS*, J. C. HORVATH**

Cancer Institute, St. Joseph's Hospital; Departments of Medicine and Medical Microbiology and Immunology, University of South Florida College of Medicine, 3001 W M. L. King Blvd, Tampa FL 33677 Tampa, FL, USA

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Poliomyelitis (PM) (polio=grey) devastated mankind for millennia (as described and depicted in the Ebers papyrus in ancient Egypt). In the 1950s its approximate worldwide incidence was 8–28/10^5 in the general population; 116/10^5 in children and with paralytic PM over 15/10^5. The virus was first transferred from patients to monkeys in 1909 (Landsteiner & Popper: "Übertragung auf Affen"); grown in tissue cultures in 1949 (Enders, Weller & Robbins); and characterized thereafter as a member of the family Picornaviridae in the genus Enterovirus (that also includes families of Coxsackieviruses and Echoviruses) [1]. PM virus might have originated eons ago from cellular mRNA; it replicates in humans in the cytoplasm of its host cells (gut, lymph nodes, anterior horn motor neurons) which die. A viral capsid protein attaches to an immunoglobulin superfamily receptor and through it the virus particle enters the cell. Spectacular 3-dimensional pictures of the virus-receptor relationship appeared in 2000 [2, 3]. The virion changes its protein structure when it attaches to its cell surface receptor (Pvr): it externalizes the lipophilic and hydrophobic amino terminus of the VPI protein. The uncoated 7400-nucleotide viral genome is translated into viral

*The senior author had the privilege to visit with Dr. Hilary Koprowski (Lederle Laboratories, Pearl River, New York) in December 1956 and January 1957 and with Dr. Albert Sabin (University Medical School, Cincinnati, Ohio) in 1957 and 1958. He cherishes the memories of their discussions and explanations as to how they tried then to achieve the attenuation of types 1, 2, 3 polioviruses, for the production of a live attenuated vaccine. He also served as visiting professor of virology at Dr. Joseph Melnick's department (Baylor College of Medicine, Houston TX) from 1980 to 1990; the discussions on the poliovaccines were daily topics. The history of poliomyelitis represents a condensed history of the entire science of virology. The authors could not resist in reciting some of the basic data that are common knowledge for virologists.

**Corresponding author, Fax: (1) 813 870 4825, E-mail: jhorvath@com1.med.usf.edu

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polyproteins (poly=many, several) and the viral RNA replicates through the formation of minus stranded RNA, mRNA and progeny RNA genomes. The polyproteins are cleaved and processed by the virus-encoded protease to form the pentamers of the viral capsid. Virus particles are assembled by encapsidation of the genomic RNA or by the pentamers nucleating around viral RNA.

The virus protects itself against interferon. When poliovirus enters the cell it shuts off all cellular mRNA→protein synthesis. Interferons induce dsRNA-activated protein kinases and ribonuclease L that would degrade viral RNA. Poliovirus induces inhibitors of these enzymes and prevails [4]. Mature viral progeny is released from disintegrating cells [5]. Of host defenses, virus-neutralizating antibodies are the most important [6]. PM viruses exist in 3 major types I. Brunhilde (Mahoney); 2. Lansing (rodent adapted); and 3. Leon. PM viruses grow well in Asian and African monkey kidney cell (MKC) cultures and in human fetal or foreskin fibroblasts. Attenuated PM viruses can circulate in the human population and by further mutations or recombinations on very rare occasions may regain their neurovirulence [4, 7–9]. For example in Dr. Sabin's laboratories a type 3 (Leon37) strain was submitted to 21 monkey passages *in vivo,* 47 rhesus monkey testicle and kidney cell passages *in vitro,* 3 plaque purification procedures in MKC cultures and 3 more passages in MKC cultures before it could be incorporated in the trivalent vaccine: P3 Leon 12ab. In the attenuated virus phenylalanine replaced serine and arginine and alanine replaced lysine and threonine. This virus on rare occasions could revert to virulence: the P3/119 revertant in which alanine was replaced by valine and arginine and leucine were replaced by lysine and methionine [4]. Sabin's attenuated types 1, 2, 3 viruses were mutated at the nonprotein-coding 5' end of the viral genome (5-UTR 480, 481, 472) which reduced their ability to replicate in nerve cells [4]. The viral protein mutations did not affect antibody-mediated neutralization of wild poliovirus virions.

Both the MKC-grown virulent but inactivated Salk vaccine and the neurovirulence-defective but replicating Sabin vaccine contained simian virus 40. The DNA SV40 vacuolates its host cells and can malignantly transform human fibroblasts *in vitro* or cause many different neoplasms in rodents (hamsters) by combining its gene product protein large T antigen with those of the tumor suppressor genes p53 and/or Rb [10, 11]. In the past decade SV40 genomic sequences and peptides encoded by them were found in some human tumors (choroid plexus ependymomas, medulloblastomas, osteosarcomas, mesotheliomas, papillary thyroid carcinomas, etc). Tumor-bearing and some tumor-free patients circulated antibodies directed at SV40 proteins. Due to unknown exposure such antibodies could be found in some exceptional human sera prior to the poliovirus vaccination campaigns. It remains unsettled if SV40 caused,

contributed to, or only used, these tumors for its replication or persistence as passenger [12, 13].

Since attenuated seed PM viruses were grown in MKC cultures both in Cincinnati (Sabin) and in Philadelphia (Wistar Institute, Koprowski), the question is posed repeatedly and in an accusatory tone [14]: if these attenuated oral poliovaccines (AOPV) contained simian or human immunodeficiency viruses (SIV, HIV)? The outbreak of the overwhelming African AIDS epidemic could be coincidental but it is used as an argument for the possible introduction of the AIDS viruses HIV-1 and -2 by Wistar's AOPV into the African population where this vaccine was extensively used to eradicate PM in the late 1950s and early 1960s. Unfortunately, the 1996 WHO world map shows Central Africa with high incidence of PM again ($>10/10^5$) certainly due to the cessation of vaccination campaigns. Of several strains of SIV, only the chimpanzee virus (SIVcpz) could be considered as the source of the first HIV-1 strain; macacus and cercopithecus SIVs are very different from HIV-1 [15] and these were the species whose kidneys were most commonly used (over 200.000 of them!) to culture attenuated PM viruses.

HIV-2 derives from sooty mangabeys (Cercocebus atys) whose kidney cells were not used to grow PM viruses but these monkeys are eaten in Africa. Several episodes of direct monkey-to-man transfers of HIV-2 occurring in the wild have been very well documented [16]. Humans are not natural hosts of HIV-1 or 2. HIV-1 evolved in the 3rd and 4th decades of this century from an ancient SIVcpzUS substrain by acquiring the new *vpu* genomic configuration (absent in all other SIVs!) enabling it to grow in the new human host [17–19]. The classical epidemiology of AIDS holds that skin-penetrating injuries were the portals of entry for the first HIV-1 from butchered (in the rapidly enlarging "bushmeat" market) chimpanzees (Pan troglodytes troglodytes) (Ptt) to man. Even if chimpanzee kidneys were shipped from the former Belgian Congo to the Wistar Institute [14], these were from Pt schweinfurthii or from Pan paniscus (bonobo: pygmy chimp), species in which SIVcpz did not evolve toward HIV-1 (no *vpu* gene) [19]. Sabin's AOPV seed viruses did not derive from chimpanzee kidney cultures; and Wistar's AOPV was also most unlikely to have incorporated an HIV-1 precursor virus: it did not cause AIDS epidemics in Croatia, Poland or Western Europe (where the Wistar CHAT seed attenuated PM virus was used but probably grown in human WI-38 fibroblasts). Further, HIV-1 would not have replicated in MKC cultures (but resident CD4 lymphocytes or monocytes-macrophages might have allowed for some viral growth in the primary MKC cultures but disappearing with passages); and it would not have readily infected through oral routes. Even those unconventional practitioners in the USA who applied AOPV to treat genital herpesvirus infections of male homosexuals in the hope that the PM virus would induce interferon production

could not have started the AIDS epidemics since the AOPV preparations were devoid of HIV-1 or 2 (for more references [20]). Once in its new host, HIV-1 M (Central Africa→worldwide), N (Cameroon) and O groups rapidly radiated mutating at their env V3 loop and recombining most frequently in M A/E and M A/G of the 11 M A-K clades [19]. Group N HIV-1 in its chimpanzee host recombined to be a SIVcpzUS/HIV-1 virus [18]. Recent socioeconomic changes of human life (African civil wars, natural disasters and movements of large segments of populations, including travel) promoted the spread of the new human virus mainly through heterosexual routes in Africa and through homosexual practices in the Western hemisphere.

Centuries ago HTLV-1 made its jump from Pan troglodytes to man and with Portuguese sailors reached Japan; with the slave trade it reached the Caribbean Islands, the Southeastern United States and Brazil; HTLV-II derives from Pan paniscus [21].

In Hungary [22, 23] Sabin's AOPV was used (never Wistar's CHAT or other AOPV) and the vaccine was screened for SV40 contamination once this virus was discovered [24–25]; it could not have been screened for the as yet undiscovered simian or human immunodeficiency viruses (personal communications from Professors István Dömök and Sándor Koch).

Current anti-PM vaccination strategies prefer the inactivated Salk vaccine that may be followed by the attenuated Sabin vaccine screened for, and free of SV40 or retrolentiviruses. Considerations are given to the future use of an inactivated Sabin virus vaccine [26]. These vaccines almost eradicated PM and hold the promise for complete eradication of this disease if the vaccines are used. There is no scientific proof for the causation of human tumors by SV40 but this virus could persist in the human host and could interact with other carcinogens; poliovaccines now in use are free of SV40. There is no proof that AOPV contained HIV; Sabin's vaccine could not have been contaminated with this virus and remaining ampules of Wistar's CHAT seed virus so far did not yield this virus (for more references [20]). Further RT-PCR assays are being conducted on vaccine samples used in the 1950s.

AOPV currently in use are free of simian retrolentiviruses and/or HIV. If scientifically not validated theories prevail and consequentially antiviral vaccines fall out of use, the world will have to face devastating epidemics of viral diseases.

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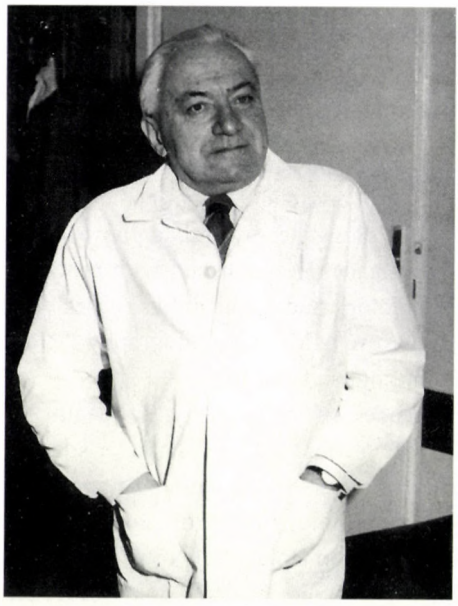
IN MEMORIAM LAJOS VÁCZI

(1917–2000)



Professor **Lajos Váczi** M. D. was born on 2.5.1917 in a peasant family of Komádi. He achieved a first-class school leaving certificate in the high school of Szeghalom. He graduated from the Medical University of Debrecen in 1942. He was the second medical doctor who's graduation was rewarded with the honour of "sub auspiciis gubernatoris" (governer's ring) in Hungary. (The first was professor György Berencsi II. M.D., who graduated from Budapest in 1936.)

He worked in the Institute of Hygiene, Medical University of Debrecen till 1944 as an unpaid junior assistant, later as a professor's assistant. Then he was commissioned to the task of Hajdú-Bihar County's medical officer of health.

From 1945 till 1951 he was the head of the professional teaching department of the Ministry of Health. From 1951 as a member of the Department of Bacteriology of the National Institute of Hygiene he became inspector of the national laboratory

network. Between 1956 and 1958 he was the head of the Department of Bacteriology. On 12.12.1958 he was appointed to professorship in the Institute of Microbiology, Medical University of Debrecen, as the head of the department.

He got the medical officer's certificate in 1947, qualification for laboratory in 1950, for public health and epidemiology in 1959, and for medical microbiology in 1979. He earned the academic candidate's degree in 1957, the academic doctor qualification of the Hungarian Academy of Sciences in 1968.

In 1943 he spent six months in the Institute of Hygiene of the University of Vienna. In 1948 he got a 6 months WHO fellowship to England and Sweden, later – in 1963 – he returned to England for 3 months.

In the first fifteen years of his career he took part in the extension of the Béla Johan founded public health network to all Hungarian counties.

On the field of bacteriology he researched the bacterial metabolism and the mechanism of antibiotic resistance. He published with professor Endre Jeney his first book, titled "Applied microbiology and its theoretical bases". His research work on the lipid metabolism of bacteria resulted is the monography "Biological role of bacterial lipids", published in 1973 in English language.

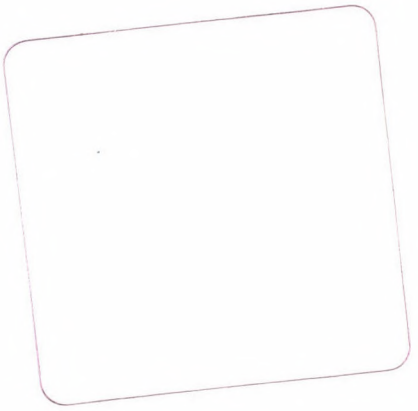
In the last two decades of his activity he dealt with the oncogenic and immunological properties of viruses and the characteristics of their replication. He initiated the professional career of several prominent representatives of Hungarian and international virology and microbiology (László Géder, Éva Gönczöl, Lajos Gergely, Mihály Fodor, Miklós Koller, István Boldogh, Ferenc D.Tóth, Ferenc Rozgonyi, Enikő Jeney, Frigyes Lehel, Antal Réti, Judit Czeglédi, Béla Szabó, etc.), who are even today active and leading persons of the profession.

During his life he was co-author of almost 200 scientific papers. His textbook with the co-authorship of Ilona Béládi, Iván Kétyi and István Nász was an interesting experiment for the introduction of "organ-specific microbiology" into the medical education (Medical Microbiology, Immunology, Parasitology, Medicina Publishing House, 1978).

He was a foundation member of the Hungarian Society for Microbiology, later between 1975 and 1985 he was its president. He was honorary member of altogether 8 different foreign universities, academies and international societies. He got the Bujvic medal from the Polish Society for Microbiology. In Hungary he was awarded with the title Eminent Physician, twice with the gold medal of the Order of Labour, the "Pro Univesitate" medal of the Medical University of Debrecen and with medals of different scientific societies (István Went, József Fodor and Rezső Manninger medals).

All of his three sons have chosen the medical profession. The last honours were given to him – chairman and professor of the Institute of Microbiology, the former

scientific prorector (1964–1970) – with loving kindness and reverence by the members of Medical and Hygiene Center of Debrecen University. In this issue the members of our journal's editorial board, the representatives of the profession, the members and leading members of different scientific societies, among whom several were his students and co-workers commemorate professor Váczi with deep respect. His remembrance will be kept also by those who observed his career only from a distance but were conscious of his encouraging support.



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