



Determination of Vancomycin and Methicillin Resistance in Clinical Isolates of *Staphylococcus aureus* in Iranian Hospitals

Abbas Farhadian¹, Qorban Behzadian Nejad¹, Shahin Najar peerayeh¹,
Mohammad Rahbar^{2,3*} and Farzam Vaziri¹

¹Department of Bacteriology, School of Medical Science, Tarbiat Modares University, Iran.

²Department of Microbiology, Iranian Reference Health Laboratory Ministry of Health and Medical Education, Tehran, Iran.

³Antimicrobial Resistance center, Iran University of Medical sciences, Tehran, Iran.

Authors' contribution

This work was carried out in collaboration between all authors. Author AF designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors QBN, SNP and FV managed the analyses of the study. Author MR managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: The aim of this study was to determine the prevalence of methicillin resistant *S. aureus* (MRSA), vancomycin resistant or vancomycin intermediate resistant *S. aureus* (aureus) (VRSA/VISA) among clinical isolates.

Study Design: *S. aureus* isolates used in this study were randomly collected from in-patient and outpatient of several hospitals of 7 cities in Iran (Tehran, Shiraz, Zahedan, Tabriz, Sannandaj, Sari, and Ahvaz) during 2006-2008.

Methodology: Antibiotic susceptibility of 250 strains of *Staphylococcus aureus* isolated from Iranian hospitals were determined by disk diffusion method. Minimum inhibitory concentration (MICs) were determined for oxacillin and vancomycin by E-test. PCRs were used by specific primers (PCR used specific primers) for detection of *mecA*, *vanA*, *vanB* genes.

Results: The percentage of resistance by disk diffusion method was as below: methicillin 46%, vancomycin 0%, penicillin 86%, erythromycin 42%, ciprofloxacin 29%, gentamicin 39% and clindamycin 33%. E-test MIC method showed that 43% isolates were resistant to

*Corresponding author: Email: rahbar_reflab@yahoo.com;

methicillin and 4% isolates were VISA ($\leq 8\mu\text{g/ml}$). The prevalence of resistance genes in the clinical isolates were: *mecA* 44%, *vanA* 0%, *vanB* 0%.

Conclusion: This study revealed that clinical isolates have rather high resistance to methicillin, erythromycin, gentamicin, penicillin and clindamycin. We did not observe resistance to vancomycin. In order to avoid a possible outbreak involving VISA), vancomycin should be used carefully as a drug for treatment of *S. aureus* infections.

Keywords: *S. aureus*; MRSA; VRSA; VISA; E-test; PCR.

1. INTRODUCTION

Staphylococcus aureus as a major cause of potentially life-threatening infections acquired in health care settings and in the community, has developed resistance to most classes of antimicrobial agents soon after their introduction into clinical use. During the recent years, the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) has increased in many parts of the world [1,2,3]. MRSA is a type of bacteria that are resistant to certain antibiotics. Resistance to methicillin in *S. aureus* is caused by the expression of penicillin-binding protein PBP2a (PBP2'), which is encoded by the *mecA* gene. This gene is located on a genetic element called the staphylococcal cassette chromosome [4,5,6,7]. Since the emergence of MRSA, options for treatment of *S. aureus* infection have been significantly limited. Vancomycin remained the last resort for MRSA treatment until recent years. Therefore, the emergence of vancomycin-intermediate *S. aureus* (VISA) in 1996 evoked great concern between health care workers around the world. These bacterial strains present a thickening of the cell wall, which is believed to deplete the vancomycin available to kill the bacteria [4]. Then the first *vanA*-mediated, vancomycin-resistant *Staphylococcus aureus* (VRSA) strain was isolated from the catheter tip of a diabetic, renal dialysis patient in a Michigan hospital in 2002. Since then, several additional occurrences have been reported [1]. VRSA is caused by the expression of *vanA* gene which is mediated by Tn1546 or closely related mobile genetic elements. The target of vancomycin is the carboxy terminal D-alanyl-D-alanine (D-ala-D-ala) of the disaccharide pentapeptide cell wall precursor, which is translocated by a lipid carrier to the outer surface of the cytoplasmic membrane. Enzymes encoded on the transposon replace D-ala-D-ala with a depsipeptide, D-alanyl-D-lactate (D-ala-D-lac) [2]. The VISA strains do not carry the enterococcal vancomycin-resistance gene *vanA*, *vanB* or *vanC* 1–3, these strains become resistant to vancomycin by producing a thick cell wall [8].

In this study we aimed to determine prevalence of MRSA and VRSA/VISA by standard microbiological methods of susceptibility testing (disk diffusion, E-test) and PCR methods in clinical isolates of *Staphylococcus aureus* in Iranian hospitals.

2. MATERIAL AND METHODS

2.1 Bacterial Isolates

A total of 250 non-duplicated *S. aureus* isolates used in this study were randomly collected from in-patient and outpatient of several hospitals of 7 cities in Iran (Tehran, Shiraz, Zahedan, Tabriz, Sannandaj, Sari, Ahvaz) during 2006-2008. The specimens of clinical consisted blood (31%), wound (20%), urine (21%), catheters (7%), sputum (12%), others (9%). Then all isolates were identified as *S. aureus* using conventional microbiological tests.

Pure stock cultures of all isolates were stored frozen at -76°C in Skim Milk broth, containing 10% glycerol.

2.2 Antimicrobial Susceptibility Testing

Disk diffusion test of Penicillin (10 U), Oxacillin (1 µg), vancomycin (30 µg), Gentamicin (10 µg), Erythromycin (15 µg), Clindamycin (2 µg), ciprofloxacin (5 µg) (Mast, Merseyside, United Kingdom), was carried out using Kirby-Bauer Method according to CLSI guidelines 2007. Mueller-Hinton agar plates were overlaid with the inoculums (turbidity equivalent to that of a 0.5 McFarland Standard) of the *S. aureus* clinical isolates. Zone diameters were measured at 24 (48 h for vancomycin) following CLSI criteria [9]. The minimal inhibitory concentration (MIC) of oxacillin and vancomycin was measured by E-test (AB biodisk dalvagen Sweden). *S. aureus* ATCC 29213 was employed as control strain for disk diffusion and E-test.

2.3 DNA Extraction

DNA was extracted from 250 staphylococcal isolates using the boiling method. Briefly, 2-3 single colonies of bacteria were washed twice with 0.5 cc EDTA-Tris buffer. Then 50λ D.W. was added and the mix was incubated at 100°C for 15 min. then it was harvested by centrifugation at 7500g for 10 min centrifugation at 8000 g for 5 min. Finally, the supernatant was used for PCR assay.

2.4 PCR

Resistance genes were detected by PCR. They included genes for methicillin resistance (10), and vancomycin resistance [1,11] The PCR primers used to detect resistance genes are listed in Table 1. The PCR mixture was prepared in a final volume of 25 ml. The amplification mixture consisted of 2.5 ml template DNA, 2ml primers, 2 ml of a 10-fold concentrate PCR buffer, 2ml dntp, 0.5 mM MgCl₂, 15 ml D.W. and 1U of Taq DNA polymerase (CinnaGen). A thermocycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) was programmed for genes with the following parameters:

Table 1. Primer Sequences for Resistance Genes

Resistance genes	Oligonucleotide sequence	Size (bp)
vanA	5'-CAT GAA TAG AAT AAA AGT TGC TGC AAT A-3' 5'-CCC CTT TAA CGC TAA TAC GAT CAA-3'	1032
vanB	5'-GTG ACA AAC CGG AGG CGA GGA-3' 5'-CCG CCA TCC TCC TGC AAA AAA-3'	628
mecA	5'-AAAATCGATGGTAAAGTTGGC-3' 5'-AGTTCTGCAGTACCGGATTTGC-3'	532

Detection of vanA: Initial denaturation step of 2 min at 94°C; 30 cycles of 15 s at 95°C, 30s at 55°C, and 30s at 72°C; and a final elongation at 72°C for 7 min.

Detection of vanB: Initial denaturation at 94°C for 10 min was followed by 30 cycles with a 30s denaturation step at 94°C, a 45-s annealing step at 50°C and a 30-s extension step at 72°C and 10 min extension step at 72°C and a holding step at 4°C until the sample was analyzed.

Detection of *mecA*: Initial denaturation at 94°C for 3 min was followed by 30 cycles of amplification with 94°C for 30 s, annealing at 55°C for 30s, and extension at 72°C for 30s (except for the final cycle, which had an extension step of 4 min).

The PCR products were submitted to electrophoresis on 1.5% agarose gel (Fermentas UAB, Vilnius, Lithuania) containing 0.4 ml/ml of ethidium bromide and visualized by using UV transillumination, and photographed (BioDoc- Analyse; Biometra, Goettingen, Germany).

Statistical Analysis

All data analyses were performed using SPSS software version 11.5 for Windows. By using the w2-test and Fisher’s exact test, P-Values <0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

Results obtained from antibiotic resistance pattern of 250 isolates of *Staphylococcus aureus* showed that 46% isolates were resistant to oxacillin, while there were no isolates resistant to vancomycin. The percentage of resistance to other antibiotics was: penicillin 86%, erythromycin 42%, ciprofloxacin 29%, gentamicin 39% and clindamycin 33% (Fig. 1).

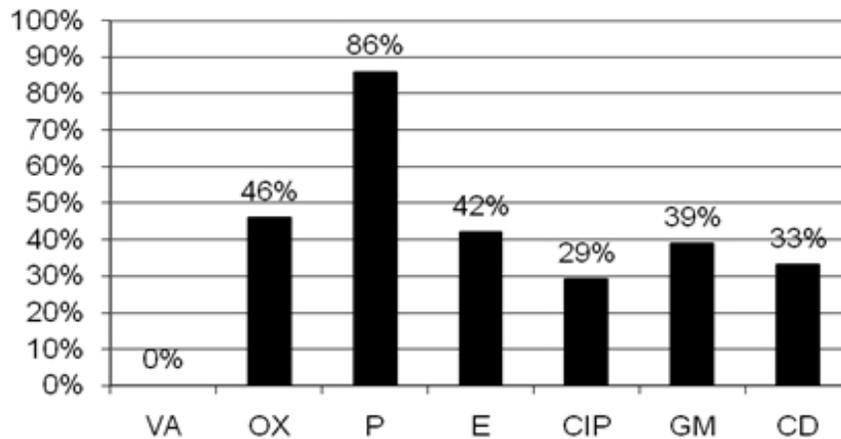


Fig. 1. Antibiotics susceptibility profiles according to disk diffusion method of 250 *S. aureus* isolates

E-test method used in this study showed that 43% isolates were resistant to methicillin (MIC $\geq 4\mu\text{g/ml}$) and 4% isolates was VISA (MIC $\geq 4\mu\text{g/ml}$) Table 2.

Resistant				Intermediate		Sensitive	
MIC($\mu\text{g/ml}$)				MIC($\mu\text{g/ml}$)		MIC($\mu\text{g/ml}$)	
4-32	32-128	128-256	>256	2-4		≤ 2	
32	58	15	3	16		126	methicillin
Isolates	isolates	isolates	isolates	isolates		isolates	
MIC($\mu\text{g/ml}$)				MIC($\mu\text{g/ml}$)		MIC($\mu\text{g/ml}$)	
16-64	64-128	128-256		4-8	2-4	≤ 2	
0	0	0		8	3	239	vancomycin
Isolates	isolates	Isolates		isolates	isolates	isolates	

All 250 isolates were screened for the presence of resistance genes (*mecA*, *vanA*, *vanB*). Amplified DNA fragments of three different sizes (1032, 628, 533 bp) were visualized using agarose gel electrophoresis (Fig. 2). Forty four percent of isolates were *mecA* positive and *vanA* and *vanB* genes were not detected. The frequencies of these genes are shown in Fig. 3. The results of the PCR assay correlated with the results of the phenotypic antibiotic resistance determination.

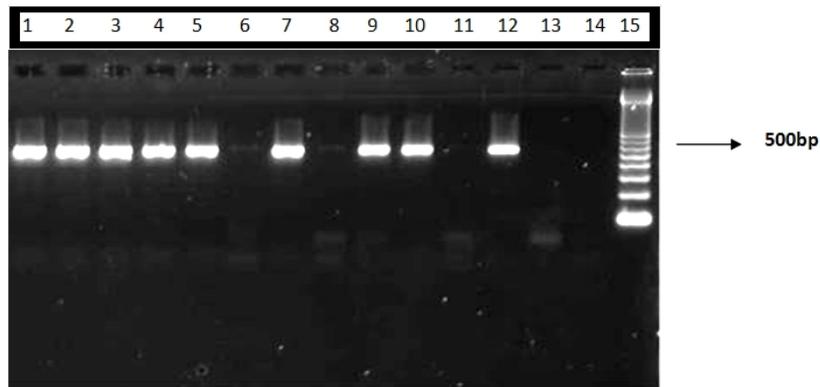


Fig. 2. Agarose gel electrophoresis of *mecA* gene

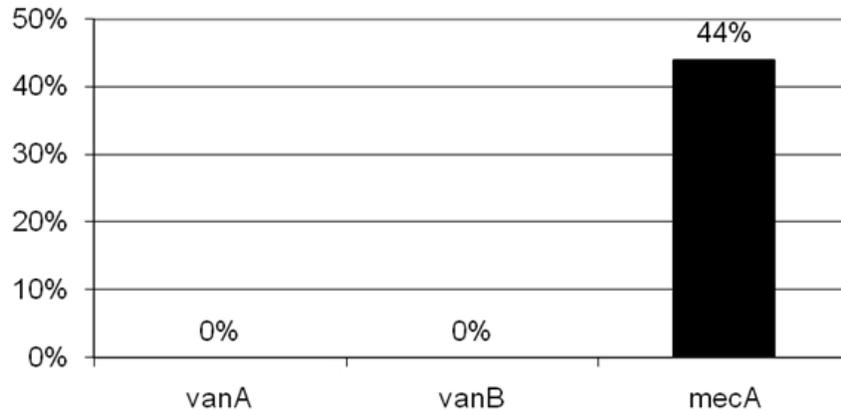


Fig. 3. The prevalence of the *mecA*, *vanA* and *vanB* genes were determined by PCR assay in 250 isolates of *Staphylococcus aureus*

Staphylococcus aureus has been reported as one of the major mortal pathogens with increasing prevalence rates around the world and can cause both nosocomial and community-acquired infection [12]. At first penicillin was used to cure patients infected by *S.aureus*, but by the 1950s, many of the antibiotics were already useless against the infection. So methicillin was developed to treat staphylococcal infections. However two years after its discovery in England in 1959, *S. aureus* had developed a resistance to Methicillin and MRSA was born. Less than several years' later resistance followed in all beta-lactam replacements—oxacillin, nafcillin and the cephalosporins—contributing to susceptibility pattern of multiple-resistance [13]. With the increase of staphylococcal resistance to methicillin, vancomycin (or another glycopeptide antibiotic, teicoplanin) was

often a treatment of choice in infections with methicillin-resistant *S. aureus* (MRSA). But in 1996 the first dreaded case of decreased susceptibility appeared, vancomycin-intermediate susceptible *S. aureus* (VISA), and then after six years, a strain that had become completely resistant to vancomycin was reported.

In this study all VISA isolates were related to blood and wound specimens while most MRSA isolates were detected from wound specimens (up to 57% of total MRSA isolates) so attention to skin and blood infections of *S. aureus* is significant in clinical centers. It is remarkable that eight isolates out of ten VISA were resistant to methicillin. In this study we also observed isolates that were methicillin-resistant by disk diffusion method although *mecA* gene was not detected by PCR method and probably this resistance phenotype is associated with modifications in native PBPs, beta-lactamase hyperproduction, or possibly a methicillinase.

Multiresistance was observed in 52% of 115 oxacillin-resistant isolates by disk diffusion method. More prevalent multiresistance profiles consisted of oxacillin–ciprofloxacin–erythromycin–gentamicin (7.2%) and oxacillin–ciprofloxacin–erythromycin (6%). In current study all isolates were negative for *vanA/vanB* gene by PCR. Therefore the absence of *vanA/B* genes in the VISA isolates does not mean that these isolates are not resistant to vancomycin.

In a 2004 article published by researchers in New York and Atlanta, the two major mechanisms of resistance, the *mecA* gene and the *vanA* plasmid were elegantly presented. It was concluded that in VRSA where both *mecA* and *vanA* resistance mechanisms exist, different sets of enzymes are employed in the formation of the organism's cell walls and it was very important for medicine [14].

Several investigations have been published in Iran about antibiotic resistance in *S. aureus*; Yadegar et al. reported 48% methicillin resistant and 0% vancomycin resistance among 100 isolates of *S. aureus* [17]. A study by Rahbar et al. showed 35.3% MRSA with 100% sensitivity to vancomycin among one hundred isolate of *S. aureus* [19]. Another study carried out by Ahmadishoar, show 38% MRSA with no vancomycin resistance among 1000 *S. aureus* isolates which were collected in Tabriz between September 2005 and July 2006 [18].

A systematic review carried out by Zarifian et al. found that at least 24 VRSA isolates have been reported from Iran up to September 2012 [20]. Out of 24 isolates only one strain was confirmed [21]. It seems that many Iranian researchers did not follow a specific guideline for reporting and confirming VRSA.

In a study that Jesús et al. carried out in Spain, they also obtained 24.5% resistance to methicillin and 0% resistance to vancomycin between in 3113 isolates of *S. aureus* [16]. Another study in India, by Hare et al. also obtained similar results. They reported 318 MRSA, two VRSA and ten VISA between 783 strains of *S. aureus* [11]. Van Griethuysen et al. showed 7.6% VISA in 250 isolates of MRSA, denoting high resistant to methicillin can decrease susceptibility to vancomycin in *S. aureus* isolates [22].

4. CONCLUSION

Because of the high prevalence of methicillin resistance and vancomycin intermediate resistance found among the *S. aureus* isolates, periodic surveillance on the resistance prevalence should be performed. It seems necessary to improve the management of

remedial and the relationship between physicians and laboratory. Since not all susceptibility testing methods can detect VISA and VRSA, laboratories should check isolates with methods such as broth microdilution, agar dilution or E-test. Establishing an Iranian reference center where studies on VRSA can be registered, evaluated and confirmed is strongly recommended.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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