



Isolation and Genetic Characterization of Vancomycin-resistant and *mecC*⁺ Methicillin-resistant *Staphylococcus aureus* Strains in Clinical Samples of Bojnurd, Northeastern Iran

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Abstract

Background: The emergence of antibiotic-resistant *Staphylococcus aureus* strains is one of the major concerns about the various staphylococcal infections. Vancomycin is one of the most important effective antibiotics on staphylococcal lethal infections. To date, vancomycin-resistant strains are increasingly isolated in different parts of the world, and it is alerting.

Objectives: The current study was designed to evaluate the prevalence, and antibiotic susceptibility pattern of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) isolates in the main tertiary hospital of Bojnurd, Iran.

Methods: *Staphylococcus aureus* isolates were collected from different clinical samples in Imam Reza Hospital of Bojnurd. After identification of isolates through using conventional methods, they were evaluated by agar screening, disk diffusion, and minimum inhibitory concentration (MIC) methods to determine resistance to vancomycin and methicillin. We also performed polymerase chain reaction (PCR) for the detection of *mecA*, *mecC*, *vanA*, and *vanB* genes. After confirmation of vancomycin resistance, genetic analysis was performed using SCC*mec*, *agr*, and *spa* typing, and multilocus sequence typing (MLST) methods on VRSA isolates.

Results: We found four vancomycin-resistant isolates (1.29%). Also, 75% of isolates were resistant to ceftiofuran. Using the PCR method, *mecA* was found in 73%, *mecC* in 0.64%, and *vanA* in 1.29% of isolates. Interestingly, we found two *mecC* positive isolates in MRSA isolates. The alpha-hemolysin (81.81%) and enterotoxin C (27%) had the highest and lowest toxins percentage, respectively. Among *mecA* positive isolates, SCC*mec IV* (37%), SCC*mec III* (31.27%), SCC*mec I* (14%), SCC*mec II* (11%), and SCC*mec V* (5.7%) were the most prevalent SCC*mec* types, respectively. It should be noted that the two *mecC* positive isolates belonged to SCC*mec XI*. *Agr I* (76.29%) was the highest *agr* type. We recognized t037 as the dominant *spa* type, and ST239, ST6, ST97, and ST8 were found in VRSA isolates.

Conclusions: In our study, the frequency of *mecA* genes in MRSA isolates was very high. It seems that the resistant isolates belonged to endemic clones of Iran.

Keywords: *Staphylococcus aureus*, Multilocus Sequence Typing, *Spa* Typing, *mecC*, Vancomycin

1. Background

Staphylococcus aureus is one of the most important opportunistic human pathogens. It's a causative agent of a wide range of infections, from skin infections to life-threatening systemic infections. This bacterium can also lead to animal infections that can transfer to a human directly or by animal products such as meat, milk, etc. (1). Its pathogenicity is related to toxins and different enzymes such as staphylococcal enterotoxins, exfoliative toxins (ETs), and toxic shock syndrome toxin-1 (TSST-1) genes (2-5). The emergence of staphylococci resistant to antimicro-

bial agents is an issue of world concern the main cause of which may be an arbitrary use of broad-spectrum and unawareness of them (6).

In most cases, antimicrobial resistance is achieved due to the resistance gene transfer between bacteria from the same or different strains. For example, the methicillin resistance is achieved by the transfer of the *mecA* gene by a mobile genetic element staphylococcal cassette chromosome (SCC), and vancomycin resistance is achieved by *vanA* gene transfer using Tn1545 transposon. This type of resistance is occasional, but it is becoming a public health challenge due to its ability of fast dissemination

(7). Recently, some European researchers found *mecA* methicillin-resistant *S. aureus* (MRSA) isolates. They found analogs named *mecC*. The origins of *mecC* are not clear yet, but there is evidence that it can be transmitted through contact with animals (8). Most of the patients with *mecC* MRSA lived in rural areas (9).

2. Objectives

Regarding the importance of *S. aureus* related community and hospital-acquired infections, the current study was designed to check the prevalence and antibiotic susceptibility pattern of MRSA and vancomycin-resistant *S. aureus* (VRSA) isolates in the main tertiary hospital of Bojnurd, Iran between 2013 and 2018.

3. Methods

3.1. Sample Collection and Identification

Samples were isolated from clinical specimens, including blood, tissue, urine, sputum, wound, peritoeum, nasal, trachea, bronchi, abscess, cerebrospinal fluid (CSF), catheter, throat, eye, and synovial. The confirmatory tests identified 308 clinical specimens as *S. aureus* isolates in Imam Reza hospital of Bojnurd. *Staphylococcus aureus* isolates were identified by standard biochemical and microbiological tests including Gram stain tests, catalase, mannitol fermentation, DNase, and slide and tube coagulase tests (10).

3.2. Antimicrobial Susceptibility Tests

Antibiotic susceptibility test was carried out on all isolates using Kirby-Bauer method based on 2018 guidelines by the Clinical and Laboratory Standards Institute (CLSI) (11). The antimicrobial disks used were vancomycin 5 µg (V), cefoxitin 30 µg (FOX), ciprofloxacin 5 µg (CIP), tetracycline 30 µg (TE), co-trimoxazole 1.25 µg (SXT), gentamicin 10 µg (GM), clindamycin 2 µg (CC), cefotaxime 30 µg (CEF), and rifampin 5 µg (R) (MAST DISKS™, UK).

3.3. Agar Screen Test

All isolates were screened for oxacillin and vancomycin resistance using the agar screening method. Methicillin resistance was defined as the capability of growth in agar screening media including 4% NaCl supplemented with 6 µg/mL oxacillin, whereas vancomycin resistance was defined as the capability of growth in agar screening media including 6 µg/mL vancomycin (12).

3.4. Determination of Minimum Inhibitory Concentration

Resistant isolates were determined by measuring the minimum inhibitory concentration (MIC) of oxacillin and vancomycin. Oxacillin resistance was defined as a MIC ≥ 4 µg/mL, and vancomycin resistance was defined as MIC ≥ 16 µg/mL (12).

3.5. Genomic DNA Extraction

Genomic DNAs of all 308 *S. aureus* isolates were extracted using a QiaAmp DNA Mini Kit (QIAGEN, Germany). According to the manufacturer's protocol for bacterial cells, we added lysostaphin at a final concentration of 30 µg/mL in the lysis buffer (13).

3.6. Detection of Resistance Genes

The presence of the *mecA*, *mecC*, *vanA*, and *vanB* genes was evaluated using the below primers as previously described (Table 1) (12-15).

Table 1. Primer Sequence Used in This Study

Gene	Primer Sequence	Product Size (bp)
<i>mecA</i>	5'-AGAAGATGGTATGTGGAAGTTAG-3'	584
	5'-ATGTATGTGCGATTGTATTGC-3'	
<i>vanA</i>	5'-GGCAAGTCAGGTGAAGATG-3'	713
	5'-ATCAAGCGGTCAATCAGTTC-3'	
<i>vanB</i>	5'-ACGGAATGGGAAGCCGA-3'	647
	5'-TGCACCCGATTTCGTTC-3'	
<i>mecC</i>	5'-CATTAAAATCAGAGCGAGGC-3'	188
	5'-TGGCTGAACCCATTTTGTAT-3'	
<i>Sec</i>	5'-GGGAATGTTGGATGAAGG-3'	900
	5'-AGGCAAGCACCGAAGTAC-3'	
<i>tstI</i>	5'-TTATCGTAAGCCCTTTGTTG-3'	398
	5'-TAAAGGTAGTCTATTGGAGTAGG-3'	
<i>pvl</i>	5'-GGAAACATTATTCTGGCTATAC-3'	502
	5'-CTGGATTGAAGTACCTCTGG-3'	
<i>hla</i>	5'-CGGTACTACAGATATTGGAAGC-3'	744
	5'-TGGTAATCATCACGAACTCG-3'	

3.7. Multiplex Polymerase Chain Reaction for Detection of Toxin Genes

The specific primers for detection of the *S. aureus* enterotoxin C (*sec*), toxic shock syndrome toxin 1 (*TSST-1*), Pantone-Valentine leukocidin (*pvl*), and alpha-hemolysin (*hla*) are listed in Table 1 (12). All *S. aureus* isolates were evaluated for these toxin genes.

3.8. Multiplex PCR for *Agr* and *SCCmec* Typing

The *agr* and *SCCmec* typing were performed on all 308 *S. aureus* isolates using Multiplex PCR as previously described (12, 16).

3.9. Multilocus Sequence Typing

Multilocus sequence typing (MLST) was performed on four VRSA and two *mecC* positive isolates based on amplification and sequencing of internal fragments of house-keeping genes *S. aureus* (*arc*, *aro*, *glp*, *gmk*, *pta*, *tpi*, and *yqi* genes) as previously described (12).

3.10. *Spa* Typing

Spa types of four VRSA and two *mecC* positive isolates were determined by PCR and sequencing of the polymorphic X region of *spa* gene as previously described (<http://spaserver.ridom.de/>) (12, 17). We also used the *spa* gene as an internal control gene for *S. aureus* verification in *mecC* and *vanA* harboring isolates.

4. Results

Using confirmatory tests, 308 clinical specimens were identified as *S. aureus* isolates. The results in antibiogram analysis showed that *S. aureus* resistance to ceftioxin (75.3%) had the highest resistance rate, followed by clindamycin (59%) and gentamicin (58%). The lowest resistance was seen for vancomycin (1.29%) (Figure 1). We found four *vanA* harboring vancomycin-resistant isolates with vancomycin MIC $\geq 16 \mu\text{g/mL}$. The *vanB* gene PCR was negative in all these VRSA isolates. Interestingly, in ceftioxin isolates, we found two *mecA*/*mecC*⁺ cases. The genetic characteristics of VRSA and *mecC*⁺ isolates are listed in Tables 2 and 3, respectively.

TSST was the most prevalent toxin in VRSA isolates, and *hla* was found in both *mecC*⁺ isolates. *Hla* was found in 81.8%, *TSST* in 33.8%, *pvl* in 29.2%, and *sec* in 27.3% of all 308 isolates. *AgrI* (76.29%) was the most prevalent *agr* type. Of four VRSA isolates, two were also MRSA and belonged to *SCCmecI* and *III*, and *mecC*⁺ isolates belonged to *SCCmecXI*. Moreover, in all MRSA isolates the most prevalent *SCCmec* types were *SCCmecIV* (37%), *SCCmecIII* (31.27%), *SCCmec I* (14%), *SCCmecII* (11%), *SCCmecV* (5.7%), and *SCCmecXI*, respectively. More details regarding VRSA and *mecC*⁺ isolates are presented in Tables 2 and 3.

5. Discussion

Due to the importance of Staphylococcal infections and their increasing antimicrobial resistance and also

the undeniable role in human hospital and community-acquired infections, in this study, we evaluated the antimicrobial resistance of *S. aureus* isolates in various clinical samples in Bojnurd, Iran, from 2013 to 2018. The prevalence of VRSA has increased in recent years in different parts of the world. Its total prevalence was 2% before 2006, 5% in 2006 - 2014, and 7% in 2015 - 2020. The prevalence of VRSA was 5% in Asia, 1% in Europe, 4% in America, 3% in South America, and 16% in Africa (18). Interestingly, we found four VRSA isolates in samples received in different years. These isolates were resistant to cefotaxime, gentamycin, tetracycline, rifampin, clindamycin, ciprofloxacin, and cotrimoxazole (Table 2).

Two isolates were MRSA, and two other isolates were not harboring the *mecA* gene. The MIC of vancomycin was 16 - 32 $\mu\text{g/mL}$, although in previously found VRSA isolates in northeastern Iran, we witnessed a higher MIC for this antibiotic (12). The vancomycin MIC in *mecA*⁺ VRSA is twofold (32 $\mu\text{g/mL}$) as *mecA*⁻ VRSA was 16 $\mu\text{g/mL}$. This may be due to the effect of *mecA* gene product on cell wall precursors (depsipeptides) that are the substrate of *vanA* gene product. The low-affinity PBP2A cannot utilize the depsipeptides, while in *mecA* negative strains PBP2 can utilize the cell wall precursors including depsipeptides (19). This isolate belonged to ST239 and *spa* t037, whereas our isolates belonged to other different ST/*spa* types in addition to ST239/t037. We have ST239/t189, ST6/t304, and ST97/t7688 isolates. It should be noted that these strains were isolated at different times, and maybe this variety indicates that the strains of our region have acquired resistance genes separately and do not have a common origin.

As discussed in many previously published articles, the source of *vanA* gene can be vancomycin-resistant enterococci or other vancomycin-resistant staphylococci (20). If the VRSA isolates are the source of resistance genes, we should find the same clones in different samples; but regarding the diversity of genetic clones of our isolates, we may predict the independent acquisition of resistance in different isolates. This is an alarming issue, as it may indicate a much higher prevalence and potential for further changes in these strains in the region. It should also be noted that Bojnurd is bordered by Turkmenistan, and it is also on the way to the city of Mashhad, which is a holy city visited by many tourists from all Islamic countries. This specific geographical position can lead to the entrance of different clones of bacteria to this area.

ST239/t037 is the endemic Asian clone previously found in different regions of Iran, and other clones such as ST97/t7688 are also found in this region. The acquisition of the *vanA* gene in endemic clones may be due to the co-infection of these clones with vancomycin-resistant enterococcus spp. as a *vanA* gene cluster source or other

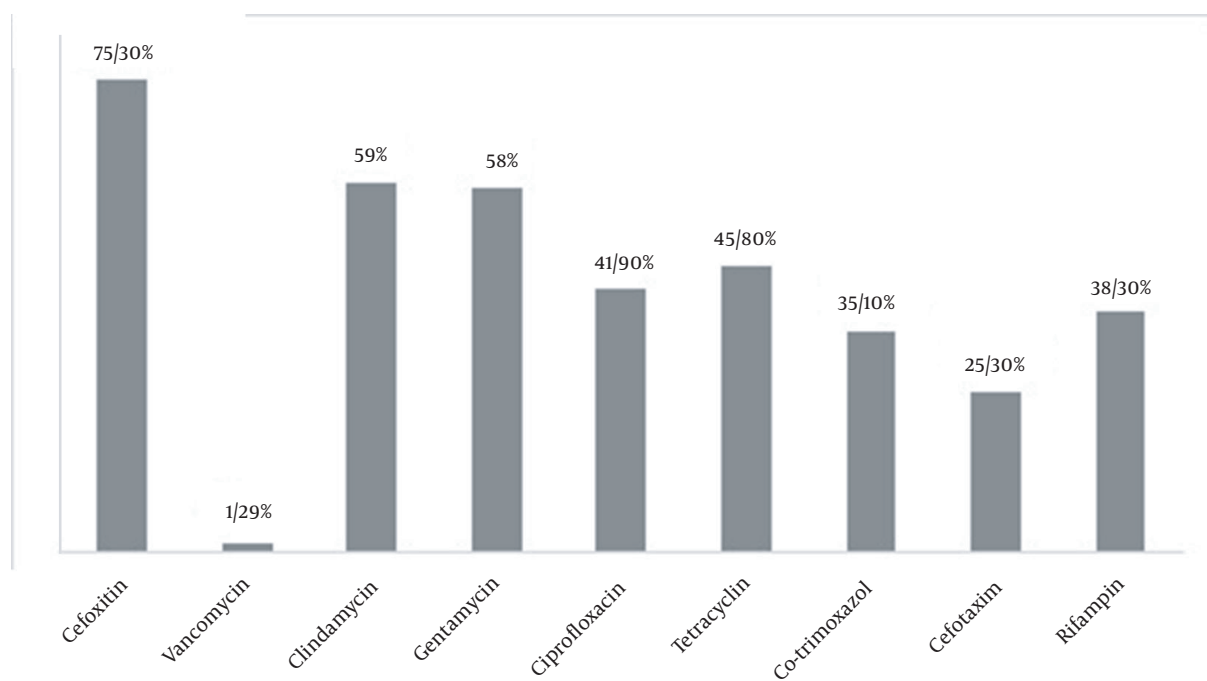


Figure 1. Antimicrobial resistance of *Staphylococcus aureus* isolates (n = 308)

Table 2. Genetic and Phenotypic Characteristics of vancomycin-resistant *Staphylococcus aureus* Isolates

	Source	Resistance	Vancomycin MIC	Resistance Gene	SCCmec Type	Agr	Toxin Gene	Spa Type	ST
VRSA1	Blood	Cef, gen, tet	32	<i>mecA, vanA</i>	III	I	<i>Pvl, TSST, etc, hla</i>	t037	ST239
VRSA2	Blood	Rif, cli	16	<i>vanA</i>	-	I	<i>TSST</i>	t189	ST239
VRSA3	trachea	Cip, cli, cot, tet	16	<i>vanA</i>	-	I	<i>TSST</i>	t304	ST6
VRSA4	wound	Cef, cli, cot	32	<i>mecA, vanA</i>	I	NT	-	t7688	ST97

Abbreviations: cef, cefotaxime; gen, gentamycin; tet, tetracycline; rif, rifampin; cli, clindamycin; cip, ciprofloxacin; cot, co-trimoxazole; NT, non typable.

Table 3. Genetic and Phenotypic Characteristics of *mecC*⁺ Isolates

	Resistance	Oxacillin MIC	Resistance Gene	SCCmec Type	Agr	Toxin Gene	Spa Type	ST
<i>mecC1</i>	cli, cef	64	<i>mecC</i>	XI	I	<i>hla</i>	t230	ST8
<i>mecC2</i>	cef, cli, cot	32	<i>mecC</i>	XI	I	<i>hla</i>	t037	ST239

Abbreviations: cli, clindamycin; cef, cefotaxime; cot, co-trimoxazole.

vancomycin-resistant staphylococci spp. Another noteworthy point is the source of strain isolation.

Two VRSA samples were isolated from the blood samples; one of them was isolated from the trachea, and the other one was isolated from a burn wound. This diversity of sources of sample isolation can indicate their high ability to spread and cause infection in different parts of the body, from superficial wound infections to systemic infections such as bloodstream infections. We previously found

VRSA in the trachea sample of a dead patient in Mashhad, northeastern Iran (12). The resistance to oxacillin in wound isolated *S. aureus* strains was reported in many reports worldwide (21-23), but resistance to vancomycin in these isolates is not prevalent (24). In recent years, some researchers reported *mecA*⁻ MRSA isolates. They found the *mecA* analogs gene (*mecC*) that acts like *mecA*. *MecC* is located within the SCCmec element, and it was found in some animal-related Staphylococci such as *S. sciuri* and *S. xylo-*

sus (25, 26). This gene has 68.7% nucleotide homology with *mecA* and is located in a new SCC*mec* element called SCC*mec* XI. It has proved to be resistant to beta-lactams phenotypically (27, 28).

The *mecC* gene was reported in isolates from humans and dairy cattle, and some recent reports showed the prevalence of *mecC* mediated methicillin resistance among human MRSA isolates (29). Some studies reported that *mecC* harboring MRSA has a lower oxacillin MIC than their *mecA* harboring isolates (8), but interestingly in our isolates, MIC was 32 and 64 mg/mL while MIC of $\geq 4 \mu\text{g/mL}$ was found in about 99% of *mecA*⁺ isolates. The *mecC*⁺ isolates were mainly found in Europe and rarely reported in other countries.

Most previously found isolates belonged to animal-related clones, but interestingly our isolates belonged to human-related endemic Asian clones, including ST239 and ST8. Thus, it could be concluded that these clones were not transferred from European sources to Iran. In the first report in Iran, *mecC*⁺ MRSA isolates belonged to ST130 and ST599 and *spa* types t843 and t5930, while our isolates belonged to ST239/t037 and ST8/t230 clones. It should be noted that most people living in North Khorasan province reside in rural areas, and animal husbandry is one of the main occupations in this area. Close human-animal contact may have led to gene transfer between human-related strains and animal-related strains. However, this assumption needs further evaluations.

5.1. Conclusions

In this study, vancomycin resistance was observed in four strains belonging to endemic clones. It's alarming because it may be a sign of genetic evolution and the spreading of these resistant isolates. We also found *mecC*⁺ MRSA isolates in human-related clones. We predict more distribution of these isolates and suggest to revise in molecular methods of MRSA detection in this area. More studies with larger sample size are required to confirm these results.

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Footnotes

Authors' Contribution: Parastoo Zarghami-Moghadam: Sample collection, laboratory analysis, and manuscript writing. Amir Azimian: Sample collection, laboratory

analysis, and manuscript writing. Abbas Akhavan Sepahi: English language editing and epidemiological analysis. Alireza Iranbakhsh: English language editing and epidemiological analysis.

Conflict of Interests: The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Reproducibility: The data presented in this study are openly available in one of the repositories or will be available on request from the corresponding author by this journal representative at any time during submission or after publication. Otherwise, all consequences of possible withdrawal or future retraction will be with the corresponding author.

Ethical Approval: This study was approved by the ethical board of North Khorasan University of Medical Sciences (IR.NKUMS.REC.1398.049).

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