High Percentage of Methicillin-Resistant *Staphylococcus aureus* Isolates with Reduced Susceptibility to Glycopeptides in The Netherlands

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While testing the in vitro activities of 14 antimicrobial agents against 107 methicillin-susceptible *Staphylococcus aureus* (MSSA) and 250 methicillin-resistant *S. aureus* (MRSA) isolates collected in The Netherlands, we found to our surprise that 19 (7.6%) MRSA isolates were suspected of having reduced susceptibilities to the glycopeptides when the Etest system (AB Biodisk, Solna, Sweden) was used with a large inoculum (no. 2 McFarland standard) and an extended incubation time (48 h) on brain heart infusion agar for MIC testing. Eventually, 15 of these isolates were classified as heterogeneously resistant to glycopeptides (heterogeneously glycopeptide-intermediate *S. aureus* [hGISA] isolates) according to the population analysis profile-area under the curve analysis. The MICs at which 50 and 90% of isolates are inhibited obtained with the Etest system comprised 247 different phage types. Three isolates were not typeable.

The 107 MSSA isolates were from cultures of blood collected between May 1998 and June 1999 from consecutive patients at the following six hospitals: St. Elisabeth Hospital and Tweesteden Hospital, Tilburg, The Netherlands; Pasteur Hospital, Oosterhout, The Netherlands; Tweesteden Hospital, Waalwijk, The Netherlands; and St. Ignatius Hospital and Hospital de Baronie, Breda, The Netherlands. Only one isolate was included from each patient per admission period.

Isolates were identified by a latex agglutination test (Staphaurex Plus Murex Diagnostics Ltd., Dartford, England), by the detection of free coagulase by the tube coagulase test with rabbit plasma (14), and by the detection of DNase (DNase agar; Oxoid Ltd., Basingstoke, England). If the results of these tests were discordant, an AccuProbe culture identification test (Gen-Probe; San Diego, Calif.) was performed according to the manufacturer’s instructions. The result of the AccuProbe test was considered the “gold standard” for the identification of *S. aureus*. At the time of collection, the blood culture isolates were classified as methicillin susceptible (oxacillin MIC ≤ 2 μg/ml) by broth microdilution susceptibility testing, performed as described by the National Committee for Clinical Laboratory Standards (NCCLS). Furthermore, no growth was observed by the oxacillin agar screen test, performed as described by the NCCLS (16).

Antimicrobial agents and MIC testing. The MICs of the following 14 antimicrobial agents were determined: linezolid, oxacillin, vancomycin, teicoplanin, gentamicin, tobramycin, quinupristin-dalfopristin, ciprofloxacin, erythromycin, clindamycin, rifampin, fusidic acid, and mupirocin. All MICs were determined with the Etest system (AB Biodisk, Solna, Sweden) according to the instructions of the manufacturer. The oxacillin Etest strip was placed onto a Mueller-Hinton agar plate supplemented with 2% NaCl, and the plate was incubated at 35°C for 24 h. For vancomycin and teicoplanin, the Etest curve analysis. The MICs at which 50 and 90% of isolates are inhibited obtained with the Etest system with McFarland standard) and an extended incubation time (48 h) on brain heart infusion agar for MIC testing. Eventually, 15 of these isolates were classified as heterogeneously resistant to glycopeptides (heterogeneously glycopeptide-intermediate *S. aureus* [hGISA] isolates) according to the population analysis profile-area under the curve analysis. The MICs at which 50 and 90% of isolates are inhibited obtained with the Etest system comprised 247 different phage types. Three isolates were not typeable.

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The prevalence of resistance to methicillin among *Staphylococcus aureus* isolates in The Netherlands is low (≤2%) (2, 4). Therefore, most infections with *S. aureus* can be treated with a semisynthetic penicillin. In many parts of the world, however, the occurrence of methicillin-resistant *S. aureus* (MRSA) is widespread, and vancomycin is used for empirical therapy for staphylococcal infections (21). However, MRSA isolates with reduced susceptibilities to glycopeptides have now been reported from many countries, including the neighboring countries of Germany and Belgium (3, 8). Until now, no MRSA isolates with reduced susceptibilities to glycopeptides have been reported in The Netherlands.

The emergence of MRSA isolates with reduced susceptibilities to glycopeptides has urged the need to create new agents for the treatment of MRSA infections (20). The ability of clinicians to choose among several antimicrobial agents with different mechanisms of action for the treatment of infections caused by these microorganisms could reduce the increasing selection pressure for resistance to glycopeptides by gram-positive microorganisms in hospitals (26). New antimicrobial agents, like linezolid, have become more and more important as alternative treatments for MRSA infections.

In this study we compared the in vitro activities of linezolid and 13 other antimicrobial agents against 107 methicillin-susceptible *S. aureus* (MSSA) and 250 MRSA isolates from The Netherlands. Furthermore, we report on the first MRSA isolates with reduced susceptibilities to glycopeptides isolated in The Netherlands. (This study was presented at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., December 2001.).

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The MIC range, the MIC at which 50% of isolates are inhibited (MIC₅₀), the MIC₉₀, and the percentage of isolates susceptible to the antimicrobial agents tested are presented in Tables 1 and 2 for the MSSA and MRSA isolates, respectively.

All S. aureus isolates showed susceptibility to linezolid and quinupristin-dalfopristin in vitro.

Only eight of the MSSA isolates showed resistance to one of the antimicrobial agents tested. One isolate was resistant to ciprofloxacin (MIC = 32 μg/ml), one isolate was resistant to eriethromycin (MIC = 256 μg/ml), and six isolates were resistant to fusidic acid (Table 1).

Highly three (1.2%) MRSA isolates showed resistance to the topical agent mupirocin, and only one of these isolates had high-level resistance (MIC > 256 μg/ml). A low percentage (7.2%) of resistance to fusidic acid was found.

According to the Etest criteria, 19 (7.6%) of the MRSA isolates, for which vancomycin and teicoplanin MICs were ≥8 μg/ml or teicoplanin MICs were ≥12 μg/ml, were suspected of having reduced susceptibilities to the glycopeptides (Table 3).

For 15 of these isolates the PAP-AUC ratio was between 0.9 and 1.3, which is the criterion for the heteroresistant phenotype (hGISA). For four isolates, the PAP-AUC ratio did not confirm the reduced susceptibilities to the glycopeptides found by the Etest. For one isolate (isolate 15 in Table 3), the PAP-AUC ratio was close to 0.90, namely, 0.88.

None of the hGISA isolates would have been detected directly by the broth microdilution method. The vancomycin MIC was 4 μg/ml for only one isolate (isolate 16) by broth microdilution testing. This isolate should have been further investigated according to NCCLS and Centers for Disease Control and Prevention guidelines, according to which strains for which the vancomycin MIC is ≥4 μg/ml should be assessed more closely (27).

While the MIC₅₀ and MIC₉₀ of vancomycin were 3.0 and 4.0 μg/ml, one isolate was resistant to mupirocin (MIC = 256 μg/ml), and six isolates were resistant to fusidic acid (Table 1).

The PAP-AUC ratio criteria used to determine vancomycin resistance, glycopeptide-intermediate resistance, and heterogenous glycopeptide-intermediate resistance were determined as described previously and are based on multiple (n = 30) tests with both Mu3 (ATCC 700698) and Mu50 (ATCC 700699) and a glycopeptide-sensitive, S. aureus strain (ATCC 29213) as controls. A ratio was then calculated by dividing the AUC for the test strain by the AUC for Mu3 (the archetype Japanese hGISA strain).

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TABLE 3. Results for 19 MRSA isolates found to have reduced susceptibilities to glycopeptides by Etest criteria

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Yr of isolation</th>
<th>Country of origin</th>
<th>Isolate source</th>
<th>MIC (µg/ml) with Etest system</th>
<th>Vancomycin MIC (µg/ml) by broth microdilution</th>
<th>PAP-AUC ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Interpretation for hGISA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1990</td>
<td>Turkey</td>
<td>—</td>
<td>6</td>
<td>1</td>
<td>1.09</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>1991</td>
<td>France</td>
<td>—</td>
<td>8</td>
<td>1</td>
<td>1.12</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>1992</td>
<td>Turkey</td>
<td>—</td>
<td>8</td>
<td>1</td>
<td>0.99</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>1992</td>
<td>Turkey</td>
<td>—</td>
<td>4</td>
<td>1</td>
<td>1.03</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>1994</td>
<td>India</td>
<td>Perineum</td>
<td>6</td>
<td>0.5</td>
<td>0.82</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>1994</td>
<td>France</td>
<td>Burn wound</td>
<td>8</td>
<td>1</td>
<td>1.16</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>1994</td>
<td>Italy</td>
<td>Pus</td>
<td>4</td>
<td>2</td>
<td>1.05</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>1994</td>
<td>—</td>
<td>Nares</td>
<td>8</td>
<td>2</td>
<td>1.07</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>1994</td>
<td>South Africa</td>
<td>Nares</td>
<td>8</td>
<td>2</td>
<td>0.84</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>1994</td>
<td>Greece</td>
<td>Skin</td>
<td>3</td>
<td>1</td>
<td>1.13</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>1994</td>
<td>Italy</td>
<td>Wound</td>
<td>4</td>
<td>1</td>
<td>1.04</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>1994</td>
<td>France</td>
<td>Pus</td>
<td>3</td>
<td>0.5</td>
<td>0.78</td>
<td>—</td>
</tr>
<tr>
<td>13</td>
<td>1995</td>
<td>Germany</td>
<td>Perineum</td>
<td>8</td>
<td>2</td>
<td>1.28</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>1995</td>
<td>Greece</td>
<td>Pus</td>
<td>6</td>
<td>2</td>
<td>1.06</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>1998</td>
<td>Argentina</td>
<td>Wound</td>
<td>12</td>
<td>1</td>
<td>0.88</td>
<td>—</td>
</tr>
<tr>
<td>16</td>
<td>1998</td>
<td>Ivory Coast</td>
<td>Perineum</td>
<td>12</td>
<td>4</td>
<td>1.23</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>1998</td>
<td>—</td>
<td>Pus</td>
<td>6</td>
<td>0.5</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>18</td>
<td>1998</td>
<td>Italy</td>
<td>Nares</td>
<td>16</td>
<td>2</td>
<td>0.97</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>1998</td>
<td>—</td>
<td>—</td>
<td>12</td>
<td>1</td>
<td>0.93</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>PAP-AUC ratio criteria for vancomycin resistance are defined in the text.

<sup>b</sup>Interpretation of PAP-AUC ratio: +, hGISA; —, glycopeptide-sensitive S. aureus.

<sup>c</sup>—, unknown.

µg/ml, respectively, for both MSSA and MRSA isolates by the Etest, the MIC<sub>50</sub> and MIC<sub>90</sub> of vancomycin were 0.5 and 1.0 µg/ml, respectively, for both MSSA and MRSA isolates by broth microdilution (Tables 1 and 2).

The MIC<sub>50</sub> and MIC<sub>90</sub> of linezolid for MSSA and MRSA isolates were equal. However, the MIC<sub>90</sub> of both quinupristin-dalfopristin and teicoplanin were higher for MRSA isolates than for MSSA isolates.

**DISCUSSION**

The purpose of the present study was to assess the in vitro activities of linezolid and 13 other antimicrobial agents against MSSA and MRSA isolates collected in The Netherlands. To our surprise we found that 15 MRSA isolates were heterogeneously resistant to glycopeptides. Staphylococci displaying this phenotype have been reported from various locations over the world, including Japan, the United States, and European countries (27), but this is the first report of S. aureus isolates with reduced susceptibilities to glycopeptides from The Netherlands. The collection of MRSA isolates used in this study is composed of isolates collected in The Netherlands between 1989 and 1998. During that period, as at present, the prevalence of MRSA isolates in The Netherlands was very low, and the MRSA strains isolated were often recovered from patients who had been hospitalized in other countries. Most isolates in the collection are of European origin (25).

The country of origin was recorded for 12 of the 15 isolates confirmed to be hGISA, and none of these isolates originated from The Netherlands. 3 isolates were from Turkey, 3 were from Italy, 2 were from France, 2 were from Greece, 1 was from Germany, and 1 was from Ivory Coast (Table 3). hGISA isolates have been reported from Greece, France, Germany, and Italy before (8, 12, 15, 17). To our knowledge, there are no reports of hGISA isolates from Turkey or Ivory Coast. The prevalence of MRSA isolates in a Turkish hospital, collected as part of the European SENTRY Antimicrobial Surveillance Program between January 1997 and December 1999, was 37.5% (4). In 1996 and 1997 the prevalence of MRSA in Ivory Coast was 16.8% (13). With this high prevalence of MRSA isolates, one can predict that the rate of glycopeptide usage is high and there is a strong chance that one would eventually find hGISA isolates.

In earlier surveillance susceptibility testing studies with part of the MRSA isolate collection used in the present study, namely, those isolates collected between 1989 and 1995, no resistance to vancomycin was described (2). In that study, however, susceptibility testing was performed by an agar dilution method according to the guidelines of the Dutch Committee on Antibiotic Susceptibility Guidelines with an inoculum of 5 × 10<sup>3</sup> CFU/spot incubated on Iso-Sensitest agar at 37°C for 24 h.

When the present study was designed, there was very little literature about the value of the Etest system for the detection of hGISA. The manufacturer, however, recommended the use of BHI agar, a larger inoculum, and a longer incubation period. Since we did not have any experience with this method and broth microdilution was the routine method for susceptibility testing in our laboratory, we tested all isolates for vancomycin resistance by the broth microdilution method as well. None of the isolates would have been correctly classified as (h)GISA by the broth microdilution method. For one isolate (isolate 16 in Table 3), the vancomycin MIC was 4 µg/ml by broth microdilution, and the isolate should have been assessed more closely according to NCCLS and Centers for Disease Control and Prevention guidelines (27). The PAP-AUC ratio for this isolate (1.23) was the second highest detected among the isolates tested.

Since this study was designed, more and more has been
published about screening methods for the detection of \textit{S. aureus} isolates heterogeneously resistant to vancomycin. Although well-standardized microdilution susceptibility testing methods are able to detect \textit{S. aureus} isolates with reduced susceptibilities to vancomycin, they cannot detect heteroresistance (22). Wootton et al. (28) found that the Etest with a large inoculum (no. 2 McFarland standard), a longer incubation time (48 h), and rich BHI medium was the only method that correctly identified Mu3, the heteroresistant isolate that was described by Hirama et al. (11). In a study comparing different methods for the detection of \textit{staphylococci} with reduced susceptibilities to glycopeptides, Walsh et al. (27) found the Etest method with a large inoculum to be a reliable and sensitive screening method for the detection of glycopeptide resistance, including heteroresistance. In the same study, the PAP-AUC ratio proved to be a reliable method for confirmation of the Etest results. In the present study, 4 of the 250 MRSA isolates (1.6%) were incorrectly identified as having reduced susceptibilities to the glycopeptides when the Etest method with the large inoculum was used. This is comparable to the 2.1% (7 of 329 MRSA isolates) rate of false-positive results described by Walsh et al. (27).

No \textit{S. aureus} isolates resistant to linezolid were detected in the present study, and as in earlier studies (1), no differences in the in vitro activities of linezolid against MSSA and MRSA isolates were found. The development of resistance to linezolid during treatment has been described in vancomycin-resistant \textit{Enterococcus faecium} isolates were found. The development of resistance to linezolid for dialysis-associated peritonitis; the linezolid-resistant MRSA isolates were, however, not related to the linezolid-susceptible isolates initially isolated.

While linezolid is bacteriostatic for \textit{staphylococci} (6), quinupristin-dalfopristin is bactericidal. However, the bactericidal activity of quinupristin-dalfopristin is variable, and \textit{S. aureus} strains that have cross-resistance to macrolides, lincosamides, and streptogramin B antibiotics were not killed by quinupristin-dalfopristin in vitro (7). Clindamycin susceptibility was found to be a good surrogate indicator of the bactericidal activity of quinupristin-dalfopristin in vitro (7). Clindamycin resistance, however, is not uncommon among MRSA isolates. In the present study, 32.8% of the MRSA isolates showed resistance to clindamycin, but even that rate is low, considering the rate of resistance to clindamycin among MRSA isolates recovered in European centers participating in the SENTRY program between 1997 and 1999, among which the rate of resistance was as high as 73.5% (4).

Interpretation of the activities of antimicrobial agents in vitro remains a difficult task. The choice of a susceptibility testing method can influence the MIC. The linezolid MICs determined by the Etest tended to be lower than those determined by broth microdilution (6). Tubau et al. (24) found that the linezolid MICs were usually 1 to 2 dilutions lower by the Etest method than by the microdilution method. In a European multicenter study, Gemmell et al. (9) found that the mean MIC of linezolid by the Etest was approximately twofold lower than the mean MIC by the broth microdilution method. It was hypothesized that this effect is due to the bacteriostatic activity rather than the bactericidal activity of linezolid. This difference between the MICs obtained by the Etest and those obtained by the broth microdilution method was not found for vancomycin. In that study, however, the tests with vancomycin were conducted with Mueller-Hinton agar, while we used rich BHI medium. Another study (18) showed that use of the Etest method with a large inoculum resulted in only a minimal difference in the vancomycin and teicoplanin MICs for susceptible American Type Culture Collection control strains compared to the MICs obtained by the agar dilution method, while the vancomycin MICs for the hGISA control strains were 1 dilution lower by the agar dilution test than by the Etest. In our study, however, the vancomycin MICs for both MSSA and MRSA isolates obtained by the Etest method with a large inoculum were increased compared to those obtained by the broth microdilution method. We did not find any MSSA isolate suspect for reduced susceptibility to glycopeptides by the macrodilution Etest method. Until now, only one MSSA isolate with reduced susceptibility to glycopeptides has been reported (18). We believe that the rich BHI medium, the large inoculum, and the longer incubation period used for the Etest method account for the higher vancomycin MIC50 and MIC90 that we found with the Etest system compared to those obtained by broth microdilution testing. This could also explain the higher vancomycin and teicoplanin MIC50 and MIC90 that we found compared to those detected in other studies, which used other susceptibility testing methods. Although use of broth microdilution techniques would have made it easier to compare our results with those of other studies, this would have meant that we would have missed the isolates with reduced susceptibilities to the glycopeptides.

Modification of the medium on which susceptibility testing is performed in order to increase the level of expression of a resistance mechanism, which makes resistant isolates easier to detect, is well accepted. For instance, for the detection of MRSA, Mueller-Hinton agar supplemented with NaCl is used to increase the chance of detecting oxacillin resistance (25).

Low levels of resistance to the agents mupirocin and fusidic acid, which are used for the eradication of MRSA carriage, were found. Only one isolate with high-level resistance to mupirocin was detected. Low-level mupirocin resistance, which was found for two other isolates, has little influence on the efficacy of treatment with topical mupirocin, while in the case of high-level resistance, it is unlikely that topical mupirocin treatment will eradicate the organism (5).

The relatively small proportion of MRSA isolates resistant to co-trimoxazole (16.4%) found in this study is comparable to the proportion of co-trimoxazole-resistant MRSA isolates reported in hospitals in Europe (23%), Canada (16%), and the United States (26%) that participated in the SENTRY program from 1997 to 1999 (4). The rate of co-trimoxazole resistance among MRSA isolates from the Latin American SENTRY program participants during the same period was much higher (65.4%).

The in vitro data presented in this and other studies, together with promising clinical results, show that linezolid is a valuable new addition to the arsenal of antimicrobial agents for the treatment of infections caused by gram-positive microorganisms. Even infections caused by resistant \textit{S. aureus} isolates, like MRSA or (h)GISA, could possibly be treated successfully with linezolid.
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