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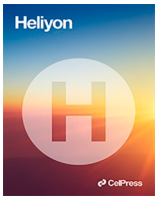

Research article

Relationship between the strength of biofilm production and the presence of *pvl* and *mecA* genes in *Staphylococcus aureus* isolated from skin and soft tissue infections

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ABSTRACT

This research sought to investigate the association between the occurrence of the *pvl* and *mecA* genes and the strength of biofilm formation, as well as to assess the efficacy of vancomycin and ceftaroline against *Staphylococcus aureus* strains obtained from skin and soft tissue infections (SSTIs). A total of 134 *S. aureus* isolates were collected from SSTI patients and identified through standard microbiological techniques. Vancomycin and ceftaroline susceptibility testing were performed using the agar dilution and disc diffusion methods, respectively. PCR analysis was conducted to identify the *nuc*, *mecA*, and *pvl* genes. Biofilm production was measured using the tissue culture plate method. Methicillin-resistant *S. aureus* (MRSA) represented 58.2 % of the isolates. All isolates displayed biofilm-forming capability, with 10.4 % classified as high-grade biofilm producers, 85.7 % of which were positive for the *mecA* gene ($P = 0.02$). 16.4 % of the isolates had *pvl* gene and 59 % of PVL-positive strains identified as MRSA. Most of the low-grade biofilm producers had the *pvl* gene ($P = 0.03$). Vancomycin susceptibility was observed in 98.5 % of isolates, with an MIC_{50} of 1 μg/mL in 51.4 % of cases. Among MRSA strains, 1.4 % exhibited intermediate resistance to vancomycin, with MICs between 4 and 8 μg/mL. No resistance to ceftaroline was found. The results demonstrate a significant association between biofilm production strength and the occurrence of the *mecA* and *pvl* genes; *mecA* correlated with increased biofilm production, while *pvl* was associated with lower biofilm levels. These findings offer valuable insights for future studies, suggesting that ceftaroline could be an effective alternative to vancomycin for treating MRSA-related SSTIs, particularly given the increasing resistance to vancomycin.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a commensal bacterium frequently colonizing the skin, nasal passages, and mucosal surfaces of healthy individuals while also serving as an important human pathogen [1,2]. *S. aureus* is a major cause of infection worldwide and can rapidly acquire antimicrobial resistance through mutation or horizontal gene transfer from other bacteria [3].

Since the 1960s, methicillin-resistant *Staphylococcus aureus* (MRSA) has become widespread in healthcare settings, with community transmission emerging in the 1990s [2]. MRSA strains harbor the *mecA* or *mecC* genes, which code penicillin-binding proteins (PBP2a

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or PBP2A$_{LGA}$) that exhibit low affinity for most beta-lactam antibiotics [4,5]. The clinical manifestations of MRSA infections range from asymptomatic colonization and skin and soft tissue infections (SSTIs) to severe invasive diseases [6,7].

S. aureus produces several virulence factors critical for skin infections, including cytolytic toxins, superantigens, immune evasion molecules, and cell wall-anchored proteins [2]. Panton-Valentine leukocidin (PVL), a toxin encoded by the pvl gene, is commonly found in S. aureus strains isolated from SSTIs and severe pneumonia cases [8,9]. The pvl gene is present in most community-acquired MRSA strains, contributing to their virulence [10].

PVL is composed of two components, LukS-PV and LukF-PV, which combine to form a pore-forming heptamer on neutrophil membranes, causing neutrophil lysis [11]. At lower concentrations, PVL can induce granulocyte apoptosis [12]. PVL-producing *S. aureus* strains are associated with recurrent abscesses in otherwise healthy young individuals; however, typical clinical presentations, diagnostic criteria, and treatment guidelines remain defined [13].

S. aureus is a predominant pathogen in biofilm-associated infections [13]. Biofilm formation provides *S. aureus* with protection against host immune responses and antibiotics, contributing to its persistence in the host and complicating clinical management of biofilm-associated infections [13]. Biofilm production in *S. aureus* is primarily regulated by the *ica* operon, which encodes proteins responsible for polysaccharide intercellular adhesion (PIA). Additionally, biofilm formation can also involve PIA-independent elements, such as biofilm-associated protein (Bap), clumping factors A and B (ClfA and ClfB), and fibronectin-binding proteins A and B (FnbpA and FnbpB) [14]. Biofilm-associated MRSA infections can range from SSTIs to deep-seated infections, including bloodstream infections, osteomyelitis, and infective endocarditis [15]. MRSA infections pose a significant challenge in intensive care units (ICUs), as many strains exhibit resistance to multiple antibiotic classes, including aminoglycosides, cephalosporins, and quinolones [15]. Consequently, vancomycin has remained the treatment of choice for severe MRSA infections [16].

Strains of *S. aureus* with reduced susceptibility to vancomycin first appeared in Japan in 1997, followed by the emergence of vancomycin-resistant *S. aureus* (VRSA) in the United States in 2002 [16]. VRSA is associated with the *vanA* gene cluster, which *S. aureus* can acquire from vancomycin-resistant *Enterococcus* species [5]. Reduced vancomycin susceptibility has been reported globally [16,17]. Hetero-resistance to vancomycin has also emerged, characterized by subpopulations with elevated vancomycin minimum inhibitory concentrations (MICs), which can lead to treatment failures and necessitate higher antibiotic doses [18].

Recent studies have highlighted ceftaroline as a potential alternative antibiotic for treating MRSA infections [19,20]. Ceftaroline, a new-generation cephalosporin, has shown efficacy against MRSA with an MIC$_{90}$ of 1–2 μg/mL and is approved for treating Acute Bacterial Skin and Skin Structure Infections (ABSSSI) and Community-Acquired Bacterial Pneumonia (CABP) [20].

Effective management of severe MRSA infections requires identifying risk factors and selecting appropriate antibiotics to enhance treatment efficacy and prevent resistance.

The objective of this investigation was to evaluate the correlation between the presence of the *pvl* and *mecA* genes and biofilm production strength, as well as to assess the antimicrobial efficacy of vancomycin and ceftaroline against *S. aureus* strains isolated from SSTIs.

2. Material and methods

2.1. Investigation population and strain Collection

A total of 134 *S. aureus* isolates, each obtained from a unique patient, were collected during routine microbiology diagnostic procedures at Razi Hospital, Tehran, Iran. Isolation and identification of *S. aureus* were performed using standard clinical microbiological tests, including tube and slide coagulase tests, deoxyribonuclease (DNase) tests, and mannitol fermentation tests [21,22]. Isolates that passed these initial tests were confirmed by amplifying the *nuc* gene with species-specific primers [23,24]. Each *S. aureus* strain was preserved at −70 °C in trypticase soy broth (TSB) containing 20 % glycerol until further testing.

2.2. Assessment of cefoxitin and ceftaroline susceptibility

MRSA isolates were identified by assessing their susceptibility to 30 μg cefoxitin (MAST, Bootle, UK) using the disc diffusion method and confirming the presence of the *mecA* gene. Ceftaroline susceptibility was evaluated with a 30 μg ceftaroline disc (MAST, Bootle, UK), following interpretation criteria based on Clinical and Laboratory Standards Institute (CLSI) guidelines [1]. For cefoxitin, isolates were categorized as susceptible (≥22 mm) or resistant (≤21 mm). For ceftaroline, isolates were categorized as sensitive (≥25 mm), sensitive dose-dependent (SDD; 20–24 mm), or resistant (≤19 mm) [25].

2.3. Vancomycin susceptibility testing

Vancomycin susceptibility was assessed using the agar dilution method with vancomycin (Sigma Aldrich, Germany) in its pure form. The investigation used vancomycin at 1 mg potency, equivalent to 1000 μg, to determine the MIC following CLSI protocols [1]. Vancomycin MIC testing concentrations ranged from 0.625 μg/mL to 1280 μg/mL. Precisely 0.0035 g of vancomycin powder was weighed using an electronic balance to test the 134 isolates. The measured dose was dissolved in 24 mL of water to achieve a 1280 μg/mL concentration, followed by serial 1:2 dilutions to produce final concentrations of 640 μg/mL, 320 μg/mL, 160 μg/mL, 80 μg/mL, 40 μg/mL, 20 μg/mL, 10 μg/mL, 5 μg/mL, 2.5 μg/mL, 1.25 μg/mL, and 0.625 μg/mL. Next, 2 mL of each dilution was mixed with 18 mL of pre-heated brain heart infusion (BHI) agar at 45 °C, poured into petri dishes to achieve final vancomycin concentrations ranging from 0.0625 μg/mL to 128 μg/mL.

Bacterial suspensions were prepared by transferring 3–5 individual overnight colonies from trypticase soy agar plates into 3 mL of 0.9 % saline, then adjusted to match the turbidity of a 0.5 McFarland standard. The suspensions were subsequently diluted 1:10 with 0.9 % saline before inoculation [26]. These prepared suspensions were inoculated onto agar plates and incubated at 35–37 °C for 24 h. Results were assessed based on the presence or absence of visible growth, with the MIC defined as the lowest concentration of vancomycin at which no bacterial growth was detected [25]. *S. aureus* ATCC 29213 was used as a negative control, while *Enterococcus faecalis* carrying the *van* gene served as a positive control.

2.4. Detection of nuc and mecA genes

DNA was extracted from 24-h broth cultures of *S. aureus* isolates using a commercial DNA isolation kit (Pouya Gene Azma kit, Iran). Multiplex PCR was performed on all *S. aureus* isolates using two sets of oligonucleotide primers: *Mec*AF-*Mec*AR to amplify a 310 bp fragment of the *mecA* gene and *Nuc*F-*Nuc*R to amplify a 279 bp *S. aureus*-specific fragment of the *nuc* gene (Table 1). PCR reactions were carried out in a 25-μL reaction volume containing 12.5 μL PCR Master Mix (Amplicon), 0.4 μM of each primer, and 5 μL of template DNA. The PCR protocol included an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min, concluding with a final extension step at 72 °C for 10 min [27]. *S. aureus* ATCC 33591 (*mecA*+; *nuc*+) and ATCC 25923 (*mecA*-; *nuc*+) were used as positive and negative controls, respectively. Water was used as a template-free control in negative PCR reactions. PCR products were visualized on a 2 % agarose gel stained with SYBR™ Safe DNA stain and observed under UV light [27].

2.5. Detection of pvl gene

The presence of the *lukS-PV* and *lukF-PV* genes, which encode the components of PVL, was detected using singleplex PCR with primers as described by Lina et al. [2] (Table 1). The PCR amplification protocol consisted of an initial denaturation at 94 °C for 5 min, followed by 36 cycles of 94 °C for 45 s, 61 °C for 45 s, and 72 °C for 45 s, with a final extension at 72 °C for 5 min to ensure complete polymerization. *S. aureus* ATCC 25923 was used as a positive control for the PCR assay. Forward strand sequencing was conducted by Bioneer Company (Korea). The nucleotide sequences obtained were analyzed using CLC Genomics Workbench 21 software, submitted to GenBank, and assigned an accession number.

2.6. Biofilm formation assay by crystal violet staining

Biofilm formation was assessed using the tissue culture plate assay. *S. aureus* isolates were grown in tryptic soy broth (TSB) supplemented with 1 % anhydrous glucose (TSBG) and incubated overnight at 37 °C. The cultures were then adjusted to an optical density (OD) of 0.2 at 600 nm and transferred to a 96-well microplate. Following a second overnight incubation at 37 °C, non-adherent bacteria were gently removed by washing each well three times with 200 μL of phosphate-buffered saline (PBS).

The plates were then air-dried, and 150 μL of absolute methanol was added to fix the remaining adherent cells. After the methanol was removed, a 1 % crystal violet solution (150 μL per well) was applied to stain the biofilm for 20 min. Excess dye was washed away with three rinses of distilled water, and the plates were air-dried in an inverted position. To dissolve the crystal violet stain, 150 μL of 33 % (v/v) glacial acetic acid was added to each well, and the OD was measured at 570 nm using a microplate reader [28,29]. Each assay included a negative control (medium-only) and *S. aureus* ATCC 25923 as a positive control. Biofilm production was categorized as high-grade positive ($OD_{570} \geq 1$), low-grade positive ($1 > OD_{570} \geq 0.1$), or negative ($OD_{570} < 0.1$) [30].

2.7. Statistical analysis

Statistical analysis was performed using IBM SPSS software, version 19 (Chicago, IL, USA). Proportions were compared using either the Chi-square test or Fisher's exact test, with a p-value of <0.05 deemed statistically significant.

Table 1
The sequences of the rimers of used in the study.

Primer name	Sequences (5′-3′)	Size of PCR product (bp)	Reference
***nuc*-F**	GCGATTGATGGTGATACGGTT	279	Louie et al., 2000
***nuc*-R**	AGCCAAGCCTTGACGAACTAAAGC		
***mecA*-F**	CCAATTCCACATTGTTTCGGTCATA	310	Sahebnasagh et al., 2014
***mecA*-R**	GTAGAAATGACTGAACGTCCGATAA		
***pvl*-F**	ATCATTAGGTAAAATGTCTGGACATGATCC	433	Lina et al., 1999
***pvl*-R**	GCATCAASTGTATTGGATAGCAAAAGC		

3. Results

3.1. Isolation and identification of S. aureus

Over 15 months, 134 *S. aureus* isolates were obtained from patients with SSTIs at Razi Hospital in Tehran, Iran. The SSTI diagnoses included pemphigus (55.2 %), bullous pemphigoid (7.5 %), eczema (14.9 %), psoriasis (6.7 %), folliculitis (6 %), hidradenitis suppurativa (3 %), panniculitis (2.2 %), pyoderma (1.5 %), lupus (1.5 %), Stevens-Johnson syndrome (SJS) and early-stage toxic epidermal necrolysis (TEN) (0.7 %), and prurigo nodularis (0.7 %). The mean age of the patients was 43.9 ± 17.6 years, with ages ranging from 6 to 88 years, and the cohort was evenly divided between male and female patients (50 % male).

3.2. Detection of nuc, mecA, and pvl genes using Multiplex and singleplex PCR

All isolates that tested positive for the species-specific *nuc* gene were analyzed further. Out of the 134 *S. aureus* isolates from SSTI patients, 78 (58.2 %) were positive for the *mecA* gene, classifying them as MRSA, while 41.8 % were methicillin-sensitive *S. aureus* (MSSA). The *pvl* gene was detected in 22 out of 134 isolates (16.4 %). Of the *pvl*-positive isolates, 13 (59 %) were MRSA and 9 (40.9 %) were MSSA (Fig. 1(A and B)). Partial sequences of the *pvl* gene were confirmed using BLASTN on the NCBI website against the *S. aureus* genome, and sequences were submitted to GenBank with accession numbers MH816970.1, MH816971.1, MH816972.1, MH816973.1, and MH816974.1.

3.3. Biofilm formation Quantification

S. aureus SSTI isolates were categorized based on biofilm production capacity into high-grade (strong) and low-grade (moderate) biofilm producers. All isolates demonstrated biofilm-forming capability; 14 out of 134 isolates (10.4 %) were classified as high-grade biofilm producers, while 120 out of 134 isolates (89.5 %) were low-grade producers. Among the high-grade biofilm producers, 12 out of 14 isolates (85.7 %) carried the *mecA* gene, and 5 (35.7 %) carried the *pvl* gene (Table 2).

Statistical analysis identified a significant correlation ($P < 0.05$) between strong biofilm production and the presence of the *mecA* gene in *S. aureus* isolates obtained from SSTI patients (Table 2). Among the 78 isolates identified as MRSA, only 14 (12 MRSA and 2 MSSA) were strong biofilm producers. In contrast, a significant correlation was found between low-grade biofilm formation and the presence of the *pvl* gene, with the majority of low- and moderate-grade biofilm producers harboring the *pvl* gene.

3.4. Ceftaroline susceptibility

Disk diffusion results (Table 3) show that 75 out of 78 MRSA isolates (96.1 %) exhibited inhibition zones ≥25 mm, with 53 isolates (67.9 %) showing zones ≥30 mm. Three MRSA isolates (3.8 %) displayed dose-dependent susceptibility with zone sizes of 20–24 mm (SDD). Among MSSA isolates, 54 out of 56 (96.4 %) were susceptible with zones ≥25 mm, while two isolates exhibited dose-dependent susceptibility with zones of 20–24 mm (SDD).

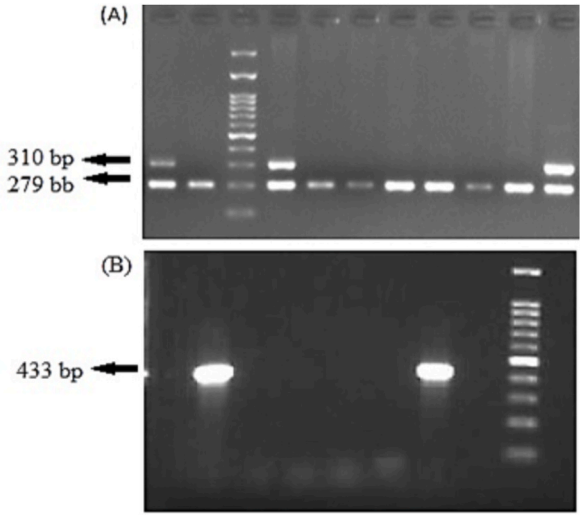

Fig. 1. PCR results for *nuc*, *mecA*, and *pvl* genes in *S. aureus* isolates caused skin and soft tissue infections (SSTIs) indicate that the amplified lengths are 279 and 310 bp for *nuc* and *mecA* genes (A), and 433 bp for *pvl* gene (B).

Table 2
Correlation between biofilm formation capacity of *S. aureus* SSTI isolates and the presence of *mecA* and *pvl* genes.

S. aureus SSTI isolates	High grade positive (14)	low-grade positive (120)	Statistical analysis
mecA+ (78)	12 (85.7 %)	66 (55 %)	Chi-square statistic is 4.8.
mecA- (56)	2 (14.2)	54 (45 %)	The p-value is 0.02
pvl+ (22)	5 (35.7 %)	17 (14.1 %)	Chi-square statistic is 4.2.
pvl- *(122)*	9 (64.2 %)	103 (85.9 %)	The p-value is 0.03

SSTI: skin and soft tissue infections.

Table 3
Disk diffusion testing of Ceftaroline against 134 *S. aureus* SSTI isolates.

Zone size (mm)	Interpretive criteria	MRSA (78) n (%)	MSSA (56) n (%)
≥30	Sensitive	53 (67.9)	39 (69.9)
25–29	Sensitive	22 (28.2)	15 (26.8)
20–24	SDD	3 (3.8)	2 (3.6)
≤19	Resistant	0	0

SSTI: skin and soft tissue infections.

3.5. Vancomycin susceptibility

MIC results (Table 4) indicate that 132 out of 134 *S. aureus* strains (98.5 %) were susceptible to vancomycin. Of these, 69 strains (51.4 %) had a MIC_{50} of 1 μg/mL, and 49 strains (36.5 %) showed MIC values of 2 μg/mL. Two strains (1.4 %), both classified as MRSA, displayed intermediate resistance with an MIC range of 4–8 μg/mL. Among the 78 MRSA isolates, 76 (97.4 %) were vancomycin-susceptible with an MIC ≤1 μg/mL, while 2 isolates (2.5 %) exhibited intermediate resistance (MIC 4–8 μg/mL). For MSSA isolates, 29 out of 56 (51.8 %) were susceptible with an MIC of 1 μg/mL, and 24 isolates (42.9 %) showed MIC values of 2–3 μg/mL. No strains demonstrated a MIC of ≥16 μg/mL, which would indicate vancomycin resistance. The control strain, *S. aureus* ATCC 29213, exhibited a vancomycin MIC of 1 μg/mL.

4. Discussion

S. aureus is a major pathogen responsible for SSTIs [31]. It colonizes around 30 % of the population, leading to infections that vary from mild skin conditions to serious systemic diseases, including sepsis, endocarditis, and pneumonia. In severe cases, standard antibiotics often fail, leading to poor clinical outcomes [32].

In the present study, more than half (58.2 %) of SSTIs were attributed to MRSA, which aligns with findings from North America and other regions where MRSA is a predominant cause of SSTIs [7,33–35].

Biofilm formation is a critical factor in microbial resistance and persistence, impacting multiple fields, including healthcare, industry, and dentistry [36]. Biofilms exhibit robust resistance to antibiotics and host immune defenses, often requiring concentrations 100 to 1000 times higher than those effective against planktonic cells [37]. Staphylococci, including *S. aureus*, are key contributors to biofilm-related invasive diseases, such as bacteremia, sepsis, osteomyelitis, pneumonia, and infective endocarditis, whether associated with foreign-body-related infections (FBRIs) or as metastatic infections [37].

In the analysis, all *S. aureus* isolates from SSTIs demonstrated biofilm-forming ability, with 10.4 % classified as high-grade biofilm producers and 89.5 % as low-grade producers. Among high-grade biofilm producers, 85.7 % carried the *mecA* gene, indicating a significant association ($P < 0.05$) between strong biofilm formation and methicillin resistance. This supports previous studies showing a link between biofilm formation and antibiotic resistance in *S. aureus*, often mediated by the *mecA* gene [38]. For example, Shah et al. reported that MRSA isolates from bovine mastitis exhibited enhanced biofilm formation and pathogenicity [14]. In contrast, Gaire et al. found that while 70 % of *S. aureus* isolates from clinical samples exhibited weak biofilm production, only 4 % were strong

Table 4
Minimum inhibitory concentrations of Vancomycin against 134 *S. aureus* SSTI isolates.

MIC: μg/L	MRSA N = 78	MSSA N = 56	Total N = 134 (%)
0.0625	0	1	1 (0.7)
0.125	0	0	0
0.25	0	1	1 (0.7)
0.5	5	7	12 (8.9)
1	40	29	69 (51.4)
2	31	18	49 (36.5)
4	1	0	1 (0.7)
8	1	0	1 (0.7)
16–128	0	0	0

SSTI: skin and soft tissue infections.

producers, and the single potent biofilm-producing MSSA isolate showed broad antibiotic resistance, except for cefoxitin and clindamycin [39]. These discrepancies may be due to differences in sample sizes or investigated populations.

Virulence factors, particularly bi-component leukocidins like PVL, play essential roles in the pathogenic success of *S. aureus* by targeting immune cells [32]. PVL is strongly associated with severe *S. aureus* infections, including necrotizing pneumonia and aggressive SSTIs [40,41]. In this investigation, 16.4 % of *S. aureus* isolates from SSTIs harbored the *pvl* gene, with a higher prevalence in MRSA (59 %) compared to MSSA (40.9 %). This is consistent with a meta-analysis conducted in Iran, which reported a *pvl* gene prevalence of 27.9 % in skin-related *S. aureus* isolates, with regional variations ranging from 7.4 % to 55.6 % [42]. Studies from other regions also report variability in *pvl* prevalence among MRSA and MSSA isolates [43–51].

A strong correlation was found between low-grade biofilm development and the presence of the *pvl* gene in the study. PVL-positive isolates were predominantly low or moderate biofilm producers. This finding is supported by an investigation by Aktas et al., which suggested that biofilm production, rather than PVL, is a key virulence factor in certain patient populations, such as those with cystic fibrosis [52]. Furthermore, environmental and metabolic factors may influence *pvl* gene expression, as shown in studies by Span et al. [32]. The observation that low biofilm producers often harbor the *pvl* gene could indicate a context-dependent expression, providing a basis for future research on PVL-mediated pathogenesis in SSTIs.

In terms of vancomycin susceptibility, two MRSA isolates (1.4 %) showed intermediate resistance with an MIC range of 4–8 μg/mL, indicating potential for future VRSA development. An investigation by Moses et al. in India reported 6.08 % VRSA and 46.08 % VISA among *S. aureus* isolates from diverse clinical specimens, highlighting the clinical challenges posed by vancomycin resistance [16]. Clinicians managing VISA and VRSA infections may need to adjust vancomycin dosages or consider alternative treatments to achieve therapeutic efficacy [16].

Ceftaroline has emerged as a promising treatment for complex skin and skin-structure infections (cSSSI) and community-acquired pneumonia (CAP), particularly MRSA-induced cases [53]. In this investigation, using a 30 μg ceftaroline disk according to CLSI guidelines [1], we found that 96.2 % of isolates were susceptible, with inhibition zone diameters $\geq$25 mm. Three isolates showed dose-dependent susceptibility (SDD) with zones between 20 and 24 mm. Alfozan et al. reported similar results, finding no ceftaroline-resistant strains [19]. Cosimi et al. demonstrated ceftaroline's clinical and microbiological efficacy in treating severe MRSA infections, further supporting its utility [54]. The findings align with other studies indicating ceftaroline's efficacy against MRSA, suggesting it may be a valuable option for managing resistant *S. aureus* infections [53].

5. Conclusion

The investigation highlights the significant associations between *mecA* and *pvl* genes with biofilm formation strength in *S. aureus* SSTI isolates. MRSA strains with the *mecA* gene were more likely to produce strong biofilms, while PVL-positive isolates typically exhibited low-grade biofilm formation. These insights into biofilm production and virulence factors could inform therapeutic strategies and underscore the potential of ceftaroline as an alternative treatment for MRSA-related SSTIs, instead of vancomycin. Future studies are warranted to explore the molecular mechanisms linking biofilm formation with virulence genes in *S. aureus* and to monitor emerging resistance trends in MRSA isolates.

CRediT authorship contribution statement

Zeinab Fagheei Aghmiyuni: Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Mohammad Hossein Ahmadi:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Horieh Saderi:** Writing – review & editing, Validation, Methodology, Investigation.

Ethics and consent

This investigation was approved by the Research Ethics Committee of Shahed University (Approval ID: IR. SHAHED. REC.1401.148). All participants provided written informed consent.

Data availability statement

Data supporting the findings of this investigation will be made available upon request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e40524.
The full, non-adjusted image files [Fig. 1(A) and (B)] have provided as supplementary materials.

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