

Evaluation of methicillin-resistant *Staphylococcus* virulence genes and antibiotics susceptibility in Iranian population

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Background: Methicillin resistance *Staphylococcus aureus* (MRSA) is one most important pathogens for human health. The ability of this organism for producing different kinds of disease is related to its virulence gene. The frequency of hemolysin alpha (hla), hemolysin beta (hly), and exfoliative toxin A (eta) virulence genes of MRSA was evaluated, and the association of these genes with antibiotics susceptibility was investigated. **Materials and Methods:** In a cross-sectional study, a total of 695 *Staphylococcus* clinical samples from seven different provinces of Iran were evaluated. MRSA was detected by cefoxitin disk. Virulence genes were detected by polymerase chain reaction. Susceptibility to clindamycin and ciprofloxacin was evaluated according to the Clinical and Laboratory Standards Institute guideline. **Results:** From a total of 695 samples, 170 (24.46%) were found to be MRSA. 142, 82, and 132 samples of MRSA were hla, hly, and eta positive, respectively. hla gene was significantly found more frequently in patients at least 18 years ($P = 0.02$). 105 (68.6%) and 93 (59.6%) of MRSA samples were resistance to ciprofloxacin and clindamycin, respectively. hly gene was significantly more resistant to clindamycin ($P = 0.04$) and ciprofloxacin ($P = 0.01$). Logistic regression analysis displayed hly-positive MRSA strains were significantly associated with ciprofloxacin (odds ratio [OR]: 3.6, 95% confidence interval [CI] = 1.637–8.00) and clindamycin (OR: 1.93, 95% CI 1.00–3.68). **Conclusion:** MRSA strains from *Staphylococcus aureus* which isolated from hospitalized Iranian patients are significantly resistant to clindamycin and ciprofloxacin and it may be because of hly virulence gene. These samples consist of both community-acquired MRSA and health-care associated MRSA, so we could not use this finding as a guide for local antibiotics usage.

Key words: Ciprofloxacin, clindamycin, methicillin-resistant staphylococcus, virulence gene

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INTRODUCTION

Staphylococcus aureus is one of the main human pathogens causing various infectious diseases with different severity.^[1] Methicillin resistance *staphylococcus aureus* (MRSA) is one of the most considerable kinds of this microorganism due to beta-lactam antibiotics resistance.^[2] During the first 30 years of recognition of this organism, MRSA caused infections in hospitalized patients in a way that healthcare-associated MRSA (HA-MRSA) was introduced

afterward.^[3,4] Furthermore, community-acquired MRSA (CA-MRSA) became widespread, especially from 1990.^[5] Universal appearance of MRSA is a major problem of health-care facilities worldwide.^[5] The ability of this kind of *Staphylococcus* to cause different infections is mostly due to virulence genes.^[6] The presence of these virulence factors facilitates invading the host immune system and different tissues.^[7]

Exotoxins and exfoliating toxins are two major virulence factors of *Staphylococcus*.^[8] Among exotoxins, hemolysin alpha (*hla*) causing neurotoxicity and dermo

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toxicity by producing pores in cell membranes of host and hemolysin beta (*hly*) degrading the sphingomyelin of outer layer of human red blood cells are two examples of known exotoxins. In contrast, exfoliations are proteases cleaving superficial layer of skin consisted of desmosomal Catherine.^[8,9]

Recent researches revealed that disease severity and antibiotics susceptibility of this genus are different in various nations.^[10,11] To the best of our knowledge, there are few studies evaluating the association of exotoxins and exfoliative toxin and antibiotic susceptibility of *Staphylococcus* (R1). Therefore, in this study, we aimed to evaluate the prevalence of *hla* and *hly* as exotoxins and exfoliative toxin A (*eta*) and its association with clindamycin and ciprofloxacin susceptibility of MRSA among Iranian patients.

MATERIALS AND METHODS

Sample selection

In a cross-sectional study done from April 2017 to April 2018, 695 *Staphylococcus aureus* specimens were gathered from seven different laboratories in seven distinct provinces of Iran which were accredited by the Iranian Health Ministry for detection of MRSA. These samples were obtained from wound, blood, tracheal aspiration, sputum, ear discharge, chest tube, bronchoalveolar lavage, bone, urine, synovial, and pleural effusion fluid, *S. aureus* isolates were obtained from/hospitalized patients with clinical signs and symptoms of colonization such as increased fever and white and blood cell counts admitted to hospital during 1 year. The study protocol was reviewed by the Ethical Committee of Shahid Beheshti University of Medical Sciences and approved by this IR.SBMU.MSP.REC.1397.631.

Detection of methicillin resistance *Staphylococcus aureus* Mueller–Hinton agar medium and 30 µg cefoxitin antibiotic disc (Mast Co., UK) were used according to CLSI (Clinical and Laboratory Standards Institute) guidelines.^[12,13] Each strain with ≤21-mm inhibition zone around the cefoxitin after 16–18 h of incubation was considered positive MecaA and consequently MRSA strains.^[13,14]

Ciprofloxacin and clindamycin susceptibility

Susceptibility to ciprofloxacin (5 µg) and clindamycin (2 µg) was performed with Kirby–Bauer method on Mueller–Hinton agar [Tables 4-6]. The results are interpreted by CLSI breakpoint. Control strain was *S. aureus* (ATCC 25923).^[13,14]

Molecular detection of virulence genes

DNA was extracted by High Pure polymerase chain reaction (PCR) Template Preparation Kit (Thermo Fisher Co., USA) according to the standard protocol. The presence

of the virulence genes including *hla*, *hly*, and *eta* was distinguished by conventional PCR and specific primers shown in below [Table 1].^[19,20] PCR reactions were performed in a final volume of 25 µL containing the 12.5 µL of master mix (Cinnaclon, Tehran, Iran), 8.5 µL of distilled water, 10 pmol of each primer, and 1 µL of template DNA (3 µg/µL). The thermal cycling condition of the PCR mixture included an initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min; 30 s at 53°C for *hla* and *hly* and 51°C for *eta* followed with primer extension at 72°C for 45 s. The final extension step was done at 72°C for another 5 min. The PCR products were analyzed by gel document after electrophoresis.^[15-17]

Statistical analysis

Categorical variables were reported as frequency (percentage). Fisher exact and Chi-square tests were used for the analysis of nominal data. All analyses were done using the Statistical Package for Social Sciences (SPSS Inc., version 21.0, Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

RESULTS

Methicillin resistance *staphylococcus aureus* prevalence

From a total of 695 *S. aureus* isolated, 170 were found to be MRSA (24.46%). One hundred and fifteen (75.25%) MRSA belonged to adult patients older than 18 years and the rest of related to pediatrics (<18-year-old). Among seven different provinces, MRSA was more prevalent in Isfahan (26.8%). Figure 1 depicts the distribution of MRSAs and their virulence genes in seven different Iranian cities. The highest frequency of MRSAs belonged to Isfahan (23.7%), and the lowest frequency of MRSAs showed up in Kordestan.

Antibiotic susceptibility and virulence genes

105 (68.6%) and 93 (59.6%) MRSA samples were resistance to ciprofloxacin and clindamycin, respectively.

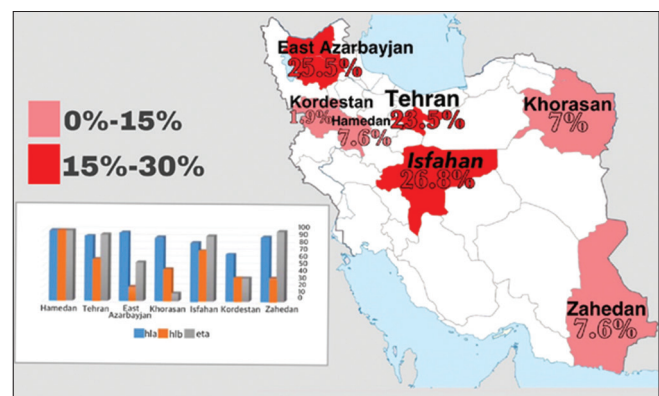


Figure 1: Distribution of methicillin resistance *staphylococcus aureus* and its virulence genes in eight different Iranian cities

Table 3 shows the distribution of clindamycin resistance MRSA according to virulence genes. Our data suggested that MRSA with *hly* gene was significantly more resistant to clindamycin ($P = 0.04$).

Data analysis provided in Table 3 also revealed the same results in terms of ciprofloxacin susceptibility ($P = 0.01$).

Virulence genes evaluation

Molecular method showed that 142, 82, and 132 samples of MRSA were *hla*, *hly*, and *eta* positive, respectively. Among virulence gene, *hla* was more prevalent in MRSA strains (90.4%).

Although virulence genes were not significantly different in females and males ($P = 0.54$), *hla* gene was significantly found more frequently in patients at least 18 years ($P = 0.02$) [Table 2].

In the evaluation of coexistence of these three virulence genes, data revealed that none of the samples were *hla* and *hly* positive, 124 (79%) were *hla* and *eta* positive, and 81 (51.6%) of them were positive for all virulence genes.

Figure 1 shows the distribution of MRSA and its virulence gene.

Moreover, logistic regression analysis displayed *hly*-positive MRSA strains were significantly associated with ciprofloxacin (odds ratio [OR]: 3.6, 95% confidence

interval [CI]=1.637–8.00) and clindamycin (OR: 1.93, 95% CI 1.00–3.68) administration.

DISCUSSION

MRSA strains can cause the vast range of the infections and make therapeutic complications because of antibiotic resistance.^[1] A few studies suggested relationship among exotoxins and exfoliative toxin and antibiotic susceptibility in MRSA strains (R1). The present study showed the frequency of MRSA among our samples was 24.46%. On the other hand, 68.6% and 59.6% of MRSA were resistant to ciprofloxacin and clindamycin, respectively. We also found that MRSA strains which had *hly* gene were 3.62 and 1.93 times more resistant to ciprofloxacin and clindamycin, respectively. *hla* virulence gene was the most frequent one found in this population suffering from MRSA infection. Moreover, we found that MRSA-isolated samples significantly consisted more *hla* virulence factor.

A meta-analysis study in 2014 revealed that the CA-MRSA prevalence was approximately 50.2% in pediatric and 42.3% in adult populations.^[16] Our study presented a lower frequency of MRSA than in the mentioned meta-analysis and other studies in North America and Europe.^[18-21] One study in Shiraz, Iran, showed that CA-MRSA prevalence was 42.3% in adult patients.^[22] However, Sedighi *et al.* in Hamadan revealed a prevalence rate of 4.1% for CA-MRSA in children between 1 and 6 years.^[23] Moreover, another study showed that CA-MRSA prevalence is lower in pediatrics than in adult population.^[24] The lower than in aforementioned studies, the prevalence of MRSA in our study might be due to study population which consisted of a combined population of pediatric and adult participants. Another possible reason for the lower frequencies in our study_ is that, although our samples were all MRSA specimens, we did not separate CA-MRSA and HA-MRSA. Furthermore, Ghosh and Banerjee in a study which did not separated the strains of MRSA find the frequency of 23.9% for MRSA in line with our finding.^[25]

Table 1: Oligonucleotide primers used in the study

Gene target	Sequences (5'-3')	Product size (bp)	Reference
<i>hla</i>	F: CTGACTACTATCCAAGAAATTCGATTG	210	[19]
	R: CTTCCAGCCTACTTTTTATCAGT		
<i>hly</i>	F: GTGCACTTACTGACAATAGTGC	310	[20]
	R: GTTGATGAGTAGCTACCTTCAGT		
<i>eta</i>	F: TTTGCTTCTTGATTGGATTG	464	[20]
	R: GATGTGTTCCGGTTTGATTGAC		

hla=Hemolysin alpha; *hly*=Hemolysin beta; *eta*=Exfoliotoxin A

Table 2: Methicillin resistance *Staphylococcus aureus* virulence gene distribution according to sex and age

	Frequency (%)	<i>hla</i> (%)		<i>hly</i> (%)		<i>eta</i> (%)	
		Negative	Positive	Negative	Positive	Negative	positive
Sex							
Female	61 (39.4)	7 (11.5)	52 (88)	33 (54.1)	28 (45.9)	14 (23)	47 (77)
Male	94 (60.6)	7 (7.4)	87 (92.6)	18 (47.4)	56.4 (20)	10.6 (4)	89.4 (34)
<i>P</i>		0.39		0.20		0.38	
Age							
≤9	28 (18.3)	6 (21.40)	22 (78.6)	14 (50)	14 (50)	3 (10.7)	25 (89.6)
10-18	10 (6.5)	1 (10.0)	9 (90)	4 (40)	6 (60)	1 (10)	9 (90)
≥18	115 (75.2)	7 (6.1)	108 (93.9)	53 (46.1)	62 (53.9)	20 (17.4)	95 (82.6)
<i>P</i>		0.02		0.89		0.14	

MRSA=Methicillin resistant *Staphylococcus aureus*; *hla*=Hemolysin alpha; *hly*=Hemolysin beta; *eta*=Exfoliotoxin A

Table 3: Clindamycin resistant methicillin-resistant *Staphylococcus aureus* and hemolysin alpha, hemolysin beta, and exfoliotoxin A

	<i>hla</i> (%)		<i>hlb</i> (%)		<i>eta</i> (%)	
	Positive	Negative	Positive	Negative	Positive	Negative
Clindamycin						
Sensitive	59 (41.8)	4 (26.7)	27 (32.9)	36 (48.6)	51 (398.9)	12 (48)
Resistant	82 (58.2)	11 (73.3)	36 (48.6)	38 (51.4)	80 (61.1)	13 (52)
<i>P</i>	0.25		0.04		0.50	

MRSA = Methicillin resistant *Staphylococcus aureus*; *hla*=Hemolysin alpha; *hlb*=Hemolysin beta; *eta*=Exfoliotoxin A

Table 4: Ciprofloxacin-resistant methicillin-resistant *Staphylococcus aureus* and hemolysin alpha, hemolysin beta, and exfoliotoxin A

	<i>hla</i> (%)		<i>hlb</i> (%)		<i>eta</i> (%)	
	Positive	Negative	Positive	Negative	Positive	Negative
Ciprofloxacin						
Sensitive	32 (23)	3 (21.4)	17 (21.2)	42 (57.5)	44 (31.7)	4 (28.6)
Resistant	107 (77)	11 (78.5)	63 (78.8)*	63 (78.8)	95 (68.3)	10 (71.4)
<i>P</i>	0.59		0.04		0.1	

MRSA=Methicillin-resistant *Staphylococcus aureus*; *hla*=Hemolysin alpha; *hlb*=Hemolysin beta; *eta*=Exfoliotoxin A

Table 5: The association of clindamycin resistance methicillin-resistant *Staphylococcus aureus* and virulence genes

Virulence gene	OR	95%CI		<i>P</i>
		Lower	Upper	
<i>hla</i>	0.50	0.153	1.66	0.26
<i>hlb</i>	1.93	1.009	3.68	0.04
<i>eta</i>	1.48	0.61	3.42	0.39

MRSA=Methicillin-resistant *Staphylococcus aureus*; *hla*=Hemolysin alpha; *hlb*=Hemolysin beta; *eta*=Exfoliotoxin A; OR=Odds ratio; CI=Confidence interval

Table 6: The association of ciprofloxacin resistance methicillin-resistant *Staphylococcus aureus* and virulence genes

Virulence gene	OR	95% CI		<i>P</i>
		Lower	Upper	
<i>hla</i>	0.41	0.16	2.11	0.41
<i>hlb</i>	3.62	1.63	8.00	0.01
<i>eta</i>	3.17	0.19	1.54	0.25

MRSA=Methicillin-resistant *Staphylococcus aureus*; *hla*=Hemolysin alpha; *hlb*=Hemolysin beta; *eta*=Exfoliotoxin A; OR=Odds ratio; CI=Confidence interval

In the present study, *Staphylococcus* strains were more resistant to clindamycin and ciprofloxacin rather than methicillin. In a study in India, 41.3% of MRSA was resistant to clindamycin.^[25] On the other hand, another study mentioned that more than 55% of MRSA were resistant to ciprofloxacin.^[26] This might be due to the more common usage of these antibiotics than oxacillin administration [Figure 1].^[27-29]

Our outcomes revealed that *hla* virulence gene in methicillin-resistant *Staphylococcus* is more prevalent in comparison with other genes, and this result was consistent with other studies in Iran and other parts of the world.^[21-24,30]

This study declared that *hla*-positive MRSA was significantly more prevalent in adult population. Multiple articles discussed regulatory systems for *Staphylococcus* toxin virulence gene expression, in which one of these systems has been suggested to be accessory gene regulator (*agr*), causing production of autoinducer peptide (AIP). Induction of AIP production by *agr A* would ultimately lead to expression of *hla gene*,^[31-33] and other studies showed that CA-MRSA has higher *agr* levels and HA-MRSA has mutation that found in *agr*.^[34] Subsequent to *agr* mutation, *agr A* expression which produces more *hla gene*, increases. Over ally, the prevalence of CA-MRSA is higher in adult population and HA-MRSA is more prevalent in children. We did not separate HA-MRSA and CA-MRSA in this study. The adult population contributed to a higher proportion of the population of the present study. Therefore, more frequent *hla gene* might be due to more adult population of our study and the higher frequency of CA-MRSA in study population.

Strengths and limitations

Sampling from different states of Iran, including different age spectrum and quite large sample size and also equal methods for sampling and molecular methods were some study strengths, but in the present study, we could not separate the CA-MRSA and HA-MRSA, therefore; the data of *Staphylococcus* resistance to antibiotics could not be used for regional antibiotics prescription guidelines.

CONCLUSION

The present study showed *hlb*-positive MRSA strain was 1.93 and 3.6 times more resistant to clindamycin and ciprofloxacin, respectively. *Staphylococcus* beta toxin which produce by *hlb* gene is contributed to biofilm formation^[35] and this could explain our results. Another possibility could

be this point that *Staphylococcus* beta toxin can stimulate interleukin 8 (IL-8) production, and this expression is associated with antibiotic resistance.^[35-37]

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Conflicts of interest

There are no conflicts of interest.

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