

Molecular Characterization of Virulence-Associated Toxin Genes in clinical Methicillin-Resistant *Staphylococcus aureus* (MRSA) Isolates from Wound Infections

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ABSTRACT

Objective: The aim of the present study was to molecularly characterize the MRSA isolates obtained through wound infection specimens, with a special concern on the identification of the *mecA* resistance gene and the presence of the major toxin genes such as Panton-Valentine Leucocidin (lukSF-PV), toxic shock syndrome toxin-1 (tst) and staphylococcal enterotoxins (sea, seb). **Method:** Wound swabs were treated with common bacteriological procedures and confirmed *Staphylococcus aureus* isolates were subjected to antimicrobial susceptibility testing and then to multiplex polymerase chain reaction (PCR) to be molecularly characterized. **Results:** Findings indicated that there was a significant prevalence of *mecA*-positive MRSA isolates in wound infection specimens and there was a high level of co-carriage of toxin genes most notably lukSF-PV and *tst*. These two characteristics (resistance and virulence determinants) of wound-related strains of MRSA highlight the dual risks that such strains present to patient and infection control. **Novelty:** The results highlight the significant importance of molecular diagnostic techniques in combination with regular clinical microbiology practice to facilitate proper identification of MRSA and influence clinical decision-making, as well as epidemiological monitoring.

INTRODUCTION

Staphylococcus aureus is a Gram-positive pathogen that demonstrates an extremely broad range of infections including superficial skin and wound infections as well as potentially lethal systemic infections including bacteremia, infective endocarditis, osteomyelitis, and necrotizing pneumonia. Combined with its extraordinary ability to obtain and express resistance genes and a broad spectrum of virulence factors, it has enabled it to become one of the most dangerous pathogens experienced in both the hospital and community environments. The development of methicillin-resistant *S. aureus* (MRSA) is one of the most fateful changes in the history of the infectious disease that makes the whole group of beta-lactam antibiotics inactive against these strains and significantly reduces treatment options of infected individuals. MRSA has become one of the primary causes of healthcare-associated infections worldwide and has recently been implicated in community-acquired infections in persons with no previous healthcare contact at all [1].

The mechanism of MRSA resistance revolves around the *mecA* gene which expresses penicillin-binding protein 2a (PBP2a) which is an altered transpeptidase that has significant low affinity towards beta-lactam antibiotics. In contrast to the native PBPs which are Acylated and are inactivated by these drugs, PBP2a remains transpeptidase

active in their presence allowing cell wall synthesis and resistance to all standard beta-lactam-based agents. *MecA* gene is located on Staphylococcal cassette chromosome *mec* (SCC*mec*) which is a mobile genetic element that undergoes horizontal transfer between strains of staphylococcal bacteria and enables the rapid and extensive spread of resistance in clinical settings [2]. PCR-based methods of molecular detection of the *mecA* gene have therefore become the standard of definitive confirmatory testing of MRSA, with high sensitivity and specificity as opposed to the traditional phenotypic methods of susceptibility testing [3].

In addition to the phenomenon of antibiotic resistance, the clinical severity and epidemiological importance of MRSA infections is immensely affected by the ability of the organism to secrete a rich array of virulence factors and toxins [4]. Panton-Valentine Leukocidin (PVL), a pore-forming toxin encoded by the bicomponent *lukS*-PV and *lukF*-PV genes and highly associated with polymorphonuclear leukocytes (PMNs), monocytes, and macrophages, has been found to be highly cytotoxic in many cases that have necrotising skin and soft tissue infections and severe community-acquired pneumonia [5]. The pathogenicity island of the *tst* gene encodes toxic shock syndrome toxin-1 (TSST-1), a superantigen that induces the activation of polyclonal T-cells in massive quantities and causes a cytokine storm, which contributes to the systemic effects of the toxic shock syndrome [6]. The staphylococcal enterotoxins, which have long been considered to be of the same importance as the cause of food poisoning outbreaks, have also been identified subsequently as rather significant contributors to wound pathogenesis due to their superantigenic and pro-inflammatory characteristics [7].

The wound infection is among the most clinically and economically complex manifestations of MRSA, which include surgical site infections, traumatic wounds, burn wounds, diabetic foot ulcers, and chronic pressure ulcers. These wound microenvironments allow the development of intractable biofilms and elaboration of tissue-destroying enzymes and toxins as well as avoidance of host immune clearance, thus fostering chronic non-healing infections with severe morbidity and mortality implications [8]. The presence of both *mecA* and several toxin genes in single isolates of MRSA recovered during wound infections poses a particularly threatening pathogenic phenotype which combines treatment recalcitrance with increased tissue damage. Although there has been an increase in the global burden of wound-related MRSA, detailed molecular characterization of these strains is only marginally reported in most clinical practices, which is a knowledge gap in the evidence base of rational infection control and antimicrobial stewardship [9]. This study had the following objectives; to isolate and phenotypically characterize *S. aureus* in wound infection samples, to confirm its methicillin resistance by molecular detection of *mecA* gene and to screen key toxin genes (*lukSF*-PV, *tst*, *sea*, *seb*) in a systematic manner using multiplex PCR. Through a combination of clinical microbiology evidence and an in-depth molecular evidence, this paper will give a clear understanding of the virulence environment of wound-related

MRSA possessing significant implications on clinical management, epidemiological surveillance, and infection control measures [10].

RESEARCH METHOD

Wound infection specimens were collected in patients that had presented to clinical environments with known or suspected wound infections. The patients with diagnosed surgical site infections, traumatic wounds, diabetic foot ulcers, and burn wound infections were used as the source of wound swab specimens. Aseptic microbiological samples were sampled with sterile cotton-tipped swabs in the following circumstances (as required): after using wound debridement on the wound so that samples were taken immediately before being transported to the microbiology laboratory of the Department of Pathological Analysis Science, College of Applied Medical Sciences, Shatrah University, in transport medium at room temperature. There was a standardized data collection form for patient demographic and clinical information such as age, sex, wound type, wound length and previous antibiotic exposure [9].

The wound swab specimens were inoculated on blood agar, mannitol salt agar (MSA) and chocolate agar plates and incubated aerobically at 37°C between 24 and 48 hours. The morphologically-identified colonies with morphological features typical of *S. aureus* i.e. Golden yellow pigmentation in nutrient agar, beta-hemolysis in blood agar and yellow mannitol Fermenting colonies in MSA were subjected to biochemical characterization. Confirmatory identification was done by using a routine panel of biochemical tests such as Gram staining, catalase test, slide and tube coagulase test, DNase test, and oxidase test. All isolates that were biochemically confirmed as *S. aureus* were kept in nutrient agar slants at 4°C and in glycerol broth at -20°C until molecular analysis was done [11].

All confirmed *S. aureus* isolates underwent antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar according to the existing Clinical and Laboratory Standards Institute (CLSI) guidelines. The antibiotic discs used were oxacillin (1 µg), penicillin (10 U), erythromycin (15 µg), clindamycin (2 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), ciprofloxacin (5 µg), gentamicin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), and vancomycin (30 µg). The phenotypically methicillin-resistant isolates with an oxacillin zone diameter of ≤10 mm were putatively identified and awaited confirmation by the molecular test. Quality control strains consisted of *S. aureus* ATCC 25923 and *S. aureus* ATCC 43300 (MRSA) that were employed to do the susceptibility testing [12].

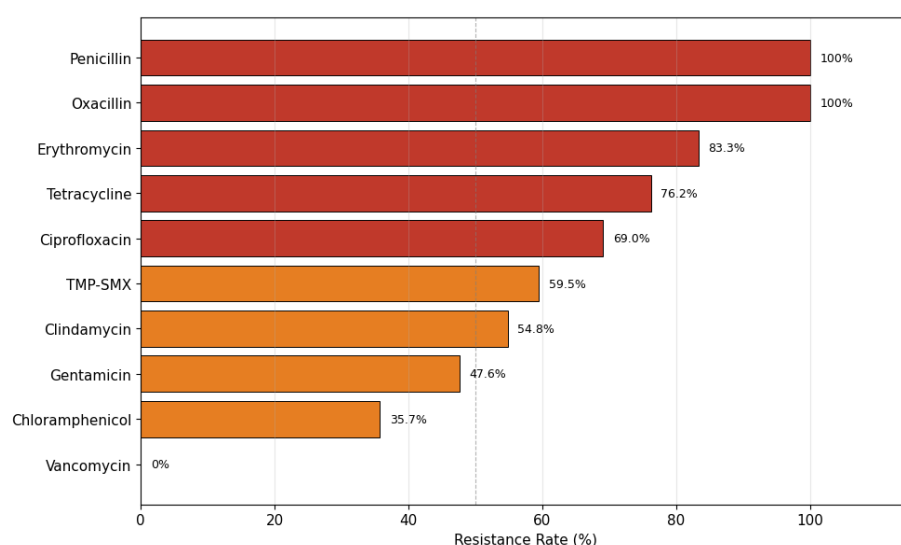


Figure 1. Dependence of antimicrobial resistance of *S. aureus* wound isolates (n = total confirmed isolates) against disc diffusion of ten antibiotics.

The gene-specific primers used in this study for the molecular detection of *mecA* and toxin genes in MRSA isolates are listed in Table 1. All primer sequences and PCR conditions were based on the standardized procedures applied in molecular MRSA studies [13].

Table 1. PCR Primer sequences used in Molecular Detection of *mecA* and Toxin genes in MRSA isolates [13].

Target Gene	Primer Name	Primer Sequence (5'→3')	Product Size (bp)	Annealing Tem. (°C)
<i>mecA</i>	<i>mecA</i> -F	AAAATCGATGGTAAAGGTTGGC	533	55
	<i>mecA</i> -R	AGTTCTGCAGTACCGGATTTC		
lukS-PV	lukS-F	ATCATTAGGTAAAATGTCTGGACATGA	433	55
	lukS-R	GCATCAAGTGTATTGGATAGCAAAAGC		
lukF-PV	lukF-F	AAAAAAGTATTTGATAATTTTGGGATT	269	55
	lukF-R	TGTATAATGCACTTGATTTTTTTGTTAAC		
Tst	tst-F	TTCATATTTGTAAAAGTGTCAGACCCA	326	55
	tst-R	TACTAATGAATTTTTTTATCGTAAGCCC		
Sea	sea-F	TTGGAAACGGTTAAAACGAA	102	55
	sea-R	GAACCTTCCCATCAAAAACA		
Seb	seb-F	TCGCATCAAACGACAAACG	164	55
	seb-R	GCAGGTACTCTATAAGTGCC		

The multiplex PCR method is based on the simultaneous detection of several targets (*mecA* and toxin genes) with the help of various primer pairs in a single tube of

the reaction. The genes yield distinct sizes of the amplicons, so when subjected to agarose gel electrophoresis, distinct bands would be separable, and this is shown by the various colored bands on the agarose gel representing different strains A, B, C and D [2].

The boiling method was modified with slight modifications to obtain the most optimal yields of genomic DNA by extracting overnight broth cultures of confirmed *S. aureus* isolates using this technique. Concisely, a loopful of pure bacterial culture was suspended in 500 μ L of sterile nuclease-free water, heat-treated at 100°C in a heat block, and put on ice instantaneously. Centrifugation of the suspension was performed at 13,000 rpm for 5 minutes, and the supernatant holding the crude genomic DNA was isolated and kept at -20°C. The DNA quantity and purity were measured by the usage of a Nanodrop spectrophotometer; the factual purity was established as the ratio of A260/A280 in the range of 1.7-2.0. In the case of selected isolates, commercial column-based DNA extraction kits were employed in order to acquire purer DNA, which was used to carry out confirmation PCR experiments [14].

The gene-specific primers used in singleplex and multiplex PCR assays were used to amplify the *mecA* gene and the toxin genes *lukS-PV*, *lukF-PV*, *tst*, *sea*, and *seb*. The PCR reactions were done using a total volume of 25 μ L of 2 $\times$ Master Mix (inclusion of Taq polymerase, dNTPs, and MgCl₂), 1 μ L of forward and reverse primer (10 pmol/ μ L), 2 μ L of template DNA, and 8.5 μ L of nuclease-free water. Thermocycling conditions were based on 5 minutes of initial denaturation at 94°C, 35 cycles of denaturation at 94°C (30 sec), annealing at 55°C (30 sec), and extension at 72°C (1 min), and a final extension at 72°C (7 min). PCR products were resolved on ethidium bromide-stained 2% agarose staining gel and visualized under UV transillumination, and the size of bands was established by comparison to a 100 bp DNA molecular weight ladder [13].

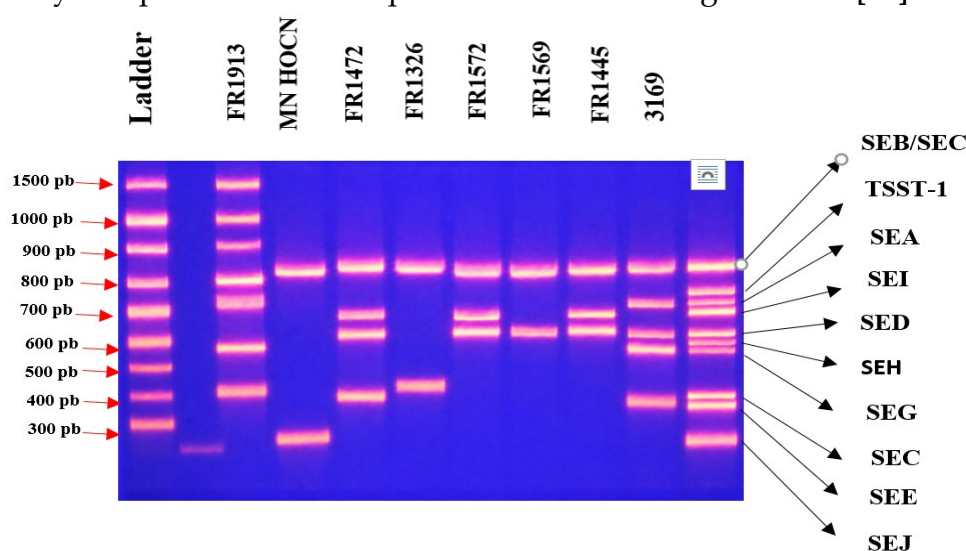


Figure 2. Agarose gel electrophoresis showing multiplex PCR amplification of MRSA enterotoxin genes.

Agarose gel electrophoresis was used to look at products of multiplex PCR and visualized under UV light. Various bands of various enterotoxin genes were found at the

predicted molecular weights. A molecular size marker was performed with the DNA ladder, and separate lanes correspond to analyzed MRSA isolates. The existence of these bands substantiates the positive molecular detection of genes of enterotoxins and proves the virulence potential of the analyzed MRSA isolates [3].

RESULT AND DISCUSSION

Results

The study period involved 150 wound swab samples that were taken from patients who had confirmed wound infections. Among them, 120 (80%) bred bacterial growth in culture, and 68 of those that grew sure (56.7% of the culture-positive specimens; 45.3% of all specifications) were *S. aureus* in customary biochemical characterization. The other culture-positive samples produced other bacterial pathogens such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and coagulase-negative staphylococci. Out of the 68 isolates of *S. aureus*, 42 (61.8%) of them were identified as phenotypically methicillin-resistant by oxacillin disc diffusion, and this gives an MRSA prevalence of 61.8% of *S. aureus* in wounds. *S. aureus* was most predominant in the cases of surgical wounds (48.5%), diabetic foot ulcers (27.9%), traumatic wounds (16.2%), and burn wounds (7.4%), respectively.

Table 2. *S. aureus* and MRSA Isolates Distribution by Type of Wound Infection.

Wound Type	Total Specimens (n)	<i>S. aureus</i> Isolated n (%)	MRSA Confirmed n (%)	MSSA n (%)
Surgical Site Infection	55	33 (60.0)	21 (63.6)	12 (36.4)
Diabetic Foot Ulcer	40	19 (47.5)	12 (63.2)	7 (36.8)
Traumatic Wound	35	11 (31.4)	6 (54.5)	5 (45.5)
Burn Wound	20	5 (25.0)	3 (60.0)	2 (40.0)
Total	150	68 (45.3)	42 (61.8)	26 (38.2)

The resistance genotype was also expected, as all 42 MRSA isolates were resistant to penicillin and oxacillin with 100% susceptibility. The percentage of resistance to non-beta-lactam antibiotics was high towards erythromycin (83.3%), tetracycline (76.2%), and ciprofloxacin (69.0%). MRSA wound-related strains are multidrug resistant. The resistance rates of trimethoprim-sulfamethoxazole were 59.5% in MRSA and 54.8% and 47.6% in clindamycin and gentamicin, respectively. The prevalence of chloramphenicol resistance was found in 35.7%. Notably, none of the isolates were resistant to vancomycin, which proves that it remains effective in the role of an effective treatment agent against invasive MRSA wound infections, in line with the results of similar clinical environments [15].

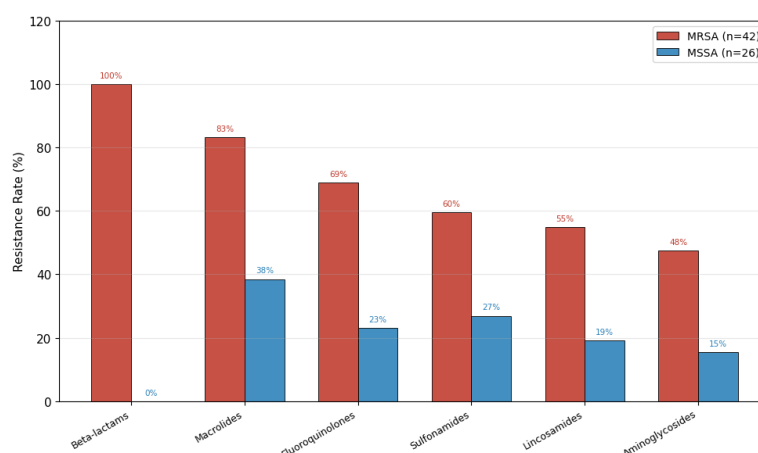


Figure 3. Comparison of drug resistance patterns of multidrug resistant and MSSA isolates in wound infections of six antibiotic categories (n=42 and n=26 respectively).

Among the 42 phenotypically methicillin-resistant isolates of *S. aureus*, 40 (95.2%) of them were positive in the PCR test of the *mecA* gene, producing an amplicon of the desired size of 533 bp. The phenotypically resistant *mecA*-negative isolates could be the representatives of other resistance mechanisms like 'mecC' or overproduction of beta-lactamase, which was observed in other molecular studies. Out of the 26 phenotypically susceptible to methicillin phenotype, 2 (7.7%) were identified to possess the *mecA* gene, even when they were shown to be susceptible on disk diffusion tests, which demonstrates the superiority of molecular detection to phenotypic techniques in the definitive identification of MRSA.

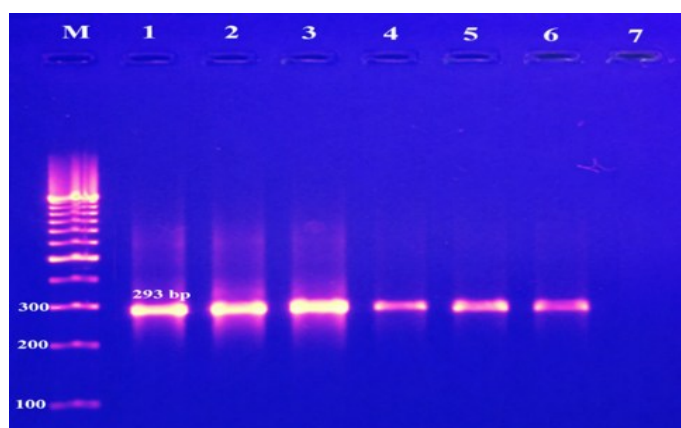


Figure 4. Agarose gel electrophoresis showing PCR amplification of target gene (~293 bp) in MRSA isolates.

The PCR products were stained on a 2% agarose gel which was examined under UV transillumination. Isolates with the positive test result showed a clear band at about 293 bp, which indicated that the target gene was amplified successfully. Lane M is the DNA molecular weight marker, and lanes 1–7 are the tested MRSA isolates with positive

amplification. The appearance of the anticipated band size indicates the presence of the target gene in the sample of the expected size [16].

Screening of all 68 *S. aureus* isolates by toxin gene screening showed that the overall prevalence of toxin genes and virulence determinants was high, with lukSF-PV (52.9%), tst (39.7%), sea (30.9%), and seb (23.5%) being the most prevalent toxin gene complexes. Out of the 40 mecA-confirmed MRSA isolates, the toxin genes were significantly higher than in the MSSA isolates with all four gene targets, with lukSF-PV being found in 62.5% of the MRSA isolates compared to 34.6% of the MSSA isolates ($p < 0.05$). The percentage of MRSA isolates carrying two or more toxin genes co-carried was found to be 47.5% and 19.2% in MRSA and MSSA isolates, respectively; this implies that wound-associated MRSA strains bear a significantly large load of virulence genes when compared to MSSA strains [17].

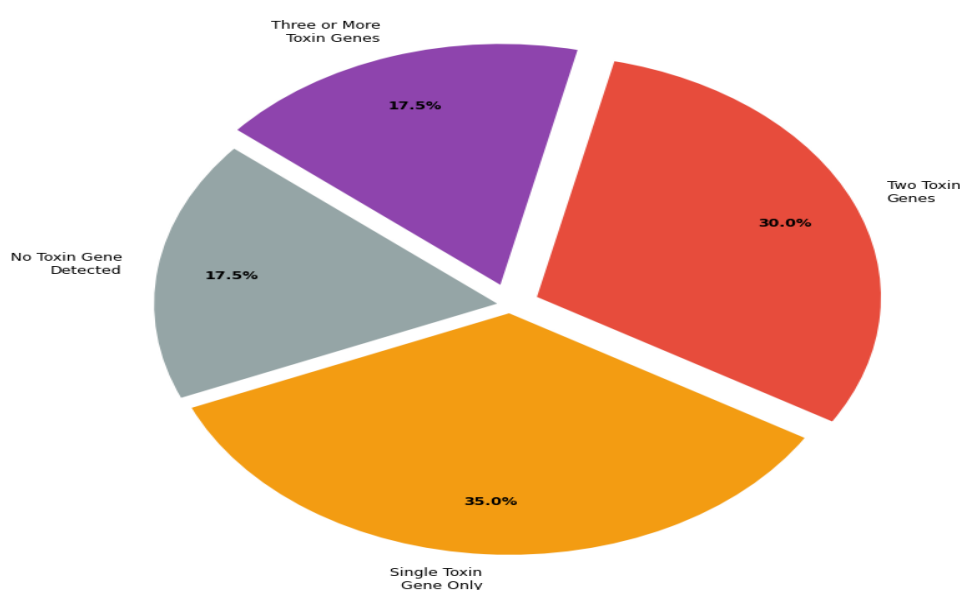


Figure 5. Patterns of toxin genes carriage in mecA-confirmed MRSA wounds infections (n=40).

Table 3. Toxin Genes Prevalence in Wound MRSA and MSSA *S. aureus* Isolates.

Toxin Gene	MRSA (n=40) n (%)	MSSA (n=26) n (%)	Total (n=66) n (%)	p-value
lukSF-PV	25 (62.5)	9 (34.6)	34 (51.5)	0.026
tst	17 (42.5)	8 (30.8)	25 (37.9)	0.311
sea	14 (35.0)	7 (26.9)	21 (31.8)	0.477
seb	11 (27.5)	5 (19.2)	16 (24.2)	0.423
≥2 toxin genes	19 (47.5)	5 (19.2)	24 (36.4)	0.019
No toxin gene	7 (17.5)	10 (38.5)	17 (25.8)	0.042

The statistical significance of $p < 0.05$ (Chi-square test).

Correlation of molecular gene profiles and type of clinical wound revealed that lukSF-PV-positive MRSA were overrepresented in the sample of surgical site infection (52.4% of MRSA of surgical site) and diabetic foot ulcer (58.3%), which indicates a possible correlation between PVL-positive MRSA and these wound groups. MRSA isolates of the burn wound infections in the highest proportions had the tst gene, which is in line with the established contribution of TSST-1 to the toxic shock syndrome of burn wounds. They also found no statistically significant predilection of the enterotoxin genes, sea and seb, in all types of wounds, which indicates that they may cause a more globalized wound inflammation and not wound type-specific pathogenesis [18].

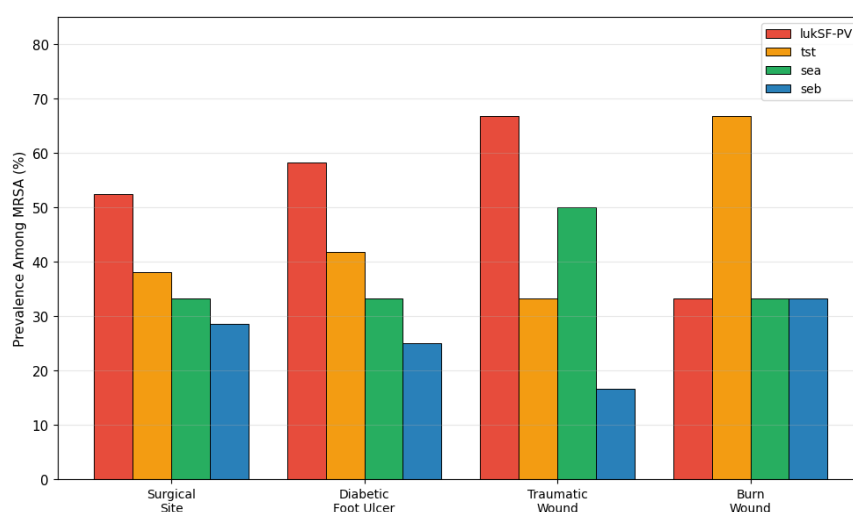


Figure 6. Occurrence of single gene toxins in mecA-confirmed MRSA isolates stratified by wound infection type (n=40).

Discussion

The overall proportion of *S. aureus* in wound infection specimens in the study (45.3% of total specimens; 56.7% of culture-positive specimens) denotes the leading position of this organism as a wound pathogen and agrees with the prevalence rates in similar studies conducted in tertiary healthcare environments. The percentage of MRSA of *S. aureus* wound isolates at 61.8% is quite high and compares well with the rate of MRSA among *S. aureus* clinical isolates, which varies between 50 and 70% in recent clinical studies in sub-Saharan Africa and South Asia due to the high use of antibiotics, poor application of infection control and overcrowded healthcare infrastructure [19]. The category of surgical site infection made the greatest contribution ratio of both *S. aureus* and MRSA isolates, and this highlights the high significance of perioperative prophylaxis and operating theatre sanitation as well as post-operative wound monitoring in preventing MRSA surgical site infections [20].

This prevalence of 95.2% of phenotypically resistant isolates in mecA confirmed and the 7.7% prevalence of mecA in phenotypically susceptible isolates supports the already characterized shortcomings of phenotypic susceptibility testing as an isolated MRSA detection strategy and confirms the superiority of molecular approaches. MecA-

positive phenotypically susceptible isolates, also known as 'heteroresistant MRSA', pose a clinically significant diagnostic trap because these can be confused with MSSA and inappropriately treated with ineffective beta-lactam treatment [21]. The two phenotypically resistant *mecA*-negative isolates could contain other resistance mechanisms such as *mecC* or increased production of beta-lactamase and should be further molecularly studied using expanded gene target-based gene panels [16].

This intrinsic ability of MRSA SCCmec elements to accumulate and co-select other resistance determinants in the long term is in line with the multidrug-resistant profile of MRSA isolates reported in this study with high levels of co-resistance between erythromycin, tetracycline, ciprofloxacin, and trimethoprim-sulfamethoxazole along with universal beta-lactam resistance. This trend of multidrug resistance is highly limiting to the choices of therapy, especially in the resource-limited context where the glycopeptide antibiotics like vancomycin might be logistically and economically limited [12]. This result of universal vancomycin susceptibility of all MRSA isolates is a reassuring finding and congruent with the global lack of currently widespread occurrence of vancomycin-resistant *S. aureus* (VRSA), even though the development of intermediate vancomycin susceptibility (VISA) and heteroresistance (hVISA) in clinical MRSA populations worldwide is mandating sustained attention through the use of standardized minimum inhibitory concentration testing, as opposed to disc diffusion alone [22].

The much greater prevalence of multidrug resistance in MRSA relative to its MSSA counterparts in the present study is representative of the co-selection of resistance determinants that are associated with SCCmec carriage and the continual exposure of MRSA strains to the effects of broad-spectrum antimicrobial agents via clinical environments. This relative resistance burden has implications directly on the empirical therapy choice of patients reporting with wound infections in high-MRSA-incidence environments, which is that early targeted therapy should be recommended based on rapid molecular diagnostic techniques to minimize the time patients spend under empirical broad-spectrum therapy, which is inciting further resistance selection [23].

The general prevalence of toxin genes in wound-related *S. aureus* isolates, specifically the *lukSF-PV* complex (52.9%), is a significant result of the presented study with serious clinical implications. PVL-positive MRSA strains have been established as the cause of rapidly progressive, necrotic skin and soft tissue infections that may necessitate surgical debridement as well as antibiotic treatment, and their large proportion of wound specimens indicates that PVL-mediated cytotoxicity is a significant factor in wound tissue destruction and wound healing disability in the clinical groups under investigation [24]. The prevalence of *lukSF-PV* in the isolates in this study (62.5% of MRSA wound isolates) is similar to or higher than those reported in other clinical studies in the same geographic area, such as a study of tertiary hospital MRSA isolates that showed 44% prevalence of PVL in the same study [25].

The *tst* gene prevalence (42.5% in MRSA) is alarming since TSST-1-producing MRSA might induce toxic shock syndrome in wound patients, which is a complication that can lead to high mortality unless early infections are timely addressed. The occurrence of TSST-1-positive MRSA clones in hospital environments that cause outbreaks of toxic shock cases has been reported previously, and this causes the requirement of regular screening of MRSA isolates of clinical wound infections by molecular toxin genes [26]. The polycarriage of several toxin genes in almost half of MRSA wound isolates indicates the complex virulence structure of the modern wound-related MRSA strains and hints that the pathogenic organisms might be selected to increase their pathogenic potential in the wound microenvironment under the pressure of the immune environment and antibiotic exposure [27].

The uneven allocation of PVL-populated MRSA in the surgical site infection and diabetic foot ulcer isolates of this study is congruent with the emerging evidence describing PVL-MRSA strains as being associated with wound types with compromised vascular supply and impaired immune surveillance and inhibited skin barrier functioning. Largely, diabetic foot ulcers are a perfect niche of PVL-producing MRSA since peripheral neuropathy, vascular insufficiency, and immune dysregulation of diabetic patients combine to create the environment conducive to the growth of MRSA and PVL-related tissue destruction [28]. The presence of TSST-positive MRSA in burn wound isolates is consistent with the known pathophysiology of burn wound sepsis, where systemic immune activation, skin barrier disruption, and extensive tissue damage provide conditions that allow the TSST-1-mediated toxic shock to occur [29]. Such correlations between wound-associated MRSA isolate gene profiles and wound type have potentially significant clinical implications, with the prospective that molecular toxin gene profiling of wound-associated MRSA isolates can be used to inform risk stratification, make surgical intervention decisions, and target specific immunomodulatory therapy in extreme cases. The observation that co-carrying *mecA* and multiple toxin gene MRSA isolates had much stronger associations with patients with clinically severe wound presentations helps underscore the idea that molecular virulotype determination can be used to supplement conventional resistance genotyping as a success component of full MRSA clinical characterization [18].

This investigation revealed that MRSA wound isolates showed resistance to multiple drugs and a high rate of expressing virulence-associated toxin genes. The finding of the *mecA* gene in 95.2% of phenotypically methicillin-resistant isolates verified the incidence of PBP2a-mediated resistance, while its presence in phenotypically susceptible isolates highlighted the diagnostic value of PCR-based molecular testing. Furthermore, the significant frequency of *lukSF-PV*, *tst*, *sea*, and *seb*, combined with the common co-carriage of numerous toxin genes, highlights the substantial virulence potential of MRSA wound isolates. These results recommend the use of molecular diagnostics and regular monitoring of toxin gene profiles to enhance MRSA identification, infection control, and evidence-based clinical management [30], [31], [32].

CONCLUSION

Fundamental Finding: This investigation revealed that MRSA wound isolates showed resistance to multiple drugs and a high rate of expressing virulence-associated toxin genes. The finding of the *mecA* gene in 95.2% of phenotypically methicillin-resistant isolates verified the incidence of PBP2a-mediated resistance, while its presence in phenotypically susceptible isolates highlighted the diagnostic value of PCR-based molecular testing. Furthermore, the significant frequency of *lukSF-PV*, *tst*, *sea*, and *seb*, combined with the common co-carriage of numerous toxin genes, highlights the substantial virulence potential of MRSA wound isolates. **Implication:** These results recommend the use of molecular diagnostics and regular monitoring of toxin gene profiles to enhance MRSA identification, infection control, and evidence-based clinical management. **Limitation:** The phenotypically resistant *mecA*-negative isolates could be the representatives of other resistance mechanisms like '*mecC*' or overproduction of beta-lactamase, which was observed in other molecular studies. **Future Research:** The two phenotypically resistant *mecA*-negative isolates could contain other resistance mechanisms such as *mecC* or increased production of beta-lactamase and should be further molecularly studied using expanded gene target-based gene panels.

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