Microbiological features of multiresistant bacteria causing nosocomial infections

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INTRODUCTION

Diagnostic and therapeutic devices have gone through a great development since the invention of Ignác Semmelweis published in 1846. Paradoxically opportunities of healthcare represent the most important factor of recent nosocomial infections.

Prevalence of hospital infections compared to the number of hospitalized patients varies between 3.5 and 12.2 % worldwide. Most frequent data are between 6 and 10 % and prevalence in Hungary is considered to be in this range as well. The incidence of nosocomial infections and the spectrum of pathogens specifically depends on the features of certain clinical wards and the existing/emerging predisposing factors. In a special group of patients, such as prematures the increasing survival due to improving healthcare results in increasing incidence of late onset sepsis caused by multiresistant microbes.

Nosocomial infections are caused even more frequently by multiresistant bacteria that used to be isolated much more seldom previously but are significant regarding hospital hygiene.

*Staphylococcus aureus* is one of the most significant nosocomial pathogens that is able to cause any kind of clinical infections. Methicillin-resistant *S. aureus* (MRSA) is a serious problem worldwide. Based on national data the proportion of bloodstream infections caused by MRSA raised from 5 to approximately 20 % since 2001. Resistance to methicillin means that not any ß-lactams should be considered as therapeutical option. Moreover in these cases bacteria show resistance against other antibiotics/groups of antibiotics such as macrolides, lincosamides, quinolones or aminoglycosides, deserving to be considered as polyresistant. Based on the differences between MRSA and methicillin susceptible *S. aureus* (MSSA) strains – regarding cell proliferation kinetics, cellular structure, slime and biofilm production, cell surface adhesins, enzyme pools, superantigenic exotoxins and the cytotoxins (particularly Panton-Valentine leukocidin and ß, ß, ß haemolysins) – the question may arise whether methicillin resistant strains are more virulent than those being susceptible for methicillin.

The increasing role of coagulase-negative *Staphylococci* (CNS) in nosocomial bloodstream infections were confirmed by lots of great studies. Among these species *Staphylococcus haemolyticus* is the second most frequent nosocomial pathogen following *Staphylococcus epidermidis*. The strains of *S. haemolyticus* are typically multiresistant thus glycopeptides represent the choice of therapy without doubt. However, *S. haemolyticus* was the first Gram-positive pathogen acquiring resistance against glycopeptides (earlier than any other *Staphylococcus* or *Enterococcus* species) and is probably able to develop glycopeptide-
resistance in a larger scale comparing to other CNS. Resistance against glycopeptides usually appears as heteroresistance requiring the completion of proper microbiological tests to avoid therapeutic failure. Sustained inappropriate serum concentration of glycopeptides during therapy is an important selective factor resulting in the emergence of resistance. Moreover, it is also frequently suggested that *S. haemolyticus* as a potential pathogen may act as a donor of resistance genes for more virulent species such as *S. aureus*.

Among the group of Gram-negative bacteria called non-fermenters, such as the most significant multi/panresistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* has also become an important nosocomial pathogen that is able to cause infections with significant lethality rate in certain groups of patients. Carbapenem therapy widely used against other nosocomial pathogens unambiguously contributed to the increasing frequency of infections caused by this microbe since these strains show intrinsic resistance against these antibiotics. In addition, it is resistant to most of the antibiotics due to other resistance mechanisms (enzymatic degradation, efflux pump systems). Since currently no specific reference method exists for the detection of antibiotic susceptibility of *S. maltophilia*, providing correct microbiological results is a great challenge. Traditionally trimethoprim/sulphamethoxazole is the drug of choice in these infections although the number of strains expressing resistance against this compound is increasing nowadays, data suggesting proportion being approximately 10%.

Fermentative bacteria belonging to *Enterobacteriaceae* family are henceforward important nosocomial pathogens, with the dissemination of strains showing specific resistance against extended spectrum cephalosporins. After the emergence of AmpC-type β-lactamases ESBLs (extended spectrum β-lactamases) especially produced by *Klebsiella* species have outstanding significance resulting in the loss of therapeutic effect of all β-lactams except carbapenems. Nowadays more than 300 different ESBL enzymes are known usually encoded by plasmids. ESBL-producing strains specifically show tendency to cause nosocomial outbreaks therefore their epidemiological surveillance is crucial.

Due to dissemination of ESBL-producing multiresistant *Enterobacteriaceae* strains carbapenems have become one of the last efficient therapeutic options in serious infections caused by Gram-negative bacteria. However, bacteria expressing resistance even against carbapenems are emerging more frequently due to increased use of these antibiotics. The first metallo-β-lactamase (MBL) producing *Klebsiella pneumoniae* strain was detected in Greece in 2002. Since then VIM-type metallo-β-lactamases were identified in several species among *Enterobacteriaceae* especially in *K. pneumoniae* strains.
The high prevalence of quinolone-resistance among enteric bacteria, especially *Escherichia coli* has already been described worldwide. Resistance against quinolones in bacteria belonging to *Enterobacteriaceae* family is usually mediated by mutations affecting genes of chromosomally encoded type II topoisomerases, efflux pumps or porin related proteins. Among *K. pneumoniae* és *E. coli* strains quinolone-resistance occurs more frequently in strains producing ESBL encoded by plasmids than ESBL-negative strains what is probably due to coordinated co-expression of certain resistance mechanisms. Recently plasmid-harboured transferable quinolone-resistance genes – *qnrA, qnrB, qnrS*, a variant of *aac(6’)-Ib-cr, qepA* and *oxqB* - have been observed in clinical isolates. Although *qnr* is only responsible for low level quinolone-resistance and does not seem to be frequent it may contribute to the development of resistance by the modification of quinolone target enzymes, activation of efflux pumps or deficiencies in porin channels in the outer membrane.

**AIMS**

1. The frequency of isolation of different bacteria highly depends on (besides its resistance) the patient population, the wards where patients are admitted and even on geographical site. We wanted to demonstrate this by collecting results of the microbiological investigations performed in our laboratory on clinical samples from various nosocomial infections. Our aim was to chart the trends observed in the last ten years concerning the spectrum of pathogenic microorganisms, the degree of resistance especially in the background of late onset sepsis cases in patients admitted to perinatal intensive care units. We also aimed to establish the prevalence of nosocomial urinary tract infections and antimicrobial resistance of uropathogens as well as determination of its dependence on patient population.

2. We performed microbiological investigations and retrospective epidemiological analyses on MSSA and MRSA strains isolated in our laboratory as well as strains isolated in Austria and Macedonia in order to compare data.

3. Since we have identified a large number of *S. haemolyticus* strains from the samples of patients admitted to the intensive care units in the clinics of Semmelweis University causing colonisation and serious infections and have considered the increasing number of *S. haemolyticus* isolates showing decreased susceptibility for teicoplanin isolated from blood cultures of patients admitted to neonatal intensive care units especially alarming we have set ourselves the aim to reveal the epidemiology of *S. haemolyticus* strains playing role in
bloodstream infections in patients hospitalized between 2002 and 2008 in the clinics of Semmelweis University. Our other aim was to determine the proportion and level of resistance against anti-staphylococcal agents, describe the epidemic phenotypic antibiotic resistance patterns and to investigate the relations between PFGE genotypes and phenotypic resistance patterns in \textit{S. haemolyticus} strains.

4. \textit{K. pneumoniae} strains producing TEM- and SHV-type ESBLs have become important pathogens in nosocomial infections due to their multiresistant character and because they cause even more hospital outbreaks, especially in neonatal intensive care units. Therefore we decided to perform a study to determine the molecular epidemiology of ESBL-producing Klebsiella species in the 2nd Perinatal Intensive Centre in a five-year period. Simultaneously we retrospectively investigated the risk factors of infections and mortality as well.

5. Besides metallo-\beta-lactamase producing \textit{P. aeruginosa} and \textit{Acinetobacter} species MBL-producing \textit{Enterobacteriaceae} strains represent an increasing worldwide problem as well. No imipenem or meropenem insensitive \textit{Klebsiella} species have been isolated in 2009 but two carbapenem-susceptible \textit{Klebsiella} strains were proven to be MBL-producers based on screening and confirmatory tests. Our aim was to characterize the first MBL-producing \textit{K. pneumoniae} and \textit{K. oxytoca} isolates in Hungary.

6. Even though quinolone-resistance is usually chromosomally encoded, the proportion of ESBL-producing strains resistant to ciprofloxacin is increasing worldwide. Recently more quinolone-resistance genes (\textit{qnrA, qnrB, qnrS} és \textit{aac(6')-Ib-cr}) carried by plasmids have been described. Our aim was to determine the prevalence of \textit{qnrA, qnrB, qnrS} és \textit{aac(6')-Ib-cr} genes in ESBL-producing strains isolated in Hungary as well as to investigate the mobility of \textit{qnr} genes.

7. \textit{S. maltophilia} is an important nosocomial human pathogen. Its high level of antibiotic-resistance and the difficulties with its determination the object we proposed to ourselves was to investigate antibiotic susceptibility by performing different methods, to compare the results with special attention towards antibacterial agents that are available for Hungarian clinicians.
MATERIALS AND METHODS

Identification and storage of bacterial strains

Species-level identification of bacteria occurred by performing classical biochemical tests according to the species as well as using automatic/semi-automatic systems such as ATB and API, VITEK I and VITEK II (BioMérieux). We stored bacterial strains in Tryptic Soy Broth (TSB) containing 20% glycerol at -80 °C until subsequent phenotypic and genotypic investigations. Since we used several strains during our work we specify their source and relevant properties together with the description of related experiments.

Antibiotic susceptibility testing

Antibiotic susceptibility tests were performed and results were interpreted according to actual annual international recommendations of the Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards (CLSI/NCCLS) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). All tests were performed according to the recommendations with control strains, controlled media, conditions based on international standards with disc diffusion and microdilution method. MIC values were also determined by using E-test strips according to the recommendations of the manufacturer (AB Biodisk, Solna, Sweden).

We investigated antibiotic resistance patterns of our S. haemolyticus isolates using recommendations as described elsewhere.

Investigation of special resistance mechanisms by phenotypic methods

Phenotypic screening of ESBL production and its confirmation were performed according to the international recommendations of CLSI.

Presumptive carbapenemase-producers were screened according to Hungarian guidelines based on CLSI recommendations as well. Probable MBL production was tested by the modified Hodge test and discs containing imipenem and/or ceftazidime alone and combined with EDTA.

We used vancomycin containing agar in order to screen strains being resistant to glycopeptides, according to the guidelines of CLSI. Modified population analysis profile was also performed using Walsh method to investigate the heteroresistance of strains against vancomycin.
**Genotypic characterization of investigated strains**

**PCR amplification and sequencing**

After overnight growth 10 µl aliquots of isolates were diluted 10-fold by water and boiled for 15 minutes and then centrifuged. Supernatants were used as bacterial template DNA for PCR. PCR was performed by using RedTaq DNA-polymerase and specific primers for different genes. 10 µl of PCR products were analyzed by electrophoresis on 0.8 w/v % gel.

The nucleotide sequences of amplified products were determined by using ABI 3700 and ABI 3100 genetic analyzers according to the manufacturer’s instructions. Sequence analysis was performed using the Lasergene DNASTAR sequencing analysis software (DNASTar). Identification of gene sequences was performed in comparison with available sequences in GenBank and EMBL databases.

**Pulsed-field gel electrophoresis (PFGE) analysis**

PFGE analysis of ESBL-producing *Klebsiella* strains was performed by using the method described by Paterson et al., for *S. haemolyticus* strains by using an internal protocol designed for CNS (a version described by Bradford et al.) whereas PFGE analysis of genomal DNA digested by *XbaI* of the MBL-producing *K. pneumoniae* strain was performed by following the instructions as previously described by Damjanova et al. Dendrograms were designed by using BioNumerics (Bio-Rad) with the application of Dice-coefficient, unweighted pair group method (UPGMA) with 1.3 % position tolerance. Relation between isolates were determined by Tenover’s criteria.

**Plasmid analysis**

We usually used alcaline lytic method for the detection of plasmids, in some cases plasmid extraction kit was applied (Wizard Plus Minipreps, DNA Purification System; Promega). During digestion we used restriction enzymes according to the manufacturer’s (BioLab) instructions. Extracted plasmid DNA of bacterial strains was transformed into either *E. coli* DH5-? cell or *E. coli* DH10B recipient cells. Transformed bacteria were cultivated on media containing antibiotics. Azide- or rifampicin-resistant *E. coli* J53 strains was used during conjugation.
Analytical isoelectric focusing

We performed the determination of isoelectric point of ESBL- and MBL-producing Klebsiella strains or quinolone-resistant enteric bacteria by previously described methods (Paterson et al.).

Collection of data required for epidemiological investigations

Clinical background of isolated and microbiologically investigated microbes as well as the presence of specific risk factors were collected by using medical charts and consulting with clinician colleagues.

RESULTS AND DISCUSSION

General epidemiology of certain nosocomial infections

Pathogen spectrum of bloodstream infections

After assessment of data regarding pathogens isolated from bloodstream infections we can establish that not any change has occurred in the frequency of the three most prevalent pathogens in the last ten years: S. aureus, coagulase-negative Staphylococci and E. coli are still in prominent position. In general the number of bloodstream infections caused by Gram-positive microbes shows slight increase that may be explained by the increasing presence of Enterococcus species. The prevalence of late onset sepsis in patients admitted to neonatal intensive care units shows promising decreasing tendency and nowadays only infections caused by coagulase-negative Staphylococci represent a problem.

Prevalence of multiresistant pathogens

The overall number of infections on the wards that we supply is decreasing, however, the increasing resistance features of specifically nosocomial strains is worrying. The prevalence of MRSA strains has increased from 5.2 % to 19.7 % between 2004 and 2009, that of vancomycin-resistant Enterococcus strains raised from 1.7 % to 3.2 %, for ESBL-producing E. coli strains from 0.8 % to 7.4 %, for multiresistant P. aeruginosa strains being only susceptible for polymyxin B from 7.2 % to 11.8 %, for S. maltophilia strains being resistant to Co-Trimoxazole from 3.2 % to 8.7 % with the emergence of Acinetobacter strains resistant to carbapenems.
Epidemiology of nosocomial urinary tract infections

There are several differences in the bacterial spectrum of urinary tract infections depending on the age, gender, immune status and other predisposing factors. Based on the levels and patterns of antibiotic resistance stricter guidelines and rules should be predominated in antibiotic policy.

Staphylococcus aureus strains

Based on our results we can assert that resistance patterns of S. aureus strains derived from distinct European countries are different. Being familiar with local resistance data is obligate since it may be the main background of empirical therapy. Emergence of multiresistant MRSA strains is a serious problem all over the continent, assigning increased significance for glycopeptides and novel anti-MRSA agents in the treatment of MRSA infections.

Patients in intensive care unit, coma centre, general surgical, nephrological and haematological wards are colonised and infected by MRSA in a large scale in Skopje. Beyond persisting disease this may be due to the intra- and interhospital dissemination of strains and the lack of strict provisions of hospital infection control.

Staphylococcus haemolyticus strains

Between 2001 and 2008 a total of 184 bloodstream infections caused by S. haemolyticus were observed in different clinics of Semmelweis University, taking approximately 6 % of all infections and 11 % of infections caused by Gram-positives. We noticed significant differences when comparing the distribution of S. haemolyticus isolates between various clinical wards. 18 % and 15 % of blood culture isolates were S. haemolyticus on the two neonatal intensive care units whereas only 4 % of isolates in „adult” intensive care units. 5-6 % rate of S. haemolyticus bloodstream infections is characteristic in surgical wards and 0-3 % for internal medicine and other wards. 97 % of S. haemolyticus isolates showed phenotypic resistance to oxacillin. All the strains were positive for mecA gene by PCR. We detected high levels of resistance against other anti-staphylococcal agents with equally elevated MIC values (Table 1.). However the multiresistance of strains did not prove to be identical. We found more phenotypic resistance patterns including four relatively frequent that simultaneously showed resistance against oxacillin, erythromycin, gentamicin and ciprofloxacin.
Table 1. Prevalence of resistance against anti-staphylococcal agents in invasive *Staphylococcus haemolyticus* strains

<table>
<thead>
<tr>
<th>Antibacterial agents</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>All</th>
<th>range of MICs</th>
<th>MIC50</th>
<th>MIC90</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxacillin</td>
<td>90%</td>
<td>100%</td>
<td>96%</td>
<td>96%</td>
<td>100%</td>
<td>92%</td>
<td>93%</td>
<td>100%</td>
<td>97%</td>
<td>4-&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>erythromycin</td>
<td>100%</td>
<td>95%</td>
<td>96%</td>
<td>100%</td>
<td>100%</td>
<td>92%</td>
<td>87%</td>
<td>94%</td>
<td>96%</td>
<td>1-&gt;256</td>
<td>128</td>
<td>&gt;256</td>
</tr>
<tr>
<td>clindamycin</td>
<td>70%</td>
<td>82%</td>
<td>66%</td>
<td>65%</td>
<td>45%</td>
<td>50%</td>
<td>47%</td>
<td>76%</td>
<td>62%</td>
<td>2-&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>gentamicin</td>
<td>80%</td>
<td>91%</td>
<td>89%</td>
<td>85%</td>
<td>89%</td>
<td>75%</td>
<td>87%</td>
<td>94%</td>
<td>87%</td>
<td>2-&gt;256</td>
<td>128</td>
<td>&gt;256</td>
</tr>
<tr>
<td>sulph./trimethoprim</td>
<td>50%</td>
<td>41%</td>
<td>36%</td>
<td>50%</td>
<td>47%</td>
<td>83%</td>
<td>67%</td>
<td>53%</td>
<td>49%</td>
<td>4-&gt;256</td>
<td>128</td>
<td>&gt;256</td>
</tr>
<tr>
<td>doxycycline</td>
<td>70%</td>
<td>27%</td>
<td>9%</td>
<td>15%</td>
<td>8%</td>
<td>8%</td>
<td>20%</td>
<td>0%</td>
<td>15%</td>
<td>2-32</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>50%</td>
<td>73%</td>
<td>84%</td>
<td>88%</td>
<td>97%</td>
<td>83%</td>
<td>80%</td>
<td>88%</td>
<td>84%</td>
<td>&lt;0.5-&gt;256</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>linezolid</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0.25-0.5</td>
<td>0.38</td>
<td>0.5</td>
</tr>
<tr>
<td>vancomycin</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>1-4</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>teicoplanin</td>
<td>40%</td>
<td>27%</td>
<td>46%</td>
<td>30%</td>
<td>64%</td>
<td>40%</td>
<td>46%</td>
<td>41%</td>
<td>42%</td>
<td>1-128</td>
<td>16</td>
<td>64</td>
</tr>
</tbody>
</table>

MIC values of vancomycin were 1.0-4.0 µg/ml for every *S. haemolyticus* strain. All the isolates were susceptible for vancomycin regarding CLSI criteria, however, 38% of them had MIC values of 4 µg/ml. Not a single strain showed growth on agar plate containing 6 µg/ml vancomycin, under special incubation conditions. None of the strains having 1 µg/ml vancomycin MIC value showed heteroresistance to vancomycin, as determined by modified population analysis profile, although 30% of the strains with 2 µg/ml vancomycin MIC values were heteroresistant and 22% were resistant to glycopeptides. Other alarming result of our investigation is the 42% teicoplanin resistance with MIC50 16 µg/ml and MIC90 64 µg/ml values. Based on these results teicoplanin should not be considered as first line antibiotic in *S. haemolyticus* infections any more. None of the teicoplanin resistans strains harboured neither vanA nor vanB gene.

Based on PFGE profile analysis strains belonged to 56 different pattern types proving heterogeneity. Several strains showing similar PFGE type or subtype were isolated from different and conversely, structurally unlinked hospital wards and at the same time several strains belonging to different PFGE types or subtypes were found in certain cases. We have not found correlations between antibiotic resistance phenotypes and PFGE genotypes therefore antibiotic resistance patterns could not be used for epidemiological studies.

**ESBL-producing Klebsiella spp. strains**

A total of 101 *Klebsiella* strains were isolated from neonates (one patient-one isolate) between January, 2001 and December, 2005 in 2nd Perinatal Intensive Centre (PIC) at Semmelweis University. We isolated 25 ESBL-producing *K. pneumoniae* and 9 ESBL-producing *K. oxytoca* strains in the first period (from January, 2001 until June, 2003). In the second period (from June to December, 2005) 12 ESBL-producing *K. oxytoca* strains were
isolated from different patients. Although being collected in two distinct periods isolates expressed considerably similar susceptibility profiles. In the first period of our study we observed the presence of SHV-5 producing *K. pneumoniae* showing three different PFGE patterns (A, B, E) and SHV-12 producing *K. oxytoca* with two different patterns (C, D). In the second period an SHV-5 producing *K. oxytoca* strain has emerged as a novel PFGE clone (F). During the epidemiological investigation of ESBL-producing strains we have not observed the presence of a single predominant strain but a significant alteration between different predominant strains during the period. SHV-12 producing *K. oxytoca* is relatively new in Hungary although it has already been isolated in France. Only a single outbreak in perinatal intensive care unit caused by (SHV-5) ESBL-producing *K. oxytoca* has been described so far.

ß-lactamase enzymes produced by ESBL-producing *K. oxytoca* and their isoelectric points as well as the results of plasmid analysis are given in Table 2.

### Table 2. Characterization of ESBL-producing *Klebsiella* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Period of the isolation</th>
<th>Number of infected patients</th>
<th>Number of colonized patients</th>
<th>PFGE</th>
<th>Isoelectric points (pI)</th>
<th>PCR and sequencing results</th>
<th>Plasmids</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em></td>
<td>2001 February - 2002 April</td>
<td>14</td>
<td>2</td>
<td>A</td>
<td>8.2</td>
<td>SHV-5</td>
<td>P1</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>2002 May - October</td>
<td>7</td>
<td>0</td>
<td>B</td>
<td>8.2</td>
<td>SHV-5</td>
<td>P1</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>2002 August - 2003 January</td>
<td>7</td>
<td>0</td>
<td>C</td>
<td>5.4, 7.6, 8.2</td>
<td>TEM-1, SHV-12</td>
<td>P2</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>2003 January</td>
<td>2</td>
<td>0</td>
<td>D</td>
<td>5.4, 8.2</td>
<td>TEM-1, SHV-12</td>
<td>P3</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>2004 January</td>
<td>2</td>
<td>0</td>
<td>E</td>
<td>8.2</td>
<td>SHV-5</td>
<td>P4</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>2005 July - November</td>
<td>7</td>
<td>5</td>
<td>F</td>
<td>7.6, 8.2</td>
<td>SHV-5</td>
<td>P5</td>
</tr>
</tbody>
</table>

Regarding the involved risk factors – gender, gestation time, twin pregnancy, birth weight, central vein catheter, ventillation, bottle-feeding, polymicrobial infection, caeserean section, transfusion – we have not found statistically significant differences between ESBL-producing and non ESBL-producing *Klebsiella* species, examining colonized and infected patients separately. Higher survival ratio was observed in patients infected by non ESBL-producing *Klebsiella* species (2/45, 4.4 %) compared to that of patients infected by ESBL-producing *Klebsiella* species (6/39, 15 %) although this different was not statistically significant.
Metallo-β-lactamase producing Klebsiella strains

In 2009 5% of non carbapenemase-producing Klebsiella spp. isolates (1.4% of Enterobacteriaceae isolates) were resistant to ertapenem in a centre in Budapest and 4.8% in another (2.4% of Enterobacteriaceae isolates). During further investigations a K. pneumoniae and a K. oxytoca strain turned out to be metallo-β-lactamase producers belonging to group B, being the first Hungarian isolates. We performed the microbiological characterization of these strains.

MIC values of K. pneumoniae KP3686 and K. oxytoca KO5294/9 isolates were determined by E-tests and showed in Table 3, as well as the genetic background of their resistance against β-lactam antibiotics and plasmid analysis results.

Strains were resistant to a wide spectrum cephalosporins. K. pneumoniae strains were resistant to most of non β-lactam antibiotics including aminoglycosides, ciprofloxacin and trimethoprim/sulphamethoxazole as well. Both strains were resistant to ertapenem.

In KP3686 strain we detected a chromosomally encoded non ESBL-type blaSHV-11 gene and a plasmid coded blacTX-M-15 as well as blaTEM-1 genes and moreover a blaVIM-4 gene situated in a class-1 integron. We only found blavIM-4 gene situated in class-1 integron in KO5294/9 strain. Both integrons carried two gene cassettes: an aac(6')-Ib (so called aacA4) gene in the first position and an ensuing blavIM-4 gene cassette. Sequencing results of integrons revealed the presence of the same VIM-4 containing class-1 integron in both isolates and furthermore that this integron was identical to those described in P. aeruginosa strains isolated in South-Hungary and the one previously described in the first MBL-producing A. hyrophilia strain. Nucleotide sequences were uploaded to GenBank database and are available under the number of GU181265 for KP3686 strain and GU181269 for KO5294/9.

By DNA hybridization we demonstrated that VIM gene was carried by approximately 90 bp plasmids in both strains. Although conjugation experiment was not prosperous we successfully managed to transform Escherichia coli DH5α strain with plasmids harbouring β-lactamase gene. PCR examination of transformed bacteria and sequencing of products confirmed the presence of blacTX-M-15 ESBL and blaTEM-1 genes on pKP3686/2 plasmid as well as blavIM-4 gene on pKP3686/1 and pKO5294/9 plasmids. Plasmids DNA extracted from transformed bacteria for fingerprint analysis were digested by PstI and EcoRI enzymes. Identity of pKP3686/1 and pKO5294/9 plasmids was confirmed by restriction analysis as well.
### Table: Characteristics of the *Klebsiella pneumoniae* KP3686, *Klebsiella oxytoca* KO5294/9, *E. coli* DH 5α and the transformants strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>PCR and sequencing</th>
<th>AMC</th>
<th>TZP</th>
<th>CAZ</th>
<th>CTX</th>
<th>FEP</th>
<th>ATM</th>
<th>ETP</th>
<th>IPM</th>
<th>MEM</th>
<th>CIP</th>
<th>AMK</th>
<th>TOB</th>
<th>GEN</th>
<th>SXT</th>
<th>TGC</th>
<th>CST</th>
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<tr>
<td>KP3686</td>
<td>TEM-1, SHV-11, CTX-M-15, VIM-4</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>32</td>
<td>64</td>
<td>8</td>
<td>16</td>
<td>1.5</td>
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<td>0.25</td>
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<td>KO5294/9</td>
<td>VIM-4</td>
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<td>&gt;256</td>
<td>64</td>
<td>64</td>
<td>48</td>
<td>0.19</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>16</td>
<td>&gt;256</td>
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<td>0.5</td>
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<tr>
<td><em>E. coli</em> DH5α</td>
<td>-</td>
<td>4</td>
<td>4</td>
<td>0.016</td>
<td>0.032</td>
<td>0.016</td>
<td>0.032</td>
<td>0.008</td>
<td>0.25</td>
<td>0.094</td>
<td>0.19</td>
<td>0.012</td>
<td>0.12</td>
<td>0.064</td>
<td>0.016</td>
<td>0.12</td>
<td>0.5</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>(pKP3686/1, ~90 kb)**</td>
<td>VIM-4</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>12</td>
<td>8</td>
<td>0.5</td>
<td>0.032</td>
<td>0.064</td>
<td>1.5</td>
<td>0.19</td>
<td>0.19</td>
<td>4</td>
<td>8</td>
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<td>0.023</td>
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<tr>
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<td>&gt;256</td>
<td>0.006</td>
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<tr>
<td><em>E. coli</em> DH5α</td>
<td>(pKO5294/9, ~90 kb)**</td>
<td>VIM-4</td>
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<td>&gt;256</td>
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<td>8</td>
<td>0.064</td>
<td>0.012</td>
<td>0.25</td>
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** Abbreviations and sizes of plasmids in transformants
PFGE and MLST investigations prove that KP3686 strain belongs to the previously described ST11 clone which has already been detected country-wide as CTX-M-15-producing epidemic clone (EC III). To our knowledge this is the first case when the internationally successful ST11 K. pneumoniae clone imported an MBL-coding integron.

Besides the clarification of molecular background there is a high responsibility during routine microbiological examinations. It is highly important to be able to verify carbapenemase production by phenotypic screening and confirmation tests – ertapenem can be used for screening carbapenemase production – in suspicious cases, later combined with molecular methods since MIC values for certain β-lactams and/or carbapenems may be in susceptible interpretation range however not any of β-lactam antibiotics should be considered as therapeutical option in these infections.

**Investigations for quinolone resistance**

In our study we investigated the presence of *qnrA*, *qnrB*, *qnrS* and *aac(6′)-Ib-cr* genes in ESBL-producing enteric bacteria (70 *E. coli*, 101 *K. pneumoniae*, 5 *C. freundii* and 61 Enterobacter strains) isolated between 2002 and 2006.

The prevalence of *qnr* determinants was relatively high in *K. pneumoniae* (8 %) and relatively low in *E. coli* isolates (1.6 %) as well as among Enterobacter spp (0 %). One of the five ESBL-producing *C. freundii* isolates was *qnr*-positive. We could detect *qnrA1*, *qnrB2* and *qnrS1* and *qnrA1* had the highest prevalence (3 %), respectively. These strains were resistant to nalidixic acid, norfloxacin, ciprofloxacin and levofloxacin with considerably high MIC values. We characterized the β-lactamase enzymes produced by *qnr*-positive strains by determining isoelectric points, PCR and sequencing data. During our investigations we found the presence of *qnrA1* gene to be linked to SHV-5, SHV-12 and CTX-M-15 enzymes (Table 4.). The conjugation test which was successful in case of three *qnrA1*-positive strains confirmed that the presence of *qnrA1* alone leads only to low-level quinolone-resistance.

The *qnrB* gene was detected in two isolates: a *K. pneumoniae* and a *C. freundii* strain. *K. pneumoniae* M95 harboured both *qnrA1* and *qnrB2* genes but only *qnrA1* was transferable by conjugation suggesting the different genetic backgrounds of these genes. MIC values of ciprofloxacin and levofloxacin were highest for *K. pneumoniae* M95 comparing to other *qnrA1*-positive strains which can be explained by the simultaneous production of *qnrA1* and *qnrB2* in the original isolate. *C. freundii* M141 harbouring solely *qnrB2* gene was susceptible for the examined fluoroquinolones with MIC values at least 8-fold lower than the breakpoints suggested by CLSI.
Table 4. Characterization of β-lactamase enzymes produced by qnr-positive strains and MIC values for quinolone resistance. ND: not determined

<table>
<thead>
<tr>
<th>Strain</th>
<th>qnr gene</th>
<th>MIC (µg/ml)</th>
<th>PCR, sequencing</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nalidixic acid</td>
<td>Ciprofloxacin</td>
</tr>
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<td>E. coli M136</td>
<td>qnr A1</td>
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<td>K. pneumoniae 124</td>
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<td>K. pneumoniae 125</td>
<td>qnr A1</td>
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<td>64</td>
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<tr>
<td>K. pneumoniae M88</td>
<td>qnr A1</td>
<td>&gt;32</td>
<td>64</td>
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<tr>
<td>K. pneumoniae M140</td>
<td>qnr A1</td>
<td>&gt;32</td>
<td>256</td>
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<tr>
<td>K. pneumoniae M143</td>
<td>qnr A1</td>
<td>&gt;32</td>
<td>32</td>
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<td>K. pneumoniae M95</td>
<td>qnr A1, qnrB2</td>
<td>&gt;32</td>
<td>&gt;256</td>
</tr>
<tr>
<td>C. freundii M141</td>
<td>qnr B2</td>
<td>2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>K. pneumoniae 96</td>
<td>qnr S1</td>
<td>&gt;32</td>
<td>4</td>
</tr>
</tbody>
</table>

The qnrS1 gene was only detected in a single K. pneumoniae strain being resistant to all examined fluoroquinolones.

Seventy six (26.6 %) of ESBL-producing isolates were positive for aac(6′)-Ib and 19 of these isolates (8 % of all) – 16 K. pneumoniae and 3 E. coli – harboured aac(6′)-Ib-cr variant. Not any qnr-positive strains harboured the aac(6′)-Ib-cr variant. The presence of aac(6′)-Ib-cr did not result in high elevation of quinolone MIC values, however significantly increased the frequency of selection of chromosomal mutants.

The wide range of MIC values in qnr-positive strains draw the attention to that phenotypical detection of qnr genes in clinical isolates may become considerably difficult.

Stenotrophomonas maltophilia strains

International recommendations are reasonably laconic concerning antibiotic susceptibility testing of S. maltophilia and strictly use the expression „under process“ when describing standardization. Thus, in order to establish a guideline for creating correct microbiological results we performed the determination of antibiotic susceptibility by using the usual methods (disc diffusion, microdilution, E-test, automated systems) and read the results after 24 and 48 hours, respectively. We evaluated our results according to interpretation criteria of CLSI and/or EUCAST on species specific level or – in the lack of these – using breakpoints for Pseudomonas spp. or occasionally for Enterobacteriaceae.

The drug of choice is trimethoprim/sulphamethoxazole which showed bimodal distribution when assessing the results of microdilution method thus it is pretty difficult to establish susceptibility breakpoints. There are two strains in the background in our study with MIC values of 4/76 µg/ml which is critical for interpretation. It is considered to be susceptible.
by EUCAST whereas resistant by CLSI. Based on our results reading the results of disc diffusion method after 24 hours of incubation correlates well to that of microdilution method. The alterations between different methods of susceptibility testing regarding every other family of antibiotics should be considered as well as the disc diffusion results read after 24 hours show towards false positivity (major fault).

Ciprofloxacin is not appropriate in the treatment of infections caused by *S. maltophilia*, only new quinolone derivatives can be administered such as levofloxacin and moxifloxacin. The effect of polymyxin B and colistin – nowadays widely used in infections caused by multiresistant nonfermentative microbes – on *S. maltophilia* is considerably poor based on our results querying their frequent use in combination therapy.

Our results show that only susceptibility for trimethoprim/sulphamethoxazole can safely be investigated by disc diffusion method evaluating after 24 hours of incubation. Either reading the results after 48 hours of incubation or unambiguously dilution method is suggested to determine the susceptibility for compounds belonging to other families of antibiotics. Automatic systems should not be applied as they can give false susceptible results since they are designed for detection of susceptibility of fast growing microbes.

**CONCLUSIONS**

1. Microbiological laboratories have to play important role in the prevention and elimination of nosocomial infections. It is crucial to be aware of the pathogen properly in case of every nosocomial infection. Moreover, microbiologists may be the first detecting outbreak by observing typical microbes and their characteristic resistance patterns on the wards and is able to help in the establishment of empirical therapeutical strategy on a certain ward. After the emergence of a special resistance mechanism the putative reasons of its appearance should be revealed as the first step by proper examination of microbe(s) as well as the determination of genetic background. That may provide informations whether further dissemination should be expected. Results can be built in the routine diagnostic work and infection control.

2. We determined the antibiotic susceptibility of *S. aureus* strains collected in our laboratory and compared data to that of Austrian and Macedonian strains. Significant differences between MSSA and MRSA strains were characterized by investigations on antimicrobial susceptibility and virulence factors. These results suggest that reclassification of MSSA and MRSA strains into two distinct subtypes should be considered.
3. We performed the first national survey on *S. haemolyticus* and investigated the teicoplanin resistance of our strains. The ability of *S. haemolyticus* for acquiring resistance and its potential pathogenicity in immunocompromised patients confirm the significance of species-level identification of CNS and the importance of investigating epidemiology. Further examinations are necessary to evaluate the significance of virulence factors playing role in the pathogenesis of sepsis caused by *S. haemolyticus*.

4. We described the molecular epidemiology of infections caused by ESBL-producing *K. pneumoniae* and *K. oxytoca* in a perinatal intensive care unit during a five-year period. We identified two different SHV-types: SHV-5 and SHV-12. We demonstrated that the epidemiology of outbreaks caused by ESBL-producing strains may vary during years: a strain causing outbreaks may dissapear and novel strains may replace it. This dynamism and multiclonality as well as being unable to prove enviromental sources lead us to the assumption that mother can be a potential source of infection in newborns by carrying ESBL-producing strains in their normal enteric flora and thus causing contamination of neonates during delivery. Based on these results we suggest the routine screening of enteric flora in women with immature neonates.

5. We characterized the first VIM-4 metallo-ß-lactamase producing *K. pneumoniae* and *K. oxytoca* strains in Hungary. We first reported the emergence of VIM-4 in *K. oxytoca*. We confirmed that the strain belonged to the previously described ST11 clone which has already been detected country-wide as CTX-M-15 producing epidemic clone (EC III). To our knowledge this is the first case when the internationally successful ST11 *K. pneumoniae* clone imported an integron coding MBL.

6. We determined plasmid-mediated quinolone-resistance in ESBL-producing *Enterobacteriaceae* in Hungary. These results suggest the slow dissemination of the three *qnr* genes as well as *aac(6′)-Ib-cr* among ESBL-producing *Enterobacteriaceae*. We established the emergence of *qnrB* gene in Europe suggesting widespread dissemination of plasmids playing role in the transfer of resistance against extended spectrum cephalosporins and quinolones in clinical isolates.

7. In the lack of international recommendations we determined the method that can safely be used in routine microbiological laboratories to examine susceptibility of *Stenotrophomonas maltophilia* for therapeutic agents that are available in Hungary. The results of our recent and previous investigations suggest that susceptibility testing can only be performed for trimethoprim/sulphamethoxazole safely by disc diffusion evaluating after 24 hours of routine incubation. Either reading the results after 48 hours of incubation or
unambiguously dilution method is suggested to determine the susceptibility for compounds belonging to other families of antibiotics.

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Sánta Eszter

LIST OF PUBLICATIONS

Papers related to the thesis published in international journals:


3. **Kristóf K**, Kocsis E, Szabó D, Kardos Sz, Cser V, Nagy K, Hermann P, Rozgonyi F: Significance of methicillin - teicoplanin resistant *Staphylococcus haemolyticus* in bloodstream infections in Hungarian University Hospitals (revízió alatt)
Papers related to the thesis published in Hungarian journals:


Papers related to the thesis published in international journals as co-author:


Papers related to the thesis published in Hungarian journals as co-author:


Citable congress abstracts related to the thesis in peer-reviewed journals with impact factors:


Book issue related to the thesis:


Other papers:


Other co-authored papers:


Other book issues:


