SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

Epidemiologic characterization of
antibiotic resistant Gram-positive cocci

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Doctoral School of Pharmaceutical Sciences

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Members of the Examination Committee: Gabriella Pásti, Ph.D.
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The Examination takes place at Department of Pharmacology, Medical and Health Science Center, University of Debrecen on 2011.09.30. 11:00.

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The Ph.D. Defense takes place at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen on 2011.09.30. 13:00.

1. INTRODUCTION

In the first half of the 20th century introduction of synthetic and semi-synthetic antibiotics caused a paradigm change in medical sciences. It was thought, that we can escape from the deadly threats of individual infections and epidemics caused by bacteria. However, the variability of bacterial genomes and the possibility of horizontal gene transfer challenged the achievements of antibiotic therapy. While from 1950 to the end of the 1980's big pharmaceutical companies introduced new antibiotics on the market regularly, in the last 20 years the progress slowed down significantly. Due to current time consuming and complicated drug licensing procedures it became less profitable to promote the discovery of new antibiotic agents. It appears that in the coming years we have to rely on the currently available panel of antibiotics, which has to be utilized more efficiently and more prudently. It is known, that one of the main reasons leading to antibiotic resistance stems from the long lasting treatment of patients with broad spectrum antibiotics selected on an empirical basis. Quite often viral infections of the upper respiratory tract are unnecessarily treated with antibiotics. In the past 50 years the excessive use of antibiotics exerted an evolutionary pressure, which resulted in the appearance of resistant bacteria followed by the propagation of the resistance genes. The genes responsible for antibiotic resistance have been in the focus of intensive research. According to recent results, many microbes living in our immediate environment poses high level resistance to antibiotics and at least some of them can be the source of clinically significant resistant strains. Modern combinatorial therapies based on the application of synergetic drugs are not suitable to solve the problem completely because multiresistant bacteria appear frequently not only as nosocomial pathogens in hospitals, but also they are causing a significant proportion of infections in the community. Fortunately, the latest antibiotics are still effective against Gram-positive bacteria, and there are encouraging results that may lead to new types of treatment (including new antibodies, as well as signal molecule and quorum sensing inhibitor based drugs). Under these circumstances the significance of bacterial diagnosis has elevated. The quick and reliable detection of resistant bacteria is an obligatory requirement for appropriate antibiotic treatment and for the introduction of proper infection control measures. Among the modern diagnostic approaches, molecular biology based methods are especially important, since they can identify the genotype unambiguously even within 24 hours. My research projects were aimed to contribute to the introduction of

modern molecular diagnostic tools in the Bacteriological and Diagnostic Laboratory of the Department of Medical Microbiology at the University of Debrecen.

1.1. Vancomycin resistance in enterococci

Secondary resistance against glycopeptides has great significance in species belonging to the *Enterococcus* and *Staphylococcus* genus. The first vancomycin-resistant enterococci (VRE) were reported in 1988 in Europe. Subsequently similar strains were found in the East coast of the United States. Concerning the epidemiology of VRE, we can conclude that the appearance and spreading of VRE strains has a different basis in Europe and in the United States. While in the USA the main cause of VRE is the widespread usage of antibiotics, in Europe, the application of avoparcin as a growth-promoting factor in animal husbandry lead to the appearance of VRE first in the gastrointestinal tract of animals, and later in humans. Fortunately, avoparcin was not used in Hungary as frequently as in other countries, therefore VRE has not caused significant outbreaks in our country until now. The use of avoparcin was banned in 1998. The first PCR based detection of VRE in Hungary was published in 2000 by Ghidán et al. Knausz and colleagues found another VRE in 2003 with the same method. In 2005, Böröcz et al. reported on the first outbreak caused by vancomycin resistant *E. faecium* in our country.

In theory, vancomycin resistance can arise in two different ways: either by the modification or by the elimination of the target molecules. According to the first mechanism the C-terminal D-alanine of the peptidoglycan precursor is replaced by D-lactate (in a reaction catalyzed by the vanA, vanB, vanD, vanF and vanM proteins) or by D-serine (caused by *vanC*, *vanE*, *vanG* and *vanL* gene products). According to the second mechanism the interaction of vancomycin with its target is prevented by the removal of the susceptible precursors that terminate in D-alanine. Two enzymes are involved in this process: the VanX D,D-dipeptidase hydrolyzes the D-Ala-D-Ala dipeptide (synthesized by the host D-Ala-D-Ala ligase), and the VanY D,D-carboxypeptidase, which removes the C-terminal D-Ala residue of late peptidoglycan precursors. In D-alanine-D-serine producing strains these functions are catalyzed by the VanXY. Classification of the resistance is based on the sequences of the structural genes coding for ligase enzymes. Until now, 9 *van* genes have been reported. The most frequent vanA type resistance (which is typically found in *E. faecalis* and *E. faecium* species) is characterized by high-levels of vancomycin and teicoplanin resistance. VanB type strains are moderately or highly resistant to vancomycin but remain susceptible to teicoplanin. The acquired VanD type resistance is due to the constitutive production of peptidoglycan

precursors ending in D-alanine-D-lactate, thus these strains are moderately resistant to both glycopeptides. The *vanF* gene cluster has not been found in enterococci yet. The most recently discovered *vanM* gene cluster was isolated from an *E. faecium* strain in 2010. *vanM* gene carrying strains are highly resistant to both vancomycin and teicoplanin. VanC type resistance against glycopeptides is caused by the D-ala-D-serine ligase. Initially vanC1 gene was identified in *E. gallinarum*, followed by the discovery of the vanC2/3 gene in *E. casseliflavus/flavescens*. These genes cause low level of constitutive or inducible resistance exclusively to vancomycin. The *vanC* operon is located on the bacterial chromosome, and is not transferable. VanE, VanG and VanL type *E. faecalis* strains exhibit low level of resistance to vancomycin only. While *vanA* and *vanB* operons can be found either on plasmids or in the chromosomes, the rest of the *van* operons are confined to the bacterial chromosome. Vancomycin dependence has been reported in some of the vanA and vanB type enterococci. These strains require the presence of glycopeptides for growth, because different mutations inactivated their authentic D-alanine-D-alanine ligases.

1.2. Vancomycin resistance in staphylococci

Although vancomycin was introduced in 1958 in the clinical practice, researchers were able to induce vancomycin resistance in staphylococci even before this date. It generated great concern when in 1997 the first *S. aureus* with reduced sensitivity to vancomycin was described in Japan. In the first publication in 1997 a methicillin-resistant *S. aureus* was described in which a part of the bacterial population had higher minimal inhibitory concentration values, thus the strain exhibited a heterogeneous phenotype (hetero-vancomycin-intermediate sensitive *S. aureus*: hVISA). In the second publication of the same year the researchers reported on the isolation of a VISA (vancomycin-intermediate sensitive *S. aureus*) strain. It turned out, that the PFGE patterns of the two strains were identical, thus it was proven that the homogeneously resistant strain stemmed from the heteroresistant precursor. These two publications were followed by similar observations from all over the world. The first hVISA strain in Hungary was described in 2008 by Tóth et al. The highly vancomycin-resistant *S. aureus* (VRSA) strains, that gained the vanA resistance from enterococci, appeared first in 2002 in the United States. Until now, there have been nine American, one Indian, and one Iranian VRSA cases in the literature.

The resistance mechanism of hVISA/VISA and VRSA strains are distinct. In the latter, the target molecule of the glycopeptides is modified just as in enterococci, while in the former the low-level resistance has multifactorial origin. It can be caused by structural changes in the

bacterial cell wall: cell wall thickening, reduced quantity or altered structure of cross-links, appearance of false antibiotic binding sites, reduced production of protein A, or the increased production of cell wall precursors. Increased concentration of PBP2 was found in some studies. In addition, the resistant strains may have elevated biofilm forming capacity and reduced autolysis. Since homogeneous moderate resistance develops in more stages than the heterogeneous, it appears less frequently. While VISA strains appear rarely, the hVISA phenotype is more common, it represents 5-6% of MRSA strains tested.

1.3. Methicillin resistance in staphylococci

Methicillin was first introduced in 1959 against infections caused by penicillin resistant *S. aureus* strains. The first MRSA was isolated two years later, in 1961 in the United Kingdom, and its incidence has been growing steadily. In the European Union, MRSA causes more than 150.000 infections annually. In Hungary, the first epidemic caused by MRSA was in 1993-1994. MRSA strains produce a modified penicillin binding protein (termed PBP2a or PBP2'), which has a reduced affinity against most of the semi-synthetic penicillins or cephem antibiotics. PBP2a is coded by the *mecA* gene, which can be found on a mobile genetic element called Staphylococcal Cassette Chromosome mec (SCCmec). Methicillin-resistant staphylococci lineages evolved by the integration of SCCmec elements into the chromosome of the sensitive strains. The first SCCmec element was characterized by Ito et al. in 1999. Until now, at least nine SCCmec types have been revealed, with many subtypes. Their common property is that they carry a mec gene complex (*mec*), a ccr gene complex (cassette chromosome recombinase: *ccr*), and have characteristic directly-repeated nucleotide sequences and inverted complementary sequences at both ends. They integrate into ISS (integration site sequence for *ccr*), which is located at the 3' of *orfX*. In addition some none essential „J" regions (junkyard or joining regions) are also present, which constitute the base of classification of different subtypes.

The majority of cells in heteroresistant strains are sensitive to low-concentrations of β-lactams, and only a small proportion (one in a million) is able to grow at 50µg/ml or higher methicillin concentrations. It represent a diagnostic challenge, that heterogeneous strains may look homogenous under certain cultivation conditions.

2. AIMS

The Bacteriological Diagnostic Laboratory of the Department of Medical Microbiology analyzes more than 30.000 samples from the hospitals of the Medical and Health Science Center, University of Debrecen annually. The goal of my work was the epidemiological and molecular characterization of the most important Gram-positive pathogenic bacteria: vancomycin resistant enterococci (VRE) and methicillin-resistant *S. aureus* (MRSA).

Investigation of vancomycin resistant enterococci

Aims:

- Screening, survey and species identification of VRE strains from the clinical samples collected between 2004-2009,
- antibiotic susceptibility testing of the strains, and the molecular identification of the genes causing antibiotic resistance,
- investigation of the genetic relationships between the strains, in order to reveal their epidemiologic importance,
- analysis of VRE infection cases,
- testing the validity of the VITEK2 system against the conventional E-test method for the determination of glycopeptide MIC values.

Investigation of methicillin-resistant *S. aureus* strains

Aims:

- Screening and survey of MRSA strains in the samples obtained from the teaching hospitals,
- detailed characterization of MRSA strains isolated in 2005: determination of antibiotic resistance profiles,
- genotyping the strains, in order to evaluate their epidemiology, and to prevent spreading of nosocomial infections,
- analysis of special antibiotic resistant *S. aureus* infections.

3. MATERIALS AND METHODS

3.1. Materials

Enterococci have been screened since January 2004 at the Bacteriological and Diagnostic Laboratory of the Department of Medical Microbiology from the samples obtained from the hospitals of the Medical and Health Science Center, University of Debrecen. Until December 31. 2009, 7271 enterococci were identified. MRSA strains collected between January 1. 2005, and December 31. 2005, were analyzed in detail. Three hVISA strains were obtained in 2007, 2008 and 2009.

Media used for cultivation of bacteria were purchased from Oxoid, Bio-Rad, Becton Dickinson and Merck. Antibiotic disks of Oxoid and E-tests of AB Biodisk were used. The rest of the chemicals and kits were obtained from Sigma, Promega, Fermentas and Invitrogen. The SP6 and T7 oligonucleotide primers for DNA sequencing were provided by the sequencing laboratory of the Szeged Biological Center HAS. All of the other oligonucleotides were from Integrated DNA Technologies Inc.

3.2. Methods
3.2.1. Screening of clinical samples and cultivation of bacteria

Clinical samples received by our laboratory were usually enriched in Brain-Heart Infusion (BHI) broth, then inoculated on blood, chocolate and eosin methylene blue agar. After 16-18 hours of incubation, Gram-positive and catalase negative enterococci growing on blood agar were inoculated on bile-esculin-azide agar. Carbohydrate fermentation was tested on arabinose and sorbitol containing media. Vancomycin resistance was tested on 6 μg/ml vancomycin containing BHI screening plates. Gram-positive, catalse positive staphylococci growing on blood agar after 16-18 hours of incubation were tested for mannitol fermentation on mannitol salt agar. Bound coagulase, or "clumping factor" was detected by the Staphaurex kit. Species identification of *S. aureus* suspect strains was also confirmed by the tube-coagulase test. Methicillin-resistance was detected by oxacillin disk diffusion, followed by oxacillin E-test to determine more accurate MICs. MRSA suspect strains were inoculated on MRSASelect screening plates on which the methicillin-resistant *S. aureus* strains grow in pink colonies. Vancomycin-resistance was detected by the conventional E-test method. Strains showing reduced vancomycin-resistance were also tested by macro E-test. hVISA/VISA suspect strains were sent to the National Center for Epidemiology for population analysis.

3.2.2. Methods for testing antibiotic resistance

Disk diffusion is the most commonly used and internationally accepted method in routine antibiotic susceptibility testing. In the Kirby-Bauer disk diffusion test, we analyzed antibiotic resistance by using filter paper disks impregnated with the antibiotic concentration corresponding to the relevant blood level, according to the manufacturer's instructions.

In some cases however, the E-test is the most appropriate method for determining MIC values, because in the disk diffusion method the diffusion of high molecular mass molecules is restricted, while in the microdilution tests low concentration of the inoculum may be suboptimal for the detection of low-level resistance. Even the quick automated systems cannot be always trusted, for example delayed expression or inducible resistance may cause problems. On the surface of the E-test strips there are well defined concentration gradients of antibiotics. After the appropriate incubation period an elliptical growth inhibition zone will develop. The MIC values correspond to the scale reading where the inhibition zone intercepts the strip.

VITEK2 is an automated system for species identification and antimicrobial susceptibility testing of bacteria. We used the software version 3.01. For the identification of enterococci we used the Gram-positive identification card, and for the susceptibility testing we used the AST (antibiotic susceptibility testing) card. Results were obtained within 8 hours.

3.2.3. Molecular biology analysis
Polimerase chain reaction and restriction analysis

Templates for the PCR reactions were bacterial lysates prepared by boiling bacterial cultures. Primers used in the Multiplex PCR reactions were specific for *E. faecalis* and *E. faecium ddl* and the *van* genes (*vanA, vanB, vanC1/2, vanD, vanE* and *vanG),* and were designed according to Depardieau et al. (J Clin Microbiol 2004, 42: 5857-60). In addition, *E. faecalis, E. casseliflavus* and *E. gallinarum* species were identified with primers specific for their superoxide dismutase (*sodA*) gene according to Jackson et al. (J Clin Microbol 2004, 42:3558-65). High level gentamicin resistance was also confirmed by PCR. The bifuncitonal aminoglycoside primer pair was modified in a few base pairs (Yean et al., BMC Microbiol 2007, 7:112). Primers for the MLST experiments were used according to the www.mlst.net website.

The *vanC* PCR products were directly digested with *Hind*III and *Sal*I enzimes. The results of the PCR reactions and the length of the digested fragments were determined by agarose gel electrophoresis.

Cloning and sequencing of PCR products

The *vanA* PCR product was extracted from the agarose gel with a Qiagen gel extraction kit. The purified DNA was ligated into the pGEM-T Easy vector with the aid of T4 DNA ligase, and competent *E. coli DH5α* cells were transformed. Plasmid DNA was isolated with a Qiaprep kit, and verified by *Eco*RI restriction digestion. To test the clones the DNA was sequenced on both strands at the Biological Research Center of the Hungarian Academy of Sciences in Szeged. For direct sequencing, PCR products were purified with a Microcon Ultracel YM 100 kit, and were sequenced with primers used for amplification at the BIOMI Ltd in Gödöllő, or at the Department of Biochemistry and Molecular Biology, Medical and Health Science Center, University of Debrecen. Sequencing of the hVISA3 strain was carried out at the Institute of Infectology and Pediatric Immunology.

3.2.4. Epidemiological studies

Multilocus sequence typing (MLST)

For MLST of *E. faecalis* strains we sequenced the internal fragments of 7 house-keeping genes (*gdh, gyd, pstS, gki, aroE, xpt, yqiL*). For *S. aureus* the 7 investigated genes were the following: *arc, aro, glp, gmk, pta, tpi, yqil*. After „trimming" the obtained sequences, we compared them with the corresponding alleles collected in the www.mlst.net website, and determined the allelic profiles and sequence types of the strains as described in the website.

Pulsed-field gel electrophoresis (PFGE)

PFGE of *S. aureus* strains was performed according to the protocol of Stephen et al. (J Clin Microbiol 2003, 41:1574-85) at the Institute of Medical Microbiology, Semmelweis University, with slight modifications. Dendrograms were analyzed with the BioNumerics program version 2.5 (Applied Maths BVBA, Sint-Martens-Latem, Belgium), and created by the UPGMA/Dice coefficients, with a band position tolerance of 1.0%.

PFGE of enterococci was performed as previously described for *S. aureus*, with slight modifications. When creating the dendrograms, we used 2% optimization and a band position tolerance of 2%.

Bacteriophage typing

Typing of MRSA strains was carried out at the ANTSZ of Csongrád county, according to Blair and Williams (Bull World Health Org 1961, 24:771–784). With the help of this

method, bacteria, which cannot be differentiated further by biochemical or serological methods were divided into different types. Phages used for phage typing lyse bacteria only if the bacteria are susceptible to them. Phage types were established according to the lysis spectrum of the typing phages.

Modified population analysis profile (PAP)

Staphylococcal strains with reduced vancomycin susceptibility were sent to the National Center for Epidemiology for modified population analysis testing (Tóth et al., J Chemother 2008, 20: 655-656). After incubation of bacteria in the presence of various concentrations of vancomycin, colonies were counted and a semi logarithmic graph of counted colony forming units versus vancomycin concentration was constructed. The area under curve (PAP-AUC) was calculated for each strain. To distinguish VISA and hVISA, a ratio of the AUC of the test strain divided by the corresponding AUC for the positive control strain was calculated. The criteria used for detection of hVISA and VISA strains were AUC ratios of ≥ 0.9 and ≥ 1.3, respectively.

Staphylococcal chromosome mec (SCCmec) typing

SCCmec typing of 3 hVISA strains isolated in Debrecen was performed at the National Center for Epidemiology using multiplex PCR (Oliveira et al., Antimicrob Agents Chemother 2002, 46: 2155-2161). The reaction contained 8 primer pairs designed for the amplification of 8 loci of the *mec* gene complex. The number and size of the PCR products defined a characteristic pattern (SCCmec type).

4. RESULTS AND DISCUSSION

4.1. Prevalence of VRE in the clinics of the University of Debrecen

The Bacteriological Diagnostic Laboratory has been screening enterococci for vancomycin resistance since 2004. During the examined period, from Januar 1. 2004. utill December 31. 2009 we identified overall 7271 enterococci from the clinical samples collected in our laboratory. Among the strains growing on the screening plates we identified one *E. faecalis* carrying the *vanA* resistance gene by multiplex PCR. In addition, we detected *vanC* positive enterococci from 15 clinical samples.

4.1.1. Characterization of *vanC* positive VRE strains

Among the 16 *vanC* gene carrying isolates 2 were vanC2 positive *E. casseliflavus* and the remaining 14 were *vanC1* positive *E. gallinarum*. Initially, we confirmed the *vanC* genes by DNA sequencing, because the *vanC* specific primers used could amplify both the *vanC1* and the *vanC2* genes. Later on, we differentiated the two genes with a new method based on restriction analysis. Instead of *Msp*I, which cleaves both *vanC1* and *vanC2* PCR fragments, we introduced two specific restriction enzymes *Sal*I and *Hind*III. *Sal*I specifically cleaves only the *vanC1*, while *Hind*III cuts the *vanC2* PCR product. With this method we found one clinical sample (No. 6130), which was affected by both enzymes, suggesting the presence of both genes. By spreading the original clinical sample and picking single colonies, we separated three subcultures. According to the *sodA* specific PCR, the three subcultures represented three different species. Of the three isolates only the *E. casseliflvaus* and *E. gallinarum* grew on the VRE screening plates, the *E. faecalis* strain was not resistant to vancomycin. The subsequent *vanC* specific PCR and restriction analysis revealed that *E. casseliflavus* carried the *vanC2*, and *E. gallinarum* contained the *vanC1* gene.

Antibiotic susceptibility of the VRE strains was determined by E-test using the following antibiotics: vancomycin, teicoplanin, linezolid, ampicillin, tigecyclin, daptomycin, gentamicin and amoxicillin/clavulanate. All 16 *vanC* positive strains were moderatley resistant to vancomycin and sensitive to teicoplanin. We found three *E. gallinarum* species, which were highly resistant to gentamicin. In the latter, we demonstrated the presence of the *aacA-aphD* bifunctional aminoglycoside resistance gene by PCR. Fortunately, the VRE were sensitive to all of the other antibiotics tested.

The *E. faecalis* strain isolated from sample 6130 was genotyped by multilocus sequence typing. By sequencing the acetyl-CoA acetyltransferase (*yiqL*) gene we found a single-nucleotide replacement of C to T at position 187 when compared with allele yiqL-8. The new allele and sequence type were termed yqiL-64 and ST-336, respectively.

To determine the genetic relatedness of the resistant strains, we performed pulsed-field gel electrophoresis. According to the dendrograms two *E. gallinarum* isolates had indistinguishable genotypes. Both strains were isolated in 2009, but the first came from the 1st Department of Surgery and the second from the 2nd Department of Surgery. The first patient received vancomycin besides several other antibiotics, but due to his old age and diseases he died 1 month after surgery. We cannot exclude the possibility that the vancomycin-resistant *E. gallinarum* contributed to the development of sepsis, and the Vancocin treatment was therefore unsuccessful. Direct contact between the two patients can be excluded. Six further strains could be grouped into one cluster, according to the >90% identity of their PFGE patterns. The strains came from different clinics, and were isolated in different time periods.

4.1.2. Characterization of one *vanA* positive case

A 50-year-old woman with known mitral valve prolapse was admitted to hospital for elective surgery. Cefuroxim plus tobramycin were administered as antibiotic prophylaxis prior to surgery, according to the local protocols. 8 days after surgery, *E. faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa* was isolated from her femoral wound, but the patient was afebrile and did not receive antibiotics. These agents only colonized the wound. The isolates were identified by conventional biochemical methods. The *E. faecalis* growing on 6 µg/ml vancomycin containing screening plates was tested in detail. For antibiotic susceptibility testing the VITEK2 automatic system was used; the strain showed resistance to streptomycin, tetracyclin, vancomycin and teicoplanin. Multiplex PCR revealed 2 lanes, corresponding to the *E. faecalis ddl* gene and the *vanA* glycopeptide resistance gene. Species level identification was confirmed by single PCR of the *E. faecalis ddl* and *sodA* genes. The presence of the resistance gene was also verified by a specific PCR. The PCR product was cloned into a plasmid vector and its sequence was determined with the T7 and SP6 plasmid specific primers at the Sequencing Laboratory of BRC HAS in Szeged. Except for one nucleotide, the sequence turned out to be identical to the corresponding segment of the *vanA* gene found in the database. For the verification of the mutation the *vanA* PCR product was also sequenced after direct purification, and was found to be 100% identical to the corresponding segment of the vanA gene in the pLW043, pVEF1, and pVEF2 plasmids.

pVEF plasmids are more frequently found in *E. faecium,* however the species was identified by several independent methods as *E. faecalis.*

4.1.3. Testing the reliability of the VITEK2 system

During our work, the performance of the VITEK2 System (bioMerieux) version 3.01 software was compared to that of the E-test (AB Biodisk) for glycopeptide susceptibility testing of the *vanA* positive strain and of the *vanC* positive strains. CLSI breakpoints were used to calculate very major, major, and minor interpretation errors between the VITEK 2 system and the E-test. Very major errors were defined when the results were obtained as susceptible by the VITEK2 system, but resistant by the E-test. Major errors were defined when the results were obtained as resistant by the VITEK 2 system, but susceptible by the E-test. A minor error was defined as discrepancy between the results of the E-test and the VITEK2 system that differed only by one interpretation category; for example: resistant instead of intermediate, or susceptible instead of intermediate. The *vanA* positive *E. faecalis* and the sensitive ATCC 29212 *E. faecalis* were used in the control experiments. The *vanA* strain was classified correctly by both systems, and no problems were found in the evaluation of teicoplanin MIC levels either. All *vanC* positive strains were susceptible to teicoplanin by both methods, however, the E-test proved to be more discriminative, because the VITEK2 levels showed a restricted distribution. As for vancomycin, minor errors were found in 10 cases. Vancomycin MICs in the resistance region were obtained in 5 of the 17 strains, and 6 of the 17 strains were determined as susceptible using the VITEK2 system. The latter 6 strains that were intermediate sensitive by the E-test appeared to be susceptible according to the VITEK2 system. In 4 cases the intermediate sensitive strains were determined by VITEK2 as resistant with interpretation of vanB phenotype. Taken together, the vancomycin susceptibilities were mistakenly determined in 10 (59%) of the 17 strains by the VITEK2 system.

4.2. Prevalence of MRSA in the clinics of the University of Debrecen

Until 2003, MRSA strains occurred sporadically at the university hospitals of Debrecen, and their prevalence varied between 2.3–3.2%. In 2004, the incidence of MRSA strains increased to nearly 4% and, despite the efforts of the nosocomial surveillance system, exceeded 7% in 2005.

The Bacteriological Diagnostic Laboratory isolated overall 339 MRSA strains from 102 patients and 17 different clinics between January-December in 2005. When preparing the

statistics we have taken into account only the data of the first isolate from each patient. The rate of MRSA strains was very high at the Departments of Surgery, Pulmonology and Paediatrics. Specimen distribution of the 102 MRSA strains was the following: bronchial exudate 23.5%, wound 17.6%, nasal discharge 13.7%, canule 9.8%, pharyngeal exudate 9.8%, and the number of blood cultures and abscesses were also as much as 7.8% (the remaining 10% was from different samples).

4.2.1. Antibiotic resistance patterns of MRSA strains

All isolates were resistant to β-lactams, and 97 of them exhibited an oxacillin MIC of 256 mg/L. The remaining 5 strain had an oxacillin MIC lower than 256mg/L, but still in the resistant region. All 102 strains were susceptible to vancomycin and teicoplanin. Resistance to erythromycin, clindamycin, ciprofloxacin and trimethoprim/sulfamethoxazole was found in 99, 99, 97 and 31% of the isolates, respectively; 39% and 49% of the isolates were resistant to amikacin and tetracycline, respectively. The strains were grouped into 20 different antibiotic resistance patterns.

4.2.2. Epidemiology of MRSA strains

The proportion of non-typable strains decreased over the years, while that of mixed strains increased, and phage-group V was gradually overtaken by group III. In 2005, more than half of the isolates (57.85%) was mixed, 34.31% of them was of group III and 7.85% was non-typable. Almost half of the strains belonged to phage type 617/622/623, while nearly 20% was typable by phage 629 only.

Out of the 84 MRSA isolates examined, 79 belonged to three main different PFGE types; A, B and C. The others were unrelated. The predominant PFGE type A included 46 strains. These strains were all sensitive to glycopeptides, but all of them were resistant to erythromycin, clindamycin and all but two were resistant to ciprofloxacin. Most frequently, they were sensitive to amikacin, trimethoprim/sulphamethoxazole and tetracycline. The vast majority of these isolates characteristically belonged to the mixed phage group. The dominant phage types were 617, 622 and 623 of the mixed group. The origin of the strains according to the clinics was very variable. The PFGE type B isolates (n=13) also had unique antibiotic resistance profiles; while most of them were sensitive to trimethoprim/sulphamethoxazole and tetracycline, all but three were also resistant to amikacin. 72.7% belonged to phage group III. The dominant phage type was 629. The majority of the PFGE type C isolates belonged to phage group III. They were variable in their resistance pattern, but, generally, these isolates

showed the highest proportion of multiresistance. Two of them were panresistant, with the exception of vancomycin and teicoplanin, and both belonged to phage group III, and showed the resistance pattern 8A. Remarkably, more than half of the PFGE type B and C isolates were derived from the Departments of 2nd Surgery, 3rd Internal Medicine, Pulmonology and Cardiac Surgery. In 2005 these departments were located in the same building and were separated from other clinics. Performing the analysis based on the phage types, a conspicuous observation was that all but one of the paediatric strains belonged to phage group III, with the predominance of phage type 623, while the other major source of these isolates was the above-mentioned separated clinical block. The majority of these strains (n=20) were of PFGE type C, and they had variable resistance patterns. Among the isolates of the mixed phage group, the most prevalent was PFGE type A, with the characteristic erythromycinclindamycin-ciprofloxacin-gentamicin-tobramycin (5B) resistance pattern. The non-typable group was very variable by all means.

4.2.3. Characterization of hVISA strains

In Debrecen three hVISA strains were isolated in 2007, 2008 and 2009. We obtained the new strains from three different patients, with these the hVISA isolates in Hungary increased from 2 to 5. First we identified the antibiotic resistance of the strains by disk diffusion according to the recommendations of the CLSI. Methicillin-resistance was determined by oxacillin E-test. The strains were resistant against the following antibiotics: erythromycin, clindamycin, ciprofloxacin, gentamicin and amikacin. The hVISA2 strain was also resistant to rifampicin. In Patient 1, the MRSA strain was isolated from blood and was confirmed by macro *E-test* and population analysis as hVISA (hVISA1). On the basis of macro *E-test* the inhibiting concentration of vancomycin and teicoplanin were 8 mg/L and 12 mg/L, respectively. The PAP-AUC ratio was 1.01. According to the MLST and SCC *mec* typing results the strain belonged to the Hungarian clone ST239-MRSA-III. The MRSA strain isolated from Patient 2 proved to be hVISA by macro *E-test* and population analysis (hVISA2). Vancomycin and teicoplanin inhibiting concentrations were both 8 mg/L. The PAP-AUC ratio was 0.97. The isolate was also tested by MLST and SCC *mec* typing, and was found to belong to the New York/Japan ST5-MRSA-II clone. We isolated an MRSA strain from Patient 3 and according to the macro *E-test* it was also intermediate vancomycin-sensitive. By macro E-test the inhibiting concentration of vancomycin and teicoplanin were both 8 mg/L; the PAP-AUC ratio was 0.93, so the population analyses profile confirmed that

it was a hVISA strain (hVISA3). We tested the isolate by MLST and SCC *mec* typing, and it was also found to belong to the New York/Japan MRSA clone ST5-MRSA-II.

In summary, we can conclude that in the hospitals of University of Debrecen the incidence of VRE and MRSA is lower than the international average. This is due to the introduction of appropriate infection control measures and also to the quick and reliable results of the diagnostic laboratory. Our immediate goal is to further improve the situation by the introduction of new molecular biology methods in the routine diagnostic procedures.

5. SUMMARY

The goal of the present study was the molecular and epidemiological characterization of vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) strains isolated from the hospitals of the University of Debrecen.

Between 2004-2009 we isolated overall 7271 Enterococci, of wich 17 were resistant to vancomycin. Among the latter we identified 1 *vanA* positive *Enterococcus faecalis*, 14 *vanC1* positive *E. gallinarum,* and 2 *vanC2* gene carrying *E. casseliflavus* strains by molecular biological methods. During this work we developed a new restriction analysis based method to differentiate the *vanC1* and *vanC2* genes from each other. Our epidemiological investigations (PFGE and MLST results) proved that the strains had no genetic relationships with each other. The comparison of the MIC values for the VRE strains obtained by E-test and by the automated VITEK2 system revealed that the version 3.01 software of VITEK2 is not suitable to detect low level glycopeptide resistance. Most of the clinical isolates were sensitive to other antibiotics tested, except for three gentamicin resistant *E. gallinarum* strains. In the latter strains we identified the bifunctional amynoglycoside resistance gene indicating that a few multiple drug resistance gene carrying Enterococci appeared in the hospitals of the University. According to our findings, the resistant enterococcus strains appeared sporadically. Still, from the epidemiologic and therapeutic point of view, it is very important to identify VRE genotypes by molecular genetic methods.

The incidence of antibiotic resistant *Staphylococcus aureus* was significantly higher. According to our survey, the rate of MRSA was 7,23% at the hospitals of the University of Debrecen in 2005. We investigated 339 MRSA strains from 102 patients in detail by epidemiological and molecular biological techniques. The results of the antibiotic sensitivity testing showed that 41,8% of the strains exhibited the 5B resistance pattern. Almost half of the strains belonged to the 617/622/623 phage type, and nearly 20% to the 629 phage type. We revealed three main PFGE types, among wich type A was predominant (55%). Thus we confirmed the clonal relationship between the strains with several methods. These epidemiological data allowed us to reduce the incidence of MRSA by introducing appropriate infection controll and hospital hygiene measures. During these studies, we also found three interesting cases of heteogeneously vancomycin-intermediate sensitive *S. aureus* (hVISA). According to MLST and SCCmec typing one of the strains belonged to the Hungarian ST239-MRSA-III clone, and two of them represented the New York/Japan ST5-MRSA-II clone.

6. PUBLICATIONS

6.1 Publications related to the thesis

Dombrádi, Zs., Dobay, O., Nagy, K., Kozák, A., Dombrádi, V., Szabó, J.: Prevalence of vanC VRE in the teaching hospitals of the University of Debrecen, Hungary. *Microbial Drug Resistance* ,"accepted by publisher", 2011.
IF:1.989

Dombrádi, **Zs.,** Bihari, Z., Illésné Horváth, K., Szabó, J.: Comparison of the VITEK 2 System with the E-test for the determintaion of glycopeptide susceptibility of VanA and VanC positive enterococci, *Acta Microbiologica et Immunologica Hungarica,* 57 (3), 157–163, 2010. DOI: 10.1556/AMicr.57.2010.3.1

Dombrádi, **Zs.,** Bodnár, F.,Orosi, P., Dombrádi, V., Szabó, J.: A case report of vancomycin-resistant *Enterococcus faecalis* colonization of a femoral wound in central europe. *Central European Journal of Medicine*, 4 (2), 259-261, 2009. DOI: 10.2478/s11536-009-0008-7
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Szabó, J., **Dombrádi, Zs.**, Dobay, O., Orosi, P., Kónya, J., Nagy, K., Rozgonyi, F.**:** Phenotypic and genetic characterisation of methicillin-resistant Staphylococcus aureus strains isolated from the university hospitals of Debrecen. *Eur J Clin Microbiol Infect Dis*. 28(2), 129-36, 2009. DOI: 10.1007/s10096-008-0588-1
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6.2. Conference presentations related to the thesis

Dombrádi, Zs., Bodnár, F., Péterffy, Á., Kónya J., Orosi, P., Dombrádi, V., Szabó, J.: Molecular analysis of vancomycin resistant *Enterococcus faecalis* strain isolated in Debrecen. 15th International Congress of the Hungarian Society for Microbiology. Budapest, Hungary, 2007. 07. 18-20.

Zs. Dombrádi, J. Szabó: Molecular analysis of vancomycin resistant *Enterococcus faecalis* strain isolated in Debrecen. Scientific-practical conference with international participation of students and young scientists. Uzhorod, Ukraine, 2008. 04. 16.

Zs. Dombrádi, J. Szabó: Phenotypic and genotypic characterisation of methicillin resistant *Staphylococcus aureus* strains isolated from university of Debrecen. Scientific-practical conference with international participation of students and young scientists. Uzhorod, Ukraine, 2008. 04. 16.

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List of publications related to the dissertation

1. **Dombrádi, Zs.**, Dobay, O., Nagy, K., Kozák, A., Dombrádi, V., Szabó, J.: Prevalence of vanC VRE in the teaching hospitals of the University of Debrecen, Hungary.
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2. **Dombrádi, Zs.**, Bihari, Z., Illésné Horváth, K., Szabó, J.: Comparison of the VITEK 2 system with the E-test for the determination of glycopeptide susceptibility of vanA and vanC positive enterococci.
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4. Szabó, J., **Dombrádi, Zs.**, Dobay, O., Orosi, P., Kónya, J., Nagy, K., Rozgonyi, F.: Phenotypic and genetic characterisation of methicillin-resistant Staphylococcus aureus strains isolated from the university hospitals of Debrecen.
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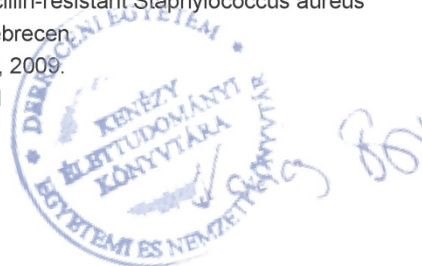
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The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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