Identification and characterization of a vancomycin-resistant *Staphylococcus aureus* isolated from Kolkata (South Asia)

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A pathogenic vancomycin-resistant *Staphylococcus aureus* (VRSA) isolate (MIC ≥64 µg ml⁻¹) was obtained from a Kolkata hospital in June 2005. Species identification was confirmed by Gram staining, standard biochemical tests and PCR amplification of the *nuc* gene, which encodes the thermostable nuclease that is highly specific for *S. aureus*. The VRSA isolate was also resistant to beta-lactams (amoxicillin, ampicillin, ceftaxime, cefuroxime, cephalaxin and meticillin), chloramphenicol, streptomycin, macrolides (erythromycin and roxithromycin), clindamycin, rifampicin and trimethoprim-sulfamethoxazole. However, the isolate was susceptible to gentamicin (an aminoglycoside) and ciprofloxacin (a fluoroquinolone). The resistance to vancomycin was inducible in vitro, because the MIC of vancomycin increased from 64 µg ml⁻¹ initially to 1024 µg ml⁻¹ during culture of this VRSA strain in the presence of vancomycin. The VRSA isolate contained a large plasmid (~53.4 kb) and four small plasmids of ~6, 5.5, 5.1 and 1.5 kb. The large plasmid of ~53.4 kb harbourd the vancomycin-resistance genes *vanHAX*, which was confirmed by PCR amplification using the same plasmid as template and, separately, primers specific for the 2.61 kb *vanHAX* gene cluster, *vanH* (969 bp), *vanA* (1032 bp) and *vanX* (609 bp). The VRSA isolate was also positive for *mecA*. Vancomycin resistance was successfully transferred from this VRSA donor to a vancomycin-sensitive recipient *S. aureus* clinical isolate by a broth mating procedure. The MIC of vancomycin for the transconjugant was 32 µg ml⁻¹, as against 2 µg ml⁻¹ for the parent strain. Nucleotide sequencing of the PCR product showed partial homology with *van* genes of an enterococcal transposon Tn1546-like element. This is believed to be the first Indian *S. aureus* isolate that has been shown to be phenotypically vancomycin-resistant, presumably due to a *vanHAX* analogue.

**INTRODUCTION**

Only four vancomycin-resistant *Staphylococcus aureus* (VRSA) isolates have been reported so far from the USA (Chang *et al.*, 2003; Tenover *et al.*, 2004; CDC, 2004; Weigel *et al.*, 2007; Perichon & Courvalin, 2006). There has been no report of a *van* gene-mediated VRSA from Asia as yet, except for vancomycin-intermediate *S. aureus* (VISA) in Japan (CDC, 1997) and Korea (Kim *et al.*, 2000). Recently, Tiwari & Sen (2006) have reported a VRSA which is *van* gene-negative. *Staphylococcus* can cause a variety of suppurative diseases in man, including skin, heart-valve, blood and bone infections (Morse, 1980). More than 90% of *Staphylococcus* strains are resistant to penicillin (Chambers, 2001), followed by increasing resistance to meticillin, aminoglycosides, macrolides and lincosamide (Dickgiesser & Kreiswirth, 1986; Levin *et al.*, 2005; Munckhof *et al.*, 2002; Schmitz *et al.*, 2000). In view of this antibiotic resistance, vancomycin has been the drug of last resort. Vancomycin, a glycopeptide antibiotic, acts against Gram-positive bacteria only, by inhibiting the incorporation of NAM-NAG-polypeptide into the growing peptidoglycan (PG) chain. It inhibits this process by reacting with D-Ala-D-Ala, which consequently blocks the release of terminal D-Ala and intrachain bond formation. Vancomycin-resistant *Enterococcus faecium* harbours the *vanA* operon, which contains five genes, *vanS*, -R, -H, -A and -X (Arthur *et al.*, 1993). *vanS* and *vanR* are the regulator genes (Wright *et al.*, 1993). VanH is a
D-hydroxyacid dehydrogenase that reduces pyruvate to D-lactate (Bugg et al., 1991a), which could be used by VanA ligase in conjunction with ATP and D-Ala to make a D-Ala-D-lactate depsipeptide (Bugg et al., 1991b), which is incorporated into the PG layer. Vancomycin binds to N-acetyl-D-Ala-D-lactate with an affinity 1000-fold lower than that of N-acetyl-D-Ala-D-Ala (Bugg et al., 1991b). VanX is a dipeptidase required for the hydrolys of D-Ala-D-Ala (Reynolds et al., 1994).

Conjugative transfer of high-level vancomycin resistance from Enterococcus faecalis to S. aureus (Noble et al., 1992), and transfer of glycopeptide- and macrolide-resistance genes by transconjugation among enterococci and from Ent. faecalis to S. aureus (Mlynarczyk et al., 2002), have been reported. Vancomycin-resistance gene acquisition by S. aureus from Ent. faecium in the clinical environment has also been reported by Weigel et al. (2007).

In this study, we have shown the emergence of vancomycin resistance in Kolkata, India, and its conjugative transfer from one clinical strain to another.

**METHODS**

**Bacterial strains.** Fifty-seven non-repeat clinical isolates of S. aureus were collected from various Kolkata hospitals, namely the Calcutta Medical College and Hospital, the School of Tropical Medicine, the Institute of Child Health, the R. G. Kar Medical College and Hospital, the Nilratan Sirkar Medical College and Hospital, and the Seth Sukhulal Karnani Medical College and Hospital, from January 2002 to December 2005. Earlier, 126 strains of S. aureus were collected from the Calcutta Medical College and Hospital during the period November 1985 to September 1988. All these strains were collected to study the antibiotic-resistance profile of S. aureus. All cultures were grown on nutrient agar (NA) medium and purified by a single colony isolation technique on NA containing 10% sodium chloride. S. aureus from the clinical isolate VRSA STM2, induced STM2, MC48 and transconjugants were prepared by sucrose-mediated conjugative transfer of high-level vancomycin resistance by mating procedure. The MIC of vancomycin was determined by a broth microdilution method using Mueller–Hinton broth (MHB; Himedia, 30 μg), cephalexin (Ranbaxy, 30 μg), cefepime (Unichem Laboratories, 30 μg), ampicillin (Biochem Pharmaceutical Industries, 10 μg), cefuroxime (Glaxo Smith Kline, 30 μg), clindamycin (Indipharma, 2 μg), erythromycin (Alembic, 15 μg), gentamicin (Nicholas, 10 μg), metillin (Himedia, 5 μg), oxacillin (Himedia, 1 μg), rifampicin (Lupin, 5 μg), roxithromycin (Alembic, 15 μg), streptomycin (Synbiotics, 10 μg), trimethoprim-sulfamethoxazole (1:5) (Piramal Health Care, 5 μg) and vancomycin (Lilly Pharma and Himedia, 30 μg). S. aureus ATCC 25923, an all-sensitive reference strain, was used as a quality control strain for the DAD test.

**Determination of MIC.** The MIC of vancomycin was determined by a broth microdilution method using Mueller–Hinton broth (MHB; dehydrated, Himedia), as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 2000).

**Isolation of plasmid DNAs.** For molecular studies, plasmid DNAs from the clinical isolate S. aureus STM2 and the reference strain E. coli V517, which contains six plasmids of molecular weights 53.4, 5.5, 5.1, 3, 2.7 and 2.1 kb (Macrina et al., 1978), were isolated using the QIAGEN Midi plasmid purification kit, following the manufacturer’s instruction of prewarming the elution buffer (Buffer EB) to 50 °C for elution of large plasmids.

**Preparation of genomic DNA.** The crude lysates of clinical isolates of S. aureus STM2 (VRSA), MC48 (vancomycin-sensitive S. aureus, VSSA) and T48 (transconjugant) were prepared by sucrose-mediated detergent lysis (Saha et al., 1989). Cell lysates were treated with proteinase K (10 μg ml⁻¹; preactivated at 37 °C for 30 min) at room temperature for 30 min. NaCl (1.44 ml, 5 M) was added to this mixture and incubated at 65 °C for 20 min. An equal volume of chloroform:isoamyl alcohol (24:1, v/v) mixture and incubated at 65 °C for 30 min. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v). The aqueous phase was again extracted twice with chloroform:isoamyl alcohol (24:1, v/v), and DNA was precipitated by the addition of chilled ethanol. The DNA pellet was washed with cold 70% ethanol and reconstituted in Tris/EDTA buffer. The genomic DNA was treated with RNase and stored at −20 °C.

**PCR.** PCR amplification was performed with an ABI 9700 thermal cycler in a volume of 50 μl. For amplification of the nuc gene and meca, the following components were used: 1.5 mM MgCl₂, 200 μM each of dATP, dCTP, dGTP and dTTP, 2 μM of each primer, 0.1 μg template DNA, and 1.25 U Taq polymerase (Invitrogen).

(i) PCR amplification of the nuc gene. A partial nuc gene was amplified using nuc gene primers (Table 1), which were selected on the basis of the published nucleotide sequence of the 966 bp nuc gene derived from the S. aureus Foggi strain (Brakstad et al., 1992). The cycling parameters consisted of 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 50 °C for 1 min, and extension at 72 °C for 1 min 30 s.

(ii) PCR amplification of meca. For amplification of meca, oligonucleotide primers (Table 1) were used (Dias et al., 2004). The reaction conditions were 30 cycles of denaturation at 94 °C for 40 s, primer annealing at 52 °C for 45 s, and extension at 72 °C for 30 s.

(iii) PCR amplification of vanHAX, vanH, vanA and vanX. The PCR amplification mixture contained the following components: 1X Phusion GC buffer containing 1.5 mM MgCl₂, 200 μM each of dNTP, 2 μM each primer, 0.1 μg template DNA, 3% (v/v) DMSO and 1 U Phusion DNA polymerase (Finnzymes). The amplification conditions were initial denaturation at 98 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s; annealing at 50 °C for 1 min; polymerization at 72 °C for 1 min 30 s for van HAX, 60 s for both vanH and vanA, and 30 s for vanX; and final extension at 72 °C for 5 min for all. The primer sequences specific for vanHAX, vanH, vanA and vanX (Donabedian et al., 2000) are given in Table 1.

**Transfer of vancomycin resistance by mating procedure.** Broth mating (Clewell et al., 1985; Philippon et al., 1983) was performed as
were edited and assembled using the Auto Assembler program. The PCR products were sequenced using an ABI PRISM 3130 Genetic Analyzer. Raw sequences were then assembled and edited using the Auto Assembler program. The resulting sequences were used for identification with the help of the NCBI BLASTN program.

RESULTS AND DISCUSSION

Source and identification of strain

The clinical isolate *S. aureus* STM2, which was found to be VRSA, was obtained from the School of Tropical Medicine (STM), Kolkata. The strain was isolated from the pus of an outpatient. No other case history could be found. Isolates MC48 and MC50 were obtained from pus samples from separate patients at the Calcutta Medical College and Hospitals.

The clinical isolates were identified using standard biochemical tests. All were catalase- and coagulase-positive. The isolates took 72 h to grow in mannitol–salt (10 % NaCl) agar medium.

The thermostable nuclease-encoding *nuc* gene is highly specific for *S. aureus*. PCR amplification of the *nuc* gene of VRSA STM2 using the gene-specific primers (Table 1) and the chromosomal DNA preparation yielded a 270 bp amplicon, as expected (data not shown). This result confirmed VRSA STM2 as an *S. aureus* strain.

PCR amplification of 16S rDNA of the clinical isolates VRSA STM2 and *S. aureus* MC48 using primers specific for staphylococcal 16S rRNA (Table 1) produced an identical...
1.5 kb amplicon of 16S rDNA in both the strains (Woo et al., 2003). The VRSA STM2 with a vancomycin MIC of 1024 \( \mu g \) ml\(^{-1}\) was named STM2-I (induced STM2). An identical 1.5 kb amplicon was also obtained from STM2-I and the transconjugant T48. These PCR results again confirmed the identity of MC48, STM2, STM2-I and T48 as *S. aureus*.

**Antimicrobial susceptibility tests**

On initial testing, the growth of VRSA STM2 on the NA screen plate containing 32 \( \mu g \) vancomycin ml\(^{-1}\) suggested possible vancomycin resistance. Further confirmation of vancomycin resistance was obtained from the DAD test, since no zone of inhibition surrounding the vancomycin disc was noted.

The VRSA STM2 isolate was resistant to many antibiotics, i.e. amoxicillin, ampicillin, cefepime, cefotaxime, cefuroxime, cephalaxin, chloramphenicol, clindamycin, erythromycin, meticillin, rifampicin, roxithromycin, streptomycin, trimethoprim-sulfamethoxazole and vancomycin. However, it was susceptible to gentamicin and ciprofloxacin, as determined by the DAD test (Table 2).

The MIC of vancomycin for the VRSA STM2 isolate was found to be 64 \( \mu g \) ml\(^{-1}\), which confirmed it as VRSA by NCCLS criteria. However, the MIC value increased to 1024 \( \mu g \) ml\(^{-1}\) after subculturing of this strain in the presence of vancomycin. This observation indicates the inducible nature of the vancomycin resistance of VRSA STM2. It has been suggested by Arthur and co-workers that the *vanA* gene cluster (\(vanR, vanS, vanH, vanA\) and \(vanX\)) carried by Tn1546 is responsible for inducible resistance to high levels of glycopeptides in Ent. faecium BM4147 and Ent. faecalis (Arthur et al., 1993; Reynolds et al., 1994). This suggestion validates our observation of an increasing MIC value for vancomycin (64 to 1024 \( \mu g \) ml\(^{-1}\)), and the inducible nature of resistance to high levels of vancomycin in VRSA STM2 might be due to the presence of the *vanA* gene cluster. The PCR and sequencing experiments further confirmed the presence of the *vanA* gene cluster (discussed below). Glycopeptide antibiotics, vancomycin and teicoplanin, are used to treat severe infections due to multidrug-resistant Gram-positive bacteria (including MRSA). However, treatment with vancomycin to alleviate this type of bacterial infection that has inducible resistance to high levels of the antibiotic itself would be a therapeutic failure, and might even be fatal for the patient.

**Plasmid analysis**

The plasmid profile of VRSA STM2 revealed five plasmids: one large plasmid of \(\sim 53.4\) kb, and four small plasmids of \(\sim 6, \sim 5.5, \sim 5.1\) and \(\sim 1.5\) kb, when compared with the molecular size of plasmid markers of the reference strain *E. coli* V517 (Fig. 1).

![Fig. 1. Agarose gel electrophoresis of plasmid DNAs isolated from VRSA STM2 and *E. coli* V517. Lanes: 1, plasmid DNAs of *E. coli* V517 as molecular size marker; 2, plasmid DNAs of VRSA STM2; 3, 100 bp DNA ladder.](http://jmm.sgmjournals.org)

Table 2. Antibiogram of clinical isolates of *S. aureus*

Results of DAD test of clinical isolates of *S. aureus*. Abbreviations: AMP, ampicillin; AMX, amoxicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; CTX, cefotaxime; CXM, cefuroxime; ERY, erythromycin; FEP, cefepime; GEN, gentamicin; LEX, cephalaxin; MET, meticillin; RIF, rifampicin; ROX, roxithromycin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin; R, resistant; S, sensitive.

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<tr>
<th>Strain no.</th>
<th>AMP</th>
<th>AMX</th>
<th>CHL</th>
<th>CIP</th>
<th>CLI</th>
<th>CTX</th>
<th>CXM</th>
<th>ERY</th>
<th>FEP</th>
<th>GEN</th>
<th>LEX</th>
<th>MET</th>
<th>RIF</th>
<th>ROX</th>
<th>STR</th>
<th>SXT</th>
<th>VAN</th>
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MIC for VAN (\(\mu g\) ml\(^{-1}\))

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>MIC for VAN</th>
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<tbody>
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<td>STM2</td>
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<tr>
<td>MC48</td>
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</tr>
<tr>
<td>MC50</td>
<td>1</td>
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http://jmm.sgmjournals.org
Transfer of vancomycin resistance by transconjugation

Twenty-one transconjugant colonies were found on LA plates containing appropriate selective antibiotics (16 µg vancomycin ml⁻¹ and 2.5 µg ciprofloxacin ml⁻¹) taking VRSA STM2 (ciprofloxacin-sensitive) as a donor, and vancomycin-sensitive ciprofloxacin-resistant S. aureus MC48 as a recipient (Table 2). However, no transconjugant colony was found on the above LA medium with VRSA STM2 as the donor and vancomycin-sensitive ciprofloxacin-resistant S. aureus MC50 (Table 2) as the recipient. No growth of recipient S. aureus strains MC48 and MC50, and the donor strain VRSA STM2, was observed on the above LA medium when inoculated separately. A selected transconjugant (named S. aureus T48) was observed to grow even after three successive overnight passages on NA plates containing 32 µg vancomycin ml⁻¹. This indicated successful and stable transfer of vancomycin resistance from the clinical isolate VRSA STM2 donor to another clinical isolate S. aureus MC48 as recipient. While the MIC of vancomycin for the transconjugant T48 was 32 µg ml⁻¹, that of the recipient S. aureus MC48 strain was 2 µg ml⁻¹.

PCR-based detection of vancomycin-resistance genes (vanHAX, vanH, vanA and vanX) and mecA in VRSA STM2

Initially, genomic DNA was used as template for PCR amplification of vanHAX, vanH, vanX, using primers specific for vanHAX (2.6 kb), vanH (969 bp) and vanX (609 bp) (Donabedian et al., 2000), as shown in Table 1. PCR products of 2.6 kb for vanHAX, 1 kb for vanH and 600 bp for vanX were obtained, which suggests the presence of the vanHAX gene cassette in VRSA STM2.

Later, the plasmid preparation obtained using the QIAGEN kit was used as template for PCR amplification of vanHAX (2.61 kb), vanH (969 bp), vanA (1032 bp) and vanX (609 bp) with the appropriate primers. Amplicons of 2.6 kb for vanHAX, ~1 kb for vanH, ~1.1 kb for vanA and ~500 bp for vanX were obtained (Fig. 2). The results confirmed the possession of van genes by the plasmid(s) of VRSA STM2. Most probably, the genomic DNA preparation had plasmid DNA contamination, and that was the reason for obtaining PCR products of vanHAX using genomic DNA as template.

Finally, the specific plasmid(s) that harboured the van genes was identified. Each plasmid was eluted from the gel, purified by using the gel-extraction kit (Promega), and used as template for PCR amplification of vanHAX, vanH, vanA and vanX separately using the specific primers for those genes (Table 1). Only in the case of the 53.4 kb plasmid were the same amplicons obtained as by PCR using the whole plasmid preparation (containing all the plasmids of different sizes) as template (Fig. 2). This result clearly established that the 53.4 kb plasmid harbours the vancomycin-resistance gene cluster vanHAX in VRSA STM2.

A 2.3 kb amplicon was also obtained from STM2-I and transconjugant T48 using primers specific for the vanHAX gene cassette. A 1 kb amplicon was obtained from VRSA STM2, STM2-I and transconjugant T48 using primers specific for the vanH gene (969 bp) (Fig. 3). However, no 2.3 or 1 kb amplicon was observed in the case of VSSA MC48 (Fig. 3). A 600 bp amplicon was also obtained from VRSA STM2, STM2-I and transconjugant T48 using primers specific for the vanX gene (609 bp) (data not shown). However, no such amplicon was observed in the case of VSSA MC48 using these primers. These results suggested the presence of a vanA gene cassette in VRSA STM2 and STM2-I that mediates high-level vancomycin resistance through alterations of the PG layer of the cell wall and cell-wall metabolism.

PCR amplification of vanHAX, vanH and vanX of transconjugant T48 (Fig. 3) clearly indicated the transfer of the same vancomycin-resistance genes vanHAX from the donor VRSA STM2 to the recipient VSSA MC48.

The meticillin resistance of VRSA STM2 was verified by PCR amplification of the mecA gene; an amplicon of 0.3 kb was obtained (data not shown).

Nucleotide sequencing

Nucleotide sequencing of 652 bases (GenBank accession no. EU019995) with the forward primer of the 1 kb PCR product of vanH showed 47% homology with the nucleotide sequence of the VanH dehydrogenase of the Tn1546-like element, and 44% homology with Tn1549 (Garnier et al., 2000). VanH dehydrogenase reduces...
Emergence of vancomycin-resistant S. aureus in India

...gene acquisition. Modification of the \( van \) gene complex in the Pennsylvania VRSA isolate (the second documented clinical VRSA isolate) has been reported by Clark et al. (2005); it has a deletion of 3098 bp, and two insertions of 809 and 1499 bp. Modification of the \( van \) gene complex in the New York VRSA isolate (the third documented clinical VRSA isolate) has also been reported by Weigel et al. (2007).

Intergeneric transfer of high-level vancomycin resistance from \( Ent. faecalis \) to \( S. aureus \) has been demonstrated by Noble et al. (1992). Intergeneric transfer of such high-level vancomycin and other antibiotic resistance from \( S. aureus \) to \( S. aureus \) has also been reported (Severin et al., 2004; Pawa et al., 2000). However, in this study, we have demonstrated intrageneric transfer of vancomycin resistance from a clinical strain of \( S. aureus \) to another clinical isolate of \( S. aureus \), which has alarming implications for the global dissemination of such high-level glycopeptide resistance in clinical settings with no known effective antibiotic therapy for some Gram-positive bacterial infections.

In summary, the major new findings of the present study are:

1. This is believed to be the first report of VRSA containing \( mecA \) and the \( vanA \) gene complex from Kolkata, India, as well as South Asia.

2. The vancomycin resistance of the clinical isolate VRSA STM2 is inducible in vitro.

3. PCR amplification and nucleotide sequencing experiments suggest the presence of a \( vanHAX \)-type gene cluster, analogous to that of the \( Tn1546 \)-like element, in VRSA STM2 and transconjugant T48.

4. The vancomycin resistance obtained in VRSA STM2 is plasmid-mediated and transferable to a sensitive clinical isolate (as found for VSSA MC48).

The emergence of inducible VRSA in Kolkata, India (as well as in South Asia), and its intrageneric transfer is alarming. This may soon become a global problem, unless antimicrobial agents are used more prudently. Scientists, clinicians and other healthcare professionals should identify and report VRSA promptly for appropriate care and treatment of patients, and start to implement infection-control precautions to prevent the spread of VRSA.

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