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Screening of antibiotic resistance genes and virulence determinants of *Staphylococcus aureus* from skin infections

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ABSTRACT

Background: Virulent strains of *Staphylococcus aureus (S. aureus)* express a series of virulence factors which cause severe infections such as skin and soft tissue infections which can be life-threatening. Additionally, extensive antibiotic resistance among nosocomial pathogens has left limited choices for their eradication. Our objective was investigation of antibiotic resistance and virulence determinants of *S. aureus* from skin infections.

Materials and methods: Two-hundred non-duplicate *S. aureus* isolates were collected from skin infections. Antibiotic susceptibility profile was evaluated using disc diffusion and methicillin resistance was confirmed. Biofilm formation was assessed by microtiter tissue plate assay. Determinants of virulence factors including *hlA-α, tsst-1, pvl, PSM's, eta* and *etb* genes and also the mupirocin resistance *mup A* gene and class I integron were detected by PCR. MLST was performed for isolates containing the *pvl* gene.

Results: The age range of patients included 12–76 years (mean ± SD = 56.34 ± 5.43). MRSA (70/200) were isolated significantly higher among ages > 50 years ($p < .001$) but not significantly different between both genders ($p = .112$). Prior antibiotic consumption and hospitalization were significantly associated with MRSA (81.42%) and MDR (84.28%) isolation. The existence of *int1* gene (44.5%) was significantly higher in multidrug-resistant (MDR) isolates ($p < .001$). Thirty-six (18%) MDR isolates carried the *mupA* gene. The existence of *mupA* gene was significantly associated with prior hospitalization ($n = 33$, 91.66%, $p < .0001$) and antibiotic consumption ($n = 30$, 83.33%, $p < .001$). Predominant virulence determinant included *PSMα* (61.5%), followed by *tsst-1* (17.5%), *hla-α* (9.5%), *pvl* (2.5%), *eta* (2.5%) and *etb* (1%). The rate of strong biofilm producers (totally 25%) was not significantly different between MRSA and MSSA. The existence of *PSM-α* gene was significantly higher among strong biofilm producers compared to biofilm non-producers ($p = .002$). PFGE exhibited no genetic relation among strains.

Conclusion: Virulence factors of *S. aureus* from skin infections contributed in severity of infection and biofilm formation. MDR phenotype was more common among older patients with history of hospitalization and prior antibiotic consumption. Mupirocin resistance has emerged among MDR and MRSA isolates. Hence suitable control strategies must be performed to inhibit the spread of these strains in healthcare and community settings.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a substantial nosocomial pathogen and a crucial health threatening agent worldwide due to possess of numerous virulence factors and developed antimicrobial resistance (Changchien et al., 2016; Cunha et al., 2013; Lee et al., 2013; Skov, and Jensen, I., 2009). Methicillin-resistant *S. aureus* (MRSA) which exhibit higher rate of antibiotic resistance and virulence, causes vast range of infections such as skin and soft tissue infections (primary and secondary or sometimes recurrent) (Control and Prevention, 1999; David, and Daum, 2010; Francis et al., 2005; King et al., 2006). In addition, community-acquired MRSA (CA-MRSA) is associated with highly invasive infections, including skin and soft tissue infection, necrotizing fascitis and necrotizing pneumonia (Di Meglio et al., 2011; Miller and Cho, 2011). MRSA, the *mecA* gene causes methicillin non-susceptibility and is transmitted by chromosomal cassette (Elements, 2009). The carriage of virulence factors such as panton-valentine leukocidin (PVL), alpha hemolysin, arginine catabolic mobile element (ACME) and

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phenol soluble modulins (PSM) contribute to the increased pathogenicity of CA-MRSA isolates (Burlak et al., 2007; Li et al., 2009; Marimón et al., 2012; Rautenberg et al., 2011; Sanchini et al., 2011). Biofilms are extracellular structural complexes consisting of a polymer matrix including bacterial cells which are attached to a biotic or abiotic surface (89). These materials protect bacteria in the host environment and cause the resistance to antibiotics, disinfectants and host defense mechanisms (90). Persistence and spread of pathogenic and MDR MRSA strains in a hospital or community setting cause an outbreak. Therefore, assessment of genetic relation among these strains in any setting is advantageous towards better understanding of occurrence of an outbreak. Pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) are efficient methods to evaluate genetic relation of strains (Enright et al., 2000; Friães et al., 2015; McDougal et al., 2003). Bacterial typing is beneficial for prevention and control of the spread of resistant isolates, identification of the sources of infections and auspicious transmission routes. Our objective was investigation of antibiotic resistance and virulence determinants of *S. aureus* from skin infections.

2. Materials and methods

Samples were taken from patients with skin infections referred to capital hospitals using sterilized swabs from the infection site during 2017–2019. The samples were inoculated into Thioglyuclate broth medium and transferred to the university's lab rapidly. After culture onto blood agar medium, suspected colonies were identified using conventional biochemical tests and thermonuclease (*nuc*) gene amplification. Subsequently, the isolates were transferred into the trypticase soy broth medium containing 30% glycerol and kept at −20 °C for further process (Chesneau and El Solh, 1992; Kateete et al., 2010; Menzies, 1977).

2.1. DNA extraction

DNA extraction was performed using the Gram-positive bacteria extraction kit from the Pishgaman gene transfer company in accordance with the manufacturer's instructions.

2.2. Molecular identification using the nuc gene

For the *nuc* gene detection, primer sequences have been depicted in Table 1 (Sahebnasagh et al., 2014). The reaction mixture included 8 µl double distilled (DD) H2O, 3 µl master mix of amplicon with 1.5% MgCl2, 1 µl of each forward and reverser primer, and 2 µl of DNA template. (See Fig. 1.)

The thermocycler program for *nuc* gene proliferation included 94 °C for 4 min, followed by 30 cycles of 94 °C for 60 s, 62 °C for 1 min, 72 °C for 60 s, final 72 °C for 7 min, and holding at 4 °C for 5 min.

2.3. Antibiotic susceptibility testing

For antibiotic susceptibility, the procedure was performed according to the CLSI 2017 using antibiotic disks cefoxitin (30 µg), gentamicin (10 µg), erythromycin (15 µg), tetracycline (30 µg), rifampin (5 µg), clindamycin (2 µg), ciprofloxacin (5 µg), trimethoprim sulfamethoxazole (25 µg), linezolid (30 µg), mupirocin (200 µg), chloramphenicol (30 µg), kanamycin (30 µg) and penicillin (10 µg) (MAST, UK). Moreover, *S. aureus* ATCC 25923 and *Escherichia coli* ATCC25922 standard strains were used for the disk quality control.

Table 1
The primer sequences of *nuc* and *mecA* genes.

Primer	Sequence (5′ → 3′)	Size (bp)	Reference
nuc-F	GCGATTGATGGTGATACGGTI	278	(Sahebnasagh et al., 2014)
nuc-R	AGCCAAGCCTTGACGAACTAAAGC		
mec-F	GTGAAGATATACCAAGTGATT	147	(Zhang et al., 2005)
mec-R	ATGCGCTATAGATTGAAAGGA		

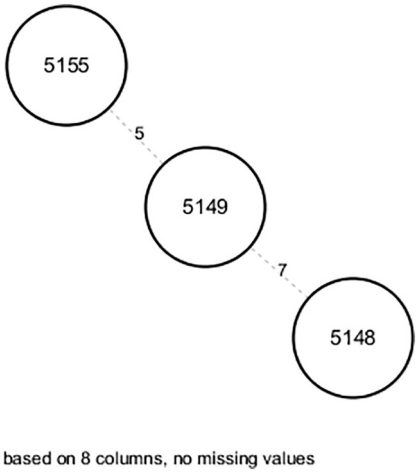

Fig. 1. The MLST results of three virulent MSSA strains.

2.4. Detection of mecA gene

The MRSA strains were confirmed by detection of the *mecA* gene using specific primers (Table 1) (Zhang, McClure, Elsayed, Louie, & Conly, 2005). The reaction mixture was prepared the same as that for the *nuc* gene. The thermocycler conditions for *mecA* gene amplification contained 94 °C for 5 min followed by 30 cycles of 94 °C for 60 s, 55 °C for 1 min, 72 °C for 60 s, 72 °C for 7 min and finally at 4 °C for 5 min.

2.5. Determination of the SCCmec types

Specific primers for SCC*mec* types are presented in Table 2 (Zhang et al., 2005). The thermocycler program for these genes included 94 °C for 5 min and then 32 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, then 72 °C for 1 min, and 4 °C for 10 min.

2.6. Detection of pvl gene

The specific primer for the *pvl* gene is shown in Table 3 (Havaei et al., 2012). The thermocycler program for this gene included 94 °C for 4 min, then 32 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, then 72 °C for 5 min, and 4 °C for 10 min.

Table 2
The primer sequences of SCC*mec* types in this study (Zhang et al., 2005).

Primer	Sequence (5′ → 3′)	Size (bp)
SCC*mec* I	*F:* GCTTTAAAGAGTGTCGTTACAGG *R:* GTTCTCTCATAGTATGACGTCC	613
SCC*mec* II	*F:* CGTTGAAGATGATGAAGCG *R:* CGAAATCAATGGTTAATGGACC	398
SCC*mec* III	F: CCATATTGTGTACGATGCG R: CCTTAGTTGTCGTAACAGATCG	280
SCC*mec* IVa	F: GCCTTATTCGAAGAAACCG R: CTACTCTTCTGAAAAGCGTCG	776
SCC*mec* IVb	F: TCTGGAATTACTTCAGCTGC R: AAACAATATTGCTCTCCCTC	493
SCC*mec* IVc	F:ACAATATTTGTATTATCGGAGAGC R: TTGGTATGAGGTATTGCTGG	200
SCC*mec* IVd	F: CTCAAAATACGGACCCCAATACA R: TGCTCCAGTAATTGCTAAAG	881
SCC*mec* V	F: GAACATTGTTACTTAAATGAGCG R: TGAAAGTTGTACCCTTGACACC	325

Table 3
Primer sequences of *pvl, tsst-1, hla-α, psm-α, Eta, Etb, int I* and *mupA* genes.

Primer	Sequence (5′ → 3′)	Size (bp)
pvl F	GGAAACATTTATTCTGGCTATAC	502
pvl R	CTGGATTGAAGTTACCTCTGG	
tsst F	TTATCGTAAGCCCTTTGTTG	326
tsst R	TAAAGGTAGTTCTATTGGAGTAGG	
F. *hla-α*	CGGTACTACAGATATTGGAAGC	744
R. *hla-α*	TGGTAATCATCACGAACTCG	
F. *psmα*	TATCAAAAGCTTAATCGAACAATTC	176
R.*psmα*	CCCCTTCAAATAAGATGTTCATATC	
eta F	TATCAAAAGCTTAATCGAACAATTC	176
atb R	CCCCTTCAAATAAGATGTTCATATC	
F. *intI*	CCTCCCGCACGATGATC	188
R.*intI*	TCCACGCATCGTCAGGC	
F. *mupA*	TATATTATGCGATGGAAGGTTGG	457

2.7. Detection of the tsst-1 gene

The thermocycler program for this gene included 94 °C for 5 min and then 32 cycles of 94 °C for 1 min, 51 °C for 1 min, 72 °C for 1 min, then 72 °C for 5 min, and maintaining at a temperature of 4 °C for 10 min. The sequence of primers has been depicted in Table 3 (Havaei et al., 2012).

2.8. Detection of hla-α

The thermocycler program for this gene included 94 °C for 5 min, then 32 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, then 72 °C for 5 min, and maintaining a temperature of 4 °C for 10 min. Has been. Positive control of clinical isolates of *S. aureus* was used in the laboratory. The sequence of primers has been depicted in Table 3 (Havaei et al., 2012).

2.9. Detection of psmα, eta and etb

The thermocycler program for these genes included 94 °C for 5 min, then 32 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, then 72 °C for 5 min, and 4 °C storage temperature Celsius is set to 10 min. The sequence of primers has been depicted in Table 3 (Ferreira et al., 2013).

2.10. Class I integron

The thermocycler program for this gene included 94 °C for 5 min, then 32 cycles of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, then 72 °C for 5 min, and holding at 4 °C for 10 min. The sequence of primers has been depicted in Table 3 (Xu et al., 2008).

2.11. Identification of mupA gene

The sequence of primers has been depicted in Table 3 (Seah et al., 2012).

2.12. Phenotypic experimentation of biofilm formation

The ability to form biofilm among isolates was evaluated using a microtiter tissue plate assay medium. TSB medium containing 1% glucose was prepared and 180 µl was divided into each well. 20 µl of bacterial suspension equal to the McFarland standard turbidity was added to the TSB broth medium. The plates were then incubated for 24 h at 37 °C. After incubation, in order to remove suspending strains, the medium was decanted and the wells were washed twice with 200 µl of DD H2O. 150 µl of crystal violet (10%) were added to each well and placed at room temperature for 45 min. Then it was washed twice with 200 µl DD H2O. After washing the plate, it was placed at room temperature for up to 24 h to dry. Then 150 µl of 98% ethanol was added to each well for 30 min to remove the dye attached to the bacteria in the biofilm and the turbidity was read at OD 490 nm using ELISA reader. The ability of biofilm formation by the isolates was calculated based on the formula including: $OD^1 > 4 \times ODc^2$ being strong (where ODc is related to the control), $2 \times ODc < OD \leq 4 \times ODc$ being moderate, $ODc < OD \leq 2 \times ODc$ being weak and $OD \leq ODc$ which meant no biofilm formation. Using the cut-off method, the results were examined. *S. aureus* (ATCC 35556) was used as a positive control of the strong biofilm producer and *S. epidermidis* ATCC 12228 was employed as the negative control (Stepanović et al., 2007).

2.13. Alpha hemolysin production

After observing the hemolysis zone, bacteria were cultured on an agar culture medium and incubated at 37 °C for 24 h to determine hemolysis pattern.

3. Genotyping using the MLST technique

In order to conduct this technique, seven genes were firstly amplified by PCR method and then sent to the desired PCR product for sequencing, and the sequencing results were analyzed. The primers used and the conditions for performing PCR at the MLST site included *S. aureus* (http://pubmlst.org/saureus/). The phylogenetic relationship between isolates was investigated by UPGMA online algorithm.

In this method, by examining the housekeeping genes, PCR genes are made for seven *S. aureus* housekeeping genes (*arcC, aroE, glpF, gmk, pta, tpi* and *yqiL*). The PCR product was sequenced using the software of these genes and compared to sequences found on the MLST website for *S. aureus* (Enright et al., 2000).

The thermocycler program for MLST was 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, 72 °C for 7 min and 4 °C for 5 min.

3.1. Pulsed field gel electrophoresis

The PFGE was performed according to t PulseNet. WWW.cdc.gov/pulsenet standard which has been applied for *S. aureus* and *Salmonella* spp. Briefly, after plaque preparation in TSB, 3 µl lysostaphin (1 mg/µl) was added to lyse bacterial isolates. After washing, enzymatic digestion was carried out using *sma1* (10 U) and lysis buffer and washing again. Then, *xba1* (10 U) was used for digestion. For electrophoresis, 0.5× buffer was used and CHEFF MAPPER XA devise (Bio Rad) was employed.

4. Results

4.1. Demographic data

The age range of patients included 12–76 years (mean ± SD = 56.34 ± 5.43) including 121 females and 79 males. Previous hospitalization was observed among 89 of them. Of totally 232 samples from skin infections, in the conventional biochemical and PCR tests, 200 *S. aureus* isolates were identified.

4.2. Antibiotic test results

The highest antibiotic resistance rate among MRSA isolates was against cefoxitin (100%), clindamycin (68%), erythromycin (46%), tetracycline (48%), and the lowest resistance to linezolid (14%), mupirocin (10.5%), ciprofloxacin (2.5%), and chloramphenicol (0%). Also, among the MSSA isolates, the highest resistance rate was observed against penicillin (96%), clindamycin (40%), rifampin (32%), erythromycin (24%), and the lowest resistance to cefoxitin (0%), gentamicin (4%), chloramphenicol and amikacin (6%). MRSA (70/200) were

isolated significantly higher among ages > 50 years ($p < .001$) but not significantly different between both genders ($p = .112$). Prior antibiotic consumption and hospitalization were significantly associated with MRSA (81.42%) and MDR (84.28%) isolation.

4.3. PCR amplification of resistance genes and virulence determinants

Among 70 MRSA from skin infections, 12 isolates contained SCC*mecI*, 12 SCC*mecII*, 16 SCC*mecIII*, 15 SCC*mecIVa*, 5 SCC*mecIVb*, 2 SCC*mecIVc*, 2 SCC*mecIVd* and 6 SCC*mecV*.

The existence of *int1* gene (44.5%) was significantly higher in multidrug-resistant (MDR) isolates ($p < .001$). Thirty-six (18%) MDR isolates carried the *mupA* gene. The existence of *mupA* gene was significantly associated with prior hospitalization ($n = 33$, 91.66%, $p < .0001$) and antibiotic consumption ($n = 30$, 83.33%, $p < .001$).

Predominant virulence determinant included *PSMα* (61.5%), followed by *tsst-1* (17.5%), *hla-α* (9.5%), *pvl* (2.5%), *eta* (2.5%) and *etb* (1%). The rate of strong biofilm producers (totally 25%) was not significantly different between MRSA and MSSA. The existence of *PSM-α* gene was significantly higher among strong biofilm producers compared to biofilm non-producers ($p = .002$).

The *pvl* gene was detected among the MSSA but not in any of the MRSA strains. Additionally, regarding the existence of *PSM-α* (86 MSSA and 37 MRSA, $p = .071$), *tsst-1* gene (16 MRSA and 24 MSSA, $p = .102$) and α-hemolysis (24 MRSA and 34 MSSA, $p = .0513$) there was no significant difference between MRSA and MSSA. 58 isolates contained the *hla-α* gene; additionally, 34 isolates of MRSA and 24 isolates of MSSA, had in the culture medium. Also, 73 (53.67%) isolates belonged to skin infections contained the *psm-α* gene. Among skin isolates, 41 (46.6%) contained the *intI* gene. Nineteen isolates (4 MRSA and 15 MSSA) carried the *mupA* gene.

Among 34 MDR strains which carried the *mupA* mupirocin resistance gene, 14 of them carried the *PSMα*, *tsst-1* and *hla-α* genes and three of them (all being MSSA) carried all virulence determinants (but not the *etb* gene), *mupA* and the *int1* genes. These strains were resistant to all the antibiotics, but not cefoxitin.

4.4. MLST results

The MLST results for three virulent and drug-resistant strains outline that they had not genetic relation, but > 80% similarity was observed between strains 5148 and 5149.

4.5. PFGE

The PFGE results also confirmed that the source of infection was not the same as no genetic relation was observed (Fig. 2).

5. Discussion

In this study, nearly half of isolates were MRSA. In some parts of the United States, CA-MRSA isolates account for 46% of the acquired infections in children (Hasibi et al., 2007). Similar to our findings, CA-MRSA isolates have been more prevalent in subjects over 40 years old (Moghadami et al., 2010; Sasan et al., 2014).

CA-MRSA strains carry SCC*mec* types II, IV and V. we observed that 12 isolates contained SCC*mecI*, 12 SCC*mecII*, 16 SCC*mecIII*, 15 SCC*mecIVa*, 5 SCC*mecIVb*, 2 SCC*mecIVc*, 2 SCC*mecIVd* and 6 SCC*mecV*. The highest antibiotic resistance rate among MRSA isolates was against cefoxitin (100%), clindamycin (68%), erythromycin (46%), tetracycline (48%), and the lowest resistance to linezolid (14%), mupirocin (10.5%), ciprofloxacin (2.5%), and chloramphenicol (0%). Also, among the MSSA isolates, the highest resistance rate was observed against penicillin (96%), clindamycin (40%), rifampin (32%), erythromycin (24%), and the lowest resistance to cefoxitin (0%), gentamicin (4%), chloramphenicol and amikacin (6%). MRSA (70/200) were isolated significantly higher among ages > 50 years ($p < .001$) but not significantly different between both genders ($p = .112$). Prior antibiotic consumption and hospitalization were significantly associated with MRSA (81.42%) and MDR (84.28%) isolation.

Higher rate of antibiotic resistance has been revealed in some other studies which is due to SCC*mec* types (Sun et al., 2013; Taha, 2013; Yao et al., 2010). Notably, low rate of resistance against some antibiotics is likely due to lower rate of consumption in an area.

The existence of *int1* gene (44.5%) was significantly higher in multidrug-resistant (MDR) isolates ($p < .001$). Thirty-six (18%) MDR isolates carried the *mupA* gene. The existence of *mupA* gene was significantly associated with prior hospitalization ($n = 33$, 91.66%, $p < .0001$) and antibiotic consumption ($n = 30$, 83.33%, $p < .001$). Resistance to high concentrations of mupirocin is due to the acquisition of the plasmid-encoded *mupA* gene. These plasmids also carry genes causing resistance to other antibiotics such as macrolides, gentamicin, tetracycline and cotrimoxazole (Patel et al., 2009).

Class I Integron genes cause resistance to multiple antibiotics (Deng et al., 2015; Ren et al., 2013) and hence, their existence and spread should be considered for control and prevention implementations.

Predominant virulence determinant included *PSMα* (61.5%), followed by *tsst-1* (17.5%), *hla-α* (9.5%), *pvl* (2.5%), *eta* (2.5%) and *etb* (1%). The rate of strong biofilm producers (totally 25%) was not significantly different between MRSA and MSSA. The existence of *PSM-α* gene was significantly higher among strong biofilm producers compared to biofilm non-producers ($p = .002$). it seems that the existence of virulence factors is not dependent on MRSA and SCC*mec* (Djahmi et al., 2013; Japoni-Nejad et al., 2013), but cause severity of infections (Yao et al., 2010; Yu et al., 2015).

We observed that the *pvl* gene was detected among the MSSA but not in any of the MRSA strains. More in-depth verifications are essential towards disclosure of any possible relation.

Additionally, regarding the existence of *PSM-α* (86 MSSA and 37 MRSA, $p = .071$), *tsst-1* gene (16 MRSA and 24 MSSA, $p = .102$) and α-hemolysis (24 MRSA and 34 MSSA, $p = .0513$) there was no significant difference between MRSA and MSSA. 58 isolates contained the *hla-α* gene; additionally, 34 isolates of MRSA and 24 isolates of MSSA, had in the culture medium. Also, 73 (53.67%) isolates belonged to skin infections contained the *psm-α* gene. Among skin isolates, 41 (46.6%) contained the *intI* gene. Nineteen isolates (4 MRSA and 15 MSSA)

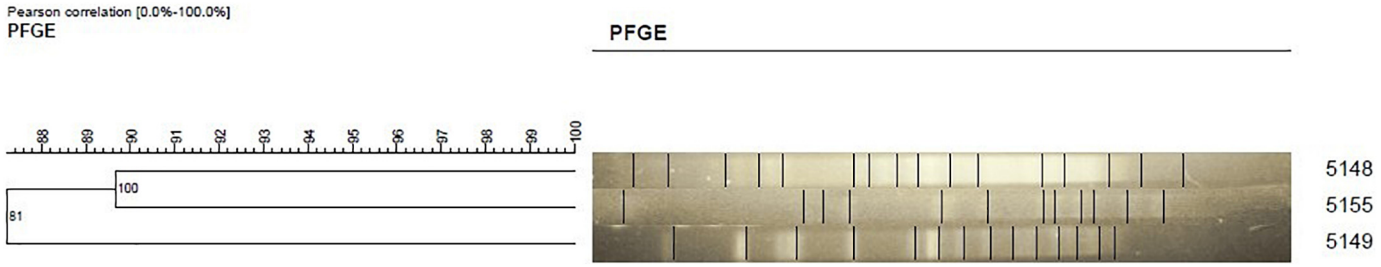

Fig. 2. The electrophoresis of bacterial DNA enzymatic digestion in the PFGE technique.

carried the *mupA* gene.

Among 34 MDR strains which carried the *mupA* mupirocin resistance gene, 14 of them carried the *PSMα*, *tsst-1* and *hla-α* genes and three of them (all being MSSA) carried all virulence determinants (but not the *etb* gene), *mupA* and the *int1* genes. These strains were resistant to all the antibiotics, but not cefoxitin.

S. aureus extracellular surface structures such as PSM-α play a primary role in bacterial colonization onto commensal epithelial cells and severe skin complications (Periasamy et al., 2012). Our study depicted that 61.5% of isolates contained this gene. It has been exhibited that several bacterial surface proteins cause bacterial attachment and colonization. Coexistence of this gene with *pvl, tsst-1, hla* and *eta/etb* genes in three strains highlighted the occurrence of severe infection.

According to MLST and PFGE results, there was no outbreak in area of study.

6. Conclusion

In this study, *S. aureus* from skin infections exhibited wide antibiotic resistance being significantly higher among MRSA strains. Spread of mupirocin resistance is a concern which needs implementation of proper control measures. In addition, the existence of various virulence factors was confirmed among these isolates. The MLST and PFGE results showed that there was no clonal relation among the strains.

Acknowledgments

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