In-vitro selection of resistance to vancomycin and teicoplanin in Enterococcus faecium and Enterococcus faecalis compared with Staphylococcus epidermidis

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Clinical isolates of Enterococcus faecium, Enterococcus faecalis and Staphylococcus epidermidis were studied for their ability to develop resistance by two selection methods, one in broth and one on agar. The MICs for enterococci after exposure to vancomycin ranged from 0.5 mg/L to 4 mg/L, and those after exposure to teicoplanin ranged from 0.25 mg/L to 1 mg/L. No significant increases occurred for E. faecalis, whilst two isolates of E. faecium showed increases of up to eight-fold (from 0.125 mg/L to 1 mg/L). Vancomycin MICs ranged from 1 mg/L to 4 mg/L and teicoplanin MICs reached 32 mg/L for S. epidermidis. Pulsed-field gel electrophoresis analysis of Smal-digested whole chromosomal DNA was performed to compare the genomic DNA of glycopeptide-exposed and wild-type strains. In-vitro exposure to vancomycin did not alter MICs significantly except in one S. epidermidis isolate for which the MIC reached 4 mg/L, whereas the teicoplanin-exposed cultures of S. epidermidis showed increases of up to 64 times the original MIC. Comparable results were achieved with the two selection methods.

Introduction

Enterococci have become a leading cause of nosocomial infections with an increasing frequency of serious infections such as endocarditis or bacteraemia.1 The emergence of glycopeptide resistance in enterococci already resistant to other antimicrobial agents like ß-lactams and aminoglycosides has been reported worldwide in the past decade.2,3 Two phenotypes of acquired resistance to vancomycin and teicoplanin, VanA and VanB, can be distinguished: the VanA phenotype is characterized by high-level resistance to vancomycin and teicoplanin,4 while VanB strains are resistant to vancomycin but susceptible to teicoplanin.5 Resistance in VanB strains is usually associated with the presence of a chromosomal vanB gene cluster,6 whereas VanA resistance is often mediated by self-transferable plasmids.7 Glycopeptide resistance occurring today may be due not only to dissemination of resistance genes, but also to selective pressure as result of widespread antibiotic usage.

The recent reports of vancomycin resistance prompted us to examine whether exposure of enterococci to vancomycin and teicoplanin in vitro can select resistant organisms, as documented for coagulase-negative staphylococci and Staphylococcus aureus.8 Twenty enterococcal species have been described to date; of all clinical isolates belonging to this genus, approximately 90% are Enterococcus faecalis or Enterococcus faecium, so in this study selection was performed with isolates of these two species. For comparison, we also studied three clinical isolates of Staphylococcus epidermidis.

Materials and methods

Bacterial strains

Randomly selected isolates of enterococci and S. epidermidis collected from the General Hospital of Vienna were identified by their appearance, Gram stain and biochemical characteristics (A P1 system; bioMérieux, Marcy l’Etoile, France). The E. faecalis strain, A TCC 29212, and an additional E. faecium strain, BM 4107 (donated by P. Courvalin), were also studied.

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Antibiotics

Vancomycin was a gift of Eli Lilly (Vienna, Austria) and teicoplanin was kindly provided by Wyeth-Lederle (Vienna, Austria).

Susceptibility testing

Antibiotic susceptibility was tested by the disc diffusion method and a standardized broth microdilution method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS).9

In-vitro selection for vancomycin and teicoplanin resistance

Vancomycin- and teicoplanin-resistant mutants were selected using agar and broth dilution techniques with two isolates of E. faecalis, two of E. faecium and three of S. epidermidis, which were randomly selected from clinical material at the General Hospital of Vienna, and with E. faecium BM 4107 and E. faecalis ATCC 29212.

The gradient plate method described by Murray & Hodel-Christian10 was used for agar selection. Each test isolate was grown in 5 mL of BHI containing vancomycin or teicoplanin at 0.5 × MIC for 24–48 h and then plated on gradient plates providing a continuous vancomycin or teicoplanin concentration gradient ranging from below to well above the MIC. Plates were incubated at 37°C for 24–48 h. Colonies visible in the area with the highest concentration of antibiotic were selected and grown again overnight in 5 mL of BHI containing vancomycin or teicoplanin. Twenty microlitres of this overnight culture were subcultured on to gradient plates. Twenty passages of each test isolate were performed. The MIC was determined after every five passages. In order to test the stability of the selected antibiotic resistance, MICs were re-evaluated after five passages in media without antibiotic, but performed exactly as before.

For broth selection, isolates were inoculated into tubes containing vancomycin or teicoplanin serially two-fold diluted in BHI in order to obtain concentrations of the antibiotic ranging from below the MIC to 256 mg/L. After incubation overnight, 10 μL was transferred from the tube containing the highest concentration of the antibiotic showing visible growth into the next set of tubes. The isolates were subjected to two cycles of broth selection; each cycle consisted of four passages in BHI with vancomycin or teicoplanin followed by two passages in BHI or on BHI agar, respectively, without the antibiotic. To ensure that cultures were not contaminated, the fifth passage of each cycle was performed on BHI agar and the growth examined for evidence of multiple colony types. Vancomycin and teicoplanin MICs were determined after each cycle.

A II tests were performed twice for each isolate. If the two replicates differed at the end of all cycles, the highest MIC was taken as the result. Both methods were tested with S. epidermidis and streptomycin as a one-step resistance antibiotic.

Cell wall and cytoplasmic membrane protein profiles

Bacterial cell walls and cytoplasmic membranes were prepared as described by Daum et al. with slight modifications. Briefly, bacteria were incubated in BHI with vancomycin or teicoplanin at 0.5 × MIC (induced) and without the drug. Cells growing logarithmically (optical density at 650 nm, c. 0.25) were harvested by centrifugation. Following washing and resuspension in sodium phosphate buffer (50 mM, pH 7), the cells were sonicated for 1–2 min at 4°C. The resultant broken cell suspension was centrifuged at 4000g for 15 min at 4°C and cell walls were collected. Membranes were separated by centrifugation at 100,000g for 60 min at 4°C. Cell wall and membrane fractions were washed twice and resuspended in buffer. Proteins in the preparations were separated by SDS–PAGE using a 5% stacking and a 10% resolving gel and visualized by staining with Coomassie brilliant blue (Pharmacia LKB, Uppsala, Sweden).

Identification of chromosomal pattern

Whole-cell DNA was extracted and embedded in agarose plugs by the method of Smith & Cantor12 with the following modifications. Plugs containing DNA were incubated in lysis buffer (EC buffer) for 5 h and then incubated in proteolysis buffer (ESP buffer) overnight at 50°C. After the plugs had been washed in TE buffer they were treated three times with 1 mM phenylmethylsulphonyl fluoride (PM SF) in TE for 10 min, washed three more times in TE buffer and digested with Smal using restriction enzyme buffers supplied by the manufacturer (A mersham plc, A mersham, UK). Pulsed-field gel electrophoresis (PFGE) was carried out in 1% agarose gels (A mresco, O H, U SA ) in 0.5 × Tris–borate–EDTA (TBE) using the Pharmacia Gene Navigator System (Pharmacia Biotech, Uppsala, Sweden). Gels were run in 0.5 × TBE at 13°C, 150 V, for 25 h with switch times of 5–35 s with linear ramping. Digestion patterns were visualized by staining with ethidium bromide and photographed in UV light.

Results

Selection of glycopeptide-resistant clones

All tested strains were susceptible to vancomycin and teicoplanin. For the enterococci, vancomycin MICs ranged from 0.25 mg/L to 2 mg/L and teicoplanin MICs from 0.125 mg/L to 0.5 mg/L. For S. epidermidis vancomycin and teicoplanin MICs were 0.5–1 mg/L and 0.5–4 mg/L, respectively.

The results of the agar resistance selection method are
summarized in Table I. In general, MICs for the enterococci increased by two- to four-fold following selection. Two isolates of E. faecium exposed to teicoplanin showed an increase from 0.125 mg/L to 1 mg/L. For S. epidermidis, the final MICs of vancomycin were the same as the initial MICs except in one case, whereas MICs of teicoplanin increased up to 64-fold, reaching the intermediate to low resistance range (MICs = 16–32 mg/L).

The results obtained with the broth resistance selection method (Table II) were similar to those obtained by the agar method. The two MIC values determined for each strain were equal or differed only by one dilution. Both selection methods were validated by exposing S. epidermidis strains to streptomycin and achieving MICs up to 64 mg/L.

After the passages in antibiotic-free medium, MICs were generally equal to or only one dilution lower than those obtained immediately after the passages in antibiotic-containing medium. No cross-resistance to vancomycin was observed for the teicoplanin-selected strains and vice versa.

### Chromosomal pattern

PFGE after SmaI digestion of chromosomal DNA revealed identical patterns for the selected strains and the analogous wild-type strains.

### Cytoplasmic membrane proteins

No differences in the cytoplasmic membrane protein pattern were noted between the selected and the wild-type strains. Even organisms that had developed high teicoplanin MICs did not differ with regard to their membrane protein profile.

### Discussion

Selection of glycopeptide resistance (vancomycin MIC > 16 mg/L; teicoplanin MIC > 16 mg/L) in various Gram-positive bacteria such as S. aureus and coagulase-negative...
staphylococci has been reported previously. Biavasco et al. showed the emergence of resistant clones of strains of S. haemolyticus exposed to teicoplanin, but also of some strains of S. epidermidis. Watanakunakorn described a 128-fold increase from 0.125 mg/L to 16 mg/L in the MIC of teicoplanin in strains of S. aureus.

In this study, enterococci and S. epidermidis were grown in the presence of vancomycin or teicoplanin using two selection methods, one on agar and the other in broth. It was found that strains of S. epidermidis with low-level resistance to teicoplanin can be easily selected by both in-vitro methods. Minor increases in vancomycin MICs were observed.

For E. faecium, teicoplanin MICs increased by two- to eight-fold with both selection methods. Despite the numerous passages, it was not possible to obtain increased vancomycin MICs in E. faecium. E. faecalis MICs showed slight increases of up to four-fold with both vancomycin and teicoplanin. These results contrast with the increasing emergence of glycopeptide-resistance in vivo in the past decade which may be due to the spread of resistant organisms from patient to patient in the clinic rather than the development of resistance in a particular patient due to glycopeptide application.

Regarding staphylococci, comparison of the two glycopeptides showed that teicoplanin differed significantly from vancomycin in developing resistance. This finding agrees with those of Radberg et al., who failed to select staphylococci resistant to concentrations higher than 1 x MIC in vancomycin-exposed cultures. The earlier report of Watanakunakorn has also described the difficulty in inducing resistance to vancomycin in S. aureus.

In contrast to this study, most of the other investigators performed a single-step selection. The multiple-step in-vitro studies of Watanakunakorn as well as the selection methods in this study simulate the conditions that may occur in vivo, when under certain circumstances only subinhibitory concentrations reach the focus of infection or the epithelia colonized by enterococci. These possibilities should be considered in clinical conditions, especially regarding the development of resistance to teicoplanin.

Although the MIC of teicoplanin for staphylococci increased to 16-32 mg/L, the organisms were still sensitive to vancomycin. Generally, with all studied strains no changes were observed in the MICs of vancomycin after selection with teicoplanin and vice versa.

In conclusion, the possibility of emergence of glycopeptide resistance in enterococci after selection in vitro seems to be low. Although exposure to teicoplanin led to certain increases in the MIC, clinically relevant resistance of enterococci was not achieved. In contrast, the development of resistance in coagulase-negative staphylococci due to selective pressure as shown in this study represents a problem of great relevance in the clinical setting.

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References

Glycopeptide resistance in enterococci


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