Antibacterial Susceptibility of Three Vancomycin-Resistant Staphylococcus aureus Strain Isolated from Northern Part of Iran

Masumeh Anvari¹*, Najmeh Ranji¹ and Fatemeh Khoshmaslak¹

¹Department of Microbiology, Faculty of Sciences, Islamic Azad University, Rasht Branch, P.O. Box 41335-3516, Rasht, Iran.
²Young Researchers Club, Islamic Azad University, Rasht Branch, Rasht, Iran.

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The emergence of vancomycin-resistant Staphylococcus aureus (VRSA) in various parts of the world has been of great concern in clinical settings. This study was carried out to isolate pathogenic S. aureus from wounds pus sample, and VRSA was identified by evaluation of resistance patterns using conventional antibiotics and vancomycin by PCR. A total 54 S. aureus were isolated from samples. All S. aureus were subjected to MIC testing (against vancomycin), Brain Heart Infusion (BHI) vancomycin screen agar test, disc diffusion testing. VRSA were confirmed by PCR amplification of the vanA and vanB genes. From this study, it was observed that isolated S. aureus strains are pathogenic, Using the disk diffusion test, most isolates (89.3%) were resistant to penicillin while the lowest resistance (7.9%) was to cephalothin. Three of the 54 isolates had a vancomycin MIC of ≥ 128 by MIC testing. All three VRSA isolates were also highly resistant to other examined antibiotics. The present study reveals for the first time emergence of VRSA from this part of Iran and indicates the antibiotic resistance in the study area.

Key Words: Antibiotic, Vancomycin-resistant Staphylococcus aureus, vanA and vanB gene.

Serious infections caused by Staphylococcus aureus are a worldwide phenomenon and occur in both the hospital and community settings ¹. In the 1980s, due to the widespread occurrence of methicillin-resistant Staphylococcus aureus (MRSA), empiric therapy for staphylococcal infections (particularly nosocomial sepsis) was changed to vancomycin in many health-care institutions and vancomycin continues to be used as a first-line antimicrobial agent for the treatment of infection with methicillin-resistant Staphylococcus aureus (MRSA) ²-³.

In 1997, the first clinical isolate of S. aureus with reduced susceptibility to vancomycin was reported from Japan ⁴. The vancomycin minimum inhibitory concentration (MIC) result reported for this isolate was in the intermediate range (8 µg/mL) using interpretive criteria defined by the National Committee for Clinical Laboratory Standards (NCCLS) ⁵.

Shortly after, two additional cases were reported from United States however, first clinical isolate of vancomycin resistant S. aureus (VRSA) was reported from United States in 2002 ⁶⁻⁷. More recently some workers have reported vancomycin resistant staphylococcal stains from different part of the world ⁸⁻¹².

* To whom all correspondence should be addressed.
E-mail: manvari1344@gmail.com
The purpose of the present study was to observe antibiotic emergence pattern of isolated *S. aureus* strains against some conventional and traditional antibiotics to identify VRSA in the Guilan (northern of Iran) for the first time.

**MATERIALS AND METHODS**

**Collection and transport of sample**

Two hundred thirty four (234) wounds pus sample were collected from patients admitted to Burn and Wound section of the most important governmental Hospital, Rasht Iran, during a three month period from January 15, 2011 to March 15, 2011. Samples were obtained using cotton tipped swabs from the pus of deep-seated wounds of patients. Swabs were transported to the laboratory in autoclaved Luria broth (LB) within 30 minutes of collection.

**Species Identification**

Identification of the clinical isolates was performed by traditional biochemical tests, including Gram staining, oxidase, catalase, coagulase, latex agglutination, motility, thermonuclease (DNase), haemolysis and mannitol fermentation tests. Antibiotic susceptibility tests were performed in a single microbiology laboratory at Islamic Azad University (Rasht – Iran).

Vancomycin was obtained from Sigma (USA, potency 1000 µg/mg) for the determination of MIC of 54 strains with the agar dilution method according to the procedure outlined by NCCLS. Vancomycin was incorporated into Mueller-Hinton agar in a Log 2 dilution series from 0.125 to 256 µg/mL.

**Disc Agar Diffusion (DAD) test**

Susceptibility of isolates to penicillin G (Pen), Oxacillin (Oxa), gentamycin (Gen), tobramycin (Tob), erythromycin (Ery), tetracycline (Tet), clindamycin (Cli), cephalotin (Cep) and vancomycin (Van) was determined by the disc agar diffusion (DAD) technique according to NCCLS.

**Growth on BHI vancomycin screen agar**

Isolates were inoculated on Brain Heart Infusion screen agar according to Subhankari Prasad Chakraborty, Santanu KarMahapatra. 6 µg/ml vancomycin containing BHI agar screen plates were prepared. Inoculums suspensions were prepared by selecting colonies from overnight growth on nutrient agar plates. The colonies were transferred to sterile saline to produce a suspension that matches the turbidity of a 0.5 McFarland standard. The final inoculum concentration of 10⁵ to 10⁶ CFU per spot was prepared by adding the sterile saline to the bacterial suspension. These suspensions were inoculated onto BHI screen agar plates and were incubated for 24 hr at 35°C in ambient air. Any visible growth indicated the vancomycin resistance. *S. aureus* ATCC 29213 and *E. faecalis* ATCC 51299 were used as vancomycin susceptible control strains and vancomycin resistant control strain, respectively.

**PCR detection**

**Plasmid DNA isolation**

Cells from *S. aureus* were lysed, and plasmid DNA was isolated as described by Tcnover et al. *S. aureus* cells were harvested from mid-log-phase cultures grown in BHI broth with 10 mg of vancomycin per ml. Cell pellets were suspended in lysis buffer containing 50 mg of lysostaphin per ml and incubated at 37°C for 30 min before beginning the standard protocol.

**Detection of van A and van B gene by PCR**

Oligonucleotide primers for *vanA* (*vanA* F 5’ CATGAATAGAATAA AGTTGCAATAA AGTGGCAATAA and *vanA* R 5’ CCCCCTTAACG CTAATACGAC GATCAA3’) and *vanB* (*vanB* F 5’ GTGACAAAC CGGAGGCGAGGAC and *vanB* R 5’ CGGCCATCC TCCTGCAAATAA 3’) genes previously reported were used.

A Bioneer DNA thermocycler was programmed with the initial denaturation, 4 min at 94°C, 30 cycles with a 45-s denaturation step at 94°C, a 45-s annealing step at 56°C and a 30-s extension step at 72°C and 2 min extension step at 72°C and a holding step at 4°C until the sample was analyzed. The PCR products were electrophoresed, stained with 10 µM ethidium bromide and visualized by using UV transillumination.

**RESULTS**

**Bacterial identification**

The results showed that 61.15% isolates were of *S. aureus*. All isolates are oxidase positive, catalase positive and coagulase positive and were non-motile and gave positivity in latex agglutination test, 100% of these had thermonuclease activity,
Table 1. MICs if vancomycin for 54 isolates of Staphylococcus aureus

<table>
<thead>
<tr>
<th>MIC (µg/ml)</th>
<th>No. of strains</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125</td>
<td>1</td>
<td>1.85</td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>14</td>
<td>25.92</td>
</tr>
<tr>
<td>1.0</td>
<td>4</td>
<td>7.40</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>59.25</td>
</tr>
<tr>
<td>4-64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>128</td>
<td>1</td>
<td>1.85</td>
</tr>
<tr>
<td>≥256</td>
<td>2</td>
<td>3.70</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Resistance pattern of vancomycin resistant staphylococcal isolates to commonly tested antimicrobial agents as determined by disc diffusion method

<table>
<thead>
<tr>
<th>Strains</th>
<th>Resistant/Intermediate</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus (MIC van 128 µg/ml)</td>
<td>Pen, Oxa, Gen, Tob, Ery, Cli, Van</td>
<td>Cep, Tet</td>
</tr>
<tr>
<td>S. aureus (MIC van 256 µg/ml)</td>
<td>Pen, Oxa, Gen, Tob, Ery, Van, Cep, Tet</td>
<td>Cli</td>
</tr>
<tr>
<td>S. aureus (MIC van 256 µg/ml)</td>
<td>Pen, Oxa, Gen, Tob, Ery, Cli, Van, Cep, Tet</td>
<td></td>
</tr>
</tbody>
</table>

Generally, antibiotics can be detected using the agar diffusion method. The results of the disk diffusion test using 8 antibiotics for 54 isolates of Staphylococcus aureus are shown in Fig. 1. Most isolates (89.3%) were resistant to penicillin while the lowest resistance was seen with cephalothin (7.9%). Minimum inhibitory concentration of vancomycin for 54 strains is shown in Table 1. NCCLS guidelines define staphylococci for which the MIC of vancomycin is ≤4 µg/mL to be susceptible, while isolates for which the MIC is 8 to 16 µg/mL are intermediate and those for which the MIC is ≥32 µg/mL are resistant. Of the 54 Staphylococcus aureus isolates, 51 (94.44%) were considered susceptible and 3 (5.55%) resistant to vancomycin.

These 3 strains had also vancomycin MIC ≥ 128 µg/mL by agar diffusion method, therefore they were considered vancomycin-resistant Staphylococcus aureus strains. All isolated VRSA were resistant to oxacillin in the disk diffusion test. These strains were also resistant to 8 examined antibiotics in the disk diffusion method.

Confirmation of VRSA by detection of vanA and vanB gene by PCR

PCR amplification of the vanA and vanB gene using the gene-specific primers and the plasmid DNA preparation of clinical isolates that was suspected to be VRSA yielded 474 bp and 800 bp amplicon respectively (data not shown).

Fig. 1. Disk diffusion test for 54 isolated of Staphylococcus aureus
DISCUSSION

The appearance of antibiotic resistant bacteria over the past decades has been regarded as an inevitable genetic response to the strong selective pressure imposed by antimicrobial chemotherapy, which plays a crucial role in the evolution of antibiotic resistant bacteria. These bacteria then pass the antibiotic resistance plasmid among other bacterial cells and species. The emergence of the glycopeptide resistance is of great concern. Since first being reported in 1997, the threat of vancomycin resistance in Staphylococcus aureus has been the topic of intensive research and discussion. Although vancomycin resistance in S. aureus remains extremely rare, there is widespread concern that vancomycin-resistant S. aureus poses, by far, the greatest risk to patients, given the virulence of the organism.

We investigated the three documented infections with S. aureus with resistance to glycopeptides in the Guilan (northern of Iran), for the first time and the second one in Iran. These VRSA strains have been isolated from the pus of three different patients who were admitted in the different wards. One VRSA (vancomycin MIC 128 µg/ml) strain was isolated from pus of a 25 years old patient and the two others VRSA (vancomycin MIC 256 µg/ml) strains were isolated from pus of a 79, 60 years old patients. These isolates were also found to be resistant to several other antimicrobials such as gentamicin, tobramycin, chloramphenicol and clindamycin (Table 2). While going through the detailed history of these patients it was found that two of them were already treated with glycopeptides for more than 17 days. Although Linezolid and quinupristin/dalfopristin were recently approved by the Food and Drug Administration and are antimicrobials with activity against glycopeptide resistant Gram positive microorganisms such as VRSA we did not examine these because they are not available in Iran.

vanA and vanB genes are highly specific for vancomycin resistant S. aureus. PCR amplification of vanA and vanB gene of the three suspected clinically isolated VRSA strains using gene specific primer and plasmid DNA preparation yielded 474 bp and 800 bp amplicon respectively. These results confirmed that these suspected clinically isolated VRSA strains were truly VRSA.

Widespread use of vancomycin to treat infections caused by MRSA and other gram-positive cocci has led to the emergence of vancomycin resistance. The large scale of development and subsequent spread of resistance to vancomycin has been perceived as a fearsome threat to the already challenging therapy of MRSA.

A strict regulation on irrational antibiotic usages might be an appropriate and effective approach in this direction. Moreover, nationwide surveillance program should be carried out to map the vancomycin susceptibility pattern in this country.

REFERENCES
8. Jones, R.N. Microbiological features of vancomycin in the 21st century: minimum inhibitory concentration creep, bactericidal/static activity, and applied breakpoints to predict