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Prevalence of *exfoliative toxin* A and B genes in *Staphylococcus aureus* isolated from clinical specimens

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KEYWORDS
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Clinical isolate

Summary
Background: The pathogenicity of *Staphylococcus aureus* is based on the production of various virulence factors. The frequency of these factors can markedly differ according to the geographical region.
Materials and methods: In this study, we investigated the prevalence of two frequent isoforms of exfoliative toxins and *mecA* genes using PCR in 197 clinical isolates obtained from clinical samples during the years 2011 and 2012. The samples were obtained from an educational hospital in northern Tehran, Iran. In addition, the antibiotic susceptibility pattern was studied for each isolate using the disc diffusion method.
Results: In this study, 186 (94.4%), 15 (7.6%) and 172 (86.3%) of the 197 isolates expressed the *eta*, *etb* and *mec*A genes, respectively. In addition, 164 (88.2%) and 12 (80%) strains, which harbored the *eta* and *etb* genes, respectively, were MRSA (methicillin resistant *S. aureus*). Furthermore, the prevalence of the *mec*A, *eta* and *etb* genes was higher among the wound samples (61.2%, 55.8% and 6.09%, respectively). We observed high rates of MDR (multi drug resistance) among our isolates. A significant correlation was detected between the presence of the *mec*A gene and the resistance to oxacillin, gentamicin, kanamycin, erythromycin, tetracycline,

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cotrimoxazole, clindamycin, and cephazolin as well as the multi-drug resistant property ($P < 0.05$). In addition to penicillin, the MDR properties and resistances to the tested antibiotics in the *etb*-positive strains were significantly lower compared to the *etb*-negative strains ($P < 0.05$).
Conclusions: The prevalence of the *eta*, *etb* and *mecA* genes might be due to the specific geographic region.

Introduction

Staphylococcus aureus is known as the main nosocomial pathogen due to its ability to generate a wide range of virulence factors, such as extracellular protein toxins and a large number of anti-microbial resistance compounds. Accordingly, the pathogenicity of the organism causes various diseases, from mild skin and tissue infections or food poisoning to severe systematic infections. In addition, harmful super-antigenic toxins, such as enterotoxins and tsst-1 and exfoliative toxin (ET), which is a pyrogenic exoprotein, result in a dangerous disorder known as staphylococcal scalded skin syndrome. This type of blistering skin disease is divided into two clinical forms, the localized and generalized forms. The generalized form, which is called Ritters disease, frequently occurs in infants and children [1]. However, the localized form, which is termed Bullous impetigo, can afflict at all ages. Moreover, in the Bullous impetigo form, the exfoliative toxin, which produces localized strains, can locally affect the skin. In contrast, in Ritters syndrome, the infecting strains are located at distant sites, and the target tissue receives the toxin via the bloodstream [2].

Three isoforms of ET, ETA, ETB and ETD, which are encoded by the *eta*, *etb* and *etd* genes, respectively, are capable of cleaving desmogelin 1, a cadherin protein, which mediates cell—cell adhesion in keratinocytes. The ETA and ETB toxins are associated with the occurrence of staphylococcal scaled skin syndrome (SSSS) [3]. Despite the genetic similarity and biological function equality, ETA and ETB differ in their immunological characteristics and location of encoding genes. The *eta* gene has a chromosomal origin that is integrated by prophages, whereas the *etb* gene is a plasmidic gene [4]. Furthermore, a higher frequency of the *eta* gene has been reported among the *S. aureus* strains [1].

To the best of our knowledge, there is no information on the prevalence of *S. aureus* strains carrying the *eta* and *etb* genes in isolations obtained from clinical samples in Iran. In this study, we aimed to assess the prevalence of *S. aureus* strains harboring the exfoliative-encoding genes in clinical isolates. In addition, the antimicrobial pattern susceptibility of each strain and the correlation of this pattern with the presence of these genes are investigated.

Materials and methods

Bacterial isolates

A total of 197 *S. aureus* strains were randomly collected from clinical samples, including wounds, blood, bail, tracheal aspirate, sputum, ear, chest tube, bronchoalveolar lavage (BAL), bone, urine, stool, synovial and pleural effusion samples, during the years 2011 and 2012 at an educational hospital in northern Tehran, Iran. The isolated *S. aureus* strains were confirmed using a gram-staining test; catalase, coagulase and DNAse activity tests; a mannitol fermentation test and the presence of hemolysis on a blood agar plate after 24 h growth at 37 °C. Standard *S. aureus* strains named MN8 and ATCC 25923 were applied as positive and negative controls, respectively.

Antimicrobial susceptibility testing

The antimicrobial susceptibility to 12 antibiotics, including oxacillin (1 μg), penicillin G (10 Units), vancomycin (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), clindamycin (2 μg), cotrimoxazole (25 μg), gentamicin (10 mg), linezolid (30 mg), cephazolin (30 mg), kanamycin (30 mg) and tetracycline (30 μg), was evaluated for each isolate. The evaluation was performed using the disc diffusion method (Kirby—Bauer) according to the CLSI (Clinical and Laboratory Standard Institute) guidelines [5].

Primer design

Primers were designed based on the *eta*, *etb* and *mec*A gene sequences of *S. aureus* obtained from Gene Bank using the Gene Runner program. To

evaluate the specificity of the designed primers, they were analyzed using BLAST. The primer were synthesized by CinnaClon Co., Iran and were as follows: *eta* forward 5′ tttgctttcttgatttggattc 3′, *eta* reverse 5′ gatgtgttcggtttgattgac 3′, *etb* forward 5′ acggctatatacattcaatt 3′, *etb* reverse 5′ tccatcgataatatacctaa 3′, *mec*A forward 5′-tgagttgaacctggtgaagtt-3′ and *mec*A reverse 5′-tggtatgtggaagttagattgg-3′. For further corroboration, the PCR product of each gene was analyzed by sequencing (Macrogen Research, Seoul, Korea).

DNA isolation and PCR amplification for gene detection

A PCR method was used to investigate the distribution of the *eta*, *etb* and *mec*A genes among the isolates. Briefly, each isolate was cultured in 5 ml of LB broth at 37 °C overnight under aerobic conditions, and the bacterial cells were subsequently harvested by centrifugation at 6000 rpm for 10 min. Next, 100 μl buffer solution (10 mM Tris·HCl, pH 8.0; 1 mM EDTA; 100 mM NaCl) and 15 μl lysozyme (0.25 mg/ml) were added to the bacterial pellet and incubated for 1 h at 37 °C. Next, 200 μl GC solution and 15 μl proteinase K solution were added and incubated for 15 min at 60 °C. Finally, the DNA was precipitated using isopropanol. The quality and quantity of the purified DNA were determined using gel electrophoresis and a NanoDrop spectrophotometer (NanoDrop-1000, Wilmington, DE), respectively. Next, 1 μl of each DNA was amplified in 25 μl of reaction mixture, which consisted of 10× reaction buffer, 0.1 mM deoxynucleoside triphosphates (dNTPs), 2 mM $MgCl_2$, 10 ρmol of designed primers and 1 U of Taq DNA Polymerase (Cinnagen, Iran). PCR was performed using the Eppendorf asterCycker (Hamburg, Germany) with an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 2 min at 54 °C, 50.9 °C and 57 °C as the annealing temperatures for the *eta*, *etb* and *mec*A genes, respectively, and 1 min at 72 °C, with a 5 min final extension at 72 °C. To determine the presence of the desired amplicon, electrophoresis was performed on 1.5% gel agarose stained with ethidium bromide, and the products were subsequently visualized using a UV transilluminator (Kiagene, Iran). The sizes of the PCR products of the amplified *eta*, *etb* and *mec*A genes were 464 bp, 200 bp and 855 bp, respectively.

Evaluation of the sensitivity and specificity of the PCR

DNA isolated from *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Clostridium perfringens* and *Clostridium difficile* was tested to assess the specificity of the designed primers. The sensitivity of the PCR tests was determined by preparing two-fold serial dilutions of the genomic DNA purified from *S. aureus* in the range of 1—32 ng. The PCR procedure was performed for each analysis according to the previously described protocol.

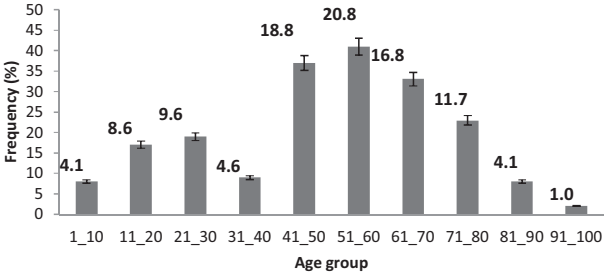

Figure 1 The frequency of patients with respect to age.

Statistical analyses

To calculate the correlation between the prevalence of the *mec*A, *eta* and *etb* genes; the drug resistance; and the demographic properties of the patients, Chi square, Fisher's exact test and multivariate analysis by logistic regression using the SPSS version 15 software package (Chicago, IL) were performed. A *p*-value less than 0.05 was considered statistically significant.

Results

Strain isolates

In this study, we evaluated the prevalence of *S. aureus*, which contained two exfoliative toxin genes, *eta* and *etb*, in the clinical isolates. Of the 197 strains, 85 (43.1%), 18 (9.13%), nine (4.56%), seven (35.5%), six (3.1%), and two (1%) were isolated from wound, blood, bile, tracheal aspirate, sputum and ear samples, respectively. In addition, one (0.5%) strain was isolated from each of the chest tube, BAL, bone, urine, stool, plural, and synovial samples. Additionally, 136 (69%) isolates were obtained from males, and the remaining isolates (31%) were obtained from females. The ages of our cases were in the range of 1—94 years and were categorized into 10 groups. Most of the cases belonged to the age group of 51—60 years. The frequency of the cases with respect to age range is shown in Fig. 1.

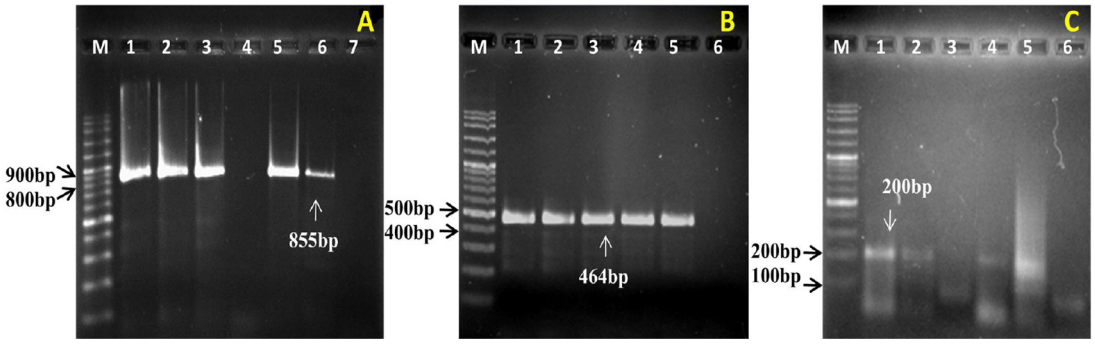

Figure 2 Gel electrophoresis of PCR products from the *mec*A (A), *eta* (B) and *etb* (C) genes. (A) The size of the amplicon of the *mecA* gene is 855 bp. M indicates the 100 bp DNA ladder. Lane 1 is the positive control (*S. aureus* ATCC 43300); lanes 2—6 show the results obtained from clinical isolates and lane 7 is the negative control (*S. aureus* ATCC 25923). (B) The PCR product of the *eta* gene is 464 bp. M: 100 bp DNA ladder; lane 1: positive control; lanes 2—5: clinical isolates and lane 6: negative control. (C) The size of the amplicon of the *etb* gene is 200 bp. M indicates the 100 bp DNA ladder. Lane 1 is the positive control, lanes 2—5 show the results obtained from clinical isolates and lane 6 is the negative control.

Distribution of the *eta*, *etb* and *mec*A genes in clinical isolates

The frequency of the *eta*, *etb* and *mec*A genes was identified using PCR. In addition, the relationship of this frequency with gender, age and sample type was investigated. A representative example of a PCR reaction for the identification of these two genes is shown in Fig. 2. In addition, the specificity of the designed primers was tested via DNA purification from *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, *Clostridium perfringens* and *Clostridium botulinum*. No PCR product was obtained from these species. Furthermore, the minimum concentration for the *eta* and *etb* genes detected by our designed primers was 2 ng.

Of the 197 isolates, 86 (94.4%), 15 (7.6%) and 172 (86.3%) expressed the *eta*, *etb* and *mec*A genes, respectively. A deficiency in both the *eta* and *etb* genes was detected in 8 (4%) isolates. In addition, 14 (7.1%) isolates expressed both the *eta* and *etb* genes, and 164 (88.2%) and 12 (80%) strains carrying the *eta* and *etb* genes, respectively, were MRSA.

The distribution of the *mec*A gene according to the type of isolates is shown in Fig. 3. The largest numbers of *mec*A-positive isolates were isolated from the wound (61.2%) and blood (17.6%) samples. Moreover, no *mec*A gene was detected in the synovial samples. The *mec*A gene was detected in 115 (84.5%) samples from males and 55 (90.1%) samples from females. No significant differences were observed between the sample type ($p = 0.3$, chi value 11.294), gender ($p = 0.7$, chi value 0.112) and age ($p = 0.054$, chi value 3.178) in the presence of the *mec*A gene.

The largest numbers of *eta*-positive strains and *etb*-positive strains were found in wound samples (55.8% and 6.09%, respectively) (Fig. 3). None of the isolates from the blood, urine, plural, stool, tracheal aspirate, sputum, synovial and BAL samples contained the *etb* gene. The isolated strains were then divided into 125 (91.91%) samples from males and 61 (100%) samples from females containing *eta* gene. In addition, 14 (10.29%) out of 136 males and only one (1.6%) female were infected by strains harboring the *etb* gene. A statistically higher prevalence of *eta*-carrying strains were isolated from female ($p = 0.019$, chi value 5.226) samples. In addition, a significantly higher number of *etb*-positive strains was found in the samples from the male population ($p = 0.024$, chi value 4.484). No significant

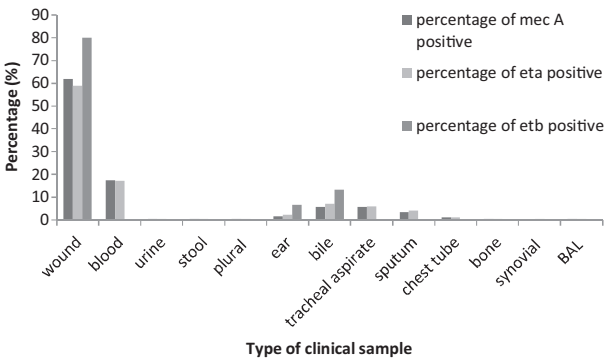

Figure 3 The frequency of the *mec*A, *eta* and *etb* genes among the isolates.

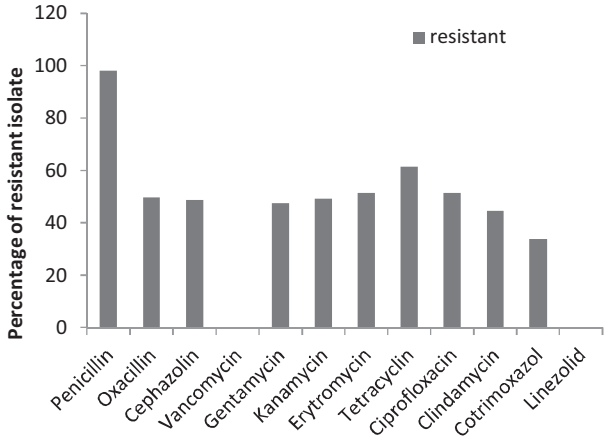

Figure 4 Prevalence of the anti-microbial resistance in the studied population.

correlation was demonstrated between the presence of the genes and age or the presence of the genes and the source of the clinical samples using multivariate logistic regression analysis ($p = 0.906$, odd ratio = 0.162).

Anti-microbial susceptibility analysis

The antibiotic-resistant properties of each isolate were determined using antimicrobial susceptibility analysis. The highest resistance to penicillin was observed among the isolates. None of the isolates were resistant to linezolid and vancomycin. The frequency of resistant isolates to each antibiotic is illustrated in Fig. 4. The multidrug resistance (resistant to more than 3 antibiotics) status of our isolates is summarized in Table 1. Statistical analyses showed no significant effect of gender, age and sample types on the prevalence of the multidrug resistance properties ($p > 0.05$).

Prevalence of the *mec*A gene and the antibiotic resistance status of the isolates are provided in Table 2. In addition to penicillin, ciprofloxacin, vancomycin and linezolid, resistance to other antibiotics increased significantly in the presence of the *mec*A gene (Table 2). Furthermore, we observed that the presence of the *mec*A gene was significantly higher among the multidrug resistant isolates ($p = 0.042$, chi value = 3.363) (Table 1). Moreover, 58 (33.7%) of the MRSA isolates were resistant to all antibiotics, excluding vancomycin and linezolid.

Furthermore, 59 (31.7%) isolates harboring the *eta* gene were resistant to all of the described antibiotics except for vancomycin and linezolid (Table 2). No significant correlation was found

Table 1 Distribution of the MRSA category and the *eta*- and *etb*-positive groups among the MDR and non-MDR clinical isolates. Values 1 through 10 indicate the number of antibiotics that showed simultaneous resistance in one isolate (MDR; multi drug resistance).

MDR		Gene							
		mecA			*Eta*			*etb*	
		Pos No (%)	Neg No (%)	*Pv*	Pos No (%)	Neg No (%)	*Pv*	Pos No (%)	Neg No (%)	*Pv*
Non-MDR	1	49 (83.1)	10 (16.9)		56 (30.1)	3 (27.3)		7 (46.7)	52 (28.6)	
	2	23 (69.7)	10 (30.3)		28 (66.7)	5 (45.5)		7 (46.7)	26 (14.3)	
MDR (number of antibiotic)	3	6 (100)	0 (0)		6 (3.2)	0 (0)		0 (0)	6 (3.3)	
	4	2 (100)	0 (0)		2 (1.1)	0 (0)		0 (0)	2 (1.1)	
	5	1 (100)	0 (0)		1 (0.5)	0 (0)		0 (0)	1 (0.5)	
	7	10 (100)	0 (0)		10 (5.4)	0 (0)		0 (0)	10 (5.5)	
	8	1 (100)	0 (0)		1 (0.5)	0 (0)		0 (0)	1 (0.5)	
	9	22 (91.7)	2 (8.3)		23 (12.4)	1 (9.1)		0 (0)	24 (13.2)	
	10	58 (95.1)	3 (4.9)		59 (31.7)	2 (18.2)		1 (6.7)	60 (33)	
Total		172 (100)	25 (100)	0.032	186 (100)	11 (100)	0.469	15 (100)	182 (100)	0.031

Table 2 Comparison of the resistance to each antibiotic in the presence or absence of the *mecA*, *eta* and *etb* genes.

Type of antibiotic resistance	Gene							
	eta			*etb*			*mecA*	
	Pos No (%)	Neg No (%)	*Pv*	Pos No (%)	Neg No (%)	*Pv*	Pos No (%)	Neg No (%)	*Pv*
Penicillin	183 (98.4)	10 (90.9)	0.207	15 (100)	178 (97.8)	0.727	169 (98.3)	24 (96)	0.422
Oxacillin	94 (50.5)	4 (36.4)	0.274	1 (6.7)	97 (53.3)	**0.000**	92 (53.5)	6 (24)	**0.005**
Cephazolin	93 (50)	3 (27.3)	0.124	1 (6.7)	95 (52.2)	**0.000**	91 (52.9)	5 (20)	**0.002**
Vancomycin	0 (0)	0 (0)	–	0 (0)	0 (0)	–	0 (0)	0 (0)	–
Gentamycin	91 (48.9)	3 (27.3)	0.138	1 (6.7)	93 (51.1)	**0.001**	89 (51.7)	5 (20)	**0.002**
Kanamycin	94 (50.5)	3 (27.3)	0.117	1 (6.7)	96 (52.7)	**0.000**	91 (52.9)	6 (24)	**0.006**
Erythromycin	98 (52.7)	3 (27.3)	0.091	3 (20)	98 (53.8)	**0.011**	95 (55.2)	6 (24)	**0.003**
Tetracyclin	115 (61.8)	6 (54.5)	0.427	5 (33.3)	116 (3.73)	**0.021**	111 (64.5)	10 (40)	**0.017**
Ciprofloxacin	96 (51.6)	5 (45.5)	0.465	1 (6.7)	100 (54.9)	**0.000**	92 (53.5)	9 (36)	0.077
Clindamycin	85 (45.7)	3 (27.3)	0.19	1 (6.7)	87 (47.8)	**0.001**	83 (48.3)	5 (20)	**0.006**
Cotrimoxazole	65 (34.9)	2 (18.2)	0.212	1 (6.7)	66 (36.3)	**0.014**	63 (36.6)	4 (16)	**0.031**
Linezolid	0 (0)	0 (0)	–	0 (0)	0 (0)	–	0	0	–
Total	169 (100)	11 (100)		13 (100)	167 (100)		172 (100)	25 (100)	

Pos, positive strains; Neg, negative strains; No., number; %, percentage.

between the resistance to the examined antibiotics and the presence of the *eta* gene ($p = 0.101$, odd ratio = 0.245). Moreover, there was no significant correlation between the distribution of the *eta* gene and the multidrug resistance pattern ($p = 0.469$, odd ratio = 0.343) (Table 1).

Except for penicillin, resistance to all of the antibiotics tested in the strains harboring the *etb* gene was significantly lower compared to the resistance in the strains deficient for the *etb* gene ($p < 0.05$). In addition, the MDR property was significantly more prevalent among the *etb* negative strains ($p = 0.031$, chi value = 13.54).

Discussion

S. aureus causes a high rate of morbidity and mortality due to severe nosocomial infections. One of the major issues is the emergence of MRSA strains and their increased prevalence in some parts of the world, resulting in some difficulties in their treatment. In addition, the multidrug resistance properties of MRSA strains and the production of various types of virulence factors cause the spread of infections. It has been proposed that resistance to antibiotics can alter the expression of genes involved in pathogenesis [6]. In this study, the genotyping outcomes demonstrated an exceedingly high prevalence of MRSA strains (87.6%) as well as an elevated rate of antibiotic resistance, which was nearly similar to several reports obtained from China (77.65—80%) [7,8]. Nevertheless, findings obtained from most studies conflict with the current study as the prevalences of MRSA observations were 41.6% (USA) [9], 16.2% (Nigeria) [10] and 18% (Russia) [11] in previous studies.

Tokajian et al. in Lebanon found that 72% of isolates were MRSA, but a lower prevalence of multidrug resistance properties (18%) was been reported [8]. However, the differences in the pattern of *mec*A gene distribution with regard to gender and age remain unclear in both MRSA and MSSA groups. In this study, the *mec*A gene was more prevalent among wound samples.

Currently, *S. aureus* strains that are resistant to numerous antimicrobial drugs are commonly isolated from nosocomial infections. The spread of multidrug-resistant strains is one of the major problems of health committees worldwide due to the complicated treatment process. In this study, the frequency of antimicrobial resistance was very high. Our results showed a high prevalence of resistance toward many antibiotics that are routinely administered for the treatment of staphylococcal infection. Similar to most regions around the

world [9—11], resistance to penicillin was high in our studied population (94.4%). Despite the high frequency of MRSA among our isolates, half of the specimens were resistant to oxacillin, which was lower than the amount reported in Belgium (99%) [12] and higher than the frequency reported in Lebanon (32%) [8] and Nigeria (16.2%) [10]. According to this result, based on the oxacillin resistance assay, some MRSA strains might not be identified using the screening test. Inconsistent with the results of some Asian and African countries, in which the percentage of resistance to erythromycin was lower than 30% [13,14] or was (0%) [10,11], erythromycin-resistant strains were more abundant in the current study (51.3%). However, higher prevalences erythromycin-resistant strains have been observed in the United Kingdom (90%) [15], China (97.8%) [16] and Australia (98%) [17]. We also observed an enhanced prevalence of tetracycline-resistant strains (61.3%) compared to previous reports from Lebanon (48% and 44%) [8,18] and USA (5%) [19]. However, Nimmo et al. and Zhang et al. reported a prevalence of 80% and 97.8% resistant isolates from Australia [17] and China [16], respectively. Moreover, the prevalence of gentamicin-resistant strains was higher (47.7%) than reports from Nigeria (14.7%) [10], China (28.1%) [9], and Russia (19%) [11]. In addition, the rates of resistance to clindamycin and ciprofloxacin were higher compared to Russia [11], Nigeria [10], Libya [20] and Lebanon [8]. Similar to the results obtained from several previous studies, the resistance to vancomycin [8,14,15,18,21] and linezolid [9,10] was not observed amongst our isolates.

In this study, we found high prevalences of *eta* and *etb* genes among our isolates. However, the occurrence of *eta* was more frequent than *etb*. In addition, strains harboring both of these genes were more abundant in the MRSA population. The low prevalence of these genes has been reported in many countries worldwide. Xie and colleagues have previously observed a low frequency of this gene [22]. In another Chinese study, the *eta* gene was detected in only one child [23]. Most of the isolates of these two studies were collected from sputum. In contrast with the reports from China, accessible findings from Czech [24], Germany [25,26], Switzerland [27] and Malaysia found a low prevalence of genes encoding exfoliative toxin isoforms. In all of the studies mentioned above, the number of wound samples was low. In another study from Colombia, which investigated the distribution of the *eta* and *etb* genes among pediatric patients, only 2% of the isolates carried *eta*, but no strain carried the *etb* gene [28]. Similar to our study, the majority of specimens collected by Demir et al. [29], and Liu et al. [16] were obtained from wound and blood. Demir and colleagues found the *eta* and *etb* genes among 19.2% and 9.2% of cases, respectively. Consistent with these findings, 43.1% of the isolates were recovered from wound samples in the current study.

Similar to most staphylococcal virulence factors, exfoliative toxins play a role in host colonization and the invasion of injured mucosa and skin. Thus, the high rates of exfoliative toxin genes in this study could be due to the high frequency of wound specimens. Our results demonstrate three important findings. First, the percentage of MRSA strains and consequent multidrug resistance has increased in our population. Second, independent of the SSSS patients' shortage among our isolates, remarkably high rates of *eta* and *etb* were found. In addition, their distribution was similar in both the MRSA and methicillin-sensitive *S. aureus* (MSSA) groups. Last, drug resistance was abundant in the analyzed population.

Despite the geographical variations, data obtained from various regions worldwide indicate a higher distribution of *eta* compared to *etb*, which could be due to the great immunogenicity of ETA [8]. Importantly, the presence of toxin genes does not demonstrate the level of related protein expression. However, among our population, no case of SSSS was detected. This observation suggested an insufficient amount of toxins that could not have caused the disease. It is thought that more antibiotic resistance is acquired, and lower virulence factor was secreted [6,30]. Thus, as a consequence of the significantly high rate of drug resistance among our isolates, it is possible that the expression of these two genes is reduced. In addition, the presence of the *eta* and *etb* genes in samples from damaged tissues (such as a wound site) is an indicator for increased susceptibility of the patient to harmful diseases, such as SSSS.

Conflict of interest

I have read and am aware of the conflict of interest policy of Journal of Infection and Public Health for the authors of this manuscript, and hereby agree to abide by this policy in all matters dealing with my responsibilities toward the Institute.

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submitted to the Applied Microbiology Research Center at Baqiyatallah University of Medical Sciences, Tehran, Iran in partial fulfillment of the requirements for the MSc in Microbiology.

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