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RESISTANCE TO β-LACTAMS AND GLYCOPEPTIDES IN STAPHYLOCOCCI AND STREPTOCOCCI

(A REVIEW)*

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Molecular mechanisms of the action of β-lactam and glycopeptide antibiotics, as well as genetic background and phenotipical features of the resistance of staphylococci, streptococci and enterococci to these antibiotics are reviewed. Furthermore, susceptibility patterns concerning β-lactam and glycopeptide drugs of staphylococcal, streptococcal, as well as enterococcal strains isolated from clinical specimens at the Semmelweis University of Medicine, Budapest, Hungary between January 1997 and December 2000 are also presented.

Keywords: β-lactam resistance, glycopeptide resistance, staphylococci, enterococci, streptococci

Introduction

Staphylococci, enterococci and streptococci are the most frequently isolated bacteria from extraintestinal clinical specimens. *Staphylococcus aureus* can cause both local, disseminated, disseminated generalized infections such as furuncle, abscess or postoperative wound infections and bacteriaemia, endocarditis, meningitis, sepsis and osteomyelitis. Exfoliative toxin producing strains can also cause scalded skin syndromes and toxic shock syndrome toxin-1 producing ones can induce toxic shock syndromes, too. Coagulase-negative staphylococci (CNS) and enterococci are common causes of any – mainly catheter associated-nosocomial infections in premature infants

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and the elderly, as well as immunocompromised patients. Enterococci are among the most frequent mircobes of nosocomial infections, and are selected by therapy with cephalosporins and other antibiotics to which they are naturally resistant. In patients, the most common sites of infection are the urinary tract, wounds, biliary tract, and blood. They may cause meningitis and sepsis in neonates, and endocarditis in adults. *Streptococcus pneumoniae* is one of the leading agents causing community-acquired bacterial pneumonia, acute otitis media and meningitis. *Streptococcus pyogenes* causes impetigo, acute tonsillitis, phlegmone and scarlet fever most frequently; whereas *Streptococcus agalactiae* is often an inhabitant of the human vaginal flora and can infect infants during delivery presenting fulminate sepsis, meningitis, or respiratory distress syndrome. In spite of the severity of the infections caused by these two latter species, they can be controlled, managed and overcome because of their sensitivity to some penicillins, cephalosporins and macrolides.

On the contrary, significant increase of resistance to antibiotics among *S. aureus*, CNS, *Enterococcus faecalis*, *Enterococcus faecium* and *S. pneumoniae* observed recently has created great therapeutic problems [1–10].

In this short review we focus the attention particularly on the β-lactam resistance and glycopeptide resistance of these "problematic" bacteria.

Mechanisms of action of β-lactam antibiotics

The site of action of β-lactams is the cell wall synthesis of bacteria [11]. Penicillins and other members of the β-lactam family kill bacteria by inactivating a set of transpeptidases that catalyze the final cross-linking reactions of peptidoglycan synthesis. Any penicillin derivate inhibits these enzymes by acting as a structural analogue, forming an irreversible penicilloyl-enzyme complex that is analogous to the transient acyl-enzyme formed during the normal transpeptidation reaction. Transpeptidases are difficult to assay and are usually detected and studied as penicillin-binding proteins (PBPs). Bacteria possess multiple transpeptidases that have different functions in the synthesis of the peptidoglycan during the cell cycle. Besides these physiologically important PBPs (PBPs with a high relative molecular mass – high M_r PBPs) bacteria contain one or more low-M_r PBPs that function as D-Alanine carboxypeptidases. Inactivation of low-M_r PBPs does not lead to killing of bacteria.

The consecutive steps of β-lactam action consist of binding of drug to cell receptor PBPs, inhibiting enzyme activities of PBPs [12], inhibiting phospholipid synthesizing enzymes, changing conformations of PBPs in membranes, changing membrane protein and lipid composition and integrity [13, 14], activating cell wall autolysins and activating murosomes [15]. As results of these activities inhibition of

transpeptidation of peptidoglycan molecules in cell wall synthesis, and deposition of uncrosslinked cell wall materials take place. The cells have structureless cross walls, thin sidewalls and enlarged cytoplasm volumes. Disturbance of selective permeability and active transport of bacterial membranes occurs, and proteins, lipids and DNA are released from the β-lactam treated bacterial cells [16]. Hyperactivity of autolysins and murosomes eventually lead to bacterial cell lysis and death.

Basic structural and physiological requirements for a β-lactam action are an intact β-lactam ring and the presence of side chains, growing bacteria, the presence of cell wall synthesis, accessible PBPs and high affinity of the drug to PBPs. In a hypertonic environment (e.g. urine in kidney, pyelon and bladder), damaged cell wall formation in bacteria leads to formation of spherical bacterial protoplasts from Gram-positive bacteria. These protoplasts are limited by the fragile cytoplasmic membranes. The pathogenetic role of these forms has still been questioned; although there are experimental evidences that abscesses in kidneys of mice infected with *S. aureus* and subsequently treated with some penicillin-contained staphylococcal protoplasts (Rozgonyi et al. unpublished data). If such protoplasts are placed in a normotonic environment they take up fluid, swell and may explode. Upon withdrawal of β-lactam from the environment they revert to cell wall synthesizing bacteria in a hypertonic environment or medium.

There are some basic factors affecting β-lactam effectiveness such as the degree of pH, binding time of the drug, the degree of affinity to PBPs and the competition ability for binding sites if more than one drug are applied (e.g. a penicillin and a cephalosporin).

Determination of β-lactam activities

As it later will be detailed, the simple disc diffusion test has a number of limitations to determine the true antibacterial activity of a given β-lactam derivate. Therefore, attempts have been made to use routinely a quantitative test such as macrodilution, microdilution or E-tests of β-lactams to measure exactly their minimal inhibitory concentrations (MICs) for a given strain of a species. All these together with the determination of the minimal bactericidal concentration are now equipped and convenient automated or semi-automated equipments, like Vitek (bioMérieux) are available to use panels or kits for the quantitative determination of the effectiveness of antimicrobials. However, all of these give static minute information about the effectiveness of the drugs. Considering the fact that all β-lactams are closely related structures, fine methods such as binding assay, growth kinetics, killing kinetics in

Bioscreen (Labsystem) and ultrastructural examinations have to be applied in order to make a more exact determination of the efficacy of these drugs against a given bacterial species.

Mechanisms of action of glycopeptide antibiotics

The glycopeptide antibiotics vancomycin and teicoplanin exert at least three consecutive or simultaneous effects on Gram-positive growing bacteria. They bind to the cell walls and cell membranes and alter the selective permeability of cells. Getting into the periplasmic space they inhibit the peptidoglycan synthesis by establishing complexes with D-Ala-D-Ala ends at the level of UDP-N-acetyl muramic acid pentapeptide formation. Both transglycosylation of disaccharides to form the polysaccharide backbone of cell walls and transpeptidation of pentapeptides to form peptide cross-bridges are inhibited either by a direct binding to transglycosylase and transpeptidase enzymes or by the above mentioned complexes preventing their access to their substrates. With similar mechanisms they eventually inhibit both RNA and DNA synthesis probably at the mesosomal-ribosomal complexes.

The effectiveness of glycopeptides also depends on a number of factors similar to those of β-lactams; however, the most critical is the penetrability through the cell wall cell-membrane barrier because of their highly hydrophobic and sterically large molecules. An outmost hydrophilic glycocalyx layer can hinder the glycopeptide penetration.

Resistance definitions

Resistance is an ability of bacteria to resist or tolerate the effect of an antimicrobial agent at therapeutic concentration. In the clinical aspect it is a quantitative measure, the MIC is higher than the maximal dose tolerable by the host.

Multiple resistance or *polyresistance* means that the bacteria are resistant simultaneously to a number of antibiotic agents with different chemical structures.

Cross resistance means that the bacterium is resistant to all derivatives coming from the same basic structure (e.g. macrolide antibiotics).

Extrinsic resistance means that the basis of the resistance is an extracellular factor excreted by the bacterium (e.g. β lactamase).

Intrinsic resistance means that the basis of the resistance is an intracellular known or unknown factor (e.g. binding protein).

β-lactam resistance in staphylococci

Antistaphylococcal β-lactams

Among the many β-lactams only few are considered as true antistaphylococcal ones; these are penicillin-G, -V, the aminopenicillins + β-lactamase inhibitors, methicillin, isoxazolylpenicillins, 1st and 2nd generation cephalosporins. Bacteria of the genus *Staphylococcus* exhibit a variety of mechanisms to resist against β-lactams.

Extrinsic resistance

Modifying or destroying the molecules of β-lactams play outstanding roles in this resistance mechanisms.

Side chain splitting

Penicillin- and cephalosporin-acylases and -amidases produced by staphylococci can split the side chains of certain preparations of penicillins and those of cephalosporins (Fig. 1) resulting in a penicillin or cephalosporin nucleus lacking side chains [11], consequently possessing only a weak antistaphylococcal effect. This type of resistance is clinically not significant probably because of the much higher affinities of the antistaphylococcal penicillins and cephalosporins to their binding sites in bacteria than those of the enzymes for their substrates.

Hydrolysis of the β-lactam ring (penicillin resistance)

In contrast to the previous mechanisms, hydrolysis of the β-lactam ring of the penicillins and cephalosporins by staphylococcal β-lactamases is a clinically very significant resistance mechanism leading to a complete destruction of the β-lactam ring and to a loss of antibacterial activity of such drugs (Fig. 1). Staphylococcal β-lactamases have a narrow spectrum which means that they hydrolyze only penicillins very efficiently, except for β-lactamase-stable penicillins such as methicillin and isoxazolyl-β-lactams. They fail to hydrolyze or very weakly hydrolyze cephalosporins and carbapenems. Staphylococci produce rarely β-lactamases constitutively, but the inductive production is very common. Both penicillins and cephalosporins may be good or bad β-lactamase inducers in staphylococci regardless of their antistaphylococcal activity.

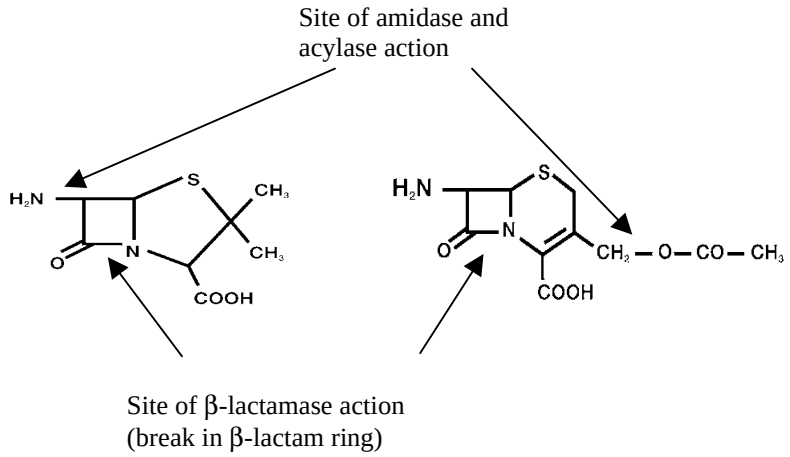

Fig. 1. Basic structures of 6-aminopenicillanic acid and 7-aminocephalosporanic acid and the sites of action of inactivating enzymes

There have been revealed approximately 50 types of staphylococcal β-lactamases on the basis of their amino acid sequences. They have been classified as types A, B, C and D on the basis of their interaction with antibodies raised against purified PC1 enzyme [17–21]. They show a variety of substrate profiles, inducibility, Vmax (μM/min) and K_M (μM) [22]. Hungarian strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* showed 86%–88% resistance to penicillin (Table 1). The activity of staphylococcal β-lactamases can be inhibited sufficiently by clavulinic acid, sulbactam and tazobactam binding irreversibly to them with high affinity. These inhibitors protect hydrolysable penicillins from destruction when they are administered in combination [11, 19].

Genetic basis of staphylococcal β-lactamases

The structural gene is the *blaZ*; it codes for the β-lactamases in staphylococci. The *blaZ* is often found on large plasmids, but there are evidences for transposons containing *blaZ*. The transposons, however, may not have specific sites for insertion and complete plasmids are capable of integrating into the chromosome. It is to be expected that the gene will be

found in many different chromosomal locations. Two of the regulatory genes *blaI* and *blaR1* are found together with *blaZ* on large plasmids. The *blaI* is the gene for the classical repressor protein *BlaI*. This protein binds specifically to the *blaZ* operator region. The *blaR1* is the gene for a putative signal sensor-transducer protein. Another regulatory gene, *blaR2* is always a chromosomal gene. For most isolates of β-lactamase producing staphylococci the enzymes are produced inducibly, only the D-type enzyme in one strain is produced constitutively. The model for induction is that the inducer's β-lactam binds to the C terminus region of *BlaR1*, causing a signal to be passed to the *BlaI* protein. As a result of the signal the *BlaI* binds no longer to the operator region, and so the transcription of *blaZ*, *blaR1* and *blaI* may start. Both the synthesis of the β-lactamases and the regulatory proteins are increased [19, 23, 24].

Detection of β-lactamases

Many tests for β-lactamase production, including either chromogenic reactions or detection of the destruction of antibiotic activity have been proposed [23]. Penicillin is the antibiotic recommended for both disc diffusion and dilution antimicrobial susceptibility testing. The disc diffusion sensitivity test can be done by using a 10-μg penicillin disc in an agar-diffusion sensitivity test. β-lactamase producing strains will show no or very narrow inhibition zones around the disc depending on the length of incubation. Strains that have a MIC greater than 0,03 μg penicillin/ml must be confirmed as susceptible by a β-lactamase detection test. The chromogenic methods are faster and more convenient. They are divided into those in which the hydrolysis of the β-lactam itself engenders a color change and those in which this change depends on a linked reaction. The first type test uses the nitrocephin as a chromogenic cephalosporin substrate, which changes on hydrolysis from yellow to pink. Nitrocephin can be used in a test tube assay or as a disc test. Linked detection systems include the iodometric and acidimetric methods. The product of the hydrolysis of β-lactams reduces iodine to iodide, this way a decolorisation of starch-iodine occurs.

Acidimetric test is based on the fact that opening the β-lactam ring generates a free carboxyl radix, and this acidity can turn bromocresol purple from violet to yellow in an unbuffered system [17, 19, 23, 25].

Table I

Susceptibility of isolates of most frequent Staphylococcus *species cultured from patients examined/treated at the clinics of Semmelweis University of Medicine, Budapest, Hungary from January 1997 to December 2000**

Antibiotic	No. of isolates tested	Percentage		
		Sensitive	Intermediate	Resistant
Staphylococcus aureus				
Penicillin	3036	13.9	0	86.1
Oxacillin	2996	73.1	1.3	25.6
Vancomycin	2940	99.6	0.1	0.3
Teicoplanin	2203	91.4	5.8	2.8
Staphylococcus epidermidis				
Penicillin	1857	9	0	91
Oxacillin	1816	44.8	1.1	54.1
Vancomycin	1806	99.5	0.2	0.3
Teicoplanin	1460	92.9	5.1	1.9
Staphylococcus haemolyticus				
Penicillin	271	16.6	0	83.4
Oxacillin	266	66.2	0.4	33.4
Vancomycin	263	99.2	0	0.8
Teicoplanin	207	83.1	8.2	8.7
Staphylococcus hominis				
Penicillin	269	15.2	0	84.8
Oxacillin	267	50.9	0.7	48.3
Vancomycin	265	100	0	0
Teicoplanin	220	94.1	4.1	1.8

* Results of disc diffusion test

Intrinsic resistance

Staphylococci have different ways to become intrinsically resistant to β-lactam antibiotics. The primary targets of β-lactam antibiotics are the PBPs. These enzymes (PBPs) are responsible for the polymerization of peptide moieties of the peptidoglycan chains, which in staphylococci are cross-linked by a

characteristic pentaglycyl side chain [26]. Staphylococci can alter their PBPs either by mutation, or more efficiently and clinically more relevant, by acquisition of a foreign DNA element, a transposon, coding for methicillin resistance (MR). MR determinant element is called *mec*. *Mec* determinant confers to the staphylococci an intrinsic cross-resistance against all β-lactams regardless of their accessibility by β-lactamases.

Phenotypic characteristics of MR

Two main phenotypes of resistance occur among MR staphylococci: homogenous resistance (class 4) and heterogeneous (classes 1–3). Clinical isolates have been divided into four classes according to MR expression by Tomasz and co-workers [27] as follows: class 1 (MIC 1.5–3.0 μg /ml for ≥ 90% of population, HRS highly resistant subpopulation, 10^{-8}–10^{-6}), class 2 (MIC 6.0–12.0 μg /ml for ≥ 90% of population, HRS, 10^{-5}–10^{-4}), class 3 (MIC ≥ 50 μg /ml for majority of population, HRS, 10^{-3}–10^{-1}), class 4 (homogeneously highly resistant, MIC > 400 μg /ml). Until now the molecular nature of the heteroresistance was not satisfactorily resolved.

Mutation to MR is accompanied by multiresistance of cocci and MR is phenotypically suppressed at elevated temperature (42°C) but is genotypically stable [28]. It is expressed best at 30°C on 2% NaCl Mueller-Hinton agar, when incubation lasts for 48h.

Properties of methicillin-resistant staphylococci

They have higher membrane lipid content in their membranes and lysozyme resistant peptidoglycans [29, 30]. They have longer generation time, consequently they show lower growth rate than their methicillin-sensitive (MS) counterparts. MR cells show retarded cell separation [31]. In experimental infections they need higher number of viable counts to cause LD50 but they show higher index of organ persistence than their MS counterparts. MR cocci show high virulence in immunocompromized hosts [32].

Genetic basis of methicillin-resistance

The *mec* determinant resides on a DNA element of more than 30 kb that is absent in susceptible staphylococci. This DNA element seems to integrate in the genome of *S. aureus* at a specific site, between the *spa* (protein A) and *purA* (adenine requirement) genes. The *mecA* gene is only a small part of this

element. It produces a low-affinity 76 kDa PBP2'. The core sequences of the *mec* element consist of this *mecA* gene and a hypervariable segment of 3'DNA that ends in an insertion-sequence like element. Upstream of *mecA*, its operator and promoter regions separate a regulatory element, which contains the *mecR1* and *mecI* genes. The gene *mecI* codes for a repressor protein (*MecI*). This protein has strong similarity to *BlaI,* the repressor of the *blaZ* gene. The product of the *mecR1* gene, the *MecR1* is similar to *BlaR1, which* is involved in the induction of β-lactamase enzymes. Because of their great similarity to *MecR1* and *MecI*, the regulatory elements *BlaR1* and *BlaI* can also regulate *mecA* transcription. In the absence of *mecI–mecR1* and β-lactamase regulatory elements, there is a constitutive PBP2' production, but some strains are strongly repressed and produce PBP2' only after induction. Methicillin and oxacillin seem to be not enough strong inducers. The resistance is established very slowly, full induction on methicillin-containing plates is seen only after 48 hours. Many clinical strains that carry the *mecI* and *mecR1* genes produce PBP2' constitutionally in high amounts, they have point mutations or deletions that inactivate the repressor.

The expression of the *mecA* gene largely depends on the activity of genes termed factors essential for methicillin-resistance (*fem).* The *femA* and *femB* is involved in the formation of the peptidoglycan pentaglycine interpeptide bridge. Mutants lacking *femA* or *femB* are able to attach only two or three glycines to the cross bridge. The precise role of these two cytoplasmic proteins is unknown, but it is known that every insert in *femA* and *femB* abolish methicillin-resistance completely [26, 33–36].

The first MRSA containing the *mec* determinant was isolated in 1960. At that time the incidence of MRSA was less than 0.1 percent of all isolates, but now they are spread all over the world, mainly in hospitals where is a constant strong antibiotic pressure. Clonal analysis of MRSA and other *Staphylococcus* species supports the hypothesis that there may be a dissemination of the *mec* determinant by horizontal transfer [26]. The MR frequency in Hungarian *Staphylococcus* strains is shown in Table 1 and in Fig. 2. The results of molecular detection of methicillin resistance are shown in Fig. 3. The MIC range of oxacillin for *Staphylococcus aureus* strains proved to be wide, in contrast to that for *Staphylococcus epidermidis* strains, which required high MICs to inhibit growth.

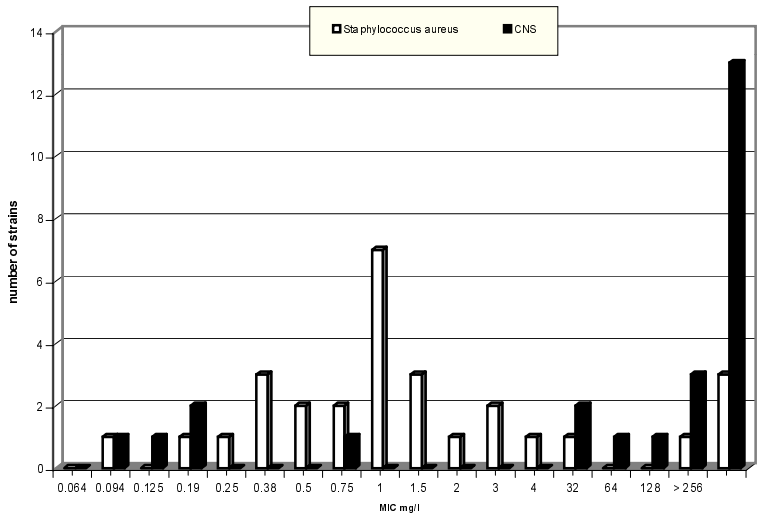

Fig. 2. Distribution of the minimal inhibitory concentrations of oxacillin for *Staphylococcus aureus* and CNS strains isolated in the year 2000 from patients treated at the clinics of the Semmelweis University, Budapest, Hungary

Detection of MR

The broth macrodilution method has proven to be the most appropriate for quantitative detection of MR. However, it is extremely laborious and time consuming. Therefore, broth microdilution is also accepted in duplicate rows. To screen MR colonies, seeding 6 μg/ml oxacillin agar is recommended. For the routine susceptibility testing, the NCCLS has approved 1 μg oxacillin disc, but in many countries higher oxacillin contents have been applied. Using any of these methods the following requirements are common and accepted: high ($\leq 10^6$ CFU/ml) inoculum, 2% NaCl in the medium, low (30–35°C) incubating temperature and prolonged incubation time (48h) [37–40]. Of course, if the MR colonies appear earlier one can report MR.

Legend

1. marker
2. no sample
3. *S. aureus* 2260
4. *S. aureus* 3282
5. *S. aureus* 2724
6. *S. aureus* 1978
7. *S. aureus* 2794
8. *S. aureus* 2260'
9. *S. aureus* 3282'
10. *S. aureus* 2724'
11. *S. aureus* BB270 positive control
12. *S. aureus* ATCC 29213 negative control

Fig. 3. Detection of the *mecA and femA* genes by multiplex PCR from *Staphylococcus aureus* isolates

Borderline oxacillin resistance

Borderline strains may be divided into two categories on the basis of whether they have *mecA* gene. Borderline strains that contain *mecA* gene are extremely heterogeneous methicillin resistant strains that produce PBP 2a [41]. Borderline strains that do not contain *mecA* gene can be differentiated phenotypically from extremely heterogeneous *mecA* positive strains, as highly resistant clones do not occur. This borderline resistance has been hypothesized to result from modification of normal PBP genes or overproduction of staphylococcal β-lactamase. Tomasz et al. reported alterations of penicillin binding by PBPs 1, 2 or 4 in β-lactamase-negative, *mecA*-negative borderline clinical strains [42]. These binding alterations are the result of point mutation in the penicillin-binding domain [43]. Overexpression of PBPs also can produce low-level resistance [44]. Borderline strains that are β-lactamase hyperproducers, and show relatively high levels of β-lactamase activity in biochemical assays, and exhibit a lowering of MICs into the susceptible range upon addition of β-lactamase inhibitors, or upon elimination of the β-lactamase plasmid [45]. As early as in 1968 Rozgonyi and Rédai reported first

that some MRSA strains could inactivate methicillin in their stationary phase of growth, while oxacillin both in the exponential and stationary phases [46]. Later Montanari et al. found a new β-lactamase that hydrolyses methicillin in the membrane fraction of clinical isolates of *S. aureus* with borderline susceptibility to this drug. Kinetic assays demonstrated that this second more specific inducible β-lactamase, the methicillinase is more likely to be responsible for the borderline phenotype than an increased amount of the penicillinase [47].

Other mechanisms of β-lactam resistance

Penicillin tolerance means that the drug can inhibit the growth of, but cannot kill the staphylococci. This results from failure of the β-lactam drug to activate murosomal and/or autolytic enzymes in the peptidoglycan break down, or the lack of these enzymes.

An overproduction of structured cell walls and/or alteration in the membrane lipid amount and composition may also lead to a high tolerance to β-lactams [30].

Another resistance mechanism is due to the absence of some penicillin receptor in the membrane. It occurs as a result of chromosomal mutation.

Glycopeptide resistance of *Staphylococcus aureus*

Since the emergence of MRSA, the use of the glycopeptide vancomycin and teicoplanin has been the only uniformly effective treatment for such staphylococcal infections. The recent appearance of glycopeptide-resistant coagulase-negative staphylococci [48–53] has heightened concern about whether or not *S. aureus* could acquire glycopeptide resistance. The emergence of such resistance could produce morbidity and mortality similar to that caused by *S. aureus* infections in the pre-antibiotic era.

The first well-documented clinical infection caused by an intermediate glycopeptide resistant strain of *S. aureus* was reported from Japan in 1996 with a MIC of 8 μg/ml [54]. According to the NCCLS, this strain is certainly not susceptible (defined as having a MIC of <4 μg/ml), but is also not yet resistant (defined as having MIC of 32 μg/ml or greater). This type of *S. aureus* was therefore described as vancomycin intermediate sensitive *S. aureus* (VISA), or more particularly to glycopeptides (GISA). Since this first isolation, there have been further three cases of clinical infections due to strains with reduced vancomycin susceptibility reported from the United States in Michigan, New Jersey, and New York [55].

In Europe, no *S. aureus* strains with reduced susceptibility have been reported yet. Lessing et al. [56] conducted a retrospective audit of altogether 137 episodes of blood stream infection due to *S. aureus* from London, United Kingdom. The authors re-tested 32 stored *S. aureus* stock-cultures (25 MSSA and 7 MRSA), but found no isolates with reduced susceptibility, or resistance to vancomycin. Despite relatively early reports about clinical isolates of glycopeptide resistant coagulase-negative staphylococci [48–52], moreover the successful *in vitro* transfer of vancomycin resistance genes from *E. faecalis* into *S. aureus* [57], as well as the emergence of GISA strains, glycopeptide fully resistant (MIC ≥ 32 μg/ml) *S. aureus* primary clinical isolate has not been reported yet. Although there is a great concern about the emergence of such strains since from one of the U.S. isolates of GISA a more resistant sub-population (MIC = 16 μg/ml) could be easily selected *in vitro* by population analysis in culture media with a frequency of 10^{-3}. After the selection, this sub-population still remained heterogeneous and contained approximately 10^{-5} bacteria capable of growing even at a vancomycin concentration of 16 μg/ml [58]. It can be easily predicted that such a selection may take place *in vivo* during vancomycin and teicoplanin therapy of infections due to GISA strains.

With Professor Waldvogel's words [59]: "*Et tu mi, fili?*" (means "You, my son, as well?"). This was Julius Caesar's outcry when, surrounded by conspirators ready to stab him to death, he discovered among them Brutus, his adopted son. Caesar's outcry was one of surprise, but also one of recognition that Brutus's betrayal could have been predicted.

Possible mechanisms of glycopeptide resistance of S. aureus

The exact mechanism(s) of glycopeptide resistance has not been revealed yet. Significant amount of data have accumulated, however, allowing to predict the resistance mechanism(s). Glycopeptide resistance may have emerged in *S. aureus* because of interspecies transfer of resistance genes (i) or selection of resistant mutants as a result of prolonged antimicrobial therapy (ii).

Ad (i), the ability of Gram-positive micro-organisms to acquire glycopeptide resistance genes became a matter of concern with the emergence of vancomycin-resistant enterococci (VRE) [60], and vancomycin resistance genes have been transferred from VRE strains to S. aureus in vitro [61]. However, none of the clinical GISA isolates have had *vanA*, *vanB*, *vanC1*, *vanC2*, or *vanC3* genes yet [62], suggesting that interspecies transfer of resistance genes from VRE is not the in vivo mechanism by which glycopeptide resistance developed in these *S. aureus* isolates.

Ad (ii), certain common factors in the cases of the two independent U.S. cases suggest that cellular modification due to prolonged vancomycin exposure was probably responsible for the emergence of glycopeptide resistance in these isolates [63]. In both cases, patients received multiple prolonged courses of vancomycin for severe MRSA infections. The patients' MRSA isolates, as well as their GISA had similar MIC to antimicrobials other than vancomycin. In addition, *S. aureus* isolates that had intermediate vancomycin resistance had increased extracellular surface material associated with the cell walls of the cocci. This finding is compatible with that observed in *S. aureus* with intermediate glycopeptide resistance induced in vitro [64–66]. The thickened extracellular surface material has been shown to sequester vancomycin [64] and to reduce the susceptibility of *S. aureus* to vancomycin [66]. The exact mechanism, however, has not been determined, yet, but these data suggest that it emerged through the selection of naturally occurring resistant mutants during prolonged exposure to vancomycin. The phenomenon that teicoplanin resistant strains of different *Staphylococcus* species are sensitive to vancomycin has also not been sufficiently explained [53]. Other observations are also pursuing the hypothesis that PBPs are overproduced, allowing them to compete with vancomycin for binding to peptidoglycan precursors [66, 67].

Elucidation of these mechanisms will be essential for the development of effective therapeutic agents in the future.

Detection of glycopeptide resistant S. aureus

The emergence of GISA strains worldwide suggests that *S. aureus* strains with full resistance to vancomycin may eventually emerge. These episodes emphasize the need to enhance laboratory capacity at the hospitals and national levels to recognize these strains. Furthermore, an effective international co-operation with exchange of national data would seem also beneficial for all parties.

Identification of the species should be carried out based on the standard reference methods [68]. Data suggest that antibiotic susceptibility tests performed by the disc-diffusion method may differ greatly from those carried out by broth microdilution techniques [63]. On disc-diffusion testing, the two U.S. isolates were read as susceptible to vancomycin at 18 and 17 mm of zone of inhibition, respectively. In contrast, they were intermediately susceptible to vancomycin with a MIC of 8 μg/ml when tested with broth microdilution [63]. Although, data concerning glycopeptide agar-gradient-diffusion susceptibility tests (E-test) in case of GISA strains as to compared to broth micro-dilution tests are not readily available, it seems that vancomycin susceptibility test should be performed with one of the above quantitative methods at least in case of high risk for vancomycin resistance [56]. All the other

antimicrobials should be tested according to the guidelines of NCCLS with the quantitative methods whenever it is possible. It is especially true for oxacillin (methicillin). If available, *mecA* PCR test should also be performed.

Although not routinely done, population analysis profile of *S. aureus* strains with intermediate susceptibility should be also performed as described by Edmond et al. [69], and Siedradzki et al. [58] to detect heterogeneous resistance. Although accumulated data about clinical isolates of GISA strains do not support the idea of the transfer of vancomycin resistance genes from VRE, because of the low number of cases this possible mechanism of resistance cannot be excluded. Consequently, it seems advisable to follow up all the GISA isolates for *vanA*, *vanB*, *vanC1*, *vanC2*, or *vanC3* genes by means of PCR techniques in suitable reference laboratories [60–62]. Molecular epidemiology studies on the isolated GISA strains (PFGE, DNA fingerprinting) should be performed in reference laboratories to assist epidemiological surveillance [58, 59, 63].

Possible origin of glycopeptide resistance

The selection of resistant mutants under the pressure of prolonged glycopeptide exposure on the normal staphylococcal and enterococcal flora seems to be the most probable origin. It may take place both *in vitro* – that has been proven – and *in vivo* – that remains to prove.

The extensive use of 3rd generation oral cephalosporins worldwide including Hungary has contributed indirectly to this selection process since staphylococci and enterococci are inherently resistant to these drugs.

Acquired resistance may be established by intra- and interspecies transfer of vancomycin resistance genes from enterococci. This has occurred so far *in vitro* only.

In Hungary phenotypically vancomycin resistant *S. aureus* strains have rarely been isolated (Table 1 and Fig. 4). However, teicoplanin resistant strains have been found in both *S. aureus* isolates and many other *Staphylococcus* species [53].

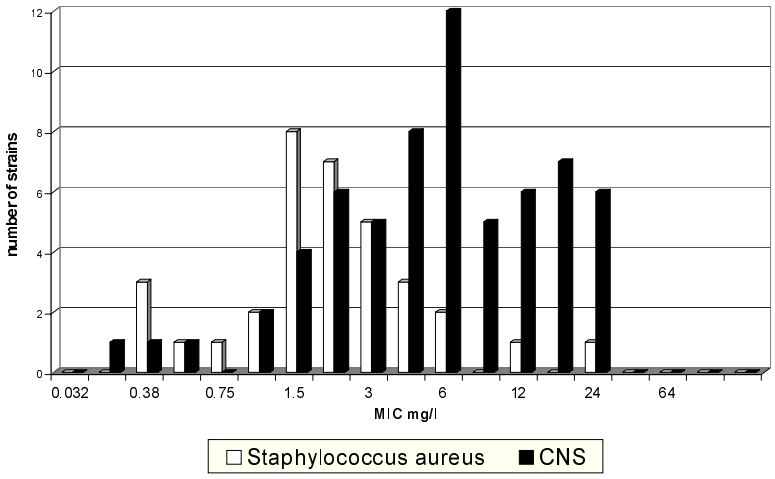

Fig. 4. Distribution of the minimal inhibitory concentrations of teicoplanin for *Staphylococcus aureus* and CNS strains isolated in the year 2000 from patients treated at the clinics of the Semmelweis University, Budapest, Hungary

β-lactam-resistance in enterococci

Mechanisms of β-lactam resistance

Intrinsic resistance

Enterococci possess relative intrinsic (naturally inherited) resistance against penicillin-G, -V, as well as the 2nd and 3rd generation cephalosporins. This resistance is attributable to one or more of the below described mechanisms. The drug cannot access the target, mostly because the cell membranes of enterococci contain high amount of lipids, furthermore the surfaces of the cocci appear to be highly hydrophobic. There may be an increased efflux of the drug from the periplasmic space, which may decrease the concentration of β-lactams to an insufficient level. Then, autolytic enzymes of the enterococcal cell walls may be deficient; consequently they fail to cause the death of the cocci. Enterococci grow relatively slow, and this phenomenon provides a relative protection against β-lactam antibiotics.

β-lactamase production

β-lactamase production in *E. faecalis* was first detected in 1983 [70]. The enzyme is a typical penicillinase. It has a narrow spectrum and is completely cell-bound, furthermore plasmid encoded, therefore transferable. Unlike in case of *Staphylococcus* strains, where enzyme production is inducible, β-lactamase is constitutively produced by enterococci. The enzyme is inhibited by clavulanic acid [71]. Nucleotide sequence of the enterococcal β-lactamase gene, *blaZ*, confirms that the enzyme is indistinguishable from some staphylococcal type A β-lactamases. The gene has also been shown to have a chromosomal location in some strains of *E. faecalis* and may be incorporated in a transposone-like element [72]. β-lactamase production has also been shown in *E. faecium* since then [73]. Although most β-lactamase producing enterococci have exhibited high level resistance to gentamicin, the mechanisms leading to both resistances are not inseparable [74].

High-level penicillin resistance

Strains of enterococci with unusually high penicillin and ampicillin MICs, in the absence of β-lactamase production, are increasingly being reported [75, 76]. This high level resistance is thought to result from overproduction of the slow reacting PBP 5, a normal PBP of enterococci, which can substitute for other PBPs in the cell wall synthesis [77]. High-level penicillin resistance may result in a loss of synergy with gentamicin, although in case of some strains this may be overcome with high concentration of penicillin [78].

Detection of β-lactam resistance

There are some important differences among species of the *Enterococcus* genus regarding penicillin, aminoglycoside and glycopeptide resistance patterns. Therefore, enterococci causing serious infections should be fully identified. The minimum requirement is the proper identification of the species isolated most frequently from clinical specimens: *E. faecalis*, as well as *E. faecium*. Classical methods (growth characteristics, colonial morphology, conventional biochemical reactions) aid the determination of different species of the genus. A number of commercially available tests also provide sufficient identification of *Enterococcus* strains [79].

Detection of β-lactamase production

When detecting β-lactamase production, one should pay attention to the fact that β-lactamase producing enterococci exhibit a marked inoculum effect, that is, penicillin resistance may not be demonstrated unless a high inoculum (e.g. 10^7 CFU/ml) is used. There are commercially available rapid tests for the detection of the enzyme. In these

tests, chromogenic substrates of the β-lactamase enzyme are incorporated into discs, which should be placed onto the plate culture of enterococci. Result of the test is then red based on the change of color of the substrate. Studies also indicate that the classical acidimetric test was less sensitive as compared with these colourimetric assays. Consequently, the use of these tests is highly recommended [80]. However, a strain of β-lactamase producing *E. faecalis* has been reported to fail hydrolyze nitrocephin substrate [81].

Detection of high-level penicillin resistance

Disc diffusion using 10 μg of ampicillin disc indentifies ampicillin and amoxicillin resistance when using *E. faecalis* ATCC 29212 control strain [82]. However, this method does not differentiate enterococci with high-level penicillin resistance (MIC ≥ 128 μg/ml) from those with lower level resistance (MIC = 16–32 μg/ml). As infections caused by the latter may still be managed by a combination of penicillin-G and an aminoglycoside, the MICs of penicillin or ampicillin or amoxicillin should be determined for enterococci causing life-threatening infections, such as endocarditis, sepsis etc. Broth microdilution method is the gold standard for the measurement of MIC of penicillin, ampicillin or amoxicillin in case of enterococci. However, the E-test also seems to be a reliable method, and an acceptable option for the detection of MICs. In a study, its quantitative accuracy compared with agar dilution results was 95% for *Enterococcus* spp. The test procedure is easy to perform, reproducible, and quantitatively and qualitatively accurate [83, 84]. Another option for the detection of high-level penicillin resistance is using commercially available panels that also allow sufficient identification of the isolate based on biochemical patterns, as well as reliable susceptibility profile for selected antimicrobial drugs (e.g. ampicillin, vancomycin) utilizing the positive breakpoint method [85]. The major advantage of these assays is that they parallel provide species identification and susceptibility data at the same time, although misinterpretation of species, and errors determining susceptibility may be observed in such tests. The frequency of β-lactam-resistant enterococcal strains in Hungary is show in Table 2.

If the patient has an allergy to penicillins, or if the enterococcal strain is resistant to ampicillin, which leads to a loss of synergism with aminoglycosides, glycopeptides, such as vancomycin or teicoplanin are the antibiotics of choice. During the past 15 years the majority of *E. faecium* strains have become resistant to ampicillin in many countries [76, 86], and a number of strains have acquired high-level resistance to aminoglycosides [77]. This has apparently occurred via the acquisition of *Tn4001*-like transposons carrying the determinant for the bi-functional AAC (6')-APH (2") enzyme and originating from staphylococci [87].

Table II

Susceptibility to some β-lactam antibiotics and vancomycin of Enterococcus *spp. and* Streptococcus pneumoniae *isolates cultured from patients treated at the clinics of the Semmelweis University of Medicine, Budapest, Hungary in the years 1997–2000**

Antibiotic	No. of isolates tested	Percentage		
		Sensitive	Intermediate	Resistant
Enterococcus faecalis				
Penicillin-G	845	0	21	79
Amoxicillin / Ampicillin	881	89	2	9
Piperacillin	705	90	2	8
Imipenem	815	94	6	0
Vancomycin	857	98.7	0.4	0.9
Enterococcus faecium				
Penicillin-G	71		21.1	78.9
Amoxicillin / Ampicillin	73	61.6	9.6	28.8
Piperacillin	61	70.0	1.6	28.4
Vancomycin	71	90.1	–	9.9
Streptococcus pneumoniae				
Penicillin-G	907	61	13.7	25.4
Amoxicillin / Ampicillin	842	84.8	3	12.2
Cefotaxime	751	92.7	2.4	4.9
Vancomycin	399	99	1	–

* Results of disc diffusion method

Glycopeptide resistance in enterococci

Resistance to glycopeptides in enterococcal clinical isolates was reported first by Leclercq et al. [88] and Shlaes et al. [89] and has been increasingly reported in recent years. By molecular typing of VRE from these outbreaks, it is possible to detect both the spread of particular strains, which is presumably related to poor hospital hygiene (exogenous infections) [90–93], and probably endogenous infections, as indicated by the variety of strains [94]. In 1993 the Centers for Disease Control [95] reported that the total resistance to glycopeptides in enterococci from nosocomial infections in U.S. hospitals increased from 0.3% in 1989 to 7.9% in 1993, enterococcal

strains from patients in intensive care units showed an increase of resistance to these antibiotics from 0.4% to 13.6% in the same period of time.

Not only because of their different pathogenic potency but also because of their variety in antibiotic resistance patterns, it is important to identify enterococci up to the species level as mentioned above [96]. *E. faecium* is more resistant to several antibiotics than *E. faecalis*, and resistance to glycopeptides is also more frequent *in E. faecium*. Enterococcal species occurring in animals preferentially can also have acquired resistance to glycopeptides [97].

Identification criteria for enterococci are that they are catalase-negative, gram-positive cocci, bile-esculin and pyrrolidonylarylamidase positive, and that they grow in 6.5% NaCl broth and possess the Lancefield group D antigen [98].

Up to now six genotypes of glycopeptide resistance are known: *vanA*, *vanB*, *vanC*-1, *vanC*-2 [97, 99], *vanD* [100] and *vanE* [101]. The *vanA* genotype is inducible and the most widespread in enterococci. It is mainly found in *E. faecium*, but also occurs in *E. faecalis*, *E. avium*, *E. durans*, and other species. This type is also the most significant one with respect to the chemotherapy options of VRE infections, since it mediates high-level resistance and cross-resistance to both vancomycin and teicoplanin used in human medicine and also to the animal food additive glycopeptide avoparcin. The *vanB* genotype expresses variable and inducible resistance to vancomycin but not to teicoplanin. The *vanC* genes are characterized by low-level resistance to vancomycin and it is specific to *Enterococcus gallinarum*, *E. casseliflavus* and *E. flavescens* [102, 103]. The *vanD* genes encode variable resistance levels to vancomycin and teicoplanin [100]. The *vanE* genes are present in a single isolate of *E. faecalis* BM4405 and susceptible to teicoplanin and resistant to low level of vancomycin [101]. The *vanA* genotype is encoded by the *vanA* gene cluster, which in some strains is integrated into the transposon *Tn1546*. This transposon can be transferred to other enterococcal strains and species at high frequency when located on conjugative plasmids.

The VanA, VanB and VanD ligase synthesize the depsipeptide D-Ala-D-Lac, instead of D-Ala-D-Ala. In VanA- and VanB-type strains three other enzymes are required for resistance: the VanH dehydrogenase reduces pyruvate to D-Lac [104], whereas the VanX D,D-dipeptidase and the penicillin G-insensitive VanY D,D-carboxypeptidase [105] prevent synthesis of UDP-MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (pentapeptide [Ala]). The VanA and VanB phenotypes of resistance, but not the VanD phenotype, are generally transferable to other enterococci by conjugation. Acquired glycopeptide resistance in enterococci is based on a modification of the target of these antibiotics, the D-alanyl-D-alanine end group of the disaccharide pentapeptide cell wall precursor. The C-terminal D-Alanine is replaced by a D-lactate. The corresponding genetic determinants encoding the ligase, dehydrogenase, D,D-

dipeptidase, and D,D-carboxypeptidase as well as two regulatory genes encoding the response regulator and the sensor/Histidine kinase are located on transposons [106, 107, 108]. The *vanA* protein, a cytoplasmic membrane protein of 39 kDa, is the main component of this resistance mechanism [104, 109, 110]. The VanA phenotype is inducible by vancomycin and teicoplanin and confers high MICs to the three glycopeptides. Strains of the VanB type have acquired inducible resistance to various levels of vancomycin but not to teicoplanin [111]. Constitutive low-level resistance to vancomycin (VanC type) is an intrinsic property of motile enterococci, *E. gallinarum*, *E. casseliflavus* and *E. flavescens* [102, 103, 112].

The actual prevalence of vanC type resistance might even be higher since phenotypic detection of low-level resistance is difficult [113, 114]. Several recent outbreaks of VRE [115, 116] emphasize the need for laboratories to be able to detect the various types of resistance.

Molecular detection of glycopeptide resistance

The PCR method has been extensively applied in bacteriological diagnosis of VRE infections [117]. It has been used for species identification of infectious agents [118–120] and specific detection of antibiotic resistance genes [57]. In the year 1999 our workgroup could detect by PCR the first glycopeptide resistant *E. faecalis* in Hungary [121]. The result is shown in Fig. 5.

Emergence of VRE outside the hospitals

VRE has been detected not only inside the hospitals but also outside them evidencing by their isolation from the wastewater of sewage treatment plants [122, 123]. Some of these plants were located in rural areas of Germany [122] where there has been no hospital in the corresponding town or where the use of glycopeptides in the local hospitals has been exceptional and intravenous.

Glycopeptides have been also used outside the hospitals, as so-called “growth promoters” in animal husbandry. For about 20 years the glycopeptide antibiotic avoparcin has been available as a feed additive in many western European countries and in Australia, although not in the U.S. or Canada. Avoparcin has a glycopeptide chemical formula similar to that of vancomycin and teicoplanin, which have been used in human medicine [124]. This has suggested that avoparcin could have been responsible for the selection of glycopeptide-resistant enterococci, since it is not absorbed from the gut, therefore it may be present at high concentration for a long period of time. In addition, the mechanism of resistance to avoparcin, similar or equivalent to that for vancomycin and teicoplanin [104, 109, 110], is likely to exist.

Avoparcin is used as a feed supplement for broiler chickens, turkeys, pigs, beef and dairy cattle, calves, goats and sheep, too.

Outlook

Experimental conjugative transfer of glycopeptide resistance has been done with *E. faecalis* [116] and also between *E. faecalis* and *S. aureus* [61]. Between *S. aureus* and enterococci horizontal gene transfer is possible under natural conditions, as demonstrated by the similarity of transposons encoding aminoglycoside resistance in *S. aureus* and *E. faecalis* [87] and by the characterization of *E. faecalis* strains carrying a β-lactamase whose origin was *S. aureus* [125, 126]. The recent detection of the *vanA* gene in *Corynebacterium haemolyticum*, *Lactococcus* and *Listeria species* [96, 127, 128], as well as that of *vanB* in *Streptococcus bovis*, indicates the potential for the spread of glycopeptide resistance to other organisms.

Phenotypically vancomycin-resistant strains of different *Enterococcus* species have been isolated in Hungary, too, and their incidence is relatively high (Table 2). However, *vanA* positive *E. faecalis* strains proven by PCR technique were isolated from two clinical cases so far [121, 129].

Penicillin resistant *Streptococcus pneumoniae* (PRSP)

Mechanism of β-lactam resistance

In the relatively few bacterial species in which β-lactamases are unknown to exist the development of high M_r PBPs with decreased affinity for β-lactam antibiotic may be one of the mechanisms that can provide increased resistance. The most spectacular example of PBP-mediated resistance is found in *S. pneumoniae*. Pneumococci that produce β-lactamase have never been reported so far, and their resistance to penicillins and cephalosporins is entirely attributable to alteration of PBPs. *S. pneumoniae* strains posses five high-M_r PBPs (1A, 1B, 2A, 2B and 2X) and the low-M_r PBP 3, which has not been implicated in the killing action of β-lactam antibiotics. The most highly penicillin-resistant isolates (MIC = 2 – 16 μg/ml benzyl penicillin) produce altered forms of PBPs 1A, 2X, 2B and 2A that have reduced affinity for β-lactams. Little is known about the biochemical base of the decreased affinity.

The recognition of conserved sequence motifs within the transpeptidase domains of high-M_r PBPs, and in the Serine β-lactamase and low-M_r PBPs, suggest that enzymes have similar structures. The sequence motifs are located around the active

center of the penicillin-interacting enzymes, and as expected the amino acid changes that reduce the affinity of PBP are within, or close to, these conserved sequences.

Fig. 5. Electrophoretic profile of the PCR product with agarose gel electrophoresis after ethidium bromide staining

Features of resistance

The reduction of affinity for different β-lactam drugs varies greatly with the individual compound. The phenomenon of hetero-resistance can easily be demonstrated among pneumococcus isolates. Intermediate PRSPs have MIC = 1–2

μg/ml, PRSPs possess MIC = 3–6 μg/ml, while high-level PRSPs exhibit a MIC > 6 μg/ml. It has also been observed that PRSP strains are often multiple resistant (macrolides, tetracyclines, chloramphenicol, sulfonamide-trimethoprim combination). The incidence of PRSP in Hungary is shown in Table 2.

Genetic basis and phenotype of resistance

The low-affinity forms of the high M_r pneumococcal PBPs have arisen by recombination rather than mutation as seen in pathogenic *Neisseria* spp., too. Pneumococci are naturally transformable, and mosaic structures in their PBP genes is believed to have arisen by interspecies homologous recombination, although it has proven difficult to identify the source, or rather sources of the divergent regions [130]. The altered PBPs have greatly reduced affinity for almost all β-lactam antibiotics, including the 3rd generation cephalosporins. Penicillin-resistant *S. pneumoniae* strains therefore show cross-resistance to other β-lactam antibiotics. In most resistant isolates, the MICs of 3rd generation cephalosporins are equal to, or just slightly less than the MICs of penicillin. The 3rd generation cephalosporins, however, may still be effective in treating pneumococcal infections caused by penicillin-resistant strains because large amount of these antibiotics can be maintained in the blood and cerebrospinal fluid. However, high-level resistance to 3rd generation cephalosporins has recently emerged [131]. In contrast to high-level resistance to penicillin, which requires alteration of four PBPs, clinical pneumococcal isolates with ceftriaxone MIC as high as 16 μg/ml have alteration of only PBPs 1A and 2X , as the other PBPs have inherently low affinity for 3rd generation cephalosporins and therefore are not involved in the killing of pneumococci by clinically relevant concentration of the compounds [132]. Most recently, strains of *S. pneumoniae* with high level penicillin and cefotaxime resistance were isolated from the respiratory tract of in- and outpatients in Hungary [133].

Detection of β-lactam resistance

β-lactam resistance can routinely be screened by 1 μg oxacillin disc (NCCLS). Susceptibility to oxacillin implies susceptibility to all the other β-lactam drugs. However, resistance to oxacillin indicates the need for the determination of penicillin MIC. Penicillin MIC is measured either by penicillin-G E-test, or by one of the dilution (micro- or macro-) methods with Mueller-Hinton broth containing 5% of horse blood as recommended by NCCLS [37]. Recently, polymerase chain reaction (PCR) techniques have also been introduced into the detection of PRSP strains. Using primers derived from the pneumolysin, autolysin, and DNA polymerase I genes, and the 16S – 23S spacer ribosomal region, PCR has been used successfully to show the presence of *S. pneumoniae* in blood, sputum, cerebrospinal fluid of patients. The nucleotide

sequences of genes coding for the native and altered PBPs (2B, 2X and 1A) of both PRSP and penicillin susceptible *S. pneumoniae* have been reported. PCR primers were designed to amplify the differential nucleotide sequences of these genes in penicillin susceptible and resistant strains. The use of PCR to differentiate between penicillin susceptible and resistant genotypes has been established [134].

Glycopeptide resistant *S. pneumoniae* has not been reported yet.

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