

# 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  $\geq 64 \mu\text{g ml}^{-1}$ ) 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, cefepime, cefotaxime, cefuroxime, cephalexin 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  $64 \mu\text{g ml}^{-1}$  initially to  $1024 \mu\text{g ml}^{-1}$  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 harboured 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  $32 \mu\text{g ml}^{-1}$ , as against  $2 \mu\text{g ml}^{-1}$  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.

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## 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

Abbreviations: DAD, disc agar diffusion; PG, peptidoglycan; VRSA, vancomycin-resistant *S. aureus*; VSSA, vancomycin-sensitive *S. aureus*.

The GenBank/EMBL/DDBJ accession numbers for the *S. aureus* sequences determined in this paper are EU019995 and EU016096.

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-acyl-D-Ala-D-lactate with an affinity 1000-fold lower than that of N-acyl-D-Ala-D-Ala (Bugg *et al.*, 1991b). VanX is a dipeptidase required for the hydrolysis 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* (Młynarczyk *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 Sukhlal 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. *Escherichia coli* V517 was obtained from National Institute of Cholera and Enteric Disease (NICED).

**Confirmation of species identification.** Identification of the clinical isolates of *S. aureus* was performed by traditional biochemical tests, including catalase, coagulase and mannitol fermentation tests, and Gram staining (Bannerman, 2003; Turk & Porter, 1978). PCR amplification of the *nuc* gene (Brakstad *et al.*, 1992) was performed only for VRSA STM2. PCR amplification of 16S rDNA was also performed for VRSA STM2, induced STM2, MC48 and transconjugant T48.

**Antibiotic susceptibility testing.** The antibiotic-resistance profile was determined by the disc agar diffusion (DAD) technique (Acar, 1980; Bauer *et al.*, 1966), using 18 antibiotic discs. Among these antibiotics, some discs were prepared in the Microbiology Laboratory, Department of Physiology, and a few were obtained commercially from Himedia. The antimicrobials, the manufacturers and the amounts ( $\mu\text{g}$ ) of antimicrobial per disc were as follows: amoxicillin (Rexcel, 30  $\mu\text{g}$ ), ampicillin (Biochem Pharmaceutical Industries, 10  $\mu\text{g}$ ), cefepime (Unichem Laboratories, 30  $\mu\text{g}$ ), cefotaxime (Alkem Laboratories, 30  $\mu\text{g}$ ), cefuroxime (Glaxo Smith Kline, 30  $\mu\text{g}$ ), cephalixin (Ranbaxy, 30  $\mu\text{g}$ ), chloramphenicol (Sigma, 30  $\mu\text{g}$ ), ciprofloxacin [Pharma(Ran), 5  $\mu\text{g}$ ], clindamycin (Indipharma, 2  $\mu\text{g}$ ), erythromycin (Alembic, 15  $\mu\text{g}$ ), gentamicin (Nicholas, 10  $\mu\text{g}$ ), meticcillin (Himedia, 5  $\mu\text{g}$ ), oxacillin (Himedia,

1  $\mu\text{g}$ ), rifampicin (Lupin, 5  $\mu\text{g}$ ), roxithromycin (Alembic, 15  $\mu\text{g}$ ), streptomycin (Synbiotics, 10  $\mu\text{g}$ ), trimethoprim-sulfamethoxazole (1:5) (Piramal Health Care, 5  $\mu\text{g}$ ) and vancomycin (Lilly Pharma and Himedia, 30  $\mu\text{g}$ ). *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  $\mu\text{g ml}^{-1}$ , 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 was added, and the aqueous layer was extracted with 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  $\mu\text{l}$ . For amplification of the *nuc* gene and *mecA*, the following components were used: 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  each of dATP, dTTP, dGTP and dCTP, 2  $\mu\text{M}$  of each primer, 0.1  $\mu\text{g}$  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: 1  $\times$  Phusion GC buffer containing 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  each dNTP, 2  $\mu\text{M}$  each primer, 0.1  $\mu\text{g}$  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 *vanHAX*, 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 (Clewel *et al.*, 1985; Philippon *et al.*, 1983) was performed as

**Table 1.** Sequences of primers and sizes of PCR-amplified products of different genes of *S. aureus*

All primers were obtained from Clonitec. The references of the primers are as follows: *nuc* (Brakstad *et al.*, 1992); 16S rRNA of *S. aureus* (Woo *et al.*, 2003); *mecA* (Dias *et al.*, 2004); *vanHAX* (Donabedian *et al.*, 2000); *vanH*, *vanA* and *vanX* (this study).

Specific gene for amplification	Primer	Primer sequence (5'–3')	Strain no.	Expected size of amplicon	Product (amplicon) obtained
<i>nuc</i>	Forward	GCGATTGATGGTGATACGGTT	VRSASTM2	270 bp	270 bp
	Reverse	AGCCAAGCCTTGACGAACATAAAGC			
16S rRNA of <i>S. aureus</i>	Forward	AGTTTGATCCTGGCTCAG	VRSASTM2	1.5 kb	1.5 kb
			MC48	1.5 kb	1.5 kb
	Reverse	AGGCCCGGGAACGTATTAC	STM2-I	1.5 kb	1.5 kb
			T48	1.5 kb	1.5 kb
<i>mecA</i>	Forward	TGGCTATCGTGTCACAATCG	VRSASTM2	310 bp	350 bp
	Reverse	CTGGAACCTTGTTGAGCAGAG			
<i>vanHAX</i>	Forward	ATGAATAACATCGGCATTAC	VRSASTM2	2.6 kb	2.6 kb
	Reverse	TTATTTAACGGGGAAATC	STM2-I		2.3 kb
<i>vanH</i>	Forward	ATGAATAACATCGGCATTAC	VRSASTM2	969 bp	1 kb
	Reverse	CTATTCATGCTCCTGTCTCC	STM2-I		1 kb
<i>vanA</i>	Forward	ATGAATAGAATAAAAGTTGC'	VRSASTM2	1032 bp	1.1 kb
	Reverse	TCACCCCTTTAACGCTAATA			
<i>vanX</i>	Forward	ATGGAAATAGGATTTACTTT	VRSASTM2	609 bp	600 bp
	Reverse	TTATTTAACGGGGAAATC-	STM2-I		600 bp
			T48		600 bp

follows. Single colonies of donor and recipient cells were inoculated separately in Luria–Bertani (LB) broth and grown overnight at 37 °C with shaking. These overnight cultures were diluted 1:100 in fresh medium, and each was grown to early exponential phase ( $OD_{600} \sim 0.6$ ). Mating mixture was prepared by adding 0.1 ml of donor cells to 0.9 ml of recipient cells, and was swirled gently for a few minutes and then incubated at 37 °C for 6 h (without shaking), followed by plating (0.2 ml per plate) on Luria agar (LA) medium containing 16 µg vancomycin ml<sup>-1</sup> and 2.5 µg ciprofloxacin ml<sup>-1</sup>. Colonies were counted after 48 and 72 h of incubation. Donor and recipient cells were also plated separately to check their disability to grow on the vancomycin plus ciprofloxacin plate, because the donor was ciprofloxacin-sensitive and the recipient was susceptible to vancomycin.

**Nucleotide sequencing.** Amplified PCR products (*vanHAX* and *vanH*) were purified with the Wizard SV Gel and PCR Clean Up System (Promega). The gel-eluted PCR products were sequenced with an ABI Prism automated DNA sequencer (Applied Biosystems) with the single primer (forward primer for *vanH*, reverse primer for *vanHAX*). The sequencing reaction was carried out using 5 pmol primer and the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), following the instructions of the manufacturer. The sequencing amplification conditions were as follows: 96 °C for 10 s, 50 °C for 10 s and 60 °C for 4 min for 25 cycles. After the sequencing PCR, the products (10 µl reaction volume) were treated with 2 µl 125 mM EDTA (pH 8.0), and then precipitated using 2 µl 3 M sodium acetate (pH 4.6) and 50 µl absolute ethanol for 20 min at room temperature (in the dark). The DNA was recovered by centrifugation, washed with 70 % alcohol, dried and resuspended in 15 µl Hi-Di formamide (Applied Biosystems). Sequencing was performed in an ABI PRISM 3130 Genetic Analyzer. Raw sequences were edited and assembled 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

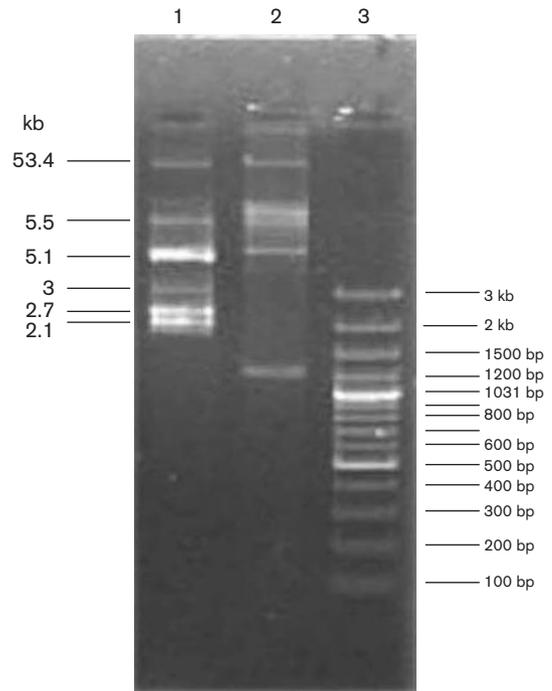
1.5 kb amplicon of 16S rDNA in both the strains (Woo *et al.*, 2003). The VRSA STM2 with a vancomycin MIC of  $1024 \mu\text{g ml}^{-1}$  was named STM2-I (induced STM2). An identical 1.5 kb amplicon was also obtained from STM2-I and the transconjugant T48. These PCR results again confirmed the identity 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  $32 \mu\text{g vancomycin ml}^{-1}$  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, cephalexin, 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  $64 \mu\text{g ml}^{-1}$ , which confirmed it as VRSA by NCCLS criteria. However, the MIC value increased to  $1024 \mu\text{g ml}^{-1}$  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  $1024 \mu\text{g ml}^{-1}$ ), 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



**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.

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).

**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, cephalexin; MET, meticillin; RIF, rifampicin; ROX, roxithromycin; STR, streptomycin; SXT, trimethoprim-sulfamethoxazole; VAN, vancomycin; R, resistant; S, sensitive.

Strain no.	Antibiotic																	MIC for VAN ( $\mu\text{g ml}^{-1}$ )
	AMP	AMX	CHL	CIP	CLI	CTX	CXM	ERY	FEP	GEN	LEX	MET	RIF	ROX	STR	SXT	VAN	
STM2	R	R	R	S	R	R	R	R	R	S	R	R	R	R	R	R	R	64
MC48	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	2
MC50	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	S	1

## Transfer of vancomycin resistance by transconjugation

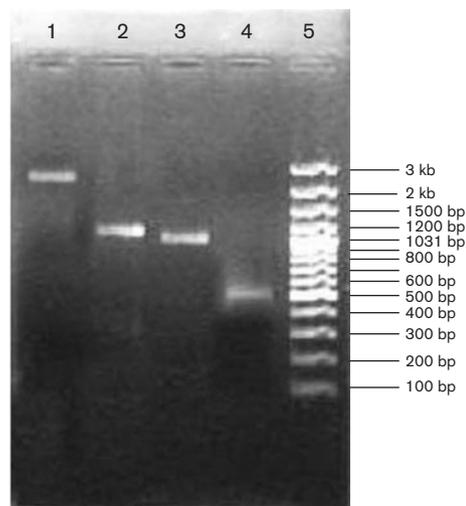
Twenty-one transconjugant colonies were found on LA plates containing appropriate selective antibiotics ( $16 \mu\text{g}$  vancomycin  $\text{ml}^{-1}$  and  $2.5 \mu\text{g}$  ciprofloxacin  $\text{ml}^{-1}$ ) taking VRSA STM2 (ciprofloxacin-sensitive) as a donor, and vancomycin-sensitive ciprofloxacin-resistant *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  $32 \mu\text{g}$  vancomycin  $\text{ml}^{-1}$ . 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  $32 \mu\text{g}$   $\text{ml}^{-1}$ , that of the recipient *S. aureus* MC48 strain was  $2 \mu\text{g}$   $\text{ml}^{-1}$ .

## 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* and *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 609 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*,  $\sim 1$  kb for *vanH*,  $\sim 1.1$  kb for *vanA* and  $\sim 500$  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.



**Fig. 2.** Agarose gel electrophoresis of PCR-amplified vancomycin-resistance genes of VRSA STM2 using plasmid DNA as template. Lanes: 1, PCR-amplified product of *vanHAX*; 2, PCR-amplified product of *vanA*; 3, PCR-amplified product of *vanH*; 4, PCR-amplified product of *vanX*; 5, 100 bp DNA ladder as molecular size marker.

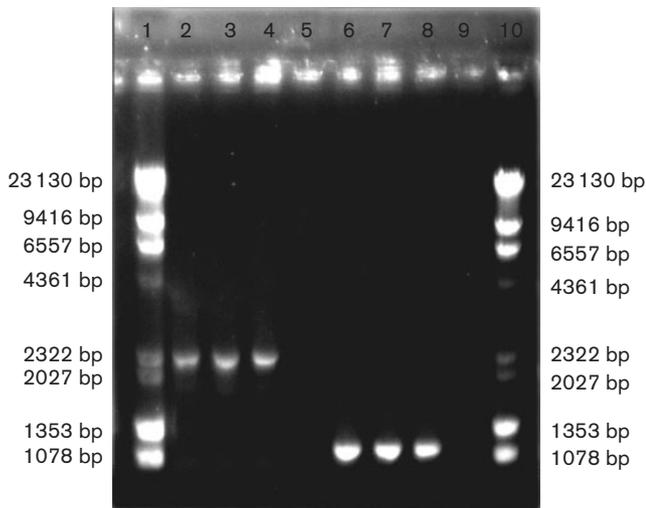
A 2.3 kb amplicon was also obtained from STM2-I and transconjugant T48 using primers specific for the *vanHAX* gene cassette. A 1 kb amplicon was obtained from VRSA STM2, STM2-I and transconjugant T48 using primers specific for the *vanH* gene (969 bp) (Fig. 3, Table 1). However, no 2.3 or 1 kb amplicon 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



**Fig. 3.** Agarose gel electrophoresis of PCR-amplified genes of VRSA STM2, induced VRSA STM2 (STM2-I), transconjugant (T48) and VSSA MC48. Lanes: 1 and 10, mix of lambda DNA *Hind*III digest and bacteriophage  $\Phi$ X174 DNA *Hae*III digest as size and mass standard; 2, 3 and 4, amplicons of *vanHAX* of STM2, STM2-I and T48, respectively; 5 and 9, MC48; 6, 7 and 8, amplicons of *vanH* of STM2, STM2-I and T48, respectively.

pyruvate to lactate, one of the building blocks of the PG layer of the cell wall of *S. aureus*. VanH dehydrogenase, which is functionally like lactate dehydrogenase, is a common metabolic enzyme required for glycolysis and the tricarboxylic acid (TCA) cycle (carbohydrate metabolism), and is ubiquitous in nature. Accordingly, in the VRSA STM2 strain, a mechanism exists that facilitates the expression of VanH dehydrogenase and VanA ligase to form D-Ala-D-lactate. Nucleotide sequencing of 272 bases with the reverse primer of the 2.3 kb PCR product of *vanHAX* produced a sequence (GenBank accession no. EU016096) that had 45% homology with, and a region of 21 bases identical to, the Tn1546-like element that contains the *vanHAX* gene cluster. This sequence also showed 56% homology with Tn1549, which is responsible for *vanB*-type vancomycin resistance in *Ent. faecalis* (Garnier *et al.*, 2000). However, the PCR product *vanA* (Fig. 2) was obtained using the 53.4 kb plasmid of VRSA STM2 as template and the primers specific for *vanA* of the Tn1546-like element. The *vanA* gene is absent in the *vanB* operon of Tn1549, which has *vanR*, *-S*, *-H*, *-B* and *-X*. Accordingly, we may conclude that the vancomycin resistance exhibited by VRSA STM2 is of the *vanA* type due to the presence of *vanHAX* analogous to that of the Tn1546-like element. These facts suggest that the VRSA isolate STM2 could have a modified *van* gene cassette that confers vancomycin resistance. The occurrence of partial sequence similarity might be due to multiple mutations that occurred during multiple replication (many thousand times) of bacterial genes having species specificity and species diversity, or during inter-species mobilization of resistance genes or

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). Intrageneric 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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