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ORIGINAL ARTICLE

Virulence Factors of *Staphylococcus aureus* Isolates in an Iranian Referral Children's Hospital

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Abstract
Objectives: The clinical importance of *Staphylococcus aureus* (*S. aureus*) is attributed to notable virulence factors, surface proteins, toxins, and enzymes as well as the rapid development of drug resistance. The aim of this study was to compare the occurrence of virulence factors produced by *S. aureus* strains isolated from children in an Iranian referral children's hospital.
Methods: The presence of genes encoding for the enterotoxins A (*sea*), B (*seb*), C (*sec*), D (*sed*), TSST-1 (*tsst*), exfoliative toxin A (*eta*), and exfoliative toxin B (*etb*) were detected by Multiplex polymerase chain reaction (PCR) using specific primers. In addition, the standardized Kirby-Bauer disc-diffusion method was performed on Mueller-Hinton agar.
Results: In total, 133 *S. aureus* isolates were obtained from different patients. Of these *S. aureus* isolates, 64 (48%) were methicillin-resistant *S. aureus* (MRSA), and all of these tested positive for the *mecA* gene. Regarding the classical enterotoxin genes, *sea* gene (40.6%) was the most prevalent followed by *seb* (19.6%), *tsst* (12.8%), *eta* (11.3%), *etb* (9%), *sed* (4.5%), and *sec* (3%). Among methicillin-susceptible *S. aureus* (MSSA) isolates, *seb* and *tsst* were the more prevalent toxins in comparison with MRSA isolates ($p < 0.05$), while the frequency of *sea*, *sed*, *eta*, and *etb* genes were higher among MRSA isolates ($p > 0.05$).
Conclusion: In our study enterotoxin A was produced by 40.6% of the isolates (48% from MRSA and 33% from MSSA isolates) which was higher than in previous reports. According to our results, strict hygiene and preventative measures during food processing are highly recommended.

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1. Introduction

Staphylococcus aureus (*S. aureus*) is the most frequently isolated bacterium among both community-acquired and nosocomial infections [1]. *S. aureus* is the causal pathogen of a wide range of infectious diseases ranging from skin and soft tissue infections to toxin-mediated diseases such as pneumonia and bacteremia [2].

The clinical importance of *S. aureus* is attributed to notable virulence factors, surface proteins, toxins, and enzymes as well as the rapid development of drug resistance [3]. The most associated virulence factors with this microorganism are large numbers of toxins including hemolysins (α, β, γ, δ), leukocidins (Panton-Valentine leukocidin; PVL, LukE/D) [4,5], heat-stable staphylococcal enterotoxins (SEs), which cause the sporadic food-poisoning syndrome or food borne outbreaks, exfoliative toxins (ETA and ETB), and the toxin of toxic shock syndrome-1 (TSST-1) [6,7], which causes food poisoning, enterocolitis, scalded skin syndrome, and toxic shock [8].

The aim of this study was to compare the occurrence of virulence factors produced by *S. aureus* strains isolated from children in an Iranian referral children's hospital.

2. Material and methods

2.1. Sample collection

Clinical *S. aureus* samples were collected from hospitalized patients at an Iranian referral children's hospital in 2012.

2.2. Identification of *S. aureus*

Standard microbiological methods for the identification of microorganisms were applied. All specimens were inoculated onto mannitol salt agar and incubated at 37°C. The identification of *S. aureus* was performed by subsequent Gram staining, the catalase test, and the coagulase test with rabbit plasma [9].

2.3. Antimicrobial susceptibility test

The standardized Kirby-Bauer disc-diffusion method was performed on Mueller-Hinton agar using the following antibiotics: oxacillin (5 μg); vancomycin (30 μg); clindamycin (2 μg); rifampicin (30 μg); amikacin (30 μg); amoxicillin/clavulanic acid (30/15 μg); penicillin (10 μg); chloramphenicol (30 μg); trimethoprim sulfamethoxazole (1.25/23.75 μg); cefazolin (30 μg); and cephalothin (30 μg). Methicillin-susceptible *S. aureus* (MSSA) strains were differentiated from methicillin-resistant *S. aureus* (MRSA) using Mueller-Hinton agar containing 2 mg/mL oxacillin with 4% NaCl [10].

2.4. DNA extraction

DNA was extracted from *S. aureus* isolates using lysostaphin digestion [11]. The pellet of a 1-mL overnight culture was resuspended with 350 μL of lysis buffer (Tris-HCL 0.01 M, EDTA 0.01 M), to which 10 μL of lysostaphin were added. The sample was incubated at 37°C overnight. Equal volumes of phenol/chloroform/isoamylalcohol (25:24:1 by volume) were added, and nucleic acid was precipitated by ethanol using a standard protocol.

2.5. Polymerase chain reaction of the *mecA*

Confirmation of *S. aureus* and methicillin-resistance was achieved by polymerase chain reaction (PCR) targeting the *mecA* gene [10].

2.6. Multiplex PCR for the detection of toxin genes

The presence of genes encoding for enterotoxins A (*sea*), B (*seb*), C (*sec*), D (*sed*),TSST-1 (*tsst*), exfoliative toxin A (*eta*), and exfoliative toxin B (*etb*) was detected by Multiplex PCR using specific primers, as previously described [12]. Multiplex PCR was performed in a total volume of 50 μL containing 25 pmol of each primer, 50 ng of total DNA, 1.5 mM $MgCl_2$, 200 μM dNTP mixture, 1× PCR reaction buffer, and 5 units of Taq DNA polymerase. The thermal cycling conditions included an initial denaturation step at 94°C for 5 minutes followed by 35 cycles of amplification comprising three steps: 2 minutes denaturation at 94°C; 1 minute annealing at 57°C; and 1 minute extension at 72°C, ending with a final extension at 72°C for 7 minutes.

2.7. Statistical analysis

A Microsoft Excel spreadsheet was used for data processing. For comparison tests between the positive isolates from each group, we used the Chi-square test and the Fischer's test for a lower number series. A p value < 0.05 was taken to be significant.

3. Results

In total, 133 *S. aureus* isolates were obtained from different patients. Fifty-seven patients (42.8%) were female (female to male ratio, 0.75), and the mean duration of a hospital stay was 13.25 ± 8.14 days.

Forty-three patients (32.3%) were from the infection unit, with the remainder from the surgical ward ($n = 27$, 20.3%), NICU ($n = 19$, 14.3%), emergency ward ($n = 10$, 7.5%), ICU ($n = 10$, 7.5%), immunology ward ($n = 10$, 7.5%), gastroenterology ward ($n = 7$, 5.2%), and others ($n = 7$, 5.2%).

Of the *S. aureus* isolates, 64 (48%) were MRSA and all of these tested positive for the *mecA* gene.

The presence of toxin genes in the strains isolated from skin and soft tissue, blood, urinary, respiratory, and eye infections is shown in Table 1. The majority of toxin-producing *S. aureus* isolates were isolated from skin and soft tissue infections. There were no significant differences between toxin genes and the type of specimen.

Table 1. The frequency of toxin-producing *Staphylococcus aureus* based on different sites of infection

Toxin	Skin & soft tissue	Eye	Blood	Urine	Respiratory
TSST	7 (41.1%)	2 (11.8%)	7 (41.1%)	0	1 (6%)
ETB	8 (66.6%)	2 (16.6%)	1 (8.3%)	0	1 (8.3%)
SEA	28 (51.8%)	4 (7.4%)	15 (27.7%)	1 (1.8%)	6 (11.1%)
ETA	11 (73.3%)	2 (13.3%)	0	1 (6.6%)	1 (6.6%)
SEB	12 (46.1%)	3 (11.5%)	6 (23%)	3 (11.5%)	2 (7.6%)
SEC	3 (75%)	0	1 (25%)	0	0
SED	2 (33.3%)	1 (16.6%)	2 (33.3%)	1 (16.6%)	0

ETA = exfoliative toxin A; ETB = exfoliative toxin B; SEA = staphylococcal enterotoxin A; SEB = staphylococcal enterotoxin B; SEC = staphylococcal enterotoxin C; SED = staphylococcal enterotoxin D; TSST = toxin of toxic shock syndrome-1.

3.1. Distribution of toxin-encoding genes

Regarding the classical enterotoxin genes, the *sea* gene (40.6%) was the most prevalent followed by *seb* (19.6%), *tsst* (12.8%), *eta* (11.3%), *etb* (9%), *sed* (4.5%), and *sec* (3%). Among MSSA isolates, *seb* and *tsst* were the more prevalent toxins in comparison with MRSA isolates ($p < 0.05$), while the frequency of the *sea*, *sed*, *eta*, and *etb* genes were higher among MRSA isolates ($p > 0.05$) (Table 2).

3.2. Antimicrobial susceptibility of toxin-producing *S. aureus* isolates

The sensitivity of the toxin-producing *S. aureus* isolates to the tested antibiotics is shown in Table 3. The sensitivity pattern showed that none of the toxin- producing *S. aureus* isolates were resistant to vancomycin, whereas all of them were resistant to penicillin. Interestingly, among these isolates, only SEC-producing isolates were susceptible to all of the tested antibiotics except penicillin, amikacin, and amoxicillin/clavulanic acid. Although the majority of the toxin-producing *S. aureus* isolates were susceptible to rifampicin, most of the SEA-producing isolates (90.8%) were resistant to this antibiotic. In addition, the most susceptible antibiotics among these isolates were chloramphenicol (76%) and clindamycin (76%). Although more than half of the toxin-producing *S. aureus* were MRSA, all TSST- and SEC-producing *S. aureus* isolates were susceptible to oxacillin.

4. Discussion

In our study SEA was produced by 40.6% of the isolates (48% from MRSA and 33% from MSSA isolates), which was higher than in previous reports [5,13,14]. In the Sina et al [5] study, among enterotoxin genes, the *seb* gene was the most prevalent (44%) followed by the *sea* gene (32%). However, the frequency of *eta* and *etb* was higher than in previous reports [5,13,14]. In the Kolawole et al [14] study in Nigeria, three (4.9%) of the isolates tested positive for the exfoliative toxin genes while no *etb gene* was detected.

In another study conducted in China of 60 isolates, 30 isolates harbored enterotoxin genes, with *sea* being the most frequent toxin gene (33%), followed by *sec* (15%), *sed* (12%), and *seb* (5%) [15]. In the Sina et al [5] study, none of the strains contained the genes responsible for ETB or SED.

In this study the TSST was found in 12.8% of the *S. aureus* isolates, all of which were MSSA. However, there are some studies that did not find the *tsst* gene in any or <1% of the strains of the isolates [5,15]. In addition, in our study, similar to the Machuca et al [16] report, the *tsst* gene was not detected in any of the MRSA isolates.

In our study 48% of MRSA isolates harbored the *sea* gene, which was lower than reported in the Wang et al [15] study where up to 84.6% of MRSA isolates harbored the *sea* gene. However, other studies have reported a lower frequency of this gene among MRSA isolates [17,18].

Similar to the Wang et al [15] study, we did not detect the *sec* gene among MRSA isolates. However, there are some studies that have reported a higher frequency of this gene [13,15,17]. In this study, *seb* (23%) and *tsst* (23%) genes were more prevalent toxins among MSSA isolates.

According to Araki et al [19], the incidence of the *sec* gene increased significantly in coagulase type II MRSA

Table 2. The frequency of virulence genes of *Staphylococcus aureus* among MRSA and MSSA isolates

	sea	*seb*	*sec*	*sed*	*eta*	*etb*	*tsst*	Total
MRSA	31 (48%)	10 (15%)	0	5 (8%)	8 (12.5%)	7 (11%)	0	64
MSSA	23 (33%)	16 (23%)	4 (6%)	1 (1%)	7 (10%)	5 (7%)	17 (23%)	69
Total	54	26	4	6	15	12	17	133

eta = exfoliative toxin A; *etb* = exfoliative toxin B; MRSA = methicillin-resistant *Staphylococcus aureus;* MSSA = methicillin-susceptible *Staphylococcus aureus; sea* = enterotoxin A; *seb* = enterotoxin B; *sec* = enterotoxin C; *sed* = enterotoxin D; *tsst* = enterotoxin TSST-1.

Table 3. Antimicrobial susceptibility of toxin-producing *Staphylococcus aureus* isolates

	OX		CF		CZ		SXT		C		P		AN		AMC		RIF		CC		V	
Toxin	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
TSST	17	100	16	94.1	14	82.3	16	94.1	14	82.3	0	0	4	23.5	5	29.4	16	94.1	15	88.2	17	100
ETB	5	41.6	8	66.6	7	58.3	8	66.6	10	83.3	0	0	1	8.3	1	8.3	12	100	10	83.3	12	100
SEA	23	42.6	22	40.7	22	40.7	17	31.5	41	76	0	0	7	12.9	5	9.2	5	9.2	41	76	54	100
ETA	7	46.6	9	60	9	60	5	33.3	12	80	0	0	4	26.6	3	20	15	100	11	73.3	15	100
SEB	16	61.5	20	61.5	20	61.5	15	57.7	21	80.7	0	0	8	30.7	3	11.5	25	96.1	4	15.3	26	100
SEC	4	100	4	100	4	100	4	100	4	100	0	0	0	0	0	0	4	100	4	100	4	100
SED	1	16.6	3	50	3	50	3	50	5	83.3	0	0	2	33.3	0	0	5	83.3	4	66.6	6	100

AMC = amoxicillin/clavulanic acid; AN = amikacin; C = chloramphenicol; CC = clindamycin; CF = cephalothin; CZ = cefazolin; ETA = exfoliative toxin A; ETB = exfoliative toxin B; OX = oxacillin; P = penicillin; RIF = rifampicin; SEA = staphylococcal enterotoxin A; SEB = staphylococcal enterotoxin B; SEC = staphylococcal enterotoxin C; SED = staphylococcal enterotoxin D; SXT = trimethoprim sulfamethoxazole; TSST = toxin of toxic shock syndrome-1; V = vancomycin.

with the *mecA* gene, whereas we detected these genes only in MSSA isolates.

Although in the Baba-Moussa et al [1] study, the majority of isolates which produced SEA, SEB, or SEC were MRSA, in our study 42.6% and 61.5% of SEA- and SEB-producing isolates were susceptible to methicillin, respectively.

In view of the high resistance rates to penicillin, amikacin, and amoxicillin/clavulanic acid, empirical treatment of toxin-producing *S. aureus* infections with these antibiotics may not be effective. In addition, the high susceptibility of toxin-producing *S. aureus* to vancomycin and rifampicin indicates that these antibiotics are effective for the treatment of *S. aureus* infections at our hospital.

It has been reported that antibiotic-resistant isolates might be transmitted to humans by the consumption of food products containing such resistant bacteria. Therefore, the extreme use of antibiotics as growth promoters in animal husbandry, especially for those which are frequently used for both humans and animals, should be avoided [6].

In addition, strict hygiene and preventative measures during food processing and also during the distribution and consumption of the final food product, in order to avoid the presence of *S. aureus* and SEs-producing strains in foods, are highly recommended.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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