Genetic Characterization of a Vancomycin-Resistant *Staphylococcus aureus* Isolate from the Respiratory Tract of a Patient in a University Hospital in Northeastern Iran

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Emergence of vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) strains has led to global concerns about treatments for staphylococcal infections. These strains are currently rare even though there is an upward trend in their reported incidence. Therefore, appropriate screening and epidemiological evaluation of VRSA strains can affect future global health care policies. Isolates of *Staphylococcus aureus* were obtained from various clinical samples and were then evaluated with agar screening, disk diffusion, and MIC methods to determine resistance to vancomycin and methicillin. After confirmation of the isolated VRSA strain, genetic analysis was performed by evaluating *mecA* and *vanA* gene presence, SCCmec, agr, and spa types, and toxin profiles. Multilocus sequence typing (MLST) and plasmid analysis were also performed. The VRSA strain was resistant to oxacillin (MIC of 128 µg/ml) and vancomycin (MIC of 512 µg/ml). Disk diffusion antimicrobial susceptibility tests showed resistance to oxacillin, vancomycin, levofloxacin, ciprofloxacin, trimethoprim-sulfamethoxazole, clindamycin, rifampin, and tetracycline. The isolate was susceptible to minocycline and gentamicin. PCRs were positive for the *mecA* and *vanA* genes. Other genetic characteristics include SCCmec type III, agr I, *spa* type 1037, and sequence type (ST) 1283. The plasmid profile shows five plasmids with a size of ~1.7 kb to >10 kb. The isolated VRSA strain was obtained from a critically ill hospitalized patient. Genetic analysis of this strain suggested that the strain was a methicillin-resistant *S. aureus* (MRSA) clone endemic in Asia that underwent some genetic changes, such as mutation in the *gmk* gene and acquisition of the *vanA* gene.

Treatment of *Staphylococcus aureus* infections has become more complicated with emergence of a methicillin-resistant *Staphylococcus aureus* (MRSA) strain in 1961. Since the 1980s, vancomycin has become the drug of choice for the treatment of serious MRSA infections (28,31). The first vancomycin-resistant *Enterococcus faecalis* (VRE) strain was reported in France in 1988 and caused great concern about the transmission of mobile genetic elements containing the *vanA* gene to *S. aureus* (5,20,39). After a publication in 1992 reporting successful transmission of the *van* element from *Enterococcus faecalis* to an MRSA strain in laboratory mice during their coinfection, concerns about this transfer in nature were increased (23). The first vancomycin-intermediate *Staphylococcus aureus* (VISA) strain was isolated from a 4-month-old newborn in Japan in 1997 (19). Afterwards, several reports concerning VISA isolation were published in the United States (37), France (27), Brazil (24), South Korea (21), and other countries (16).

The thickened and poorly cross-linked cell wall layer of a VISA strain presents increased amounts of D-Ala-D-Ala building blocks of cell wall as a binding site for vancomycin, resulting in diminished effects of vancomycin due to competition. A high level of resistance to vancomycin occurs due to the function of the *van* gene complex. The product of the *vanA* gene is a ligase that produces D-Ala-D-lactate (Lac), a substitution for D-Ala-D-Ala which is a building block for peptidoglycan synthesis, with much less affinity to glycopeptides such as vancomycin and teicoplanin. The *vanA* gene is not found in any VISA strain (26,28); however, in 2002, the first vancomycin-resistant *Staphylococcus aureus* (VRSA) strain was reported in Michigan (8), and in the same year, a second VRSA strain was found in Pennsylvania (6). Subsequently, several other cases of VRSA were reported in the United States (6,7,38), India (29,35,36), and Iran (1,12). Here, we genetically characterized a VRSA strain isolated from a respiratory tract sample of a patient hospitalized in Emam Reza Hospital, Mashhad, Iran.

**CASE REPORT**

*Staphylococcus aureus* was isolated from a bronchial aspirate of a 26-year-old man who had a history of Crohn’s disease and had been hospitalized for 3 months to treat an ileal perforation and peritonitis. During the course of his hospitalization, he underwent three abdominal operations. He received hydrocortisone, ciprofloxacin, metronidazole, and ketoconazole during this period. Hydrocortisone was prescribed for the reduction of inflammation in abdominal mucosal tissue caused by Crohn’s disease. The other

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three antibiotics were prescribed to prevent peritonitis and sepsis. Three months after hospitalization, symptoms of respiratory distress appeared, and S. aureus was isolated from tracheal wash samples. The laboratory of the hospital primarily reported the isolate as a VISA strain based on the disk diffusion method, and therefore vancomycin was then prescribed as an S. aureus medication; one month later the patient was expired. Our subsequent analysis of the S. aureus isolate for the purpose of molecular epidemiology showed that it was a VRSA strain. Notably, a blood culture obtained 2 days before the patient’s death was positive for Acinetobacter baumannii. Based on the report from the hospital, the isolated Acinetobacter baumannii was a multidrug-resistant strain.

MATERIALS AND METHODS
Strains of Staphylococcus aureus were isolated from patients admitted between 23 September 2011 and 21 December 2011 at Emam Reza Hospital, a 918-bed university teaching hospital in Mashhad, Iran. These isolates were obtained from blood, urine, sputum, wound, abscess, nose, throat, eye, and respiratory tract samples. S. aureus isolates were identified by conventional biochemical methods including Gram staining, tests for catalase, mannitol fermentation, and DNase, and slide and tube coagulase tests.

Screening for methicillin and vancomycin resistance. All S. aureus isolates were screened for oxacillin and vancomycin resistance using the agar screening method. The isolates that had grown in vancomycin agar screening medium were retested with phenotypic tests to confirm the identifications. It is noteworthy that the data obtained from spa typing, considered to be highly specific for S. aureus, were aligned with phenotypic data and confirmed the isolated strain as S. aureus.

Antimicrobial susceptibility tests. Antibiotic susceptibility testing was carried out using the disk diffusion method (Mast disks) according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (10, 11). The following antibiotics were used: oxacillin, vancomycin, minocycline, levofloxacin, ciprofloxacin, tetracycline, cotrimoxazole, gentamicin, clindamycin, and rifampin. S. aureus ATCC 25923 was used as a control.

MIC determination. The Etest method (bioMerieux strips) was used for the MIC determination for the only S. aureus strain capable of growing in the agar screening medium. Methicillin resistance was defined as a MIC of ≥4 µg/ml for a strain with the ability to grow in agar screening medium containing 4% NaCl plus 6 µg/ml oxacillin, whereas vancomycin resistance was defined as a MIC of ≥16 µg/ml for a strain with the ability to grow in agar screening medium supplemented with 6 µg/ml vancomycin (6). Staphylococcus aureus ATCC 29213 and Enterococcus faecalis ATCC 52199 strains were used as controls.

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

PCR. PCR amplification was performed with a TaKaRa Gradient PCR TP600 thermal cycler in a volume of 50 µl. We used an EmeraldAmp Max PCR Master Mix (TaKaRa, Japan) for all PCRs.

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

(ii) Multiplex PCR for detection of toxin genes. The primers used for amplification of the Panton-Valentine leukocidin (pvl), toxic shock syndrome toxin 1 (tst1), alpha-hemolysin (hla), and enterotoxin C (sec) genes are listed in Table 1. PCR was performed with the following thermal settings: 5 min at 94°C for initial enzyme activation followed by 40 cycles of amplification consisting of denaturation at 94°C for 40 s, annealing at

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<th>Target</th>
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<th>Product size (bp)</th>
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Table 1: Oligonucleotide primers used in this study
Antimicrobial susceptibility test results were as follows: the strain was resistant to oxacillin (MIC of 128 μg/ml), vancomycin (MIC of 512 μg/ml), levofloxacin, ciprofloxacin, tetracycline, cotrimoxazole, clindamycin, and rifampin; it was susceptible to minocycline, and gentamicin. Moreover, genetic evaluation results for this strain produced the following profile: positive for the mecA, vanA, sec, and hla genes and negative for pvl and tst1; agr group I; SCCmec type III; ST1283; spa type t037.

The sequenced vanA PCR product (Fig. 2) of the isolated VRSA strain showed very high similarity to the vanA gene in Tn1546. The plasmid profile of the isolated strain revealed five plasmids, including one large plasmid of >10 kb and four small plasmids of ~7, ~5, ~3, and ~1.7 kb. The vanA gene was located on the >10-kb plasmid.

Subsequent enzymatic digestion with HindIII showed seven bands ranging from ~1 to >10 kb (Fig. 3).

**DISCUSSION**

We found a VRSA strain in a patient who had underlying conditions including immunosuppressive therapy, long-time hospitalization, and serious disease of the gastrointestinal tract that resulted in several surgeries. He finally died after respiratory distress. The patient conditions were in agreement with previous findings about VRSA infections (14, 33). The isolated strain had a vancomycin MIC of 512 μg/ml, consistent with other reported VRSA strains that had vancomycin MICs in the range of 32 to 1,024 μg/ml (2). This high level of resistance may be due to selective pressure caused by continuous use of vancomycin in Iranian hospitals for any S. aureus infection, and, therefore, the development of VISA and VRSA strains is a probable outcome. Périchon and colleagues noted that vanA-type resistance is inducible by glycopeptides (26).

In contrast to previously reported VRSAs isolates that were susceptible to many of the antibiotics in the disk diffusion method (4, 6, 7, 8), this strain was resistant to most of the tested antibiotics except minocycline and gentamicin. Most previously isolated VRSA strains in the United States belong to sequence type 5 (ST5); isolates within this lineage are the most common health-care-associated MRSA strains (3, 40). Our isolate belongs to ST1283.
that is a single locus variant of ST239, differing from its sequence type by a single point mutation in position 483 of the guanylate kinase gene (gmk) with the replacement of thymine by guanine (T → G). ST1283 was reported for the first time in Malaysia in 2008 (17). In fact, other genetic characteristics of our isolate are similar to those of its Malaysian counterpart, including spa type t037, agr I, and SCCmec type III, suggesting that the Iranian isolate has a strong relationship with the MRSA ST1283 Malaysian strain. Based on information from the patient’s family concerning the history of his travel to Malaysia, we found that neither he nor his family traveled to Malaysia or any other Asian country. It should be noted that due to the small difference between ST1283 and ST239 (an endemic clone in most Asian countries except South Korea and Japan) and also other genetic similarities of ST239 with ST1283, including the t037, agr I, and SCCmec III/IIIa profile (9, 22, 30), we should not rule out the possibility of a mutation in the gmk gene of endemic ST239 that results in the generation of ST1283 in Iran rather than its entrance from Malaysia. Moreover, considering the patient’s conditions, such as a history of several operations, immunosuppressive therapy, and long-time hospitalization, and the SCCmec type III of the isolated VRSA strain, his infection can be classified as a hospital-acquired infection that could have been obtained from health care workers, other patients, or the hospital setting.

Regrettfully, we do not have enough information about the other patients hospitalized in the same ward as our patient. Evaluation of toxin genes including pvl, hla, tst1, and sec with PCR revealed that this strain is positive for hla and sec and negative for tst1 and pvl. This finding is consistent with most other hospital-acquired MRSA (HA-MRSA) strains that are negative for the pvl gene. This toxin is related to SCCmec types IV and V which are representative for community-acquired MRSA (CA-MRSA) (32). In most previous cases of VRSA isolation, VRE strains were also isolated (26, 40). Members of the Enterococcus species, especially E. faecalis, can act as a donor of the carrier plasmid for Tn1546 and subsequent transfer of the van gene complex to a receiver S. aureus strain (23, 26). In our case, all cultures from different samples of the patient, including blood, feces, surgical wound, abdominal abscess discharge, and respiratory tract samples, during the approximately 3 months of his hospitalization were negative for E. faecalis.

To determine the approximate origin of the van gene in this strain, we examined the plasmid content and restriction profile of the extracted plasmids using HindIII restriction endonuclease (Fig. 3). As our results show, the plasmid profile of our strain is almost similar to the one of a VRSA strain isolated in south Asia that has five plasmids with the sizes of 5.1, 5.5, 5.6, 6, and 5–1.1, and 1.5 kb (29). The VRSA strain that was isolated in Pennsylvania has a completely different plasmid profile and contains two plasmids of 4 and 120 kb, and the latter of these is uncommon for S. aureus. This large plasmid could be either an enterococcal plasmid or a cointegrate of enterococcal and staphylococcal plasmids (34). So far, other isolated VRSA strains show relatively high degrees of plasmid profile differences in comparison to our strain (14, 15, 29, 40). By comparing the restriction profile of our plasmids with other reported profiles, we suppose that our profile has more similarity with staphylococcal plasmid profiles than with enterococcal ones even though further experiments are required to elucidate the matter (14, 15, 29, 40).

The findings of this study can be an advance warning about the dissemination of VRSA strains in various parts of the world. To date, most of the reports have been confined to the United States, with few from other parts of the world. Further studies are necessary to elucidate the prevalence, potential sources, and transmission routes of VRSA. Also, health officials in developing countries should be warned about uncontrolled antibiotic prescription and should adhere to the principles of infection control in their health care systems.

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