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ORIGINAL RESEARCH

Genetic characterization of two vancomycin-resistant *Staphylococcus aureus* isolates in Kerman, Iran

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Aim: The aim of this study was the genetic characterization of two clinical vancomycin-resistant *Staphylococcus aureus* (VRSA) isolates.

Materials and methods: Resistance to vancomycin was determined by phenotypic method. PCR was used for detection of *mecA, vanA, ermA, ermB, ermC, msrA/B, aph*(2")-Ic, *aph*(3′)-IIIa, *pvl*, Immune Evasion Cluster [*sea, sep, chip, sak* and *scn*] genes and biofilm operon *ica*ABCD. On the other hand, multilocus sequence typing and *agr* typing methods were performed for the determination of clonal relationship and *van* operon was detected and sequenced.

Results: Vancomycin-resistant *Staphylococcus aureus* strain 1 (VRSA-1) was positive for *vanA, ermA, ermC, aph*(2")-Ic, *aph*(3′)-IIIa, *sea, sep, ica*D genes, belonging to *agr* type I; SCC*mec* type III; *spa* type t030; and ST239. However, the genetic characterization of Vancomycin-resistant *Staphylococcus aureus* strain 2 (VRSA-2) revealed the presence of various types of resistance genes *vanA, ermA, ermC, aph*(2")-Ic, *aph*(3′)-IIIa, *sea*, i*caD*, relating to *agr* type I; SCC*mec* type III; *spa* type t459; and ST239. The presence of transposon Tn*1546* was determined by PCR sequencing.The Basic Local Alignment Search Tool analysis of *van* operon in the VRSA isolates showed 99.6% sequence homology to Tn*1546* in vancomycin-resistant enterococci, indicating the *vanA* operon has an enterococcal origin.

Conclusion: In conclusion, the ST239 is one of the most common clones of MRSA isolates which involved the hospital-associated infections, therefore, the emergence of VRSA isolates with ST239 increased the spread of resistance to vancomycin in the hospital settings.

Keywords: VRSA, MLST, SCC*mec*, *spa*, *agr* type, *van* operon

Introduction

Staphylococcus aureus is one of the most destructive causes of bacterial infections in hospital settings.[1] The rapid spread of this type of bacterial infections has been accompanied by a rise in antibiotic-resistant strains.[2] The high-level vancomycin-resistant *S. aureus* (VRSA) was reported in 2002, Michigan, USA, for the first time and was subsequently detected in different hospitals around the world.[3,4] However, VRSA strains are rarely reported from all over the world.[5] So far, the VRSA strains have been reported, belonging to sequence types (STs) including ST1, ST5, ST8 ST85, ST231, ST239, ST250, ST371, ST923, and ST1283, and ST231.[5–7] The VRSA strains belonging to ST5, ST8, ST239 and ST1283 were recently reported in Iran.[6,7] The sequence type 239 is the most common clone of methicillin-resistant

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S. aureus (MRSA) which has been reported in the hospital settings around the world that would be resistant to the widespread antibacterial agents.[8,9]

Transposon Tn*1546*, a Tn3-related transposon,causing vancomycin resistance, was associated with a cluster of seven genes including *vanS, vanR, vanH, vanA, vanX, vanY,* and *vanZ*.[10] Generally, the horizontal transfer of Tn*1546* transposon from vancomycin-resistant *Enterococci spp* to *S. aureus* strains is responsible for resistance to vancomycin in *S. aureus* strains as well as the emergence of VRSA.[5] Herein, we reported the genetic characterization of two vancomycin-resistant *S. aureus* strains which were collected from hospitalized patients, Kerman, Iran.

Materials and methods

Patients and isolates sources

In our previous studies, from April 2015 to March 2017, we detected two VRSA strains from 205 non-duplicate of *S. aureus* isolates. The two VRSA isolates were collected, VRSA-1 belonged to *spa* type t030; SCC*mec* III and VRSA-2 belonged to *spa* type t459; SCC*mec* III. The minimum inhibitory concentration (MIC) of isolates to vancomycin was ≥64 µg/mL and the isolates were negative for panton-valentine leukocidin (*pvl*) gene and were resistant to gentamicin, amikacin, erythromycin, clindamycin, tetracycline, ciprofloxacin, penicillin, cefoxitin, and trimethoprim/sulfamethoxazole[11,12] The isolates were stored in Trypticase Soy Broth plus 15% Glycerol at −80°C for more investigation.

The VRSA strains were isolated from two patients with a long history of hospitalization. Briefly, the VRSA-1 was associated with the bronchoalveolar lavage sample from a 76-year-old female patient, hospitalizing in ICU at Afzalipour hospital (425 beds) in Kerman along with clinical manifestations including pneumonia, fever, with a history of diabetes mellitus and hemodialysis,who died without including infections treated. The VRSA-2 isolate was obtained from a surgical wound infection of a 21-year -old male patient, hospitalized in orthopedic unit in Bahonar hospital (375 beds) in Kerman. (Other data about patient was not available.)

Antibiotic susceptibility test

The MIC of the isolates to linezolid and daptomycin was determined according to Clinical & Laboratory Standards Institute guidelines and also, disk diffusion method was used for determination of susceptibility of isolates to rifampin.[13]

Detection of virulence, resistance genes, and molecular typing of the VRSA isolates

In the following previous studies,[11,12] the isolates were confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS, Bruker, Daltonics, Medical Microbiology and Clinical Microbiology Department of Umea University, Umea, Sweden). Resistance genes including *ermA, ermB, ermC, msrA/B*, aminoglycoside modifying genes [(*aac* (6′)-Ie-*aph*(2′′)-I, *aph*(2′′)-Ib, *aph*(2′′)-Ic, *aph*(2′′)-Id, *aph*(3′)-IIIa, *ant*(4′)-Ia)], and *agr* type of the isolates were determined by PCR method in FlexCycler2 PCR-Thermocycler (Analytik Jena, Co, Jena, Germany). On the other hand, the virulence genes including *sea, sep, chip, sak, scn, icaABCD* were detected by PCR. Table 1 shows the primers that were used in PCR amplification of virulence, resistance genes, and *agr* typing. Multilocus sequence typing was performed by PCR sequencing of seven housekeeping genes (*arc, aro, glp, gmk, pta, tpi*, and *yqi*) according to https://pubmlst.org/saureus/.

Detection and sequencing of *van* operon

The *van* operon including *vanR, vanS, vanH, vanA, vanX, vanY,* and *vanZ* were detected and sequenced by primer walking method, using 12 specific primer pairs (Table 2) that were designed by primer designing tool in NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast) for different regions of Tn*1546* by PCR technique. We used the Tn*1546* sequences with accession numbers HM565172.1, M97297, KR349520, KR047792, and KX496042.1 in GenBank for primer designing (https://www.ncbi.nlm.nih.gov/nuccore). The PCR products for *van* genes were sequenced by Applied Biosystems 3730/3730Xl DNA Analyzers (Bioneer, Co, South Korea). The sequences were assembled by Lasergene package software (DNASTAR) and were analyzed by the basic local alignment search tool (BLAST) in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Open reading frames (ORFs) of *van* genes were determined by Open Reading Frame Finder software (https://www.ncbi.nlm.nih.gov/orffinder).

Table 1 The primer sequences were used in this study

Gene target	Primer sequence(5′-3′)	Annealing temperature (°C)	Product (bp)	Ref
nuc	F-GCGATTGATGGTGATACGGTT	60	279	11,12
	R-AGCCAAGCCTTGACGAACTAAAGC			
mecA	F-TCCAGATTACAACTTCACCAGG	56	162	11,12
	R-CCACTTCATATCTTGTAACG			
vanA	F-CATGAATAGAATAAAAGTTGCAATA	50	1030	12
	R-CCCCTTTAACGCTAATACGATCAA			
erm A	F-TATCTTATCGTTGAGAAGGGATT	56.5	139	11,12
	R-CTACACTTGGCTTAGGATGAAA			
erm B	F-CTATCTGATTGTTGAAGAAGGATT	56.5	142	11,12
	R-GTTTACTCTTGGTTTAGGATGAAA			
erm C	F-AATCGTCAATTCCTGCATGT	55.5	297	11,12
	R-TAATCGTGGAATACGGGTTTG			
msrA	F-GCAAATGGTGTAGGTAAGACAACT	56.5	402	11,12
	R-ATCATCATGTGATGTAAACAAAAT			
aac(6′)-Ie-aph(2″)-I	F-CAGGAATTTATCGAAAATGGTAGAAAAG	60	369	14
	R-CACAATCGACTAAAGAGTACCAATC			
aph(2″)-Ib	F-CTTGGACGCTGAGATATATGAGCAC	60	867	14
	R-GTTTGTAGCAATTCAGAAACACCCTT			
aph(2″)-Ic	F-CCACAATGATAATGACTCAGTTCCC	60	444	14
	R-CCACAGCTTCCGATAGCAAGAG			
aph(2″)-Id	F-GTGGTTTTTACAGGAATGCCATC	60	641	14
	R-CCCTCTTCATACCAATCCATATAACC			
aph(3′)-IIIa	F-GGCTAAAATGAGAATATCACCGG	60	523	14
	R-CTTTAAAAAATCATACAGCTCGCG			
ant(4′)-Ia	F-CAAACTGCTAAATCGGTAGAAGCC	60	294	14
	R-GGAAAGTTGACCAGACATTACGAACT			
chp	F-GAAAAAGAAATTAGCAACAACAG	48	410	15
	R-CATAAGATGATTTAGACTCTCC			
sak	F-AAGGCGATGACGCGAGTTAT	50	223	15
	R-GCGCTTGGATCTAATTCAAC			
sea	F-AGATCATTCGTGGTATAACG	50	408	15
	R-TTAACCGAAGGTTCTGTAGA			
sep	F-AATCATAACCAACCGAATCA	50	500	15
	R-TCATAATGGAAGTGCTATAA			
scn	F-AGCACAAGCTTGCCAACATCG	49	258	15
	R-TTAATATTTACTTTTTAGTGC			
icaA	F-TCTCTTGCAGGAGCAATCAA	60	188	15
	R-TCAGGCACTAACATCCAGCA			
icaB	F-ATGGCTTAAAGCACACGACGC	61	526	15
	R-TATCGGCATCTGGTGTGACAG			
icaC	R-CTCTCTTAACATCATTCCGACGCC	63	1013	15
	F-ATCATCGTGACACACTTACTAACG			
icaD	F-GAACCGCTTGCCATGTGTTG	61	483	15
	R-GCTTGACCATGTTGCGTAACC			
pvl	F-TCATTAGGTAAAATGTCTGGACATGATCCA	55	433	11,12
	R-GCATCAASTGTATTGGATAGCAAAAGC			
agr	Pan-ATG CAC ATG GTG CAC ATG C	50	441	16
	I-GTCACAAGTACTATAAGCTGCGAT		575	
	II-TATTACTAATTGAAA AGT GGC CATAGC		323	
	III-GTA ATG TAATAGCTTGTATAATAATACCCAG		659	
	IV-CGATAATGCCGTAATACCCG			

Table 2 Primers used for amplification and sequencing of *van* operon

Name of primers	Primer sequence(5′-3′)	Annealing temperature (°C)	PCR product (bp)	Ref
Tn1	F-CCATTTTCGCTCCTCTAACG R-CGAATCTGGAAAGCGAAAAG	50.5	1025	This study
Tn2	F-CTTTTCGCTTTCCAGATTCG R-AGAATGGTGTGGGAAGCAAG	51	802	
TnRe	F-GATGGATGCTGCGAGGTAAT R-ATCCATTCCGATCTCGTTCA	51.5	849	
Tn,Re,VanR	F-CCCGTTTGGAAAAAGTGAAG R-TCGGCAATTTCATGTTCATC	50	1074	
VanRS	F-GTATTCCGCTAATGGGCAAT R-GATCCAATCCCCAAGTTTCC	50	1027	
VanS	F-TTCAATGATCCGAGGGAAAC R-CGCTGGAAGCTCTACCCTAA	52	1009	
VanS,IS1251	F-ACGCGGAAAGCAATGATAAC R-TATTGTGCGTTGGGGTACAA	52	1297	
IS1251,VanH	F-GCTGACAAGCTTTGCATTTG R-CGCTATTTTGCAGCAACTCA	52	1203	
VanHA	F-GCTTATAGTCGCAGCCGAAG R-TTTTGCCGTTTCCTGTATCC	52.5	1273	
VanAX	F-TTATAACCGTTCCCGCAGAC R-TGGGGTATGGTTCGTCTCTT	52.5	847	
VanX,IS1216E	F-CATGGAAAACAGTGGGTTTG R-ACGGCACAATCTCGTTTGA	51.5	1200	
VanY	F-TTGTCAACAGGCAGTTCAGC R-TTTCATTCCGCCATCCTTAC	51	1143	
VanYZ	F-CACCATTCATGTGCCGACTA R-TTTTCCCCTCACTTCACACC	52	862	
Rev	F-CCCTTCACGTTGTCTCATCC		Pan	

Results

Genetic characterization of isolates

The isolates were resistant to rifampin and sensitive to linezolid and daptomycin with MIC ≤0.5 µg/mL (antibiotic resistance profile to other antibiotic is given in "Material and methods" section). The expected molecular size 1030 bp product referring to the *vanA gene* was detected and sequenced in both VRSA strains as shown in Figure 1. The sequence presented 100% identity with *vanA* gene of vancomycin-resistant enterococci (VRE), on Tn*1546* as indicated in GenBank database. Furthermore, the VRSA strains were analyzed for the presence of resistance and virulence genes. VRSA-1 was positive for *ermA, ermC, aph* (2")-Ic, *aph* (3′)-IIIa, *sea, sep, ica*D genes. The strain belonged to SCC*mec* III, *agrI, spa* type t030 and ST239. VRSA-2 was positive for *ermA, ermC, aph* (2")-Ic, *aph* (3′)-IIIa genes, as well as the virulence genes including *sea, ica*D were detected. The VRSA-2 belonged to SCC*mec* III, *agr*I, *spa* type t459, and ST239.

Molecular characterization of *van* operon in VRSA strains

The amplification and sequencing of *van* operon showed that the operon contains 9 ORFs, harboring a transposase (*tnp*) and resolvase (*rev*) genes, corresponding to *vanR, vanS, vanH, vanA, vanX, vanY,* and *vanZ* operon, respectively. The analysis of the sequence results indicated that it is closely related to Tn*1546* in VRE. Similar to Tn*1546* sequence in VRE in the current work, the insertion sequences including IS*1251* and IS*1216E* were also distinguished. Phylogenetic and distance analyses in BLAST revealed that Tn*1546* in our VRSA strains had 99.6% similarity to Tn*1546* in VRE. The sequence of Tn*1546* was submitted in GenBank under accession number MG592387 (https://www.ncbi.nlm.nih.gov/nuccore/mg592387).

Discussion

Recently, due to the rapid prevalence of MRSA, reporting in health care settings and also the exposure of selective

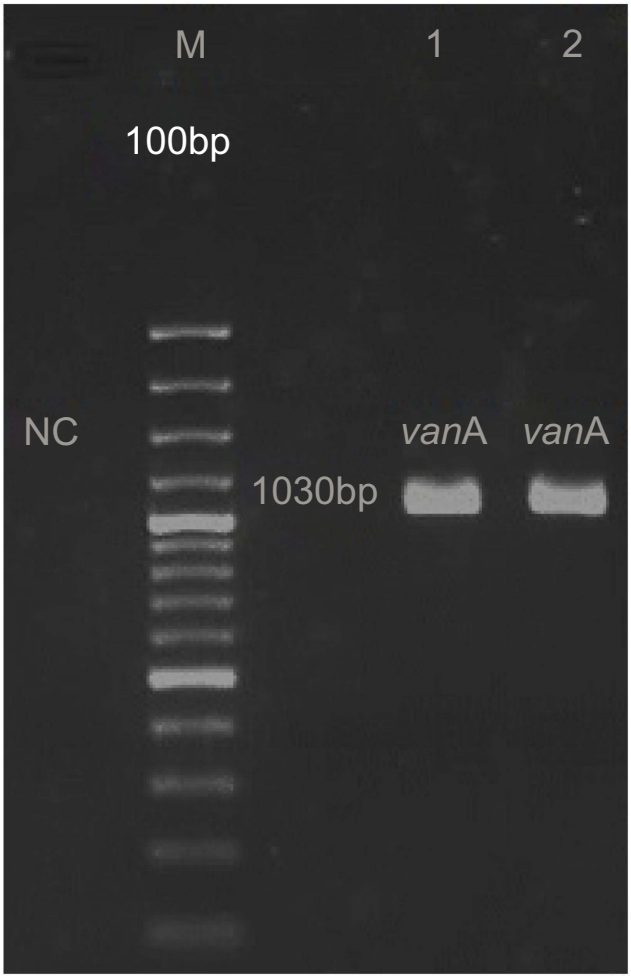

Figure 1 Agarose gel electrophoresis of *vanA* gene detected in two VRSA strains.

pressure of antibiotics, the emergence of VRSA strains, showing high level of resistance to antibiotics is unavoidable.

The emergence of VRSA isolates has been a major concern for the physicians and hospital management systems; however, VRSA isolates are rarely reported around the world and the total reported VRSA strains in the world have been reported to be less than 20 strains since 2002.[5] Long-time hospitalization, prolonged treatment with vancomycin, colonization with enterococcal as well as MRSA isolates, diabetes, chronic skin ulcers, and hemodialysis are the risk factors for colonization and infection by VRSA.[17,18] This study showed VRSA isolates from two patients who suffered from diabetes, chronic skin ulcers, underlying long-time hospitalization, and prolonged vancomycin therapy. The Centers for Disease Control and Prevention reported 9 out of 13 VRSA strains in the USA that were isolated from diabetic patients.[17,18]

The *van* operon is commonly located on transposon Tn*1546* and causes high-level resistance to vancomycin, initially reported in hospital strains VRE.[8,9] In the present study, both the isolates carried *vanR, vanS, vanH, vanA, vanX, vanY,* and *vanZ* genes on a *van* operon. Furthermore, we align the *van* genes sequence in BLAST, and results indicated high identity with Tn*1546* in VRE. Therefore, it has been suggested that the *van* operon has probably been transferred from VRE to *S. aureus* strains in simultaneous infections or colonization of VRE along with *S. aureus*, although we did not distinguish any evidence of mixed infection or colonization with VRE in this study. In the present work, the VRSA as well as VRE strains were not isolated from roommates, other patients, and also health care workers. Furthermore, the presence of the IS elements including IS*1251* and IS*1216E* in Tn*1546* confirmed the transfer of resistance to vancomycin from VRE to *S. aureus*. Similar to present study, some VRSA strains were reported in the USA, harboring IS*1251* and IS*1216E* on Tn*1546*.[5,19] A study which was carried out in Kerman hospitals showed that vancomycin resistant-*Enterococcus faecalis* isolates harbored *vanA gene*.[20]

CC5 and CC8 are the most common MRSA clones around the world. ST239 from CC8, ST5 from CC5, and ST22 from CC22 are the most prevalent VRSA sequence types which were reported in Iran, USA, and Brazil.[3–7] The sequence type (ST) 239 from CC8 is the most common clone of MRSA in hospital-acquired infections in Asia.[7] According to literature, the ST239 is the epidemic clone with resistance to a wide range of antibacterial agents in hospital settings around the world.[7] In recent years, the VRSA isolates belonging to ST5, ST8, ST239, and ST1283 were reported in our country.[6,7] According to eBURST results, ST8 and also ST1283 are the single locus variant of ST239, and these findings indicated that, in our country, the VRSA isolates are closely related to the same clone (Figure 2). Also in the present study, the molecular typing results indicated that the genetic background of the VRSA isolates (*agr* type I; SCC*mec* type III; ST239 and *pvl*-negative isolates) was closely related to health-care-associated MRSA strains.

ST239 is the common clone of the MRSA isolates that involved the hospital-associated infections especially in ICU-associated infections, and so the emergence of VRSA isolate, belonging to ST239 in health care settings,

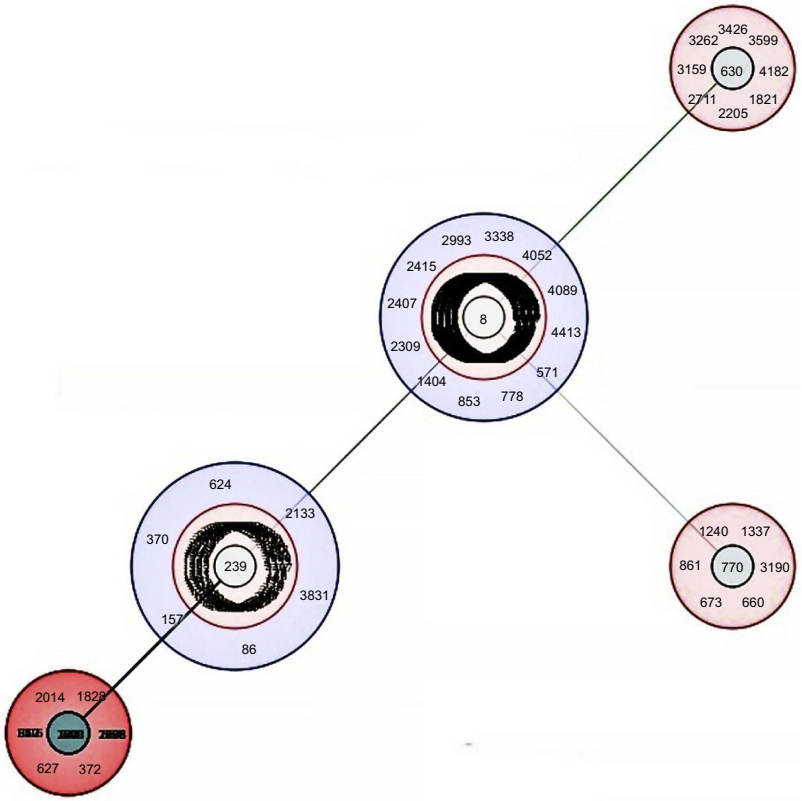

Figure 2 A population snapshot of the entire *S. aureus* with 5 or more nearest match of seven allelic profiles with ST239 in http://bigsdb.pasteur.fr MLST database showing clonal complexes (CCs) viewed using eBURST.

would be prevalent more than the other clones of MRSA (another clone of MRSA). Therefore, clinical laboratories have an important role in the diagnosis, isolation, and infection control with VRSA strains. So, we suggest that the surveillance of long-time hospitalized patients and the use of vancomycin agar screening for detecting VRSA and VISA strains in different hospital wards especially in ICU which probability of MRSA carriage and prolonged vancomycin therapy in them is high.

Ethical statement

The *S. aureus* strains were originally taken as part of routine hospital procedure, and then specifically recovered for this work. This study was approved by ethical numbers: IR.KMU.REC.1395.859 in ethical committee of Kerman University of Medical Sciences.

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Author contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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