Investigation of glycopeptide (vancomycin and teicoplanin) tolerance in coagulase-negative staphylococci isolates.

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Running title: glycopeptide tolerance in coagulase-negative staphylococci.

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ABSTRACT

Tolerance to vancomycin and teicoplanin was investigated in 90 clinical coagulase-negative staphylococci (CoNS) isolates by time-kill curve methodology. Only six strains, belonging to the Staphylococcus lugdunensis species, exhibited tolerance. The seven other S. lugdunensis tested displayed weaker susceptibility to bactericidal activity of glycopeptides when compared to the other CoNS species. These phenomena are of concern since S. lugdunensis is recognized as one of the most pathogenic CoNS.
Coagulase-negative staphylococci (CoNS) are involved in infections which require bactericidal treatment such as indwelling foreign body-related infections, endocarditis and meningitis (4, 10). As CoNS become more and more resistant to beta-lactams (2), glycopeptides are often considered as antibiotics of last resort (12). Some investigators, however, have reported glycopeptide tolerance for sporadic CoNS (16, 23). Antibiotic tolerance describes a particular “type of resistance” in bacteria capable of surviving, but not growing, in the presence of a normally lethal dose of a given bactericidal antibiotic (20, 21). As early screenings for glycopeptide tolerance in CoNS have been performed by the controversial MBC/MIC determinations (1, 14, 19, 21), the present study was designed to examine vancomycin and teicoplanin tolerance in a collection of clinically significant CoNS isolates by using the killing curve method, which is considered to be the most reliable by the Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS (14).

An initial set of 79 clinically significant CoNS isolates from 79 individual patients attending the Rouen University Hospital between January 1999 and April 2001 was studied. Strains were identified to the species level with the ID32Staph system (bioMérieux, Marcy l’Etoile, France) and by a gap gene PCR-RFLP assay (24). This set reflected the current epidemiology of CoNS (11), with Staphylococcus epidermidis as a very dominant species (n=66, i.e., 84% of the isolates) and some less frequently encountered species, i.e., S. hominis (n=4), S. capitis (n=3), S. lugdunensis (n=2), S. warneri (n=2), S. haemolyticus (n=1), and S. pasteuri (n=1).

The MICs of vancomycin (Eli Lilly & Co., Saint-Cloud, France) and teicoplanin (Sanofi-aventis, Romainville, France) were determined by the agar dilution method in accordance with CLSI guidelines (15). S. aureus ATCC 29213 was used as a reference control strain. The replicator prong delivered approximately $10^4$ CFU per spot. All the isolates were susceptible to vancomycin (MICs, ≤4 µg/ml) according to the breakpoints of the Comité de l’Antibiogramme de la Société Française de Microbiologie (CA-SFM) (6) and to those of the CLSI (5). Fifty-two isolates were
susceptible to teicoplanin (MICs, \( \leq 4 \mu g/ml \)), 22 isolates showed intermediate susceptibility (MICs, \( = 8 \mu g/ml \)) and 5 isolates were resistant (MICs, \( =16 \mu g/ml \)) according to the CA-SFM’s breakpoints. This categorization corresponds to 74 isolates susceptible to teicoplanin (MICs, \( \leq 8 \mu g/ml \)) and 5 showing intermediate susceptibility (MICs, \( >8 \mu g/ml \) and \( \leq 32 \mu g/ml \)) according to the CLSI’s breakpoints.

Time-kill curves were performed according to CLSI guidelines (14), with mean starting inoculum at 5.6 log\(_{10}\) CFU/ml (standard deviation, 0.1), flasks containing 50 ml of Mueller-Hinton broth (Becton Dickinson, Le Pont de Clayes, France) and antibiotic at 10 times the MIC. Bacterial counts were performed just before and at 6 and 24 h after the addition of antibiotics. To prevent carry-over effects (14, 19), 0.5 ml samples were removed from the flasks, diluted 10-fold and subcultured (0.1 ml aliquots in duplicate) on pre-warmed blood agar plates. Tolerance was defined as a <3-log\(_{10}\) reduction of bacterial count after 24 h according to CLSI (14) and also as a <1-log\(_{10}\) reduction of bacterial count after 6 h according to May et al. (13).

Only two of the 79 isolates tested were found tolerant to glycopeptides: *S. lugdunensis* 111A53, tolerant to vancomycin, and *S. lugdunensis* 111A91, tolerant to teicoplanin (Table 1). Of note, these two isolates were the only *S. lugdunensis* isolates of the 79 CoNS studied. For these two isolates, additional time-kill curves were performed using antibiotic concentrations of 5, 10 and 20 times the MIC to detect a potential Eagle (or paradoxical) effect (14, 21). The latter phenomenon was excluded for both glycopeptides (Table 2) and these additional results confirmed a glycopeptide tolerance. As tolerance has also been defined by a MBC/MIC ratio \( \geq 32 \), MBC/MIC ratios of both glycopeptides were determined for the two *S. lugdunensis* isolates in triplicate according to CLSI recommendations (14), with a starting inoculum between \( 10^5 \) and \( 10^6 \) CFU/ml in Mueller-Hinton broth. The quality control strain *S. aureus* ATCC 25923 was tested along within each assay (14) The MICs were comparable to those determined by the agar dilution procedure (data not shown). Despite disparities between MBC/MIC ratios obtained (Table 2), vancomycin
tolerance of isolate 111A53 (MBC/MIC≥32, 2 of 3 assays) and teicoplanin tolerance of isolate 111A91 (MBC/MIC≥32, 3 of 3 assays) were confirmed.

The frequency of glycopeptide tolerance observed among CoNS in this set (2/79, i.e. 2.5%) is markedly lower than those reported in two previous studies (3/10, i.e. 30% and 17/50, i.e. 34% respectively) (16, 23). These studies are not, however, strictly comparable since the CoNS identification methods were not described, and tolerance screening tests consisted only in MBC/MIC ratios determination. Furthermore, one of these studies (23), involving 50 S. epidermidis isolates, used a less stringent threshold (MBC/MIC≥16) than that which is now recommended (MBC/MIC≥32 ) (14).

Our data prompted us to search for tolerance by killing curves among an additional set of 11 S. lugdunensis isolates including three reference strains (ATCC 43809, ATCC 49576 and ATCC 700328) and eight clinical isolates (3/8 from the Versailles General Center Hospital). Tolerance was found for four of these additional strains (Table 1). Overall, nearly half of the S. lugdunensis strains tested (6/13) met the bacteriological criteria for tolerance to either vancomycin or teicoplanin. In addition, glycopeptides displayed a weaker and, above all, slower bactericidal activity against the seven other S. lugdunensis isolates than against the other CoNS tested (mainly S. epidermidis). In fact, after 6 hours, the reduction in bacterial counts due to vancomycin and teicoplanin was in average 2-log_{10} CFU/ml weaker for the S. lugdunensis strains than for the 77 other CoNS (statistically significant difference, Mann-Whitney U test with P values <0.05) (Fig.1). Of note, all these 13 isolates were fully susceptible to vancomycin (MICs: 0.5-2 µg/ml) and to teicoplanin (MICs: 0.5-1 µg/ml).

This study shows a defect in the bactericidal activity of glycopeptides against CoNS of the S. lugdunensis species. Since its description in 1988 (8), this species, shown to be part of the normal skin flora, has been described as one of the most pathogenic CoNS (9). Indeed, S. lugdunensis
resemble *S. aureus* infections (9) in terms of virulence, tissue destruction, and clinical course, particularly for endocarditis (22). Current *S. lugdunensis* isolates usually remain susceptible to methicillin and other antistaphylococcal antibiotics (9). Thus, the use of glycopeptides for *S. lugdunensis* infections is usually limited to the initial days of empiric treatment when a possibly methicillin resistant *Staphylococcus* has to be considered and to patients with a beta-lactams allergy. The fact that *S. lugdunensis* appears less affected by the bactericidal activity of glycopeptides reinforces the need to identify CoNS to the species level for serious infections as well as to consider tests for detection of tolerance when glycopeptides have to be used for a *S. lugdunensis* infection.

This study also confirms that time-kill curves have the crucial advantage of providing dynamic data (14) and are the most reliable approach to detect tolerance (1, 14), especially by bacterial count reduction after 24 h (14). Expanded use of time-kill curves should lead to increased appreciation of the magnitude of the glycopeptide tolerance phenomenon in CoNS and thus permit relevant comparisons between studies.

Tolerance mechanism remains elusive to this day, even if recent works on *Streptococcus pneumoniae* and *S. aureus* have suggested the involvement of impaired autolysins regulation systems (3, 18) or modification in the cell wall composition (7, 17). Studies should be undertaken to explore the mechanism of the *S. lugdunensis* tolerance phenomenon to glycopeptides observed in the present work and to evaluate its clinical implications.

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We wish also to express our gratitude to M.F. Hellot and M. Etienne for their assistance with statistical analysis.
REFERENCES


TABLE 1. Variations in bacterial counts of 90 isolates of coagulase negative staphylococci (CoNS) after 6 and 24 h of glycopeptide exposition at 10 x MIC

<table>
<thead>
<tr>
<th>Strains</th>
<th>Vancomycin mean (SD) log CFU/ml after 6 h</th>
<th></th>
<th></th>
<th>Teicoplanin mean (SD) log CFU/ml after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>First set strains (n=79)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>77 CoNS</td>
<td>- 2.98 (1.04)</td>
<td>- 4.80 (0.98)</td>
<td>- 3.22 (0.98)</td>
<td>- 5.33 (0.74)</td>
</tr>
<tr>
<td><em>S. lugdunensis</em> 111A53</td>
<td>- 0.31 (0.08)</td>
<td>- 1.94 (0.18)</td>
<td>- 0.66 (0.16)</td>
<td>- 3.77 (0.23)</td>
</tr>
<tr>
<td><em>S. lugdunensis</em> 111A91</td>
<td>- 0.31 (0.08)</td>
<td>- 3.53 (0.20)</td>
<td>- 0.34 (0.10)</td>
<td>- 2.26 (0.16)</td>
</tr>
<tr>
<td>Additional set strains (n=11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 <em>S. lugdunensis</em></td>
<td>- 1.17 (0.74)</td>
<td>- 4.10 (0.70)</td>
<td>- 0.49 (0.26)</td>
<td>- 4.42 (0.62)</td>
</tr>
<tr>
<td><em>S. lugdunensis</em> ATCC49576</td>
<td></td>
<td>- 3.91 (0.17)</td>
<td>- 0.37 (0.11)</td>
<td>- 2.40 (0.33)</td>
</tr>
<tr>
<td><em>S. lugdunensis</em> 111A223</td>
<td>- 0.67 (0.23)</td>
<td>- 1.65 (0.24)</td>
<td>- 0.46 (0.07)</td>
<td>- 2.81 (0.11)</td>
</tr>
<tr>
<td><em>S. lugdunensis</em> ATCC43809</td>
<td></td>
<td>- 2.18 (0.44)</td>
<td>- 0.12 (0.06)</td>
<td>- 1.29 (0.33)</td>
</tr>
<tr>
<td><em>S. lugdunensis</em> 111A229</td>
<td>- 2.24 (0.47)</td>
<td>- 2.42 (0.25)</td>
<td>- 0.31 (0.21)</td>
<td>- 2.33 (0.31)</td>
</tr>
</tbody>
</table>

* Experiences performed in duplicate for each strain. Bold data correspond to a tolerance phenomenon according to the CLSI’s criterion.
TABLE 2. Evaluation of glycopeptides tolerance in 2 *S. lugdunensis* isolates by time-kill curves and MBC/MIC ratios.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Antibiotic concentration</th>
<th>Time-kill curves</th>
<th>MBC/MIC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Variation in bacterial numbers (mean log CFU/ml) after:</td>
<td>Three values from three independent assays</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 h</td>
<td>24 h</td>
</tr>
<tr>
<td><em>S. lugdunensis</em> 111A53</td>
<td>5 x MIC</td>
<td>-0.05</td>
<td>1.72</td>
</tr>
<tr>
<td>vancomycin tolerant isolate</td>
<td>10 x MIC</td>
<td>-0.31</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>20 x MIC</td>
<td>-0.07</td>
<td>1.74</td>
</tr>
<tr>
<td><em>S. lugdunensis</em> 111A91</td>
<td>5 x MIC</td>
<td>-1.16</td>
<td>3.59</td>
</tr>
<tr>
<td>teicoplanin tolerant isolate</td>
<td>10 x MIC</td>
<td>-0.31</td>
<td>3.53</td>
</tr>
<tr>
<td></td>
<td>20 x MIC</td>
<td>-0.56</td>
<td>2.83</td>
</tr>
</tbody>
</table>

Bold data correspond to a tolerance phenomenon according to the criterion of each methodology.

^a first assay; ^b second assay; ^c third assay.
FIG. 1. Comparative killing of glycopeptides after 6 and 24 hours of exposition at 10 times the MIC against 2 populations of coagulase negative staphylococci: 77 non *S. lugdunensis* isolates versus 13 *S. lugdunensis* isolates. Errors bars indicate standard deviations and asterisks indicate statistically significant differences (p<0.05).