Emergence of vancomycin-intermediate \textit{Staphylococcus aureus} in a Belgian hospital: microbiological and clinical features

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In 1999, all clinical \textit{Staphylococcus aureus} isolates from patients admitted to a Belgian University hospital were tested for decreased vancomycin susceptibility. Three vancomycin-intermediate \textit{Staphylococcus aureus} (VISA) and four hetero-VISA strains were detected among 2145 isolates tested. They emerged from strains that belonged to locally endemic methicillin-resistant \textit{S. aureus} (MRSA) genotypes in three patients who had received repeated courses of vancomycin therapy. A cystic fibrosis patient with MRSA/VISA-associated broncho-pulmonary exacerbation was successfully treated by continuous vancomycin infusion plus fusidic acid followed by oral minocycline–fusidic acid. Two other patients had VISA recovered from specimens of undetermined clinical significance. Emergence of VISA variants of endemic MRSA strains in Belgium warrants active microbiological surveillance and careful monitoring of vancomycin therapy. Therapy with high-dose vancomycin administered by continuous infusion in combination with other antimicrobials may be a therapeutic option worth investigating for VISA infection.

Keywords: \textit{Staphylococcus aureus}, VISA, Belgium, vancomycin

Introduction

\textit{Staphylococcus aureus} is a major pathogen causing a diversity of infections including bacteraemia, pneumonia, skin, soft tissue and osteo-articular infections.\textsuperscript{1} In the past two decades, the prevalence of methicillin-resistant \textit{S. aureus} (MRSA) among both nosocomial and community-acquired infections has increased throughout the world.\textsuperscript{2,3} In Belgium, \textit{S. aureus} bacteraemia was due to MRSA in 22\% of cases in 1999.\textsuperscript{3} Many MRSA strains are co-resistant to other antibiotic classes, including macrolides, aminoglycosides and fluoroquinolones. Until recently, glycopeptides were the last uniformly effective drugs available for treatment of MRSA infections.

Since 1997, infections caused by MRSA strains with intermediate susceptibility to vancomycin (VISA) (MIC 8–16 mg/L) have been reported from Japan, France, the United States, Korea and Germany.\textsuperscript{4–10} These strains were recovered from patients who failed therapy with vancomycin that had often been used for prolonged periods of time. Other strains, named hetero-VISA, appear to be borderline susceptible to vancomycin (MIC 2–4 mg/L) but exhibit low-level subpopulations (10\textsuperscript{-6} cells) able to grow at vancomycin concentrations of 4–8 mg/L.\textsuperscript{11} Such strains have been described in Europe, Asia and Brazil.\textsuperscript{12–20} Hetero-VISA strains could represent first-step mutants that are precursors of VISA strains in patients receiving prolonged courses of vancomycin. Both VISA and hetero-VISA strains belong to a restricted range of epidemic MRSA genotypes.\textsuperscript{10–15,17–20}

We performed a prospective study to assess the incidence and characteristics of VISA strains recovered from patients...
admitted to a Belgian University hospital and we report the clinical course of VISA infections.

Materials and methods

Definitions

VISA was defined as an S. aureus strain with: (i) reproducible growth on brain-heart infusion (BHI) agar containing 6 mg/L vancomycin; (ii) a vancomycin MIC > 4 mg/L by Etest on Mueller–Hinton (MH) agar and by broth microdilution; (iii) a population analysis profile similar to homogeneous VISA control strains Mu50 and HIP5827.4,6

Hetero-VISA was defined as an S. aureus strain with: (i) a vancomycin MIC ≤ 4 mg/L by Etest on MH agar or by broth microdilution; (ii) a population analysis profile similar to hetero-VISA strain Mu3 with detectable subpopulation growing at 4 mg/L vancomycin.11

Bacterial strains

Between 1 January 1999 and 31 December 1999, all clinical isolates (n = 2145) of S. aureus from patients admitted to Erasme University hospital, an 858 bed tertiary care teaching hospital, were studied. Identification of S. aureus was performed by coagulase test with human plasma. MRSA strains were confirmed by multiplex PCR for mecA and nuc genes as described previously.21

Antimicrobial susceptibility testing

All isolates were tested by a vancomycin screen agar method.22 Briefly, 10 μL of 0.5 McFarland suspension of the first pure subculture was inoculated on to BHI agar supplemented with 6 mg/L vancomycin (BBL, Becton Dickinson, USA). Isolates showing growth after 24 or 48 h on this medium were subcultured on BHI agar supplemented with 1 mg/L vancomycin before determination of the MIC of vancomycin.

The MIC of vancomycin was determined by a broth microdilution method according to NCCLS guidelines and by two Etest methods (AB Biodisk, Solna, Sweden). In the first protocol, 3 mL of McFarland 0.5 suspension was flooded on to MH agar and incubated for 24 h at 35°C.23 In the second, BHI agar was inoculated with 100 μL of a McFarland 2 suspension and incubated for 48 h at 35°C.24 MICs were interpreted according to NCCLS breakpoints.

Strains with vancomycin MICs > 4 mg/L were characterized by population analysis.11 Briefly, 100 μL of an overnight suspension (McFarland 2) was spread on to agar plates supplemented with 0, 2, 4 and 8 mg/L vancomycin. The number of colonies was counted after incubation for 48 h at 35°C. Each assay included the following control strains: vancomycin-susceptible S. aureus strain ATCC 29213, hetero-VISA strain Mu3 and VISA strains Mu50 and HIP5827.4,6,11

Susceptibility to other antimicrobials was determined by a disc diffusion method (Rosco, NeoSensitab, Taastrup, Denmark) using interpretative inhibitory zone size according to NCCLS recommendations. The antibiotics tested included penicillin G (P), rifampicin (R), erythromycin (E), clindamycin (C), minocycline (M), doxycycline (D), fusidic acid (F), gentamicin (G), amikacin (A), ciprofloxacin (C), co-trimoxazole (SXT) and mupirocin (M). Oxacillin (O) was tested on oxacillin screen agar (6 mg/L) according to NCCLS recommendations. Oxacillin MICs were determined by Etest on MH agar supplemented with 2% NaCl incubated for 24 h at 35°C.

VISA strains were examined by multiplex PCR for the presence of vanA, vanB and vanC-1/2/3 genes coding for vancomycin resistance in enterococci.25

Electron microscopy

All cultures were grown to an OD600 of 0.6 in BHI broth prior to processing for electron microscopic examination, as described by Mani et al.26 The strains were cultured at 37°C in 10 mL of BHI broth in two series of tubes containing for each strain either no antibiotic or 2 mg/L vancomycin. Cells were harvested in the post-exponential growth phase (18–24 h → OD600 of 0.6) and fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2)–MgSO4 0.05% for 1.5 h, then rinsed twice in cacodylate buffer 0.1 M. They were treated with 1% osmium tetroxide in 0.2 M cacodylate buffer for 2 h at 4°C. Cells were dehydrated with graded concentrations of ethanol and embedded in Epon A+B. Ultra-thin sections were stained with uranyl acetate and lead citrate, then examined with a transmission electron microscope (TEM) (JEOL 1200MX) at different magnifications and photographed.

Pulsed-field gel electrophoresis (PFGE)

Macrogenic restriction (Smal) and PFGE analysis were performed as described previously.21 Smal patterns were normalized and compared using the Dice coefficient and UPGMA clustering method with BioNumerics software version 1.0 (Applied Maths, Ghent, Belgium). Patterns differing by one to six DNA fragments were considered as subtypes (designated by a letter suffix) and those distinguished by seven or more DNA fragments were labelled as distinct types (designated by numeral).21

Infection control and epidemiological investigation

The isolation procedures for patients colonized with MRSA followed Belgian national guidelines.27 Briefly, carriers were
placed in single rooms. Healthcare workers wore disposable gloves and gowns before entering the patient’s room. In addition, they wore face masks for aerosol-generating procedures. Hand disinfection with hydro-alcoholic solution was undertaken after glove removal. In addition, patients with VISA colonization/infection were cared for by dedicated nursing staff. Compliance with the isolation procedures was strictly monitored by infection control personnel. Patients and healthcare workers in contact with patients colonized with VISA were screened for colonization to assess potential transmission of VISA. Swabs obtained from anterior nares and hands were inoculated on to sheep blood agar and into MRSA selective broth medium containing colistin (32 mg/L), oxacillin (2 mg/L) and 2.5% NaCl. S. aureus isolates were identified by the coagulase test and tested for susceptibility to oxacillin and vancomycin by agar screen methods.

Results

Vancomycin susceptibility

Of 2145 isolates tested, of which 881 (41.1%) were oxacillin resistant, 146 (4.9%) S. aureus (of which 107 were MRSA) isolated from 106 patients grew on vancomycin screen agar after 24 or 48 h (Figure 1). By the heavy inoculum Etest procedure on BHI agar, 73 (50%) of these strains showed decreased susceptibility to vancomycin (MIC 6–16 mg/L). By Etest with a standard inoculum on MH agar, only four strains showed decreased susceptibility to vancomycin (MIC 6–8 mg/L). By the heavy inoculum Etest procedure on BHI agar, the distribution of MICs for the 146 strains showed a four-fold greater modal MIC value (8 versus 2 mg/L; P < 0.001) than that determined by standard inoculum on MH agar. By broth microdilution, four strains showed intermediate susceptibility to vancomycin (MIC 8–16 mg/L) (Table 1). No VISA strains carried van resistant genes.

Population analysis indicated that only the three strains with intermediate MIC by Etest MH agar and microdilution (P1V44, P2V136, P3V156) had a vancomycin resistance profile similar to homogeneous VISA reference strains Mu50 and HIP5827 (Table 1 and Figure 2). Four additional isolates, P1V69, P4V144, P5V133 and P6V6419, had a population profile overlapping with heterogeneous VISA reference strain Mu3 (Table 1 and Figure 2).

Proportion of VISA isolates

The proportion of hetero-VISA strains was 0.1% of S. aureus and 0.4% of MRSA strains, whereas the proportion of VISA strains was 0.1% of S. aureus and 0.3% of MRSA strains.

Antimicrobial susceptibility of VISA and hetero-VISA strains

All strains with reduced susceptibility to vancomycin possessed the meca gene and exhibited multiple resistance to other antimicrobials (Table 1). Hetero-VISA and two VISA strains, P2V136 and P3V156, were susceptible to minocycline, doxycycline, fusidic acid and co-trimoxazole (Table 1). VISA P1V44 strain was susceptible to clindamycin, gentamicin, minocycline, doxycycline, fusidic acid, co-trimoxazole and rifampicin. This isolate was borderline susceptible to oxacillin (MIC 2 mg/L) but possessed the meca gene by PCR. This VISA strain derived from a high-level oxacillin-resistant MRSA (MIC > 256 mg/L) progenitor strain P39575 isolated previously from patient 1.

PFGE analysis

By PFGE, VISA and hetero-VISA strains belonged to either of the two multidrug-resistant MRSA types A and D, both of which are endemic in our hospital. These PFGE types were distinct from VISA strains from Japan and the USA (Mu50 and HIP5827). VISA isolate P1V44 and hetero-VISA isolate P1V69 recovered from patient 1 in 1999 were indistinguishable by PFGE from MRSA strain P39575, a fully vancomycin-susceptible strain isolated during a previous hospitalization in December 1998.

TEM analysis

Three MRSA strains isolated consecutively from patient 1 (vancomycin-susceptible MRSA strain P39575, hetero-VISA strain P1V69 and VISA strain P1V44) were examined by TEM (Figure 3). MRSA strain P39575 and hetero-VISA strain P1V69 showed normal cell wall thickness but an uneven cell surface, irregular cell shape and abnormal septation (Figure 3c and d). These abnormalities were more pronounced in hetero-VISA strain P1V69. Strains P1V69 and
P39575 showed increased cell wall thickness when grown in 2 mg/L vancomycin. P1V44 strain showed a thickened cell wall (Figure 3b), irregular cell shape and abnormal septation in contrast to the thin and regular cell wall morphology of *S. aureus* ATCC 29213 strain (Figure 3a). When grown in the presence of vancomycin (2 mg/L), the abnormalities of strain P1V44 were slightly enhanced, whereas strain ATCC 29213 showed no morphological change (not shown).

**Case reports of VISA infection**

**Patient 1.** An 18-year-old woman with cystic fibrosis, colonized with MRSA since 1993, presented three successive pulmonary exacerbations attributed to MRSA (strain P39575) and *Pseudomonas aeruginosa* during the winter 1998–1999. Each episode required hospital admission and intravenous (iv) treatment with a combination of vancomycin, ceftazid-
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Figure 3. Transmission electron micrographs of (a) S. aureus ATCC 29213, (b) VISA strain P1V44, (c) hetero-VISA strains P1V69 and (d) MRSA P39575. Magnification ×60 000.

Patient 2. A 54-year-old woman was admitted to the intensive care unit (ICU) in November 1999, for multiple trauma with hepatic and pulmonary contusions. On day 11, she developed a primary MRSA bacteraemia. She was given intermittent vancomycin for 11 days adapted to serum levels, which were all in the expected range (trough, 5–10 mg/L; peak 30–40 mg/L). She improved but fever and bacteraemia recurred on day 22. Rifampicin was added to intermittent vancomycin for 10 more days. Because of the emergence of rifampicin resistance, rifampicin was then discontinued and replaced by iv fusidic acid (1.5 g/day). During this treatment (day 47), she presented with a second recurrence of bacteraemia due to an MRSA strain that was susceptible to vancomycin. Concomitantly, a VISA strain (P2V136) was isolated from a decubitus ulcer. From day 56 to 112, she was given continuous infusion of vancomycin (2–3 g/day) in combination with fusidic acid (1.5 g/day). During continuous infusion, serum levels of vancomycin ranged between 24.2 and 51.2 mg/L, with a median of 33.3 mg/L. She presented on day 233 with a new episode of bacteraemia due to an MRSA strain that was susceptible to vancomycin (MIC 1 mg/L) attributed to pulmonary infection. Sepsis resolved rapidly under treatment with continuous vancomycin infusion combined with fusidic acid. She died suddenly on day 243 of a non-infectious cause.

Patient 3. A 46-year-old man underwent a liver transplant for alcoholic cirrhosis in January 1999. The post-operative course was complicated by splenic haemorrhage, acute renal failure and graft rejection. On day 14, he developed a deep-seated surgical infection and bacteraemia due to MRSA. This strain was susceptible to vancomycin (MIC 3 mg/L). Intermittent vancomycin was administered from day 14 to 21 and dosing was adapted to serum levels. His clinical state deteriorated and surgical drainage was performed on day 20. Treatment was altered thereafter to continuous iv infusion of vancomycin (1.5 g/day) combined with rifampicin (900 mg/day). During continuous infusion, serum levels of vancomycin ranged between 18.5 and 54.6 mg/L, with a median of 33.3 mg/L. She presented on day 233 with a new episode of bacteraemia due to an MRSA strain that was susceptible to vancomycin. Concomitantly, a VISA strain (P2V136) was isolated from a decubitus ulcer. From day 56 to 112, she was given continuous infusion of vancomycin (2–3 g/day) in combination with fusidic acid (1.5 g/day). During continuous infusion, serum levels of vancomycin ranged between 24.2 and 51.2 mg/L, with a median of 33.3 mg/L. She presented on day 233 with a new episode of bacteraemia due to an MRSA strain that was susceptible to vancomycin (MIC 1 mg/L) attributed to pulmonary infection. Sepsis resolved rapidly under treatment with continuous vancomycin infusion combined with fusidic acid. She died suddenly on day 243 of a non-infectious cause.
Discussion

Well-documented infections caused by VISA strains have been reported from Asia and the USA.3–6,8 To our knowledge, our study reports the first clinical descriptions of cases of well-documented VISA infection from Europe with a rigorous set of criteria. The cases reported previously from Europe were associated with VISA strains that were not confirmed by population analysis or electron microscopy.5,10 Previous reports of VISA infection described patients who had received prolonged or repeated courses of vancomycin for MRSA infection, often administered for several months.4,6 In many of these patients, venous administration was irregular due to renal failure and, in some cases, vancomycin underdosing appeared as a contributing factor. In most cases, vancomycin failed to cure the infection and, in four cases, shift to a combination of other anti-staphylococcal drugs was reported to be successful. However, the majority of these patients had many risk factors for treatment failure, including infection of indwelling devices, undrained abscesses, and underlying pathology such as cancer and end-stage renal failure. Likewise, the shift to a non-glycopeptide was concomitant with surgical drainage or removal of the infected indwelling device, therefore making interpretation of the vancomycin failure uncertain. In the patients studied here, the VISA strains also emerged following vancomycin therapy. In our study, only patient 1 was considered infected by a VISA strain. This cystic fibrosis patient had hetero-VISA and VISA variants of MRSA that were isolated repeatedly from her sputum samples. As in previous reports of vancomycin failure for VISA infection, she showed only a transient response to intermittent or continuous high-dose vancomycin therapy. However, her pulmonary infection with VISA was treated successfully with high-dose continuous vancomycin in combination with fusidic acid, followed by suppressive oral treatment with a combination of minocycline and fusidic acid. To our knowledge, this is the first documented attempt to treat VISA infection with continuous infusion of high-dose vancomycin to achieve a blood level above MICs. However, as this therapy was administrated in combination with other anti-staphylococcal antibiotics it was not possible to conclude with certainty that the high-dose vancomycin was clinically effective. Further investigation of this therapeutic approach may be useful.

In contrast to the rarity of reported cases of VISA infection, the proportion of S. aureus with heterogeneous expression of reduced susceptibility to vancomycin ranges widely between surveys worldwide, including those from the European region: Spain 56%, France 20%, Germany 2–14%, Italy 1%, Japan 5–26%, Brazil 4%, England 0–16%, Greece 1%.10,11,13–15,17,18,20,24,28,29 The results from these studies are difficult to compare because most investigations used a retrospective study design and many restricted their analyses to a limited number of strains, usually from selected groups of patients. More importantly, definitions of VISA and methods for MIC determination of vancomycin differ markedly between investigators. For example, hetero-VISA strains are variously defined by different authors as S. aureus strains that exhibit a cell subpopulation growing at either 4 or 8 mg/L vancomycin.11,13,24,30 Using more restricted criteria and in an unselected population, we observed a low incidence of 0.3% VISA and 0.4% hetero-VISA among the patients in our hospital colonized or infected by MRSA.

The low-level resistance of VISA strains to vancomycin (MIC 8 mg/L) makes them difficult to detect by standard susceptibility test methods.23 A reversible vancomycin resistance phenotype observed with some VISA strains may compound this difficulty.31 Tenover et al.23 have shown that the disc diffusion method is inadequate to detect VISA strains. In our study, we used BHI agar supplemented with 6 mg/L vancomycin for VISA screening as recommended by the NCCLS, but with prolonged incubation (48 h) instead of the recommended 24 h.22 Using this modified technique, vancomycin agar screening had a low specificity in our study, since 95% of strains that grew after 48 h on screen agar were not confirmed as VISA. Other authors have reported artefactual growth of occasional vancomycin-susceptible S. aureus isolates on a vancomycin agar screen when using media that were prepared in-house.23,32 Our finding of low specificity using this screening method is in contrast to the report of Walsh et al.,33 who found it specific. The Etest method using a heavy inoculum on BHI agar with prolonged incubation also proved to be of low specificity in the present study (90% of false positive). This was also reported by Aucken et al.29 but it is again at variance with the findings of Walsh et al.31 In addition to technical differences, these discrepancies between studies could be explained by differences in study design. Our study looked at consecutive S. aureus clinical isolates, whereas Walsh et al.33 examined a selected collection of strains referred for reduced susceptibility to glycopeptides. Hetero-VISA strains are even more difficult to detect than VISA strains. They do not grow reproducibly on vancomycin screen agar and appear to be susceptible by Etest on MH agar with a standard inoculum and by microdilution MIC testing. The population analysis profile is at present the only reliable confirmation method but is too time consuming for routine use.

Many VISA and hetero-VISA strains are related to epidemic gentamicin-resistant MRSA clones that are disseminated in hospitals in Europe, Brazil and Japan.10–15,17,18,24,34 The VISA and hetero-VISA isolates in the present study belonged to two different MRSA types that are endemic in our hospital and unrelated to the US and Japanese VISA strains. One of these genotypes has been widely disseminated in western Europe since the mid-1980s and has been reported in more than 80% of Belgian hospitals.21 Outbreaks of S. aureus with reduced susceptibility to glycopeptides have been described
in French hospitals among patients who had not received glycopeptides.\textsuperscript{30,35} It appears that these strains are hetero-
VISA. In our study, the three patients with VISA infection or colonization showed no space–time clustering. According to local policy, the patients were already cared for using MRSA isolation precautions, including placement in a private room and use by healthcare personnel of gowns, gloves and masks.\textsuperscript{27} When the VISA strains were identified, nursing staff were increased in number for the care of VISA carriers to consolidate compliance with the isolation precautions according to the published recommendations.\textsuperscript{36}

The mechanism of decreased susceptibility of \textit{S. aureus} to glycopeptides is not yet well understood. Like other VISA strains described previously, the VISA strains studied here lacked the \textit{vanA} and \textit{vanB} genes that are responsible for glycopeptide resistance in enterococci. The strains that we examined by TEM showed the same abnormality of cell wall structure that has been described in other strains.\textsuperscript{4,6–7,9,37} Strains Mu50 and Mu3 have an accelerated cell wall turnover with an increased proportion of glutamine non-amidated muropeptides and overexpression of penicillin-binding proteins 2 and 2\textsuperscript{\textprime}.\textsuperscript{38–40} These abnormalities suggest increased production of D-Ala-D-Ala residues acting as false targets, which trap the antibiotic away from its lethal target site of cell wall synthesis adjacent to the membrane.\textsuperscript{38} Moreover, all VISA strains have a loss of detectable PBP4 activity, which leads to a decrease in cell wall cross-linking.\textsuperscript{41} Interestingly, in one of the VISA clinical isolates detected in our study, strain P1V44, the decreased susceptibility to vancomycin was accompanied by a parallel loss of expression of oxacillin resistance. This phenomenon has been reported previously to occur \textit{in vivo} by Sieradzki \textit{et al.}\textsuperscript{42} It was reproduced \textit{in vitro} by selection of a mutant VISA strain from an MRSA strain exposed to increasing vancomycin concentrations.

In conclusion, our study describes the first cases of infection and colonization with VISA and hetero-VISA strains in Belgium. These VISA strains emerged during vancomycin therapy for infections due to MRSA strains that are endemic in Belgian hospitals. Although these strains appear to be rare, they were associated with therapeutic difficulties, as reported previously. Therefore, it is desirable to improve the methods of routine detection of VISA strains to ensure their epidemiological surveillance. The optimal management of patients infected with VISA strains is not well defined. We concur with the recommendation that isolation precautions used for control of MRSA nosocomial transmission should be strictly enforced. Therapy with high-dose vancomycin administered by continuous infusion in combination with other anti-staphylococcal drugs may be a therapeutic option worth investigating in the case of VISA infection, but the removal of any foreign body or undrained collections should always be the first and foremost intervention.

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