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VanA and *VanB* Positive Vancomycin-resistant *Staphylococcus aureus* Among Clinical Isolates in Shiraz, South of Iran

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Abstract

Objective: The purpose of this study was to determine the prevalence of vancomycin-resistant *Staphylococcus aureus* isolated from clinical samples in Shiraz hospitals.

Methods: From March to December 2012, 100 *S. aureus* isolates (mainly from wound and blood) were collected from three hospitals in Shiraz, south of Iran. After identification of *Staphylococcus aureus* by biochemical, microbiological and molecular methods, antibiotic susceptibility testing was performed by Kirby-Bauer disc diffusion test for 13 different antibiotics. Vancomycin-resistant *Staphylococcus aureus* isolates were determined by vancomycin agar screening test and PCR for vancomycin resistant genes (*vanA* and *vanB*).

Results: The lowest and highest resistance was seen for quinupristin-dalfopristin (n=1) and ampicillin (n=95), respectively. Vancomycin agar screening test showed that 37 isolates can grow on these media. Further study by PCR also detected *vanA* and/or *vanB* genes in all of these strains. Also, 19 isolates showed either *vanA* or *vanB* but were susceptible according to vancomycin agar screening test. In total, *vanA* and *vanB* resistant genes were detected in 34% and 37% of clinical isolates, respectively.

Conclusion: The results showed that the frequency of vancomycin resistance genes (*vanA*, *vanB*) is very high in *Staphylococcus aureus* strains isolated from patients in south of Iran. Thus, urgent interventions are needed to keep the emergence and transmission of these isolates to a minimum.

Keywords: *Staphylococcus aureus*; Vancomycin Resistance; *vanA*; *vanB*; Iran.

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Introduction

Staphylococcus aureus is a major human pathogen which is known for more than a century. Primarily as a colonizer in one-third of general population, *S. aureus* can also cause life-threatening infections both in the community and healthcare settings. *Staphylococcal* infections range in severity from uncomplicated skin and soft tissue infections (such as folliculitis) to the more severe infections like necrotizing pneumonia and endocarditis.[1,2]

Resistance of staphylococci to antimicrobial agents is an issue of worldwide concern with a history of almost 70 years. Penicillin was successfully used to treat *S. aureus* until 1942, when penicillin-resistant *S. aureus* appeared.[3] In 1961, methicillin-resistant *S. aureus* (MRSA) was reported from England and is now a common cause of hospital-acquired infections.[4]

Vancomycin is the main antimicrobial agent available to treat serious infections caused by MRSA. In May 1996, the first documented clinical infection due to *S. aureus* with the intermediate resistance to vancomycin (minimum inhibitory concentration [MIC] equal to 8 µg/ml) was reported from Japan.[5] Later, vancomycin-intermediate *S. aureus* (VISA) strains were isolated in USA, Australia, Europe and other Asian countries.[2]

The first clinical MRSA isolate exhibiting high-level resistance to glycopeptides (vancomycin MIC>256 µg/ml; teicoplanin MIC=128 µg/ml) due to acquisition of the *vanA* operon was detected in 2002 from Michigan.[6] Although there are few reports of vancomycin-resistant *Staphylcoccus aureus* (VRSA) worldwide, it seems that Iran is a hot spot region for the emergence of these isolates.[7-10]

The present study aimed to determine the antimicrobial susceptibility patterns of *S. aureus* strains isolates from patients in Shiraz hospitals in south of Iran with emphasis to the possible presence of vancomycin resistance.

Methods

A total number of 220 staphylococcal samples were collected from laboratories of Shiraz hospitals (Shahid-Faghihi, Namazi and MRI) from March to December 2012. The strains were collected from various clinical specimens including pus, pharynx, sputum, wound swabs, skin, urine and blood. One hundred *S. aureus* isolates were selected after identification by standard biochemical and

microbiological tests, including Gram staining, oxidase, catalase, coagulase, latex agglutination, motility, DNase, haemolysis and mannitol fermentation tests.[11]

To confirm the strains, PCR amplification of the *nuc* gene was performed for all isolates with these primers; *nuc*F 5'GCGATTGATGGTGATACGGTT3' and *nuc*R 5'AGCCAAGCCTTGACGAACTAAAGC 3', according to Brakstad et al. (1992).[12]

Staphylcoccus aureus ATCC 29213 and *Enterococcus faecalis* ATCC 29212 strains were used as vancomycin-susceptible controls. Vancomycin-resistant *Enterococcus faecalis* ATCC 51299 was used as a positive control.

Disc agar diffusion (DAD) test was carried out using Kirby-Bauer method according to CLSI procedure with the following discs, which are all from MAST company, Liverpool, UK: penicillin G (10μg), ampicillin (10 μg), amoxicillin (20 μg), vancomycin (30 μg), clindamycin (2 μg), rifampin (5 μg), ciprofloxacin (5 μg), oxacillin (1 μg), tetracycline (30 μg), erythromycin (15 μg), linezolid (30 μg), quinupristin-dalfopristin (15 μg), and teicoplanin (30 μg). Mueller–Hinton agar plates were overlaid with the inoculum (turbidity equivalent to that of a 0.5 McFarland standard) of the *S. aureus* clinical strains. The zone diameter of bacterial growth inhibition surrounding the disc was measured and compared with a standard for each drug. *S. aureus* ATCC 25923 was used as quality control strain for the DAD test.[13]

Vancomycin agar screen plates were prepared In-house by addition of 6 mg/L vancomycin to brain heart infusion (BHI) agar (Merck, Germany). Inoculum suspension was prepared by transferring colonies from overnight growth on nutrient agar plate to sterile saline to produce a suspension that matches the turbidity of a 0.5 McFarland standard. Then, 0.1 ml of this suspension was spread on BHI agar containing 6 mg/L of vancomycin (BHI6V), the vancomycin agar screen plate, and was incubated for 24h at 35°C in ambient air. *E.fecalis* ATCC 29212 was used as a vancomycin-susceptible and *E.fecalis* ATCC 51299 was used as a vancomycin-resistant control, according to the CLSI guideline [14].

Genes encoding the vancomycin resistance determinants, *vanA* and *vanB*, were investigated by PCR using specific primers (Table 1).[15] PCR amplification was carried out in a 20μl reaction mixture with each primer as the following steps: an initial denaturation step at 98°C for 2 min; followed by 35 cycles of 98°C for 10 sec, 50°C for 1 min and 72°C for 90 sec for *vanA* gene, and an initial denaturation step at 94°C for 10 min; followed by 30 cycles of 94°C for 30 sec, 50°C for 45 sec and 72°C for 30 sec for *vanB* gene, then finally elongation step at 72°C for 10 min. The PCR products were electrophoresed in a 1.5% agarose gel which was stained with ethidium bromide and visualized by using UV transillumination.[15]

This PCR was performed to rule out the contamination by *Enterococcus spp*. Oligonucleotide primers were directed to the $ddl_{E.\ faecalis}$, and $ddl_{E.\ faecium}$ genes are shown in table 1. The *ddl* primers yielded a product of 429bp for *E. faecalis* and 688 bp for *E. faecium*. PCR amplification was programmed as follows: 10 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; and 10 min at 72°C. Samples were held at 4°C until the products could be analyzed. Ten-microliter samples of the PCR products were electrophoresed through a 1.5% agarose gel for 45 min at 150 V. The gels were stained with ethidium bromide and photographed under UV light[16].

DNA Sequencing was carried out on PCR products by Takapoozist Company in Iran. The sequences were aligned and compared with reference sequences achieved using GenBank with the BLAST system.

Results

In this study, 100 strains were isolated from patients and all were confirmed as *S. aureus* by amplification of *nuc* gene. The age of patients ranged from 5 to 60 years with an average age of 30.4 ± 42.4 and 56% out of them were male. The number of isolated staphylococci from each ward and their antimicrobial resistance rates are detailed in table 2. Most of the isolates were from internal medicine (n=29), surgery (n=28) and ICU (n=16) wards. Resistance rates were highest for ampicillin (n=95) and lowest for quinupristin-dalfopristin (n=1) (Table 2).

Amplification of nuc, $ddl_{E.\ faecalis}$ and $ddl_{E.\ faecium}$ targets produced distinct bands corresponding to their respective molecular sizes that were easily recognizable (Fig. 1).

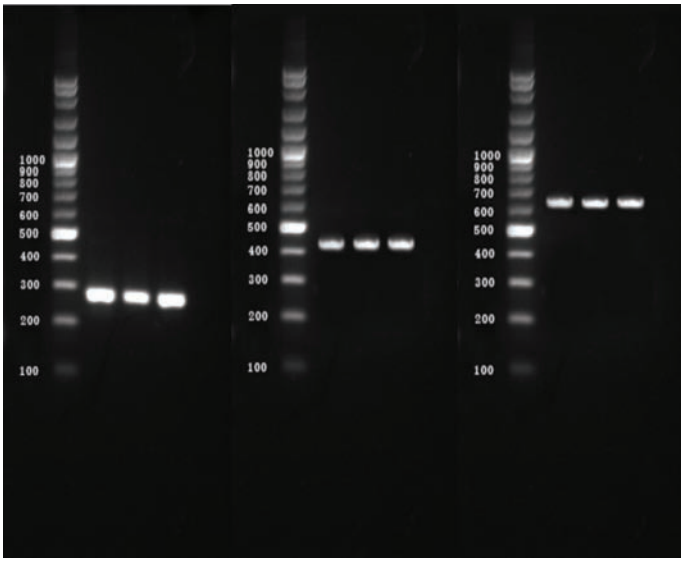

Figure 1: Agarose gel electrophoresis of PCR-amplified *nuc* and *ddl* genes (*E.faecalis* and *E. faecium*). Lanes: M, 100-bp ladder; 1-3: *S. aureus* isolates showing 279 bp *nuc* amplicon; 4-6: *S. aureus* isolates showing 429 bp *ddl* genes (*E.faecalis*) amplicon,7-9 *S. aureus* isolates showing 688 bp *ddl* genes (*E. faecium*) amplicon.

There were 3 van gene containing S. aureus (VRSA) strains according to disc diffusion test; one strain was isolated from a 35-year old female patient in dermatology ward. It was resistant to all antibiotics tested except ciprofloxacin. Two other VRSA isolates were also found; one isolated from blood of a patient in NICU and another from a wound of a patient in surgical ward (Data not shown).

Table 1: Primers used in this study.

Target	Primer	Sequence (5'to3')	Product size (bp)	Reference
nuc	Forward	GCG ATT GAT GGT GAT ACG GTT	279	12
	Reverse	AGC CAA GCC TTG ACG AAC TAA AGC		
vanA	Forward	ATG AAT AGA ATA AAA GTT GC	1032	15
	Reverse	TCA CCC CTT TAA CGC TAA TA		
vanB	Forward	GTG ACA AAC CGG AGG CGA GGA	430	15
	Reverse	CCG CCA TCC TCC TGC AAA AAA		
($ddl_{E. faecium}$)	Forward	CAGTGCATGTGCCATGGATA	429	16
	Reverse	ACTTCGGCTGGAATCTGCAT		
($ddl_{E. faecalis}$)	Forward	CAGAAGTGAAGAGCACGATG	688	16
	Reverse	AGGTAAAGTCGTACGGACAT		

Table 2: Antimicrobial resistance rates of *S. aureus* isolates in different wards

Antimicrobial Agent	Resistant rates (%)								
	Internal Medicine (n=29)	Surgery (n=28)	ICU (n=16)	NICU (n=7)	Dermatology (n=7)	Outpatient (n=7)	Emergency (n=4)	Dialysis (n=2)	Total (n=100)
Ampicillin	93.1	96.4	100	100	100	71.4	100	100	95
Penicillin G	100	92.9	100	100	85.7	71.4	100	50	94
Amoxicillin	96.1	89.3	100	100	71.4	85.7	75	100	92
Tetracycline	51.8	50	50	57.1	28.6	14.3	75	0	47
Ciprofloxacin	48.3	42.9	50	71.4	28.6	28.6	75	0	46
Erythromycin	37.9	46.4	43.7	71.4	28.6	28.6	75	50	44
Oxacillin	44.8	46.4	43.7	57.1	28.6	28.6	75	0	44
Clindamycin	34.5	35.7	43.7	57.1	28.6	28.6	75	0	38
Rifampin	37.9	17.9	31.2	14.3	28.6	28.6	75	0	29
Vancomycin	0	3.7	0	14.3	14.3	0	0	0	3
Linezolid	0	3.7	0	0	14.3	0	0	0	2
Teicoplanin	0	0	0	0	14.3	0	0	0	1
Synercid*	0	0	0	0	14.3	0	0	0	1

* *Synercid = Quinupristin-dalfopristin*

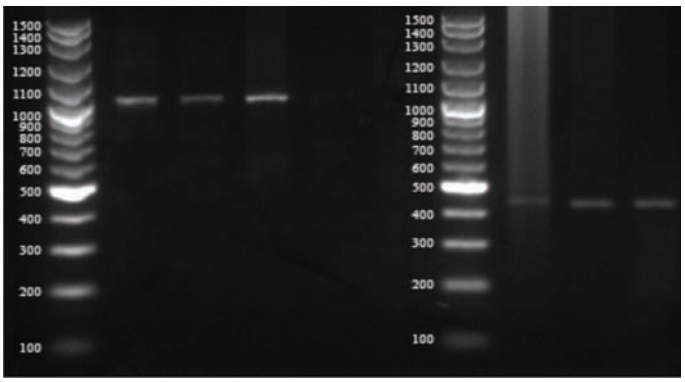

Figure 2: Agarose gel electrophoresis of PCR-amplified vancomycin resistance genes (*vanA* and *vanB*). Lanes: M, 100-bp ladder; 1-3: *S. aureus* isolates showing 1032 bp *vanA* amplicon; 4-6: *S. aureus* isolates showing 430 bp *vanB* amplicon.

Table 3: Results of *vanA* and *vanB* PCR compared to vancomycin screening agar test.

		vanA		*vanB*		(*vanA* + *vanB*)	
		+	–	+	–	+	–
Screening agar	+	28	24	28	24	19	33
	–	6	39	9	37	0	48
Total		34	63	37	61	19	81

Screening for vancomycin resistance showed that 37% isolates can grow on BHI6V. Further studies by PCR also detected *vanA* and/or *vanB* genes in all of these strains (Fig. 2). Also, 19 isolates showed either *vanA* or *vanB* but were susceptible according to

vancomycin agar screening test. Totally, *vanA* and *vanB* resistant genes were detected in 34% and 37% of clinical isolates, respectively (Table 3).

Discussion

During the past decade VRSA did not spread rapidly and there were only a few reports of this superbug. Until the end of 2012, 33 cases of *vanA*-type VRSA have been reported worldwide: 13 from the United States, 16 from India, 3 from Iran (2 from Tehran, 1 from Mashhad) and 1 from Pakistan.[7] Limited spread of VRSA is attributed to the highly-costly *vanA* operon for *S. aureus*, which can be acquired from enterococcal conjugation.[17]

Recent articles show that VRSA is now reported in at least four continents (i.e. Asia, Europe, North and South America).[7,18,19] The European VRSA was isolated in May 2013 from pus of the toe amputation wound of a 74-year-old female in a Portuguese hospital. The patient had multiple co-morbidities and her culture grew *Pseudomonas aeruginosa*, vancomycin-resistant *Enterococcus faecalis*, and methicillin-resistant VRSA.[18] There is also another report from Brazil which describes a 35-year-old male with a history of diabetes mellitus and Sezary syndrome who had blood culture positive for methicillin-resistant VRSA. Vancomycin resistant *E. faecalis* was also isolated from the patient and he died despite vancomycin therapy.[19] Unfortunately, we do not have enough clinical information regarding our VRSA strains.

In contrast to previous studies, we found a high number of *vanA* and/or *vanB*-VRSA confirmed by PCR which were phenotypically susceptible to vancomycin. Recently, Banerjee et al also found two *vanA*-VISA strains which none of these two expressed *vanA* operon. This finding can at least partly explain our results.[20]

There is only one report of *vanB*-VRSA in the literature. Also, VRSA isolates simultaneously with *vanA* and *vanB* genes have been found only in the mentioned study.[21] In a study from India, Chakraborty and colleagues found eight VRSA strains from 30 *S. aureus* isolated from pus samples. All eight isolates had simultaneously *vanA* and *vanB* genes and were also resistant to vancomycin according to vancomycin macro-broth dilution. Compared to this study, we found all types of vancomycin resistance including *vanA*, *vanB* and *vanA*+*vanB* and some of our isolates were phenotypically susceptible to vancomycin.

In our study, about 40% of the isolates harbored at least one of the *van* genes. There is a possibility that these infections were caused by dissemination of a few clones of VRSA circulating in south of Iran but, we can neither confirm nor exclude this possibility.

Previously, all VRSA isolates were considered to be susceptible to newer antimicrobial agents such as linezolid and quinupristin-dalfopristin. Saravolatz et al reported that all VRSA reported in the United States were susceptible to these agents.[22] In our study, one of the *vanA*-VRSA isolates from dermatology ward was resistant to linezolid and quinupristin-dalfopristin. Further studies of this isolate with E-test also confirmed our findings (Data not shown). Interestingly, this isolate was susceptible to ciprofloxacin which is unusual and has been also reported in a study which has reported VRSA from Kolkata.[23,24] Recently, Alzolibani et al have reported VRSA among children with atopic dermatitis in Saudi Arabia but the authors did not confirm their isolates by PCR of *van* genes.[25] Although we found several VRSA in our study, these isolates remain very rare and are not usually detected.[26,27]

Conclusion

Our results showed that the frequency of vancomycin resistance genes (*vanA*, *vanB*) is very high in *Staphylococcus aureus* strains isolated from patients in Shiraz hospitals and multidrug-resistant VRSA is also emerging. Thus urgent interventions are needed to keep the emergence and spread of these isolates to a minimum.

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