



## RESEARCH ARTICLE

# Distribution of virulence genes in clinical isolates of hospital-associated and community-associated methicillin-susceptible *Staphylococcus aureus* from Terengganu, Malaysia

Che Hamzah, A.M.<sup>1</sup>, Yeo, C.C.<sup>2</sup>, Puah, S.M.<sup>3</sup>, Chua, K.H.<sup>3</sup>, A. Rahman, N.I.<sup>2</sup>, Ismail, S.<sup>2</sup>, Abdullah, F.H.<sup>4</sup>, Othman, N.<sup>4</sup>, Chew, C.H.<sup>1\*</sup>

<sup>1</sup>Faculty of Health Sciences, Universiti Sultan Zainal Abidin, 21300, Kuala Nerus, Terengganu, Malaysia

<sup>2</sup>Centre for Research in Infectious Diseases and Biotechnology, Faculty of Medicine, Universiti Sultan Zainal Abidin, 20400, Kuala Terengganu, Terengganu, Malaysia

<sup>3</sup>Department of Biomedical Science, Faculty of Medicine, Universiti Malaya, 50603, Kuala Lumpur, Malaysia

<sup>4</sup>Department of Pathology, Hospital Sultanah Nur Zahirah, 20400, Kuala Terengganu, Terengganu, Malaysia

\*Corresponding author: [chewch@unisza.edu.my](mailto:chewch@unisza.edu.my)

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## ABSTRACT

*Staphylococcus aureus* is a common bacterial pathogen known to cause various kinds of infections due to its repertoire of virulence factors. This study aimed to investigate the distribution of 19 types of virulence genes among clinical isolates of methicillin-susceptible *S. aureus* (MSSA) using the polymerase chain reaction. A total of 109 MSSA isolates, i.e., 63 hospital-associated (HA) and 46 community-associated (CA) were collected from Hospital Sultanah Nur Zahirah, the main tertiary hospital in Terengganu, Malaysia, from July 2016 to June 2017. The most frequent virulence genes detected were *hla* (78.9%,  $n=86$ ) and *hld* (78.0%,  $n=85$ ) encoding hemolysins, *lukED* (56.9%,  $n=62$ ) encoding leukotoxin ED, followed by *seb* (26.6%,  $n=29$ ) and *sea* (24.8%,  $n=27$ ) encoding enterotoxins. Among 34 (31.2%) isolates carrying six or more virulence genes, only five were multidrug resistant (MDR) while the remaining isolates were susceptible. Significant associations were discovered between the *hld* gene with CA-MSSA ( $p=0.016$ ) and the *seo* gene with HA-MSSA ( $p=0.023$ ). However, there is no significant association between virulence genes among the different types of infection. The clinical MSSA isolates in Terengganu showed high prevalence and high diversity of virulence gene carriage.

**Keywords:** Methicillin-susceptible *Staphylococcus aureus*; virulence genes; CA-MSSA; HA-MSSA.

## INTRODUCTION

*Staphylococcus aureus* has long been recognized as one of the most important human pathogens that causes a great variety of infections, ranging from benign skin infections to potentially life-threatening conditions due to its repertoire of virulence factors. Approximately 10% of the secretome of *S. aureus* consists of exotoxins, a diverse group that can be broadly categorized as cytotoxins, superantigens and cytotoxic enzymes (Tam & Torres, 2019). These exotoxins play various roles in *S. aureus* infection. The pore-forming cytotoxins induce cell lysis and death through the efflux of metabolites and molecules (Kim, 2019). In addition to causing cellular damage, cytotoxins also contribute to the pathogenesis of certain infections. For example, leukotoxin ED (LukED) plays an important role in *S. aureus* bloodstream infection, impetigo and antibiotic-associated diarrhea (He *et al.*, 2018), while Panton-Valentine leukocidin (PVL) is associated with skin and soft tissue infections, particularly those caused by community strains of methicillin-resistant *S. aureus* (MRSA) (Shallcross *et al.*, 2013). In addition, hemolysins have also been implicated in the pathogenesis of pneumonia and corneal

infections ( $\alpha$ -toxin), chronic osteomyelitis and respiratory infections ( $\beta$ -toxin), as well as skin diseases and atopic dermatitis ( $\delta$ -toxin) (Berube & Wardenburg, 2013; Salgado-Pabón *et al.*, 2014; Chung *et al.*, 2022).

Generally, *S. aureus* is one of several bacterial species known to produce superantigens. These superantigens, which include enterotoxins and toxic shock syndrome toxin 1 (TSST-1), are potent T cell mitogens that trigger oligoclonal T cell activation, leading to immense cytokine release (Abdurrahman *et al.*, 2020). The enterotoxins are potent emesis-inducing toxins, that require only minute quantities to be toxic in humans (Fisher *et al.*, 2018). While they are commonly implicated in food-borne illnesses, studies have also shown that enterotoxins are associated with infections of the respiratory tract, sepsis-related infections and autoimmune diseases (Ortega *et al.*, 2010; Fisher *et al.*, 2018). The TSST-1 causes an acute-onset, potentially life-threatening systemic infection that involves multiple organ systems. Additionally, *S. aureus* also secretes cytotoxic enzymes such as exfoliative toxin, the serine protease that cleaves desmoglein-1, contributing to the development of staphylococcal scalded skin syndrome (Hubiche *et al.*, 2012).

The vast majority of research conducted on *S. aureus* focuses mainly on MRSA, resulting in a scarcity of data on methicillin-susceptible *S. aureus* (MSSA). In Malaysia, *S. aureus* is identified as one of the top priority pathogens, as reported annually in the Malaysian Ministry of Health's National Surveillance of Antibiotic Resistance (NSAR) reports ([https://imr.nih.gov.my/MyOHAR/index.php/site/archive\\_rpt](https://imr.nih.gov.my/MyOHAR/index.php/site/archive_rpt)). The majority of these infections are caused by MSSA, with prevalence rates ranging from 80.2% – 94.0% from 2012 to 2022 indicating its role as a major cause of infection in the country (Che Hamzah *et al.*, 2019a; Ministry of Health Malaysia, 2023). Furthermore, MSSA also demonstrated the potential to develop multidrug resistance (MDR), severe infections and even death cases (Che Hamzah *et al.*, 2019b). The study of virulence factors contributes to our understanding of the pathogenesis of an infection, its possible complications as well as the prognosis. Understanding the pathogenesis is an important aspect for the development of potential novel antibiotics or even vaccines (Li *et al.*, 2019; Mirzaei *et al.*, 2021). In this study, we aimed to investigate the occurrence of 19 types of genes encoding virulence factors among 109 clinical MSSA isolates obtained from a tertiary hospital in Terengganu, Malaysia.

## MATERIALS AND METHODS

### Ethical approval

From July 2016 to June 2017, 109 clinical MSSA isolates and clinical data were collected in accordance with the Declaration of Helsinki from the Microbiology Laboratory of Hospital Sultanah Nur Zahirah (HSNZ) (Che Hamzah *et al.*, 2019b), with approval from the Malaysian National Medical Research Registry and the Medical Research Ethics Committee (NMRR-MREC) of the Ministry of Health Malaysia [NMRR-15-2369-28130 (IIR)]. Clinical data were obtained with approval from the Clinical Research Centre of HSNZ and were used to categorize the isolates as either hospital-associated (HA) or community-associated (CA). In brief, the Centers for Disease Control and Prevention (CDC) defines CA cases as isolates obtained within the first 48 hours of hospitalization from patients with no prior history of hospitalization, surgery, residence in a long-term care facility or dialysis within the past 12 months, no presence of percutaneous device or indwelling catheter, and no previous infection or colonization. Isolates that do not meet the mentioned criteria are classified as HA (Centers for Disease Control and Prevention, 2016).

### Bacterial identification and DNA extraction

Standard microbiological procedures for isolation and identification of the MSSA isolates were carried out at the Microbiology Laboratory of HSNZ. The MSSA isolates were cultured on mannitol salt agar and isolates showing mannitol fermentation were extracted for their DNA using a simple boiling method. Polymerase chain reaction (PCR) was conducted to validate the isolates as *S. aureus* by screening for the presence of *nuc* gene (279 bp) using previously established primer (Brakstad *et al.*, 1992). Confirmed MSSA isolates were cryopreserved in LB broth containing 15% (v/v) glycerol stock and kept at -80°C for long-term storage (Che Hamzah *et al.*, 2019b).

### Antimicrobial resistance profiling

The 109 MSSA isolates in this study were tested for resistance against 26 antibiotics from 18 antimicrobial classes as follows:  $\beta$ -lactams (penicillin, oxacillin, and ceftazidime); fluoroquinolones (ciprofloxacin and moxifloxacin); macrolide (erythromycin); lincosamide (clindamycin); aminoglycosides (gentamicin and amikacin); folate inhibitor (co-trimoxazole); fusidans (fusidic acid); tetracyclines (tetracycline, doxycycline, and minocycline); glycolylglycylines (tigecycline); phenicols (chloramphenicol); monoxycarboxylic acid (mupirocin); ansamycin (rifampin); aminocoumarins (novobiocin); glycopeptides (vancomycin and teicoplanin); oxazolidinones (linezolid); phosphonic acid

(fosfomycin); 'streptogramins' only (quinupristin-dalfopristin); and anti-MRSA cephalosporins (ceftaroline) (Che Hamzah *et al.*, 2019b). Based on the antimicrobial resistance profile, isolates that conferred resistance to three or more antimicrobial classes were categorized as MDR (Magiorakos *et al.*, 2012).

### Screening of virulence genes

The presence of 19 staphylococcal virulence genes which included genes encoding for enterotoxins (*sea*, *seb*, *sec*, *seg*, *seh*, *sei*, *sel*, *sem*, *sen*, *seo*, and *ser*), TSST (*tst*), exfoliative toxin (*eta*), leukotoxin ED (*lukED*), Panton-Valentine leukocidin (PVL) (*lukPV*), and hemolysins (*hla*, *hlb*, *hld*, and *hlg*), were screened by PCR assay using previously established primers (Puah *et al.*, 2016). The PCR conditions were optimized accordingly. For the genotyping assay, positive control (laboratory collection of positive isolates) and negative control (sterilized double-distilled water) were included in each run of PCR for results validation. The PCR products were visualized using 2% (w/v) SYBR Safe-stained agarose gel and documented using an Atto Printgraph (Atto, Japan) documentation system.

### Statistical analysis

Statistical analyses were performed using the chi-square or Fisher's exact test using IBM SPSS Statistics version 27.0. Fisher's exact test with the Freeman-Halton extension was employed for larger contingency tables. Post hoc analysis was conducted using standardized residuals, and multiple comparisons correction was performed using the Holm-Bonferroni method (Stańkowska *et al.*, 2022). A *p*-value less than 0.05 was considered to be statistically significant.

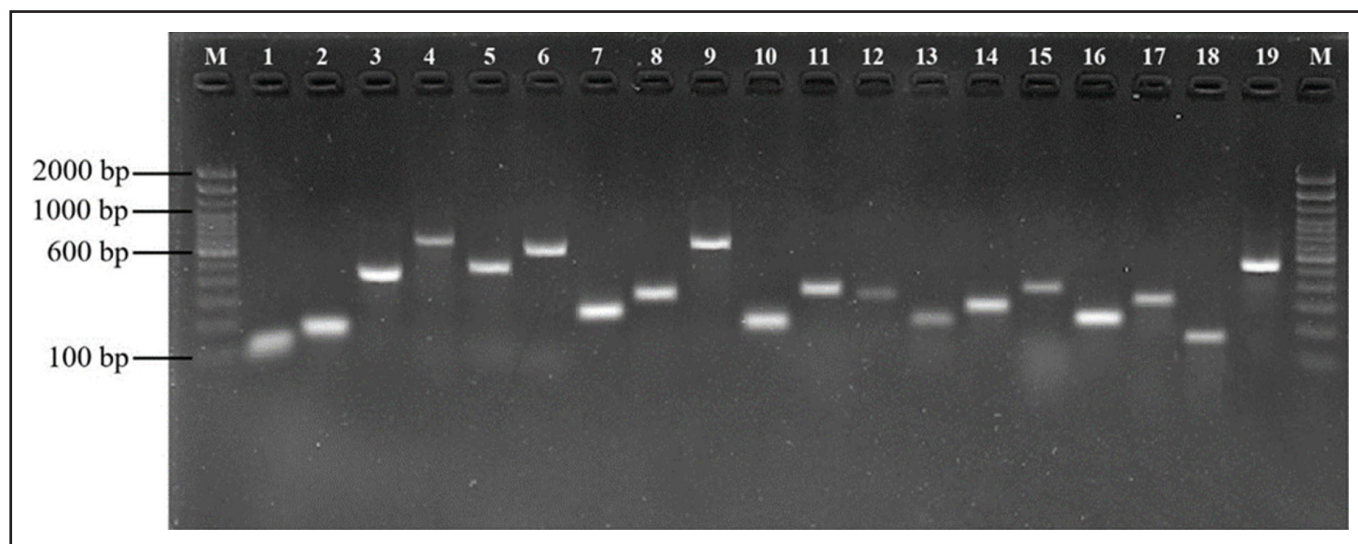
## RESULTS

### Distribution of virulence genes

Based on the medical records, the MSSA isolates were categorized into HA-MSSA (*n*=63) and CA-MSSA (*n*=46). The presence of virulence genes and respective amplicon sizes are shown in Figure 1. The prevalence and distribution of the 19 virulence genes are shown in Table 1 and Figure 2. Overall, the carriage of virulence genes is high, with 94.5% of the 109 MSSA isolates (92.1% of the HA isolates and 97.8% of the CA isolates) harboring at least one type of virulence gene. Across the 109 MSSA isolates, the hemolysin genes, *hla* (78.9%, 86/109) and *hld* (78.0%, 85/109) were identified as the most prevalent virulence genes. The leukotoxin gene, *lukED* was detected in over half of the isolates (56.9%, 62/109), while *lukPV*, which encodes for PVL was present in 15.6% (17/109) isolates. Among the enterotoxin genes, *seb* (26.6%, 29/109), *sea* (24.8%, 27/109), and *sel* (21.1%, 23/109) were the most common, with all three genes sharing almost similar prevalence rates. On the other hand, the TSST gene and the exfoliative toxin gene were found to be the least common with *tst* being detected in just two (1.8%) isolates (SA121 and SA149), whereas the *eta* gene was absent. Most virulence genes showed a higher prevalence in HA-MSSA isolates (Table 1). Notably, the enterotoxin gene, i.e., *seo* (15.9%, 10/63, *p*=0.023) showed a significant association with HA-MSSA, while the hemolysin gene, i.e., *hld* was significantly associated with CA-MSSA (89.1%, 41/46, *p*=0.016).

### Diversity and co-occurrence of virulence genes

Thirty-four (31.2%) isolates carried six or more virulence genes, of which 17.4% (*n*=19) were HA-MSSA and 13.8% (*n*=15) were CA-MSSA (Figure 2). One (0.9%) HA-MSSA isolate (SA121) had the highest number of virulence genes, with 10 of the 19 genes detected. Two (1.8%) isolates, i.e., SA41 (also a death case) and SA71, each had nine virulence genes and were identified as HA-MSSA and CA-MSSA, respectively. Conversely, six (5.5%) isolates were found negative for all 19 virulence genes. The virulence gene profiles of the MSSA isolates were highly diverse, with a total of 71 distinct patterns



**Figure 1.** SYBR Safe-stained 2% (w/v) agarose gel showing the 19 virulence genes screened in this study. Note that the *eta* gene was absent in all isolates, and the positive band depicted here was from a positive control isolate. Lane M contains the DNA Ladder (Invitrogen™ 100 bp DNA Ladder). Lanes 1, *sea* (102 bp); 2, *seb* (164 bp); 3, *sec* (451 bp); 4, *seg* (704 bp); 5, *seh* (495 bp); 6, *sei* (630 bp); 7, *sel* (240 bp); 8, *sem* (326 bp); 9, *sen* (680 bp); 10, *seo* (180 bp); 11, *ser* (367 bp); 12, *tst* (326 bp); 13, *eta* (190 bp) from positive control; 14, *lukED* (269 bp); 15, *lukPV* (433 bp); 16, *hla* (209 bp); 17, *hlb* (309 bp); 18, *hld* (111 bp); 19, *hlg* (535 bp).

**Table 1.** Distribution of virulence genes among methicillin-susceptible *Staphylococcus aureus* isolates

Virulence gene	Total (n=109) n (%)	HA-MSSA <sup>1</sup> (n=63) n (%)	CA-MSSA <sup>2</sup> (n=46) n (%)	p-Value
<b>Enterotoxin</b>				
<i>sea</i>	27 (24.8)	17 (27.0)	10 (21.7)	0.531
<i>seb</i>	29 (26.6)	19 (30.2)	10 (21.7)	0.326
<i>sec</i>	16 (14.7)	10 (15.9)	6 (13.0)	0.680
<i>seg</i>	11 (10.1)	7 (11.1)	4 (8.7)	0.679
<i>seh</i>	18 (16.5)	10 (15.9)	8 (17.4)	0.833
<i>sei</i>	9 (8.3)	6 (9.5)	3 (6.5)	0.574
<i>sel</i>	23 (21.1)	14 (22.2)	9 (19.6)	0.737
<i>sem</i>	12 (11.0)	7 (11.1)	5 (10.9)	0.968
<i>sen</i>	9 (8.3)	8 (12.7)	1 (2.2)	0.076
<i>seo</i>	11 (10.1)	10 (15.9)	1 (2.2)	0.023*
<i>ser</i>	10 (9.2)	5 (7.9)	5 (10.9)	0.740
<b>Toxic shock syndrome toxin</b>				
<i>tst</i>	2 (1.8)	2 (3.2)	0 (0.0)	0.508
<b>Exfoliative toxin</b>				
<i>eta</i>	0 (0.0)	0 (0.0)	0 (0.0)	–
<b>Leukotoxin ED</b>				
<i>lukED</i>	62 (56.9)	31 (49.2)	31 (67.4)	0.058
<b>Panton-Valentine leucocidin</b>				
<i>lukPV</i>	17 (15.6)	8 (12.7)	9 (19.6)	0.329
<b>Hemolysin</b>				
<i>hla</i>	86 (78.9)	49 (77.8)	37 (80.4)	0.737
<i>hlb</i>	14 (12.8)	7 (11.1)	7 (15.2)	0.527
<i>hld</i>	85 (78.0)	44 (69.8)	41 (89.1)	0.016*
<i>hlg</i>	3 (2.8)	2 (3.2)	1 (2.2)	0.752
Death <sup>3</sup>	9 (8.3)	6 (9.5)	3 (6.5)	–

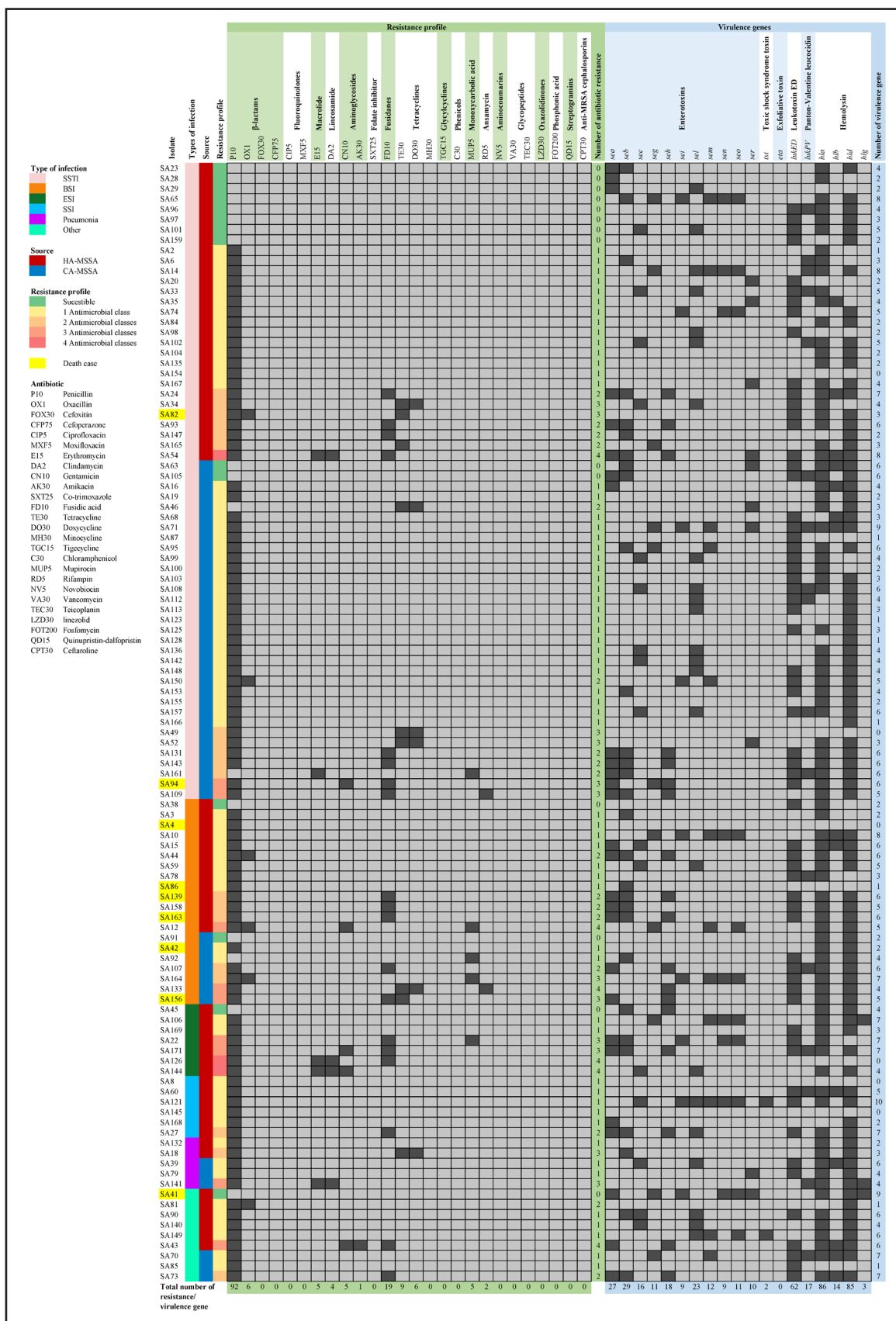
<sup>1</sup>HA-MSSA, hospital-associated methicillin-susceptible *S. aureus*; <sup>2</sup>CA-MSSA, community-associated methicillin-susceptible *S. aureus*; <sup>3</sup>Number (%) of death case; \*, statistically significant.

detected among the 109 isolates (Figure 2). Overall, *hla-hld* was the most common pattern found in eight isolates, followed by *sea-seb-seh-lukED-hla-hld* (six isolates), *lukED-hla-hld* (five isolates) and *sec-sel-hla-hld* (four isolates).

#### Virulence genotypes in relation to multidrug resistance and isolate type

Antimicrobial resistance profile indicated that only 11.0% (12/109) of MSSA were categorized as MDR, with nine isolates resistant to three antimicrobial classes and the other three isolates were resistant to four antimicrobial classes (Figure 2). From the 34 isolates that harbored six or more virulence genes, only five were MDR isolates, four of which were resistant to three antimicrobial classes and positive for six and seven virulence genes (two isolates each), and one was resistant to four antimicrobial classes and carried eight virulence genes (Figure 2). Four fully-susceptible isolates possessed six (two isolates), eight (one isolate), and nine (one isolate) virulence genes, respectively. The single isolate (SA121) that harbored 10 virulence genes and another isolate (SA71) with nine virulence genes were non-MDR, being resistant to only one antimicrobial class (Figure 2).

The MSSA isolates were obtained from patients with various clinical infections, including skin and soft tissue infections (SSTI) ( $n=62$ ), bloodstream infections (BSI) ( $n=20$ ), exit site infections (ESI) ( $n=7$ ), surgical site infections (SSI) ( $n=6$ ), pneumonia ( $n=5$ ), and other infections ( $n=9$ ), which included those involving the urinary tract, eyes, ears, and isolates from the endotracheal tube (Table 2 and Figure 2). While SSTI was the most common type of infection in this study, none of the screened virulence genes were found to be more prevalent in this group. In contrast, *sea*, *seh*, and *lukED* genes were found at higher levels among BSI isolates, while SSI isolates showed higher levels of *sei*, *sel* and *tst*. Notably, five genes, namely *ser*, *lukPV*, *hla*, *hld*, and *hlg* were observed at higher levels in isolates obtained from pneumonia patients. However, statistical analysis did not reveal any significant associations between the virulence genes and the types of infections.



**Figure 2.** Heatmap showing the antibiotic resistance profile and the distribution of virulence genes across the 109 methicillin-susceptible *Staphylococcus aureus* (MSSA) isolates, organized by types of infection. Isolate names highlighted in yellow indicate death cases. The types of infections are categorized as follows: SSTI, skin and soft tissue infections; BSI, bloodstream infections; ESI, exit site infections; SSI, surgical site infections; pneumonia; other infections (urinary tract, eyes, ears, and endotracheal tube). The sources of infection are classified into hospital-associated (HA) and community-associated (CA) following the CDC definition (Centers for Disease Control and Prevention, 2016). The resistance profiles were determined by the number of antimicrobial classes to which the isolates exhibit resistance, with multidrug resistance (MDR) indicating resistance to three or more antimicrobial classes (Magiorakos et al., 2012). Antibiotic-resistant and presence of virulence gene are indicated by dark grey scales, while light grey scales denoted antibiotic-susceptible and absence of virulence genes.

**Table 2.** Distribution of virulence genes among the different types of infection

Virulence gene	SSTI <sup>1</sup> (n=62) n (%)	BSI <sup>2</sup> (n=20) n (%)	ESI <sup>3</sup> (n=7) n (%)	SSI <sup>4</sup> (n=6) n (%)	Pneumonia (n=5) n (%)	Other <sup>5</sup> (n=9) n (%)	p-Value
<b>Enterotoxin</b>							
<i>sea</i>	13 (21.0)	7 (35.0)	2 (28.6)	2 (33.3)	0 (0.0)	3 (33.3)	0.552
<i>seb</i>	15 (24.2)	7 (35.0)	3 (42.9)	1 (16.7)	1 (20.0)	2 (22.2)	0.824
<i>sec</i>	9 (14.5)	2 (10.0)	1 (14.3)	1 (16.7)	1 (20.0)	2 (22.2)	0.906
<i>seg</i>	6 (9.7)	2 (10.0)	1 (14.3)	0 (0.0)	0 (0.0)	2 (22.2)	0.721
<i>seh</i>	7 (11.3)	6 (30.0)	2 (28.6)	1 (16.7)	0 (0.0)	2 (22.2)	0.240
<i>sei</i>	4 (6.5)	2 (10.0)	1 (14.3)	1 (16.7)	0 (0.0)	1 (11.1)	0.592
<i>sel</i>	15 (24.2)	1 (5.0)	1 (14.3)	2 (33.3)	1 (20.0)	3 (33.3)	0.288
<i>sem</i>	5 (8.1)	3 (15.0)	1 (14.3)	1 (16.7)	0 (0.0)	2 (22.2)	0.490
<i>sen</i>	3 (4.8)	2 (10.0)	2 (28.6)	1 (16.7)	0 (0.0)	1 (11.1)	0.174
<i>seo</i>	3 (4.8)	3 (15.0)	2 (28.6)	1 (16.7)	0 (0.0)	2 (22.2)	0.083
<i>ser</i>	8 (12.9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	1 (11.1)	0.367
<b>Toxic shock syndrome toxin</b>							
<i>tst</i>	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	1 (11.1)	0.064
<b>Exfoliative toxin</b>							
<i>eta</i>	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	–
<b>Leukotoxin ED</b>							
<i>lukED</i>	36 (58.1)	13 (65.0)	3 (42.9)	3 (50.0)	2 (40.0)	5 (55.6)	0.866
<b>Panton-Valentine leucocidin</b>							
<i>lukPV</i>	11 (17.7)	2 (10.0)	1 (14.3)	1 (16.7)	1 (20.0)	1 (11.1)	0.969
<b>Hemolysin</b>							
<i>hla</i>	47 (75.8)	18 (90.0)	6 (85.7)	3 (50.0)	5 (100.0)	7 (77.8)	0.308
<i>hlb</i>	7 (11.3)	2 (10.0)	0 (0.0)	1 (16.7)	1 (20.0)	3 (33.3)	0.333
<i>hld</i>	48 (77.4)	15 (75.0)	5 (71.4)	4 (66.7)	5 (100.0)	8 (88.9)	0.804
<i>hlg</i>	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	1 (20.0)	1 (11.1)	0.190
<b>Death<sup>6</sup></b>							
	2 (3.2)	6 (30)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	9 (8.3)

<sup>1</sup>SSTI, skin and soft tissue infections; <sup>2</sup>BSI, bloodstream infections; <sup>3</sup>ESI, exit site infections; <sup>4</sup>SSI, surgical site infections; <sup>5</sup>other infections (urinary tract, eyes, ears, and endotracheal tube); <sup>6</sup>Number (%) of death case.

## DISCUSSION

In Malaysia, there is a scarcity of research on virulence genotyping of MSSA isolates, and to the best of our knowledge, there are only five studies so far. Four of these studies took place in Kuala Lumpur, the capital of Malaysia, using 237 (Neoh *et al.*, 2017) and 880 (Sapri *et al.*, 2020) clinical isolates from Hospital Canselor Tuanku Muhriz (HCTM), formerly known as Universiti Kebangsaan Malaysia Medical Centre (UKMMC), 252 isolates, i.e., 142 clinical isolates from the Hospital Kuala Lumpur (HKL) and 110 nasal-carriage community-acquired isolates from various groups of healthy individuals (Ghasemzadeh-Moghaddam *et al.*, 2011), and 67 isolates, i.e., 29 in-patient admitted to University of Malaya Medical Center (UMMC) and 38 community-acquired isolates from healthy undergraduate students (Lim *et al.*, 2012a). The fifth study was conducted in Terengganu, using 21 isolates obtained from the same hospital as this study (Lim *et al.*, 2012b). All five studies focused on MSSA isolates collected between 2008 – 2010.

In the present study, the *sea* (24.8%) and *seb* (26.6%) were the two most predominant enterotoxin genes, followed by *sel* gene (21.1%). While the rest of the screened enterotoxin genes ranged around 8.3% to 16.5%. Similarly, Lim *et al.* (2012b) also reported *sea* (28.6%, 6/21) as the predominant enterotoxin gene. In contrast, *seb* gene was found in only two strains (9.5%) with the absence of the *seg*, *seh*, and *sei* genes among 21 clinical MSSA isolates collected from HSNZ (Lim *et al.*, 2012b). In contrast, about half of the UKMMC isolates carried *sei* (54.9%, n=130) and *sea* (49.4%, n=117) genes, followed by *seg* (41.8%, n=99) and *seb* (30.0%, n=71) enterotoxin genes (Neoh *et al.*, 2017). Ghasemzadeh-Moghaddam *et al.* (2011)

reported that *sei* (36.9%, n=93) and *seg* (36.5%, n=92) were the most prevalent enterotoxin genes followed by *sea* (24.2%) and *seb* (23.8%) genes among 252 isolates obtained from clinical and community sources. In our neighboring country, Thailand, the prevalence of the *sea* and *seb* genes were reportedly much higher at 63.9% and 36.1%, respectively (Indrawattana *et al.*, 2013). Meanwhile, Iranian isolates recorded lower prevalences of 16.0% for *sea* and absence of *seb* (Goudarzi *et al.*, 2020).

The absence of the *eta* gene in this study is also consistent with two Malaysian studies that collected samples from UMMC (Lim *et al.*, 2012a) and HSNZ (Lim *et al.*, 2012b). Low carriage of the *eta* gene (1.7% and 2.3%) in two other Malaysian studies also indicate its rarity (Ghasemzadeh-Moghaddam *et al.*, 2011; Neoh *et al.*, 2017). In contrast, the occurrence of *tst* gene (1.8%) among our isolates was notably lower. Earlier Malaysian studies reported *tst* prevalence between 3.4 to 6.8% (Ghasemzadeh-Moghaddam *et al.*, 2011; Lim *et al.*, 2012a; Sapri *et al.*, 2020). Interestingly, Indrawattana *et al.* (2013) found a higher prevalence of *eta* gene in MSSA isolates from Thailand (8.3%), while the *tst* gene was not detected.

The prevalence of the nine virulence genes, i.e., enterotoxins (*sel*, *sem*, *sen*, *seo*, and *ser*), leukotoxin ED (*lukED*), and hemolysins (*hla*, *hlb*, and *hld*) among the Malaysian MSSA clinical isolates was first reported in this study. As for leukotoxins, carriage of *lukED* gene (56.9%) in this study was slightly lower than that reported in MSSA isolates from Türkiye (67.0%) (Bayirli *et al.*, 2021). Similarly, the prevalence of *lukPV* gene (15.6%) in this study was much lower than that reported in Iranian isolates (45.3%) (Goudarzi *et al.*, 2020), but similar to Turkish isolates (14.6%) (Bayirli *et al.*, 2021).

Among the 19 virulence genes investigated in this study, the hemolysin genes, i.e., *hla* (78.9%) and *hld* (78.0%) were the most frequently detected. High rates of *hla* and *hld* genes have been observed elsewhere, implying that these genes are ubiquitous in MSSA. For example, in China, the prevalence of *hla* gene was found to be 98.8% (Li *et al.*, 2018), whereas in Brazil, it was reported at 97.5% (Pereira-Franchi *et al.*, 2019). Similarly, multiple studies showed that the *hld* gene was found in more than 90% of MSSA strains, demonstrating its widespread presence (Liu *et al.*, 2015; Pereira-Franchi *et al.*, 2019; Bayirli *et al.*, 2021). The absence and low carriage (2.8%, 3/109) of *hlg* gene were reported in two batches of MSSA isolates from HSNZ, which is located on the east coast of Peninsular Malaysia. In contrast, 48.3% (14/29) of UMMC clinical isolates were reported to harbor this gene (Lim *et al.*, 2012a). These findings were unable to rule out any regional variation due to their small sample sizes and another two studies carried out using HKL and UKMMC isolates, which are located in the central region of the west coast of Peninsular Malaysia did not screen for the *hlg* gene.

The present study identified notable differences in the prevalence of certain virulence genes among various types of infections. Isolates from BSI had higher rates of *sea*, *seh*, and *lukED* genes, while SSI isolates had a higher prevalence of *sei*, *sel* and *tst* genes. Additionally, *ser*, *lukPV*, *hla*, *hld*, and *hlg* genes were observed to be more common among pneumonia isolates. Statistical analysis did not reveal any associations between the virulence genes and the infection types, which might be due to limitations in sample size, particularly for some infection types like SSI ( $n=6$ ) and pneumonia ( $n=5$ ). Nonetheless, previous studies have linked the virulence factors encoded by these genes to specific infections. For instance,  $\alpha$ - and  $\gamma$ -hemolysin encoded by the genes *hla* and *hlg*, respectively, contribute to lung infections (Kebaier *et al.*, 2012; Ishii *et al.*, 2014), while toxic shock syndrome associated with surgical wound infections, despite being a rare occurrence, has also been documented (Abuzneid *et al.*, 2021). Furthermore, the leukotoxin gene *lukED* serves as a critical virulence factor that contributes to the lethality of *S. aureus* associated with BSI (Alonzo *et al.*, 2012). When comparing the distribution of virulence genes in relation to the staphylococcal origin, it was found that the *seo* enterotoxin gene was significantly higher in HA-MSSA (15.9%,  $p=0.023$ ), whilst *hld* hemolysin gene was significantly associated with CA-MSSA (89.1%,  $p=0.016$ ). This indicates that these genes could have toxin characteristics and contribute to the pathogenicity of HA- and CA-MSSA isolates, respectively, in this region.

Amongst the 109 MSSA isolates, a total of nine (8.3%) deaths were reported (Che Hamzah *et al.*, 2019b). A review of the clinical data indicated that most of the death cases were from blood isolates (30%, 6/20) with three of them (15%, 3/20) attributed to MSSA sepsis (Che Hamzah *et al.*, 2019b). The MSSA bacteremia mortality observed in our study was lower than that reported by another Malaysian study, which was 25% (Nordin *et al.*, 2021), but comparable to that reported in Thailand, which was 12.5% (Chuamuangphan *et al.*, 2014). Of the nine death cases, four of the MSSA isolates carried six and more virulence genes. SA94, which was isolated from a patient with necrotizing fasciitis, as well as SA139 and SA163, which were blood isolates from patients with bacteremia complicated with lung abscess and catheter-related infection, respectively, all harbored six virulence genes. One HA-MSSA, SA41, was identified with nine virulence genes and was isolated from the tracheal aspirate of an intensive care unit patient who died due to motor vehicle accident. Interestingly, among the three deaths attributed to MSSA sepsis, two isolates (SA86 and SA156) carried one and five virulence genes, respectively, while another isolate (SA4) did not carry any of the 19 virulence genes. This finding suggests that the number of virulence genes carried by MSSA isolates might not influence mortality, as isolates like SA4, without the carriage of any of the virulence factors that were screened, were still capable of causing fatal infections. As highlighted by Horváth *et al.* (2020),

the outcome of infections appears to be more related to antibiotic resistance, clonality, and patient-specific factors. Supporting this notion, all three patients had underlying medical conditions or comorbidities, suggesting these factors may have contributed to the fatal outcomes.

The HSNZ MSSA isolates exhibited diversity and most had high carriage of virulence genes, which was consistent with earlier studies reporting on the abundance of virulence genes in MSSA (Jiménez *et al.*, 2011; Hoseini Alfatemí *et al.*, 2014; Liu *et al.*, 2015). Among the 34 isolates harboring six or more virulence genes, only five were MDR while the rest were susceptible strains. The fitness cost associated with multi-resistant strains may explain why only a small percentage of resistant organisms have a large number of virulence genes (Jiménez *et al.*, 2011; Beceiro *et al.*, 2013; Vogwill & Maclean, 2015). It was theorized that the production of virulence genes often compete with the evolution of antibiotic resistance in bacterial pathogens, as the energy needed to express resistance reduces the capacity of multi-resistant strains to express virulence (Beceiro *et al.*, 2013; Vogwill & Maclean, 2015; Li *et al.*, 2019).

## CONCLUSION

The clinical MSSA isolates in Terengganu showed high prevalence and high diversity of virulence genes, with carriage of *seo* and *hld* genes being possible characteristics of HA-MSSA and CA-MSSA isolates, respectively, in this region. While most HSNZ MSSA isolates tend to be susceptible to antibiotics, they still carry an abundance of virulence genes, demonstrating their potential for pathogenicity.

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## Conflict of Interests

The author declares that they have no conflict of interest.

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