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A B S T R A C T S

BLOCK 1: Direct diagnostics and microbial identification

M. Vlk, J. Svoboda (Institute for Microbiology, Faculty of Medicine of Masaryk University and St. Anna's Faculty Hospital, Brno, Czech Republic):

Significance of the test of decapsulation for the identification of the bacterial species *Staphylococcus aureus*

At present the most used test for the identification of the species *Staphylococcus aureus (STAU)* is the detection of the coagulase-production, both of its free (by means of the tube test) and its bound (using slide tests) fractions. We are capable to distinguish different coagulase-positive species of staphylococci using a variety of other (mostly simple) tests. In clinical materials of human medicine we confirm *STAU* with a high probability rate.

Staphylococci produce, however, a number of other enzymes, some of them being produced only by certain species.

In our work we focused on hyaluronidase-production in staphylococci. We tried to find out the number of hyaluronidase-positive *STAU* on one hand and that of the other staphylococci producing this enzyme on the other hand. In other words, we wished to be able to take a position on the potential of hyaluronidase-detection (i. e. the test of decapsulation) for differentiation of *STAU* from other staphylococci.

The chosen method of hyaluronidase-detection is based on its ability to split the hyaline capsule of the bacterial species *Streptococcus equi.* The whole procedure is fast, carried out in a simple way and its result is evident. Compared with other currently used tests this one is substantially cheaper. Its disadvantage is reading test results only after 18 hours.

We had not found enough information on the value of the test in literature. That is why we examined staphylococci, of clinical origin, using three different methods:

• test of decapsulation for hyaluronidase-detection

• third-generation-latex-test for the detection of clumping-factor (CF) (combined with PYR-test in order to distinguish *STAU* from other CF-positive strains)

• tube test for the revelation of free coagulase (completed by the test of anaerobic mannitol-fermentation) – in our work the reference method for *STAU* identification

The results of our research show a relatively high correlation of all three tests. This fact, together with very low costs of the test of decapsulation can make us take into account its eventual introduction in the procedure of *STAU* identification. However, an essential condition for this should be that sufficient specificity and sensitivity of the test of decapsulation is confirmed.

We can conclude that the problem of hyaluronidase-presence in staphylococci remains an open issue that needs in future to be kept exploring, discussing and documenting on much larger amount of strains.

L. Mejzlíková (Institute for Microbiology, Faculty of Medicine of Masaryk University and St. Anna's Faculty Hospital, Brno, Czech Republic):

Isolation of haemophili from clinical material

The species of *Haemophilus* require to their cultivation the V-factor (nicotinamid-adenin dinucleotide) and X (haemin). The optimal media for their growth are blood agar with the staphylococcal line and chocolate agar. The basal of this work is the comparison of both cultivation media used to hemophili detection.

M. Sládeková, J. Hanzen, M. Lisálová, P. Milošovič (HPL Ltd. - Microbiological Laboratory, Bratislava, Slovakia):

Serological typing of *Haemophilus influenzae* strains in clinical materials

In the Slovak Republic, the obligatory vaccination against *Haemophilus influenzae* type b (Hib) is now in progress. The purpose of the vaccination is to decrease the occurrence of the invasive diseases caused by pathogenous strains of Hib.

The authors submit the set of the strains of *Haemophilus influenzae* (Hi) which were isolated, identified and classified by agglutinating serum a, b, c, d, e, f during more than one year. The aim of this work was to map the situation in the region of Bratislava before the beginning of the obligatory vaccination against Hib. This vaccine should increase the immunity of the threatened age category. The tables and graphs present the analysis of the occurrence of the individual serotypes Hi according to the kind of the material, to the age category and to the type of diagnosis. The work also presents the review of the sensibility of antiinfection substances and we have laid stress upon the group b.

L. Slobodníková, A. Jánošková, I. Kozínková, Ĺ. Klokočníková, D. Kotulová (Institute of Microbiology of School of Medicine and Faculty Hospital, Bratislava, Slovakia):

CHROMagar Orientation in the diagnostics of urinary tract infections

Urine samples create a considerable part of biological materials cultured in laboratories of clinical microbiology. Appropriate culture media play an important role in their precise, reliable and time-efficient examination. The aim of this study was to analyse 445 mid-stream urine samples of patients with symptoms of urinary tract infections hospitalised in the Faculty Hospital in Bratislava and to compare the culture results obtained on blood agar (BA) in combination with Endo agar (EA) with those obtained after a parallel cultivation on CHROMagar Orientation, BBL (CHA). Urine samples were processed following standard procedures and evaluated after an overnight cultivation at 36°C. Isolation of no more than three various microbial species and cell counts of 10^3 per ml were considered clinically significant. 61 samples (13.7 %) yielded mixed culture of more than 3 species at least on one of the compared media (18 on BA/EA only, and 33 on CHA only). All these specimens were considered to be contaminated with normal flora in the process of sampling. Absence of any microbial growth was detected identically on all tested media in 105 samples (23.6 %); 18 samples yielded growth only on BA (2 in significant cell count) and 54 only on CHA (22 in significant cell count). Nonsignificant bacteriuria was detected identically in 33 samples (7.4 %). Different cell counts in consideration to the clinical significance were detected in 55 cases, with significant counts mainly on CHA. Of the 143 specimens (32.1 %) yielding microbial growth in

significant cell count on all media, 147 samples yielded growth of 1 species in significant count on CHA and 128 on BA/EA; 34 samples yielded growth of two species in significant count on CHA and 27 on BA/EA. Three species in significant cell count were isolated on CHA only (from 6 samples). The majority of discrepant results was caused by more precise differentiation of microbial cultures on CHA. The differences concerned mainly the *Enterococcus* genus, which grows on CHA in easily detectable blue colonies; its growth on BA may be hidden by the colonies of gramnegative bacteria or staphylococci. Additional advantage of CHA is its ability to inhibit swarming of *Proteus* and to give correct culture results in the presence of this genus at any amount. The results documented very good potential of CHA to differentiate micro-organisms in urine cultures, which allowed for safe and easy detection of urine specimens contaminated during sampling, and reliable detection and enumeration of micro-organisms in mixed cultures.

E. Durnová et al. (Regional Institute for Public Health, Ostrava, Czech Republic):

Identification of bacteria species on a profile of whole cell fatty acids – Sherlock system, Midi Inc

The Sherlock system is able to identify bacteria species on the basis of presence of fatty acids in their cell lipoid structures, which is species specific.

Fatty acids are picked up from freshly growing culture of bacteria cells by means of heated alkaline hydrolysis, followed by acidifying of the arising salts. As fatty acid methyl esters (FAME), these are then transferred into a mixture of organic solvents. The FAME profile of an unknown strain, which is present in the mixture, gained by the gas chromatography, is then compared with the sample fatty acids profiles of the fatty acid samples of the reference strains in a computer library.

The presence and amount of each FAME in the bacteria cell are to a great extent dependent on the selected medium, temperature, and period of cultivation. Unknown strain can be identified not only if the culture is pure, but also the standard conditions should be kept. Besides the common aerobic and anaerobic bacteria, the Sherlock system is able to identify yeast, moulds and mycobacteria, too.

Results obtained during one year show the ability of this identification method for the routine diagnostic of some microbial groups in a clinical laboratory (e. g. gramnegative rods, anaerobic bacteria).

F. Růžička (Institute for Microbiology, Faculty of Medicine of Masaryk University and St. Anna's Faculty Hospital, Brno, Czech Republic):

Detection of elastase at *Candida albicans*

Elastase enzyme was found in many microbial species. Production of this enzyme was proven in many fungal species too (*Cryptococcus*, *Aspergilus*, *Trichophyton*, *Epidermophyton* etc.).The elastase inhibits phagocytosis of macrophages and myeloperoxidase activity of polymorphonuclears, decomposes immunoglobulins of classes IgA and IgG and degrades many tissue components, especially elastin, laminin, fibrin, human collagen of types III and IV etc.. The attribute is considered as potential factor of microbial virulence responsible for invasivity.

The aim of this study was to evaluate the production of elastase in *Candida albicans* strains isolated from different clinical materials. These strains were tested for the proof of elastolytic activity using the pulverisation variant of the classical method which is based on sprinkle of colonies with fine granules of elastin with Congo red.

BLOCK 2: Serological and virological methods in microbial diagnostics

M. Kobidová[1], K. Schwarzová, I. Čižnár ([1]Faculty of Science of Comenius University & Institute of Preventive and Clinical Medicine, Bratislava, Slovakia):

Immunochemical analysis of *Borrelia burgdorferi* s. l. strains, isolated from patients in Slovakia

Strains of *Borrelia burgdorferi* s. l. isolated from the blood and spinal fluid of patients with Lyme borreliosis were analysed by immunochemical methods. Whole cell antigens of these isolates

were tested using Ouchterlony's method, crossed immunoelectrophoresis and standard immunoelectrophoresis. Strains PK1 and LeMo isolated from blood and spinal fluid of patients with erythema migrans and neuritis reacted with hyperimmune rabbit antisera against *B. garinii* and *B. afzelii* forming thick diffusion bands. These bands indicated that they could be produced by LPS complex.

The antigen spectrum detected by these methods pointed out to some differences between individual species. Thus, this method could serve as a helpful tool for differentiation of species.

G. Vozárová[1], *F. Ondriska*[1], *R. Mego*[2], *M. Ondrušková*[3], *D. Moravčíková*[3], *M. Noskovičová*[4] ([1]HPL Ltd., Parasitological Laboratory, Bratislava, Slovakia, [2]Children's Faculty Hospital, Department of Neurology, Bratislava, Slovakia, [3]Dérer's Hospital, Clinic of Infectious Diseases and Geographical Medicine, Bratislava, Slovakia, [4]Slovak Institute of Cardiovascular Diseases, Department of Transplantation, Bratislava, Slovakia):

Laboratory diagnostics of toxoplasmosis and its importance for clinical diagnosis

Toxoplasmosis is one of the most widespread parasitic human diseases. The causative agent is *Toxoplasma gondii*. In the most postnatal infections in healthy immunocompetent people toxoplasmic infection occurs asymptotically in spite of the fact that parasite survives in the host's organism during its whole life. Most of all it potentially endangers two human groups: foetus and immunosuppressed patient. Is it therefore understandable that earlier diagnosis with appropriate chosen methods for proof of toxoplasmosis and efficient therapy are the limited criteria for further fate of these patients.

The following two cases of the most hazardous groups show actual possibilities of serological diagnostics of toxoplasmosis in routine parasitological laboratory. The first case presents the proof of congenital toxoplasmosis occurring in fourth months old child whose illness manifested with a neurological and an oftalmological symptomatology. The finding of the specific antibodies of IgA, IgM and borderline IgG avidity in liquor and serum confirmed by Western-blot (IgM), with the proof of their intrathecal synthesis (according to Reiber) and the proof of total antibodies (CFT) in high levels confirmed acute toxoplasmosis with pathological process in CNS. Retrospective serological examination of mother proved primoinfection in pregnancy.

The second case documents the importance and effectivity of serological monitoring of the patients on toxoplasmosis before and after transplantation of heart. Of 172 examined patients the infection of *Toxoplasma gondii* was proved after the transplantation of heart in one case (seronegative before). It was probably acquired by the transplant. The disease was manifested with fever and sinusitis. Using serological diagnostics high levels of IgM (1 : 3835) and IgA (1 : 4500) antibodies and seroconversion of total (CFT) and specific IgG antibodies was proved. After the therapy the patient's condition ameliorated, with levels of antibody decreased.

In both cases we determined the stage of the disease on the base of a single sample of serum and liquor. The fast laboratory diagnostics with the optimum combination of serological methods and interpretation of the results proved aetiology of the disease, which especially in the second case, did not clinically perspicuously manifest.

Ľ. Vozárová, D. Hučková, J. Predný (HPL Ltd., Microbiological Laboratory, Bratislava, Slovakia):

Our experience with diagnostics of cytomegalovirus (CMV) in immunocompromised patients

In our work we present results for one year, which we acquired in laboratory diagnostics of CMV infection in immunocompromised patients. We ensure investigation for several groups of these patients – pathological new-borns, child oncological patients and especially persons integrated to transplant programs. The last group concerns the investigation of donors and recipients and posttransplant monitoring after transplantation of bone marrow, heart, lung and kidneys.

Among the methods of the direct detection of CMV we chose the determination of CMV pp65 antigenemia in peripheral blood leukocytes and PCR assay.

We investigated 251 plasma samples from 88 patients by PCR assay. Three heart transplant recipients, one lung transplant recipient, one kidney transplant recipient and two patients with other diagnosis were PCR positive. We investigated 219 blood samples from 90 patients by antigenemia assay. CMV antigen was detected in only one patient who was not transplanted.

Our experience confirmed the necessity of application more methods for detection of active CMV infection. PCR assay proved more for monitoring of possible active CMV infection and for timely initiation of preemtive antiviral therapy than antigenemia.

M. Stará, S. A. Bopegamage (Institute of Preventive and Clinical Medicine, Bratislava, Slovakia):

Demonstration of antibodies against Coxsackie B3 virus in orally infected mice using Western blot

Coxsackie B viruses are important human pathogens frequently associated with acute diseases, but also with serious chronic diseases like myalgia, myocarditis, IDDM. Therefore the rapid diagnosis of these infections is desired.

For study of some aspects of pathogenesis of coxsackieviral infection 3 weeks old Swiss albino mice were used. The mice were given CVB3 by oral ravage. The blood samples were collected by direct heart puncture, at different time intervals after infection. Anti-coxsackieviral antibodies in the sera of these mice were detected using the Western blot technique developed in our laboratory. For separation of capsid viral protein was used gel with 9–12 % concentration of polyacrylamid (gradient gel). Comparison of the results obtained using of single antigen strips (CBV 3) and mixed antigen (CVB1–6) showed a slight difference. The antibodies were detected in tests with the mixed antigen on day 4 p. i. and a number of reacting bands were seen and on day 5 p. i. with a clear single reacting band in the single antigen strip. The results were comparable with the results obtained in virus neutralisation tests.

The Western blot method developed in our laboratory showed to be a rapid and sensitive method for preliminary diagnosis and screening of coxsackie virus infections.

K. Hejcmanová, J. Smola (Institute for Microbiology and Immunology, Veterinary and Pharmaceutical University, Brno, Czech Republic):

Serological diagnostics disease of animals, invite with bacterial species *Lawsonia intracellularis*

The bacterial species *Lawsonia intracellularis* is the causal of persistent disease, which is resulted by proliferative enteritis. The disease is detected not only in pigs but also in other animals including primates. This hypothesis was determined on the bases of PCR results and consequently by genetic analysis of DNA. For diagnostics purpose is used PCR as main direct method. Additionally it can be used indirect methods to detected the causative agent. The aim of this work is to detect serological positivity in pigs. During this study was determined serum of other possible host species. It was a possibility to proof a new serological kit of indirect imunofluorescence method, which is produced by ELANCO. It was analysed 489 blood serum samples of pigs and 66 % of this were positive. Simultaneously 35 serum samples from wild pigs were determined and 51 % of this were positive. More of this 41 serum samples from horses were evaluated and 80 % of it were positive. Also after investigation of serum samples of other animals was registered that, 74 % positivity in dogs, 29 % positivity in *Mesocricetus auratus* were seen with specific IgG antibodies. Negative results were registered in human serum. The present of antibodies in serum of domestic and wild pigs confirms the extensive expansion of this disease in natural habitat. Detection of antibodies in other animals showed the wide range of host determination.

J. Chumchalová, J. Šmarda (Department of Biology, Medical Faculty, Masaryk University, Brno, Czech Republic):

Colicins and their effect on malignant cells

Colicins are generally characterised as proteins synthesised by *Escherichia coli* and other *Enterobacteriaceae* with specific antibiotic effects towards other strains of the same species. Bacterial receptors are generally considered to be decisive for a role in binding colicins to bacterial cells and mediate their specific antibacterial effects.

We can sort colicins into three large groups on the bases of their inhibition effects. 1st group: colicins form pores in the plasma membrane of sensitive cells, thus interfering with the membrane potential, 2nd group: colicins that destroy bacterial DNA by acting as non-specific DNA endonucleases, 3rd group: colicins that block protein synthesis in sensitive cells acting as a specific 16S rRNAase. Colicins exert inhibition effects not only on normal sensitive bacterial cells, but on their protoplast-like stable L-form cells as well. This fact led to testing colicins for inhibition of eukaryotic cells which do not have cell wall. ämarda (1978) found inhibition effect of colicin E3 on mouse fibroblasts and human HeLa cells. Farkas-Himsley and Cheung (1976) reported toxic effects of colicin, vibriocin and pyocin on established mouse neoplastic cell line L60T. Saito and Watanabe (1979) found normal cells to be less sensitive to colicins than tumour transformed cells. Animal transformed cells were more sensitive than human malignant cells.

We tested four colicins (A, E1, E3 and U) on 11 cancer cell lines with characterised status of protein p53 and on one normal cell line. Sensitivity of these lines to colicins in vitro was quantified by MTT assay. We also tested effect of three colicins on 5 selected lines and their cell cycle. Colicins E3 and U did not show any inhibition effect on the cell lines tested, but colicins E1 and A inhibited most of them. Colicin E1 had no inhibition effect on the normal cell line. Tumour line HT 29 was more resistant than the normal one. Colicin A showed an inhibition effect about 35 % on the normal cell line. The most sensitive were the carcinoma lines BT549 (56 %) and SKUT (72 %) and the most resistant was BT474 (- 10 %).

We pursued changes in cell cycle by flowcytometry on five selected lines MRC5, MCF7, HS913T, HOS and MDA-MB-231, after treating them by three colicins with affect plasma membrane: U, A and E1. Colicin U had no significant changes in cell cycle, but colicins A and E1 changed cell cycle of the cell lines treated. We observed increase of cell number in G1 phase in lines with wt p53 for 23–35 %, but of cell lines which mutations in the gene p53 only for 5–16 %. Number of cells in apoptosis after exposure to colicin A was increased for 7–28 %, except for the cell line HS913T. After treating the cells by colicin E1, their cycle was altered only in 2 lines: number of MCF7 cells in G1 phase was increased for 58 % and their number in apoptosis for 58 %, too, and in HS913T for 17%. These results correlate with those of chemosensitivity assays, in which colicin U had no effect and the effect of colicin A on most cell lines tested was stronger than that of colicin E1.

K. Kollárová, I. Čižnár (Institute of Preventive and Clinical Medicine, Bratislava, Slovakia):

Cross-reactivity of whole-cell antigens of *Plesiomonas shigelloides* and members of family *Enterobacteriaceae* and *Vibrionaceae* with rabbit antiserum

Antigenic similarity between Plesiomonas shigelloides and strains from family Enterobacteriaceae (Citrobacter sp., Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, Provindetia rettgeri, Salmonella Enteritidis, Shigella boydii, Shigella flexneri, Shigella sonnei S and R form) and Vibrionaceae (Vibrio cholerae El Tor Ogawa, Vibrio cholerae El Tor Inaba, Vibrio cholerae non-O1, Vibrio fluvialis, Vibrio parahaemolyticus, Vibrio metschnikovii) was studied by crossed immunoelectrophoresis.

Cells were cultivated in a peptone water with neutral pH for *Enterobacteriaceae* and alkaline pH for *Vibrionaceae* at 37 °C for 18 hours. The cells were harvested by centrifugation of the culture and after washing them in PBS (pH 7,2) they were disintegrated by ultrasound. Hyperimmune rabbit serum was obtained after i.v. immunisation with whole-cell antigens of *Plesiomonas shigelloides* O48 H3.

The experiments showed that number of common antigens among members of family *Enterobacteriaceae* and *Vibrionaceae* reacting with antibodies against *Plesiomonas shigelloides* was in range from 3 to 6. Some antigens formed bands of similar shape and position on immunoelectrophoretogram. However, some of the antigens formed bands of different characteristics. The largest number of common antigens was with *Shigella sonnei*.

R. Melková, I. Čižnár (Institute of Preventive and Clinical Medicine, Bratislava, Slovakia):

Identification of antigen adsorbed by Mycoplasmas from media

We have studied antigenic profile of *Mycoplasma hominis, Mycoplasma fermentans* and *Ureaplasma urealyticum*. For this purpose immunochemical methods – double immunodiffusion in agar gel, immunoelectrophoresis and crossed immunoelectrophoresis – were applied. The antiserum used in this study was obtained by i. v. immunisation of rabbits with whole-cell antigens of the Mycoplasmas. The cultivation medium for Mycoplasmas was enriched by horse serum.

The adsorption studies with media components showed that the dominating antigen, which was identical for all three Mycoplasmas, originated from horse serum. The precipiting band formed by this antigen disappeared from the immunoelectrophoretic picture when the rabbit antiserum was adsorbed with the horse serum and used in the immunochemical studies.

D. Kotulová[1], L. Slobodníková[1], L. Klokočníková[1], A. Longauerová[1], J. Horvát[2], I. Kozánková[1] ([1]Institute of Microbiology, [2]1st Department of Orthopaedics, School of Medicine of Comenius University and University Hospital, Bratislava, Slovakia)

Bacterial infections of hip prostheses

An objective of the study was to analyse the spectrum of bacterial agents causing hip prostheses infections in patients with clinical signs of inflammation in the implanted area, with consideration to the type of plastic material of implanted prostheses.

In the period from July 1995 to December 1999, samples from prosthetic hip joints of 124 patients of the University Hospital in Bratislava with inflammation in the implanted area were collected and examined for the presence of aerobic and anaerobic bacteria. The quantitative antibiotic susceptibility of isolated and identified strains was tested. Individual sets of the tested antibiotics were chosen according to the bacterial species.

Samples of 82 (66 %) patients yielded positive culture results. Coagulase negative staphylococci were isolated most frequently (in 48 % of positive cases). Hybrid total hip prosthesis was the implant type most frequently infected with this group of bacteria.

Infection of 33 patients in the study was cured after the intensive complex antibiotic therapy. 57 % of patients with therapeutic failure were infected with *Pseudomonas aeruginosa* and other gram-negative non-fermenting bacteria.

Coagulase-negative staphylococci and gram-negative non-fermenting bacteria were the most frequent bacterial infecting agents of the prosthetic hips of investigated patients, and gram-negative non-fermenting bacteria caused the most therapeutic difficulties. Despite of antibiotic therapy based on susceptibility data, there was only temporary clinical improvement in the majority of patients in the study.

BLOCK 4: Genetical methods in microbial diagnostics

[1]*R..Hrstka,* [1]*V. Růžičková,* [1]*R. Pantůček,* [2]*P. Petráš and* [1]*J. Doškař* ([1]Department of Genetics and Molecular Biology, Faculty of Science, Masaryk University, Brno, Czech Republic, [2]National Institute of Public Health, NRL, Prague, Czech Republic):

Detection of prophages in TSST-1-positive *Staphylococcus aureus* strains by means of specific probes

The strains of *Staphylococcus aureus* producing TSST-1 toxin can induce human disease called Toxic Shock Syndrom (TSS). Production of staphylococcal toxins is based on the function of genes, which are usually localised in chromosome of host bacterium, but sometimes they can be a part of

prophage genome. Temperate bacteriophages of *S. aureus* can move genes by transduction and/or mediate lysogenic conversion of bacterial cells, which leads to increased ability of toxin production, usually of enterotoxins A and E.

In this study 25 strains of *S. aureus* were analysed for presence of prophages of the serological groups A, B and F.

The presence of prophages was determined using hybridisation by means of specific probes to *Sma*I and *Hin*dIII DNA restriction patterns of *S. aureus* strains. Probes were prepared from genomic sequences of phages 77 (serological group F), 53 (serological group B) and 3A (serological group A).

Positive signal in *Sma*I restriction patterns was found in 92 % strains when probe 77A was used. On the other hand probes 53B and 3A hybridised to 45 % strains only.

The presence of at least one of prophages (of three tested serological groups) was detected in *Hin*dIII restriction patterns of 96 % strains. The prophages of serological group F were detected in 84 % strains. Therefore we suppose that TSST-1 positive strains contain mostly prophages of serological group F. The occurrence of prophages of serological groups A and B is less abundant.

In conclusion we suppose that there is a certain correlation between presence of prophages of serological group F in the bacterial cells and their ability to produce TSST-1.

A. Liptáková, M. Sabol, H. Sehnálková, L. Siegfried (Institute of Medical Microbiology of Medical Faculty of Šafarik University, Košice, Slovakia)

Detection of genes encoding main virulence factors in STEC

There is increased number of epidemics in European countries and North America due to shiga-like toxin producing *Escherichia coli* (STEC). In our country there are sporadic cases of diseases caused by STEC. Selective sorbitol-McConkey agar with supplement (SMAC) was used for detection suspicious strains among children 0–14 years old suffering from diarrhoea. After positive cultivation on SMAC, determination of somatic antigen, cell cytotoxicity testing for shiga-like toxins, multiplex PCR was used to detect gene sequences encoding four virulence factors – intimin, enterohaemolysin, shiga-like toxin 1 and shiga-like toxin 2 - associated with STEC. 107 *E. coli* strains were investigated, 28 of 107 were able to grow on SMAC and only 4 of 107 possess all 4 investigated gene sequences. Three of them were untypable and one belonged to O157. Another one strain was positive in *eae* gene encoding intimin receptor belonging to O44.

L. Dvorská, I.. Parmová, L. Mátlová, M. Bartoš, I. Pavlík: (Veterinary Research Institute, Brno, Czech Republic):

Differentiation of *Mycobacterium avium* complex strains by PCR and RFLP in animals and human patient

Mycobacterium avium complex (MAC) strains, originally isolated from birds, infect also pigs and cattle and become an important pathogen in immunocompromised human, especially AIDS patients. As diagnostics and differentiation of MAC strains by standard methods are very difficult, the new molecular methods are used, for example PCR detection of specific insertion elements IS901 or IS1245, and RFLP analysis.

We have tried to determine the prevalence of MAC in cattle lymphnodes. During three years (1996–1998) we have isolated 79 strains of atypical mycobacteria (98.7 %) and only one strain *R. equi* (1.3 %) from 248 lymphnodes and organs (liver, testes) examined. We have found that 95 % of mycobacterium strains belonged to MAC. The strains of MAC were detected in 35.5 % of intestinal lymphnodes, in 9.1 % of pulmonary lymphnodes and in 15.8 % of not exactly identified lymphnodes. By serotyping the most dominant virulent serotype 2 was discovered in 59 % which suggests that the sources of infection could be through contaminated feed of animals. Using probe IS901, 8 different DNA patterns were detected in 27 *M. avium* strains, the most frequent DNA profile was identified in 43 % of strains. Identical RFLP patterns were found in the isolates from different farms. The results suggest the cattle were infected from environmental origins. With IS1245 probe 8 *M. intracellulare* strains were analysed, the high heterogeneity with more than 10 copies was found. In three *M. avium* strains mixed infection with *M. avium* and *M. intracellulare* was found. We have also analysed strains

from clinical case from HIV-negative patient. During 7 months of therapy 8 independent isolates were isolated from sputum. After isolation of individual colonies 20 RFLP types with IS1245 were identified. The result suggests an environmental origin of infection.

P. Švástová, L. Dvorská, M. Bartoš, I. Pavlík (Veterinary Research Institute, Brno, Czech Republic):

PCR in diagnostics of selected mycobacterium strains

The genus *Mycobacterium* consists of a diverse group of acid-fast bacilli which include a number of human and animal pathogens. The definitive diagnosis of mycobacterium infections rests on isolation by culture followed by identification. Owing to the long generation time of mycobacteria, culture-based isolation techniques require 3 weeks to several months to complete. A variety of biochemical, phenotypic and molecular methods have been established for the rapid identification of mycobacteria.

For example insertion elements IS*901* and IS*1245* and IS*901*-flanking region (FR) have facilitated the differentiation of strains belonging to the *Mycobacterium avium* complex, which is a serious bacterial pathogen of domestic and wild animals.

Due to the fact that PCR is an enzymatic process with extreme sensitivity, false-positive and false-negative results may occur. False-negative results depends on inhibition of the amplification (by different inhibitors, inefficient extraction of mycobacterium DNA or low number of bacteria present in the sample). The efficiency and sensitivity of the amplification can be controlled by including of internal standards that are coamplified in the same tube with the same primers as the target DNA. A size difference between the target DNA and the competitor template allow to corresponding bands to be discriminated.

Internal standards corresponding to IS*901*, IS*1245* and FR were produced by PCR of the alfalfa genome segment (Accession No. AF222048) inserted into pCR2.1 plasmid (Invitrogen, The Netherlands). The size difference between amplicons obtained from IS*901* (1108bp), IS*1245* (427bp) and FR (300bp) and from corresponding internal standards ISIS*901* (1336bp), ISIS*1245* (583bp) and ISFR (488bp) respectively, allowed easy discrimination. Internal standard amplicons were still visible on agarose gel with only 10–100 starting molecules. The system was tested to define the amount of internal standards that could be used in the PCR without affecting the amplification of the specific genome segment. Aliquots of the correctly diluted internal standards were kept at - 20 °C for more than 3 months and used for analysis. It was concluded that using the internal standards for coamplification could be a useful tool for screening material especially in avoiding false-negative results.

A. Müllerová, L. Mezenský (Department for microbiology of TBC, Regional Institute for Public Health, Brno, Czech Republic):

Contribution of molecular biology for identification of Mycobacteria

Because of the slow growth of Mycobacteria, bacterial diagnosis of mycobacterial disease is demanding. Applications of molecular biology make it easier and quicker.

Methods based on molecular biology used in clinical laboratories are amplification techniques for direct detection of Mycobacteria in clinical specimens and nucleic acid probes that make possible the identification of *Mycobacterium* species during 1 hour. The probe assays could be performed on acid acid-fast bacilli positive cultures recovered both in egg/based media and in liquid media from automated detection systems. (We have experience with automated detection systems MB/BacT and MGIT 9600 in our laboratory).

These systems have brought lessening of the time to detection of positive mycobacterium culture (about 10 days) but there is disadvantage – inability to observe typical colony morphology. It is useful to perform the probe assays in these cases to eliminate or confirm the growth of the most consequential pathogen (*M. tuberculosis*) = important information for clinicians.

There is described about 80 *Mycobacterium* species now. Several of them other than M. tuberculosis have emerged as important pathogens – *M. avium-intracelulare*, *M. kansasii*, *M. xenopi*, *M. fortuitum* are the most often. Restricted number of probes can be used in clinical laboratories for the culture confirmation of *Mycobacterium* species (*M. tuberculosis* complex = *M.*

tuberculosis, *M. bovis*, *M. bovis* - BCG, *M. africanum* and *M. microti*, *M. avium* complex = *M. avium* and *M. intracelulare*, *M. avium*, *M. intracelulare*, *M. kansasii*, *M. gordonae).* W e have done 300 assays in our laboratory (sensitivity 98 %, specificity 99 %).

If the identification by nucleic acid probes is negative, there are conventional methods to reach the result. It takes 3 – 6 weeks.

K. Hrochová, L. Plíšková (Institute of Clinical Biochemistry and Diagnosis, University Hospital of Hradec Králové, Czech Republic)

Atypical mycobacteria – ways of identification of the genus *Mycobacterium* in clinical laboratories

The rate of infections with atypical mycobacteria increases. Consequently also increase requirements for rapid and correct identification of *Mycobacterium spp.* Culture is time consuming, especially in slow-growing mycobacteria. The application of molecular biology methods for rapid detection and identification of mycobacteria has remarkably improved the diagnosis in routine laboratory.

Moreover, PCR (polymerase chain reaction) is a very useful tool to identify different types of mycobacteria. The PCR techniques are based on the amplification of sequences common in all mycobacteria. However, to detect and distinguish *Mycobacterium* species in clinical samples, PCR must be supplemented by hybridisation assays with non-radioactive labelled specific probes or by restriction enzyme analysis (RFLP).

Another simple method is PCR with specific primers for different species of mycobacteria presented in samples. These different species can be differentiated in one PCR run.

These methods are a quick, cost efficient, easy and reliable tool to identify mycobacteria other than the *Mycobacterium tuberculosis* complex in routine clinical laboratory. In our laboratory we have been using molecular biology methods since 1997.

Additional methods such as sequencing, fingerprinting, HPLC are powerful techniques of differentiating species; however, they are labour-intensive and difficult to be implemented for use in clinical laboratories.

K. Herčík, O. Melter, P. Branny: (Institute for Microbiology, Czech Academy of Science, Prague, Czech Republic)

Molecular biological diagnostic methods for *Bartonella henselae* caused disease

Bartonella henselae is a short, aerobic, Gram – rod, first found in patient with AIDS in 1990. It is a major causative agent of „cat scratch disease“ which is the most frequently manifested as a lymphadenopathy. The other diseases causing by *B. henselae* are – bacillary angiomatosis, bacteremia, endocarditis, peliosis hepatitis or some neurologic manifestations.

Routine laboratory diagnosis of these diseases associated with *B. henselae* infections are quite difficult. *B. henselae* is very fastidious, its primary cultivation is time consuming, can take more than 45 days, often is not successful, and routine biochemical tests are negative. For this reason it is absolutely necessary to use other diagnostic methods such as: serologic detection of *B. henselae* antibodies in sera (IFA, EIA, IPA), histology staining, or PCR based methods.

We have succeeded to develop a new useful diagnostic method for detection bacterial DNA of *B. henselae* in morphologically preserved tissue or whole cells using specific probe. The probe was prepared by PCR with dUTP-digoxigenine label according to the 16S–23S intergenic spacer region of *B. henselae* and this type of probe is highly specific for *B. henselae* DNA. The method is useful for identification of whole cells of *B. henselae* in suspension or tissue cultures as well.

M. Dendis, R. Horvát, J. Michálek, F. Růžička, J. Benedík: (Genetic Laboratory of Center for Cardiosurgery and Transplant Surgery, Brno, Czech Republic):

Early diagnosis and treatment of mycotic infection by PCR

Early diagnosis and treatment of mycotic infection can prevent dissemination and systemic spread of yeast and moulds in immunocompromised cancer patient.

In a group of 24 children aged 1 - 18 years with cancer and febrile neutropenia, the invasive mycotic infection was monitored using cultivation in combination with fungal DNA detection by polymerase chain reaction.

Fungal DNA was found in peripheral blood in 11 episodes of febrile neutropenia in 10 patients while cultivation from blood cultures (*Candida albicans, C. tropicalis, C. glabrata*) was positive in only 5 out of 11 PCR-positive episodes of febrile neutropenia. The aspergilloma has developed in a patient with bronchopneumonia and was confirmed by both histology and PCR.

The examination of fungal DNA by polymerase chain reaction is reliable, fast and highly sensitive method for the detection of invasive mycotic infection enabling early targeted antifungal therapy in patients with cancer, especially during episodes of febrile neutropenia.

D. Molinková, V. Celer (Institute for Microbiology and Immunology, Veterinary and Pharmaceutical University, Brno, Czech Republic):

Isolation and partial characterisation of Equine herpesvirus type 1 (EHV-1) in Czech republic

Equine herpesvirus type 1 (EHV-1) is an important pathogen of horses and causes important loses in the horse industry. An abortion storm occurred in 1997 in several breeds in Czech republic. From one aborted foetus we have isolated a virus, which was subsequently characterised as EHV-1 by serological means and PCR. In order to characterise this isolate in greater details we have cloned and sequenced genes coding for the glycoproteins B and D. Phylogenetic analysis showed a high degree of conservation of both glycoprotein genes when compared with standard abortogenic isolate Kentucky A.

BLOCK 5: Experimental genetics of micro-organisms

J. Kadlec, J. Janata, J. Spížek (Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic):

Function of *lmbJ* gene product in biosynthesis of lincomycin

Lincomycin, produced by *Streptomyces lincolnensis,* and its derivates (e. g. clindamycin) are important, clinically used antibiotics. Lincomycin biosynthesis proceeds via two intermediates, propyl-L-proline (derived from L-tyrosine) and methylthiolincosamide. These moieties become condensed to N-demethyllincomycin (NDL) which is methylated by NDL methyltransferase to yield lincomycin A.

The *lmbJ* product could be a determinant of the NDL methyltransferase activity (Chung *et al.* 1997). However, the authors assumed that the enzyme also includes additional subunits, as the DNA fragment containing the *lmbJ* gene did not induce any methyltransferase activity. In order to confirm the NDL methyltransferase activity of LmbJ we developed an efficient system for overexpression of His tag-LmbJ protein in *Escherichia coli* BL21 (DE3). Then the fusion protein was purified by metal-affinity chromatography and cleaved by thrombin into its native form. In presence of S-adenosyl methionine, LmbJ protein alone converts NDL to lincomycin. The measured kinetic parameters are in good agreement with those published for the enzyme isolated from the natural source (Chung *et al.* 1997). It was thus directly demonstrated that the LmbJ protein is an N-demethyllincomycin methyltransferase. In addition, by using yeast two-hybrid analysis, we found that the LmbJ protein tends to form homodimers or homooligomers. By means of gel filtration, we showed that the LmbJ protein forms a complex of apparent molecular weight 272 kDa. Further we identified minor forms of apparent MW 144 kDa and 70 kDa. Finally, electron microscopy revealed four-fold symmetry complexes in the negatively stained samples of the purified LmbJ protein. Combination of these data may explain the high molecular weight of the native NDL methyltransferase multimeric complex and indicates that the enzyme is an octamer.

L. Najmanová, J. Janata, P. Novák, F. Kalousek and J. Spížek (Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic):

DNA-dependent RNA polymerase a subunit in streptomycetes

All eubacteria have a single type of DNA-dependent RNA polymerase (E.C., 3.2.1.1., later only RNAP), which exists in two basic forms. The core enzyme (‘$_2$) is able to polymerise and complete

the nascent RNA chain. However, only the holoenzyme which contains additional subunit(s) s (‘$_2$s), recognises the promoter sequence and initiates the transcription accurately. The variability of s proteins is one of very important regulatory mechanisms. Recently it was demonstrated, that a subunit (later only RNAP) is also able to recognise and bind the promoter sequence, specifically its upstream element, and thus regulate the rate of transcription. The RNAP C-terminal domain also contains multiple binding sites for various transcription regulators.

Using western blot and immunochemical detection we demonstrated the presence of two different a subunits in spores of *Streptomyces granaticolor*, only the bigger one of which was also found in vegetative cells. Tryptic cleavage experiments confirmed that the proteins are highly similar to each other as well as to other bacterial RNAP a subunits, and that the smaller one is shortened from its C-terminus, i. e. the part of the protein, responsible for binding of DNA and transcription regulators. The gene for RNAP from *S. granaticolor* was cloned and sequenced and the protein was overproduced in *Escherichia coli*. *In vitro* experiments with purified RNAP confirmed that the proteolytic activity is present in the cell free extract from spores of *S. granaticolor* but not in vegetative cells. The nature of the protease responsible for the proteolytic cleavage was studied using various protease inhibitors. The hypothetical function of a 14 aminoacid fragment, which is cut off in spores, was assessed using 3D modelling.

One can speculate that this might be a novel mechanism of transcription control in streptomycetes.

A. Šebková, R. Karpíšková, I. Rychlík (Veterinary Research Institute, Brno, Czech Republic):

Biological properties of low molecular weight plasmids in *Salmonella* Enteritidis

Low molecular weight plasmids are frequently used as epidemiological markers in *Salmonella*. However, their biological role is largely unknown. After development of a system for exact identification of individual plasmids in *S.* Enteritidis we proceeded to sequence characterisation of selected plasmids. Using DNA hybridisation we showed that there are at least three completely different kinds of low molecular weight plasmids in *S.* Enteritidis. We have completely sequenced representatives of two of these groups. The 4053 bp plasmid “represents the most frequent group of plasmids in *S.* Enteritidis. It encodes 5 possible ORFs, two of which are involved in plasmid replication and maintenance in bacterial cell. The remaining three ORFs are specific for plasmid”. ORF4 was identified as retron reverse transcriptase. This enzyme catalyses production of linear low molecular weight DNA which accumulates in cytoplasm. Some of the results support the hypothesis that this protein is also involved in protection of bacterial cells against phage infection. Second completely sequenced plasmid (2096 bp) encodes for a single ORF homologous to Rep proteins. Its biological role is currently unknown and is under investigation.

This study has been supported by the Czech Ministry of Agriculture, grant EP6076/96.

M. Ševčík, L. Cardová, I. Rychlík (Veterinary Research Institute, Brno, Czech Republic):

RpoS expression in respiration deficient mutants *cyd*A and *nuo*G of *Salmonella* Typhimurium by monitoring of rpoS expression

During transition into stationary phase of growth, multiple changes in bacterial metabolism are under the control of RpoS (stationary phase specific sigma subunit of RNA polymerase). Strains defective in RpoS are of reduced adaptation to stationary phase and prolonged survival. Similarly, mutants defective in respiration are of similar phenotype in stationary phase survival. The aim of this study was therefore to determine whether defects of respiration mutants are caused by modification of rpoS expression. Using rpoS-luxAB transcription fusions, rpoS expression was monitored under fully aerobic and microaerophillic conditions, in the wild type *Salmonella* Typhimurium strain and in its isogenic mutants nuoG and cydA (nuoG, $NADH_2$ dehydrogenase and cydA, cytochrom d oxidase). We found that rpoS is more expressed under fully aerobic than in microaerophillic conditions. Next we observed that rpoS is suppressed in the nuoG mutant but overexpressed in the cydA mutant. Because NuoG catalyses the reduction of NADH2 to NAD, the

decreasing $NADH_2$: NAD ratio can serve as signal for increased rpoS expression. CydA activity leads to oxygen consumption and simultaneously to suppression of the rpoS expression. Therefore, presence of oxygen may serve as a second signal for upregulation of the rpoS.

This work has been supported by the Czech Grant Agency, grant 524/98/1089.

J. Volf, M. Ševčík, I. Rychlík (Veterinary Research Institute, Brno, Czech Republic):

Function of *lux*S and *sdi*A genes in intercellular communication of *Salmonella* Typhimurium

Quorum sensing, i. e. sensing of bacterial density through production and sensing of specific secondary metabolites, has been confirmed in many bacterial species. Different functions have been attributed to quorum sensing systems. In *Pseudomonas aeruginosa* and *Erwinia carotovora*, they are, for example, related to regulation of virulence factors expression in dependence on bacterial population density. Homology search identified in the *Salmonella* Typhimurium genome two genes, sdiA and luxS, which are similar to genes involved in quorum sensing in other bacterial species. In *Salmonella*, protein SdiA was found to participate in cell division and virulence regulation. To further determine functions of these genes in *S.* Typhimurium, deletion strains sdiA and luxS and strains with transcription fusions sdiA-luxAB and luxS-luxAB were constructed. The expression of sdiA was stimulated during growth in cell free supernatants prepared from 24 hours of aerobic bacterial cultures. The stimulation of expression was observed in supernatants after control strain and both the deletion strains, *S.* Typhimurium luxS and sdiA. Expressions of luxS was strongly suppressed by 24 hour supernatants of all strains.

This work has been supported by the Czech Grant Agency, grant 524/98/1089.

I. Rychlík, M. Ševčík, A. Šebková, J. Volf, H. Havlíčková, F. Šišák (Veterinary Research Institute, Brno, Czech Republic):

Quorum sensing in *Salmonella* Typhimurium

Salmonella spp. can inhabit two extreme ecological niches. After oral infection of a host it multiplies in a rather anaerobic environment rich in nutrients and quickly reaches a concentration of 108–109 CFU per gram of intestinal content. The opposite extreme is found outside the host in fully aerobic conditions where nutrients are usually scarce. Both of these situations are characteristic by constant concentration of bacterial cell, similar to in vitro situation when bacteria reach stationary phase of growth. Therefore we have investigated the physiology of bacteria entering stationary phase of growth. By deletion mutagenesis we prepared strains with disrupted rpoS, sdiA and luxS genes. All of these genes are involved in *Salmonella* metabolism during transition into stationary phase of growth. In vitro characteristics of all of the mutants were very similar. They grew in rich broths as well as in minimal medium. Only rpoS strain grew more quickly during logarithmic phase of growth but reached lower concentration in stationary phase. After experimental oral infection of one day old chickens, mutant sdiA behaved in animal experiments similarly to the control virulent strain. rpoS and luxS mutants, two days after infection, reached higher concentrations per gram of caecal content when compared with control virulent strain. Six days post infection, *Salmonella* concentrations in all experimental groups were the same. Thirteen days post infection lower *Salmonella* counts were observed in the caecum of chickens infected with the rpoS and luxS mutants. Therefore we propose that rpoS and luxS genes are necessary for colonisation of caecum but the reduction of virulence is not high enough to consider these mutants as candidates for live attenuated vaccine.

This work has been supported by the Czech Grant Agency, grant 524/98/1089.

R. Burdychová, M. Bartoš (BioVendor – Laboratory Medicine, Ltd., Brno, Czech Republic):

Cloning and expression of human myostatin gene in *Pichia pastoris*

Myostatin is a secreted growth and differentiation factor that has been shown to be involved in the regulation of skeletal muscle mass in both mice and cattle. Targeted disruption of the myostatin gene in mice and a 11bp deletion mutation in the third exon of the myostatin gene in double-muscled Belgian Blue cattle breed result in skeletal muscle hyperplasia in both cases. Myostatin expression

has also been detected in the muscle of foetal and adult hearts, specifically in Purkinje fibers and cardiomyocytes. Following myocardial infarction, myostatin expression is upregulated in the cardiomyocytes surrounding the infarct area. Therefore, myostatin plays an important role in skeletal and cardiac development and physiology.

We report the cloning and expression of recombinant human myostatin exon 3 in yeast *Pichia pastoris*.

Pichia pastoris is a methylotrophic yeast, capable of metabolising methanol as its sole carbon source. Metabolism of methanol depends on enzyme alcohol oxidase. The promoter regulating the production of alcohol oxidase is used to drive heterologous protein expression. The secretion requires the presence of a signal sequence to target the protein to the secretory pathway. The major advantage of expression of heterologous proteins as secreted proteins in *Pichia pastoris* is that this yeast secretes very low levels of native proteins. Therefore the secreted heterologous protein comprises the vast majority of the total protein in the medium.

The exon 3 of the myostatin gene was amplified from total genomic DNA from skeletal muscle. For oriented cloning restriction sites for two restrictases and desirable sequences for direction of secretion were added following the first round amplification. The resulting fragment was digested by the two restriction endonucleases and cloned to shuttle vectors. After propagation of recombinant plasmids in *Escherichia coli*, the presence of insert was confirmed by PCR screening and sequencing. The correct recombinant plasmids were transformed to competent yeast cells. Integration of fragment to yeast chromosome was confirmed by PCR directly from cell culture. Expression and secretion was studied in selected integrants.

M. Mokrejš, M. Pospíšek (Faculty of Science, Charles University, Prague, Czech Republic)

dsRNA virus of yeast-like organism *Endomyces magnusii*

We report here some new observation concerning the toxin of yeast-like organism *Endomyces magnusii* which secretion is thought to be in connection with the presence of dsRNA virus in cells. In order to get some evidence supporting this hypothesis we made some attempts to cure cells off the virus. Experiments using UV-light irradiation or 5-fluoro-uracil treatment were not successful. To follow toxin production we developed microtitre plate assay for easy routine detection of the toxin secreted by *E. magnusii* into liquid medium. We determined a time-course of the toxin production in aerated liquid culture and compared it with growth curves of *E. magnusii.* This project was supported by grants No. 204/98/1995 and 204/97/P009 from the Czech Grant Agency. We would like to thank R. Šípošová for technical assistance.

BLOCK 6: Experimental microbiology

S. Sethi, I. Horák, H. Hahn, O. Liesenfeld (Institute for Infection Medicine, Department of Microbiology, Berlin, Germany):

Interferon consensus sequence binding protein deficient mice display a Th2 type cytokine profile after peroral infection with *Toxoplasma gondii*

Toxoplasma gondii is transmitted via the oral route. We have investigated the role of the intestinal immune response in orally infected ICSBP$^{-/-}$ mice which show impaired IL-12 p40 production. After peroral infection with 10 cysts of *T. gondii*, all ICSBP$^{-/-}$ mice died, whereas all control mice survived. ICSBP$^{-/-}$, but not control, mice showed dramatic necroses and infiltrations by mononuclear cells and granulocytes in livers and lungs. ICSBP$^{-/-}$ mice exhibited unchecked parasite replication in liver, lung and ileum, whereas only few parasites were observed in control mice. Analysis of cytokine production in mesenteric lymph nodes (MLN), Peyer's patches (PP), and spleens on day 5 post infection revealed upregulation of IFN- and IL-12p40 production in control, but not in ICSBP$^{-/-}$ mice. In contrast, ICSBP$^{-/-}$ mice showed elevated IL-4 and IL-5 levels in MLN, but not in PP and spleens, indicating a compensatory local Th-2 response. In summary, ICSBP$^{-/-}$ mice are highly susceptible to oral infection with *T. gondii*. Infection is accompanied by a local, but not systemic, Th-2 type response. The functional role of this shift to a Th-2 type immune response is under investigation.

O. Gakh[1], J. Adamec [3], T. Obšil[2], F. Kalousek[1], J. Spížek[1], E. Amler[2], and J. Janata[1]. (Institute of [1]Microbiology and [2]Physiology, Czech Academy of Sciences, Prague, Czech Republic, [3]Mayo Clinic and Foundation Department of Adolescent and Paediatric Medicine, Rochester, USA):

The yeast mitochondria processing peptidase: Modelling and biochemical study

The mitochondria processing peptidase (MAP) is the primary peptidase responsible for the removal of leader peptides from mitochondria proteins encoded by nucleus. The MAP is a metallopeptidase and it forms a heterodimer with structurally related a and b subunits. Both subunits are indispensable for the activity of MAP. We used an improved system for individual overexpression and purification of both yeast MAP subunits in *E. coli*. It allows production of soluble a- but not b-MAP, which may be then solubilised by urea and renatured into functional subunit. Lifetime analysis of tryptophan fluorescence has been used for protein-protein interaction study. Results proved that substrate binding evoked a conformational change of the a-MAP subunit while no significant response was detected with the b-MAP subunit. Interestingly, tryptophan fluorescence decay of the a-MAP subunit revealed about 11 % of steady-state fractional intensity due to the long-lived lifetime component, indicating that at least one tryptophan residue is partly buried at the hydrophobic microenvironment. To understand how MAP functions in recognising and removing mitochondria presequences, we created a model of MAP based on its homology to the chicken cytochrome bc_1 complex and introduced several point mutations in both MAP subunits which decreased or disturbed the activity of this enzyme. We found that deletion of 30 amino acid residues forming putative C-terminal helix of the -MAP results in the complete loss of the enzyme activity. The MAP model suggests that the glycine-rich region of the -MAP is exposed to the catalytic centre of the -MAP as well as to a likely site of the preprotein substrate. It appears that the essential function of a-MAP is localised just in this region.

L. Nováková, R. Nádvorník a P. Branny (Institute for Microbiology, Czech Academy of Sciences, Prague, Czech Republic)

Analysis of eukaryotic-like protein kinases expression during the life cycle of streptomycetes

Streptomyces spp. are saprophytic soil bacteria which undergo complex morphological and physiological differentiation. They grow as a mycelium of branching hyphae which differentiate and form aerial hyphae and finally long strings of hydrophobic spores. Spores are the only unicellular stage in the life cycle of *Streptomyces*. Therefore, *Streptomyces* can be viewed as a simple and attractive model of multicellular organism. The morphological differentiation of their mycelium is accompanied with the production of variety of secondary metabolites including antibiotics. The need of various kinds of cell-cell interactions, various levels of regulation and various types of signal transduction mechanisms in order to regulate these complex processes is obvious.

Transmission of a signal by protein phosphorylation at serine, threonine and tyrosine residues plays a major role in differentiation and cell-cell communication in eukaryotes. Similar signalling cascades in prokaryotes have not been described so far. However Ser/Thr kinases were found in many bacteria supporting the hypothesis that signalling cascades similar to those of eukaryotes could operate in prokaryotes.

We have identified three genes named pkg2, 3 and 4 encoding eukaryotic-like protein kinases from *Streptomyces granaticolor*. The genes were cloned and expressed in *Escherichia coli* and we proved that proteins Pkg2 and Pkg4 possess catalytic activity and are capable of autophosphorylation. Inactivation of the genes resulted in changes in the morphology of aerial hyphae.

We have studied a time-correlated expression of these genes using two different approaches. We used transcription fusions of a promoter-less reporter gene xylE to putative promoter regions of these protein kinase genes. For direct RNA analysis we performed RT-PCR. Monitoring of promoter activities showed that pkg2 is expressed constitutively during the life cycle in contrast with pkg3 and 4 which are transcribed in the late stages only. However, transcription analysis performed by

RT-PCR proved the presence of mRNA of pkg2 and pkg4 during the whole life cycle. Following this result we suggest that regulation of expression of Pkg2, 3, and 4 is not restricted to a transcription level only.

L. Strašák, V. Vetterl, J. Šmarda (Faculty of Science and Faculty of Medicine, Masaryk University, Brno, Czech Republic)

Effect of low-frequency magnetic fields on bacteria *Escherichia coli*

This work is based on our previous results with a magnetic field produced by a solenoid. We found that a magnetic field (frequency 50 Hz, maximal amplitude of magnetic induction Bm = 10mT), produced by a cylindrical coil and relatively homogenous in the position of a sample, acts on bacteria *Escherichia coli* K12-Row, similarly as non – homogenous fields in our previous work. Number of colony forming units decreases proportionally with the higher time of exposure (0–12 min) and with magnetic induction (0–10 mT). We observed a difference between growth curves of control bacteria and bacteria exposed to the field during measurement of growth dynamics, the number of bacteria in control samples is increasing with a higher velocity than in exposed ones. We found that the decrease of oxidoreductive activity of exposed samples in the tetrasolium test was due to the decrease of the number of living bacteria in culture. We suppose that magnetic field has no effect on the metabolism intensity of bacteria.

I. Kyselová (Faculty of Biological Sciences, The University of South Bohemia, Czech Republic, and Institute of Parasitology of Czech Academy of Sciences, České Budějovice, Czech Republic):

Amoebae of the genus *Naegleria* isolated from organs of freshwater fishes

Most *Naegleria* spp. have been described from water environment but no isolates have been reported from organs of freshwater fishes. For our morphometric analysis of cysts and trophozoites we used 18 *Naegleria* strains isolated from organs of freshwater fishes. Using the difference in size of the cysts the strains were divided into 6 groups. From each group one strain was used for a sequence analysis of small subunit ribosomal DNA (SSU rDNA). The SSU rDNA was amplified by PCR with specific primers. In a control *N. fowleri* strain a band with the expected DNA length of 2.0 kb was obtained, which also was found in our CB2B/I strain. In the other five strains a band of 3.3 kb was observed corresponding to the presence of a 1.3 kb intron in SSU rDNA. Sequence analysis of the introns revealed that they were group I introns. Sequence analysis of SSUr DNA revealed homology of strain CB2B/I to *N. australensis australensis*. Restriction-fragment-length polymorphism analyses of SSU rDNA and of introns showed homology of the 5 strains to *Naegleria clarki* and confirmed homology of strain CB2B/I to *N. australensis australensis*. Phylogenetic analysis based on SSU rDNA and introns separated these strains as a sister group of *N. clarki*, *N. gruberi* and *N. italica*.

M. Veverková (Faculty of Biological Sciences, The University of South Bohemia, České Budějovice, Czech Republic, and Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic):

Pathogenic potential of *Acanthamoeba* strains isolated from organs of freshwater fishes

Pathogenic potential of selected, well defined strains of *Acanthamoeba* was studied in experimental infections of three species of fishes: common carps including hybrids of isogenic homozygous line (*Cyprinus carpio*), goldfishes (*Carassius auratus*) and tilapias (*Oreochromis niloticus*). Morphological criteria were applied to assess pathogenicity of individual strains. Attempts were made to increase susceptibility of experimental fish with immunosuppressive agents. The results showed that only four out of six tested strains were able to induce infection and minor histhopathological changes. The immunosuppressive agents used had no influence on susceptibility of the experimental fish to *Acanthamoeba* infections. The fish were able to cope with large ineffection doses up to $3{,}8 \, . \, 10^7$ *Acanthamoeba* cells per ml.

I. Fiala (Faculty of Biological Sciences, The University of South Bohemia, České Budějovice, Czech Republic, and Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic):

Protozoan and myxosporean fauna of brackish ornamental fishes

Nine species of brackish-water fishes imported from south-east Asia, Africa and South America as ornamental specimens were sampled in order to assess marine and freshwater elements in their protozoan and myxosporean fauna. Tissues from organs were examined in fresh mounts, using light and electron microscopy. Five species of fishes were infected with 23 species of myxosporeans and 4 species of protozoans. Thirteen of these myxosporean species appear to be new for science. The most interesting findings were those in *Scatophagus argus*. This fish species was infected with five myxosporean and one microsporidian species. The microsporidian was found in the kidney of *S. argus,* where numerous spores were localised in plasmodia of a myxosporean parasite, *Caudomyxum* sp. This suggests hyperparasitism by the microsporidian. In the kidney of the same host were also found xenoma formations representing extrasporogonic stages of a myxosporean, *Ortholinea* sp.

BLOCK 7: Biotechnological applications of microbiology

J. Navrátilová[1], *L. Kotoučková*[1], *J. Nečas*[2] ([1]Department of Microbiology, Faculty of Science, Masaryk University in Brno, [2]Veterinary Research Institute, Brno, Czech Republic)

Isolation and characterisation of 4-nitrocatechol degrading bacteria

4-Nitrocatechol (4-NC) is the intermediate of bacterial metabolism of 4-nitrophenol, bacteria degrade them fast in the case of activity of all enzymes for complete mineralisation of 4-nitrophenol, but some bacterial strains accumulate 4-nitrocatechol (*Corynebacterium* sp. 8/3 and *Pseudomonas* sp. ATCC 29354, for example). The aim of this study was to isolate bacterial strain(s) capable to degrade 4-nitrocatechol in high concentration, resulting from accumulation of this compound by microbial conversion of 4-nitrophenol.

Several 4-nitrocatechol-growing bacterial strains were isolated from soil by selective enrichment with 4-nitrocatechol. The isolated strains belong mostly to irregular nonsporing gram-positive rods. Decolorization of yellow-orange colour of 4-NC in the mineral medium was used for detection of degradation in the course of isolation. Concentration of substrate and presence of metabolites was determined by HPLC. All strains use 4-NC as sole source of carbon and energy, growth in minimal medium supplemented by 4-NC is accompanied by complete degradation of 4-NC.

Isolated strains differ one another by substrate specificity of degradation enzymes, their capability to degrade other nitroaromatic compounds was tested (2-, 3-, 4-nitrophenol, 4- and 5-nitroguaiacol). Two isolated strains do not degrade 4-nitrophenol, the pathway of 4-nitrocatechol degradation is not a part of mechanism of degradation of 4-nitrophenol, in contrast to all strains described previously.

M. Rosenberg, H. Miková, Ĺ Krištofíková (Department of Biochemical Technology, Faculty of Chemical Technology, Slovakian Technical University, Bratislava, Slovakia):

Production of malic acid by the yeast *Dipodascus magnusii*

Malic acid is widely used in the food and pharmaceutical industry. It can be produced either by chemical synthesis (hydration of fumaric or maleic acid) or by the bioconversion of fumaric acid by the action of fumarase. Recently, variety yeast strains from genus *Saccharomyces*, *Pichia*, *Candida* and *Paecilomyces* have been used for L-malic acid production.

In this work, the yeast *Dipodascus magnusii* were used for bioconversion of fumaric acid to L-malic acid. In comparison with baker's yeast, the fumarase activity of *D. magnusii* (7.5 U/mg) is approximately 10-times higher in the intact cells. After disintegration of the cells was the total fumarase activity 75 U/mg. The basic characteristics and properties of fumarase, the possibilities of fumarase activity stimulation during propagation of the strain and also during the bioconversion were studied in more details. The optimal temperature for the bioconversion is 37 °C and the optimal pH is around 7.5. In the conversion performed by the intact cells, only 10 % of total

fumarase activity is utilised. Therefore, we tested the permeabilisation of the cells by the addition of detergents. About 100 % increase in the fumarase activity was observed when Triton X-305 and sodium taurocholate (0.1 %) were added to the reaction mixture.

M. Strouhal, M. Němec (Department of Microbiology, Faculty of Science, Masaryk University in Brno, Czech Republic):

Heavy metals and dehydrogenase activity of cells *Yarrowia lipolytica* CCM 4510

The strain of yeast *Yarrowia lipolytica* CCM 4510 was used for determination of influence of heavy metals on dehydrogenase activity of cells. The strain was isolated from soil contaminated by petroleum. In experiments the yeast cells were exposed to cadmium, zinc, cobalt and nickel. Metals were added into the medium at start of cultivation at concentrations from zero to minimal inhibition concentration (MIC). Cells were cultivated 20 hours at 25°C under intensive shake. After cultivation the collected cells were washed in phosphate buffer, sonicated and required parameters were established.

The all of used metals have effect on measured parameters at different rate. MIC for this strain was 9.62 mmol/l of zinc, 2.45 mmol/l of nickel, 2.43 mmol/l of cobalt and 0.39 mmol/l of cadmium. Biomass production was direct proportional to used metal concentration. Cadmium decrease amount of wet biomass from 12.6 to 6.3 mg/ml of medium (50 %) at concentration 37.5 µmol/l. The same effect was made by 1000 µmol/l of nickel and 1500 µmol/l of cobalt. Zinc decrease production of biomass about 50 % at much higher concentrations (4800 µmol/l, 12.6 to 5.9 mg/ml of medium). Cadmium had also the highest effect on total dehydrogenase activity, 37.5 µmol/l of cadmium decrease the activity versus control about 87 %. Cobalt subsequently increased total dehydrogenase activity with maximum at the highest used concentration 1000 µmol/l (253 % enhancement). Nickel had not significant effect on total dehydrogenase activity at used concentrations. Zinc stimulated that at low concentration, and when the concentration crossed 2400 µmol/l the activity decreased about 63 %. The major part of individual dehydrogenases (malate, glucose-6-phosphate, lactate and succinate dehydrogenase) were stimulated. Ascending concentrations of the metals slowly decreased their activities. Only lactate dehydrogenase in presence of nickel, malate and glucose-6-P dehydrogenases in presence of cadmium were inhibited. Relation of dehydrogenase activities against metal concentration was almost linear.

S. Marvanová[1,2]*, K. Hynková*[1,2]*, J. Damborská*[1] ([1]Laboratory of Biomolecular Structure and Dynamics, [2]Department of Environmental Chemistry and Ecotoxicology, Faculty of Science, Masaryk University, Brno, Czech Republic):

Optimal method of spectrophotometry for screening of haloalkane dehalogenases

Halogenated organic compounds are widely used in industry and agriculture. Haloaliphatics are also formed as the by-products and intermediates of chemical syntheses or as the products of chemical transformations. A great attention is devoted to the fate of these compounds in the environment because of their toxicity, carcinogenicity and persistence. Microbial dehalogenation is one of the ways for their detoxification.

This work is focused on hydrolytic dehalogenation of halogenated aliphatic hydrocarbons. The main step of the enzymatic reaction is removal of the halogen atom from the carbon atom and its substitution by hydroxyl. At the same time, the halide ion and the hydrogen proton are released. The enzymes catalysing this reaction are called halidohydrolases. Gas chromatography determining the concentration of reaction products (primary alcohol) is commonly used for the quantification of the dehalogenase activity. Spectrophotometric method by Iwasaki et al. determines the concentration of the other reaction product – halide ion. The spectrophotometric method by Holloway et al. was optimised for the purposes of fast dehalogenase screening in this work. This method is based on the decrease of pH in a weakly buffered medium due to the increase of proton concentration and consumption of with hydroxyl anions. The method was optimised for dehalogenase activity measures in the microplates.

Purified haloalkane dehalogenase LinB from bacterium *Sphingomonas paucimobilis* UT26 was used for the optimisation. The enzyme activity was tested with 16 different halogenated aliphatic

compounds. The absorbency was measured in 30 sec intervals and provided straight lines for the plot of absorbency vs. time. The slopes of the lines quantified the dehalogenase activity and were compared with values of catalytic constants (k_{cat}) obtained from gas chromatography analyses. The comparison showed that the screening method is suitable for semi-quantitative determination of the dehalogenase activity. Presently, the screening method is used for characterisation of the substrate specificity of four mutant enzymes LinB. This procedure will be applied for the screening of dehalogenases in bacterial isolates in the future.

BLOCK 8: Antimicrobial therapy

G. Novotná, J. Janata, P. Tichý (Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic):

Detection of MLS resistant determinants in methicillin resistant *Staphylococci*

Macrolide, linkosamide and streptogramin antibiotics (MLS) are chemically distinct but have a similar mode of action based on proteosynthesis inhibition in elongation step of translation. Accordingly, also some mechanisms of resistance common to this group of antibiotics. MLS antibiotics have narrow spectrum of activity including also gram-positive cocci.

So far in *Staphylococcus* sp. three mechanisms of resistance were identified, which are represented by following genetics determinants. *ErmC*, *ermM* and *ermA* genes encoding methyltransferase, modifying the target site of all MLS_B antibiotics, *msrA* and *msrB* protein products actively pump 14-member ring macrolides and type B streptogramins by efflux mechanism, *linA* and *linA'* responsible for specific inactivation of lincomycin. Using disk diffusion and agar dilution methods we tested 117 clinic isolates of methicillin resistant Staphylococci, co-resistant to erythromycin (macrolide) 78.6 %, lincomycin 67.5 % or clindamycin 30.7 %. By Southern hybridisation analysis, we identified the presence of genes *ermC, ermM* (appropriates to the same group) and *ermA*. The specific primers for PCR identification of these genetics determinants were designed and some of them we have identified.

Optimalisation of the PCR conditions and preparation of methods for detection of other resistance determinants will enable the construction of the PCR system for fast and accurate detection of bacterial resistance in clinical practice.

O. Melter[1, 2]*, I. Sanches*[1, 3]*, J. Schindler*[4]*, M. Aires de Sousa*[1]*, R. Mato*[1]*, V. Kovářová*[4]*, H. Zemličková*[4]*, H. de Lencastre* [1, 3, 5] ([1]Unity of Molecular Genetic, Institute for Chemical Technology and Biotechnology of New University of Lisbon, Oeiras, Portugal, [2]National Institute of Public Health, Prague, Czech Republic, [3]Faculty of Science and Technology, New University of Lisbon, Monte da Caparica, Portugal, [4]3rd Faculty of Medicine, Charles University, Prague, Czech Republic, [5]Laboratory of Microbiology, The Rockefeller University, New York, USA):

Methicillin-Resistant *Staphylococcus aureus* Clonal Types in the Czech Republic

Molecular surveillance studies have documented the extensive spread of methicillin-resistant *Staphylococcus aureus* (MRSA) clones. Studies carried out by Centro de Epidemiologia Molecular – Network for Tracking Gram Positive Pathogenic Bacteria (CEM/NET) led to the identification of two international multidrug-resistant strains, which were designated as the Iberian and Brazilian MRSA clones and which were defined by multiple genomic typing methods; these included *Cla*I restriction digest hybridised with *mec*A and Tn*554* specific DNA probes and pulsed-field gel electrophoresis (PFGE). The genotypic characteristics of these clones are distinct: the Iberian clone is defined as *mec*A type I, Tn*554* type E (or its variants), and PFGE pattern A (I:E:A), whereas the Brazilian clone is defined as *mec*A type XI (or its variants), Tn*554* type B, and PFGE pattern B (XI:B:B). In this study, we characterised 49 single-patient isolates of MRSA collected during 1996 and 1997 at seven hospitals located in Prague and five other cities in the Czech Republic by using methodologies mentioned above and by using ribotyping of *Eco*RI and *Hin*dIII digest hybridised with a 16S-23S DNA probe. The Brazilian MRSA clone (XI:B:B) was the major clone

(80 %) spread in two hospitals located in Prague and one located in Brno; the Iberian MRSA clone (I:E:A or its variants I:DD:A), although less representative (12 %), was detected in two hospitals, one in Prague and other in Plzen. Strains belonging to clone XI:B:B corresponded to a unique ribotype E1H1(*Eco*RI, *Hin*dIII), whereas strains of I:E:A and I:DD:A clonal types corresponded to ribotype E2H2 (*Eco*RI, *Hin*dIII).

O. Zahradníček, J. Svoboda, L. Mejzíčková (Institute for Microbiology, Faculty of Medicine of Masaryk University and St. Anna's Faculty Hospital, Brno, Czech Republic):

Comparison of susceptibility of respiratory pathogens to azithromycin a klarithromycin

Macrolid and azalid antibiotics are a very quickly developing group of antimicrobial drugs. The new generation preparations lost the side effect of the classical preparates, keep the advantages (e. g. the application usually do not lead to allergies) and even have many better characterictics: an intracellular effect, and also a broader spectre of susceptible organisms.

In our study, we have compared in-vitro effect of azithromycin (AZI) and clarithromycin (CLA) on bacteria found in samples from respiratory tract. Besides classical potential pathogens - *Streptococcus pneumoniae* (SRPN), *Haemophilus influenzae* (HEIN), *Staphylococcus aureus* (STAU), *Branhamella catarrhalis* (BRCA) - we have also tested *Haemophilus parainfluenzae* (HEPA), which is considered to be rather normal flora; however, under some condition it is able to act as pathogen. Another reason for testing was that it is much more common than it relative *Haemophilus influenzae* and so we were able to get more strains (so it served us as a model organism).

We have tested for antibiotic susceptibility 157 strains of HEPA 68 strains of STAU, 38 strains of HEIN, 24 strains of SRPN and 5 strains of BRCA. We have used diffusion disc test with Sanofi-Pasteur discs on Mueller-Hinton agar (STAU), Mueller-Hinton agar with red blood cells (SRPN, BRCA) and Levinthal agar (HEIN, HEPA). We have compared the zones of susceptibility. We found, that AZI gave larger inhibition zones in 100 % of HEIN, HEPA and BRCA strains, while for both grampositive the zones were slightly larger for CLA (80.9 % of STAU strains, 92,3 % of SRPN strains). The average differences (AZI - CLA) of zone diameter were 7.2 mm for HEPA, 6.7 mm for HEIN, 5.2 mm for BRCA, - 2.9 mm for STAU and - 4.0 for SRPN. According to Urbášková (1) the minimal zone interpretable as „susceptible“ is 12 mm (AZI), resp. 13 mm (CLA) for hemofili and 18 mm (both AZI and CLA) for grampozitive cocci. After this criterion, all the tested HEIN strains were susceptible to both AZI and CLA. Nevertheless, an at least small difference was found (probably thanks to bigger amount of strains) in HEPA: here in CLA two strains (1 %) were found to be resistant, in eight strains (5 %), still evaluated as susceptible, the size of the zone was sharply equal to borderline 13 mm. On the other hand, in AZI all the strains were sensitive, and the smallest observed zone was 18 mm (i. e. 6 mm more than the borderline). In STAU we have found seven strains (10 %) resistant to both tested antibiotics, all the other strains were sensitive to both of them. In SRPN two strains (8 %) were found to be resistant to AZI and CLA, and here, too, all the other strains were sensitive to both antibiotics. In BRCA it was difficult to make relevant conclusions of so few specimens. So, seen from one point of view, it is clear, that AZI and CLA are not so substantial that they would influence the ratio of susceptible strains, on the other hand, it is evident, that gramnegative bacteria are better susceptible to azithromycin, while gramnegative to clarithromycin. This is in relations with literature data (2). Of course, these results are not fully transferable to in vivo situation, at least for two reasons: 1) clarithromycin does not act only as the original drug, but also as products of its metabolism, 2) azithromycin increases its intracellular concentrations more than clarithomycin, so the same dosage of antibiotic leads to an increased intracellular concentration.

P. Hejnar (Department of Microbiology, Faculty of Medicine, Palacký University, Olomouc, Czech Republic):

Susceptibility of *Stenotrophomonas maltophilia* clinical strains to -lactams and evaluation of activity of -lactam / -lactamase inhibitor combinations

A total of 127 *Stenotrophomonas maltophilia* clinical strains were identified using the NEFERMtest (Lachema) and supplementary tests. Susceptibility to 11 different -lactams was tested by means of a standard dilution micromethod and minimum inhibitory concentrations (MICs) were assessed. In -lactam/-lactamase inhibitor combinations the synergism was defined as at least four-fold decrease of the MIC value, the antagonism as at least four-fold increase of this value also in inhibitor's presence. In other cases the effects were evaluated as indifferent.

The tested strains were most often susceptible to cefoperazone/sulbactam (81.8 % of susceptible strains), cefoperazone (50.4 %), piperacillin/tazobactam (47.1 %), piperacillin (41.6 %) and ceftazidime (40.9 %).

Antagonistic effect was not recorded in some of -lactam / -lactamase inhibitor combinations used. Synergism occurred in 40.8 % of cases using ampicillin/sulbactam, in 14.9 % at cefoperazone/sulbactam and in 4.1 % at piperacillin/tazobactam.

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Z. Šándorčínová, M. Kmeťová, V. Takáčová, L. Siegfried (Institute of Medical Microbiology, Medical Faculty of Šafarik University, Košice, Slovakia):

Detection of ESBL production in clinical isolates *Escherichia coli and Klebsiella pneumoniae*.

Production of -lactamases is a significant cause of resistance of gram-negative bacteria to -lactam antibiotics.

Since November 1999 up to April 2000 a total of 310 *Klebsiella pneumoniae* and 50 *Escherichia coli* strains were isolated in the three ICUs at University Hospital in Košice. Production of -lactamase was detected by using the iodometric method and in the -lactamase positive isolates double disc synergy test (DDST) was carried out. Production of extended spectrum -lactamases (ESBL) was verified by means of ratios of minimum inhibitory concentration of ceftazidime and combined ceftazidime with clavulanic acid determined by the E-test.

On the basis of given tests 41 *K. pneumoniae* strains were detected to be the ESBL producers. In case of *E. coli* the production of ESBL was not recorded, but the ”common“ beta-lactamase production was detected in 13 strains.

M. Hábeková[1], M. Mokráš[2], D. Staneková[1]. ([1]National Reference Centre for HIV/AIDS Prevention, Institute for Preventive and Clinical Medicine, Bratislava, Slovakia, [2]Clinic of Infectous and Geographical Diseases, Dérer Hospital Bratislava, Slovakia):

Observing the effectivity of antiretroviral therapy in HIV infection

According to the recommandations for the antiretroviral therapy (ART) of HIV infection the clinical observation and laboratory markers determination are necessary to decide about the ART optimal mode ART. For this purpose the examinations of clinical, immunological and viral status (CD4 counts and viral load) of HIV infected individuals have been regularly investigated in the National Reference Centre for HIV/AIDS prevention in Bratislava.

In attempts to elucidate the suboptimal treatment response or treatment (virological) failure and to obtain the data about possible transmission of resistant mutants as well, the resistance HIV to drugs used for treatment has been examined. With the collaboration with Virological laboratory of the Hôpital Pitié – Salpetriere in Paris and according to the protocol of ARMS Q151M of the multicentric study of the prevalence of Q151M mutation (Centre Hospitalier de Luxembourg, Luxembourg) the genome of RT HIV RNA was genotyped and screened for the presence of resistance mutations.

Z. Krakovská, E. Piecková, Z. Jesenská (Institute for Preventive and Clinical Medicine, Bratislava, Slovakia):

Tolerance of Environmental Micromycetes to fluconazole

Many environmental microfungi are unexpected pathogens and are capable to infect immunocompromise patients (e. g. AIDS, transplantation, cancer etc.). In vitro testing of susceptibility, tolerance and resistance of microfungi against antimycotics is getting into the interest of experts in some last years.

The aim of this study was to test activity of fluconazole – a modern antimycotics used in prophylaxis and therapy of deep mycoses – against soil fungi.

Working concentration of fluconazole selected according to clinical treatment schedules (15, 20, 30, 40, 60, 80, 100, 120 µg/ml) were added into the Sabouraud agar with Bengal Rose (150 mg/l). The diluted soil samples were inoculated onto the surface of the plates and cultivated 7 days at 25 and 37 °C.

Strains *Absidia* sp., *Cunninghamella* sp., *Geomyces pannorum*, *Mortierella* sp., *Mucor* sp., *Penicillium* sp., *Trichoderma* sp. *Zygorhynchus heterogamus* were able to grow on the medium with the highest concentration of fluconazole (120 Ķg/ml) at 25 ĮC. *Aspergillus flavus*, *Aspergillus* gr. *A. niger*, *Aspergillus terreus*, *Fusarium* sp., *Mucor* sp., *Neosartorya fischeri*, *Penicillium* sp. a *Trichoderma* sp. were growing on the medium with 120 Ķg/ml of fluconazole at 37°C. Strains are already know as agents of human infections, usually fatal ones.

It's clear that a lot of environmental microfungi are tolerant/resistant against fluconazole and there is high probability of patient's infections by them.

In vitro tests of susceptibility, tolerance and resistance of microfungi against antimycotics should be added to the official protocols and the experts should take a different view of pharmacological studies carried out with new antimycotics.

BLOCK 9: Microbiology in clinical and epidemiology

L. Ryöková[1], M. Morávková[1], T. Česák[2], V. Lebedová[3] ([1]Department of Microbiology, University Hospital, Hradec Králové, Czech Republic [2]Department of Neurosurgery, University Hospital, Hradec Králové, Czech Republic, [3]National Reference Laboratory, National Institute of Public Health, Prague, Czech Republic)

***Haemophilus aphrophilus* as a cause of brain abscess**

The authors report a case of brain abscess due to *Haemophilus aphrophilus*.

A 42-year-old female was admitted because of headache, vomiting and focal neurological symptoms. Computed tomography demonstrated multiple brain abscesses. A craniotomy and an evacuation of the abscess cavity located in parietooccipital part of the brain was performed. Gram negative bacilli isolated from aspirated pus were identified as *Haemophilus aphrophilus*. The patient responded well to appropriate antibiotics treatment.

The brain abscess is a life-threatening disease and it is important to identify the causative agents and their antibiotic susceptibility. We report the first isolation of this bacterial strain in our laboratory.

R. Hubač, R. Chlíbek, M. Hartmanová, M. Špliňo (Department of Epidemiology, Purkyně Military Medical Academy, Hradec Králové, Czech Republic):

Is the Czech Army ready to diagnose all the hazardous agents?

The problems of biological warfare agents and generally detection of hazardous infections are ranked among priorities of all NATO armies, especially after experience in Iraq and other regions.

In my presentation I would like to inform you about contemporary possibilities of the Czech Army microbiologists and epidemiologists in the field of the hazardous infection diagnostics.

Among potentially misusable infections we rank bacterial, viral, mycotic infections, and diseases caused by toxins. In the frame of the Czech Army there exists nowadays only one microbiological laboratory which can diagnose some agents even by express methods. But it is

unable to identify some of them by any method. The situation would be much worse under emergency situations when mobile equipment should be used. Some sets are namely outdated and don't postulate the present requirements.

M. Šimončičová[1], *L. Slobodníková*[1], *M. Borovský*[2] ([1]Institute of Microbiology and [2]1st Department of Gynaecology and Obstetrics, Medical Faculty of Comenius University and Faculty Hospital, Bratislava, Slovakia):

Occurrence of non-fastidious micro-organisms in vaginal and cervical swabs of patients, with vaginitis and cervicitis

Inflammatory vaginal and cervical diseases are considerably frequent. Almost every woman suffers from this diseases at least once in her life. Aerobic and anaerobic bacteria are the main cause of this disease, however, to the frequent diseases of the female genital tract belongs also chronic and recurrent candidoses.

Aim of the study was to investigate the occurrence of non-fastidious micro-organisms with possible role in the chronic and recurrent vaginitis and cervicitis in materials from inflamed area.

From March 1999 to March 2000, vaginal and cervical swabs were sampled from patients (at reproductive age) of the 1st Department of Gynaecology and Obsterics of The Faculty Hospital in Bratislava and transported to microbial laboratory in transport medium. Specimens were cultivated by usual methods on enriched blood agar and on Endo agar. Isolated micro-organisms were identified according their colony morphology, microscopical morphology and biochemical tests. Yeast were identified by germ-tube test (*Candida albicans*), and according their growth on CHROMagar Candida (BBL) medium. Growth of pathogenic or potentially pathogenic micro-organism in quantitative prevalence and the absence or highly decreased amount of lactobacilli in both types of samples parallel were considered to be significant.

Out of 2810 examined samples, 1149 (48.9 %) were positive. *Streptococcus agalactiae* was isolated from 209 samples, *Enterococcus sp.* from 292 samples, *Escherichia coli* from 195, *Staphylococcus aureus* from 24, *Klebsiella sp.* from 23, *Proteus mirabilis* from 27, and gramnegative nonfermenting bacteria from 38 samples. Among yeast *Candida albicans* was the most frequently isolated species (239 samples). Other yeast species were isolated from 108 materials.

The most frequently isolated micro-organisms in females of reproductive age were *Candida albicans* and *Streptococcus agalactiae.*

D. Vaňková (Regional Institute for Public Health, Ostrava, Czech Republic):

Diphyllobothriosis and echinococcosis – case reports

Two brief case reports of imported parasitic infections. The first case is diphyllobothriosis of a man of the Russian nationality, the other case is echinococcosis of a man of the Macedonian nationality. The findings are supported with the pictures of eggs of *Diphyllobothrium latum* and contents of hydatid cysts in liver.

Posters:

R. Burdychová[1], *M. Bartoš*[2] ([1]Dept. of Alimentary Chemistry and Biotechnology, Faculty of Chemistry, Technical University Brno, Czech Republic, [2]BioVendor – Laboratorny Medicine, Ltd., Brno, Czech Republic):

Rapid and easy PCR detection of heterogeneous gene insertion into genome of yeast *Pichia pastoris*

Cloning and expression of human proteins in bacterial expression systems often yields unsatisfactory results because of different mechanisms of translation, protein folding, processing and posttranslation modification in prokaryotes and eukaryotes. For this reason yeast, insect or mammalian cell cultures or baculovirus or adenovirus are used in expression of genes from higher eukaryotes.

The eukaryote *Pichia pastoris* shares many of the advantages of higher eukaryotic expression systems, while being as easy to manipulate as *Escherichia coli* or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive for use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture, and generally gives higher expression levels.

Several shuttle vectors has been developed for the expression of secreted and intracellular recombinant proteins in *Pichia pastoris*. Recombinant plasmids are propagated in *E. coli*, linearised and transformed into *P. pastoris*. Stable transformants are generated by homologous recombination between the transforming DNA and regions of homology within the genome. Such integrants show extreme stability in the absence of selective pressure.

The screening of stable transformants of *P. pastoris* involves a time consuming and expensive method of DNA isolation from yeast cells. We have developed easy and inexpensive method, which is based on quick disruption of cells and direct amplification of crude lysate. Different disruption conditions were used for cell lysing and Taq polymerase from two suppliers and own recombinant Taq polymerase were tested in amplification. The best conditions for the cell disintegration was achieved in 80 °C / 10 min and good amplification results were generated using HotStar Taq polymerase (QIAGEN). The system was tested on two *Pichia* integrants containing genes for human leptin and exon 3 from human myostatin.

E. Bogyiová, L. Siegfried, M. Kmeťová (Institute of Medical Microbiology of Medical Faculty of Šafárik University, Košice, Slovakia):

Frequency of Cytotoxic Necrotising Factor (CNF1) in *Escherichia coli* isolates from patients with Urogenital Infections

The CNF1 is one of the bacterial toxins reported to modify several cytoplasmatic proteins by specific deamidation of glutamine. These modified proteins disturb control machinery of the actin cytoskelet synthesis and its destruction. Moreover, overexpresion of host-cell adhesins may occur as a consequence of the CNF1 presence in host-cell environment. All these cytological changes may support invasion of the carcinomas and their metastasing. We inspected the frequency of CNF1 – positive *Escherichia coli* strains in various types of urogenital infections. *E. coli* urinary and vaginal strains were collected from 80 patients (pyelonephritis 20, cystitis 20, vaginal isolates 30). Fecal samples of 30 healthily donors were collected as controls. CNF1 status was detected by PCR, using specific primers for *cnf1* gene.

M. Sabol, A. Liptáková, M. Kmeťová, L. Siegfried (Institute of Medical Microbiology of Medical Faculty of Šafárik University, Košice, Slovakia):

Estimation of Shiga-like toxin activity on cell cultures

The goal of this paper is to present the state of art of Shiga-like toxin (Stx) activity detection and our experiences with cell culture assay. Strains of *Escherichia coli* producing shiga-like toxins are known to cause both intestinal and extraintestinal manifestations of infection seen as hemorhagic colitis and the hemolytic uremic syndrome in humans. There are two major Stx types (Stx-I and Stx-II) described in literature. However, a large number of variations in the coding sequence for Stx-II and several chromosomally encoded variants of Stx-I have been demonstrated in recent years. In relation to the fact that Stx producing *E. coli* vary in their capacity to cause serious diseases in humans and that this is a function of the type and/or amount of Stx produced the impact of cell culture assay results should be judged carefully. Generally, more severe clinical manifestations are associated with Stx-II than Stx-I and thus neutralisation assay with monoclonal Ab against Stx-I and Stx-II should be unavoidable part of assay. The amount of Stx produced is measurable with MTT assay and cell culture assay gain by this way also quantitative feature. Both above mentioned approaches increase the validity of test. In spite of some discrepancies, the cell culture assay remains clinical significance and should be used as a confirmation for Stx production in addition to PCR and latex agglutination test.

A. Holubářová, P. Müller, O. Nečas, A. Svoboda (Department of Biology, Medical Faculty, Masaryk University, Brno, Czech Republic):

A response of yeast cells to heat shock: the effect of osmotic, radiation and mild heat stress

On the model of yeast *Saccharomyces cerevisiae* CCY 21-4-59 the effects of heat, osmotic and radiation stresses on cells exposed to the lethal heat shock were studied.

Thermoresistance or thermosensitivity of cells was detected by their ability to form colonies (CFU) at 25 °C. After the transfer from 25 °C to 46 °C most of cells lost their viability during 10 min of incubation and this temperature was then referred as lethal heat shock. It was found that a 30 min preincubation of cells at 37 °C or 41 °C (referred here as a mild heat stress) lead to the increase of their resistance to the lethal heat shock. Western blotting technique + ECL revealed that during mild heat stress the concentration of Hsp104 in cells increased, while in controls only traces of this protein were detected. The Hsp104 synthesis was apparently adaptive, because cykloheximid present during mild heat stress effectively prevented the increase of cell thermoresistance. Accumulation of Hsp104 was detected also by indirect immunofluorescence technique in cytoplasm of cells after mild heat stress.

We found that the resistance to heat shock 46 °C could be induced also by previous osmotic shock (KCl, 1 mol . l^{-1}) or short UV radiation. However, the stress proteins seemed to form slowlier, because the cells became thermoresistant only after 3 hours adaptation following heat shock application.

In control cells the lethal heat shock 46°C caused rapid disintegration of spindle and cytoplasmic microtubules leaving preserved only point fluorescence of spindle pole bodies. Mild heat stress (37–41 °C) did not lead to the degradation of microtubules and, surprisingly, the microtubules from these pre-treated cells remained stable even after the transfer of cells to 46 °C. Similarly, microtubules from mild stressed cells remained stable after the transfer of cells to 0 °C, while in controls this cooling lead to the microtubule disintegration. Both osmotic and radiation stresses caused a rapid degradation of microtubules. These re-appear within 2 to 3 hr of incubation and remained then stable at 46 °C. Actin structures (cables and patches) were more sensitive: actin cables disappeared and polar localisation of actin patches were disrupted even in mild stressed cells.

It can be concluded that the stress proteins, including Hsp104, formed under condition of mild stress, take part in the maintenance cell integrity during heat shock.

M. Střítecká (Institute for Microbiology, Faculty of Medicine of Masaryk University and St. Anna's Faculty Hospital, Brno, Czech Republic):

Maintenance of *Haemophilus* spp. within routine laboratory conditions

This work concerns problems which occur during storage of *Haemophilus* spp. I have found out that when using a suitable cultivation medium for re-inoculation, the results are much better when temperature 8 °C is applied than the room temperature. 100 % species remained vital for 3–4 days. On the contrary, *Haemophilus* stored under temperature 22 °C required a subculture every day. Amies medium turned out to be the most suitable. Out of 36 species, no one remained vital for more than 28 days while storing under both temperatures and on various media.

To send the species successfully via postal transport, it is necessary to use well sealed tube with slant medium CABS. All the tested species remained vital for 28 days at room temperature.

The goal was to find out the best way how to preserve *Haemophilus* within routine microbiological laboratory conditions for one year. I have used three media: Levintal's broth, broth with glycerol and serum, medium LSPQ. I have also applied different storage temperatures: – 20 °C and – 70 °C. I strongly recommend not to use preservation at – 20 °C. If still this temperature has to be used, LSPQ seems to be the most suitable.

The best results were observed while using broth with glycerol and serum at temperature – 70 °C. After one year of preservation the number of vital cells dropped roughly from 10^9 to 10^7 CFU on average (0.27 %).

Š. Kavánová (Institute for Microbiology, Faculty of Medicine of Masaryk University and St. Anna's Faculty Hospital, Brno, Czech Republic):

Detection of antibodies against *Haemophilus influenzae* and *Haemophilus parainfluenzae*

Both types of *Haemophilus* dealt in our study are isolated especially from human respiratory tract. While *H. influenzae* is a demonstrable pathogen, at least in some cases, *H. parainfluenzae* is thought to be only a commensal. In recent years *H. influenzae* has been very often isolated from children's pharyngeal swabs in the Institute for Microbiology, Faculty of Medicine of Masaryk University and St. Anna's Faculty Hospital Brno.

One of the possibilities how to prove that *H. parainfluenzae* doesn't act as a pathogen was detection of antibodies. In this work the method of fluorescence immuno – assay (FIA) and ELISA was used.

Having used FIA method, we have found out that hyperimmune rabbit sera had cross-reactions with both of antigenes, but nevertheless there were little differences in the intesity of fluorescence. Differences in the intensity of fluorescence in sera of adult patients were observed in 26 % samples. 12 % had higher titer of antibodies against *H. influenzae* and 14 % had higher titer of antibodies against *H. parainfluenzae*.

Differentiation of antibody's levels was following: 7 % of population had no antibodies, 57 % had low level of antibodies, 31 % population had middle level of antibodies and 5 % had even high titer of antibodies. Sera of children with respiratory infection and with cultural finding of *Haemophilus* were found different in the intensity of fluorescence in 55 % samples. 23 % samples had higher titer of antibodies against *H. influenzae* and 32 % had higher titer of antibodies against *H. parainfluenzae*.

The results of FIA concurred with cultural finding only in 32 % samples. Findings of higher titers against *H. parainfluenzae* indicate, that this micro-organism is able to act as a pathogen. However, this method has turned out not to be satisfactory, as it does not distinguish *H. influenzae* from *H. parainfluenzae* sufficiently.

Using ELISA (by comparison of positivity indexes obtained after incubation with both antigenes), 64 % samples had the same results as cultural findings showed. *H. influenzae* was found to be an ethiological agent for 18 % patients with cultural finding of common flora or micro-organism different than *H. influenzae*. For 4,5 % samples the results were negative, and for 9 % the results were disputable.

Low ability to distinguish between similar antibodies has been observed while using ELISA method, but if the positivity indexes are counted, we are able to distinguish antigenes *H. influenzae* from *H. parainfluenzae*.

D. Molinková, V. Celer (Institute for Microbiology and Immunology, Veterinary and Pharmaceutical University, Brno, Czech Republic):

Expression of EHV-1 glycoprotein D in Escherichia coli

Glycoprotein D is an important component of herpesvirus particle. It is involved in the binding of virus on the surface of host cells and it expresses antigenic structures responsible for induction of neutralising antibodies. Gene coding for glycoprotein D of equine herpesvirus type 1 (EHV-1) was cloned into several plasmid vectors and bacterial cells were transformed by these DNA constructs. Resulting protein was expressed as a recombinant molecule with polyhistidine, glutathion-S-transferase or thioredoxin tag. Purification of soluble protein and protein in inclusion bodies was performed using appropriate methods based on affinity chromatography.

R. Filip[1], *L. Chihu-Amparan*[2], *J. Silva-Sanchez*[2] ([1]University of Medicine and Pharmacy, Iaļi, Romania, [2]Center for Investigation of Infectious Diseases, Cuernavaca, Mexico):

Study of outer membrane proteins (OMPs) and their role in co-modulation resistance in non-typhoidic *Salmonella* strains isolated in Romania

Purpose was to characterise the OMP profile of the *Salmonella* strains and to study the possible interference with other resistance determinants.

Sixteen non-typhoidic *Salmonella* strains, isolated in 1999 from faeces of paediatric patients, were selected according to their resistance phenotype. They all displayed clear resistance to ampicillin (AMP), amoxycillin / clavulanic acid (AMC) and third generation cephalosporines – ceftriaxone (CRO) and ceftazidime (CAZ). Identification at species' level and sensitivity testing were performed by using the DADE Micro Scan System. b-lactamase characterisation was performed by using isoelectric phocusing (IEF) in minigels, developed with nitrocefin and bioassayed versus ceftazidime (1; 0. 5; 0. 25 ug/ml). OMPs were studied on membrane preparations on SDS-Urea gels.

Isoelectric points were variable between the isolates, majority of them had the association 5.4 + 7.6 (3/16) or 5.4 + 8.2 (6/16). The second b-lactamase (7.6 and 8.2) has the capacity to hydrolyse CAZ. We found three different patterns of OMPs: pattern I: 30, 29 and 25 kDa; pattern II: 30, 26 and 24 kDa; pattern III: 30 and 25 kDa. We couldn't find any correlation between the OMPs profile and the resistance pattern of the isolates, showing that b-lactamase production is the only responsible for the resistance showed by these strains.

The study shows that there is high frequency of extended-spectrum b-lactamases (ESBL) between the non typhoidic *Salmonella* isolates; the association between two enzymes TEM type and ESBL diminishes considerably the therapeutic resources – inhibitor associated combinations are non effective. In non typhoidic *Salmonella*, the porins seem to have no influence in co-modulation the resistance.

C. Tuchiluţ, D. Bosnea, A. Poiata, R. Filip, I. Badicut, D. Buiuc (Microbiology Department, University of Medicine and Pharmacy, Iaţi, Romania):

Detection of extended-spectrum b-lactamases in clinical isolates from eastern Romania

Extended-spectrum -lactamases (ESBL) are an increasing cause of resistance to third-generation cephalosporins in *Enterobacteriaceae*.

The aim of the present work was to study ESBL production by *Enterobacteriaceae* ceftazidime-resistant strains.

Bacterial strains: 11 clinical isolates (5 strains of *Escherichia coli* and 6 of *Samonella* spp.) selected among 670 enterobacteria according to their susceptibility spectrum (M2-A4 NCCLS 1999 standard). The identification of the species by their biochemical properties was done using rapid methods – API 20E and API 32E. ESBL production was established by using both double disk synergy test (DDT) and Expert computer program of mini API (bioMerieux). The isoelectric point (pI) of ESBL obtained by chloroform extraction was determined by isoelectric focusing in polyacrylamide gel and revealed by adding iodine solution. Isoelectric points were estimated by comparison to references b-lactamases, with known pI.

Results: We revealed ESBL production for all selected strains. By isoelectric focusing we identified ESBL with pI = 6.3 at all studied strains, excepting one *Salmonella* spp. strain, which had an ESBL with pI = 5.9.

Conclusions: The *E. coli* studied strains and five strains of *Salmonella* spp. producing a TEM-3 ESBL. The ESBL with pI = 5.9, produced by *Salmonella* spp. strain is TEM-6 type.

A. G. Anghelescu-Dogaru[1], *G. C. Chitanu*[1], *A. Poiata*[2], *A. Carpov*[1] ([1] „Petru Poni“ Institute of Macromolecular Chemistry, Iaţi, Romania, [2] “Gr.T. Popa“ University of Medicine and Pharmacy, Iaţi, Romania):

Synthesis and characterisation of maleic copolymers with pendant disinfectant and/or odorant molecules and their stability in aqueous medium

The controlled release of functional compounds by means of different types of physical, chemical or biochemical degradation provide a variety of release rates. The synthesis of some polymeric systems containing disinfecting and/or odorant molecules attached to maleic anhydride copolymers via ester link is advantageous due to the mild conditions of synthesis and to the advanced purity of the products.

In our study the parent copolymers were synthesised by radical copolymerisation of maleic anhydride (MA) with vinyl acetate (VA), styrene (St) or N-vinylpyrrolidone (NVP). All the

polymers were alternating 1:1 (moles) copolymers, with molecular weight between 33 000 and 436 000. Maleic anhydride copolymers were reacted with thymol, eugenol, phenylethyl alcohol or citronellol at low temperature without catalyst. The esters were purified and characterised by elemental analysis, conductometric titration, IR spectra and TG analysis. It was established that conversions between 20 and 40 % were attained.

The behaviour and the stability in aqueous medium of the maleic derivatives were also studied.