INTRODUCTION

*Staphylococcus aureus* is a pathogenic bacterium that is one of the most common causative agents of suppurative skin inflammation, osteomyelitis, meningitis, sepsis and other infections (Otto, 2010). The rapid emergence and spread of drug-resistant organisms, such as meticillin-resistant *S. aureus* (MRSA), in both the healthcare setting and the community, creates new challenges. The glycopeptide antibiotic vancomycin was first introduced in 1958, as the most effective treatment for MRSA infections. In 1997, the first strain of *S. aureus* with reduced susceptibility to vancomycin was reported in Japan (Hiramatsu *et al.*, 1997). Soon thereafter, a report of two additional cases from the USA was published, and since then there have been several reports of vancomycin-intermediate *S. aureus* (VISA) and heterogeneous vancomycin-intermediate *S. aureus* (hVISA) infections from around the world. These reports have shed some light on the microbiological characteristics of these organisms, although they have also led to some confusion regarding laboratory detection.

Vancomycin-susceptibility-reduced *S. aureus* (VISA and hVISA) usually develop changes in morphology and biological characteristics, such as cell wall thickening (van Hal *et al.*, 2011), slow growth, smaller colonies, decreased pigment formation, less or no haemolysis and reduced coagulase activity (Hiramatsu *et al.*, 1997). More to the point, coagulase, a major phenotypic determinant of *S. aureus*, is often used to identify *S. aureus* (Wilcox *et al.*, 1996). If the coagulase test is inaccurate, conventional testing methods may wrongly identify *S. aureus* as coagulase-negative staphylococci (CoNS). For this purpose, we used *S. aureus*-specific genes to test 24 vancomycin-susceptibility-reduced CoNS strains identified by conventional biochemical methods. If the strains contained *nuc*, *coa* and 16S rRNA genes, they were passaged on nonselective medium for 15 days. Revertant isolates were identified by conventional biochemical testing methods. This suggests that biochemical changes in *S. aureus* strains with reduced vancomycin susceptibility should be highlighted and that the detection of these strains requires more attention and improved techniques.

METHODS

**Screening for vancomycin-susceptibility-reduced CoNS.** CoNS strains were identified by conventional biochemical tests (Kloos & Bannerman, 1995), including the tube coagulase test; identity was further confirmed by the Vitek 32 (bioMérieux). CoNS strains were screened for potential vancomycin resistance as described elsewhere (Hiramatsu *et al.*, 1997). We carried out a one-step resistance selection experiment by using vancomycin-salt agar. Vancomycin-salt
agar was prepared by incorporating 4 mg vancomycin ml\(^{-1}\) and 4 % NaCl into Mueller–Hinton (MH) agar (Oxoid). Overnight cultures were adjusted to a 0.5 McFarland unit suspension, and 10 µl of bacterial suspension was inoculated onto vancomycin-salt agar. The plates were incubated at 35 °C for 48 h. If confluent growth of cells was apparent within 24 h, the strain was considered a potential vancomycin-resistant staphylococcus (VRS). If a countable number (1–30) of colonies was apparent within 48 h, the isolate was designated h-VRS (heterogeneous VRS). The vancomycin MICs for these subclones were first determined by Etest (AB Biodisk) and then confirmed by the broth microdilution method if the vancomycin MIC was ≥4 mg l\(^{-1}\) by Etest. Vancomycin-resistant CoNS (VRCoS) status was confirmed by a vancomycin MIC of 8 mg l\(^{-1}\) or above; heterogeneous vancomycin-resistant CoNS (h-VRCoS) status was considered definite if the strain produced a subclone(s) with a vancomycin MIC of 8 mg l\(^{-1}\) or above, with the stability of the strain persisting beyond 9 days in a drug-free medium.

**Population analysis of h-VRCoS.** To confirm h-VRCoS strains, the strains were tested by population analysis. In brief, strains were first cultured overnight in brain heart infusion (BHI) broth. Then, 50 µl of the bacterial suspension was transferred to 4 ml BHI broth and incubated at 37 °C. After the optical density had been adjusted to 0.5 McFarland units, 10-fold dilutions of cell suspensions were used to inoculate MH agar plates containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 mg vancomycin l\(^{-1}\). The plates were incubated at 37 °C for 48 h, and then the colonies were counted. h-VRCoS was defined as a staphylococcus strain that gave vancomycin resistance at a frequency of 10\(^{-9}\) colonies or higher (Hiramatsu et al., 1997).

**Morphological observation and identification.** The vancomycin-susceptibility-reduced CoNS isolates were plated on fresh blood agar, and the growth of bacteria, colony size, colony colour and haemolysis were observed. The coagulase and catalase tests were applied, and bacterial identification was performed by using the Vitek 32 (bioMérieux).

**Coagulase test.** The coagulase test was performed as described elsewhere (Moreira et al., 1997). The isolate was plated on fresh blood agar and incubated overnight at 37 °C. Rabbit coagulase plasma (bioMérieux) was used according to the manufacturer’s instructions. Briefly, the rabbit coagulase plasma was reconstituted by adding distilled water to the vial. The vial was mixed by gentle rotation to ensure complete dissolution and 0.5 ml reconstituted coagulase plasma was added to a test tube. Two to four colonies were suspended in the tube, mixed and incubated at 37 °C for more than 4 h and then at room temperature overnight. Tubes were examined for clot formation at 30 min intervals until 24 h.

**Electron microscopic examination.** For electron microscopy, an *S. aureus* isolate (10827-R) was fixed in 3 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.8, containing 0.1 M sucrose and 1 % tannin for 4 h at 4 °C. Then the cells were dehydrated through an ethanol series and propylene oxide, and embedded in Agar 100 resin (Agar Scientific). The embedded samples were sectioned (80 nm thick) and stained with lead citrate. Transmission electron microscopy (JEM-1230 transmission electron microscope, JEOL) and scanning electron microscopy (ISM-6700 scanning electron microscope, JEOL) were used to enlarge to ×60 000 and ×30 000 for observation. *S. aureus* ATCC 25923 was used as the reference strain.

**Detection of the nuc, coa and 16S rRNA genes.** The 24 isolates were suspended in lysostaphin (20 µg ml\(^{-1}\)) and proteinase K (0.5 mg ml\(^{-1}\)), incubated at 37 °C for 1 h, then boiled and centrifuged. The supernatants were used as DNA templates. DNA was amplified by PCR with specific primers as described elsewhere (Geha et al., 1994; Kalorey et al., 2007). For *S. aureus* the primer sequences for nuc were: forward, 5’-GGTAGCCATCTATTGTAGTTGTTG-3’; reverse, 5’-CTGTGTGTTAGCTTTATTTTGGTCG-3’; coa: forward, 5’-GTTCAAGGTCCGGATTCTT-3’, reverse, 5’-CTAGGC-CCTATGTCGGAGT-3’; 16S rRNA: forward, 5’-GAATTCTAAA (T/G, 1:1)GAATTGCGGGGCC-3’, reverse, 5’-GGGATCCCGG-CCGCGAAGTATTAC-3’. The reaction mixture was subjected to denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, and then a final extension step of 72 °C for 10 min was done.

**Passage.** The six strains that were positive for the nuc, coa and 16S rRNA genes were serial-passaged daily on nutrient agar without vancomycin supplementation. After 15 continuous passages, vancomycin-susceptible revertants were obtained. Revertant isolates were identified by conventional biochemical and automated testing methods.

**RESULTS**

In this study, 24 vancomycin-susceptibility-reduced CoNS strains (including 22 *Staphylococcus haemolyticus* strains and two epidermal *Staphylococcus* strains) were retested by PCR-based detection of *S. aureus*-specific genes (nuc, coa and 16S rRNA). Six strains contained 16S rRNA, coa and nuc genes, and these strains were coagulase-negative, mannitol-negative, lactose-negative and identified as *S. haemolyticus* by conventional methods. The morphological features of these strains included slow growth, smaller colonies, decreased pigment formation and less or no haemolysis.

As shown in Table 1, the revertant isolates showed biochemical characteristics different from the original isolates, such as faster growth, bigger colonies, increased pigment formation and haemolysis, and were coagulase-positive, mannitol-positive and lactose-positive. They were susceptible to vancomycin with MICs of 0.5–2 µg ml\(^{-1}\) and were identified as *S. aureus* by conventional methods.

As shown in Fig. 1, the vancomycin-susceptibility-reduced isolate (10827-R) was much thicker than *S. aureus* ATCC 25923 by transmission electron microscopy. Scanning electron microscopy showed that 10827-R had lost the typical cell wall surface smoothness and regularity. Instead, its cell wall surface appeared rough with irregular raised spots.

**DISCUSSION**

Although the first reports of acquired resistance to glycopeptides in CoNS were made in 1983 (Tuazon & Miller, 1983), these reports did not gain much attention, as CoNS are considered to be relatively avirulent organisms and are not thought to cause severe infection. In contrast to CoNS, *S. aureus* is a dangerous pathogen, causing serious infections such as pneumonia and bacteraemia (Otto, 2010). However, significant concern in the medical community was generated after initial reports of reduced vancomycin susceptibility in clinical isolates of *S. aureus* from Japan in 1997 (Hiramatsu et al., 1997).

Controversy still exists regarding the mechanisms of vancomycin resistance in *S. aureus*, while many studies...
**Table 1.** Growth characteristics and bacterial identification results for six strains before and after the reversion test

Abbreviations: VAN, vancomycin; B, before reversion test; A, after reversion test; COA, coagulase test; MAN, D-mannitol test; LAC, lactose test.

<table>
<thead>
<tr>
<th>Strain code</th>
<th>Specimen source</th>
<th>VAN MIC (µg ml⁻¹)</th>
<th>Growth in broth tube</th>
<th>Colony size</th>
<th>Colony colour</th>
<th>Haemolysis</th>
<th>VITEK 32 identification profile number and result</th>
<th>Variation in biochemistry tests</th>
<th>nuc, coa and 16S rRNA gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>86-B</td>
<td>Pus</td>
<td>6</td>
<td>Precipitate</td>
<td>Small, not uniform</td>
<td>Beige</td>
<td>None</td>
<td>76121031240, 93% <em>S. haemolyticus</em></td>
<td>COA (−), MAN (−)</td>
<td></td>
</tr>
<tr>
<td>86-A</td>
<td>1 Homogeneous</td>
<td>1</td>
<td>Bigger</td>
<td>Yellow</td>
<td>β</td>
<td>76125031241, 99% <em>S. aureus</em></td>
<td>COA (+), MAN (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>117-B</td>
<td>Blood</td>
<td>8</td>
<td>Precipitate</td>
<td>Smaller</td>
<td>White</td>
<td>None</td>
<td>76425031040, 92% <em>S. haemolyticus</em></td>
<td>COA (−)</td>
<td></td>
</tr>
<tr>
<td>117-A</td>
<td>1 Homogeneous</td>
<td>1</td>
<td>Bigger</td>
<td>Yellow</td>
<td>β</td>
<td>76425031041, 99% <em>S. aureus</em></td>
<td>COA (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23837-B</td>
<td>Sputum</td>
<td>12</td>
<td>Precipitate</td>
<td>Bigger</td>
<td>White</td>
<td>None</td>
<td>77521030240, 93% <em>S. haemolyticus</em></td>
<td>COA (−), MAN (−), LAC (−)</td>
<td></td>
</tr>
<tr>
<td>23837-A</td>
<td>2 Homogeneous</td>
<td>2</td>
<td>Bigger</td>
<td>Yellow</td>
<td>β</td>
<td>77527030241, 99% <em>S. aureus</em></td>
<td>COA (+), MAN (+), LAC (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10827-R</td>
<td>Sputum</td>
<td>16</td>
<td>Precipitate</td>
<td>Smaller</td>
<td>White</td>
<td>None</td>
<td>76121031040, 72% *S. haemolyticus, 17% <em>Staphylococcus hominis</em></td>
<td>COA (−), MAN (−)</td>
<td></td>
</tr>
<tr>
<td>10827-P</td>
<td>2 Homogeneous</td>
<td>2</td>
<td>Bigger</td>
<td>Yellow</td>
<td>β</td>
<td>76125031041, 99% <em>S. aureus</em></td>
<td>COA (+), MAN (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23488-R</td>
<td>Sputum</td>
<td>8</td>
<td>Precipitate</td>
<td>Small, not uniform</td>
<td>Beige</td>
<td>Narrowed</td>
<td>76121031040, 93% <em>S. haemolyticus</em></td>
<td>COA (−), MAN (−)</td>
<td></td>
</tr>
<tr>
<td>23488-P</td>
<td>1 Homogeneous</td>
<td>1</td>
<td>Bigger</td>
<td>Yellow</td>
<td>β</td>
<td>76125031241, 99% <em>S. aureus</em></td>
<td>COA (+), MAN (+)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>383-R</td>
<td>Prostatic fluid</td>
<td>6</td>
<td>Precipitate</td>
<td>Small, not uniform</td>
<td>Yellow</td>
<td>Narrowed</td>
<td>76521030040, 77% *S. haemolyticus, 5% <em>S. hominis</em></td>
<td>COA (−)</td>
<td></td>
</tr>
<tr>
<td>383-P</td>
<td>0.5 Homogeneous</td>
<td>0.5</td>
<td>Bigger</td>
<td>Yellow</td>
<td>β</td>
<td>76521030041, 98% <em>S. aureus</em></td>
<td>COA (+)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
have focused on the cell wall changes associated with reduced vancomycin susceptibility in *S. aureus* that are not related to the acquisition of *vanA*. Apart from cell wall rearrangements, biochemical and morphological changes can be found in *S. aureus* isolates with reduced vancomycin susceptibility, including decreased coagulase activity, reduced pigmentation, smaller colony size, reduced haemolytic activity, lower growth rate, larger cell diameter and decreased susceptibility to lysozyme. Whether these changes affect the identification of *S. aureus* isolates with reduced vancomycin susceptibility by routine methods has not been reported. In this study, six isolates originally identified as *S. haemolyticus* using the Vitek 32 were later determined to be *S. aureus* by molecular methods. *S. haemolyticus* and *S. aureus* display largely similar biochemical reactions, and the most important is the coagulase test. However, it has been reported that coagulase activity decreases in *S. aureus* isolates with reduced vancomycin susceptibility, occasionally leading to misidentification if the coagulase test is incubated for less than 4 h (Moreira et al., 1997). In our study, we found that vancomycin-susceptibility-reduced *S. aureus* strains showed negative plasma coagulation activity even when the incubation period was extended to 24 h. The reason for this is unknown. It may be due to low expression of the plasma coagulation enzyme gene or it may be that the enzyme activity is affected by the thickening of the bacterial cell wall. In addition, other changes characteristic of isolates with reduced vancomycin susceptibility were also found (Table 1).

Several studies (Froggatt et al., 1989; Kristóf et al., 2011) have reported that reduced susceptibility to vancomycin is more common in *S. haemolyticus*. This species displayed a higher incidence of reduced susceptibility to vancomycin compared with other CoNS species, even reaching the 42% reported by Froggatt et al. (1989). Interestingly, unlike the cases of vancomycin-susceptibility-reduced *S. haemolyticus*, it appears that VISA isolates are seldom detected in hospital microbiology laboratories. However, in clinical specimens there are more *S. aureus* isolates than *S. haemolyticus* isolates. The varying prevalence rates for *S. aureus* with reduced susceptibility to vancomycin are due mainly to methodology differences, but possibly could be due to the misidentification of isolates as CoNS.

In brief, phenotypic changes in *S. aureus* isolates with reduced vancomycin susceptibility leading to wrong identification as *S. haemolyticus* may be the reason for the underestimated incidence of vancomycin-susceptibility-reduced *S. aureus* strains. Even more worrying is the fact that vancomycin-resistant *S. aureus* isolates may become nosocomial pathogens, with disastrous consequences, if the isolates are wrongly identified as CoNS and effective control measures for preventing nosocomial transmission are not implemented. Therefore, the detection of *S. aureus* strains with reduced vancomycin susceptibility requires more attention and improved techniques.

**REFERENCES**


