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# Emergence of Vancomycin-Resistant *Staphylococcus aureus* in the Southwest of Iran

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ABSTRACT

**Aims:** Following the emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, the use of other antibiotics especially vancomycin in *S. aureus* infections has become inevitable, leading to the emergence of vancomycin-resistant *S. aureus* (VRSA) strains, which is considered as a major public health concern. This study aimed to determine the vancomycin susceptibility patterns of *S. aureus* clinical isolates in order to evaluate the current status of vancomycin resistance in the southwest of Iran.

**Materials & Methods:** In this study, 100 *S. aureus* clinical strains were collected from the hospitals of Khuzestan province in the southwest of Iran. Next, antibiotic susceptibility, vancomycin resistance, and the presence of *mecA*, *vanA, vanB, vanC*, and *vanD* genes were investigated in these isolates.

**Findings:** It was found that 1 and 2 isolates were vancomycin-intermediate *S. aureus* (VISA) and VRSA, respectively. All three strains showed methicillin-resistance pattern and carried *mecA* gene. *vanA* gene was detected in VRSA strains, whereas *vanB*, *vanC*, and *vanD* genes were detected in none of these isolates.

**Conclusion:** This study findings could be alarming regarding the emergence and spread of VRSA strains; therefore, the principles of infection control should be employed in the healthcare systems to prevent the spread of VRSA strains in healthcare facilities.

**Keywords:** Vancomycin; *Staphylococcus aureus*; Antibiotic resistance; Methicillin-resistant *Staphylococcus aureus*

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## Introduction

*Staphylococcus aureus* as an important human pathogen could lead to a variety of infectious diseases ranging from soft tissue infections to life-threatening diseases, such as endocarditis and pneumonia [1-2]. Resistance of *S. aureus* to antimicrobial drugs has become a global concern in the past eight decades. Penicillin-resistant *S. aureus* emerged for the first time in 1942, and nineteen years later in 1961, methicillin resistance was reported. Finally, in 2002, the first vancomycin-resistant *S. aureus* (VRSA) strain was detected [3]; however, this type of resistance has not been reported in many countries.

The high prevalence of MRSA, beside the increased use of vancomycin, has led to the emergence of two types of glycopeptide resistance among *S. aureus* strains. The first type, known as vancomycin-intermediate *S. aureus* (VISA), is produced by a thickened and poorly cross-linked cell wall layer, resulting in the accumulation of D-Ala-D-Ala building blocks, which are the targets of vancomycin in the periphery, and the diminishment of vancomycin effects [4-6]. Research has showed that chemotherapy failure may be significantly correlated with a mild increase in vancomycin MIC for MRSA strains recovered from patients, even when vancomycin is not used for treatment [7-10].

The second type is VRSA with a high level of vancomycin resistance due to the acquisition of *vanA* gene from *Enterococcus* species [4, 11-12]. Generally, *vanA* operon is encoded on transposon Tn1546, which is a part of a conjugative plasmid related to vancomycin-resistant *Enterococci* (VRE) [11]. Resistance to vancomycin in *S. aureus* may arise in two ways. Vancomycin resistance plasmid could be acquired by *S. aureus* during conjugation events, leading to the vancomycin resistance plasmid or Tn1546 transfer from VRE plasmid into a resident staphylococcal plasmid [4, 13]. VanA is a ligase producing D-Ala-D-Lac, a substitution for D-Ala-D-Ala, which shows a reduced affinity to glycopeptides, including vancomycin and teicoplanin [4].

To date, transferable resistance mechanisms including *vanA* gene has not been detected in VISA examined, and this phenotype has not been found in the presence of vancomycin. Evidence suggests that VISA strains pose a lower public health risk in comparison with VRSA strains; however, it is still of clinical significance and needs to be controlled [5]. The first VISA strain was isolated in Japan in 1997, followed by the United States, France, United Kingdom, and other countries, confirming the emergence of these strains on a global scale [14]. There are several types of *van* genes, including *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, and *vanG*. The operons of *vanA* and *vanB* are located on plasmids or chromosomes, while *vanC, vanD*, *vanE*, and *vanG* operons are only located on chromosomes [4, 15]. Although the prevalence of VRSA is very low, the high prevalence of MRSA strains and the overuse of vancomycin could be considered as risk factors for the emergence and dissemination of VRSA strains [16].

**Objectives**: This study aimed to investigate the emergence of VISA and VRSA strains in *S. aureus* clinical isolates and to determine the susceptibility patterns of these strains in order to evaluate the current status of vancomycin resistance in the southwest of Iran.

## Materials and Methods

**Bacterial isolates**: A total of 100 *S. aureus* isolates were collected from different hospitals of Khuzestan province, Iran, during seven months from December 2015 to June 2016. The strains were isolated from various clinical specimens, including urine, blood, trachea, and wound. *S. aureus* isolates were confirmed by conventional biochemical methods such as Gram staining, catalase, oxidase, DNase, mannitol fermentation, slide



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and tube coagulase, furazolidone (100 µg) susceptibility, and bacitracin (0.04 unites) resistance tests [3].

**Antimicrobial susceptibility testing**: Kirby-Bauer disk diffusion method was carried out to detect the antibiotic susceptibility pattern of the isolates according to the Clinical Laboratory Standards Institute (CLSI) guidelines [17]. Antibiotic disks used in the test contained penicillin (10 unites), gentamicin (10 µg), ciprofloxacin (5 µg), cefoxitin (30 µg), tetracycline (30 µg), kanamycin (30 µg), and amikacin (30 µg).

**Detection of VISA and VRSA isolates**: To screen VRSA strains, all the isolates were cultured on in-house-prepared brain heart infusion (BHI) agar (Merck, Germany) containing 6 µg/mL of vancomycin (BHI6V). Next, direct colony on nutrient agar plate was transferred and suspended in sterile saline to obtain 0.5 McFarland turbidity. Then 10 µL of suspension was inoculated onto BHI6V agar surface and incubated for 24 hours at 35°C in ambient air. *E. faecalis* ATCC 29212 was considered as negative control (vancomycin-susceptible), and *E. faecalis* ATCC 51299 was used as positive control (vancomycin-resistant). Isolates producing one or more colony on BHI6V medium were further investigated by measuring vancomycin minimum inhibitory concentration (MIC). To screen VISA isolates, vancomycin MIC was determined by the preparation of two-fold dilutions of vancomycin (2-1024 µg/mL) in Mueller-Hinton broth (MHB). According to the CLSI guidelines, MIC breakpoints for VSSA, VISA, and VRSA were defined as follows: 2 µg/mL; 4-8 µg/mL; and ≥ 16 µg/mL, respectively [17].

**Detection of *mecA*, *vanA*, *vanB*, *vanC* and *vanD* genes by PCR assay**: DNA of the isolates was extracted by a Gram positive DNA extraction kit (CinnaGen; Iran) according to the manufacturer's instructions. The PCR primers and conditions related to *mecA*, *vanA*, *vanB*, *vanC,* and *vanD* genes were applied as recommended by Emaneini et al. (2013), Clark et al. (1993), Clark et al. (1998), and Perichon et al. (1997) [18-20]. VRSA and VISA isolates were confirmed genotypically by DNA sequence analysis of 16S rRNA gene [21] (Table 1).

**Ethical Permissions**: The study was approved by the research committee of Shahid Chamran University of Ahvaz and performed in accordance with the ethical standards laid down in the Declaration of Helsinki in 1964.

## Findings

**Antibiotic resistance**: All the isolates were confirmed as *S. aureus* by conventional morphological and biochemical methods. The antibiotic susceptibility profile of all the isolates was assessed against seven antibiotics. The highest resistance rate was related to penicillin (99%), followed by cefoxitin (95%), tetracycline (90%), kanamycin (87%), amikacin (72%), and gentamicin (61%). Also, *mecA* gene was detected in 95 (95%) isolates.

**VISA and VRSA strains**: Based on the MIC results, one (1%) isolate showed intermediate resistance to vancomycin (MIC: 8 µg/mL); however, none of the vancomycin resistance genes were detected in this isolate. Two strains were confirmed as VRSA with MIC of 512 µg/mL. These two isolates carried *vanA* gene and showed resistance to all the investigated antimicrobial agents. One of these two VRSA isolates generated two forms of small and pinpoint colonies when cultured on vancomycin-free agar plates; however, both colonies were similar in terms of the antibiotic resistance pattern, the presence of *vanA* gene, and MIC for vancomycin. On the other hand, *vanB, vanC*, and *vanD* genes were found in none of the isolates. All three isolates were confirmed as *S. aureus* by DNA sequence analysis of 16S rRNA gene. Demographic data and antibiogram profiles of these strains are presented in Table 2.



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**Table 1)** Names and sequences of primers used in this study

| Primer Name | Sequence (5'→3') | References |
|---|---|---|
| *vanA* | CATGAATAGAATAAAAGTTGCAATA<br>CCCCTTTAACGCTAATACGATCAA | 19 |
| *vanB* | GTGACAAACCGGAGGCGAGGA<br>CCGCCATCCTCCTGCAAAAAA | 19 |
| *vanC* | GAAAGACAACAGGAAGACCGC<br>ATCGCATCACAAGCACCAATC | 20 |
| *vanD* | TAAGGCGCTTGCATATACCG<br>TGCAGCCAAGTATCCGGTAA | 4 |
| 16SrRNA | CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG<br>CCCGGGATCCAAGCTTACGGTTACCTTGTTACGACTT | 21 |
| *mecA* | TCCAGATTACAACTTCACCAGG<br>CCACTTCATATCTTGTAACG | 18 |

**Table 2)** The characterizations and antibiotic susceptibility profiles of VISA and VRSA isolates

| Sample Code | Size of Colony | Gene Detected | MIC | PEN | GM | KAN | VAN | AM | FOX | TET | CP |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 48 | Small | No | 8 | R | I | I | I | R | R | R | I |
| 142 | Small and pinpoint | *vanA* | 512 | R | R | R | R | R | R | R | R |
| 148 | Small | *vanA* | 512 | R | R | R | R | R | R | R | R |

MIC: Minimum Inhibitory Concentration; PEN: penicillin; GM: gentamicin; KAN: kanamycin; VAN: vancomycin; AM: amikacin; FOX: cefotaxime; TET: tetracyclin; CP: ciprofloxacin; R: resistant; S: sensitive; I: intermediate

## Discussion

With the emergence of methicillin resistance in *S. aureus* strains, the use of other antibiotics especially vancomycin in *S. aureus* infections is inevitable. Accordingly, resistance of these MRSA isolates to vancomycin is a major public health threat [22]. The genetic basis of VISA strains is largely unknown as no gene or operon has been linked to VISA. However, previous reports indicated that frequent use of high doses of vancomycin may lead to the emergence of strains with





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chromosomal mutations. According to the Center for Disease Control and Prevention (CDC) recommendations about the MIC and vancomycin screen agar methods for VISA and VRSA detection, both methods were applied to detect vancomycin-susceptible isolates.

In the present study, one strain showed a VISA phonotype and had no vancomycin resistance gene; although it carried *mecA* gene. This strain showed MDR pattern. On the other hand, a few VRSA cases have been reported worldwide. So far, several reports have been published about VRSA in Iran [3, 5, 23]. Azimian et al. (2012) detected one VRSA strain in the northeastern region of Iran. Its genetic characteristics revealed that this strain was a MRSA clone endemic in Asia with some genetic changes, including mutation in *gmk* gene and acquisition of *vanA* gene [5]. VRSA strains isolated by Saadat et al. (2014) in Shiraz, south of Iran, carried *vanA* and/or *vanB* resistance genes [3]. The existence of VRSA and VISA strains in Tehran, capital of Iran, was reported by Shekarabi et al. (2017), *vanA* gene was detected in all VRSA isolates [23]. In the present study, two VRSA strains were detected in the southwest of Iran for the first time. Both isolates carried *vanA* gene and were resistant to all antibiotics tested with MDR phenotype; they also carried *mecA* gene, which is in accordance with the previous studies results [24-25]. VRSA strains detected in other studies were also resistant to multiple antibiotics including methicillin, quinolones, macrolides, and cephalosporins; therefore, MDR is a common phenotype in these isolates [7].

VRSA strains were reported in some countries including Pakistan, Iran, USA, Nigeria, and India [26]; however, according to these reports, it seems that the prevalence of VRSA isolates is increasing in Iran, and this country is becoming a hotspot region for the emergence of VRSA isolates [23]. Consistent with the previous reports on VISA and VRSA strains, one of the two VRSA strains isolated in the present study generated small and large colony variants [27-28]. Generally, VRSA and VISA strains have phenotypically smaller colonies compared to their susceptible counterparts [29].

Although simultaneous resistance to beta-lactams and glycopeptides has a high biological cost for *S. aureus* [30], in the absence of an inducer (vancomycin), the biological cost is low for VRSA; therefore, the spread risk of such clinical isolates should not be underestimated [4, 30]. So far, there have been only few reports of VRSA strains; thus, it is difficult to determine the risk factors associated with the emergence of VRSA strains. According to the literature, these strains have been isolated from dissimilar diseases. Nonetheless, continuous exposure to a glycopeptide antibiotic could play an important role in the emergence of these strains [7]. The development of antibiotic resistance could be attributed to the antibiotics overconsumption, availability even without prescription, misuse in hospitals, and uncontrolled use in livestock and agriculture industries [31].

## Conclusion

The emergence and dissemination of VRSA strains is one of the most important life-threatening events in clinical settings; therefore, special attention is needed to be paid on the risk factors associated with VRSA in any region. To prevent the prevalence of this type of resistance, physicians should perform vancomycin susceptibility tests for *S. aureus* infections, prescribe an appropriate antibiotic, and employ the principles of infection control in the healthcare systems to prevent the spread of VRSA in healthcare facilities [5, 14].

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**Conflicts of interests:** The authors declare that they have no competing interest.

**Authors' contribution:** SER and VA participated in searching for subjects and data and also performing the research. SER conducted the research. SER and HM analyzed and interpreted data. All authors read, revised, and approved the final manuscript.

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