

**Original Article****Molecular Characterization of Vancomycin, Mupirocin and Antiseptic Resistant *Staphylococcus aureus* Strains**Mahtab Hadadi¹, Hamid Heidari¹, Hadi Sedigh Ebrahim-Saraie¹ and Mohammad Motamedifar^{1,2}¹ Department of Bacteriology and Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.² Shiraz HIV/AIDS Research Center, Institute of Health, Shiraz University of Medical Sciences, Shiraz, Iran.**Competing interests:** The authors have declared that no competing interests exist.

Abstract. Background. *Staphylococcus aureus* is a common cause of nosocomial infections leading to a broad spectrum of diseases. Increasing antibiotic resistance among *S. aureus* strains, particularly methicillin-resistant *S. aureus* (MRSA), is a serious concern. In addition, the emergence of antiseptics resistance in MRSA helps the organism to persist and spread in healthcare environments easily. The aim of this study was to determine the molecular characteristics of vancomycin, mupirocin, and antiseptic resistant *S. aureus* strains.

Materials and Methods. This cross-sectional study was performed on a total of 120 MRSA isolates collected from two major hospitals in Shiraz, Iran. Minimum inhibitory concentrations (MICs) of vancomycin and mupirocin were determined by E-test method according to CLSI and Eucast guidelines. Presence of resistance genes was investigated by PCR method.

Results. Antibacterial susceptibility tests for MRSA isolates showed that three isolates (2.5%) were vancomycin-intermediate *S. aureus* (VISA), seven isolates (5.8%) were vancomycin-resistant *S. aureus* (VRSA), and 15 isolates (12.5%) were high-level mupirocin-resistant (MuH). None of the isolates had vancomycin resistance gene (*vanA*), but the frequency of mupirocin resistance gene was significant, and 55 (45.8%) isolates carried the *mupA* gene. Moreover, *norA*, *smr* and *qacA/B* genes were detected in 110 (91.7%), 55 (45.8%) and 36 (30%) strains, respectively.

Conclusion. This study showed the existence of VISA and VRSA strains in our region, and we also found a high frequency of mupirocin and biocide resistance genes among them.

Keywords: MRSA, VRSA, Antiseptics, Antibiotic resistance, Mupirocin.

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Introduction. *Staphylococcus aureus* is an important nosocomial pathogen that can cause superficial and life-threatening infections. Resistance to antibiotics has made this organism more problematic.¹ From the 1990s, that methicillin-resistant *Staphylococcus aureus*

(MRSA) strain has become responsible for one-third of all *S. aureus* infections worldwide, and vancomycin is the drug of choice.¹ However, its increased usage has led to the surge of glycopeptide-resistant *S. aureus*, VISA and VRSA, and the resistance mechanisms which have

been identified for VISA and VRSA strains are quite different, which are not fully understood. For VISA strains, thickening of the bacterial cell wall is the proposed mechanism of resistance, and for the VRSA strain acquiring *vanA* gene from *Enterococcus* spp.

The worldwide increase in antimicrobial resistance in *S. aureus* strains has led to increased mortality and morbidity in human, which highlights the importance of infection control practices. With this respect, mupirocin and biocides are increasingly being used in healthcare systems to eradicate MRSA in individuals who carry it. However, this increasing usage has led to the occurrence of microorganisms with reduced susceptibility to them.

Mupirocin (pseudomonic acid A) is an effective topical antibiotic which is widely used to eliminate MRSA strains among patients and healthcare workers and is a part of a comprehensive infection control program to reduce the risk of infection among the patients who are high risk MRSA carriers.² Moreover, it has been used to control the widespread of MRSA strains among patients during outbreaks. Another intervention strategy used in clinical practice to prevent the spread of nosocomial infections is the use of biocides (including disinfectants and antiseptics), which play a major role in controlling and preventing nosocomial infections. A wide variety of biocidal agents, including quaternary ammonium compounds (QACs), such as benzalkonium chloride and benzethonium chloride and divalent cations like chlorhexidine digluconate are commonly used in hospitals and healthcare facilities. In staphylococci, at least 12 biocide resistance genes have been identified: *qacA* - *qacJ*, *smr* and *norA*.³ These determinants encode multidrug resistance efflux pumps that can mediate reduced susceptibility to either antibiotics or biocides. In *S. aureus*, *qacA*, *qacB*, *smr* and *norA*, which encode multidrug-transporter proteins, have been identified as antiseptic-resistance genes.⁴

Given the importance of VRSA strains which are life-threatening, the spread of MuR strains, and the resistance to the most common and important antiseptics in MRSA strains, we aimed to determine the molecular characteristics of vancomycin, mupirocin and antiseptic resistant *S. aureus* strains obtained from two teaching

hospitals affiliated to Shiraz University of Medical Sciences.

Materials and Methods. *Bacterial isolates.* In the present study, 120 clinical MRSA isolates were collected from October 2012 to March 2013 from two teaching hospitals in Shiraz, Iran. Outpatient specimens and duplicate isolates were not included. The isolates were identified as *S. aureus* using conventional methods (colony morphology, gram stain, catalase activity, growth on mannitol salt agar, DNase test, and tube coagulase) [5]. Moreover, all these isolates were investigated for *femA* and *mecA* genes for molecular confirmation of MRSA.⁶

Determining the minimum inhibitory concentration. The MICs of vancomycin (0.016-256 µg/ml) and mupirocin (0.064-1024 µg/ml) were determined by the E-test method on Mueller-Hinton agar (HiMedia, India) using E-test strips (Liofilchem, Italy) according to the clinical and laboratory standards institute (CLSI) and Eucast guidelines.^{7,8} Because of the importance of resistance to vancomycin among the isolates, MICs determination of vancomycin was done twice for resistant strains. *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 strains were used as the control strains.

Detecting resistance genes. Bacterial DNA was extracted from the isolates, using the small-scale phenol-chloroform method, as described previously.⁹ Genes encoding the mupirocin and biocide resistance *mupA*, *qacA/B*, *smr* and *norA* were investigated by polymerase chain reaction (PCR), using specific primers, and the seven isolates that were phenotypically resistant to vancomycin were evaluated to determine *vanA* gene.^{2,10,11} All of the mupirocin-resistant isolates that were negative for the *mupA* gene were investigated for the presence of the *mupB* gene. The products were separated by electrophoresis in 1.5% agarose gels with 1 X TAE (Tris/Acetate/EDTA) buffer, stained with KBC (Kawsar Biotech Company) load dye and bands were observed by ultraviolet irradiation.

Results. During the study period (6 months), MRSA strains were collected from different infection sources, in which the most were related to the respiratory tract infection (RTI), 51 isolates

(42.5%), followed by skin and soft tissue infection (SSTI) 22 isolates (18.3%), blood stream infection (BSI) 19 isolates (15.8%), urinary tract infection (UTI) 15 isolates (12.5%), sterile fluid infection 7 isolates (5.8%), eye infection 3 isolates (2.5%), and the others 3 isolates (2.5%).

Determining vancomycin MIC showed that out of 120 studied isolates, 110 (91.7%) were susceptible; 3 isolates (2.5%) with MIC= 4 µg/ml were intermediate (VISA) and seven isolates (5.2%) were resistant to vancomycin (VRSA) by MIC 256 µg/ml. Resistant strains were evaluated by detecting *vanA* gene, but all of them were negative for this gene.

By determining mupirocin MIC, with MIC= 8-256 µg/ml the frequency of low-level resistance was 3 (2.5%), and with MIC >256 µg/ml the high-level resistance was 15 (12.5%). Molecular test to

determine the frequency of *mupA* gene showed a prevalence of 45.8%. The distribution of Low-level mupirocin resistant (MuL) and High-level mupirocin resistant (MuH) isolates among different sources of infection is shown in **Table 1**. Moreover, we found that among MuH strains only seven isolates carried the *mupA* gene, and the other eight isolates were examined to determine *mupB* gene, but all of them were negative for this gene.

Results of antiseptic resistant genes revealed that the intended genes, *norA*, *smr* and *qacA/B* were detected in 110 (91.7%), 55 (45.8%) and 36 (30%) MRSA strains, respectively. *norA* gene was the most frequent resistance genes among MuL and MuH isolates (**Table 2**). **Table 3** shows the co-presence of resistance genes among different sources of infections.

Table 1. Frequencies of MuL and MuH strains according to type of infection.

Phenotype	RTI N (%)	SSTI N (%)	BSI N (%)	UTI N (%)	Sterile fluid N (%)
MuL	-	3 (100%)	-	-	-
MuH	5 (33.3%)	5 (33.3%)	3 (20%)	1 (6.7%)	1 (6.7%)

SSTI, Skin and Soft Tissue Infection; BSI, Blood Stream Infection; UTI, Urinary Tract Infection; RTI, Respiratory Tract Infection; MuL, Low-level mupirocin resistance; MuH, High-level mupirocin resistance.

Table 2. Distribution of resistance genes among high and low levels of resistance to mupirocin.

Phenotype	<i>mupA</i> N (%)	<i>norA</i> N (%)	<i>smr</i> N (%)	<i>qacA/B</i> N (%)
MuL (3)	1 (33.3%)	3 (100%)	1 (33.3%)	0
MuH (15)	7 (46.7%)	12 (80%)	10 (66.7%)	5 (33.3%)

MuL, Low-level mupirocin resistance; MuH, High-level mupirocin resistance.

Table 3. Co-presence of resistance genes among the strains.

Gene	RTI (51)	SSTI (22)	BSI (19)	UTI (15)	Sterile Fluid (7)	Eye (3)	Other (3)	Total (120)
<i>norA</i>	48 (94.1)	18 (35.2)	18 (35.2)	14 (27.5)	6 (11.8)	2 (3.9)	3 (5.9)	110 (91.7)
<i>mupA</i>	25 (49)	10 (19.6)	8 (15.7)	5 (9.8)	4 (7.8)	1 (1.9)	2 (3.9)	55 (45.8)
<i>qacA/B</i>	13 (25.5)	8 (15.7)	6 (11.8)	4 (7.8)	4 (7.8)	-	1 (1.9)	36 (30)
<i>Smr</i>	26 (50.9)	10 (19.6)	9 (17.6)	5 (9.8)	3 (5.9)	-	2 (3.9)	55 (45.8)
<i>norA + mupA</i>	22 (43.1)	9 (17.6)	8 (15.7)	5 (9.8)	4 (7.8)	1 (1.9)	2 (3.9)	51 (42.5)
<i>norA + qacA/B</i>	13 (25.5)	6 (11.8)	6 (11.8)	4 (7.8)	3 (5.9)	-	1 (1.9)	34 (28.3)
<i>norA + smr</i>	26 (50.9)	9 (17.6)	9 (17.6)	4 (7.8)	3 (5.9)	-	2 (3.9)	53 (44.2)
<i>norA + mupA + qacA/B</i>	7 (13.7)	2 (3.9)	3 (5.9)	3 (5.9)	3 (5.9)	-	1 (1.9)	19 (15.8)
<i>norA + mupA + smr</i>	13 (25.5)	4 (7.8)	3 (5.9)	3 (5.9)	3 (5.9)	-	1 (1.9)	28 (23.3)
<i>norA + qacA/B + smr</i>	8 (15.7)	4 (7.8)	4 (7.8)	2 (3.9)	3 (5.9)	-	1 (1.9)	22 (18.30)
<i>mupA + qacA/B + smr</i>	3 (5.9)	2 (3.9)	1 (1.9)	2 (3.9)	3 (5.9)	-	1 (1.9)	13 (10.8)
<i>norA + mupA + qacA/B + smr</i>	3 (5.9)	3 (5.9)	1 (1.9)	2 (3.9)	3 (5.9)	-	1 (1.9)	12 (10)

SSTI, Skin and Soft Tissue Infection; BSI, Blood Stream Infection; UTI, Urinary Tract Infection; RTI, Respiratory Tract Infection.

Discussion. Since the first report of vancomycin-resistant *Staphylococcus aureus* (VRSA) strains, many studies have been conducted to determine the prevalence of these strains.¹² However, to date the rate of strains with complete resistance to vancomycin (*vanA* positive strains) are rare (16 cases from India; 14 cases from the U.S.; 6 cases from Iran, and one case in Pakistan), but the frequency of VISA is relatively high.¹³ As the results showed, we found seven strains that had MIC more than 256 µg/ml and were considered as VRSA. Therefore, we expected to find *vanA* gene in these strains as the most common and important cause of high-level resistance to vancomycin, but in the molecular test, all of these strains were negative for *vanA* gene. Some other studies reported similar cases; for example, in a study performed by Aligholi et al. in Tehran, Iran among 149 examined MRSA strains two strains were VRSA strains, one of which was negative for *vanA* gene, but the other was positive.¹⁴ In another study carried out in Tehran in 2017, Shekarabi et al. reported 4 VRSA strains, one of which was negative for *vanA* gene and the others harbored this gene.¹⁵ Thati et al. reported that among all VRSA strains, only one was negative for *vanA* gene.¹⁶ In Tiwari et al.'s study, out of 783 examined strains in their research, two strains were found to be vancomycin resistant with no trace of *vanA* gene.¹⁷ It is supposed that in the absence of *vanA* gene the resistance might be expressed through other mechanisms, such as increase in cell wall thickness based on “vancomycin trapping” theory, which states that the production of large amounts of peptidoglycan layers can cause the vancomycin molecules to intercept the monomers and peptidoglycan layers; as a result, antibiotics are suppressed before they reach the cell membrane, where the cell wall synthesis occurs and cannot apply its effect.¹⁷ In

addition to the vancomycin trapping theory, another theory called “affinity trapping” was presented by Hiramatsu, which states that accumulation of vancomycin molecules in a thickened wall greatly delays the time to completely inhibit the cell wall synthesis by preventing sufficient penetration of vancomycin molecules through thickening of the cell wall layers.¹⁷

However, in the present study no *vanA* gene was found amongst the detected VRSA strains, but to date, six cases of *vanA* positive VRSA strains have been reported from other parts of Iran, and according to the latest information, 17 VISA strains have been reported in addition to the present study.^{14,15,18-20} By comparing the frequency of these strains present in our work and worldwide literature, there is evidence that our country is at risk of increasing resistance to this critical antibiotic. Hence, more attention to managing the resistance process is necessary, especially for VISA strains, associated with persistent infections, poor clinical outcomes, prolonged vancomycin treatment or treatment failure.

Furthermore, we investigated different indices on the seven VRSA isolates and found that most isolates were isolated from respiratory tract infections (42.8%), followed by bloodstream infections (28.5%). Also, we found that all VRSA strains are of high-level resistance to mupirocin, except for one other isolate which carries the *norA* gene (**Table 4**).

As mentioned previously, increased use of mupirocin has led to the occurrence of mupirocin resistance strain which is considered as a significant alarm. Hence, it is crucial to determine the frequency and distribution of these resistant strains. In the present study, we found 18 mupirocin-resistant strains, 15 of which showed MuH phenotype. The molecular test showed that

Table 4. Resistance patterns of VRSA strains.

No.	Infection source	Level of mupirocin resistance	<i>vanA</i>	<i>mupA</i>	<i>norA</i>	<i>qacA/B</i>	<i>smr</i>
1	SSTI	MuH	-	-	+	+	+
2	BSI	MuH	-	-	+	+	+
3	UTI	MuH	-	-	+	-	-
4	BSI	MuH	-	+	+	-	+
5	RTI	MuH	-	-	+	-	+
6	RTI	MuH	-	+	-	-	-
7	RTI	MuH	-	-	+	-	+

SSTI, Skin and Soft Tissue Infection; BSI, Blood Stream Infection; UTI, Urinary Tract Infection; RTI, Respiratory Tract Infection; MuL, Low-level mupirocin resistance; MuH, High-level mupirocin resistance.

among 15 MuH strains, only seven strains carried *mupA* gene and the eight others lacked this gene. Since the *mupB* gene was discussed recently as another contributing factor in resistance to mupirocin,² we also considered the possibility of the presence of this gene, but all the isolates were negative for the *mupB* gene. Actually, we examined all the isolates for the presence of the *mupA* gene, and 55 of them (45.8%) carried *mupA* gene.

The results of the present study showed that resistance to mupirocin (12.5%) in our region has increased since in the previous scientific literature no resistance to mupirocin was reported.²¹ This frequency was high in comparison with other reports in the country. In the study performed in Tehran, the incidence of MuL strains was 3.5%, MuH was 1%, and *mupA* gene was 5.8%.²² A study in Arak reported that the frequency of MuH was 7.3%, and that of the *mupA* gene was 6%.²³ Goudarzi et al. reported among burn patients with bacteremia in Teheran that the frequency of MuH was 19.8% and 6.5%, respectively.^{24,25} However, the results of our study are in line with those of Nepal in which the frequency of *mupA* gene was 48.3%, but the prevalence of MuH strains was 51%. Consequently, it is suggested that this significant difference might be due to the pattern of consuming this antibiotic, different geographic regions, and the study population. According to these results and the increase in antibiotic consumption that causes resistance, it is necessary to pay more attention to the manner and rate of using mupirocin to prevent further resistance.

The frequency of *norA* gene, as a biocide resistance gene, in these strains was notable and all (100%) of MuL strains and 80% of MuH isolates harbored it. *norA* encoded efflux pumps can create resistance either to biocides or antibiotics. Resistance to mupirocin might be related to such efflux pumps. Moreover, out of 55 isolates carrying *mupA*, 48 were susceptible to mupirocin; cases like this have been reported,²⁶ and the probable cause has been announced to be lack of gene expression.

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The emergence of antibiotics resistant *S. aureus* has made it difficult to treat and control the infections caused by this organism. Also, this ability gives them the potential to stabilize in healthcare settings as well as their transmission among patients. Consequently, prevention and control have reduced the presence of these problematic strains through appropriate measures and proper use of proper disinfectants. Nonetheless, studies have shown that prolonged and inappropriate use of disinfectants can lead to the development of resistant strains.²⁷ The most abundant and critical anti-disinfectant determinants studied are *qacA/B*, *smr* and *norA* genes.²⁸ In the present study, the frequencies of *norA*, *smr* and *qacA/B* genes were 91.7%, 45.8%, and 30%, respectively. The frequency of *norA* gene was the highest, and that of *smr* was higher than *qacA/B*, which was similar to a previous study.²⁹ The rate of *qacA/B* gene in different countries ranges from 2% in Canada to 61% to 83% in China, and that of *smr* gene varies from 37.4% to 59% in China. In fact, to date, these genes have been reported most frequently in China.¹⁰ Many other studies, as well as ours, reported the presence of both genes together,^{10,30-32} where the prevalence of these strains was 19.1%.

Conclusions. MIC tests of vancomycin showed that 91.7% of isolates were susceptible. The frequencies of VISA and VRSA were 2.5% and 5.2%, respectively. *vanA* gene was not detected in any of resistant strains. Mupirocin MIC tests showed that the frequency of low-level resistance (MuL) was 2.5% and high-level resistance (MuH) was 12.5%. The distribution of *mupA* gene was 45.8%. Only seven MuH isolates carried *mupA* gene, and all of them were negative for *mupB* gene amplification. Investigation of antiseptic resistant genes revealed that *norA*, *smr* and *qacA/B* were detected in 91.7%, 45.8% and 30% of MRSA strains, respectively. According to the data, more attention is required when using these antibiotics, and antiseptics are suggested to control the range of resistance.

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