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Characteristics of Prophage Patterns and Virulence Gene Profiles among Methicillin-Resistant *Staphylococcus aureus* Isolates from Patients with Diabetic Foot Infections in a Referral Hospital in Tehran, Iran

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ABSTRACT

Backgrounds: Diabetic patients are at risk of developing serious foot infections with methicillin-resistant *Staphylococcus aureus* (MRSA) strains, which are associated with high morbidity and mortality. This study aimed to investigate the frequency of different prophage types and virulence factors among MRSA strains isolated from patients with diabetic foot infections (DFIs) in a referral hospital in Tehran, Iran during 2019 and 2020.

Materials & Methods: A total of 238 *S. aureus* isolates were collected and confirmed using specific primers. The presence of staphylococcal enterotoxins (*sea-seq*) and *hlb*, *sak*, *eta*, *etb*, and *tsst-1* genes among MRSA isolates was tested using separate polymerase chain reaction (PCR) assays. Also, multiplex PCR was employed for prophage typing of MRSA isolates.

Findings: A total of 73 (31%) isolates were confirmed as MRSA, among which four prophage types and 13 different prophage patterns were identified, and prophage type SGF and prophage pattern 7 consisting of SGB, SGF, SGFa, and SGFb types were the dominant ones. Also, 11 enterotoxin-encoding genes and four virulence factor genes were detected among the isolates. All MRSA isolates were positive for *sea*, *sek*, *seq*, and *hlb* genes. Moreover, out of 12 different enterotoxin patterns, most MRSA isolates were classified into enterotoxin pattern 1, harboring three enterotoxin genes (*sea*, *sek*, and *seq*).

Conclusion: This study results indicated the presence of different prophage types and virulence factor genes among MRSA strains isolated from DFI patients, which enable them to produce a variety of diseases.

Keywords: Methicillin-resistant *Staphylococcus aureus*, Virulence factor patterns, Prophage typing, Diabetic foot infection..

CITATION LINKS

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Introduction

Foot ulcers are a common problem in patients with diabetes mellitus. Infection of these open wounds is a common (40-80%) complication that significantly increases the risk of hospitalization and lower limb amputation [1]. Also, diabetic foot infections (DFIs) impose a substantial clinical and economic burden on healthcare systems worldwide comparable to the costs associated with cancer, depression, and pulmonary and musculoskeletal diseases [2, 3]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the main pathogens isolated from DFIs, which has emerged as a growing public health concern in recent decades due to its widespread distribution, virulence factors, and ability to overcome antimicrobial agents [4]. Based on previous studies in Iran, a high percentage of *S. aureus* isolates (43%) that could cause infection are methicillin-resistant [5]. At present, MRSA infections are resistant not only to all beta-lactam antibiotics but also to vancomycin (the last resort antibiotic for treating resistant staphylococcal infections) and newer agents such as linezolid and daptomycin, which have recently been developed [6]. The production of a large number of virulence factors by MRSA strains makes host tissues favorable for bacterial growth, invasion, and development of severe infections. The production of virulence factors is the result of phenotypic changes caused by lysogenic bacteriophages which could convert non-pathogenic strains to pathogenic strains through horizontal gene transfer [7, 8]. *Staphylococcus* prophages carry genes that code diverse virulence factors such as staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), staphylokinase, (Pantůček, #22)β-hemolysin (Hlb), and exfoliative toxins (ETs) [9, 10]. Toxigenic strains often cause more severe and systemic infections, while non-toxigenic strains cause localized deep infections and are involved in diabetic foot osteomyelitis. Therefore, determining the virulence profile of MRSA strains in wounds seems to be a reliable method to predict the behavior of these bacteria in host organs [11]. Strain typing could provide important information about the diversity and sources of the most prevalent strains in DFIs for effective infection control [12]. Among different typing methods, prophage typing is a simple, quick, inexpensive, reliable, and reproducible technique that requires no specialized equipment [13] and could provide crucial information about phage therapy as an alternative treatment method for MRSA infections [14].

Objectives: This study aimed to determine the presence of different prophage patterns and also virulence gene profiles among MRSA strains isolated from patients with DFIs in a referral hospital in Tehran, Iran.

Materials and Methods

Sample collection and identification of bacterial strains: During 2019 and 2020, a total of 238 *S. aureus* isolates were collected from patients with DFIs in a referral hospital in Tehran, Iran. The collected isolates were identified at the species level using primers specific for the *nuc*A gene as described previously [15]. Moreover, the resistance of *S. aureus* isolates to cefoxitin (30 µg) (Rosco Diagnostica, Denmark) was evaluated according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [16]. Also, the strains were confirmed as MRSA based on the presence of the *mec*A gene [15].

DNA extraction: Boiling method was used to extract bacterial DNA. For this purpose, a loopful of each pure bacterial culture was suspended in 200 µL of sterile distilled water and boiled for 20 min. After centrifugation at 13000 × g for 15 min, the supernatant was collected and used as a DNA template in the PCR reaction mixture [9].

Prophage typing: Multiplex-PCR assay was carried out to identify different prophage types (SGA, SGB, SGF, SGD, and SGL) and subtypes (SGFa and SGFb) among MRSA isolates using specific primers as described previ-

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Table 1) Frequency of different prophage patterns among MRSA isolates

Pattern	Prophage Types							No (%)
	SGA	SGB	SGF	SGFa	SGFb	SGFL	SGFD	
1	+	+	+	+	+	+	-	2 (3)
2	+	-	+	+	+	+	-	2 (3)
3	+	+	+	+	+	-	-	2 (3)
4	-	+	+	+	+	+	-	1 (1)
5	+	-	+	+	+	-	-	1 (1)
6	-	-	+	+	+	+	-	1 (1)
7	-	+	+	+	+	-	-	41 (56)
8	+	-	+	-	+	+	-	1 (1)
9	-	-	+	+	+	-	-	15 (21)
10	+	-	+	-	+	-	-	2 (3)
11	-	+	+	-	+	-	-	1 (1)
12	-	-	+	-	+	-	-	2 (3)
13	-	+	-	-	-	-	-	2 (3)

ously [17]. The primers (Sinaclon Company, Tehran, Iran) used for prophage typing of MRSA isolates are listed in the Supplementary Table.

Identification of virulence genes: All bacterial MRSA isolates were investigated for the presence of 16 enterotoxin-encoding genes (*sea, seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *sek*, *sel*, *sem*, *sen*, *seo*, *sep*, and *seq*) as well as toxic shock syndrome toxin 1 (*tsst-1*), exfoliative toxin (*eta* and *etb*), staphylokinase (*sak*), and β-hemolysin (*hlb*) genes using PCR reactions. PCR conditions and sequences of specific primers were according to previously published studies [18, 19].

Findings

Identification of strains: Among all 238 *S. aureus* isolates that were tested in this study and were positive for the presence of *nuc*A gene, 73 (31%) isolates showed resistance to cefoxitin. All cefoxitin-resistant isolates harbored the *mec*A gene based on the PCR test results and were confirmed as MRSA.

Prophage typing: The results indicated the presence of four prophage types and two prophage subtypes among MRSA isolates, in which prophage types SGF (97%) and SGB (67%) as well as subtypes SGFb (97%) and SGFa (89%) were the most prevalent types, while the SGD prophage type was not found in any of the isolates (Table 1). In total, 13 prophage patterns were observed amongst MRSA isolates, and prophage pattern 7 consisting of SGB, SGF, SGFa, and SGFb prophage types was the predominant one (56%), followed by prophage pattern 9 consisting of SGF, SGFa, and SGFb (21%). All SGA-positive MRSA isolates were resistant only to penicillin and showed low-level resistance to oxacillin (MICs ranged from 4 to 16 µg/mL) (unpublished data).

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Table 2) Prevalence of enterotoxin genes among MRSA strains

Pattern	Staphylococcal Enterotoxin (se) Gene Patterns																No of Genes	No (%)
	sea	*seb*	*sec*	*sed*	*see*	*seg*	*seh*	*sei*	*sej*	*sek*	*sel*	*sem*	*sen*	*seo*	*sep*	*seq*		
1	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	3	49 (67)
2	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	4	1 (1.3)
3	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	4	1 (1.3)
4	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	4	1 (1.3)
5	+	-	+	-	-	-	-	-	-	+	+	-	-	-	-	+	5	1 (1.3)
6	+	-	-	-	-	-	-	-	-	+	-	+	+	-	-	+	5	1 (1.3)
7	+	-	-	-	-	+	-	+	-	+	-	-	-	-	-	+	5	1 (1.3)
8	+	-	-	-	-	+	-	-	-	+	+	-	-	-	-	+	5	11 (15)
9	+	-	-	-	-	+	-	+	-	+	+	-	-	-	-	+	6	2 (3)
10	+	-	+	-	-	+	-	+	-	+	+	-	-	-	-	+	7	1 (1.3)
11	+	-	-	-	-	+	-	+	-	+	-	+	+	+	-	+	8	3 (4)
12	+	-	+	-	-	+	-	+	-	+	+	+	+	+	-	+	10	1 (1.3)

Identification of virulence genes: Out of 16 different enterotoxin-encoding genes evaluated in this study, 11 enterotoxin genes were detected among MRSA isolates (Table 2). All MRSA isolates (100%) were positive for *sea*, *sek*, and *seq* genes, while *seb*, *sed*, *see*, *seh*, and *sej* were detected in none of the isolates. Moreover, 12 different enterotoxin patterns were found among MRSA isolates based on the presence of enterotoxin genes. Most MRSA strains (n=49, 67%) were classified into enterotoxin pattern 1 which contained three enterotoxin genes *sea*, *sek*, and *seq*, followed by enterotoxin pattern 8 (n=11, 15%) which contained *sea*, *seg*, *sek*, *sel*, and *seq* genes. Moreover, only one strain was positive for 10 virulence genes (pattern 12), and six (8.3%) strains harbored 6-8 virulence genes. On the other hand, the *hlb* gene was detected in 100% of MRSA strains, whereas all the strains were negative for the gene encoding exfoliative toxin B (*etb*). The prevalence of *sak*, *eta*, and *tsst-1* genes was limited to 60 (82%), 10 (14%), and three (4%) MRSA strains. The present study revealed that the prevalence of MRSA isolates in DFIs was as high as 31%, which is in agreement with the previous report [20].

Discussion

MRSA strains play an important role in the development of foot infections in diabetic patients, causing increased length of hospital stay, cost of management, additional risks of amputation (43% MRSA vs 9% non-MRSA), and mortality (43% MRSA vs 20% non-MR-

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Supplementary Table. Sequences of primers used in this study

Genes	Sequence	Molecular Weight (bp)	Reference
nucA	F: 5′-AGTTCAGCAAATGCATCACA	400	15
	R: 5′-TAGCCAAGCCTTGACGAACT		
mecA	F:5′-GTAGAAATGACTGAACGTCCGATAA	310	15
	R: 5′-CCAATTCCACATTGTTTCGGTCTAA		
SGA	F: 5′-TATCAGGCGAGAATTAAGGG	744	17
	R: 5′-CTTTGACATGACATCCGCTTGAC		
SGB	F: 5′-ACTTATCCAGGTGGYGTTATTG	405	17
	R: 5′-TGTATTTAATTTCGCCGTTAGTG		
SGF	F: 5′-CGATGGACGGCTACACAGA	155	17
	R: 5′-TTGTTCAGAAACTTCCCAACCTG		
SGFa	F: 5′-TACGGGAAAATATTCGGAAG	548	17
	R: 5′-ATAATCCGCACCTCATTCCT		
SGFb	F: 5′-AGACACATTAAGTCGCACGATAG	147	17
	R: 5′-TCTTCTCTGGCACGGTCTCTT		
SGD	F: 5′-TGGGCTTCATTCTACGGTGA	331	17
	R: 5′-GTAATTTAATGAATCCACGAGAT		
SGL	F: 5′-GCTTAAAACAGTAACGGTGACAGTG	748	17
	R: 5′-TGCTACATCATCAAGAACACCTGG		
sea	F: 5′-TAAGGAGGTGGTGCCTATGG	180	19
	R: 5′-CATCGAAACCAGCCAAAGTT		
seb	F: 5′-TCGCATCAAACTGACAAACG	478	19
	R: 5′-GCAGGTACTCTATAAGTGCC		
sec	F: 5′-ACCAGACCCTATGCCAGATG	371	19
	R: 5′-TCCCATTATCAAAGTGGTTTCC		
sed	F: 5′-TCAATTCAAAAGAAATGGCTCA	339	19
	R: 5′-TTTTTCCGCGCTGTATTTTT		
see	F: 5′-TACCAATTAACTTGTGGATAGAC	170	19
	R: 5′-CTCTTTGCACCTTACCGC		
seg	F: 5′-CCACCTGTTGAAGGAAGAGG	432	19
	R: 5′-TGCAGAACCATCAAACTCGT		

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Supplementary Table. Sequences of primers used in this study

Genes	Sequence	Molecular Weight (bp)	Reference
seh	F: 5′-TCACATCATATGCGAAAGCAG	463	19
	R: 5′-TCGGACAATATTTTTCTGATCTTT		
sei	F: 5′-CTCAAGGTGATATTGGTGTAGG	529	19
	R: 5′-CAGGCAGTCCATCTCCTGTA		
sej	F: 5′-GGTTTTCAATGTTCTGGTGGT	306	19
	R: 5′-AACCAACGGTTCTTTTGAGG		
sek	F: 5′-ATGAATCTTATGATTTAATTTCAGAATCAA	545	19
	R: 5′-ATTTATATCGTTTCTTTATAAGAAATATCG		
sel	F: 5′-CACCAGAATCACACCGCTTA	204	19
	R: 5′-CTGTTTGATGCTTGCCATTG		
sem	F: 5′-ATGAAAAGAATACTTATCATTGTTGTTTTATTG	720	19
	R: 5′-CTTCAACTTTCGTCCTTATAAGATATTTC		
sen	F: 5′-ATAAAAAATATTAAAAAGCTTATGAGATTGTTC	777	19
	R: 5′-ACTTAATCTTTATATAAAAATACATCAATATG		
seo	F: 5′- TATGTAGTGTAAACAATGCATATGCA	685	19
	R: 5′-TCTATTGTTTTATTATCATTATAAATTTGCAAAT		
sep	F: 5′-TTAGACAAACCTATTATCATAATGGAAGT	618	19
	R: 5′-TATATAAATATATATCAATATGCATATTTTTAGACT		
seq	F: 5′-GGAAAATACACTTTATATTCACAGTTTCA	539	19
	R: 5′-ATTTATTCAGTTTTCTCATATGAAATCTC		
hlb	F: 5′-AGCTTCAAACTTAAATGTCA	525	18
	R: 5′- CCGAGTACAGGTGTTTGGTA		
sak	F: 5′- GTGCATCAAGTTCATTCGAC	383	18
	R: 5′- TAAGTTGAATCCAGGGTTTT		
eta	F: 5′-CTAGTGCATTTGTTATTCAA	119	18
	R: 5′-TGCATTGACACCATAGTACT		
etb	F: 5′-ACGGCTATATACATTCAATT	200	18
	R: 5′-TCCATCGATAATATACCTAA		
tsst	F: 5′-ATGGCAGCATCAGCTTGATA	350	18
	R: 5′-TTTCCAATAACCACCCGTTT		

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SA) [20]. The present study revealed that the prevalence of MRSA isolates in DFIs was as high as 31%, which is in agreement with the previous report [20]. Available data on the epidemiology of MRSA strains represent that their diversity mainly depends on the presence of mobile genetic elements, many of which are prophages or phage-related genomic islands [21]. Also, epidemic MRSA strains that contain certain prophage types are more virulent and could spread rapidly in hospital environments. These prophage types are responsible for the development of infections that may vary from time to time and hospital to hospital in terms of their antibiotic susceptibility patterns [22]. Thus, in the next step, the most prevalent prophage types among MRSA strains isolated from the study population were determined. The findings revealed that all the studied prophage types, except SGD, were present among the isolates, and the SGF prophage type was the predominant type. Also, SGFa and SGFb were the dominant subtypes among the isolates, which is consistent with previous reports in Iran [15, 17, 18, 23-25]. In *S. aureus* strains isolated from human sources, SGF-type prophages are associated with the immune evasion cluster [26], which includes enterotoxin A (*sea*) [26], enterotoxin K [7], enterotoxin Q (*seq*), beta-hemolysin (*hlb*), staphylokinase (*sak*), chemotaxis inhibitory protein, and staphylococcal complement inhibitor [26, 27]. Therefore, MRSA strains isolated in the current study could be considered as reservoirs of virulence-associated genes circulating in Iran. The findings revealed the presence of 13 different prophage patterns among all MRSA strains. Several studies conducted in other countries as well as Iran have reported almost different numbers of prophage patterns compared to the present study findings [15, 17, 18, 23-25, 28, 29], which could be due to differences in ecological settings and locations of these studies.

Studying the virulence profile of bacteria could determine the occurrence and progression of *S. aureus* infections [30]. On the other hand, the potential of *S. aureus* to cause DFI is especially dependent on secreted toxins, including pore-forming toxins, exfoliatins, and superantigen exotoxins [11]. Hence, the presence of these virulence factor genes in MRSA strains isolated from patients with DFIs was evaluated in this study. The results indicated the presence of 11 different staphylococcal enterotoxin genes and four virulence factor genes among MRSA isolates. Staphylococcal enterotoxins, known as pyrogenic toxin superantigens (PTSAgs), induce cytokine production by both T-lymphocytes and macrophages [11]. None of the MRSA isolates in this study possessed *seb*, *sed*, *see*, *seh,* and *sej* genes. Different prevalence rates for these enterotoxin genes have been reported worldwide, which is probably due to the origin and genetic structure of the isolates [31, 32]. In the current study, all MRSA isolates harbored genes encoding enterotoxins A, K, and Q (SEA, SEK, and SEQ). Among them, SEA could be used as a potent marker to differentiate colonization from infection as well as to determine the foot ulcer grade (it is often common in wound grades 2-4) [30]. Therefore, the detection of SEA indicates that patients suffer from deeper ulcers which put them at a higher risk of developing osteomyelitis, gangrene, and even amputation. Enterotoxin pattern 1 consisting of enterotoxins A, K, and Q was the most frequent enterotoxin pattern, which is consistent with previous publications in 2018 [18, 19]. The gene encoding TSST-1, as another superantigen of *S. aureus*, was found in 4% of bacterial MRSA isolates, which may also predispose patients to more serious disease consequences [33]. Compared to our previous report, the frequency of *sak* (a fibrin-specific activator of human plasminogen) and *eta* (a secreted protease facilitating bacterial skin invasion) genes was significantly decreased among MRSA isolates [18].

Conclusion

MRSA strains isolated from patients with

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DFIs in a referral hospital in Tehran, Iran, showed high diversity in virulence gene profiles. Identifying these virulence factors plays a critical role in evaluating the pathogenicity and transmission rate of these bacteria and thereby finding effective and reliable strategies to manage, control, and treat MRSA infections. Although *sea*, *sek*, *seq*, and *hlb* genes were observed in all the studied MRSA strains, the putative relationship between these genes and resistance to antibiotics in MRSA isolates is still unknown. On the other hand, bacteriophages could enable MRSA strains to cause various diseases by transferring virulence factor-encoding genes among them. Thus, the prevalence of such virulence factors could be predicted using the prophage typing method.

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