The prevalence of \textit{vanA} gene in clinical isolates of vancomycin-resistant \textit{Staphylococcus aureus} in a hospital in Mazandaran, Iran

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ABSTRACT

\textit{Staphylococcus aureus} is one of the most important causes of infections in hospitals. Although vancomycin is often prescribed for the treatment of methicillin-resistant \textit{S. aureus} (MRSA), vancomycin-resistant \textit{S. aureus} (VRSA) isolates remain a major problem in hospitals. In this study, we investigated the prevalence of vancomycin-resistant \textit{S. aureus} isolates and also \textit{vanA} gene in these clinical isolates in a hospital in Mazandaran Province, Iran. In this cross-sectional study, a total number of 60 \textit{S. aureus} samples were identified from different clinical specimens after standard biochemical and microbiological tests. Disk agar diffusion test using Kirby-Bauer method was applied for antibiotics against \textit{S. aureus} isolates. The presence of \textit{vanA} gene was investigated in VRSA and intermediate resistance (VISA) isolates by PCR method using specific primers. Over 40\% of isolates were resistant to commonly-used antibiotics, including gentamicin (46.67\%), ceftazidime (45\%) and carbenicillin (43.34\%). Only few, however, were sensitive to gentamicin (33.33\%) and ceftazidime (35\%). Vancomycin was the most effective antibiotic against \textit{S. aureus} isolates (56.66\% sensitivity). Eleven isolates (18.34\%) were resistant (VRSA) and 15 isolates (25\%) were intermediate resistance (VISA) to vancomycin. Molecular analysis of \textit{vanA} gene in 11 VRSA and 15 VISA showed that 8 VRSA (72.72\%) and 6 VISA (40\%) isolates were positive for \textit{vanA} gene. The incidence of VRSA and VISA strains, as well as the frequency of \textit{vanA} gene in these isolates are high and emerging in Mazandaran hospitals. There is a need to keep the emergence and spread of these strains to a minimum level.

Key words: Clinical isolates, Iran, \textit{Staphylococcus aureus}, \textit{vanA} gene, vancomycin-resistant.

INTRODUCTION

\textit{Staphylococcus aureus} is one of the most important human pathogenic bacteria which causes various complications such as skin and tissues infections, surgical wound infections, bacteremia, endocarditis, and pneumonia (Hoban \textit{et al}. 2003, Roberts & Chambers 2005). Although several antibiotics such as penicillin and methicillin were introduced during the last decades for the treatment of \textit{S. aureus} infections, these isolates became rapidly resistant to these antibiotics (Wenzel \textit{et al}. 1991). Methicillin-resistant \textit{S. aureus} (MRSA) isolates can be also acquired during exposure to hospitals and causes serious healthcare-associated infections (Loomba \textit{et al}. 2010). So that, MRSA isolates have become a significant threat to human health worldwide (McGuinness WA \textit{et al}. 2017). In recent years, vancomycin has been introduced as an effective antibiotic for treatment of MRSA infections (Asadi 2016, Habibi \textit{et al}. 2018). Unfortunately, \textit{S. aureus} clinical isolates with complete (VRSA) and intermediate resistance (VISA) to vancomycin have emerged within the two past decades due to an increase in the use of this antibiotic for other infections in hospitalized patients (Ena \textit{et al}. 1993, Hidayat \textit{et al}. 2006). The first VISA was reported in 1997 from Japan (Hiramatsu \textit{et al}. 1997), and then in USA, Europe, Australia and...
Asia countries (Howden BP et al. 2010). Thus, this is an important alarm for the medical community, as *S. aureus* causes life-threatening infections in hospitalized and non-hospitalized patients (SK 2001).

The exact mechanism of vancomycin resistance *S. aureus* clinical isolates is unclear. Recent studies have indicated that vanA operon, consisted of vanA, vanH, vanX, vanS, vanR, vanY, and vanZ genes, is likely a major factor required for vancomycin resistance phenotype and pathogenesis of these isolates (Hong et al. 2008). vanA gene is necessary for synthesizing the depsipeptide D-Ala-D-lactate peptidoglycan precursors which are critical for the pathogenesis of these isolates (Bugg et al. 1991). Incorporation of altered D-Ala-D-lactate into peptidoglycan yields a cell wall which is resistant to vancomycin (McGuinness et al. 2017).

Although the VRSA and VISA isolates are increasing, only few studies have reported the prevalence of these isolates is some parts of a country. Recent studies in different parts of Iran have demonstrated that this country may be a spot region for the emergence of these isolates (Aligholi et al. 2008, Askari et al. 2013). The current study aimed to determine the antimicrobial susceptibility patterns of *S. aureus* isolates from patients in Mazandaran hospitals in north of Iran with emphasis to the possible presence of vancomycin resistance and also vanA gene in these strains.

**MATERIALS AND METHODS**

**Samples collection**

In this cross-sectional study, a total number of 100 clinical samples were collected from laboratories of Imam Hospital (Behshahr City, Mazandaran Province) from January to August 2017. Bacterial strains were collected from different clinical specimens, including urine, sputum, wound swab and blood. Isolates were cultured in blood agar medium at 37 °C for 24 h. Standard biochemical and microbiological tests were performed for the identification of *S. aureus* isolates (TL 2003) (Table 1).

<table>
<thead>
<tr>
<th>Biochemical tests</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram staining</td>
<td>Gram positive, cocci- shaped</td>
</tr>
<tr>
<td>Coagulase test</td>
<td>+</td>
</tr>
<tr>
<td>Catalase test</td>
<td>+</td>
</tr>
<tr>
<td>DNase test</td>
<td>+</td>
</tr>
<tr>
<td>Bacitracin test</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Novobiocin test</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

**Antibiotic susceptibility test**

Disk agar diffusion test using Kirby-Bauer method according to CLSI procedure was applied for the assessment of antibacterial effects of different antibiotics against *S. aureus* isolates (Bauer et al. 1996). The clinical *S. aureus* isolates (0.5 McFarland) were spread onto the surface of the Muller Hinton Agar (MHA) with a sterile swab. Gentamicin (10 μg), kanamycin (20 μg), cefotaxime (30 μg), amikacin (30 μg), piperacillin (100 μg), carbenicillin (100 μg), ceftazidime (30 μg), cefalotin (30 μg), and vancomycin (30 μg) disks were used as antibiotics. Vancomycin was purchased from the Sigma Aldrich Company (USA), while the other antibiotics were purchased from the PADTAN TEB Company (Tehran, Iran). The agar plates were incubated at 37 °C for 24 h and the diameter of the zone of inhibition for each microorganism was measured. All tests were performed as triplicate.

**Detection of vanA gene by PCR**

The *S. aureus* vancomycin resistance gene (*vanA*) was investigated in vancomycin-resistant and intermediate resistance isolates by PCR method using specific primers (Table 2). DNA was extracted with boiling method. Briefly, 100 μl of bacterial suspension in PBS were placed into a labeled micro-tube and boiled at 100 °C for 10 min. The micro-tube was then centrifuged at 10000 g for 5 min. Supernatants were collected and transferred into another sterile micro-tube. The quantity and quality of extracted DNA were evaluated with Nanodrop (Thermo 2000) and agarose gel electrophoresis methods, respectively. The supernatants containing the DNA were stored at -20 °C further analyses.

PCR amplification was carried out in a 25 μL reaction mixture with each primer (contained 13 μL Master Mix; 1.2 μL each primer; 2.6 μL DNA sample; and 7 μL dH2O) as the following steps: an initial denaturation step at 95°C for 60 sec; followed by 30 cycles denaturation at 95°C for 30 sec, annealing at 56 °C for 30 sec and extension at 72 °C for 60 sec and a final extension at 72 °C for 5 min (Bamigboye et al. 2018). The PCR products were
electrophoresed in a 1% agarose gel for 20 min at 100 V. The gels were then stained with ethidium bromide and visualized using UV trans-illuminator.

Table 2. Primers used for vanA amplification.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5'-CTGGAGAGACTAAGCCCTCC-3'</td>
<td></td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-ATTACTGACGCTGATTGTGC-3'</td>
<td>425 bp</td>
</tr>
</tbody>
</table>

Statistical analysis
Descriptive statistics was applied for the analysis of frequencies among patients group. Data were analyzed using SPSS software (IBM, version 19).

RESULTS
In this study, S. aureus was isolated from 4 different clinical samples of 100 patients with different infective conditions. After standard biochemical and microbiological tests analysis, 60 clinical samples were positive for S. aureus (Table 1). The antibiotic susceptibility of isolated S. aureus assessed by the disk agar diffusion method is shown in Table 4. More than 40% of isolates were resistant to commonly used antibiotics, including gentamicin (46.67%), ceftazidime (45%) and carbenicillin (43.34%). Only few, however, were sensitive to gentamicin (33.33%) and ceftazidime (35%). Approximately, 50% of isolates were sensitive to vancomycin, kanamycin, and piperacillin. Vancomycin was the most effective antibiotics against S. aureus isolate (with 56.66% sensitivity). Totally, 11 isolates (18.34%) were resistant to vancomycin (VRSA) and 15 isolates (25%) were intermediate resistance (VISA) (Table 3). Fig. 1 shows the molecular analysis of vanA gene. Agarose gel electrophoresis revealed a major band with 425 bp in positive isolates with vanA gene. Molecular analysis of vanA gene in 11 VRSA and 15 VISA showed that 8 VRSA (72.72%) and 6 VISA (40%) were positive for vanA gene (Fig. 1).

Table 3. Antimicrobial resistance rates of S. aureus isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Sensitive (S)</th>
<th>Intermediate resistance (I)</th>
<th>Resistant (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>20 (33.33%)</td>
<td>12 (20%)</td>
<td>28 (46.67%)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 (50%)</td>
<td>18 (30%)</td>
<td>12 (20%)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>26 (43.34%)</td>
<td>14 (23.23%)</td>
<td>20 (33.33%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>24 (40%)</td>
<td>20 (33.33%)</td>
<td>16 (26.67%)</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>32 (53.34%)</td>
<td>13 (21.66%)</td>
<td>15 (25%)</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>24 (40%)</td>
<td>10 (16.66%)</td>
<td>26 (43.34%)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>21 (35%)</td>
<td>12 (20%)</td>
<td>27 (45%)</td>
</tr>
<tr>
<td>Cefalotin</td>
<td>28 (46.67%)</td>
<td>14 (23.33%)</td>
<td>18 (30%)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>34 (56.66%)</td>
<td>15 (25%)</td>
<td>11 (18.34%)</td>
</tr>
</tbody>
</table>

Fig. 1. Agarose gel electrophoresis of PCR-amplified vancomycin resistance gene (vanA). Lanes: M, 100-bp ladder; 1-5: S. aureus isolates showing 425 bp vanA amplicon.
DISCUSSION

Staphylococcus aureus has become a major cause of infections, morbidity and mortality in hospitals because of its rapidly acquired resistance to various antibiotics such as penicillin, methicillin and vancomycin (Friäes et al. 2015). So that, information about the incidence of vancomycin-resistant S. aureus in a hospital or society and its treatment have been of great concern for public health. This has made the study of VRSA and VISA as a significant topic for clinical research. However, a few studies have investigated the prevalence and clinical infections caused by S. aureus throughout the world (Thati et al. 2011). In the present study, we investigated the prevalence of vanA gene in clinical isolates of vancomycin-resistant S. aureus in Mazandaran Province, Iran. We detected VRSA in 11 (18.34%) and VISA in 15 (25%) out of 60 S. aureus clinical isolates. Further molecular analysis by PCR has revealed the presence of vanA gene in 8 VRSA (72.72%) and 6 (40%) VISA isolates.

There are a few studies with different reports of VRSA and VISA prevalence throughout the world. For example, Bamigboye et al. (2018) detected VRSA in 1 (1.4%) out of 73 S. aureus-clinical isolates in a hospital in Nigeria. This isolate did not contain vanA and vanB genes. There is also another study from Nigeria worked on the prevalence's of VRSA and VISA clinical isolates, reporting that 25 (51.0%) out of 49 clinical isolates were susceptible to vancomycin (MIC ≤ 2 µg ml⁻¹), while 18 (36.7%) isolates were VISA and 6 (12.2%) were VRSA (Taiwo et al. 2011). A recent study has reported the first case of infection with vancomycin-resistant S. aureus in Europe, in a Portuguese hospital (Melo-Cristino et al. 2013). There is also another study in Brazil reporting that a 35-year-old male had with blood culture positive for methicillin-resistant VRSA (Makhdoomi 2018).

In contrast to these reports, we found higher prevalence of VRSA and VISA clinical isolates harboring vanA gene in our hospital. In another study in Iran, Saadat et al. (2014) found that the prevalence of VRSA clinical isolates was 37%. Interestingly, vanA and vanB genes were found approximately in all vancomycin-resistant strains. Further molecular analysis exhibited that vanA and vanB genes were detected in 34% and 37% of all clinical isolates, respectively. Firouzi et al., (2016) studied the prevalence of VISA strains of S. aureus in 447 healthcare staff and inpatients in Iran, finding that 31.31% of the isolates were resistant to methicillin (MRSA), while 16.1% were VISA. In another study, Hadadi et al. (2011) evaluated the prevalence of methicillin- and vancomycin-resistant S. aureus among hospitalized patients in Sina Hospital in Tehran, Iran. MRSA was detected in 50% of isolates, while VISA in one isolate (1.17%) out of 85 clinical ones. These data indicate the higher incidence of vancomycin-resistance strains in clinical specimens in Iran compared to the other countries. More importantly, these findings implicate that VRSA and VISA isolates are increasingly emerging in several parts of the world, especially in Iran. Therefore, there remains a public health threat from these increasing S. aureus isolates which can no longer be ignored (Bamigboye et al. 2018).

The molecular mechanisms underlying for the pathogenesis and resistant pattern of VRSA to different antibiotics are not well-understood. It seems that vanA and vanB genes play an important role in the pathogenesis of VRSA strains. In the present study, 72.72% of VRSA and 40% of VISA isolates contained vanA gene suggesting the critical role of this gene in the pathogenesis of this strain.

CONCLUSION

In conclusion, our results exhibited that the prevalence of vancomycin- and intermediate-resistant S. aureus strains isolated from patients in Mazandaran hospitals is high and emerging. More importantly, the frequency of vanA gene in these isolates, particularly in VRSA isolates, was very high which may be a reason for its pathogenesis and multiple-antibiotic resistance. Therefore, there is a need to keep the emergence and spread of these strains to a minimum level through an appropriate antibiotic prescribing and also initiating infection control measures.

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چکیده

استافیلوکوکوس اورتوس (MRSA) مقطعی مقاوم به ونکومایسین به عنوان یک مشکل اساسی در بیمارستان‌ها محسوب می‌شوند. در این مطالعه شیوع استافیلوکوکوس اورتوس مقاوم به ونکومایسین در بیمارستان‌های مازندران بررسی شده است. vanA در این مطالعه توصیفی- مقطعی تعداد 60 سویه استافیلوکوکوس اورتوس با استفاده از آزمایش‌های بیوشیمیایی و میکروب شناسی در نمونه‌های مختلف بیمارستان‌های مازندران، روشن کرده است. همچنین در این سویه به آمیتا که به باکتری‌های استافیلوکوکوس اورتوس مقاوم و نیمه حساس به ونکومایسین با روش PCR جناتامپسین و سنتاز دریم و نیمه حساس به ونکومایسین با روش vanA به آمیتا نسبت به آنی بیوتیک‌های رایج شمل جناتامپسین (۴۶/۶۷%) و کاربنی سیلین (۴۵/۷%) مقدار بوده. در جناتامپسین و سنتاز دریم میزان vanA در سویه به آمیتا برابر ۱۵ سویه (۱۵/۸۲%) و میزان آمیتا به آمیتا در سویه به آمیتا برابر ۲۵ سویه (۲۵/۸۳%) بوده. در نهایت مطالعه بررسی شد که در این سویه فراوانی ژن vanA در نمونه‌های استافیلوکوکوس اورتوس بالا و در حال افزایش است. بنابراین، به حداکثر رساندن انتشار این سویه ضروری است.

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