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Detection of Toxins and Antibiotic Resistance Genes Profile among Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolates and their types of Infection in a Tertiary Hospital in Malaysia

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ABSTRACT

Objective: *This study aimed to determine the molecular characteristics of virulence and antibiotics resistance genes profile of MRSA isolates and to compare between the conventional and molecular patterns of antibiotic resistance and determine the association between the MRSA virulence genes with their types of infection.* ***Methods:*** *A total of 60 MRSA isolates were collected from the Microbiology Laboratory of Selayang Hospital, a tertiary hospital in Malaysia. Antimicrobial susceptibility tests were performed by the disc diffusion method. The virulence and the antibiotic resistance genes were determined by PCR.* ***Results:*** *Among the sixty clinical isolates, there were six types of MRSA infections including cellulitis (30%), diabetic foot ulcer (28.3%), necrotizing fasciitis (13.3%), osteomyelitis (15%), catheter-related bloodstream infection (10%) and pneumonia (3.3%). Overall, lukS genes were detected in 60% followed by staphylococcal enterotoxins A (sea) gene (45%), Toxic Shock Syndrome Toxin (TSST) gene (43.3%), and 8.3% for each exfoliative toxin A (ETA) and α-hemolysin (hla) genes. A significant association was found between sea and hla genes with all the types of infection. The sea genes were significantly associated with cellulitis, diabetic foot ulcer, and osteomyelitis. The detection of antibiotic resistance by the molecular and conventional methods was comparable.* ***Conclusion:*** *This study showed that there were multiple virulences and antibiotic-resistant genes involved in the pathogenicity of MRSA infections. Both sea and hla genes have a significant association with the various types of infections. Our outcomes showed an elevated rate of lukS gene among MRSA isolates. Vancomycin-resistance was not detected among the MRSA isolates.*

Keywords: MRSA, Virulence genes, lukS, antibiotic resistance, sea

INTRODUCTION

Staphylococcus aureus is among the most common human pathogens and can cause numerous and serious infections. The highly virulent strain can cause severe diseases, which may lead to fatality. They can produce a wide range of toxins and exhibit resistance to single or multiple antibiotics. Most of these traits are located on mobile genetic elements of the bacterial genome [1]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a worldwide pathogen leading to hospital-acquired infections and is commonly resulted in significant mortality, morbidity, and increase of hospital stay cost burden [2]. Recently, MRSA infections have emerged in the community and also from livestock; however, they vary not only in their clinical aspects and molecular biology but also their antimicrobial susceptibility and treatment [3]. MRSA strains have evolved by acquiring a genetic element known as Staphylococcal Cassette Chromosome (*SCC*) *mec*, which contains the mobile *mecA* gene. Acquiring of the *mecA* gene codes for an altered Penicillin-Binding Protein (PBP) that has a lower affinity for binding β-lactams [4]. Risk factors associated with MRSA infection include prolonged hospitalization, previous antibiotic administration, and invasive procedures in addition to MRSA colonization [2]. A higher incidence of MRSA infection is also reported among healthcare workers who are dealing with

patients infected with this pathogen [3]. MRSA can cause a variety of infections involving skin and subcutaneous tissues, to invasive infections like osteomyelitis, meningitis, pneumonia, lung abscess, and infective endocarditis [5].

A wide range of virulence factors, including toxins, immune-evasive surface factors, and enzymes that promote tissue invasion are produced by MRSA [6]. The virulence genes of interest in this study were collectively targeted for the first time in Malaysia including staphylococcal enterotoxin A gene (*sea*), toxic shock syndrome toxin gene (*tsst*), exfoliative toxin A gene (*eta*), Panton-Valentine leukocidin gene (*lukS*), and α-hemolysin gene (*hla*).

MRSA can produce a variety of Staphylococcal Enterotoxins (SEs). These toxins have a potent superantigenic role and are consisted of nine types (SEA to SEI). Few of these SEs have a vital role in epidemics of food poisoning and other infections that are septic-related. These SE proteins have a significant ability to resist heat and acid. Therefore, they may not be denatured by simple cooking of contaminated food [7].

Staphylococcal enterotoxins act as strong gastrointestinal toxins and as superantigens that spur non-specific T-cell proliferation [8]. Though these are two distinct actions located on detaching domains of the proteins, there is a high association between these actions i.e., a loss of superantigen activity (due to genetic mutation) results in loss of enterotoxic activity also [9]. Another toxin produced by MRSA is the toxic shock syndrome toxin encoded by the *tsst*-1 gene. The *tsst*-1 gene encodes a 21.9 KDa extracellular toxin causing toxic shock syndrome. The *tsst*-1 triggers a T cell-dependent shock syndrome resulting in high lethality by stimulating the release of cytokines, including interleukin-6 and tumor necrosis factors [10].

Exfoliative toxins (ETA, ETB, and ETD) are produced by MRSA which act as proteases that hydrolyze desmoglein-1 (DG-1), an important keratinocyte help in cell-cell adhesion in the superficial epidermis. Hydrolysis of desmoglein caused dissociation of keratinocytes in humans a condition called staphylococcal scalded skin syndrome [11].

Panton-Valentine Leukocidin (PVL) is another important cytotoxin made by MRSA strains, which is encoded by two separate genes, *lukS*-PV and *lukF*-PV. The PVL is a synergohymenotropic toxin, and it acts through the synergistic activity of 2 non-associated secretory proteins, component S and component F that induces pores in the membranes of cells [12]. PVL production was initially linked to furuncles, cutaneous abscesses, and severe necrotic skin infections, though; severe cases of necrotizing hemorrhagic pneumonia and septicemia have also been reported. PVL genes are carried mainly by Community-Acquired MRSA (CA-MRSA). However, PVL genes are seldomly present in hospital isolates; hence it is known as a marker of CA-MRSA. Epidemiological records indicate that high severity of CA-MRSA is reported with PVL genes [13].

Red blood cell lysis by MRSA is mainly mediated by the different hemolysins known as alpha, beta, delta toxins, and gamma hemolysins. The alpha-hemolysin is a pore-forming toxin encoded by the *hla* gene. α-hemolysin is the most characterized virulence factor of *S. aureus* that forms heptameric pores in host cell membranes, leading to lysis of the cell [14]. Also, α-hemolysin has been shown to affect innate immune effector cells, accelerate a hyper-inflammatory response characteristic of bacterial pneumonia, and disrupt epithelial and endothelial barriers. The *hla* expression is controlled by a complex regulatory network and its expression has been reported to be up-regulated during infection [15].

Mobile genetic elements carrying antibiotic resistance genes have been acquired by MRSA on multiple independent occasions and it reflects the strong selective pressures within a hospital environment [6]. Antibiotic resistance genes which were selected in this study included vancomycin-resistant gene (*vanA*), linezolid resistant gene (*cfr*), mupirocin resistant gene (*mupA*), rifampicin-resistant gene (*rpoB*), fusidic acid-resistant gene (*fusA*), and clindamycin resistant gene (*ermA*) represented the common genes in MRSA.

The high rate of antibiotic resistance and increasing multi-drug resistant bacteria is a promising challenge. Biocompatible nanostructures or nanocomposites such as reduced graphene oxide have been reported to exhibit strong antibacterial activity toward both Gram-positive and Gram-negative bacteria and make it an interesting candidate to incorporate into wound bandages to treat and/or prevent microbial infections [16,17].

In Malaysia, only a few studies had targeted MRSA virulence and antibiotics resistance characteristics and the infections they caused. Hence, this study was conducted to determine the virulence and antibiotics resistance profiles of the MRSA strains and the association with their types of infection.

MATERIALS AND METHODS

This was a cross-sectional study that was carried out from March to June 2018 in Hospital Selayang, a tertiary, 800-bedded hospital in the suburb of Kuala Lumpur after approval was obtained from the Research and Ethical Committee. This study complies with the Declaration of Helsinki, Informed consent was obtained from the participants. A total of 60 MRSA isolates from clinical samples received at the microbiology laboratory of the hospital were included in the study. The clinical samples were from both adults and pediatric patients admitted to the hospital. Only one MRSA isolate per patient was included. The patients' demography and types of infections were obtained from their medical records.

Conventional Methods

Identification of *S. aureus* was determined by standard microbiology practices based on colonial morphology, Gram stain, catalase test, and coagulase test. The Disc-diffusion agar method was used for the differentiation of MRSA from Methicillin-Sensitive *S. aureus* (MRSA) strains. The isolates were suspended in a broth medium of 0.5 McFarland standard concentrations and then grown in Muller Hinton agar. Cefoxitin (30 µg) discs were used as a control. All plates were incubated overnight at 37°C. The diameter of the inhibition zone around the discs was recorded and interpretation was in accordance with the Clinical Laboratory Standard Institute (CLSI) guideline with inhibition zone diameter ≤ 21 specified MRSA and inhibition zone diameter ≥ 22 suggested MSSA [18].

Conventional Identification of Antibiotic Resistance

All the isolates were tested for antibiotic susceptibility testing towards clindamycin (2 µg), linezolid (30 µg), mupirocin (5 µg), rifampicin (5 µg), fusidic acid (10 µg) using disc-diffusion agar method while vancomycin using the MIC E-test® strips (BioMérieux, Marcy l'Étoile, France). Both the methods were performed following the CLSI [18].

Method for the Identification of Virulence and Antibiotic Resistance Genes

DNA extraction: One bacterial colony was suspended in 200 µl of TE buffer (Tris-HCl [10 mM]: EDTA and boiled for 10 minutes then centrifuged for 5 minutes at 14,000 rpm at room temperature. The supernatant containing DNA (100 µl) was used for PCR assay.

PCR assay: All the 60 MRSA samples were tested for virulence (*sea*, *tsst*, *eta*, *lukS,* and *hla*) and antibiotic resistance (*vanA*, *cfr*, *mupA*, *rpoB*, *ermA,* and *fusA*) genes by PCR using previously described primers Table 1 [19-25].

Table 1 The oligonucleotide sequences and amplicon size of the virulence and antibiotic resistance genes in MRSA

Oligonucleotide sequence (5'-3')			Amplicon size, bp	Reference
sea	*sea*1 (F)	GGTTATCAATGTGCGGGTGG	102	[20]
	*sea*2 (R)	CGGCACTTTTTTCTCTTCGG		
tsst	*tsst*1 (F)	ACCCCTGTTCCCTTATCATC	326	[20]
	*tsst*2 (R)	TTTTCAGTATTTGTAACGCC		
eta	*eta*1 (F)	GCAGGTGTTGATTTAGCATT	93	[20]
	*eta*2 (R)	AGATGTCCCTATTTTTGCTG		
lukS	*pvl*1 (F)	ATCATTAGGTAAAATGTCTGGACATGATCCA	443	[20]
	*pvl*2 (R)	GCATCAAGTGTATTGGATAGCAAAAGC		
hla	*hla*1 (F)	CTGATTACTATCCAAGAAATTCGATTG	209	[20]
	*hla*2 (R)	CTTTCCAGCCTACTTTTTTATCAGT		
vanA	*vanA*1 (F)	ATGAATAGAATAAAAGTTGC	1031	[25]
	*vanA*2 (R)	TCACCCCTTTAACGCTAATA		
cfr	*cfr*1 (F)	TGAAGTATAAAGCAGGTTGGGAGTCA	746	[22]
	*cfr*2 (R)	ACCATATAATTGACCACAAGCAGC		
mupA	*mup*1 (F)	TATATTATGCGATGGAAGGTTGG	456	[21]
	*mup*2 (R)	AATAAAATCAGCTGGAAAGTGTTG		

rpoB	*rpoB*1 (F)	GTCGTTTACGTTCTGTAGGTG	432	[23]
	*rpoB*2 (R)	TCAACTTTACGATATGGTGTTTC		
ermA	*ermA*1 (F)	GTTCAAGAACAATCAATACAGAG	421	[19]
	*ermA*2 (R)	GGATCAGGAAAAGGACATTTTAC		
fusA	*fusA*1 (F)	CGCGGATCCTATCGTATTTATTCAGTAAT	2100	[24]
	*fusA*2 (R)	AAGGATCCCTTGTATTTTAACCTAGGCTA		

Standard strains known to be positive for the respective genes were used as positive controls (Table 2).

Table 2 The control strains used in the detection of virulence and antibiotic resistance genes among MRSA isolates

Strains	Target genes
S. aureus ATCC 14458	*lukS*
S. aureus N315	*hla*, *eta*
S. aureus JCSC/4469	*tsst/sea*
E. faecium BM4147	*vanA*
S. aureus NRS 119	*cfr*
S. aureus ATCC BAA1708	*mupA*
S. aureus ATCC BAA44	*rpoB*
S. aureus ATCC 700699	*fusA*
S. aureus ATCC BAA977	*ermA*

The optimal concentrations of primers for each gene 1 pmol were used in the multiplex PCR. The other PCR components used included 25 mM $MgCl_2$, 10 × PCR buffer, 5 U Taq DNA polymerase, and 10 µM dNTPs. The PCR was accomplished using a Mastercycler Gradient Eppendorf, Hamburg, and Germany. The initial cycle of denaturation at 95°C for 4 min was followed by 30 cycles of denaturation at 95°C for 30 s, annealing for 30 s at range (59°C-45°C based on each primer), extension at 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were mixed with 1 µL of loading buffer solution and neatly loaded in the agarose gel wells (1.5% plus Floro Red Acid Stain (1st Base, Singapore)) and electrophoresed at 75 V for 90 minutes.

Statistical Analysis

The data were analyzed by Statistical Package for Social Sciences (SPSS) software, version 25. Chi-square test was used to define the association among the two groups of variables with $p<0.05$ considered to be significant statistically.

RESULTS

Among the 60 MRSA isolates 39 (65%) were isolated from Malay patients, followed by Chinese 11 (18.33%), Indian 9 (15%), and others 2 (1.7%). The results showed higher MRSA infections in male patients, with a sex ratio male to female of 1.3:1. The mean age of patients was 44.9 years and ranged from (1-72) years old. The MRSA infections were classified into six types of infections. Among the 60 clinical isolates; 18 (30%) were cellulitis 18 (30%), 17 (23.8%) were diabetic foot ulcer, 8 (13.3%) were necrotising fasciitis, 9 (15%) were osteomyelitis, 6 (10%) were catheter-related bloodstream infection and 2 (3.3%) were pneumonia. Antibiotic susceptibility testing revealed all the isolates were susceptible to linezolid, vancomycin, and mupirocin. Fifty-two (86.7%) of the isolates were susceptible to rifampicin, 54 (90.0%) susceptible to fusidic acid, and 57 (95.0%) susceptible to clindamycin (Table 3).

Table 3: Comparison of antibiotic resistance of MRSA isolates by conventional and molecular methods

Antibiotic (µg)	Conventional n (%)	Resistance gene	Molecular n (%)
Vancomycin *	0 (0)	*vanA*	0 (0)
Linezolid (30)	0 (0)	*cfr*	0 (0)

Mupirocin (5)	0 (0)	*mupA*	0 (0)
Rifampicin (5)	8 (13.3)	*rpoB*	5 (8.3)
Fusidic acid (10)	6 (10)	*fusA*	5 (8.3)
Clindamycin (2)	3 (5)	*ermA*	1(1.7)
*Determination of MIC by E-test			

The current study revealed five common virulence genes detected among the MRSA clinical isolates. The most common virulence gene was *lukS* (60%), followed by *sea* (45%), *tsst* (43.3%), *eta* (8.3%, and *hla* (8.3%). The *sea* gene was found in 27 (45%) isolates and 26 (43.3%) isolates possessed the *tsst* gene. The *eta* and *hla* genes were found in 5 (8.3%) isolates. The *lukS* was found in 36 (60%) isolates.

Table 4 shows the association between clinical diagnosis and the presence of virulence genes. The *sea*, *tsst,* and *lukS* genes were detected in a higher proportion in patients with MRSA infection. There is no significant difference between the various types of infections and the presence of *tsst*, *eta,* and *lukS* genes. The sea and luk*S* genes were detected in a higher proportion in patients with cellulitis, diabetic foot ulcer, and osteomyelitis. The *sea* genes were significantly associated with cellulitis, diabetic foot ulcer, and osteomyelitis ($p<0.05$). But the *hla* gene was not found in previous infections ($p=0.006$).

Table 4 Association between MRSA virulence genes and types of infection

Virulence genes	No. of positive isolates (n=60)	Virulence genes present in MRSA isolates according to infection types n (%)						
		Cellulitis (n=18)	Diabetic foot ulcer (n=17)	Necrotising fasciitis (n=8)	Osteomyelitis (n=9)	CRBSI (n=6)	Pneumonia (n=2)	p-value
sea	27 (45)	10 (55.6)	8 (47.1)	2 (25)	6 (66.7)	0 (0)	1 (50)	0.049
tsst	26 (43.3)	8 (44.4)	6 (35.3)	3 (37.5)	6 (66.7)	3 (50)	0 (0)	0.423
eta	5 (8.3)	1 (5.6)	0 (0)	1 (12.5)	3 (33.3)	0 (0)	0 (0)	0.101
lukS	36 (60)	13 (72.2)	8 (47.1)	4 (50)	7 (77.8)	3 (50)	1 (50)	0.512
hla	5 (8.3)	0 (0)	0 (0)	3 (37.5)	0 (0)	2 (33.3)	0 (0)	0.006
CRBSI: Catheter-Related Blood Stream Infection								

In this study, the virulence and antibiotic resistance genes were detected by PCR. All the detected genes were correctly matched to their base-pair according to the primers used from earlier studies. Figure 1 showed positive samples for sea genes from clinical MRSA isolates.

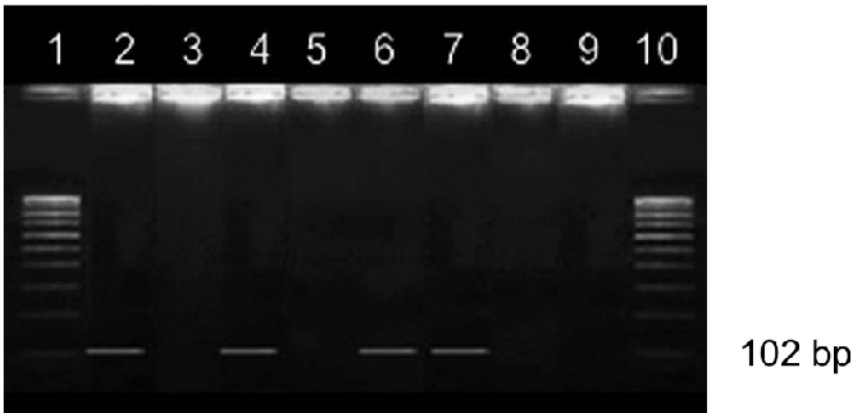

Figure 1 PCR assay. Lane 1 and 10: DNA ladder (100 bp marker); lane 2, positive control; lane 3, negative control; lane 4-9, MRSA isolates showing positive genes in lane 4, 6 and 7

DISCUSSION

In the study, the higher proportion of MRSA was among the Malay race (65%) which was due to the higher proportion of this ethnic group admitted to the Selayang Hospital. We had detected a range of antibiotic resistance and virulence genes among the MRSA isolates. Antibiotic-resistant testing of the MRSA isolates by molecular method showed resistance to rifampicin (8.3%), fusidic acid (8.3%), and clindamycin (1.7%). These results were in concordance with previous studies [19,23,24]. On the other hand, all MRSA isolates didn't show a resistant gene band for vancomycin, linezolid, and mupirocin. These findings were similar to earlier studies by Thati, et al. in Hyderabad, Kehrenberg, et al. in Germany, and Akpaka et al. in Trinidad [22,25,26]. The presence of antibiotic resistance and virulence genes frequency differ globally and within the same country and even between different cities or hospitals. The difference in the percentage of these genes could be due to variations in geographical situations of each population or region or part of the hospital where the samplings were collected [20].

The study showed an insignificant discrepancy in the detection of antibiotic resistance patterns via disc diffusion and molecular methods. The Disc-diffusion method showed slightly higher resistance rates to rifampicin, fusidic acid, and clindamycin than the molecular method. This might be attributed to other mechanisms of antibiotic resistance that were not coded by the genes that were tested. Furthermore, it is known that there are other mechanisms of antibiotic resistance that contribute to the same antibiotic-resistant pattern such as decreased cell permeability, active efflux, enzymatic inactivation of the antibiotic, modification of the drug-receptor site, and synthesis of the resistant metabolic pathway. All the MRSA isolates were susceptible to vancomycin, linezolid, and mupirocin by both disc-diffusion and molecular methods. Similar findings were reported by current studies conducted in Hospital Tuanku Jaafar in Negeri Sembilan and University Malaya Medical Centre in Kuala Lumpur [27,28]. Fortunately, Vancomycin-Resistance *S. aureus* (VRSA) has not been documented in Malaysia thus far. However, many VRSA strains were already reported in neighboring countries from Indonesia and Thailand with a vancomycin MIC of more than ≥ 16 µg/mL [29,30]. Hence, we should constantly be vigilant of the VRSA strains as it is anticipated that they would eventually emerge in Malaysia.

Earlier studies have stated that the *lukS* and *sea* genes are the most common toxin genes in MRSA isolates [31]. Similarly, the *lukS* gene was the predominant gene (60%) detected in this study, which was also consistent with previous studies conducted in Malaysia which showed the *lukS* gene detection rate ranged between 3.8% to 11.1% in three different hospitals in the country [32-34]. The higher *lukS* generate in our study could be attributed to the types of infection as the MRSA infections in the patients were mostly acquired from the community whereby the presence of *lukS* gene is well documented or it might reflect a possible clonal expansion of those CA-MRSA into the hospital environment. Moreover, among the 60 clinical MRSA isolates 71.6% were of skin and soft tissue infections which were known to be highly associated with the presence of *luk*S gene [35]. The second highest virulent toxin which was detected from MRSA isolates was staphylococcal enterotoxin type A (*sea*) with 45%. Although, enterotoxin A is associated with the sporadic food-poisoning syndrome and foodborne outbreaks *sea* gene was the most common staphylococcal enterotoxin gene present among Malaysian MRSA strains which were isolated from invasive samples [36]. A previous study by Lim, et al. showed a significant increase in the prevalence of sea genes in 2008 strains compared with 2003 strains. These toxins can also cause toxic shock-like syndromes and have been implicated in several allergic and autoimmune diseases [8].

To the best of our knowledge, studies on *tsst* in Malaysia were scarce. Surprisingly, this study has demonstrated a high percentage of *tsst* gene among MRSA isolates i.e., 26 (46%) out of 60 isolates. The finding in this study was similar to a study conducted in Japan by Parsonnet and his colleagues; but lower than another study from Iran which showed 68% [37,38]. However, none of the MRSA isolates showed *tsst* gene in two earlier studies conducted in Kuala Lumpur and Terengganu [39,40]. The difference between the findings in this study and other literature may be due to a difference in geographic regions and also due to the differences in techniques as most of the previous studies evaluated the presence of anti-TSST antibodies.

Although the frequency of the *tsst* gene within Methicillin Sensitive *Staphylococcus aureus* strains (MSSA) is known to be higher, recently, it was reported that *tsst* expression was independent of the sensitivity of *S. aureus* to methicillin; also the number of MRSA strains harboring this gene has increased [37]. Several studies have also stated that staphylococcal toxins yielded vary considering the existence of different genotypes. Furthermore, it has been indicated that the virulence gene profiles of *S. aureus* in specific isolates may be influenced by the origins of their geographical place for example there was a high detection rate of *tsst* and *sea* genes in the United Kingdom and the United State of

America [41,42]. An association between the MRSA virulence genes and the type of infections revealed no significant difference in the distribution of *lukS* among different types of MRSA infections. Although, *hla* has the lowest rate among other genes it showed a significant association with clinical MRSA cases (p-value<0.05). The result was not in concordance with the previous finding by Rossato, et al. who found the coexistence of *hla* genes in 87.6% among MRSA isolates obtained from hospitals in Porto Alegre, Brazil [43].

This study has several limitations including its small sample size. Also, the protein expression by virulence genes was not studied. Previous reports stated that the expression of drug resistance genes in MRSA strains might reduce the expression of virulence protein [44]. Another limitation is that this study used Chi-square in analysis, which does not provide the strength of the association between those two groups of variables.

CONCLUSION

The study revealed different characteristics of toxins and antibiotic resistance genes profiles between different MRSA infections. Both molecular and conventional methods for the detection of antibiotic resistance were comparable. All MRSA strains were sensitive to vancomycin, linezolid, and mupirocin. Both *sea* and *hla* genes have a significant association with the types of infection. Our outcomes showed a high rate of *lukS* gene among the MRSA isolates. Further studies needed to be carried out on the pathogenesis of these genes and their association with the types of MRSA infections.

DECLARATIONS

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Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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