



## Frequency of Reduced Vancomycin Susceptibility among Clinical *Staphylococcus aureus* Isolated in Ahvaz Iran

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### ARTICLE INFO

**Article type:**  
Original Article

### Article history:

Received: 15 Feb 2015  
Revised: 19 Feb 2015  
Accepted: 9 Mar 2015

### Keywords:

Methicillin-resistant  
*Staphylococcus aureus*,  
*mecA* gene,  
Polymerase chain  
reaction, Vancomycin,  
Staphylococcal  
infections

### ABSTRACT

**Introduction:** One of the most important agents in hospital-acquired infections is *Staphylococcus aureus*. Treatment of methicillin-resistant *S. aureus* (MRSA) infections with decreased susceptibility to vancomycin has recently been more difficult. The aim of this study was to evaluate the possible presence of vancomycin intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) and also to determine the frequency of MRSA in clinical specimens.

**Methods:** In this study, 195 *S. aureus* isolates were collected from the patients were examined. All of the isolates were identified using standard biochemical tests. Susceptibility of *S. aureus* isolates against 10 antibiotics was detected by disk diffusion method and was followed by E-test and vancomycin screen agar methods. Minimum inhibitory concentration (MIC) of vancomycin was determined according to the CLSI guidelines. Also, detection of *mecA* gene was performed by PCR and finally, the results were compared.

**Results:** All of the isolates were susceptible to vancomycin (i.e. MIC range of vancomycin was between 0.25-2 µg/ml). Out of 195 *S. aureus* isolates, 99 isolates (50.8%) were resistant to methicillin, and *mecA* gene was detected in 96 isolates. These results also showed that the highest and lowest resistance rate of isolates was to penicillin (96.9%) and chloramphenicol (0%), respectively.

**Conclusion:** Our findings showed that vancomycin can still be used as a valuable drug for treatment of *S. aureus* infections in our region. However, periodic evaluation of vancomycin MIC of *S. aureus* isolates is critical for monitoring MRSA and preventing the spread of VISA or VRSA among patients.

- **Please cite this paper as:** Moosavian M, Torabipour M. Frequency of Reduced Vancomycin Susceptibility among Clinical *Staphylococcus aureus* Isolated in Ahvaz Iran. *J Med Bacteriol.* 2015; 4 (1, 2): pp.1-7.

## Introduction

*Staphylococcus aureus* is the most clinically significant species which is responsible for a number of infections (1). Recently, some of reports have indicated that the most prevalent bacterial isolates, after *Escherichia coli* was *S. aureus* (30.7%) among children with suspected septicemia (2). The incidence of methicillin-resistant *S. aureus* (MRSA) has been raised in the past two decades (3). MRSA is one of causative agents of hospital-acquired infections which is costly and poses a serious threat for human health. In the past, MRSA was associated mainly with the hospitalized patients (4). Since the late 1990s, several outbreaks of community-acquired MRSA infections have been reported (5, 6). The use of vancomycin as a drug of choice for treatment of MRSA infections was increased which caused decreased susceptibility to vancomycin and other glycopeptide antibiotics (7, 8). In 1997, the first vancomycin-intermediate *S. aureus* (VISA) strains were reported from Japan (9). The first true vancomycin-resistant *S. aureus* (VRSA) was reported in the United States in 2002 (10). The similar reports from other countries have also found these strains in their studies. In Iran, *S. aureus* with reduced susceptibility to vancomycin has been reported before by Sadari *et al.*, Emaneini *et al.* and others (11-13). This study was performed to evaluate the possible presence of reduced susceptibility to vancomycin among clinical *S. aureus* isolates. We also aimed to determinate the drug resistance patterns of *S. aureus* isolates to oxacillin and other antibiotics in Ahvaz city, Iran.

## Materials and Methods

### Bacterial isolates

From March to December 2011, 195 *S. aureus* from different clinical specimens such as pus, burn wound catheter, blood, sputum and CSF

were isolated. These specimens were collected from hospitalized patients in three teaching hospitals affiliated to Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

### Identification and preparation of a pure culture

After Gram staining and culturing on blood agar (Merck-Germany), all of the isolates were identified using standard biochemical tests such as catalase, coagulase, DNase and mannitol salt agar (14). The isolates that were confirmed as *S. aureus*, were stocked in deep freezer for later tests.

### Antibiotic susceptibility testing by disk diffusion method

Susceptibility of *S. aureus* isolates to antimicrobial drugs was determined by disk diffusion based on the Clinical and Laboratory Standards Institute (CLSI) recommendations (15). Disks purchased from MAST Co.(UK) were penicillin (10 U), oxacillin (1 µg), gentamicin (10 µg), ciprofloxacin(5 µg), chloramphenicol (30µg), erythromycin(15 µg), vancomycin(30 µg), rifampin(30µg), clindomycin (30µg) and cephalotin(30µg). In this test, *S. aureus* ATCC 29213 was used as a reference strain (16).

### Oxacillin agar screening test

The test was performed according to CLSI guidelines for phenotypic detection of MRSA among all isolated *S. aureus* (15). Mueller-Hinton agar containing 6 µg /ml oxacillin (Sigma – Aldrich) was prepared in house for this test.

### Determination of minimum inhibitory concentration (MIC) by E-test

The MIC of vancomycin was determined by E-test (BioMerieux) according to the manufacturer's instructions.

We used E-test with vancomycin on Mueller-Hinton agar (Merck-Germany) with an inoculum of 0.5 McFarland. The plates were incubated at 35°C for 24-48 h and then were interpreted according to the CLSI guidelines (15). *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 51299 were used as negative (vancomycin-susceptible) and positive (vancomycin-resistant) control strains, respectively (16, 17).

#### Screening for reduced vancomycin susceptibility on BHI Agar

The BHI agars (Merck, Germany) containing 2, 4 and 6 µg/ml of vancomycin were prepared in the laboratory. By transferring colonies from an overnight growth to the sterile Tryptic Soy Broth medium (Merck-Germany), the inoculum suspension was prepared. The turbidity of these bacterial suspensions were matched the same as a 0.5 McFarland. Then 10 µl of each bacterial suspension was inoculated as a spot onto BHI agar plate and was incubated for 24 h at 35°C. Any growth which was visible onto BHI agar plate was considered as vancomycin-resistant. *S. aureus* ATCC 29213 and *E. faecalis* ATCC 51299 were used as vancomycin susceptible and resistant control strains, respectively.

#### Detection of *mecA* gene by PCR

DNA extraction from phenotypically MRSA isolates was done by boiling method according to the recommended instructions (18). The used oligonucleotide primers for *mecA* gene were (*mecA* F 5' GTA GAA ATG ACT GAA CGT CCG ATG A 3' and *mecA* R 5' CCA ATT CCA CAT TGT TTC GGT CTA A 3') as described previously (17). The thermocycler was programmed with initial denaturation at 94°C for 4 min, followed by 35 cycles each including of 94°C for 60 sec, annealing step at 62°C for 60 sec, an extension at 72°C for 45 sec, and final extension at 72°C for 5 min (17). The holding step was 4°C until the samples were analyzed. The PCR products were electrophoresed on 2% agarose gel.

The DNA bands were visualized by staining with ethidium bromide and photographed by UV transilluminator. The *S. aureus* strains ATCC 29213 and ATCC 33591 were used as *mecA* negative and positive controls, respectively.

## Results

The results of screening method on BHI Agar showed that all 195 *S. aureus* isolates (100%) were susceptible to vancomycin. MICs of vancomycin for these isolates were between 0.25-2 µg/ml to 2µg/ml by E-test. None of isolates grew on 4 or 6 µg/ml of vancomycin BHI screen agar. The MIC rate of *S. aureus* isolates to vancomycin by E-test is shown in Table 1. Susceptibility rates of isolates to antibiotics used in this study were as follows: vancomycin (100%), chloramphenicol (100%), clindamycin (89.2%), rifampin (73.8%), cephalothin (60%), ciprofloxacin (49.7%), oxacillin (49.2%), gentamicin (53.3%), erythromycin (%48.2) and penicillin G (3.1%).

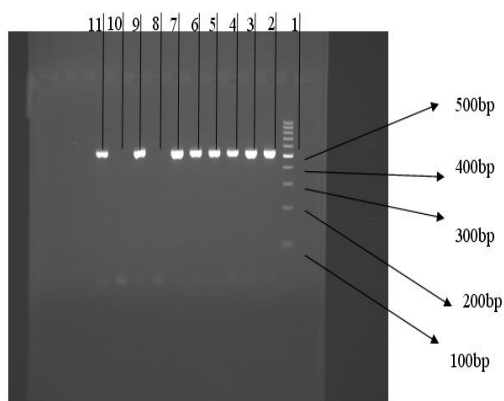
**Table 1.** MIC rate of *Staphylococcus aureus* isolates to vancomycin resistant strains.

MIC (µg/ml)	No. of Isolates	Percentage
0.25	1	0.5
0.5	9	4.6
0.75	22	11.3
1.0	90	46.2
1.5	68	34.9
2.0	5	2.6
Total	195	100

Out of 195 *S. aureus* isolates, 99 isolates (50.8%) were phenotypically resistant to methicillin (Table 2). They grew on 6 µg/ml oxacillin screen plates, while the remainder (i.e. 49.2%) were susceptible to methicillin, and did not grow on these plates (Figure 1). However, detection of *mecA* gene by PCR confirmed that 96 out of these 99 MRSA isolates (96.6%) were *mecA* positive (Figure 2).

**Table 2.** Frequency clinical isolates of MRSA, in 6 µg /ml oxacillin screen plates

Growth on oxacillin agar	No of isolates	Percentage
Negative	96	49.2%
Positive	99	50.8%
Total	195	100%

**Figure 1.** The growth of *S. aureus*, susceptible (Down) and resistant (Up) to methicillin, on Mueller-Hinton agar containing 6 µg/ml of oxacillin**Figure 2.** Agarose gel electrophoresis of PCR-amplified methicillin resistance gene (*mecA* gene 500bp). Lines: 1: 100-bp ladder/ 2-7 & 9: MRSA (*mecA*+)/ 8: *mecA* negative/ 10 & 11: negative (ATCC 29213) and positive (ATCC 33591) controls.

## Discussion

In the last decade, methicillin-resistant *S. aureus* (MRSA) strains have been spread and now became endemic in hospitals worldwide (17). Based on European Antimicrobial Resistance System (EARSS) report (Oct. 2006), isolation of MRSA in Belgian hospitalized patients has been also increased from 22% in 1999 to 31.4% in 2005 by blood cultures(19). Also, a report from the United States (3), showed 16.3% increase in MRSA among 21009 pediatric. With head and neck *S. aureus* infections occurred during a 6 year-period. Askari *et al.* (2012) in an epidemiological review of MRSA in Iran showed that the mean prevalence of MRSA was 52.7 +/- 4.7% (20). Also, the prevalence rate of MRSA in our study was 50.8% (99 out of 195 *S. aureus* isolates) which is comparable with Askary *et al.* study. The results of antibiotic susceptibility testing in our study showed that 100% of *S. aureus* isolates were susceptible to vancomycin and chloramphenicol. Likewise, many studies have failed to find VISA or VRSA among their isolates (21). However the most of isolates in our study were known as MRSA isolates. In present study, among of 99 phenotypically-resistant MRSA isolates, 96 (96.6%) were positive for *mecA* gene, however, the PCR reaction could not confirm *mecA* gene in only 3 *S. aureus* isolates (3.4%) which were resistant to methicillin phenotypically by disk diffusion and E- test. The mechanism of resistance may be due to other mechanisms such as high production of beta-lactamase enzymes or a normal penicillin-binding protein (PBP), however with altered binding capacity (22). Widespread emergence of MRSA has made vancomycin as an important treatment option (23, 24). However, shortly after the first report from Japan about a strain of *S. aureus* with reduced susceptibility to vancomycin, additional strains were reported from other countries (8).

For example vancomycin-Intermediate *S. aureus* (VISA) strains with vancomycin MIC of 8 µg/ml have been reported from United State (25, 26), France (27), and Germany (28). Tiwari and Sen (2006) from India reported (17) only 2 strains of VRSA (MIC = 32-64 µg/ml) and 6 strains of VISA (MIC 8-16 /ml) between 783 *S. aureus* isolates. Also, Rossi *et al.* (2014) in Brazil reported an isolated strain of MRSA that was susceptible to vancomycin, but after antibiotic therapy of the patient acquired the *van A* gene cluster and became resistant to vancomycin (29). Recently, some of reports from our country have also shown a few isolate of *S. aureus* strains as VISA and VRSA (12, 13 and 30). Recent review and meta-analysis studies have emphasized on vancomycin MIC which has been significantly associated with a higher mortality rate in the patients with bloodstream infections due to MRSA (31, 32). In our study, no *S. aureus* isolate grew on the BHI agar containing either 4 µg/ml and 6 µg/ml vancomycin E-test results showed vancomycin MIC of *S. aureus* strains ranging from 0.25-2 µg/ml. According to the newer CLSI definition (33) which has been introduced in 2006, all staphylococci requiring concentrations of vancomycin of  $\leq 2$  µg/ml for growth inhibition are considered as “susceptible” and those requiring 4 µg to 8 µg/ml for inhibition as “intermediate” and those requiring concentrations of  $\geq 16$  µg/ml are considered as “resistant”. Thus, all of 195 *S. aureus* isolates in our study were susceptible to the vancomycin. In a recent report, Liu *et al.* showed that none of 984 *S. aureus* examined were resistant to the vancomycin (5).

## Conclusion

The present study showed that all *S. aureus* isolates were susceptible to the vancomycin. This drug can still be used as a valuable treatment option for infections due to MRSA in our region. However, periodic evaluation of vancomycin MIC of *S. aureus* isolates is critical

for monitoring MRSA and preventing the spread of VISA or VRSA among patients.

## Acknowledgments

This paper has been extracted from an approved research plan (No.88115). We thank Vice- chancellor for research affairs and Infectious & Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences which financially supported this plan and, especially Research Consultancy Center (RCC) for technical support.

## Conflict of interest

None declared conflicts of interest.

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