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Enterotoxin and Exfoliative Toxin Genes Among Methicillin-Resistant *Staphylococcus aureus* Isolates Recovered From Ilam, Iran

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Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is one the most important pathogens across the world, associated with increased mortality rate compared to other Staphylococcal species.
Objectives: The present study aimed to investigate different virulence determinants among MRSA isolates from Ilam, Iran.
Materials and Methods: Overall, 100 MRSA isolates were collected from selected hospitals of Ilam, Iran. Oxacillin screening test and molecular detection of *mecA* were carried out by polymerase chain reaction (PCR) to confirm the methicillin resistance. Presence of the virulence genes *et A, B, tst, sea a, b, c, d* and *e* were assessed using multiplex PCR.
Results: All the tested isolates were susceptible to vancomycin, but resistant to penicillin (100%), erythromycin (31%), clindamycin (27%), ciprofloxacin (27%), gentamicin (21%), and amikacin (16%). The frequencies of antibiotic resistance and virulence genes were as follows: *tst* 46%, *eta* 1%, *etb* 3%, *sea* 41%, *seb* 2%, *sec* 14%, and *see* 31%.
Conclusions: Results of the present study showed that the *tst* and *sea* genes had high frequencies among the MRSA isolates. The increased prevalence of MRSA isolates containing different virulence genes, probably accompanied by antimicrobial resistance, can complicate the therapy of MRSA isolates.

Keywords: Methicillin-Resistant *Staphylococcus aureus*; Virulence Determinant

1. Background

Staphylococcus aureus is one the most common pathogens causing community-acquired infections and a major concern for public health (1). *S. aureus* is present in food products (2) and is one of the causes of bacterial food poisoning across the world. This organism possesses host damaging toxins such as lipase, thermonuclease, hyaluronidase and haemolysin, which are accounted as the microorganism invasive factors to the host tissues (3). Heat-resistant enterotoxins are probably the most virulent factors of this bacterium which are able to cause the food poisoning syndrome (4). Staphylococcal enterotoxins (SEs) are a part of *S. pyogenes* exotoxins (PT), which are homologous by means of structure, function and sequence and have phylogenic relationships. These toxins can be the causes of toxic shock syndrome, food poisoning, some allergic syndromes and autoimmune diseases. The members of this family include SEs, two types of toxic shock syndrome toxin proteins, and *PT* groups (5). SEs as well as gastrointestinal toxins act as superantigens and induce nonspecific proliferation of T-cells. Although these two actions are performed through two independent active sites on the protein, there is still a strong connection between the toxic properties of these proteins as enterotoxins and superantigens, so that losing the superantigenic properties through mutation can lead to loss of enterotoxic properties of the proteins (6).

Antibiotic resistance is also of great concern for public health authorities. The incidence of antibiotic resistance among bacterial species increases the costs of antibiotic therapy as well as treatment of infection-derived complications. *S. aureus* is also able to grow in food products. Apart from the pathogenic properties of this organism as a concerning matter, *its* species can quickly become resistant to a variety of antibiotics such as methicillin. The incidence of methicillin-resistant *S. aureus* (MRSA) isolates was initially reported in 1961, in the same year that methicillin was introduced to the market (7). Currently, MRSA isolates have become the most common causative agents

of hospital-acquired infections, leading to increased mortality rates. Methicillin resistance in *S. aureus* is due to acquisition of the *mecA* gene, which encodes penicillin binding proteins (PBP2a) with a low affinity against β-lactams. This gene makes PBP2a producing species resistant to all β-lactams (8). By emergence of MRSA isolates which are resistant to quinolones and other antibiotics as well as to β-lactams, limited antibiotic choices are left to control these microorganisms.

2. Objectives

The present study aimed to investigate different virulence determinants among the MRSA isolates from Ilam, Iran.

3. Materials and Methods

Through a descriptive study in 2012, clinical isolates of MRSA were recovered from wound secretions, urine samples, and blood and urine cultures after coordination with central hospitals of Ilam, Iran. Following sample collection, the bacterial strains were confirmed using conventional tests.

3.1. Antibiotic Resistance

According to the standards published in 2012 by Clinical and Laboratory Standards Institute (CLSI) in America (9), the isolated strains were cultured in Mueller-Hinton agar-containing plates; then, the antibiotic disks were placed on the agar medium, considering the appropriate distance between each two disks. The bacterial cultures were incubated for 24 hours in 37°C, after which the inhibition zone surrounding the disks were measured. The following antibiotics were used in the current study: tetracycline (TE) 10 μg, gentamycin (GM) 10 μg, oxacillin (OX) 1 μg, erythromycin (E) 5 μg, vancomycin (VAN) 30 μg, penicillin (P) 10 U, synercid (SYN) 15 μg, amikacin (AK) 30 μg, imipenem (IMP), linezolid (LZ) 10 μg, tigecycline (TG) 15 μg, ciprofloxacin (CIP) 5 μg, clindamycin (CD) 2 μg, tobramycin (TO) 10 μg, and rifampin (RF) 15 μg (MAST, UK).

3.2. DNA Extraction

DNA extraction was carried out using the phenol chloroform isoamyl alcohol method according to previous reports. The methicillin resistance phenotype was detected among all the isolates using a 1-μg oxacillin disk and the results were evaluated according to the CLSI standards. Polymerase chain reaction (PCR) detection of the *mecA* gene was then carried out to confirm the phenotypic results.

3.3. Detection of Virulence and Antibiotic Resistance Genes Using Multiplex Polymerase Chain Reaction

Since the primers in this study were previously employed in former studies (Tables 1 and 2), they were tested only for primer BLAST and used after confirmation.

Table 1. Primers for Polymerase Chain Reaction Detection of Methicillin-Resistant and Erythromycin-Resistant *Staphylococcus aureus* Isolates [a]

Gene	Primer Sequence (5' to 3')	Product Size, bp
femA	F-AAA AAA GCA CAT AAC AAG CG	132
	R-GAT AAA GAA GAA ACC AGC AG	
mecA	F-ACT GCT ATC CAC CCT CAA AC	163
	R-CTG GTG AAG TTG TAA TCT GG	
ermA	F-TAT CTT ATC GTT GAG AAG GGA TT	139
	R-CTA CAC TTG GCT GAT GAA A	
ermC	F-AAT CGT CAA TTC CTG CAT GT	299
	R-TAA TCG TGG AAT ACG GGT TTG	
mup	F- CCC ATG GCT TAC CAG TTG A	1158
	R- CCA TGG AGC ACT ATC CGA	
blaZ	F-ACT TCA ACA CCT GCT GCT TTC	172
	R-TGA CCA CTT TTA TCA GCA ACC	

[a] The primers were acquired from reference (10).

Table 2. Primers for Detection of Virulence Factors Among Methicillin-Resistant *Staphylococcus aureus* Isolates by Multiplex Polymerase Chain Reaction [a]

Gene	Primer Sequence (5' to 3')	Product Size, bp
sea	F- GGT TAT CAA TGT GCG GGT GG	102
	R- CGG CAC TTT TTT CTC TTC GG	
seb	F- GTA TGG TGG TGT AAC TGA GC	164
	R-CCA AAT AGT GAC GAG TTA GG	
sec	F- AGA TGA AGT AGT TGA TGT GTA TGG	451
	R- CAC ACT TTT AGA ATC AAC CG	
sed	F- CCA ATA ATA GGA GAA AAT AAA AG	278
	R- ATT GGT ATT TTT TTT CGT TC	
see	F- AGG TTT TTT CAC AGG TCA TCC	209
	R- CTT TTT TTT CTT CGG TCA ATC	
eta	F-GCA GGT GTT GAT TTA GCA TT	93
	R-AGA TGT CCC TAT TTT TGC TG	
etb	F-ACA AGC AAA AGA ATA CAG CG	226
	R-GTT TTT GGC TGC TTC TCT TG	
tst	F-ACC CCT GTT CCC TTA TCA TC	326
	R-TTT TCA GTA TTT GTA ACG CC	

[a] The primers were acquired from reference (10).

3.4. Polymerase Chain Reaction Product Analysis

Following the PCR that was carried out according to the previous reports, the products, mixed with loading buffer, were run on an electrophoresis gel for 45 minutes with a voltage of 100 V. Therefore, the molecular weights of the products by were compared with that of an expected product. The gel was illustrated using an UV transilluminator and data analysis was consequently performed

according to presence or absence of each gene, primary data of the patients including gender, city and hospital setting of the sample collection, and the source of bacteria (wound secretions, urine culture, blood culture, or sputum culture). These data were entered into SPSS software and analyzed using descriptive statistical tests.

4. Results

Overall, 37 (37%) and 63 (63%) isolates belonged to the outpatients and inpatients, respectively. Among the strains, 100% and 87% possessed the *mecA* gene (methicillin resistance encoding gene) and *femA* gene, respectively. The MRSA frequencies based on the sample types were as follows: 43% in wound samples, 37% in blood cultures, 14% in urine cultures, and 6% in tracheal secretions. All the strains were susceptible to vancomycin (n = 100; 100%), but some were resistant to penicillin (100%), oxacillin (100%), erythromycin (31%), clindamycin (27%), ciprofloxacin (27%), gentamicin (21%), amikacin (16%), tetracycline (15%), rifampin (11%), imipenem (2%), linezolid (1%), and tigecycline (1%).

4.1. Polymerase Chain Reaction Results

Of 100 MRSA isolates tested in this study, 11 (11%) and 57 (57%) isolates possessed the *ermA* and *ermC* genes, respectively. Among these 100 MRSA isolates, 100 (100%) possessed the *blaZ* gene, but lacked the *mup* gene. Only 1 (1%) had the *eta* gene and 3 (3%) possessed the *etb* gene. A total of 46 (46%) isolates had the *tst* gene, 41 (41%) had the *sea* gene, 2 (2%) possessed the *seb* gene, and 14 (14%) had the *sec* gene, whilst 0 (0%) had the *sed* gene. A total of 31 (31%) isolates possessed the *see* gene.

5. Discussion

Antibiotic resistance has become one of the most important problems of medical sciences in the 21st century. Infections caused by *S. aureus* have always been one of the main challenges in antibiotic treatments, and usually a short time after introduction of an antibiotic, *S. aureus* strains become resistant to it (11, 12). In the recent years, the study of genetic principles of methicillin resistance has led to a better understanding of this phenomenon. The *S. aureus* cassette chromosome mec (SCCmec) has been the only known carrier of the *mecA* gene to date (13). The *mecA* gene is able to cause resistance against bleomycin, aminoglycosides and streptomycins, as well as to all β-lactams (14). All the isolates assessed in this study possessed the *mecA* and *blaZ* genes. These results were similar to those reported by Lim and colleagues, who worked on 188 MRSA isolates recovered from Malaysia (15). In the current study, frequency of the *ermA* gene was 11%, which is less than that reported by previous studies. For example, in a study carried out by Ghaznavi-Rad and colleagues on 189 MRSA isolates, the *ermA* gene frequency was reported 95.1% (16). This difference was probably due to the antibiotic consumption pattern in the past in Malaysia.

All the isolates assessed in the present study were sensitive to vancomycin, after which the maximum susceptibility was found against linezolid and tigecycline (with 99% susceptibility for both) and imipenem (98% susceptibility). These findings were similar to those reported by previous investigations such as the study of Wang L.X et al. who worked on 60 *S. aureus* isolates recovered from blood culture samples in China (17). According to the findings of the current study, 87% of the MRSA isolates were susceptible to rifampin, which was in contrast with the investigations reporting high frequencies of rifampin resistance. As an instance, in a study carried out by Adebayo O Shittu in southern Africa, the prevalence of rifampin resistance among MRSA isolates was 73.8% (18). In another study carried out during 1996-1997, the prevalence of rifampin resistance in African countries was 54% and in an increasing state (19).

The reason behind this difference might be the increased consumption of rifampin in African countries. An interesting point in the results of the current study was the unexpected susceptibility of the isolates to erythromycin (69%), which was in contrast with the findings of Zeinali and his colleagues in 2007, who carried out a similar study in Kashan (20). This difference might be due to the distance between the study locations. All the isolates in this study were resistant to penicillin, which was in concordance with other similar studies. After penicillin, the isolates were most susceptible to oxacillin (21%), which was similar to an earlier report (21).

Although some investigation in the recent years have reported increasing numbers of vancomycin-resistant MRSA isolates (22, 23), the results of the current study showed that vancomycin is still the main antibiotic choice for treatment of serious and threatening infections caused by MRSA isolates. Surveys have indicated that in cases that the minimum inhibitory concentration (MIC) of vancomycin for MRSA isolates has been higher than 1 μg/mL, the mortality rate, treatment failure, and time of hospitalization for each patient have increased proportionally.

Therefore, consumption of vancomycin is recommended for serious and threatening infections and for limited infections oral therapy could be applied. In Australia, the oral treatment of choice for nonthreatening infections is the combination of rifampin and fusidic acid (24, 25), whilst it is trimetoprim-sulfamethoxazole with or without rifampin in America (26). The findings of this study also considered rifampin as an appropriate oral treatment for nonthreatening infections caused by MRSA isolates.

In the current study, 55% of isolates carried at least one type of enterotoxin. This result was inconsistence with the results obtained by Satari and colleagues, studying 100 MRSA isolates obtained from burn patients admitted to Shahid Motahari Hospital in Tehran during 2008-2009. Our results were in accordance with Imanifooladi et al. study, performed on 200 *S. aurous* isolates obtained from patients with skin diseases during 2007, of which, 45% carried enterotoxin (10). These discrepancies might be due to different study locations. In the current study,

the most common enterotoxin coding gene was *sea* with 41% prevalence, followed by *see* with 31% prevalence. The frequencies of enterotoxin-coding genes in the current study were in accordance with Wang et al. study, performed on 60 *S aurous* isolates detected from blood culture samples during 2006-2011 (17). The most common enterotoxin coding gene in their study was *sea* with a prevalence of 33%, followed by *sec* with 15% prevalence (17).

In a study by Lim and others from Malaysia performed on 100 MRSA isolates, the frequencies of enterotoxin-coding genes were 38% for *sea* and 19% for *sec* (9). However, the prevalence of enterotoxin-coding genes reported by different studies was generally different, which could be associated with variety of geographical and health conditions. For example, in the study performed by Satari et al. the enterotoxin-coding gene of *sec* was the most prevalent enterotoxin, present among 35% of the isolates. In that study, 17% of the isolates had both enterotoxin-coding genes of *sea* and *see* simultaneously. These results were inconsistent with those reported by Akineden and others in 2008. They reported that none of the isolates carried two enterotoxin-coding genes simultaneously (28). SEs are similar in structural characteristics and biological activities; however, they are different in their produced amounts and mechanisms of actions. For example, *seb* and *sea* enterotoxins compared to others are more produced (27). In addition, enterotoxins generated by the *sea* and *sec* genes, compared to other enterotoxins, could create more severe immunological responses and consequently more tissue damages (28).

All the evaluated isolates in the current study were sensitive to vancomycin (100%), followed by linezolid and tigecycline (99% sensitivity to each) and imipenem (98%). These results were in accordance with the previously reports and the study by Wang and coworkers on 60 *S. aurous* isolates detected from blood culture samples in China (17).

In the current study, the presence of enterotoxin-coding genes was evaluated by genotyping method. Although, several factors including host, pathogen and genetic variables are important for pathogenesis of *S. aurous* or any other bacteria; however, determination of the exact role of each factor is difficult. The existence of the enterotoxin coding gene does not indicate the ability of bacteria for enterotoxin production and pathogenesis; but generally, isolates with more virulence genes show higher pathogenesis abilities, resulting in higher treatment costs, longer hospitalizations, and higher mortality rates for the involved patients. Genetics investigations of clinical MRSA could provide a global and comprehensive aspect of risk prediction, which can be suitable for short-term and long-term health care policies.

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