ISSN: 2588-4115; Infection Epidemiology and Microbiology. 2019;5(2):9-16

The Frequency of Methicillin and Vancomycin Resistance Genes in *Staphylococcus aureus* Strains Isolated from Clinical Specimens in Marand, Iran

ARTICLE INFO

Article Type
Original Research

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How to cite this article
Imeni N. Ghiami Rad M. Farah Bakhsh H., Jafari-Sales A. The Frequency of Methicillin and Vancomycin Resistance Genes in *Staphylococcus aureus* Strains Isolated from Clinical Specimens in Marand, Iran. Infection Epidemiology and Microbiology. 2019;5(2):9-16

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Article History
Received: March 1 ,2019
Accepted: April 10 ,2019
ePublished: August 30 ,2019

ABSTRACT

Aims: Given the prevalence of methicillin-resistant *Staphylococcus aureus* infections and the importance of antibiogram pattern in the treatment of these infections, the present study aimed to evaluate the methicillin and vancomycin resistant *Staphylococcus aureus* clinical isolates.

Materials & Methods: *S. aureus* isolates were diagnosed using proprietary cultivation environments and standard biochemical methods by isolating 130 *Staphylococcus* samples from patients' clinical specimens. The isolates antibiotic susceptibility pattern was determined by disc diffusion method. MRSA isolates were identified using cefoxitin discs, and the E-test method was used to determine the minimum inhibitory concentration (MIC) of vancomycin antibiotic. Furthermore, the multiplex PCR method was used to study the frequency of *mecA* and *vanA* genes.

Results: In the present study, 57 out of 130 *Staphylococcus* isolates were diagnosed as *S. aureus*. According to the antibiogram test results, the isolates showed the highest resistance to penicillin (92.98%) and the lowest resistance to ciprofloxacin (10.52 %). In addition, the resistance to methicillin was reported as 21.56 % using cefoxitin disc. According to the E-test results, 90% of the isolates were susceptible to vancomycin, and 10% showed heterogeneous resistance to vancomycin. The molecular analysis indicated that *mecA* gene was present in 35.08% of the isolates, but no isolate contained *vanA* gene.

Conclusion: Despite the lack of resistance to vancomycin, the isolates showed a high resistance to methicillin. Therefore, the present study results emphasized the necessity of performing antibiotic sensitivity tests before the drug administration.

Keywords: *Staphylococcus aureus*; Methicillin resistance; Vancomycin resistance

CITATION LINKS

[1] Mechanisms of vancomycin resistance... [2] Molecular investigation of antibiotic resistance genes in... [3] Prevalence of vancomycin intermediate *Staphylococcus*... [4] Vancomycin tolerant, methicillin-resistant *Staphylococcus aureus* reveals the effects of vancomycin on cell wall thickening. [5] Evolution of peptidoglycan biosynthesis under the... [6] Prevalence and antimicrobial susceptibility pattern of ... [7] Study of antibiotic resistance pattern in methicillin-resistant... [8] Detection of the antibiotic resistance pattern in *Staphylococcus aureus* isolated... [9] Antimicrobial resistance: the example of *Staphylococcus aureus.* [10] Characterization and persistence of *Staphylococcus*... [11] Clinical and Laboratory Standards Institute... [12] Novel multiplex PCR assay for characterization and concomitant subtyping of staphylococcal cassette ... [13] Molecular detection of human bacterial pathogens. USA, Florida: Taylor and... [14] Staphylococcal cassette chromosome (SCCmec) typing of methicillin-resistant... [15] Identification of virulence gene panton-valentine leukocidin (PVL)... [16] Molecular and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* isolates... [17] Clinical and Laboratory Standards Institute. M100-S21: Performance standards for... [18] Detection of vancomycin resistance among... [19] A comparison of clinical virulence of ... [20] Methicillin-resistant *Staphylococcus aureus* in... [21] FemA, a host-mediated factor essential for methicillin resistance in... [22] Guidelines for the laboratory diagnosis and... [23] Activities of ceftobiprole, linzolid, vancomycin and daptomycin against ... [24] Emergence of vancomycin resistance *Staphylococcus* ... [25] Methicillin-resistant Staphylococcus... [26] Sensitivity study of *Staphylococcus aureus* ... [27] Prevalence of vancomycin resistance in methicillin-resistant *Staphylococcus aureus*. [28] Epidemiology of mecA-methicillin-resistant ... [29] Vancomycin sensitivity of methicillin-resistant... [30] Molecular detection of nine antibiotic resistance genes in methicillin-resistant... [31] High-level vancomycin-resistant *Staphylococcus aureus* (VRSA) in ...

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[DOR: 20.1001.1.25884107.2019.5.2.1.4]

Introduction

Staphylococcus aureus is the most important pathogen in humans and one of the main causes of hospital acquired infections in the community [1]. This bacterium is the cause of a wide range of human infections including mild skin infections and severe systemic infections such as sepsis, toxic shock syndrome, osteomyelitis, food poisoning, and endocarditis [2]. Penicillin and methicillin were used to treat infections caused by this bacterium, but resistance to these antibiotics and the creation of methicillin-resistant *S. aureus* isolates led to the administration of vancomycin as the main antibiotic for the treatment of methicillin-resistant *S. aureus* (MRSA) associated infections since the last 50 years [3]. Vancomycin through binding to the terminal d-Ala-d-Ala moiety of the bacterial peptidoglycan precursors, prevents transglycosylation and transpeptidation steps in the production process of peptidoglycan [1]. The presence of *mecA* gene encoded by a sequence of genes in a chromosomal region called staphylococcal chromosomal cassettes *mec* (*staphylococcal* chromosomal cassettes mec: SCCmec), is the main cause of methicillin resistance. The length of *mecA* gene is 1.2 kb in this region, encoding a protein called PBP2a (penicillin-binding protein 2a). This protein which is responsible for methicillin resistance has a low affinity for β-lactam drugs [4]. The *fem* gene (A/B/C) in the genome is known as another cause of methicillin resistance. These genes encode a 48-kD protein involved in the formation of the pentaglycine bridges. Deactivation of these genes causes the bacterial sensitivity to methicillin as well as other beta-lactam antibiotics [5]. The frequent use of vancomycin in the treatment of staphylococcal infections has increased the emergence of MRSA strains, by reducing the susceptibility to vancomycin and producing VISA (vancomycin intermediate *S. aureus*) and VRSA (vancomycin resistant *S. aureus*) strains [1]. The VISA strain was first reported in Japan in 1996. Furthermore, the first strain of VRSA was reported in 2002 in the United States [6]. From the microbiology perspective, the intermediate resistance to vancomycin is associated with many changes in the bacterial physiology, including increased cell wall diameter, decreased growth, and changes in the gene amplification [7, 8]. The vancomycin resistance mechanism occurs by changes in the target site of drug binding in the cell wall through the genetic transfer of resistance (*vanA* gene) from *Enterococcus faecalis* to *S. aureus* via a conjugation mechanism, resulting in the conversion of D-Alanine-D-Alanine into D-Alanine-D-Lactate [9]. According to the recent reports on the isolation of *staphylococcus* strains with reduced susceptibility and increased resistance to vancomycin and methicillin from different regions of world, including Iran, and given the importance of these antibiotics as the first line in the treatment of infections caused by this bacterium, it is important to conduct a continuous examinations on the status of these strains in different countries in order to be able to adopt suitable therapeutic strategies. **Objectives:** Accordingly, the present study was conducted to investigate resistance to methicillin and vancomycin phenotypically, and to determine the antibiotic resistance encoding genes (*mecA* and *vanA*) in *S. aureus* strains isolated from clinical specimens of patients referring to Ayatollah Kuh-Kamari hospital in Marand city, Iran.

Materials and Methods

Collection and identification of samples: In the present descriptive cross-sectional study conducted during 2016-2017, 130 *Staphylococcus* samples were collected from the diagnostic lab of Ayatollah Kuh-Kamari hospital during a year. All clinical specimens

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obtained from admitted patients were sub-cultured on Blood agar media. The isolates were re-cultured on BHIA (Brain-Heart Infusion Agar) (Merck, Germany) media for purification. Subsequently, the isolates were phenotypically identified and detected using a Gram staining method, catalase, oxidase, DNase, slide coagulase, culture on mannitol salt agar, and VP (Voges Proskauer) tests in accordance with the standard microbiological methods [10].

Antibiotic susceptibility testing using a disc diffusion method: A bacterial suspension was prepared from 24-hour-cultured samples according to 0.5 McFarland turbidity standard and then cultured on the Mueller-Hinton agar medium (Merck, Germany). The penicillin (10µg), tetracycline (30µg), cephalexin (30µg), ciprofloxacin (5µg), and clindamycin (2µg) (Himedia, India) discs were located on the medium and incubated at 37°C for 18-24 hrs. Results were interpreted according to the standard tables of CLSI (Clinical & Laboratory Standards Institute) [11].

Determining MRSA isolates using the disk diffusion method with cefoxitin disc: Cefoxitin disc (30µg) (Hi-Media, India) was located on the MHA medium and incubated for 24 hrs after preparing the bacterial suspension and culturing on the BHI agar medium (Merck, Germany). The diameter of bacterial growth inhibition region was interpreted according to CLSI standard tables [11]. The standard strain of methicillin-sensitive *S. aureus* ATTC 25923 was used for the quality control testing.

Determination of susceptibility to vancomycin using the E-test method: The bacterial suspension was prepared equivalent to 0.5 McFarland turbidity standard and cultured on the Muller-Hinton agar medium using a cotton swab. Finally, the specimens were incubated at 35°C for 24 hrs after planting E-test strip and vancomycin disc (Liofilchem, Italy). The results were read and evaluated in accordance with the CLSI guidelines [11]. The standard strain of vancomycin-sensitive *E. faecalis* ATCC 29212 was used as the quality control strain.

DNA extraction and multiplex-PCR technique for detecting the resistance genes: The DNA was extracted by boiling in the sterile Tris buffer for 18 min and the centrifugation at 10000 g. The supernatant was used as a sample containing bacterial DNA. To confirm the purity and concentration of extracted DNA, all samples were purified in the ratio of 260 to 280 nm in the range of 1.8-2%, and their concentrations were measured at 50 ng/mL using a Nano drops device. The presented sequences in Table 1 were used to identify genes responsible for resistance. The multiplex-PCR process was performed in a final volume of 25µL containing 12.5µL of Master Mix (containing Taq Polymerase, $MgCl_2$ and dNTP), 3µL of mixed primers (GENERAY, Korea), 8.5µL of dH_2O, and 1µL of the studied DNA sample using a thermal program, including an initial denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, extension

Table 1) Primers used for detecting *mecA* and *vanA* genes in *S. aureus* isolates.

Genes	Primers Sequence (5′ ... 3′)	Size (bp)	Reference
mecA	F: TTAGTGAACCATATAGAAGTG R: ATGCGCTATAGATTGAAAGGAT	147	[12]
vanA	F: ATAACGTTGAAAATAAGATAAGTAG R: CCCCTTTAACGCTAATACGATCAA	1030	[13]

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at 72°C for 1 min, and a final extension at 72°C for 10 min in a Thermocycler device (BIORAD, United States). The PCR products were electrophoresed on 2% agarose gel for 75 min at 90 volts, stained with DNA Safe Stain, and analyzed under the UV light in a Gel Doc.

The standard strains were as follows: vancomycin-sensitive *E. faecalis* ATCC 29212 as a negative control, methicillin-resistant *S. aureus* ATCC 33591 as a positive control, and methicillin-sensitive *S. aureus* ATCC 29231 as a negative control. A 100bp+3K marker was used to determine molecular weights.

Findings

In the present study, 57 (43.85%) out of 130 *Staphylococcus* isolates were identified as *S. aureus*. Among which, 39 (68.42 %) samples were isolated from females, and 18 (31.58 %) samples were isolated from males. Also, 32 (56.14 %) samples were related to inpatients, and 25 (43.86 %) samples were related to outpatients. The patients' mean age was 60.2 ± 21.3 with a minimum of 3 years and maximum of 82 years.

The isolates antimicrobial pattern showed the highest resistance to penicillin (92.98%), followed by cephalexin (29.82%), clindamycin (28.07%), tetracycline (24.56%), and ciprofloxacin (10.52%). Also, methicillin resistance was reported as 21.56% using cefoxitin disc. Moreover, the results of E-test used for determining the vancomycin resistance showed that 30% of the isolates had MIC=1.5 mg/mL, and 60% had MIC=2 mg/mL. In accordance with CLSI standards, these isolates were identified as vancomycin-sensitive *S. aureus* isolates. In addition, 10% had MIC=3.2 mg/mL, which were also identified as heterogeneous vancomycin-intermediate *S. aureus* (hVISA) isolates. Moreover, no isolate was identified as VRSA via this method (Figure 1).

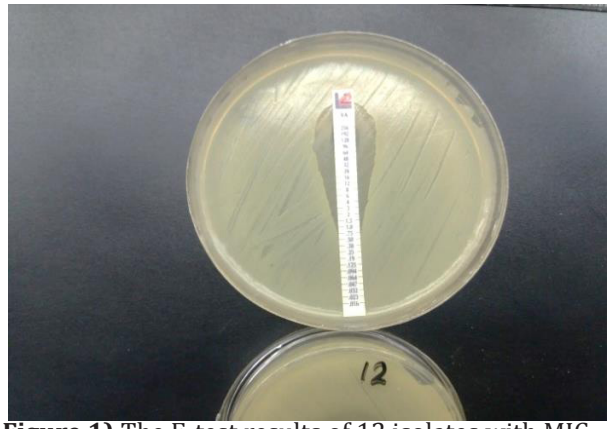

Figure 1) The E-test results of 12 isolates with MIC= 1.5 mg/mL

The evaluation of the distribution of antibiotic resistance-encoding genes indicated that *mecA* gene had a frequency of 35.08% (20 isolates), but none of the isolates contained vancomycin resistance gene (Figure 2).

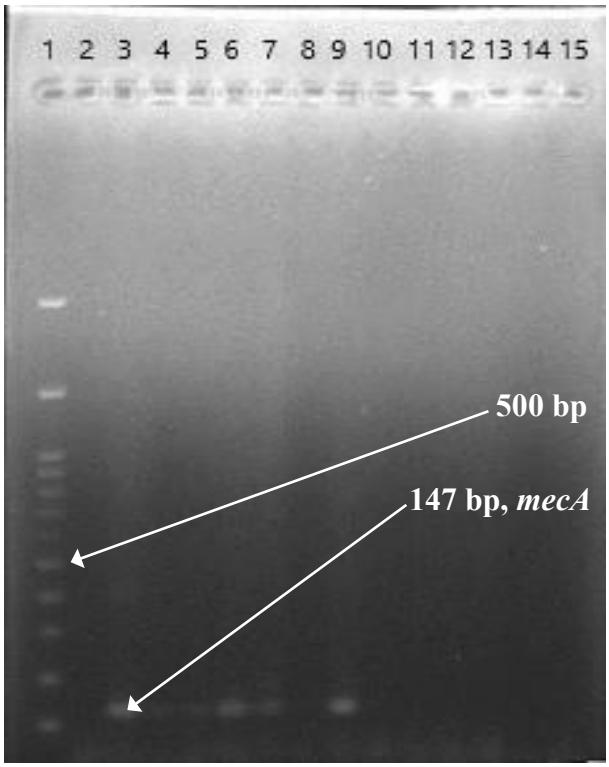

Figure 2) Electrophoresis results of *mecA* and *vanA* genes amplification by a multiplex-PCR technique for clinical and standard strains. Column 1: 100-bp marker; Column 2: *E. faecalis* ATCC 29212 (negative control for *mecA* and *vanA* genes); Column 3: *S. aureus* ATCC 33591 (positive control for *mecA* gene); Column 4: *S. aureus* ATCC 29213 (positive control for *mecA* gene); and column 6 and 8: positive isolates.

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Discussion

In the present study, the antibiotic resistance pattern was investigated in *S. aureus* clinical samples using a disk diffusion method. The results indicated the highest resistance to penicillin (92.98%). In Ebadi et al. (2018)'s study, the highest resistance was also reported to penicillin (100%) [14]. In another study by Alizadeh et al. (2015), the highest resistance was reported to linezolid (35%) [15]. Vall et al. (2016) reported the highest resistance to Co-trimoxazole (20.37 %) [16]. Different results in these studies conducted on antibiotic sensitivity could be attributed to the selective pressure of antibiotic therapy in target zones, infection locations, patients' status (inpatient or outpatient), and previous antibiotics used.

The methicillin resistance was evaluated in a study using oxacillin and cefoxitin discs due to sustained effects of these antibiotics over time and the high probability of detecting heterogeneous resistant strains in the microbiology lab [17].

In the present study, using a disk diffusion method, the antimicrobial resistance testing indicated that among the 57 *S. aureus* isolates, 21.55% were resistant to methicillin using cefoxitin disc.

In a study by Rengaraj et al. (2014) [18], 45.875% of the isolates were resistant to cefoxitin. In a study in Chicago, the prevalence of MRSA was reported as 19% [19]. In Australia, the prevalence of methicillin resistant *S. aureus* isolates was reported as 14.9 % [20]. MRSA prevalence level varies in different regions of the world. Different prevalence rates of MRSA in the these studies may have various causes, including the antibiotic self-therapy, experimental and extreme consumption of drugs in the community, and thus centers failure to comply fully with health regulations in dealing with patients with *S. aureus* infections.

Routine microbiology laboratories use the disc diffusion method to detect resistance to vancomycin because of no need to expensive equipment. However, the problems arising from the use of the disc diffusion method in detecting resistance to vancomycin, result from the poor diffusion of glycopeptide antibiotics in agar culture media; hence, it is recommended to use minimum inhibitory concentration (MIC) methods such as E-test that effectively detect VISA, hVISA, and VRSA strains[17]. On the other hand, reports indicated that failure in the treatment with vancomycin leads to the emergence of hVISA strains [21]. Therefore, it is recommended to use tests approved by CDC and CLSI to detect VISA/hVISA strains, including dilution broth, screening test in the BHI agar containing vancomycin, and E-test [22].

In the present study, E-test method was used to evaluate the MIC of vancomycin. The results indicated that 90% of the isolates were sensitive to vancomycin and had a MIC of 1.5 to 2 mg/mL. In addition, 10% of the isolates were hVISA. In a study by Leonard et al. (2007) in the United States, not only was no VISA strain detected, but also the MIC range of vancomycin was reported as 0.25-2 μg/mL [23]. Tiwari et al. (2006) reported 6 and 2 isolates of VISA and VRSA out of 783 *S. aureus* isolates in India, respectively [24].

In a study by Sancak et al. (2005), MIC values of vancomycin were reported to be 0.4-12 μg/ml, and all the isolates were sensitive to vancomycin [25]. Another study in Tabriz, Iran, found no resistance or intermediate sensitivity to vancomycin and reported that the MIC of vancomycin was about 1.5-3μg/mL [26]. Mohajeri et al. (2014) indicated that the frequency of hVISA isolates was 49.9% in Kermanshah, Iran, but no VISA or VRSA isolate was observed using the same method [27]. In this study, there was no vancomycin-resistant strain according to the literature.

In the present study, the frequency of *mecA* gene in *S. aureus* clinical isolates was 35.08%

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by PCR, but none of the isolates contained *vanA* gene. Askari et al. (2012) reviewed published articles on the prevalence of methicillin-resistant *S. aureus* strains in Iran. According to their study findings, 2690 full-text articles and abstracts were about the MRSA in Iran, totally examining 7464 *S. aureus* samples genetically for the presence of *mecA* gene. In a study, the incidence of MRSA varied from 20 to 90%, but its prevalence was between 50 to 60% in Tabriz [28]. In the present study, the frequency of MRSA isolates by molecular method was in range 20 to 90%. In a study by Ebadi et al. (2017), 75.7% of the isolates contained *mecA* gene [16]. In a study in Tabriz, Bohlouli et al. (2016) reported that all 100 *S. aureus* isolated strains contained *mecA* gene [29]. In a study by Adwan et al. (2014), all 55 *S. aureus* strains isolated during 2011-2013 contained *mecA* gene [30].

The difference in the *mecA* gene frequency could be due to different distribution of genes in various locations, their expression method, and different sampling locations. In this study, it was shown that all the isolates with *mecA* gene were resistant to methicillin through a phonotypic method. However, the presence of this gene in the isolates does not necessarily mean the resistance to methicillin; other agents or genes may be also involved. Therefore, more studies is needed to be conducted about the distribution of resistance genes in *S. aureus* strains.

In another study by Askari et al. (2012), reviewing all published articles on the prevalence of VRSA strains in Iran, the results indicated that 24 VRSA isolates were reported in various studies in Iran until 2012; for example, this strain was observed in Tehran, Isfahan, Rasht, Mashhad, Gorgan, Sari, and Karaj, but no VRSA strain was reported in Tabriz [31]. In the present study, no VRSA isolate was observed using PCR. However, it was found that *S. aureus* sensitivity to vancomycin was decreased using the E-test, resulting in the emergence of VISA and hVISA strains The non-judicious use of vancomycin develops hVISA and VISA strains and converts them into VRSA strains. Therefore, the non-prescribed and unnecessary administration of available antibiotics should be avoided in order to prevent the increased resistance to these antibiotics and other common antibiotics. Furthermore, the results of this study clarified the necessity of physicians' attention to vancomycin as a main drug in the treatment of staphylococcal infections.

Conclusion

The obtained results from the E-test and PCR techniques indicated no vancomycin-resistant isolate. Therefore, this antibiotic can be used for the treatment of staphylococcal infections compared with other commonly-used antibiotics. On the other hand, several reports on the emergence of VISA and VRSA strains in Iran and other countries emphasize the need for molecular detection of antibiotic resistance genes in order to be able to accurately detect drug resistance genes and to select an appropriate drug for treatment.

Acknowledgments: The authors would like to appreciate the personnel of the Department of Microbiology, Ayatollah Kuh-Kamari hospital for their assistance in the collection of isolates.

Ethical permissions: Ethical permissions was approved and issued by Islamic Azad University, of Marand.

Conflict of Interests: There was no conflict of interest between authors.

Authors' Contribution: All of authors contribute to this study.

Fundings: None to declare.

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[DOR: 20.1001.1.25884107.2019.5.2.1.4]

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