Session VII. New methods in microbiology

VII.P.1

New insights into the investigation of marine microbial diversity of the Black Sea surface water

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The Black Sea is unique marine ecosystem with high microbial biotechnology potential. The microbial communities are the integral part of the ecosystem and play the key role in all biological processes occurring in it. The data on the Black Sea bacterial diversity is mainly obtained by cultural methods. However few prokaryotes are capable of cultivation and it's known about candidate phyla without cultured representatives. The aim of research was to investigate the Black Sea biodiversity by metagenomics 16S rRNA analysis. Provided study is a complex investigation of the seawater microbiota based on isolation of environmental DNA from marine biome and further sequencing. Marine water was collected and filtered during July 2014 from 6 sites along the seacoast in Odessa region. Total DNA was isolated from filters. Primer design followed Kozich *et al.* (2013) with dual indexing, Read1, Read2, Index1 primers and 16S V4 variable DNA region specific primer. PCR reactions were performed to obtain a 16S clone library that was quality controlled, purified and quantified. Sequencing was performed on Illumina MiSeq platform. QIIME workflow (Caporoso *et al.*, 2010) was used for computer analysis of data. The 16S rRNA gene sequencing and bioinformatics analysis showed dense Bacterial community among samples. Obtained sequences were identified up to genus level. Taxonomic phylum Proteobacteria was the most abundant among Bacterial Domain. The composition of Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria and Deltaproteobacteria classes varied depending on the source of sample. Sequences of Cyanobacteria, Bacteroidetes, Actinobacteria, Planctomycets and Verrucomicrobia phyla were identified in high amounts as well. The members of Fusobacteria, Tenericutes and Firmicutes phyla were less represented in samples. The results of research identified members of candidate divisions such as SR1, OD1, OP3, OP8, TM6, TM7 and others. Some of the determined bacterial species were described for other parts of World Ocean in similar metagenomics investigations.

Key words: 16S rRNA analysis, metagenomics, marine microbiota, Black Sea

VII.P.2

Proteomic approach in identification of factors associated with virulence of *Staphylococcus aureus*

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Staphylococcus aureus is a major pathogen of human and veterinary hosts. It secretes a range of proteins into extracellular milieu, many of which act as virulence factors. Our previous study on chicken embryo model revealed that the pathogenic potential of poultry-originated strains varies among the isolates and correlates with their genotype [1]. This suggests the existence of factors associated with virulence.

The aim of the study was to investigate extracellular proteomes of avian *S. aureus* strains in order to verify whether the differences in virulence are reflected at the proteome level, and to identify proteins responsible for high- or low-virulence phenotype. For this purpose extracellular proteomes of five virulent (VS) and five non-virulent (NVS) *S. aureus* strains were compared. Precipitated proteins were labeled with fluorescent dyes and separated using two-dimensional difference gel electrophoresis in pairs VS *versus* NVS. Differentiating protein spots were cut out from the gels, trypsin digested and subjected to mass spectrometry analysis.

The obtained results reveled that exoproteomes were highly heterogeneous irrespective of strain virulence, as only a few proteins were identified in all VS and all NVS proteomes. The number of differentiating spots was higher in VS than in NVS proteomes (263 *versus* 176). However the number of proteins identified as a unique for VS and NVS proteomes was opposite (28 *versus* 48). Especially in VS exoproteomes the same proteins occurred in many spots, indicating their high abundance, but also posttranslational processing. Moreover, high complexity of exoproteomes of NVS suggests that staphylococcal virulence is driven not only by the production of virulence factors but also by the secretion of proteins that may attenuate virulence. It indicates that although the array of proteins responsible for high virulence of staphylococcal strains is wide and heterogeneous, even more proteins may be involved in development of commensal phenotype.

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1. Polakowska K, Lis M, Helbin WM, Dubin G, Dubin A, Niedziolka J, Miedzobrodzki J, Wladyka B (2012). The virulence of *Staphylococcus aureus* correlates with strain genotype in a chicken embryo model but not a nematode model. *Microbes Infect* **14**: 1352–1362.

Key words: 2D DIGE, host-preference, proteomics, *Staphylococcus aureus*, virulence factor

VII.P.3

Fluorescence optical respirometry – an interesting method for testing antimicrobial compounds

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The development of the analysis methodology for testing the susceptibility of bacteria to different antimicrobial compounds has a long history, from analyses based on phenotypic properties of microorganisms using the dilution method, by numerous modifications of diffusion methods, to the technology for genotyping (Wheat, 2001). Therefore, growth analysis of microorganisms remains still the most effective method to analyse the impact of chemicals on bacteria and fungi. An interesting method of growth analysis of microorganisms in cultures using so-called optical respirometry has been proposed (Wodnicka *et al.*, 2000, Hałasa *et al.*, 2014). This method is based on analysis of the fluorescence of an oxygen-sensitive sensor. An analysis of the usefulness of the fluorescence optical respirometry test method to study several antimicrobials was performed. An oxygen-sensitive sensor: ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride $Ru(DPP)_3Cl_2$, the fluorecence of which is quenched by molecular oxygen, was synthesised according to a method modified by us and then applied. Analyses of the impact of various antimicrobial chemical factors were performed. It was shown that optical respirometry allows for analysis of the culture growth kinetics of bacteria and fungi and determination of cell growth parameters. It was shown also that MIC values obtained by fluorescence optical respirometry are consistent with the results of the MIC determinations made by serial dilution method (traditional MIC testing using CLSI). The method allows the time to obtain results to be significantly reduced and allows the effect of concentrations below the MIC for the metabolic activity of microorganisms to be monitored. The sensitivity of the method allowed for the volume reduction of the tested samples. Fluorescence optical respirometry allows for the rapid detection and evaluation of the action of various chemical compounds on the metabolic activity of microorganisms in real-time measurement of fluorescence intensity.

Key words: fluorescence optical respirometry, antimicrobials, oxygen sensor

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Wheat PF (2001) History and development of antimicrobial susceptibility testing methodology. *J Antim Chemioth* **48** (Suppl S1): 1–4.
Wodnicka M, Guarino RD, Hemperly JJ, Timmins MR, Stitt D, Pitner B, (2000) Novel fluorescent technology platform for high throughput cytotoxicity and proliferation assays. *J Biomol Screen* **5**: 141–152.
Hałasa R, Turecka K, Orlewska C, Werel W (2014) Comparison of fluorescence optical respirometry and microbroth dilution methods for testing antimicrobial compounds. *J Microbiol Methods* **107**: 98–105.

VII.P.4

Overproduction of stable isotope labeled proteins

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Nuclear magnetic resonance (NMR) spectroscopy is one of the leading methods of 3D protein structure determination. However, it has a limited potential due to the difficulty of interpretation of NMR spectra containing broad overlapping signals. To overcome this problem methods utilizing stable isotope as labels are often used. Two main approaches for labeling are cell-free protein expression (protein stereo-array isotope labeling) and protein expression in bacterial cells [1].

The aim of this research was to establish and optimize a method of stable isotope protein labeling which would allow to label a protein with three isotopes at one time. It should allow to incorporate stable isotopes of carbon, nitrogen and hydrogen into amino acid residues forming the protein, utilizing protein expression in bacterial cells. The method was optimized on the example of human cystatin C protein which structure is being resolved as part of another research.

The protein was expressed in properly selected strain of *Escherichia coli* bacterial cells (competent strain BL21, genotype: *fhuA2 [lon] ompT gal [dcm] ΔhsdS*). For the purpose of labeling modified compositions of microbiological media were established. During protein expression media containing labeled glucose (^{13}C), ammonium chloride (^{15}N) and deuterium oxide (^{2}H) were used. The replacement of water with deuterium oxide in all used media effected in prolongation of expression process due to the slower growth of bacterial cells. Thus the expression time optimization was required.

As a result of the optimization process a method which allows to obtain a triple labeled protein was established. The yield of the method is low due to addition of deuterium oxide to media but sufficient for NMR studies.

Key words: bacteria, *E. coli*, protein, expression, labeling, stable, isotope

Reference:
1. Kainosho M, Torizawa T, Iwashita Y, Terauchi T, Ono AM, Guntert P (2006) Optimal isotope labelling for NMR protein structure determinations. *Nature* **440**: 52–57.

VII.P.5

Shedding light on *Candida parapsilosis* and *Candida tropicalis* secretome and surfaceome with the use of cell surface shaving method and shotgun proteomic approach

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The initiation and further spreading of the fungal infections in the human organism depend on the interactions of pathogens' surface-exposed or secreted proteins with the host proteins and tissues. Surprisingly, only a very limited information is available about secretome and proteinaceous components of the cell wall of important opportunistic pathogens from the genus *Candida*, particularly the species, other than the most widespread *C. albicans*, such as *C. parapsilosis* and *C. tropicalis*. The incidence of severe diseases caused by these two species has alarmingly increased in the last few decades and recently reached a level of approximately 20% of all invasive candidiases.

The aim of this study was to identify proteins from the cell wall of live filamentous forms of *C. parapsilosis* and *C. tropicalis*, as well as proteins secreted during fungal growth and morphogenesis, considering the fact that both can act as important virulence factors.

By a rapid treatment with trypsin – the method known as "cell surface shaving" – we identified several proteins from the surface of *C. parapsilosis* and *C. tropicalis* pseudohyphae. In *C. parapsilosis*, true cell wall proteins, including cell surface mannoprotein MP65 and GPI-anchored cell wall proteins Ecm33, Crh1 and chitinase 2, as well as other structural cell wall proteins including Pir1 and glycosidase Phr2 were identified. In *C. tropicalis*, the most notable of the indentified proteins included a typical adhesin from agglutinin-like sequence family, Rbt1 similar to *C. albicans* hyphal wall protein 1 and hyphally regulated cell wall protein, and, in addition, two atypical proteins, alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase.

Moreover, a number of cell wall proteins, including few of those mentioned above, were also found in the yeast culture medium, apparently having been released from the cell surface during the growth and proliferation of the fungi. Some of those identified molecules can be taken into consideration in the future perspective as potential diagnostic markers or vaccine components.

Key words: cell wall, virulence, candidiasis

VII.P.6

Specific detection of *Alternaria alternata* by PCR and real-time PCR

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Fungi of *Alternaria* genus are cosmopolitan organisms, which spores can be found in the air, soil, water, clothing and food. They commonly occur as saprotrophs on the plant remains, contributing to the decomposition of organic matter. Additionally, they are components of the normal human and animal skin flora. *Alternaria* spp. are also known human allergens, causing hay fever and allergic reactions that can lead to the development of asthma. They are more and more frequently found as etiological factors of superficial, organ and disseminated infections in humans and animals. The most prevalent species of *Alternaria* genus isolated from infection sites is *A. alternata*.

The aim of this work was to develop PCR and real-time PCR assays that enable specific detection of *A. alternata* DNA.

Research methods included fungal DNA isolation, design of *A. alternata* – specific primers based on the alignment of β-tubulin gene sequences from different fungi, PCR followed by amplicons electrophoretic analysis and real-time PCR followed by amplicons melting curve analysis.

Both PCR and real-time PCR assays accurately identified all tested *A. alternata* strains. Developed assays are rapid, easy to interpret, and can be useful tools for detection of *A. alternata*.

Key words: *Alternaria alternata*, detection, diagnostics, fungi, PCR, real-time PCR

VII.P.7

Application of a novel extended RFLP-based approach for molecular typing of rare Staphylococci species

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Staphylococcal strains, apart from being a part of human and animals' microflora, are commonly encountered in the natural environment. The clinical importance of many non-S*taphylococcus aureus* species is constantly growing as the antibiotic resistance and their transmission from environmental to human strains do as well. There are numerous molecular typing methods developed for staphylococci, however theses are mostly suited to *S. aureus*. This study presents a new approach for staphylococci typing which utilises *saoC* gene and its usefulness in typing of different clinical as well as environmental isolates [1]. The method has also been validated in a clinical case study on *S. pettenkoferi* isolate. In the course of the study for 10 environmental isolates the PCR product of *saoC* gene was obtained. Afterwards each product was separately digested with four restriction enzymes (TaiI, Tsp509I, AluI and MseI). The samples were subsequently separated in agarose gel to obtain the restriction fragment length polymorphism (RFLP) patterns. In the case of the blood isolate of *S. pettenkoferi* the PCR product and the digestion pattern was also obtained. The identity of this isolate was additionally confirmed using *gap* gene AluI RFLP and *16S rRNA* and *rpoB* genes' sequencing as well.

For all analysed strains the digestion patterns were successfully obtained. What is important, in contrast to the state-of-art methods the *saoC*-based method allowed to type environmental strains, which were genetically atypical when compared to human or animals'. Moreover, the application of *saoC* gene RFLP for the clinical isolate supported its identification as *S. pettenkoferi* and thus proved to be well suited to this species as well.

Considering the atypical strains molecular identification, the RFLP-based method of *saoC* gene analysis proved to be a useful, reliable and relatively cheap tool for identification of coagulase-negative staphylococci.

Reference:
1. Bukowski M, Polakowska K, Ilczyszyn WM, Sitarska A, Nytko K, Kosecka M, Miedzobrodzki J, Dubin A, Wladyka B (2015) Species determination within Staphylococcus genus by extended PCR-restriction fragment length polymorphism of *saoC* gene. *FEMS Microbiol Lett* **362**: 1–11.

Key words: RFLP, staphylococci, typing

VII.P.8

Farnesol sensitizes *Staphylococcus aureus* cells to lethal photosensitization with TMPyP

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Introduction: Farnesol (FRN) is a natural sesquiterpenoid inducing pleiotropic effect in *Staphylococcus aureus* cells, including staphyloxanthin synthesis, biofilm formation and cell wall synthesis inhibition. For research purposes, it is commonly used as an adjuvant for antimicrobial agents. We have observed, that FRN accelerates the bactericidal effect of photodynamic inactivation with the use of photosensitizer 5.10.15.20-tetrakis(1-methyl-4-pyridinio)porphyrin tetra(*p*-toluenesulfonate), TMPyP. This study aims to characterize the mechanism of farnesol-dependent TMPyP sensitivity of *S. aureus*.

Methods: *S. aureus* strains SH1000 and RN6390 were used. Photodynamic effect of TMPyP was assessed in cells treated with sub-inhibitory concentration of trans-trans-farnesol, illuminated with red light emitting LED lamp (Δ_{max}=624 nm). Chemical interactions of FRN and TMPyP was evaluated spectrophotometrically. Biological effect of FRN in *S. aureus* strains was analyzed in the context of staphyloxanthin level by carotenoid extraction, TMPyP accumulation by spectroscopic measurements and cell membrane fluidity by fluorescence anisotropy analysis.

Results: Photodynamic inactivation experiments showed increased effectiveness of TMPyP in farnesol-treated cells as compared to no farnesol pre-treatment procedure (up to more than 99.99% reduction). TMPyP absorption spectra analysis revealed no physical interactions during titration with FRN, suggesting no toxic product formation, which was confirmed by the lack of bactericidal effect in the darkness. FRN effectively inhibited carotenoid accumulation in SH1000 to the level of non-pigmented RN6390. Lower staphyloxanthin amount was confirmed to significantly reduce cell membrane fluidity. However, FRN treatment enhanced photodynamic effect in both pigmented and non-pigmented cells, suggesting involvement of additional mechanism. TMPyP accumulation was shown to be unaffected by FRN treatment.

Conclusion: Our observations suggest, that farnesol action in accelerating TMPyP photodynamic effect depends neither on TMPyP uptake nor solely on carotenoid synthesis inhibition. This implies engagement of other biochemical factors and/or impairment of particular defense mechanisms, which could be useful in further analysis of specific TMPyP action in *S. aureus*.

Key words: farnesol, photodynamic inactivation, *Staphylococcus aureus*; TMPyP

VII.P.9

New method of *Mycobacterium tuberculosis* complex strains differentiation using polymorphic GC-rich sequence genes

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Tuberculosis (TB) is one of the leading infectious disease. Molecular biology methods used for tracking the evolution of an outbreak strain from person to person can be helpful in early detection and mapping the transmission of strains and allow for determination of whether the recurring TB is due to relapse or recurrence. In this work new genotyping method of *Mycobacterium tuberculosis* (MTB) complex strains is presented. The method is addressed to the analysis of polymorphic GC-rich sequence (PGRS) genes encoding some virulence and antigenic properties of strains. Four genes Rv3345c, Rv3507, Rv0747 and Rv3511 showing variation in length depending on MTB strain were selected for designing of primer sequences flanking variable regions. HGDI index for 27 *M. tuberculosis* genomes analyzed including 21 *M. tuberculosis*, 1 *M. africanum* and 5 *M. bovis* strains was equal to 0.923. Specific sizes of some PCR bands identified allowed to find out species-specific differences between *M. tuberculosis*, *M. africanum* and *M. bovis* strains. Characteristic of proteins encoded by selected genes based on motifs revealed the presence of NodO calcium binding signature in Rv3507, Rv0747 and Rv3511 amino-acid sequences. This domain indicates putative haemolytic activity of analyzed proteins. Furthermore, tumor necrosis factor family signature identified in Rv3507 and Rv0747 proteins reveals their cytotoxin activity. In addition, translocated intimin receptor (Tir) signature identified in all four analyzed proteins indicates participation in secretion of virulence factors from the pathogen directly into the host cells. Moreover, surface eggshell of *Schistosome mansoni* and dense granule Gra6 protein signatures identified in four analyzed proteins indicate antigenic properties of analyzed proteins and their contribution in the immune response. In conclusion, identification of 16 genotypes among 27 analyzed genomes showed usefulness of our genotyping method in differentiation of MTB genomes based on virulence and antigenic properties of strains using only four PGRS genes.

Keywords: *Mycobacterium tuberculosis* complex, PGRS, genotyping

VII.P.10

Determination of bacterial chemical markers in urine using gas chromatography coupled with tandem mass spectrometry

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Urinary tract infections (UTIs) are very common in the whole human population. UTIs represent 10–20% of all community-acquired infections, and 40–50% of nosocomial infections. The microbial etiology of urinary tract infections has been regarded as well characterized. The major cause of UTIs are Gram-negative bacteria from the gastrointestinal tract. Infections caused by Gram-positive bacteria do not exceed 10% of all urinary tract infections. The majority of UTIs are caused by *Escherichia, Klebsiella, Enterobacter* and *Proteus* species.

Microorganisms can be detected directly in clinical specimens by determination of their specific compounds, called bacterial chemical markers. Lipopolysaccharides (LPS) are major components of the outer membrane of Gram-negative bacteria. LPS molecule consists of three regions differing in biosynthesis and structure, namely lipid A, core region and repeating O-antigen subunits. Kdo (3-deoxy-D-*manno*-2-octulosonic acid) is one of the inherent constituents of core oligosaccharide. Nearly all LPS molecules contain at least one Kdo residue, what indicates that this sugar can be used as a specific chemical marker for detection of bacteria in urine specimens from infected individuals. Detection of Kdo and 3-hydroxy fatty acids has already been applied in various biological studies. Gas chromatography-tandem mass spectrometry (GC-MS/MS) is a very useful analytical method for the determination of specific bacterial markers directly in clinical or environmental samples, without a need for cultivation of bacteria.

In the present work we describe our studies on mass-spectrometric method used for chemical marker analysis in urine samples, where we detect peracetylated methyl ester of Kdo methyl glycoside. GC-MS/MS, tuned for a specific ion of m/z 375, has been used to detect that derivative.

Key words: bacterial chemical marker, Kdo, GC-MS/MS

VII.P.11

Analysis of variability of uropathogenic *Escherichia coli* strains based on their infrared spectra

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Infrared spectroscopy (IR) is a method useful in identification and differentiations of microorganisms. In our previous studies we reported that susceptibility of the uropathogenic *E. coli* strains (UPEC) to cephalothin can be determined using the Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR/FT-IR) method and artificial neural network. One of advantages of ATR/FT-IR is a holistic view of all molecules of bacterial cells. The aim of presented study was to compare variability of 128 UPEC strains collection by ATR/FT-IR technique. Bacteria were grown in standard condition (37°C for 24 h on Luria-Bertani Agar). After the time a single colony was collected from the Perti dish for IR spectrum scanning. The measurements were performed at least in three independent replications using ATR/FT-IR technique. Differentiation D-index was used for the evaluation of UPEC strains spectral variability. The D-index was calculated independently for each of the five analyzed windows (W1-W5) in IR spectrum. The analyzed UPEC strains turned out to be most various in the range between 900–750 cm^{-1} (window 5) – the average D index was estimated as 412. A high value of the D index indicates that W5 is the strain specific fingerprint region. The strains spectral analysis showed that the smallest variation was notice within the range W2 (1700–1500 cm^{-1}), where the average D index was 2 only. Windows W2 represented vibrations from peptides and proteins present in UPEC cells. In conclusion – the results suggest that the UPEC strains have similar protein profiles. This fact might be explained by identical for UPEC strains environmental living conditions in human urinary tract.

Key words: *E. coli*, infrared spectroscopy

VII.P.12

Links between hypertrophy, reproductive potential and longevity in yeast *Saccharomyces cerevisiae*

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Despite many controversies, *Saccharomyces cerevisiae* yeast became model organisms for studying the mechanisms of aging. The main problem with the use of these unicellular fungi is an incorrect definition of "longevity" unit. In case of all organisms, lifespan is expressed as a unit of time, not as the number of produced daughter cells. Our recent studies with the use of "longevity mutants" (with increased reproductive potential) showed no significant differences in their time of life. In spite of the significant differences in the reproductive potential of daughter cells, the life time remains the same as in wild type strain. These conclusions completely change the classification of long-lived mutants. However, our study additionally showed that there is a inverse correlation between reproductive potential and postreproductive lifespan (the time when the cell does not reproduce, but was still alive).
The aim of this study is to analyze the relations between the reproductive potential, longevity and changes in cell volume during subsequent cell cycles. We used *Saccharomyces cerevisiae* deletion mutants: *fob1Δ, sch9Δ* and *sfp1Δ* in BMA64-1A genetic background. BMA64-1A strain (derived from strain W303-1A) was selected for analysis because it has a numerous of specific features, mainly its size. Our results clearly support the hypertrophy hypothesis. Excessive cell volume results in the loss of reproductive capacity. The lifetime of all the tested mutants, except *sch9Δ* was at the level of wild-type strain. The lifetime of all analyzed mutants, except the *sch9Δ*. was the same as in the case wild-type strain. Knockout *SCH9* gene in BMA64-1A genetic background leads to extension of doubling time, reproductive as well as total lifespan. Interestingly, despite the increase in *fob1Δ* mutant's reproductive potential by 121% compared to the *sfp1Δ* mutant, does not result in the prolongation of its time of life (total lifespan).

Key words: yeast, longevity, genetic background

VII.P.13

Genetic methods to identify the microbiome

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Microorganisms are commonly found in our environment. They are present on our skin, in the nose, ears, digestive tract and reproductive organs and in the environment around us. All microorganisms that inhabit the data environment is called microbiome. It is estimated that the material of microorganisms in the human intestine weighs about 1–2 kg. The universality of their occurrence shows a huge role they play in our lives. The environment is a source of both beneficial and harmful microorganisms. Most of them are essential to human health and well-being. The cause of many diseases is the imbalance of the composition and diversity of microorganisms present in our body. This indicates a highly developed interactions between species living in the human body and those found in the environment.

Plant products are an important component of the human diet. Plants, such as the human body are habitat to a variety of microorganisms, both beneficial and pathogenic. Beneficial species can promote the growth and development of plants and protect them against pathogens. This affects the productivity of the crop and the functioning of the ecosystem. By the consumption of plant products microorganisms living on them get to our gastrointestinal tract. Plants as components of the human diet can influence the improvement of human health or cause diseases. To date, the role of microorganisms in human life was limited to the knowledge of pathogenic species. Now modern genetic methods allow to examine all of the microorganisms that live on plants and affect human health.

Key words: microbiome, microorganisms, human health

VII.P.14

Computer prediction of the potential allergenicity of selected leguminous plants

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Food allergy became the problem of human civilization. The knowledge concerning this problem is considered taking into attention medical, nutritional and clinical aspects. Information technologies may be found useful in broadening the knowledge about the food allergies and allergens. Particular attention is paid to the computer databases of allergens, including those originating from food. The computer (bioinformatic) tools possess calculation functions allowing for the analysis of a food component (e.g. protein) as the source of potential allergen(s).

The aim of the study was the computer analysis of potential allergenicity of the leguminous plant proteins. We analyzed sequences derived from garden pea (*Pisum sativum*), broad beans (*Vicia faba*), soybean (*Glycine max*), french beans (*Phaseolus vulgaris*), lentil (*Lens culinaris ssp. orientalis*) and chickpea lectin (*Cicer arietinum*). The above-mentioned sequences were derived from the BIOPEP (i. e. the database of protein and bioactive peptide sequences and allergenic proteins and their epitopes) and UniProt databases. Programs AlgPred and SDAP were applied for the allergenicity prediction.

The analysis of protein sequences carried out by e. g. AlgPred program indicated that legumin and convicilin derived from garden pea were found to be as potentially allergenic. The protein sequences from french beans possessed ambiguous allergenic potential. According to SDAP (Structural Database of Allergenic Proteins) all protein sequences analyzed contained fragments (epitopes) that may decide about their allergenicity.

Our results allow to get the preliminary data about the potential of food allergens. Moreover, such data can enrich the existing knowledge about food allergy and obtained results are less time and cost consuming comparing to the research carried out in-house experiments. The advantage of databases is their availability to allergenic people who can acquire the information about the food products that should not be recommended in their diet.

Key words: allergy, proteins, epitopes, bioinformatics

VII.P.15

A new assay for simultaneous identification and differentiation of *Klebsiella oxytoca* strains

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Bacterial strain typing, or identifying bacteria at the strain level, is particularly important for diagnosis, treatment, and epidemiological surveillance of bacterial infections. This is especially the case for bacteria exhibiting high levels of antibiotic resistance or virulence, and those involved in nosocomial or pandemic infections. Strain typing also has applications in studying bacterial population dynamics.

In this study, the combination of Ligation Mediated PCR fingerprint method (LM PCR) and species-specific PCR amplification of polygalacturonase gene (*pehX*) was used for simultaneous identification of the *Klebsiella oxytoca* species and its intraspecific differentiation (*pehX*-LM PCR/*Xba*I).

The LM PCR method, that is widely used for genotyping bacteria strains, is based on the size-differences of the DNA restriction fragments after the whole genome digestion. We found that *Xba*I restriction enzyme digests Enterobacteriaceae genomic DNA at no more than 40 sites and generates up to 10 fragments of length from 300 to 3000-bp. These fragments are ligated to the adapter, amplified by PCR using adapter-specific primer, and then separated by agarose gel electrophoresis forming strain-specific band pattern.

To differentiate LM PCR-patterns of *K. oxytoca* from LM PCR-patterns of other Enterobacteriaceace we added in PCR amplification during LM PCR analysis an additional pair of primers which are specific to *pehX* gene in *K. oxytoca* strains. After pehX-LM PCR/*Xba*I analysis only within *K. oxytoca* patterns there is an extra 197-bp band, which indicate the species. For other Enteobacteriaceae there is lack of PCR products of less than 300 bp.

To verify the usefulness of *pehX*-LM PCR/*Xba*I method over than 200 *K. oxytoca* strains and 50 strains of other species of Enterobacteriaceae family were tested. The typing results were confirmed by REA-PFGE and PCR MP. The new *K. oxytoca* identification/differentation assay could be useful for both clinical and ecological monitoring of *K. oxytoca* strains.

Key words: LM PCR, *Klebsiella oxytoca, pehX*, genotyping

VII.P.16

Novel application of terminal restriction fragments length polymorphism method (t-RFLP) for identification of six clinically important *Candida* sp.

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Terminal restriction fragments length polymorphism method (t-RFLP) is a modified classical RFLP method. The main difference is based on carrying out PCR using a fluorescently labeled primer or primers. The result of such modification is that after digestion of amplicons only terminal fragments of PCR product (containing fluorescent dye) are visualized. T-RFLP method has found application in environmental microbiology and epidemiology. It is mostly used for genotyping and study of microbial diversity and bacterial community structure in different environmental samples. In this study a method to differentiate six *Candida* species using PCR t-RFLP technique was elaborated. This work shows the new application of t-RFLP in molecular diagnostic of fungi. Homocitrate synthase gene was chosen as a molecular target. This gene encodes enzyme involved in the first reaction in lysine biosynthesis pathway. The protein is characteristic for fungi and several *Archaea*. Specific forward primer was labeled with fluorescein. During this study new sequence for homocitrate synthase gene fragment was obtained for *C. krusei* and submitted to NCBI GenBank. Three endonucleases were chosen for digestion of amplicons: *Dra*I, *Rsa*I and *Bgl*II. Elerophoretic pattern of bands obtained for three enzymes gives unequivocal result for determinig the *Candida* species. Designed method is flexible for differentiation of each combination of investigated *Candida* species and only one or two enzymes may be used for diagnostic purpose.

Key words: *Candida* sp., PCR t-RFLP, homocitrate synthase

VII.P.17

Growth-dependent cell wall proteome and secretome of *Candida glabrata*

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Candida glabrata is a second major cause of human candidiasis after *C. albicans*. However, in contrast to the latter most widespread fungal pathogen, *C. glabrata* is a haploid yeast that does not demonstrate the morphological dimorphism. Whereas the yeast-to-hyphae transition is important for *C. albicans* virulence, significant changes in *C. glabrata* cell wall proteome and secretome, related only to the growth phase and medium, but not morphological changes, have been reported and considered essential for the pathogenicity of this fungus.

The aim of this study was to analyze the changes in *C. glabrata* cell wall proteome and secretome under different environmental conditions, including temperature and the composition of the medium in which yeast cells were cultivated to the exponential or stationary growth phase.

A relatively novel gel-free approach – the "cell surface shaving", relying on a short treatment of fungal cells with trypsin – was used to identify surface-exposed proteins. Alternatively, cell-wall associated proteins were released with β-1,6-glucanase. Proteins, secreted to the medium during yeast growth were also collected. Thereafter, all protein fractions were analyzed with liquid chromatography-coupled tandem mass spectrometry.

The composition of the *C. glabrata* cell wall proteome and secretome differed, depending on the culturing medium (YPD vs. RPMI-1640). We observed that some proteins that were initially localized mainly to the cell wall, were shed to the medium after several hours of yeast growth. For example, typical adhesins from Epa (epithelial adhesin) family were only found at the cell surface in the RPMI-1640 medium, whereas in the YPD medium they occurred exclusively in the secretome, together with YPS3 protease. These findings demonstrate the dynamic nature of *C. glabrata* cell wall proteome and a complex regulation of yeast secretome, the processes that help *C. glabrata* to adapt to various conditions encountered in the infected host.

Key words: *Candida glabrata*, secretome, cell wall proteome

VII.P.18

MIRU-VNTR typing in epidemiological study of *Mycobacterium kansasii*

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Nontuberculosis mycobacteria (NTM) of *Mycobacterium* genus are opportunistic pathogenes widely spread in the environment. NTM group comprises of more than 100 species of bacteria including 25 recognised as the etiological factors for the condition called mycobacteriosis. *M. kansasii* bacillus, described by Buhler and Polloc in 1953, belong to most frequently isolated NTM bacillus. Diagnostics of NTM bacillus is difficult and often ambiguous, *M. kansasii* infections are often mistook for tuberculosis. Therefore correct identification of these bacteria in the diagnostic process is extremaly important. The epidemiological analysis of strains belonging to *M. kansasii* require a new methods which could allow to differentiate environmental strains and those responsible for infection in a given patient.

MIRU-VNTR (Mycobacterial Interspresed Repetitive Unit – Variable Number of Tandem Repeats) typing is successfully applied in molecular epidemiology of bacteria including *M. tuberculosis* and seems useful in *M. kansasii* genotyping. MIRU-VNTR consists of amplification of the specific locus, with appropriate starters complementary to flanking sequences in the given MIRU region, followed by separation of fragments on agarose gel and then by analysis of their size. The size of product depends on the number of repeats of the basic sequence. The number of MIRU sequence repeats in each locus gives the final numeric code for strain.

The present research identified in *M. kansasii* genome the most hyper-variable loci, starters were created, verified and applied in epidemiological tests of collected *M. kansasii* strains. Genotyping of *M. kansasii* isolates based on the polimorphism of selected VNTR loci can be useful in epidemiological study of *M. kansasii*.

Key words: *Mycobacterium kansasii*, mycobacteriosis, MIRU-VNTR