ORIGINAL ARTICLE

Oman Medical Journal [2018], Vol. 33, No. 2: 134-140

The Relationship Between Prevalence of Antibiotics Resistance and Virulence Factors Genes of MRSA and MSSA Strains Isolated from Clinical Samples, West Iran

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ARTICLE INFO

Article history:
Received: 12 October 2017
Accepted: 25 November 2017

Online:
DOI 10.5001/omj.2018.25

Keywords:
Methicillin-Resistant *Staphylococcus aureus*; Antibiotic; Staphylococcal Enterotoxins.

ABSTRACT

Objectives: We sought to evaluate the relationship between the prevalence of antibiotics resistance and virulence factors genes in methicillin-resistant *Staphylococcal aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) strains from clinical samples taken in west Iran. ***Methods:*** We performed a cross-sectional study using 100 MRSA and 100 MSSA samples isolated from clinical specimens. We used biochemical methods to identify the isolates, which were confirmed by the polymerase chain reaction (PCR) assay. Antibiotic susceptibility testing was performed using disk diffusion. PCR detected the presence of virulence factors, including enterotoxin genes, toxic shock syndrome toxin-1 (*TSST-1*), and exfoliative toxin. ***Results:*** The majority of MRSA isolates exhibited a high level of resistance to common antibiotics and susceptible to vancomycin, while most MSSA isolates were also resistant to erythromycin and ciprofloxacin. The prevalence of Staphylococcal enterotoxins (SEs) were reported 147 (73.5%). Among 100 MRSA samples, 92 (92.0%) harbored *SAg* genes. The most frequent toxin gene was *sea* (45.0%) followed by *sec* (39.0%). Among 100 MSSA isolates, 89 (89.0%) harbored *SAg* genes and the most prevalent genes were *sea* (42.0%), *sek* (38.0%), *sec* (35.0%,), and *TSST-1* (10.0%). The prevalence of *TSST-1* and exfoliative toxin genes in MRSA samples were 12 (12.0%). The association of *SAg* genes with MRSA and MSSA isolates showed a high prevalence of enterotoxin *seq*, *seg,* and *sei* in MRSA than MSSA with a statistically significant difference ($p < 0.050$). ***Conclusions:*** The prevalence of MRSA and the association of pathogenic agents with antibiotics resistance genes can lead to the emergence of strains with higher pathogenicity and less susceptibility.

Staphylococcus aureus isolates have evolved as common pathogens linked to serious community and hospital-acquired infections and have been considered as a major public health problem throughout the world for a long time.[1] These organisms express a wide range of virulence factors implicated in bacteria pathogenesis including surface attachment proteins which facilitate their adherence to injured tissues.[2] Depending on the strain, *S. aureus* is capable of secreting enzymes such as thermonuclease, lipase, hyaluronidase, and hemolysin. *S. aureus* is often present in food products,[3] and is one of the causes of bacterial foodborne intoxications via the output of staphylococcal enterotoxins (SEs), which are heat-stable and act as superantigens.[4] Furthermore, *S. aureus* is a human commensal colonizer and the nasal carriage is a frequent route of nosocomial infection transmission.

S. aureus can quickly gain resistance to a variety of antimicrobials such as methicillin. The first evidence of methicillin-resistant *S. aureus* (MRSA) isolates arrival was in 1961.[5,6] Nowadays, MRSA has become a major cause of hospital-associated infections worldwide.[7] There is an alarming increase in community-acquired MRSA (CA-MRSA) infections.[7] Most strains of *S. aureus* code superantigens, including *SEA* to *SEE*, *SEG* to *SER*, *SEU,* and toxic shock syndrome toxin -1 (*TSST-1*).[8] Most of these superantigens are encoded by genes located on mobile genetic elements or comprising insertion elements or plasmids or transposons,

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so-called pathogenicity islands (e.g., *TSST-1* and some enterotoxins) or lysogenic bacteriophages.[9]

Superantigens that induce vomiting in a primate model are designated the classical SE type. Those that lack the activity or have not been implicated in this condition are classified as the SE-like (SEls) type.[10] The staphylococcal enterotoxin F (SEF) lacks emetic activity, but it is associated with *TSST-1*.[11] Other studies have exhibited that staphylococcal phage Q3 carries *sea* (in strain Mu50), *sep* (strain N315), or in case of *sea-sek-seq* (strain MW2) genes.[12] On the other hand, a family of pathogenicity islands carry *seb-sek-seq* (e.g., SaPI1 in strain COL), *TSST-sec3-sel* (SaPI2 in strains N315 and Mu50), or *sec-sel* (SaPI3 in strain MW2).[13] The locus encoding the enterotoxins SEG, SEI, SEM, SEN, and SEO is currently known as *egc* (enterotoxin gene cluster).[14] It is intriguing that the prevalence of *egc* gene in isolate of *S. aureus* seems to be negatively correlated with the severity of infection. For SEA, the situation is precisely the opposite: the toxin gene is significantly more often present among invasive isolates.[15]

This study deals with development of a rapid, reliable and low-cost polymerase chain reaction (PCR)-based protocol for the detection of most known *SE* genes, the *TSST-1* gene, exfoliative toxins, and *egc* in *Staphylococcus* spp.

METHODS

Two-hundred clinical samples were collected from July 2013 to June 2014 in Beheshti, Besat, Sina, and Fatemieh Hospitals, Hamadan, Iran, as shown in Table 1. The isolates were identified at species level using routine microbiological methods and *S. aureus* ATCC 25423 and *S. epidermidis* were used as positive and negative controls, respectively. PCR targeting of *nuc* and *mecA* genes were used to confirm phenotypic speciation and methicillin resistance.[16]

Antibiotic susceptibility testing were carried out using the Kirby-Bauer disk diffusion technique according to Clinical and Laboratory Standard Institute 2013 guidelines,[17] Applied antibiotics (Mast, England) including cefoxitin 30 µg, tetracycline (TE) 10 µg, gentamycin (GM) 10 µg, erythromycin (E) 15 µg, vancomycin (VAN) 30 µg, amikacin (AK) 30 µg, imipenem (IMP) 10 µg, linezolid (LZ) 10 µg, tigecycline (TG) 15 µg, ciprofloxacin (CIP) 5 µg, clindamycin (CD) 2 µg, tobramycin (TO) 10 µg, rifampin (RF) 5 µg, and trimethoprim/sulfamethoxazole (TMP/SMX) 1.25/23.75 µg. Methicillin resistance was examined using cefoxitin disk and confirmed by the *mecA*-specific PCR.

Total DNA was extracted using the DNA extraction Kit (BioFlux Co., Japan), according to the manufacturer's instructions. Quality of extracted DNA was assessed spectrophotometrically by the Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, DE, USA). Following the DNA extraction, PCR targeting of *nuc* gene was used for confirmation of *S. aureus* strains. PCR was carried out in a final volume of a 20 µL reaction mixture containing 10 µL of 2X Taq premix Mastermix (Parstous Biotech Co., Iran), 5 µL of sterile double distilled water, 1 µL of forward primer, 1 µL of reverse primer, and 3 µL of DNA sample. The DNA of samples, as well as the DNA of positive control (*S. aureus* ATCC 25423) and a negative control (*S. epidermidis*), were amplified by an initial denaturation step for 5 minutes at 94 °C followed by 35 cycles of 94 °C for 60 seconds, 50 °C for 60 seconds, and 72 °C for 1 minute and a final extension step at 72 °C for 10 minutes in a Bio-Rad Thermal Cycler (Bio-Rad Laboratories, Inc., USA). The products PCR were subjected to 1.5% agarose gel electrophoresis.

The PCR assay targeting *mecA* gene coding methicillin resistance was performed for all isolates. Primer sequences included: forward (5'-GTAGAAATGACTGAACGTCCGATAA-3') and reverse (5'- CCAATTCCACATTGTTTCG GTCTAA-3'). *S. aureus* ATCC 25923 was included as the positive control. PCR was carried out in a final volume of a 20 µL. The initial denaturation was at 92 °C for 5 minutes. Denaturation at 94 °C for 60 seconds, annealing at 56 °C for 1 minute, and extension at 72 °C for 1 minute was maintained for 35 cycles.

Each isolate was tested by seven multiplex PCRs according to the method of Holtfreter for the genes: (i) *sea*, *seh,* and *seo*; (ii) *sed*, *etd,* and *eta*; (iii) *see*, *seb,* and *sem*; (iv) *sen*, *seq,* and *sej*; (v), *ser*, *seu,* and *sep*; (vi) *sel*, *sei,* and *TSST*; and (vii) *seg*, *sek,* and *sec*. Primers sequences are provided in Table 2. The reaction mixture for the PCR assay was 20 µL, and was prepared as previously described. DNA denaturation at 95 °C for 5 minutes was followed by 35 cycles of amplification (95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 60 seconds), ending with a final extension at 72 °C for 10 minutes.[18,19]

Table 1: Primer sequences.

Gene	Target genes	Primer sequence (5'-3')	PCR product (bp)
Multiplex 1	*sea*	F:GAAAAAAGTCTGAATTGCAGGGAACA	560
	seh	R : CAAATAAATCGTAAT TAACCGAAGGTTC	780
	seo	F : CAATCACATCATAT GCGAAAGCAG	180
		R: CATCTACCCAAAC ATTAGCACC	
		F: AGTTTGTGTAAGAAG TCAAGTGTAGA	
		R: ATCTTTAAATTCAGC AGATATTCCATCTAAC	
Multiplex 2	*sed*	F: GAATTAAGTAGTACC	492
	etd	GCGCTAAATAATATG	358
	eta	R: GCTGTATTTTTCC TCCGAGAGT	190
		F: CAAACTATCATGTAT CAAGGATGG	
		R: CCAGAATTTCCC GACTCAG	
		F: ACTGTAGGAGCTA GTGCATTTGT	
		R: TGGATACTTTTGTCT ATCTTTTTCATCAAC	
Multiplex 3	*see*	F: CAAAGAAATGCTTTA	482
	seb	AGCAATCTTAGGC	404
	sem	R: CACCTTACCGCCAAAGCTG	326
		F: ATTCTATTAAGGACA CTAAGTTAGGGA	
		R: ATCCCGTTTCATAAGGCGAGT	
		F: CTATTAATCTTTGG GTTAATGGAGAAC	
		R: TTCAGTTTCGACAG TTTTGTTGTCAT	
Multiplex 4	*sen*	F: CGTGGCAATTAGACGAGTC	474
	seq	R:GACTCGTCTAATTGCCACG	204
	sej	F: ACCTGAAAAGCTTCAAGGA	138
		R: CGCCAACGTAATTCCAC	
		F: TCAGAACTGTTGTTCCGCTAG	
		R:GAATTTTACCAYCAAAGGTAC	
Multiplex 5	*sep*	F:GAATTGCAGGGAACTGCT	182
	seu	R:GGCGGTGTCTTTTGAAC	215
	ser	F:AATGGCTCTAAAATTGATGG	363
		R:ATTTGATTTCCATCATGCTC	
		F:AGCGGTAATAGCAGAAAATG	
		R:TCTTGTACCGTAACCGTTTT	
Multiplex 6	*TSST*	F:TTCACTATTTGTAAA	180
	sel	AGTGTCAGACCCACT	234
	sei	R: TACTAATGAATTTTT TTATCGTAAGCCCTT	461
		F: GCGATGTAGGTCCAGGAAAC	
		R: CATATATAGTACGA GAGTTAGAACCATA	
		F: CTAGCGGAACAACAGTTCTGA	
		R: AGGCAGTCCATCTCCTG	
Multiplex 7	*seg*	F:TCTCCACCTGTTGAAGG	323
	sek	R:AAGTGATTGTCTATTGTCG	134
	sec	F:ATGCCAGCGCTCAAGGC	275
		R:AGATTCATTTGAAAA TTGTAGTTGATTAGCT	
		F:CTTGTATGTATGGAGG AATAACAAAACATG	
		R:CATATCATACCAAAA AGTATTGCCGT	

PCR: polymerase chain reaction; TSST: toxic shock syndrome toxin.

One sample of each enterotoxin, as well as *mecA* PCR products (amplicons), was sequenced by Bioneer Co., Korea mediated by Takapouzist Co., Iran and the data were analyzed using the Chromas software.

Table 2: The distribution of isolates among clinical specimens.

Samples	MRSA	MSSA	Total
Sputum	19	9	28
Pus	3	1	4
Blood	40	18	58
Puncture	15	6	21
Urine	21	11	32
Pharyngeal swabs	2	5	7
Nasal swabs	0	50	50
Total	100	100	200

MRSA: methicillin-resistant staphylococcal aureus; MSSA: methicillin-sensitive S. aureus.

Data was analyzed using SPSS Statistics (SPSS Inc. Released 2007. SPSS for Windows, Version 11.0. Chicago, SPSS Inc). Descriptive statistical methods were used to determine the frequency, percentage, and mean. Chi-square test was used to compare the qualitative results, and independent *t*-test to compare quantitative data. A *p*-value ≤ 0.050 was considered significant in comparative data.

RESULTS

Among the 200 specimens, the total number of samples from males was 105 (52.5%) and 95 from females (47.5%). The distribution of isolates amoung the samples is shown in Table 2.

The results of antibiotic resistance of MRSA and MSSA isolates have been presented in Table 3. Chi-squared analysis of the presence of the virulence and resistance genes showed preferential distribution to MRSA in comparison with MSSA isolates ($p \leq 0.050$). There was a significant relationship between the pathogenicity of MSSA and MRSA and the presence of antibiotics resistance agents.

In our study, MRSA isolates were more prevalent in males (54.0%) than females (46.0%), whereas MSSA isolates were more prevalent in females (59.0%) than males (41.0%) with no significant statistical difference ($p = 0.109$). There was no significant association of MRSA and MSSA with age ($p = 0.126$).

The distribution of *SAg* genes among MRSA and MSSA strains is presented in Table 4. Among MRSA isolates, 92 (92.0%) harbored *SAg* genes and the most frequent toxin gene was *sea* (45.0%), followed by *sec* (39.0%). Among MSSA isolates, 89 (89.0%) harbored *SAg* genes and the most prevalent genes were *sea* 42 (42.0%), *sek* 38 (38.0%), *sec* 35 (35.0%), and *TSST-1*, which were harbored by 10.0% of strains. None of the investigated isolates carried the *eta* gene. The *etd* gene in MRSA was detected only in two strains that belonged to puncture samples. In MSSA, *etd* was detected in two strains isolated from blood and puncture samples. The association of *SAg* genes with MRSA and MSSA isolates showed a higher prevalence of enterotoxin *seq*, *seg*, and *sei* in MRSA than MSSA with significant difference ($p = 0.014$), ($p = 0.001$) and ($p = 0.009$), respectively [Table 4]. There were no significant difference among MRSA and MSSA with other *SAg* ($p > 0.050$).

The results of the basic local alignment search tool (BLAST) of intended genes product indicated

Table 3: The pattern of antibiotics resistance among 100 MRSA and 100 MSSA isolates.

Antibiotics	Antibiotic resistance of MRSA and MSSA clinical isolates, n (%)			
	MRSA	MSSA	Overall resistance	*p*-value
Tetracycline	91 (91.0)	52 (52.0)	143 (71.5)	< 0.001
Gentamycin	90 (90.0)	25 (25.0)	115 (57.5)	0.009
Erythromycin	92 (92.0)	68 (68.0)	160 (80.0)	< 0.001
Vancomycin	-	-	-	-
Cefoxitin	100 (100)	-	100 (50.0)	< 0.001
Clindamycin	80 (80.0)	46 (46.0)	126 (63.0)	0.001
Ciprofloxacin	95 (95.0)	66 (66.0)	161 (80.5)	< 0.001
TMP/SMX	85 (85.0)	66 (66.0)	151 (75.5)	< 0.001
Rifampin	85 (85.0)	45 (45.0)	130 (65.0)	< 0.001

TMP/SMX: trimethoprim-sulfamethoxazole.; MRSA: methicillin-resistant staphylococcal aureus; MSSA: methicillin-sensitive S. aureus.

Table 4: Prevalence of *SAg* genes among 100 MRSA and 100 MSSA strains, n (%).

Toxin gene	MRSA	MSSA	Total	*p*-value
sea	45 (45.0)	42 (42.0)	87 (43.5)	0.097
seb	34 (34.0)	31 (31.0)	65 (32.5)	0.190
sec	39 (39.0)	35 (35.0)	74 (37.0)	0.067
sed	32 (32.0)	28 (28.0)	60 (30.0)	0.066
see	23 (23.0)	22 (22.0)	45 (22.5)	0.351
seh	34 (34.0)	24 (24.0)	58 (29.0)	0.297
sek	34 (34.0)	38 (38.0)	72 (36.0)	0.771
sel	20 (20.0)	22 (22.0)	42 (21.0)	0.581
sej	15 (15.0)	18 (18.0)	33 (16.5)	0.186
sem	19 (19.0)	21 (21.0)	40 (20.0)	0.911
sen	17 (17.0)	20 (20.0)	37 (18.5)	0.472
seo	22 (22.0)	20 (20.0)	42 (21.0)	0.071
sep	24 (24.0)	22 (22.0)	46 (23.0)	0.945
seq	34 (34.0)	22 (22.0)	56 (28.0)	0.014
ser	14 (14.0)	16 (16.0)	30 (15.0)	0.413
seu	26 (26.0)	23 (23.0)	49 (24.5)	0.852
seg	21 (21.0)	13 (13.0)	34 (17.0)	0.001
sei	21 (21.0)	15 (15.0)	36 (18.0)	0.009
etd	2 (2.0)	2 (2.0)	4 (2.0)	0.360
TSST-1	12 (12.0)	10 (10.0)	22 (11.0)	0.058

MRSA: methicilin-resistant staphylococcus aureus; MSSA: methicilin-resistant S. aureus; TSST: toxic shock syndrome toxin.

that it has the same DNA sequences and all PCR assay results were confirmed.

DISCUSSION

In this study, the frequency of MRSA among *S. aureus* isolates was 50.0%. Our results are in agreement with another study from Shiraz that reported a MRSA prevalence of 42.3%.[20] Among the 100 confirmed MRSA isolates, 46 (46.0%) and 54 (54.0%) were isolated from females and males, respectively, which is similar to previous findings in Shiraz, Iran.[20] In addition, higher numbers of MRSA was observed in patients aged 45–60 and 15–30 years old, which is consistent with the results from a Malaysian study.[21] Studies on the prevalence of MRSA isolates in clinical samples are inconsistent. Some studies, such as our results, indicated a higher prevalence of MRSA in blood (40.0%) and very low rates (5.0%) in pus and pharyngeal swabs samples. In the study performed by Alfatemi et al,[20] most cases of MRSA (39.79%) were obtained from sputum and the fewest number (2.05%) were gathered from eye and nose samples. A higher percentage of MRSA was found in blood and urine than other specimens was similar to other studies.[21,22]

Most MRSA isolates were multiple-drug resistant. Resistance to TE and GM was higher in MRSA isolates than MSSA isolates, which in conflict with Asadollahi et al,[23] study. Although some investigations in the recent years have reported increasing number of VAN-resistant MRSA isolates, the results of the current study showed that VAN is still the main antibiotic of choice for treatment of serious and threatening infections caused by MRSA and MSSA isolates. Our results also demonstrated that all isolates were susceptible to VAN, which is in agreement with another study from Iran.[23]

Studies on the prevalence of enterotoxin gene in MRSA and MSSA isolates are inconsistent. We observed a significant association among the blood samples and high prevalence of enterotoxin in blood ($p = 0.001$) were observed, while there was no significant relationship between *SAg* with other samples ($p > 0.050$). The frequency of the *sea* gene in MRSA isolates from various specimens in our study was 45.0%. This figure is similar to studies performed in Tehran (32.07%),[24] Gorgan (58.8%),[25] and Shiraz (27.39%),[20] Iran, but higher than that

of a study from Germany (12%),[17] Ilam (17%),[23] Iran, and Korea (17.5%).[26] Report of *sea* (35.4%) gene prevalence in China was consistent with our result.[19] In our study, the frequency of the *seb* gene was 34.0%, which was inconsistent with other studies in Iran (Gorgan 61.3%,[25] Ilam 2%,[23] and Tehran 73.58%)[27] and studies from other countries (China 5%,[28] Canada 15.78%).[29] These differences could be associated with geographical differences, number of samples, and year. The frequency rates of *sed* gene in studies performed in Tehran,[28] Shiraz,[20] Korea,[30] and Columbia,[31] were 3.77%, 2.05%, 2.9%, and 7%, respectively.

In this study, 90.5% of isolates carried at least one type of enterotoxin. This result was inconsistent with the results obtained by Asadollahi et al,[23] and in accordance with Alfatemi et al.[20] These discrepancies might be due to the different study locations. There was no significant correlation between enterotoxins of MRSA and MSSA strains observed, except for *seq*, *seg,* and *sei,* which had a statistically significant difference $p = 0.014$, $p = 0.001$ and $p = 0.009$, respectively.

The frequency of *TSST-1* gene in MRSA strains in our study was 12.0%, which were obtained from blood (8%) and nasal septal (4%). Our results were consistent with a study from Shiraz (11.64%)[20] and Germany (9.14%),[17] and conflicting with those from Ilam (46%)[23] and several other countries (Korea 72.2%, Czech 50%, and 0–78% in the US). In our study, none of the investigated isolates harbored the *eta* gene. The frequency rates of *eta* gene in different studies from Iran were very low (Shiraz and Ilam were 0.68% and 1%, respectively).[20,23] This was also the finding in other countries including Germany, Turkey, and Colombia[31] (2%, 19.2%, and 3%, respectively). Furthermore, in our study, the frequency rate of *etd* gene in all strains of *S. aureus* was 2.0%, while the *etd* gene was not found in any of the isolates tested by Wu et al.[19] The frequency of MRSA enterotoxin genes in different countries and even within a country in different cities or hospitals, have not been determined to be the same. This could be due to differences in the geographical features of each country, the hospital wards where the specimens collected from, number of samples, patients, and health conditions.

One of the strengths of our study compared to similar studies is the number of clinical samples taken from different locations and also the application of seven multiplex PCR complex for detection more than of 20 Staphylococcus toxin genes. Our study is limited by not using advanced typing methods such as pulsed-field gel electrophoresis or multilocus sequence typing for epidemiological interpretation.

CONCLUSIONS

Our isolates exhibited a high rate of resistance to CIP and TMP/SMX. There is a significant relationship between virulence genes and antibiotic resistance patterns. Genetic investigations of clinical MRSA and MSSA could provide a global and comprehensive aspect of risk prediction, which can be suitable for short-term and long-term health care policies.

Disclosure

The authors declared no conflicts of interest. The authors would like to acknowledge the Vice Chancellor of Hamadan University of Medical Sciences for funding of the current study.

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