

Antibacterial Susceptibility of Three Vancomycin-Resistant *Staphylococcus aureus* Strain Isolated from Northern Part of 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 settings¹. 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 Japan⁴. The vancomycin minimum inhibitory concentration (MIC) result reported for this isolate was in the intermediate range (8 $\mu\text{g/mL}$) 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 2002⁶⁻⁷. More recently some workers have reported vancomycin resistant staphylococcal stains from different part of the world⁸⁻¹².

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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 tests¹³⁻²⁰. 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 NCCLS²¹.

Growth on BHI vancomycin screen agar

Isolates were inoculated on Brain Heart Infusion screen agar according to Subhankari Prasad Chakraborty, Santanu KarMahapatra²². 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 Tcover et al.²³. *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'CATGAATAGAATAAA AGTTGCAATA3' and *vanA* R 5'CCCCTTTAACG CTAATACGAC GATCAA3') and *vanB* (*vanB* F 5'GTGACAAAC CGGAGGCGAGGA3' and *vanB* R5'CCGCCATCC TCCTGCAAAAAA 3') genes previously reported were used²⁴.

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 transillumination²⁵.

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 of vancomycin for 54 isolates of *Staphylococcus aureus*

MIC ($\mu\text{g/ml}$)	No. of strains	Percentage
0.125	1	1.85
0.25	0	0
0.5	14	25.92
1.0	4	7.40
2	32	59.25
4-64	0	0
128	1	1.85
≥ 256	2	3.70
Total	54	100

mannitol fermentation activity, haemolytic activity.
Antibiotic susceptibility testing

The results of the disk diffusion test using 8 antibiotics for 54 isolates of *S. 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 $\leq 4 \mu\text{g/mL}$ to be susceptible, while isolates for which the MIC is 8 to $16 \mu\text{g/mL}$ are intermediate and those for which the MIC is ≥ 32

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

Strains	Resistant/Intermediate	Sensitive
<i>S. aureus</i> (MIC van 128 $\mu\text{g/ml}$)	Pen, Oxa, Gen, Tob, Ery, Cli, Van	Cep, Tet
<i>S. aureus</i> (MIC van 256 $\mu\text{g/ml}$)	Pen, Oxa, Gen, Tob, Ery, Van, Cep, Tet	Cli
<i>S. aureus</i> (MIC van 256 $\mu\text{g/ml}$)	Pen, Oxa, Gen, Tob, Ery, Cli, Van, Cep, Tet	

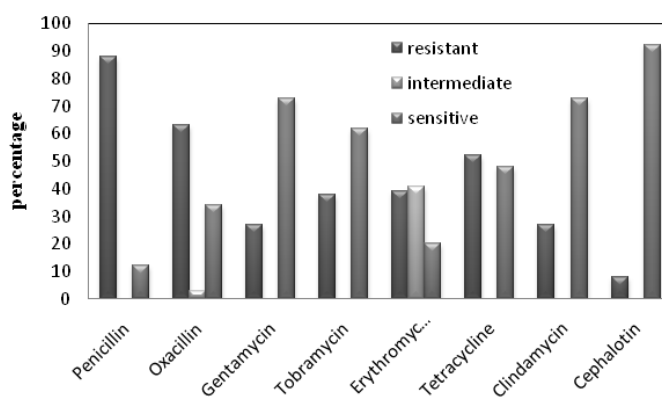
$\mu\text{g/mL}$ are resistant. 3 Of the 54 *S. aureus* isolates, 51 (94.44%) were considered susceptible and 3 (5.55%) resistant to vancomycin.

These 3 strains had also vancomycin MIC $\geq 128 \mu\text{g/mL}$ by agar diffusion method, therefore they were considered vancomycin-resistant *S. 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 species²⁵. The emergence of the glycopeptide resistance is of great concern. Since first being reported in 1997, the threat of vancomycin resistance in *S. 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 organism²⁶.

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 glycopeptideresistant Gram positive microorganisms such as VRSA we did not examine these because they are not available in Iran²⁷⁻²⁸.

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 MRSA²⁹.

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 country³⁰.

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