Research Article

Genetic Characterization of Methicillin Resistant and Sensitive, Vancomycin Intermediate Staphylococcus aureus Strains Isolated from Different Iranian Hospitals

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Background. Global concerns have been raised due to upward trend of Vancomycin Intermediate Staphylococcus aureus (VISA) and Vancomycin Resistant Staphylococcus aureus (VRSA) reports which mean casting doubt on the absolute effectiveness of the last line of antibiotic treatment for Staphylococcus aureus, vancomycin. Hence, epidemiological evaluation can improve global health care policies.

Methodology. 171 isolates of Staphylococcus aureus were collected from different types of clinical samples in selected hospitals in Isfahan, Mashhad, and Tehran, Iran. Then, they were evaluated by agar screening, disk diffusion, and MIC method to determine their resistance to vancomycin and methicillin. The isolated VISA strains were then confirmed with genetic analysis by the evaluation of mecA and vanA genes, SCCmec, agr, and spa type, and also toxin profiles. MLST was also performed.

Results and Conclusion. Our data indicated that 67% of isolated Staphylococcus aureus strains were resistant to methicillin. Furthermore, five isolates (2.9%) had intermediate resistance to vancomycin (VISA). In contrast to usual association of VISA with MRSA strains, we found two isolates of MSSA-VISA. Therefore, our data suggests a probable parallel growing trend of VISA towards MSSA, along with MRSA strains. However, more samples are required to confirm these primarily data. Moreover, genetic analysis of the isolated VISA strains revealed that these strains are endemic Asian clones.

1. Introduction

Staphylococcus aureus is one of the major causes of hospital and community acquired infections that can vary from mild superficial lesions to acute deep and systemic infections. Increasing antimicrobial resistance in Staphylococcus aureus has led to major concerns about the future of antimicrobial therapy of its infections. Finding methicillin resistant Staphylococcus aureus (MRSA) strains, just two years after the clinical use of beta lactamase resistant betalactams, put specialists into trouble with treating these infections. About twenty years later in 1980s, alternative effective anti-Staphylococcal antibiotics such as vancomycin were introduced [1, 2]. Thence, for many years vancomycin was the drug of choice for MRSA infections, until the first Staphylococcus aureus isolate with reduced sensitivity to vancomycin (vancomycin intermediate Staphylococcus aureus (VISA)) was reported in Japan in 1997 [3]. Thereafter, several reports of VISA from USA [4], France [5], Brazil [6], Korea [7], and other parts of the world [8] were published and resulted in increasing concerns.
about the effectiveness of vancomycin therapy in serious Staphylococcal infections. Reduced sensitivity to vancomycin in Staphylococcus aureus occurs due to several genetic and phenotypic alterations in wild-type bacteria including altered expression of regulatory genetic elements, thickness of cell wall, changes in the penicillin binding protein (PBP) profiles, and decreased cell wall autolysis [9]. Since 2002, there have been also several reports of vancomycin resistant Staphylococcus aureus (VRSA) strains, mainly from the United States, India, and Iran [10–19]. The majority of the reported VISA isolates are MRSA strains with reduced susceptibility to vancomycin even though a few reports have also shown this phenomenon in methicillin sensitive Staphylococcus aureus (MSSA) isolates [9, 20–22]. Data obtained from genetic analysis of VISA isolates was rarely reported in Asian countries, particularly from Iran. Here, we reported genetic characteristics of five VISA strains, isolated from admitted patients in three teaching hospitals in Isfahan, Mashhad, and Tehran, Iran, in the autumn of 2011.

2. Methodology

171 isolates of Staphylococcus aureus were obtained from patients admitted between September 23 2011 and December 21, 2011 at selected hospitals in Isfahan, Mashhad, and Tehran, Iran.

These isolates were taken from blood, urine, sputum, wound, abscess, nose, throat, eye, and respiratory tract samples. S. aureus isolates were identified by conventional biochemical tests including gram staining, catalase, mannitol fermentation, slide, and tube coagulase test and DNase.

2.1. Screening for Methicillin and Vancomycin Resistance. All S. aureus isolates were screened for oxacillin and vancomycin resistance using agar screening method. The isolates that had grown in vancomycin agar screening media were retested with phenotypic tests to confirm the identification. It is noteworthy that the data obtained from spa typing, considered to be highly specific for S. aureus, was in align with phenotypic data and confirmed the isolated strains as S. aureus.

2.2. Antimicrobial Susceptibility Test. Antibiotic susceptibility test was carried out using disk diffusion method (MAST DISSKS) according to guidelines of Clinical Laboratory Standards Institute (CLSI) [23, 24]. The list of the antibiotics used in the test is presented in Table 2. Strain of S. aureus ATCC 25923 was used as control.

2.3. MIC Determination. Oxacillin and vancomycin agar dilution was used for all S. aureus isolates according to guidelines of Clinical Laboratory Standards Institute (CLSI). E-Test method (Biomerieux Strips) was used for redetermination of MIC results for S. aureus strains capable of growing in the vancomycin agar screening plate and/or having vancomycin MIC 4–8 μg/mL. Methicillin resistance was defined as the capability of growth in agar screening media including 4% NaCl + 6 μg/mL oxacillin and MIC ≥ 4 μg/mL and vancomycin intermediate resistance was defined as vancomycin MIC of 4–8 μg/mL [23]. Strains of Staphylococcus aureus ATCC 29213 and Enterococcus faecalis ATCC 52199 were used as controls.

2.4. Genomic DNA Extraction. Genomic DNAs of S. aureus isolates were extracted using QIAamp DNA minikit. According to manufacturer’s protocol for bacterial cells, we added lysostaphin at the final concentration of 30 μg/mL in lysis buffer.

2.5. PCR. PCR amplification was performed with a TAKARA Gradient PCR TP600 thermal cycler in a volume of 50 μL. We used EmeraldAmp MAX PCR Master Mix (Takara, Japan) for all PCR reactions.

(i) PCR Identification of mecA and vanA Genes. The primers used for amplification of mecA and vanA genes are listed in Table 3. PCR was performed with the following thermal setting: 5 min at 94°C for initial enzyme activation followed by 40 cycles of amplification (denaturation at 94°C for 30 sec for mecA and 1 min for vanA; annealing at 57°C for 45 sec for mecA and at 55°C for 1 min for vanA; extension at 72°C for 30 sec for mecA and 2 min for vanA) and final extension at 72°C for 5 min.

(ii) Multiplex PCR for Detection of Toxin Genes. The primers used for amplification of Panton-Valentine Leukocidin (pvl), Toxic Shock Syndrome Toxin-1 (tst1), alpha hemolysin (hla), and Enterotoxin C (sec) genes are listed in Table 3. PCR was performed with the following thermal setting: 5 min at 94°C for initial enzyme activation followed by 40 cycles of amplification (denaturation at 94°C for 40 sec; annealing at 60°C for 40 sec; extension at 72°C for 1 min) and final extension at 72°C for 5 min.

(iii) Multiplex PCR for SCCmec and agr Typing. SCCmec and agr typing were performed as previously described [25, 26]. The primers used for the PCR are listed in Table 3.

2.6. MLST. Multilocus Sequence Typing was carried out by PCR and sequencing of the internal fragments of arc, aro, glp, gmk, pta, tpi, and yqi genes of S. aureus as previously described [27].

2.7. spa Typing. Determination of spa type was performed by PCR and sequencing of polymorphic X region of spa gene was carried out as previously described [28].

2.8. Nucleotide Sequencing. Amplified PCR products were purified with QIAquick Gel Extraction Kit. The purified PCR products were sequenced with an ABI 3730XL DNA analyzer (Applied Biosystems) in both directions. The sequences were used for both confirmation and sequence-based typing methods (MLST and spa typing).

3. Results

Of 171 S. aureus isolates analyzed, we found that 115 isolates (67%) were MRSA and 5 isolates (2.9%) showed
Staphylococcal Cassette Chromosome, spa

vancomycin is considered as of the world. Resistance to vancomycin has led to global intermediate resistance to vancomycin. The demographic data and antibiogram results of these strains are listed in Table 2. Moreover, genetic evaluation results including mecA and vanA genes PCR, Sccmec types, agr groups, toxin profiles, spa types and Sequence Types (STs) are presented in Table 1.

### 4. Discussion

Isolation of VISA strains has been reported in many parts of the world. Resistance to vancomycin has led to global concerns owing to the fact that vancomycin is considered as the last effective drug of choice for Staphylococcal infections [3–8]. The isolation of heteroresistant VISA (hVISA) and VISA strains has been increasingly reported from different Asian countries with various prevalence rates [3, 7, 29, 30]. There are not many reports concerning the prevalence rate of VISA strains in Iran; however, in 2008, Saderi et al. reported the mean prevalence rate of 1.8% for VISA isolated from clinical samples based on evaluation of four teaching hospitals in Tehran [31]. In our study, we found five VISA strains (2.9%) amongst 171 S. aureus isolates. Our data shows an upward trend in the isolation of VISA strains in Iran that might be due to the high and uncontrolled vancomycin prescription rate, even though more research is needed to elucidate the matter.

Some reports have been published concerning the antimicrobial susceptibility patterns of VISA strains, isolated in various parts of the world [32, 33]. In most of these studies, the majority of the isolates were resistant to clindamycin but were susceptible to most of the other anti-Staphylococcal antibiotics [34–36]. Most of VISA strains in this study were also resistant to clindamycin. In contrast, Levofloxacin, Ciprofloxacin, Cotrimoxazole, and Gentamycin were effective on most of the VISA isolates (Table 2).

The majority of the previously reported VISA strains were resistant to methicillin; however, there have been also a few reports about methicillin sensitive Staphylococcus aureus-VISA (MSSA-VISA) strains [9]. Two isolates of our five VISA strains, both obtained from the same hospital in Mashhad, were susceptible to methicillin; however, one of them was isolated from throat sample of a hospitalized patient in internal ward and another was isolated from wound sample of an outpatient in urgent ward that had not had any history of hospitalization. With regard to genetic characteristics, the two strains were similar; for instance, both of them belonged to agr group II and sequence type 8 (ST8) and therefore, the relation between agr group, type of infection, geographical area, and resistance to some antibiotics, particularly vancomycin, is highlighted in some articles [37]. Impaired function of accessory gene regulator (agr) has been associated with the development of VISA strains [9]. agr I or II is associated with VISA strains isolated from diverse geographical regions [32, 33, 37, 38]. Our findings are in concordance with these reports. Both of our MSSA-VISA strains belonged to agr II but the other three MRSA-VISA strains had agr I.

All the patients with MRSA-VISA either had a previous history of hospitalization or were being hospitalized at the

| Table 1: Genetic characteristics of VISA isolates. |
|-----------------------------------------------|
| VISA1 | agr | Sccmec | pvl | hla | etc | tsI1 | mecA | vanA | spa | type | spa repeat profile | ST | CC | MLST allelic profile |
|-------|-----|--------|-----|-----|-----|-----|------|------|-----|-----|---------------------|----|----|---------------------|
| VISA2 |     |        |     |     |     |     |       |      |     |     | 15-12-16-02-15-27-17-24 | ST-1283 | 8   | 2-3-1-8-4-4-3       |
| VISA3 |     |        |     |     |     |     |       |      |     |     | 15-12-16-02-15-27-17-24 | ST-585 | 8   | 2-1-1-4-4-3       |
| VISA4 |     |        |     |     |     |     |       |      |     |     | 11-19-12-19-17-24-24 | ST-239 | 8   | 2-3-1-4-4-3       |
| VISA5 |     |        |     |     |     |     |       |      |     |     | 07-23-12-11-21-17-34 | ST-8  | 8   | 3-3-1-4-4-3       |

pvl: Panton-Valentin Leukocidin, sec: Enterotoxin C, tsI1: Toxic Shock Syndrome Toxin1, hla: alpha Hemolysin, agr: accessory gene regulator, Sccmec: Staphylococcal Cassette Chromosome, spa: Staphylococcal protein A.

| Table 2: Phenotypic characteristics of VISA isolates. |
|----------------------------------------------------|
| Gender/age | city | Sample/ward | Oxa | Van | Min | Lev | Cip | Tet | Cot | Gen | Cli | Rif | Oxa MIC | Van MIC | Van Agar screen |
|------------|------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------|---------|-----------------|
| VISA1 | M/15 | Mashhad | Blood/neurology | R | S | S | S | R | S | S | S | 128 | 8 | + |        |
| VISA2 | M/39 | Isfahan | Throat/out patient | R | S | R | R | R | R | R | R | 128 | 4 | + |        |
| VISA3 | M/50 | Isfahan | Blood/neurology | R | S | R | R | S | S | S | S | 64 | 4 | + |        |
| VISA4 | M/52 | Mashhad | Wound/urgent | S | R | S | S | S | S | S | S | 2 | 4 | – |        |
| VISA5 | M/71 | Mashhad | Throat/Internal | S | S | I | R | R | R | S | R | 2 | 4 | + |        |

Oxa: oxacillin, Van: vancomycin, Min: minocyclin, Lev: levofloxacin, Cip: ciprofloxacin, Tet: tetracycline, Cot: cotrimoxazole, Gen: gentamycin, Cli: clindamycin, Rif: rifampicin, R: resistant, S: susceptible.
Table 3: Primers used in this study.

| Target | Primer | Sequence (5′-3′) | Product size (bp) | Reference |
|--------|--------|-----------------|------------------|-----------|
| mecA   | F      | AGAAGATGGTATGTTGGAAGTTAG | 583             | This study |
|        | R      | ATGTATGGCGATTGATATTGC |                 |           |
| pvl    | F      | GGAACATTTTACCTGGCTATAC | 502             | This study |
|        | R      | CTGGATGTAATTACCTCTTGG |                 |           |
| hla    | F      | CGTGACTACAGATAATTGGAAAGGC | 744             | This study |
|        | R      | TGGTAATCATACGAACTGCTG |                 |           |
| sec    | F      | GGAATGTGGTAGAAAGG | 900             | This study |
|        | R      | AGGCAAGCACGAGTAC |                 |           |
| tst1   | F      | TTATCGTAAAGCCCTTGTGG | 398             | This study |
|        | R      | TAAAGGTAATTTCTTGGTAGG |                 |           |
| agr    | Pan F  | ATGCACATGGTGCAATGC | 439             | [26]      |
|        | R I    | GTCAACAGACTATAAAGCTGGAT | 572             |           |
|        | R II   | GATTCTAATTGAAAAAGTGCCATAGC | 406           |           |
|        | R III  | CTTGAAAAGTCACTAAAAGCTC | 657             |           |
|        | R IV   | CAGTAATGGCGATAATACCCG |                 |           |
| SCCmec | F β    | ATCGCCTTGATAATAGCCTCT | 937             |           |
|        | R α3   | TAAAGGATCAATGCACACACT |                 |           |
|        | F ccrC | CGTCTATTACAAAGTGTAAAGGATAAT | 518     |           |
|        | R ccrC | CCTTTATAGACTTGATTTCAAATTAT |          |           |
|        | F 1272 | GCCACTCATAACATATGGGA | 415             | [25]      |
|        | R 1272 | CATCCGGATGAAACCCAAA |                 |           |
|        | F 5RmecA | TATACCCAAACCGACAACTAC | 359             |           |
|        | R 5R431 | CGGCTACAGTGATAACATCC |                 |           |

time of sampling. Moreover, their SCCmec types belonged to types I and III. Collectively, the data indicates that the isolated MRSA-VISA strains were hospital-acquired MRSA (HA-MRSA). VISA1 strain was positive for all evaluated toxin genes including pvl, hla, sec, and tst1 which can increase its potential pathogenicity; however, this isolate was susceptible to most of the tested antibiotic disks (Table 2). Interestingly, pvl gene was found in HA-MRSA “VISA1” despite the fact that it is mostly found in CA-MRSA strains. VISA 2, 3 did not have any major toxin genes.

All of our MRSA-VISA isolates (two isolates from Isfahan in center of Iran and one isolate from Mashhad in northeast of Iran) had spa t037 and their STs were ST1283, ST585, and ST239 for VISA 1, 2, and 3, respectively. ST1283 and ST585 are single-locus variants (SLV) of ST239, the endemic strain of Asia (Figure 1) which our data suggest to be the probable common ancestor of our MRSA-VISA isolates. All of our five VISA isolates belonged to clonal complex 8 (CC8) that is the major endemic S. aureus clone (particularly MRSA) in Asia and also is one of the pandemic MRSA clones [33, 39–44].

Increasing antibiotic resistance in major S. aureus clones intensifies precautionary policies for public health care systems. In this study, we have shown the upward trend of MSSA- and MRSA-VISA clones in Iran. Moreover, findings of our study suggest that the isolated VISA strains most probably belong to endemic Asian clones. In addition, these data suggest that vancomycin resistance has the potential to become a widespread problem in both MRSA and MSSA strains.

Conflict of Interests
S. A. Havaei, A. Azimian, H. Fazeli, M. Naderi, K. Ghazvini, S. M. Samiee, and Z. Masoumi have no conflict of interests.

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