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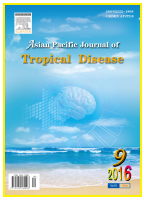

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Molecular characterization of vancomycin-intermediate *Staphylococcus aureus* isolates from Tehran

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ABSTRACT

Objective: To determine the prevalence and some genetic characteristics of clinical isolates of *Staphylococcus aureus* (*S. aureus*) with reduced susceptibility to vancomycin.
Methods: A total of 414 isolates of *S. aureus* were collected from clinical specimens from hospitals in Tehran. Vancomycin-intermediate *S. aureus* (VISA) was determined by brain heart infusion agar containing 4 μg/mL vancomycin screening plate and confirmed via E-test. VISA isolates were analysed for *vanA, vanB, mecA*, staphylococcal cassette chromosome *mec* types, surface protein A *(Spa)* types and *agr* specific groups.
Results: Brain heart infusion agar containing 4 μg/mL vancomycin screening tests revealed that 17.14% (n = 71) of *S. aureus* isolates were VISA phenotype. Ten of the 71 isolates were confirmed by E-test method (minimal inhibitory concentration was 4 to 8 μg/mL). All VISA isolates were susceptible to linezolid and 6 isolates (60%) were methicillin-resistant *S. aureus*. Five isolates belonged to *agr* Group II, 4 belonged to *agr* Group I and 1 belonged to agr Group III. *Spa* type t030, and staphylococcal cassette chromosome *mec* Type III were dominant among VISA isolates.
Conclusions: This study provides further evidence of the global dissemination of VISA isolates and emphasizes to vancomycin susceptibility testing prior to antibiotic therapy.

1. Introduction

Staphylococcus aureus (*S. aureus*) is an important bacterium responsible for community and hospital acquired infections. Most of *S. aureus* infections are caused by methicillin-resistant *S. aureus* (MRSA) isolates, which the glycopeptide antibiotic vancomycin is considered the effective antimicrobial for these infections. Unfortunately, widespread empirical use of vancomycin has led to emergence of strains with reduced susceptibility to vancomycin. Most infections caused by clinical isolates intermediate *S. aureus* (VISA) strains occur in patients with serious underlying diseases such as diabetes and malignancy. Other diseases such as endocarditis or infection of a prosthetic joint with a high bacterial load may also predispose an individual to the development of VISA infection during glycopeptide therapy[1-3].

Because of the difficulty of testing methods, the exact prevalence of VISA and heterogeneous VISA (hVISA) remains uncertain[1,4-6]. According to the Clinical and Laboratory Standards Institute guidelines, the minimum inhibitory concentration (MIC) of vancomycin for susceptible, intermediate, or resistant strains is 2 μg/mL, 4–8 μg/mL or 16 μg/mL, respectively[7]. A subpopulation of cells in hVISA strains with MIC of 4 μg/mL for vancomycin could not detected via reference methods such as broth microdilution, agar dilution and standard E-test methods[4]. The population analysis profile-area under the curve (PAP-AUC) method is the gold standard for detection of hVISA, but it is labor-intensive, costly and also impractical to perform for a large number of isolates[4,8,9]. Riederer *et al*. in 2011 reported that brain heart infusion (BHI) agar supplemented

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with 3 or 4 μg/mL vancomycin is a useful alternative screening method for detecting hVISA and VISA respectively[10]. The sensitivity and specificity of their methods were 100% and 94.6% for BHI-supplemented with 3 μg/mL vancomycin, and 100% and 99.2% for BHI-supplemented with 4 μg/mL vancomycin[10].

After the first hVISA and VISA strains appeared in 1996 in Japan, reduced susceptibility to vancomycin in clinical isolates of *S. aureus* have been reported in many parts of the worlds, however, there are only a few reports of hVISA and VISA strains from Iran[3-6,11,12]. Therefore in this study, we detected prevalence of VISA strains among clinical isolates of *S. aureus* collected in teaching hospitals over a 3 years period. Our study may also provide genetics information of VISA strains including *spa* types, *mecA* gene, staphylococcal cassette chromosome *mec* (SCC*mec*) types and *agr* specificity groups.

2. Materials and methods

2.1. Bacterial strains

A total of 414 non-consecutive clinical isolates of *S. aureus* which were collected during 2009 to 2012 from teaching hospitals in Tehran, were screened for vancomycin susceptibility. All isolates were identified by conventional bacteriological methods (Gram-positive cocci, catalase-positive, mannitol-fermenting, slide and tube coagulase-positive and deoxyribonuclease-positive). All *S. aureus* strains were stored at –70 ˚C in BHI broth containing 20% (v/v) glycerol.

2.2. Detection of VISA

All *S. aureus* isolates were screened for VISA strains on BHI agar containing 4 μg/mL of vancomycin (BHI-4V) as previously described[10]. The growth of one or more colonies after 48 h was considered positive. A positive isolate on BHI-4V screening plates was further analysed by the MICs using E-test (AB Biodisk, Solna, Sweden). The isolate was considered VISA if the MIC of vancomycin was 4 to 8 μg/mL. MIC of isolates which displayed a VISA profile on the E-test was further confirmed by the agar dilution method[7].

2.3. Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed as recommended by the Clinical Laboratory Standards Institute using disk diffusion method for all isolates. Antimicrobial disks (Mast. UK) tested included oxacillin (1 μg), gentamycin (10 μg), amoxicillin (30 μg), ciprofloxacin (5 μg), tetracycline (30 μg), co-trimoxazol (1.25 μg + 23.75 μg), erythromycin (15 μg), rifampin (5 μg), clindamycin (10 μg) and linezolid (30 μg). *S. aureus* ATCC 25923 was used as a control strain[7].

2.4. PCR for detection of mecA, vanA and vanB genes

Genomic DNA of VISA isolates were extracted using DNA extraction kit (GeneAll, Korea). Lysostaphin at the final concentration of 20 μg/mL in lysis buffer [Tris-Hcl (50 mmol/L), 1% sodium dodecyl sulfonate (w/v) and ethylene diamine tetraacetic acid (100 mmol/L)] was used. The DNA was used as the template in all PCRs experiments. All VISA strains were analyzed for the *mecA*, *vanA* and *vanB* genes. PCR Red Master Mix (Amplicon, Denmark) was used for all PCR reactions in an Eppendorf thermal cycler (Mastercycler® gradient, Germany). Amplification program consisted of initial denaturation at 94 ˚C for 5 min, 30 cycles of denaturation at 94 ˚C for 60 s, annealing at 55 ˚C for 60 s for *mecA*, 57 ˚C for *vanA* and 52 ˚C for *vanB*, extension at 72 ˚C for 60 s and a final step of 72 ˚C for 5 min. The PCR products were analyzed by electrophoresis in a 1.4% agarose gel and stained with gel red (Biotium, USA)[13,14]. The primers and size of the expected amplification products were listed in Table 1.

Table 1
List of primers used in this study.

Targets	Primers	Sequence*	Product size (bp)	References
agr	Pan F	ATGCACATGGTGCACATGC		
groups	R I	GTCACAAGTACTATAAGCTGCGAT	439	[15]
	R II	GTATTACTAATTGAAAAGTGCCATAGC	572	
	R III	CTGTTGAAAAAGTCAACTAAAAGCTC	406	
	R IV	CGATAATGCCGTAATACCCG	588	
mecA	F	GTG AAG ATA TAC CAA GTG ATT	147	[13]
	R	ATG CGC TATAGATTGAAA GGA		
vanA	F	CATGAATAGAATAAAAGTTGCTGCAATA	1 030	[14]
	R	CCCCTTTAACGCTAATACGATCAA		
vanB	F	GGGGGG AGGATGGTGGGATAGAG	530	[14]
	R	GGAAGATACCGTGGCTCAAAC		
SCC*mec*	Type I-F	GCTTTAAAGAGTGTCGTTACAGG	613	
	Type I-R	GTTCTCTCATAGTATGACGTCC		
	Type II-F	CGTTGAAGATGATGAAGCG	398	
	Type II-R	CGAAATCAATGGTTAATGGACC		
	Type III-F	CCATATTGTGTACGATGCG	280	
	Type III-R	CCTTAGTTGTCGTAACAGATCG		[13]
	Type IVa-F	GCCTTATTCGAAGAAACCG	766	
	Type IVa-R	CTACTCTTCTGAAAAGCGTCG		
	Type IVb-F	TCTGGAATTACTTCAGCTGC	493	
	Type IVb-R	AAACAATATTGCTCTCCCTC		
	Type IVc-F	ACAATATTTGTATTATCGGAGAGC	200	
	Type IVc-R	TTGGTATGAGGTATTGCTGG		
	Type IVd-F5	CTCAAAATACGGACCCCAATACA	881	
	Type IVd-R6	TGCTCCAGTAATTGCTAAAG		
	Type V-F	GAACATTGTTACTTAAATGAGCG	325	
	Type V-R	TGAAAGTTGTACCCTTGACACC		

*: Sequence of primer as synthesized 5′ to 3′.

2.5. Multiplex PCR for SCCmec typing

All VISA strains were analyzed for SCC*mec* using multiplex PCR. The primers and size of the PCR products were listed in Table 1. The cycling parameters were as follows: an activation

step at 95 ˚C for 5 min, followed by 30 cycles of initial denaturation at 94 ˚C for 30 s, 57 ˚C for 1.5 min and 72 ˚C for 1.5 min, and a final extension at 72 ˚C for 10 min[13].

2.6. Duplex PCR for agr typing

The *agr* specificity groups for VISA strains were determined by two duplex PCR[15]. A forward primer, pan-agr, according to conserved sequences from the *agrB* gene, was used in all reactions. Four reverse primers, each specific for amplification of a single *agr* group based on nucleotide polymorphism of *agrD* or *agrC* genes, were used. The primers and size of the PCR products were listed in Table 1. The cycling parameters were as follows: 94 ˚C for 5 min followed by 30 cycles at 94 ˚C for 1 min, 50 ˚C for 30 s and 72 ˚C for 1 min, and a final extension at 72 ˚C for 7 min.

2.7. Spa typing

All VISA strains were analyzed by staphylococcal protein A *(spa)* typing as previously described[16]. The short sequence repeat X region of the *spa* gene was amplified using the primers F (5′-AGACGATCCTT CGGTGA GC-3′) and R (5′-GCTTTTGCAATGTCATTTACTG-3′). PCR cycling conditions were as follows: an initial denaturation at 94 ˚C for 3 min; 30 cycles at 94 ˚C for 30 s, at 55 ˚C for 30 s, and at 72 ˚C for 1 min, and a final extension at 72 ˚C for 10 min. Amplified PCR products were purified with QIAquick gel extraction kit. Purified PCR products were sequenced commercially by an ABI 3730XL DNA analyzer (Applied Biosystems) in both directions. *Spa* sequences were determined using BioNumerics v7.1 (Applied Maths) software and the SpaServer website.

3. Results

3.1. Bacterial isolates

A total of 414 non-duplicates *S. aureus* isolates included in this study. The isolates recovered from tracheal aspiration (n = 210, 50.7%), blood (n = 71, 17.1%), wound (n = 49, 11.8%), bronchoalveolar lavages (n = 24, 5.7%), catheter (n = 16, 3.8%), urine (n = 14, 3.3%), sputum (n = 10, 2.4%) or others (n = 20, 4.8%).

3.2. Detection of VISA

A total of 71 (17.14%) isolates were grown on BHI-4V plates. The E-tests of vancomycin MIC distribution among those 71 isolates were 2 μg/mL for 51 isolates, 3 μg/mL for 10 isolates, 4 μg/mL for 5 isolates, 6 μg/mL for 2 isolates and 8 μg/mL for 3 isolates. The E-test results were confirmed with repeat testing. The agar dilution of vancomycin MIC for those 10 VISA isolates was concordantly positive. The antimicrobial susceptibility, MICs, isolation source of VISA stains and clinical characteristics of patients with VISA infection were shown in Tables 2 and 3.

Table 2
Phenotypic characteristics of VISA isolates.

Isolates	Samples	Oxa	Tet	Cip	Gen	Rif	SXT	Ery	Cli	Am	BHI-4V	Van MIC (μg/mL)
TMU 1	Drainage	R	R	R	S	S	R	R	R	R	+	8
TMU 2	Blood	S	S	S	S	S	S	S	S	R	+	8
TMU 3	Wound	R	R	R	R	S	R	R	R	R	+	4
TMU 4	Wound	S	S	S	S	S	S	S	S	R	+	4
TMU 5	Trachea	R	R	R	R	R	R	R	R	R	+	4
TMU 6	Trachea	R	R	S	R	S	R	R	R	R	+	4
TMU 7	Trachea	R	R	S	R	R	R	R	R	R	+	8
TMU 8	Blood	S	R	S	R	S	S	S	S	R	+	6
TMU 9	Wound	R	R	R	R	R	S	R	R	R	+	4
TMU 10	Wound	S	S	S	S	S	S	I	R	S	+	6

R: Resistant; S: Susceptible; Oxa: Oxacillin; Tet: Tetracycline; Cip: Ciprofloxacin; Gen: Gentamicin; Rif: Rifampin; SXT: Co-trimoxazole; Ery: Erythromycin; Cli: Clindamycin; Am: Amoxicillin; Van: Vancomycin.

3.3. Antibiotic susceptibility testing

The disk diffusion test was performed for all 414 *S. aureus* (Table 3). All isolates were susceptible to linezolid. The antimicrobial resistance of VISA isolates were 60.0% for oxacillin, 70.0% for erythromycin and tetracycline, 80.0% for amoxicillin and clindamycin, 40.0% for ciprofloxacin, 50.0% for co-trimoxazole and 30.0% for rifampin. The antimicrobial susceptibility assay revealed that non-VISA isolates (Table 4) were mostly resistant to oxacillin (61.1%), tetracycline (46.6%)

Table 3
Clinical characteristics of patients with VISA infection.

VISA isolates	Patient age (yr) and sex	Underlying disease or condition	Type of infection	Antibiotic	Additional antibiotic	Death
TMU 1	74, M	Neurosurgery	CVL infection	Vancomycin	Gentamicin	Yes
TMU 2	68, M	Diabetes mellitus	Bacteremia	Ceftriaxone	Rifampin	No
TMU 3	53, F	Abdominal surgery	Surgical wound infection	Vancomycin	Gentamicin	Yes
TMU 4	38, M	Multiple trauma	Surgical wound infection	Cephalotin	Vancomycin	Yes
TMU 5	42, M	Chronic renal failure	VAP	Vancomycin	Rifampin	No
TMU 6	72, F	Intracerebral hematoma	VAP	Vancomycin	Gentamicin	Yes
TMU 7	57, M	Cirrhosis	VAP	Vancomycin	Gentamicin	Yes
TMU 8	32, M	Intravenous drug use	CVL infection	Vancomycin	Gentamicin	No
TMU 9	63, M	Diabetes mellitus	Osteomyelitis	Vancomycin	Ciprofloxacin	Yes
TMU 10	28, F	Leukemia	Bacteremia	Cephalotin	Vancomycin	Yes

M: Male; F: Female; CVL: Central venous line; VAP: Ventilator associated pneumonia.

and erythromycin (41.6%). Of 343 vancomycin-susceptible *Staphylococcus aureus* isolates (no growth on BHI-4V, Table 4), the highest resistance was observed for gentamicin (55.9%) followed by amoxicillin (53.3%), oxacillin (51.3%) and tetracycline (47.5%).

Table 4
In vitro antibiotic resistance pattern of 414 *S. aureus* isolates to 9 antimicrobial agents [*n* (%)].

Antibiotics	No growth on BHI-4V (*n* = 343)	Growth on BHI-4V (*n* = 71)	
		Non-VISA (*n* = 61)	VISA (*n* = 10)
Oxacillin	176 (51.3)	37 (61.6)	6 (60.0)
Ciprofloxacin	97 (28.2)	19 (31.6)	4 (40.0)
Erythromycin	114 (33.2)	25 (41.6)	7 (70.0)
Tetracycline	163 (47.5)	28 (46.6)	7 (70.0)
Amoxicillin	183 (53.3)	22 (36.6)	8 (80.0)
Gentamicin	192 (55.9)	18 (30.0)	6 (60.0)
Clindamycin	136 (39.6)	11 (18.3)	8 (80.0)
Co-trimoxazole	55 (16.0)	14 (23.3)	5 (50.0)
Rifampin	56 (16.3)	13 (21.6)	3 (30.0)
Linezolid	0%	0%	0%

3.4. Identification of mecA, vanA, vanB, agr groups and SCCmec genes

All of the 10 VISA isolates were evaluated for the *mecA* gene using PCR. The *mecA* gene was found in six (60%) of 10 VISA strains and *vanA* and *vanB* genes were not found in any of the VISA strains. The presence of *agr* specificity groups in VISA isolates was determined by PCR. Most of VISA isolates belonged to *agr* Group II (50%), followed by *agr* Group I (40%) and *agr* Group III (10%). All of the VISA strains with resistance to methicillin (VISA-MRSA) were examined by multiplex PCR for SCC*mec* types. One isolate was found to be SCC*mec*-Type I and five isolates were Type III (Table 5).

3.5. The spa typing

Seven *spa* types (t030, t230, t037, t586, t1149, t2467 and t12925) were identified in the 10 VISA isolates (Table 4). The most prevalent *spa* type was t030 (50%). One new repeat sequences was found and it was assigned (t12925) (spaServer. ridom.de).

4. Discussion

In this study, 414 non-repetitive *S. aurous* isolates were analyzed to determine the prevalence of VISA isolates. The result showed that VISA clinical isolates of Tehran hospitals were relatively low (2.41%). The prevalence of VISA in previous study from Iran was a slightly higher (2.9%) than our results[11]. In Asia, prevalence of VISA in Turkey (2.4%) was similar to our data, but in China (0.5%), Japan (0.24%), and Korea (0.09%) were lower that of our result[17-20]. It also found that VISA strains increased from 2009 (one isolate) to 2012 (five isolates) (Table 5).

While the majority of detected VISA isolates were MRSA strains, in this study we showed that 2.06% of methicillin sensitive *S. aureus* (MSSA) isolates were VISA[14,21,22]. The high occurrence of VISA in MRSA strains was reported by Hu *et al*.[18] and sun *et al*.[23], whereas in this study, we showed that the occurrence of VISA among MRSA (2.72%) and MSSA (2.06%) strains were approximately the same. The occurrence of VISA strains among MRSA and MSSA isolates indicates a potential for development of vancomycin resistant *S. aurous* isolates and it is an important to pay an attention to detect of VISA in both MSSA and MRSA population.

Most VISA isolates in this study were identified from wound infection (four isolates), followed by trachea (three isolates), blood (two isolates) and drainage (one isolate). Therefore, diverse clinical specimens should be considered for isolating VISA strains.

Linezolid was fully active on VISA and all *S. aureus* in this study. Since the number of VISA isolates was low, we did not compare the rate of resistance between VISA strains and other isolates, but according to Table 3, VISA strains was found more resistant to multiple antibiotics, including erythromycin, tetracycline, clindamycin, rifampin and co-trimoxazole.

We used BHI-4V plate for screening VISA isolates. BHI-4V plate screening method was suggested by Riederer *et al*. as an alternative to PAP-AUC for detection of VISA isolates[10]. A total of 71 isolates were grown on BHI-4V plates in this study and by using E-test, we found only 10 isolates with vancomycin MIC between 4 and 8 μg/mL. The study was performed according to Chung *et al*. and 18 of

Table 5
Genetic characteristics of VISA isolates.

Isolates	Date (mo/day/yr) of isolation	*agr* group	*mecA*	*vanA* and *vanB*	SCC*mec*	*spa*[2] type	*spa* repeat
TMU 1	3/11/09	I	+	-	III	t030	15:12:16:02:24:24
TMU 2	6/15/10	II	-	-	-	t230	08:16:02:16:34
TMU 3	5/21/11	I	+	-	III	t037	15:12:16:02:25:17:24
TMU 4	7/17/11	II	-	-	-	t586	26:16
TMU 5	9/14/11	II	+	-	I	t2467	11:10:34:22:25:25
TMU 6	2/17/12	III	+	-	III	t030	15:12:16:02:24:24
TMU 7	2/19/12	II	+	-	III	t030	15:12:16:02:24:24
TMU 8	3/01/12	I	-	-	-	t030	15:12:16:02:24:24
TMU 9	3/28/12	I	+	-	III	t1149	08:16:34:24:34:17:17
TMU 10	4/17/12	II	-	-	-	t12925	26:23:13:23:31:31:29:17:25:17:25:28

The *spa* types were listed based on the Ridom SpaServer website.

the 33 isolates that were grew on BHI-4V were confirmed as hVISA via the PAP-AUC method and 15 were identified as VISA[20]. Hence, we suggest that it is an important to evaluate the results of BHI-4V via the PAP-AUC method for discriminating VISA and hVISA isolates. In this study, we confirmed VISA isolates via E-test.

The SCC*mec* typing revealed that most prevalent genotype among our VISA strains was SCC*mec* Type III and one isolate was SCC*mec* Type I. In the previous study from Iran, these SCC*mec* genotypes for VISA strains were also reported[11]. SCC*mec* Type III and SCC*mec* Type I are related to nosocomial infections. The majority of previous studies have indicated that VISA strains were more associated with SCC*mec* Type II, yet the study of Havaei *et al*.[12], and Hsueh *et al*.[24], from Taiwan showed that SCC*mec* Type III was predominant. The SCC*mec* Type III is the main SCC*mec* genotype in Iranian MRSA isolates[25-28]. Given this, the association between reduced vancomycin susceptibility and SCC*mec* genotype is more likely dependent to predominant SCC*mec* gene cassette types in each country. On the other hand, while *agr* Group II has been related with reduced vancomycin susceptibility, in the present study, 40% of VISA strains belonged to *agr* Group I. All VISA strains in the study of Hsueh *et al*. were *agr* Group I and these *agr* types are common in Iran and in Taiwan[24,29-32].

Molecular characterisation of all VISA isolates showed seven different *spa* types and four of them have been reported elsewhere; two of them (t586 and t2467) are new in Iran, and one of them is new allele (t12925). While six *spa* types were detected only once, the *spa* t030 was dominant and accounted for 40% of all our VISA isolates. The *spa* t030 was detected in 100% isolates during 2006–2008 in Ankara and also in Turkey, 70.3% of MRSA isolates during 2011 was t030[33]. The high prevalence (80.1%) of *spa* t030 was also reported in Chinese MRSA isolates in 2013.[34]. Three of four *spa* t030 strains in this study were SCC*mec* Type III. The *spa* t030 is also highly associated with the SCC*mec* Type III in China and Turkey. SCC*mec* Type III–*spa* t030 clone was the most common MRSA clone in Turkey during the 6 years of the study period and it is also represents a major public health problem in China[33,34]. As Ridom SpaServer, *spa* t030 is more frequently associated with ST 239 and ST 249, and related to CC8/239.

Vancomycin treatment failure was common in MRSA infections and was more pronounced in patients infected with VISA isolates[35,36]. A total of 10 patients with VISA infections, with a mean age of 52.7 years comprising 7 men and 3 women, were included in this study. The medical records regarding demographics, underlying diseases, history of exposure to antimicrobials and outcomes were shown in Table 3. One patient with VISA-MSSA bacteraemia after an initial failure ceftriaxone therapy was treated with rifampin. The other patient with VISA-MSSA central venous line infection after an initial failure vancomycin therapy were treated with gentamicin. Ventilator associated pneumonia with VISA-MRSA isolate was also treated with rifampin after an initial failure vancomycin therapy. Unfortunately, the outcome for seven other patients with VISA infection was hospital mortality due to underlying diseases and/or persistent infections and others factors (*i.e.*, age and immune status and so on).

In conclusion, both MSSA and MRSA isolates with reduced vancomycin susceptibility infections are associated with higher rates of treatment failure and mortality. Therefore, early recognition via MIC susceptibility testing by E-test or dilution methods will help to select antimicrobial therapy and the clinical management of *S. aureus* infections.

Conflict of interest statement

We declare that we have no conflict of interest.

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