Original Article

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Genomic analysis of vancomycin-resistant *Staphylococcus aureus* VRS3b and its comparison with other VRSA isolates

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Summary **High-level vancomycin resistance among *Staphylococcus aureus* poses a grave threat to global health as the treatment options for this pathogen are very limited. A detailed evaluation of the genetic background of vancomycin-resistant *S. aureus* (VRSA) is expected to facilitate the understanding of its origin and pathogenicity. In this study, we performed the genetic analysis of the clinical VRSA isolates and identified the genetic basis of resistance to multiple antibiotics among these strains, based on the available draft genome sequences. In addition, we generated the draft genome of the strain VRS3b, which was considered to be same as VRS3a based on its isolation from the same patient. We found that strain VRS3b did not harbor the genes responsible for tetracycline and gentamicin, which was further confirmed by the sensitivity towards these antibiotics. Our results suggest that the strains VRS3a and VRS3b are different from the view of antibiotic resistance and highlight the possibility of generation of two distinct VRSA strains from the same patient.**

Keywords: *Staphylococcus aureus*, resistance, vancomycin, methicillin, genomics

1. Introduction

Staphylococcus aureus, a gram-positive bacterium, can reside in the human body both as a commensal or as an opportunistic pathogen and accounts for the majority of the deaths and hospitalization globally. Staphylococcal infections caused by methicillin-sensitive *S. aureus* can easily be cured with penicillins or cephalosporins (*1*). Resistance to methicillin (defined as minimum inhibitory concentration (MIC) of oxacillin ≥ 4 μg/mL) (*2*), mediated mainly by the *mecA* gene, was observed shortly after the introduction of methicillin (*3*). Since then, methicillin-resistant *S. aureus* (MRSA) developed itself as a leading cause of death and major clinical threat with more than 60% *S. aureus* isolates becoming resistant to methicillin (*4*). Vancomycin, a glycopeptide antibiotic produced by *Amycolatopsis orientalis*, has been the mainstay of treatment against infections caused by methicillin-resistant *S. aureus* (*1*). In 1996, *S. aureus* with reduced susceptibility to vancomycin (MIC 8 μg/mL), referred as vancomycin-intermediate *S. aureus* (VISA), was isolated in Japan (*5*). The first case of vancomycin-resistant *S. aureus* (VRSA) was first isolated in 2002 in the USA and following it, at least 14 cases of VRSA have been identified in the United States, and the first case in Europe was reported in 2013 (*6*). *vanA* type vancomycin resistance is the most prevalent vancomycin resistance and has been associated with the synthesis of an alternative, vancomycin-resistant pentatdepsipeptide peptidoglycan precursor (*7*). Genomic analysis of the strains resistant to vancomycin is critical to understand the genetic background and identify the difference between the strains, thus facilitating the development of novel anti-VRSA drugs.

S. aureus VRS3b was co-isolated with *S. aureus* VRS3a from nephrostomy tube exit site of a 64-year old female in New York, USA. As these two strains were isolated from the same patient, they were considered to be identical, and the characterization has been performed mainly for VRS3a. However, the vancomycin-resistant phenotype of VRS3b is more stable than VRS3a (*8*). Recently, the genome of

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multiple VRSA strains has been sequenced (*9*) and the study excluded the strain VRS3b considering it to be same as the strain VRS3a. In this study, we sequenced and analyzed the draft genome of VRS3b strain to reveal the genetic basis of drug resistance and its difference with the strain VRS3a.

2. Materials and Methods

2.1. *Draft genome sequences of VRSAs*

Strain VRS3a and VRS3b were obtained from BEI Resources and grown at 37°C aerobically in tryptic soy broth containing 6 µg/mL vancomycin. Genomic DNA was isolated using Qiagen DNA-blood Mini Kit (Qiagen, Hilden, Germany) (*10,11*). One hundred ng of the DNA was subjected to fragmentation using Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific, Waltham, MA, USA) to prepare 400 bp reads according to manufacturer's recommended protocol. The libraries were then enriched in an Ion Chef (Thermo Fisher Scientific) and subsequent sequencing was performed in Ion PGM System (Thermo Fisher Scientific). The reads were first assembled in the Ion Torrent Server (Thermo Fisher Scientific) and then analyzed in the CLC Genomics Workbench ver 9.5.3. (CLC bio, Aarhus, Denmark). The draft sequences were downloaded from NCBI using following GenBank accession numbers AHBK00000000 – VRS1; AHBL00000000 – VRS2; AHBM00000000 – VRS3a; NBCP00000000 – VRS3b; AHBN00000000 – VRS4; AHBO00000000 – VRS5; AHBP00000000 – VRS6; AHBQ00000000 – VRS7; AHBR00000000 – VRS8; AHBS00000000 – VRS9; AHBT00000000 – VRS10; AHBU00000000 – VRS11a; AHBV00000000 – VRS11b; and JICL00000000 – BR-VRSA.

2.2. *Genomic analysis of VRSAs*

2.2.1. *Sequencing typing*

The draft genomes (*9,11*) were analyzed in the CLC Genomics Workbench. For multi locus sequence typing (MLST), the sequence of seven housekeeping genes: *arcC* (carbamate kinase); *aroe* (shikimate dehydrogenase); *glpf* (glycerol kinase); *gmk* (guanylate kinase); *pta* (phosphate acetyltransferase); *tpi* (triosephosphate isomerase); and *yqil* (acetyl coenzyme A acetyltransferase) was collected and trimmed to extract the seven loci by using the standard sequence of a typical *S. aureus*. The trimmed sequences were then concatenated and submitted to clustalW for the generation of phylogenetic tree and to the MLST server (*http://saureus.beta.mlst.net/#*) for sequence typing. The trimmed sequence used for MLST can be found in the supplementary information (Supplementary Data, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=10*).

2.2.2. Analysis of resistance genes

The recent database to find resistance was downloaded from the server (*12*). The database consisted of 2,156 genes known to be involved in the resistance against multiple antibiotics. The presence of resistance gene on the draft genome was scanned with a minimum identity

a.

Strain	Isolation year	Loci							ST
		arcc	*aroe*	*glpf*	*gmk*	*pta*	*tpi*	*yqil*	
NWMN		3	32	1	1	4	4	3	254
USA300		3	3	1	1	4	4	3	8
VRS1	2002	1	4	53	4	12	1	10	371
VRS2	2002	1	4	1	4	12	1	10	5
VRS3a	2004	1	4	1	4	12	1	10	5
VRS3b	2004	1	4	1	4	12	1	10	5
VRS4	2005	N/A	4	1	4	12	1	10	N/A
VRS5	2005	1	34	1	4	12	1	28	231
VRS6	2005	31	4	1	4	12	1	10	85
VRS7	2006	1	34	1	4	12	1	28	231
VRS8	2007	1	4	1	4	12	1	10	5
VRS9	2007	1	4	1	4	12	1	10	5
VRS10	2009	1	4	1	4	12	1	10	5
VRS11a	2010	1	4	1	4	12	1	10	5
VRS11b	2010	1	4	1	4	12	1	10	5
BR-VRSA	2012	3	3	1	1	4	4	3	8

b.

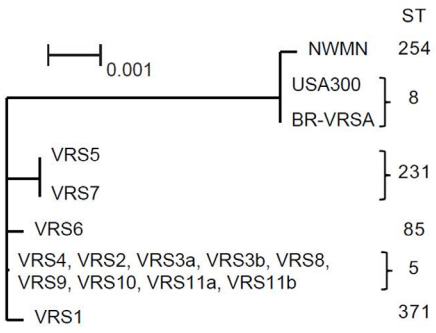

Figure 1. Multi locus sequence typing (a), and phylogenetic tree (b) of vancomycin-resistant *Staphylococci*. N/A: not analyzed. The *arcC* locus of strain VRS4 could not be analyzed as the size of the allele was different from standard allele size of 465. The distance scale of 0.001 in the phylogenetic tree indicates 0.1% differences between the groups. The nine strains from ST5 group had a highly similar concatenated sequence used for the tree construction.

Table 1. Genes involved in antibiotic resistance present in clinical isolates of VRSAs

	Staphylococcus aureus strain													
MRSA	VRSA													
USA300	VRS1	VRS2	VRS3a	VRS3b	VRS4	VRS5	VRS6	VRS7	VRS8	VRS9	VRS10	VRS11a	VRS11b	BR-VRSA
mecA	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *vanR* *aadD* *spc* *aac(6')-aph(2")* *blaZ* *erm(A)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *vanR* *aadD* *spc* *aac(6')-aph(2")* *ant(6)-Ia* *aph(3')-III* *blaZ* *erm(A)* *erm(B)* *mph(C)* *msr(A)* *tet(K)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *aac(6')-aph(2")* *ant(6)-Ia* *aph(3')-III* *blaZ* *erm(B)* *mph(C)* *msr(A)* *tet(U)* *tet(S)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *aph(3')-III** *blaZ* *erm(B)* *mph(C)* *msr(A)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *aadD* *spc* *erm(A)* *erm(B)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *vanR* *aadD* *spc* *erm(A)* *erm(B)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *vanR* *aadD* *spc* *erm(A)* *erm(C)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *vanR* *aadD* *spc* *aac(6')-aph(2")* *ant(6)-Ia* *aph(3')-III* *blaZ* *erm(A)* *erm(B)* *mph(C)* *msr(A)* *tet(K)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *aadD* *spc* *aac(6')-aph(2")* *erm(A)* *erm(C)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *aadD* *spc* *aac(6')-aph(2")* *ant(6)-Ia* *aph(3')-III* *blaZ* *erm(A)* *mph(C)* *msr(A)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *aadD* *spc* *blaZ* *erm(A)* *erm(B)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *aadD* *spc* *blaZ* *erm(A)* *erm(B)*	*mecA* *vanZ* *vanX* *vanR* *vanY* *vanH* *vanA* *vanS* *aac(6')-aph(2")* *blaZ* *erm(C)* *dfrG*

Genes conferring resistance: methicillin – *mecA*; vancomycin – *van* genes; aminoglycosides – *aadD*, *spc*, *aac(6')-aph(2")*, *ant(6)-Ia*, *aph(3')-III*; beta lactams – *blaZ*; erythromycin – *erm* genes, *msr(A)* ; streptogramin B – *msr(A)*; macrolide – *mph(C)*; tetracycline – *tet* genes; trimethoprim – *dfrG*. *: part of the gene was detected in VRS3b.

Table 2. Minimum inhibitory concentrations of various antibiotics against VRS3a and VRS3b

Strain	MIC (µg/mL)			
	Vancomycin	Tetracycline	Kanamycin	Gentamicin
VRS3a	> 128	16	128	128
VRS3b	> 128	< 0.125	128	0.5
MSSA1	1	< 0.125	4	0.5

Table 3. List of the genes present in VRS3b that did not map in the VRS3a reads

Contig	ORF ID	Putative function
22	B6A35_13980	Arsenical efflux pump membrane protein ArsB
	B6A35_13985	ArsC
	B6A35_13990	Lactococcin 972 family bacteriocin
	B6A35_13995	Bacteriocin associated protein
23	B6A35_14125	Hypothetical protein
38	B6A35_14765	Arsenic transporter
	B6A35_14770	ArsC
	B6A35_14775	Lactococcin 972 family bacteriocin
	B6A35_14780	Bacteriocin associated protein
43	B6A35_14855	Hypothetical protein
	B6A35_14860	Hypothetical protein
	B6A35_14865	Hypothetical protein
44	B6A35_14870	Hypothetical protein

of 98% and minimum length of 60% in the CLC Genomics Workbench.

2.3. *Determination of minimum inhibitory concentration*

The MIC was determined by broth microdilution assay (*13-15*). Briefly, *Staphylococci* were grown with aeration in 50-mL falcon tube containing 5 mL Tryptic Soy Broth (TSB; Becton Dickinson and Company, Franklin Lakes NJ, USA) in a shaker maintained at 37°C. The medium was supplemented with 6 µg/mL vancomycin as required. The overnight culture was diluted with cation-adjusted Muller-Hinton Broth (MHB; Becton Dickinson and Company) to have approximately 5×10^4 colony forming units (CFU) per 100 µL in each well in a round bottom 96-well plate. Serial dilutions of antibiotics were added to each well to obtain a final concentration of 128 µg/mL to 0.0125 µg/mL. The plates were further incubated at 37°C for 20 h and MIC was determined as the lowest concentration that did not allow visible growth of cell.

3. Results

3.1. *The phylogeny of VRSAs*

We found that the 14 VRSAs were categorized among five sequence types (STs): ST5, ST8, ST85, ST231, and ST371. ST5 was the most predominant group with eight VRSAs falling within this group (Figure 1a). The strain VRS4 could not be typed as the *arcC* locus in this strain had a deletion of a nucleotide and the trimmed length was different from the standard length. Previous reports have characterized the strain VRS4 as ST5 (*9*). Further, we constructed a phylogenetic tree based on the sequences used for sequence typing and found that VRS4 clustered with other VRSAs of ST5 group. The strain BR-VRSA was categorized to be ST8, same as the methicillin-resistant, vancomycin-sensitive strain USA300 strain and based on the phylogenetic tree, it also claded with the USA300 rather than other VRSAs (Figure 1b).

3.2. *Presence of antibiotic resistance genes in VRSAs*

When checked for the presence of antibiotic resistance genes, we found that all the strains of VRSAs harbored *mecA* gene, involved in resistance to methicillin. Further, multiple genes involved in the resistance against clinically used antibiotics such as vancomycin, aminoglycosides, streptogramins and macrolides were identified in the draft genome (Table 1). This finding, in fact, indicated the complexity of VRSA treatment in the clinic. Interestingly, the drug resistance genes were not the same among these strains. This difference might reflect the clinical setting where most of the

patients from whom these VRSAs were isolated had a history of several underlying conditions and were exposed to multiple antibiotics during isolation. Among the VRSAs, VRS3a and VRS3b were considered to be same as these two strains were isolated from the same patient source. Our analysis indicated that antibiotic resistance genes were not the same among these two strains.

3.3. *Comparative analysis of strains VRS3a and VRS3b*

We found that VRS3b did not harbor the putative genes that conferred resistance to tetracycline and aminoglycosides such as kanamycin and gentamicin. To confirm this, we determined the MIC of these two strains and found that VRS3a was resistant to these antibiotics whereas VRS3b was sensitive to tetracycline and gentamicin but resistant to kanamycin (Table 2). Resistance to kanamycin but not to gentamicin in *S. aureus* might be ascribed to *aph(3')-III* gene (*16*). Given that the sequence we generated was a draft genome, we performed a blast search for this gene in all contigs generated from the VRS3b sequence. We found a part of *aph(3')-III* at the terminal position of a contig less than 1,000 bp suggesting that this might be responsible for resistance to kanamycin but not gentamicin in VRS3b. These results further suggested that these two strains were different. To confirm this notion, we mapped the reads obtained from *de novo* sequencing of VRS3a against the VRS3b contigs. We found that some of the genes present in VRS3b were missing from the VRS3a reads (Table 3).

3.4. *Comparative analysis of Tn1546*

Transposon *Tn1546*, obtained from vancomycin-resistant *Enterococcus* (*17*), has been shown to be one of the responsible elements of vancomycin resistance in VRSAs (*7*). The analysis of the genome indicated that all the VRSAs but VRS3a, VRS3b, and BR-VRSA harbored complete sets of genes from *Tn1546*. To further confirm this result, we independently mapped the reads from strains VRS3a and VRS3b and found that these strains did not harbor transposase and part of resolvase from *Tn1546* (Figure 2).

4. Discussion

The clinical isolates of MRSA with decreased susceptibility to vancomycin (VISA) and high-level vancomycin resistance (VRSA) pose a serious threat to public health. Comparative genomic analysis of the clinical isolates of VRSA will facilitate our understanding of how these strains acquired the antibiotic resistance gene. Successful growth of these isolates in mixed culture and their ability to overcome the continuous antibiotic selection pressure in the

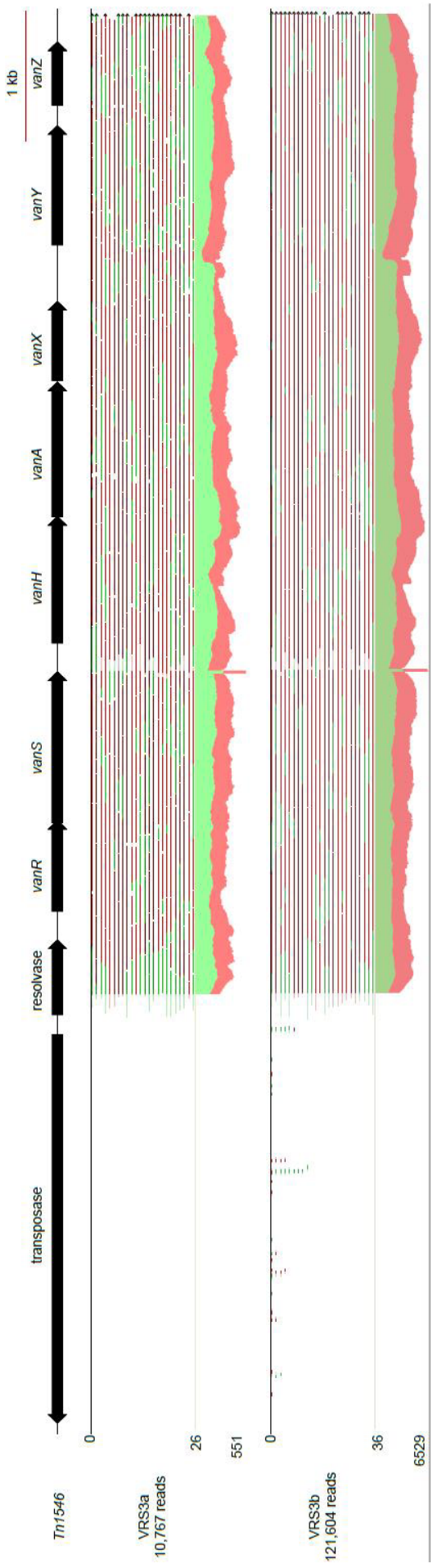

Figure 2. Mapping of VRS3a and VRS3b reads against transposon *Tn1546* reference sequence.

hospital further highlights the importance of this issue. Here, we showed that the genes conferring resistance to methicillin, vancomycin and erythromycin were common in all the VRSAs and the genes conferring resistance to other antibiotics such as beta lactams, macrolide and tetracycline were distributed. Besides, we found that the strain VRS3b was typically different from VRS3a. Furthermore, we demonstrated that some of the genes present in VRS3b were missing in VRS3a. Although we could not precisely predict whether these two strains independently acquired vancomycin-resistance trait or diverged after the acquisition of the vancomycin resistance, our results suggested that these two strains acquired vancomycin-resistance in a similar manner. Our findings highlighted the possibility of generation of two different VRSA strains from the same source.

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