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Molecular Characteristics of Community-Associated Methicillin-resistant *Staphylococcus aureus* (CA-MRSA) Isolates from Clinical Specimens in Iraq

Alaa H. Al-Charrakh[1*], Huda H. Al-Hassnawi[1] and Jawad K. Al-Khafaji[1]

[1]Department of Microbiology, College of Medicine, Babylon University, Hilla, Iraq.

Authors' contributions

This work was carried out in collaboration between all authors. Author AHA managed the literature searches, designed the study, wrote the first draft of the manuscript and supervised the work. Author HHA isolated CA-MRSA from clinical specimens, performed the experiments and wrote the protocols. Author JKA supervised the work. All authors read and approved the final manuscript.

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ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have been recognized for decades as hospital acquired MRSA (HA-MRSA). Nowadays, MRSA is also recognized as a worldwide emerging community-associated pathogen. Community associated-MRSA (CA-MRSA) has been shown to be more virulent with a high degree of severity of disease when compared to HA-MRSA.
Objectives: The study was designed to assess the occurrence and the molecular detection of HA-MRSA and CA-MRSA isolates obtained from clinical specimens in Iraq.
Methods: HA-MRSA and CA-MRSA isolates were obtained from clinical specimens in three main hospitals in Hilla city/Iraq during the period, March to June 2011. MRSA isolates obtained primarily from clinical specimens of skin and soft tissue infections (SSTs) were subjected to genetic study. PCR was used for detection of genes responsible for methicillin resistance (*mec*A, SCC*mec* type

**Corresponding author: Email: ahani67@gmail.com, aalcharrakh@yahoo.com;*

IV) and genes responsible for toxin production (*pvl*, *luk*ED). Statistical analysis was performed using chi-square (X2) test to assess intergroup significance, inpatients, and outpatients with respect to all genes used in present study.
Results: Out of 301 clinical samples, 24 MRSA isolates were obtained. All these MRSA isolates (100.0%) were mecA gene positive. Twenty three (95%) were found to be carrying *SCCmec* type IV, 19 (79%) had positive result for *pvl* toxin gene, and 20 (83%) had *luk*ED toxin gene.
Conclusion: The majority of MRSA isolates belonged to SCC*mec* IV. *pvl* and *luk*ED toxin genes are also found in the MRSA isolates among the CA-MRSA isolates.

Keywords: CA-MRSA; mecA gene; SCCmec type IV; pvl gene; PCR; HA-MRSA.

1. INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have been recognized for decades as hospital acquired or healthcare associated [1]. The possibility of transmission of healthcare associated MRSA (HA-MRSA) to the community was unavoidable [2]. Now-a-days MRSA is also recognized as a worldwide emerging community-acquired pathogen (CA-MRSA) [3]. It was shown that Panton-Valentine leukocidin (PVL) positive MSSA are a likely reservoir for the development of PVL positive MRSA [4] via integration of *Staphylococcus* cassette chromosome mec (SCC*mec*) elements including the *mec*A gene conferring methicillin resistance [5]. MRSA strains that have been clinically identified as CA-MRSA have been shown to be more virulent with a high degree of severity of disease when compared to HA-MRSA [6]. This is due to the production of the PVL toxin. PVL toxin is associated with deep skin and soft tissue infections, such as furunculosis and abscesses, however, necrotizing tissue infections and lethal hemorrhagic pneumonia have been reported [7]. The prevalence rate of the PVL toxin in CA-MRSA strains varies with different studies and countries [8]. Some studies reported a prevalence of 77% to 100% for the PVL toxin in Minnesota, USA in 2000 [9] while a prevalence of less than 5% was reported in Western Europe [10].

The *mec*A gene resides on a genomic island termed the staphylococcal cassette chromosome mec (SCC*mec*) [5]. Most outbreaks of MRSA involve CA-MRSA rather than HA-MRSA, thus monitoring the SCC*mec* type is important in determining the epidemiologic trend of the MRSA strains in clinical settings [11]. The presence of PVL toxin in CA-MRSA strains can be confirmed by co-amplification of the *lukS/F-PV* genes [11]. In comparison to previous detection methods such as Southern blotting and pulsed-field gel electrophoresis (PFGE), PCR assays can provide a rapid amplification, detection and typing tool for MRSA strains [12]. Outbreaks in closed living communities, jail inmates, military recruits, and gay community, have been reported in the United States [13]. The aim of this study was to assess the occurrence of HA-MRSA and CA-MRSA isolates and to detect the PVL genes in MRSA isolates obtained from clinical specimens in Hilla/Iraq.

2. MATERIALS AND METHODS

2.1 Study Design and Bacterial Isolates

This cross sectional study was designed to assess the occurrence of HA-MRSA and CA-MRSA isolates obtained from clinical specimens and to detect the PVL genes in MRSA isolates. At the beginning of this study, 301 different clinical samples were collected during the period of March to June 2011 from the main three hospitals in Al-Hilla city/Iraq (Hilla Teaching hospital, Margan Teaching hospital, and Childhood and gynecology hospital), in addition to some private clinics. Clinical isolates were as follows: ear swabs (37), burns (12), skin infections (65), wounds (25), sputum (23), urine (70), vaginal swabs (4), and CSF (65).

Out of 301 clinical samples, forty six *S. aureus* isolates were obtained. These bacterial isolates were identified as *S. aureus* based on their morphology, Gram-staining, catalase properties in addition to coagulase test [14] and as suggested previously [15].

In the light of antibiotic susceptibility results of our previous study [15], 13 MRSA isolates were detected using phenotypic methods. They were oxacillin and cefoxitin resistant. The MRSA isolates were also SCC*mec* typed to differentiate between HA-MRSA and CA-MRSA isolates using a PCR. The remaining 11 samples from specific skin infections (abscesses, boils), which phenotypically identified as oxacillin sensitive

and cefoxitin resistant [15], were selected for genetic study to detect other genes that are considered as a marker for CA-MRSA isolates.

2.2 DNA Extraction and PCR Assay

One colony of each strain (cultured on solid medium) was inoculated into 5 mL of BHI (Broth Heart Infusion) and grown overnight at 37°C. From these cultures, DNA was purified from bacterial cells using Genomic DNA Mini kit (Geneaid, UK). Chromosomal DNAs obtained were used as templates for all PCR experiments. The PCR reactions were carried out in a thermal cycler (Clever, U.K). Before PCR assay, and in order to quantify the DNA concentration (ng μL^{-1}), the quantification of DNA samples was carried out by means of a spectrophotometric reading using 1 µL aliquots of Genomic DNA with a NanoDropTM spectrometer (NanoDrop Technologies), adopting the manufacturer's recommendations. The concentration of DNA was estimated from absorbance at 260 nm. DNA profiles were performed using bacterial DNA and loading buffer according to the manufacturer instructions (Bioneer, Korea).

2.3 Polymerase Chain Reaction Protocols

The DNA extracts of *S. aureus* isolates were subjected to different genes by PCR. The protocols used were according to manufacturer's instructions. Different primers of antibiotic resistance and toxins genes were used (Bioneer, Korea) in an attempt to differentiate MRSA isolates (Table 1). All PCR components were assembled in PCR tube and mixed on ice bag under sterile conditions. The PCR tubes were placed on the PCR machine and the accurate PCR cycling program parameters conditions were installed (Table 2).

2.4 Statistical Analysis

Statistical analysis was performed using chi-square (X^2) test to assess intergroup significance, inpatient (hospital acquired) and outpatient (community acquired) with the respect to all genes used in present study. P value less than 0.005 was considered as statistically significant and P- value less than 0.001 was considered as highly significant.

3. RESULTS AND DISCUSSION

3.1 Detection of *mec*A and SCC*mec* IV Genes by PCR

Chromosomal DNA was extracted from twenty four clinical strains. Results of this study revealed that a high frequency of *mec*A gene (100%) among all *S. aureus* isolates was detected (Fig. 1). All hospital (100%) and all community isolates (100%) carried *mec*A gene (Table 3).

Using phenotypic methods, a total 13 out of 24 isolates were determined previously as MRSA [10]. The *mec*A gene is responsible for mediating methicillin resistance in Staphylococci. These results indicate that there is a large dissemination of MRSA in the community (62%) and not restricted to the hospitals as expected [20] and as reported by other authors [7,21] who found that most MRSA isolates were associated with nosocomial infections. This high prevalence of *mec*A gene in the community may be due to horizontal gene transfer (HGT) from MRSA to MSSA isolates by transduction [22] since the *mec*A gene is carried on the staphylococcal cassette chromosome. SCC*mec* is inserted into the *S. aureus* chromosome near the origin of replication [23].

In a local study in Iraq, the presence of *mec*A gene in isolates of CA-MRSA from holy shrine in Najaf city was detected [24]. In Europe, MRSA strains account for less than 5% of *S. aureus* isolates in the community setting and for less than 11% of isolates from patients with skin and soft tissue infections (SSTI) in France [25]. The CDC created a definition for a CA-MRSA infection: any MRSA infection diagnosed for an outpatient or within 48 h of hospitalization if the patient lacks the following health care-associated MRSA risk factors: hemodialysis, surgery, residence in a long-term care facility or hospitalization during the previous year, the presence of an indwelling catheter or a percutaneous device at the time of culture, or previous isolation of MRSA from the patient [26]. All other MRSA infections were considered to be HA-MRSA.

The *mec*A gene for methicillin resistance resides on the chromosome. Accurate detection of *mec*A mediated resistance to methicillin and other penicillinase stable penicillins (PSPs) like oxacillin, nafcillin, cloxacillin, dicloxacillin, flucloxacillin, and mecillinam is necessary to ensure appropriate antimicrobial chemotherapy of staphylococcal infections, particularly those from community associated infections.

Table 1. Primers of antibiotic resistance and toxins genes used in the present study

Primer	Primer Sequence (5'-3')	Product size (bp)	Ref.
*mec*A-F	GTG GAA TTG GCC AATACA GG	1339	[16]
*mec*A-R	TGA GTT CTG CAG TAC CGG AT		
SCCmec typeIV-F	TTTGAATGCCCTCCATGAATAAAAT	450	[17]
SCCmec typeIV-R	AGAAAAGATAGAAGTTCGAAAGA		
pvl- F	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	[18]
pvl- R	GCATCAASTGTATTGGATAGCAAAAGC		
LukED-F	TGAAAAAGGTTCAAAGTTGATACGAG	269	[19]
*Luk*ED-R	TGTATTCGATAGCAAAAGCAGTGCA		

Table 2. PCR thermocycling conditions

Monoplex Gene	Temperature (°C)/ Time					Cycle No.
	Initial denaturation	Cycling condition denaturation	annealing	Extension	Final extension	
lukS/F	95/5min	94/30 sec	55/30 sec	72/1 min	72/10 min	35
*luk*ED	95/5 min	95/2min	58/1min	72/2 min	72/10 min	30
*mec*A	94/5 min	94/1 min	58/1 min	72/1 min	72/10 min	35
SCCmec type IV	94/5 min	94/1 min	55/1 min	72/1.5min	72/1.5 min	35

However, antimicrobial susceptibility tests using oxacillin are often difficult to read despite changes in techniques to improve the discrimination between oxacillin susceptible and resistant results [27]. Since conventional identification and antibiotic resistance detection often take more than 48 h, molecular based detection techniques, including conventional PCR, have been developed for the rapid and accurate identification and characterization of MRSA isolates [22].

SCC*mec* typing is one of the most important molecular tools available for understanding the epidemiology and strain relatedness of MRSA [28]. In this study, *Staphylococcus* Cassette Chromosome type IV (SCC*mec* IV) was also detected, and the detection of this was used as stable marker of CA-MRSA [3]. In this present study almost all MRSA isolates were carrying SCC*mec* IV (95%) (Fig. 2). This single SCC*mec* IV negative isolate was recovered from the community as well; the lack of this cassette chromosome from this isolate may be due to newly discharged patient from hospital designated as HA-MRSA; however, marker genes SCC*mec* type I, II and III of HA-MRSA were not detected. The results showed that out of 23 MRSA isolates, 9 (39.1%) were HA-MRSA carrying this gene and 14 (60.8%) for CA-MRSA isolates (Table 3). It is clear that all HA-MRSA were carrying SCC*mec* IV cassette.

The high frequency of SCC*mec* IV gene in hospitals (100%) may be due to dissemination of CA-MRSA in the hospital area since the SCC*mec* IV had smaller size comparing with type II and III elements which may serve as an evolutionary advantage by making them more amenable to horizontal spread among a bacterial population [29]. However, CA-MRSA strains have now been found in association with nosocomial infections [30]. Irrespective of the characteristics of the population or the setting, community-onset MRSA carrying the SCC*mec* type IV element poses a real threat and will likely continue to emerge as a major public-health concern. CA-MRSA strains harbor the smaller SCC*mec* type IV element which grow faster and achieve higher infection burdens than nosocomial MRSA strains [17].

These results confirm a tendency seen in previous studies from the University hospitals of Zurich (45%; type IV) and Basel (58%; type IV) and in other studies [31,32] that reported the spread of CA-MRSA SCC*mec* type IV strains in hospital settings. The carriage of *SCCmec* IV in community acquired infections is reported to be due to the lack of antibiotic pressure outside of the hospital environment as well as the lack of resistance plasmids or transposons downstream of *mec*A [7]. SCC*mec* typing is essential because it can distinguish between HA-MRSA and CA-MRSA [33]. Rapid and accurate identification and characterization is needed for the detection of these strains, as they are ecologically fit to reside both in the community and health-care facilities [32].

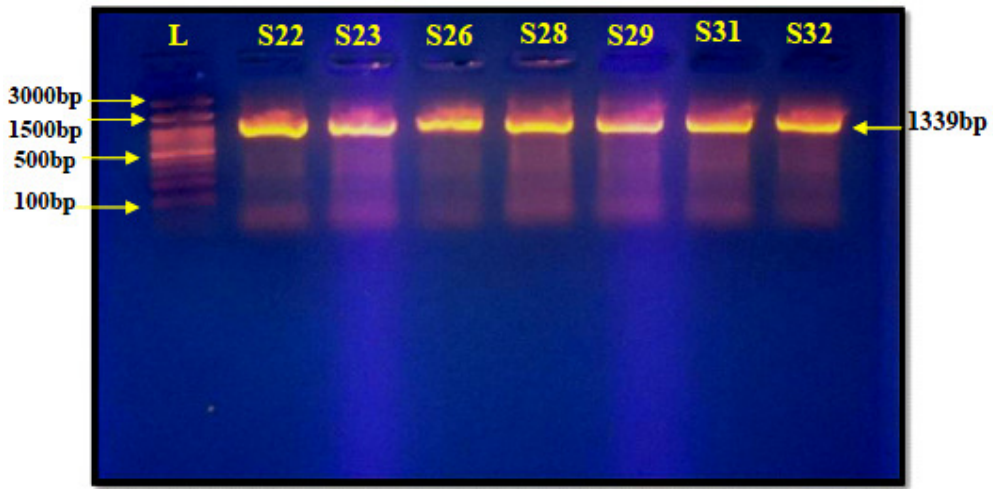

Fig. 1. Gel electrophoresis of PCR of *mec*A amplicon product

Lane L: Ladder (3000-bp ladder), Lanes (S22, S23, S26, S28, S29, S31, and S32) represent isolates from skin. Negative control without amplicon and positive control with the expected band were not shown

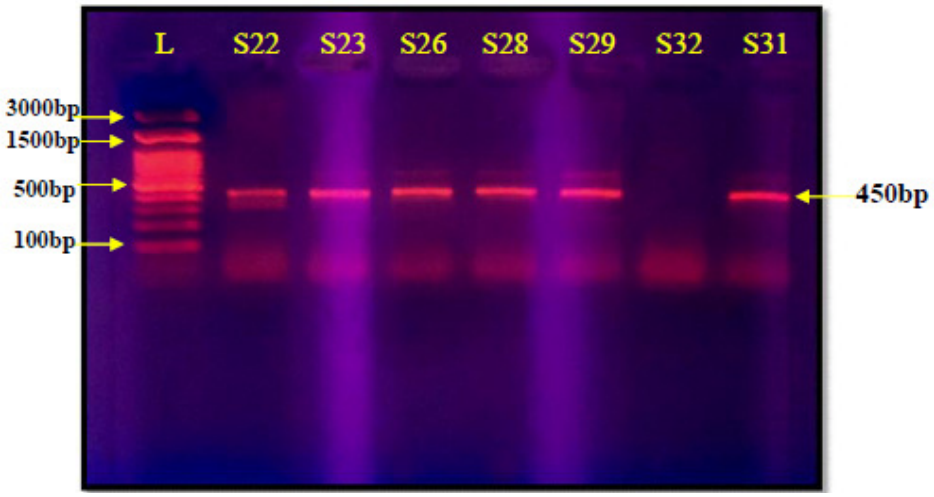

Fig. 2. Gel electrophoresis of PCR of SCC*mec* IV amplicon product

Lane L: Ladder (3000-bp ladder), Lanes (S22, S23, S26, S28, S29, and S31) represent isolates from skin. Lane S32 shows negative result

3.2 Molecular Detection of Virulence Genes by PCR

PCR was used to determine the bi-component leukocidin PVL gene in all 24 MRSA isolates. However, PVL gene by PCR was seen in 19 (79.1%) isolates. PVL gene was not detected in 5 (20.8%) isolates (Fig. 3). All these isolates were *mec*A gene positive and identified as MRSA by genotypic test (Fig. 3). The results also showed that *mec*A: PVL ratio was 24:19 and *mec*A is a peptidoglycan transpeptidase and this protein is expressed at the external surface of cytoplasmic membrane, where it could interact with the extracellular protein pvl. In this case it can be given the name CA-MRSA to this MRSA isolates, as these isolates harbored PVL and SCC*mec* IV genes [3].

The high rate of pvl gene in the present study was due to involvement of purulent skin infections (boils, abscesses) because there is a close correlation between this gene and suppurative infections [18]. Out of these 19 positive isolates, 8 (42.1%) isolates were found in HA-MRSA while 11 (57.8%) isolates were found in CA-MRSA isolates.

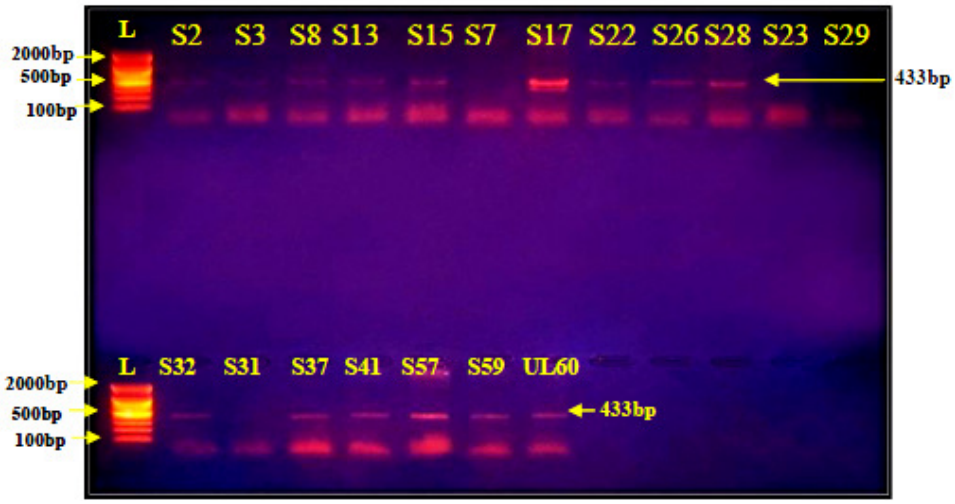

Fig. 3. Gel electrophoresis of PCR of pvl amplicon product

Lane L: Ladder (3000-bp ladder), Lanes (S2, 3, 8, 13, 15, 17, 26, 28, 32, 22, 37, 41, 57, 59) represent isolates from skin, Lane (Ul60) represents isolate from ulcer, Lane (W79) represents isolate from wound

In Iraq, little attention has been paid to the prevalence of pvl gene in MRSA isolates, except in some reports [24]. There is also no professional center available for studying or research about prevalence, distribution, and purification of this toxin. The prevalence of PVL gene in the present study (79%) was more than that obtained by Al-Mohana et al. [24] who found that PVL genes were detected in 27.2% of CA-MRSA isolates. However, the high prevalence of PVL gene in this study was due to high numbers of selected skin samples.

Lina et al. [18] showed an association between PVL genes and cutaneous infections (85%), confirming earlier findings by other workers [34]. In other studies, Badiou et al. [35] found that MRSA isolates harbored PVL gene were more prevalent in cutaneous MRSA isolates (83%), and 70% of *mec*A positive isolates harbored PVL gene, that closely correlated with the present study results.

It has been suggested that only few *S. aureus* strains are susceptible to infection with PVL-converting phages. Different strains of *S. aureus* have been shown to harbor different PVL-carrying phages [36]. PVL is rarely produced by *S. aureus* (less than 5% of strains); however, the PVL gene is frequently detected from community-acquired skin infections [18], which was also noted in the present study results. An interesting finding of this study was the presence of PVL genes in MRSA isolates recovered from urine which was not reported previously [18]. However, the result showed that PVL-containing MRSA not only existed in the community (57.8%) but also found in hospitals (42.1%) (Table 3).

It has been shown that PVL gene is not universally present in CA-MRSA isolates [37]. However, Havaei et al [38] found that 61.8% of HA-MRSA isolates had PVL genes. This may be due to dissemination of CA-MRSA isolates in the hospitals, acquisition of gene by phage infection, and integration into the chromosome of hospital isolates. It has been well established that PVL genes (*luk*S- and *luk*F-PV) of CA-MRSA are harbored by a bacteriophages. Furthermore, these toxin genes may be transmitted easily to other HA-MRSA strains [7]. It is possible that the PVL- (negative) strain originated from the PVL+ (positive) strain via loss of prophage from the genome of the latter [7].

*luk*ED gene was detected in 20 (83%) MRSA, however, it was not detected in 4 (16.6%) MRSA isolates (Fig. 4). Out of 20 positive MRSA isolates, 9 (45%) and 11 (55%) isolates were detected from hospital and community settings respectively (Table 3).

The results also revealed that all HA-MRSA isolates (100%) were harboring *luk*ED gene. This result was in agreement with results obtained by Vandenesch et al. [39], who found that *luk*ED gene was detected in most HA-MRSA isolates. However, in hospital PVL and *Luk*ED positive MRSA isolates were 42.1% and 45% respectively (Table 3). From these 20 positive isolates for *luk*ED, 17 (85%) MRSA isolates shared both genes (PVL and *luk*ED).

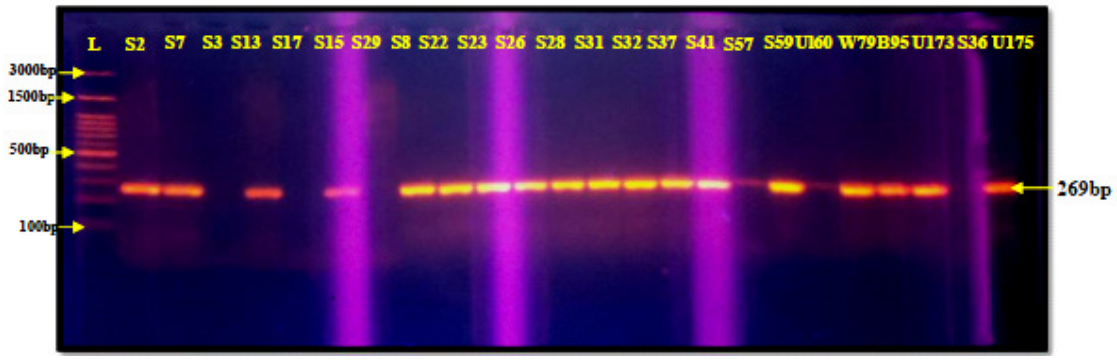

Fig. 4. (A) Gel electrophoresis of PCR of lukED amplicon product
Lane (S7, 8, 13, 15, 2, 22, 23, 26, 28, 31, 32, 36, 37, 41, 57, 59, S3, 17, 29), Lane (B95), Lane (Ul60) isolate from ulcer, Lane (W79) isolate from wound

Table 3. Numbers and percentages of genes among HA-MRSA and CA-MRSA isolates

Genes	HA no.(%)	CA no.(%)	Total positive	Negative	P value
*mec*A	9 (37.5%)	15 (62.5%)	24	0	p>0.05
Scc*mec* IV	9 (39.1%)	14 (60.8%)	23	1	
lukS/F	8 (42.1%)	11 (57.8%)	19	5	
*luk*ED	9 (45%)	11 (55%)	20	4	

Baba-Moussa et al. [40] found that 40% of MRSA isolates produced simultaneously the PVL and *Luk*ED from isolates with antibiotic associated diarrhea (AAD). In Iran, Khosravi et al. [41] found that prevalence was 33.3% for both genes (PVL, *luk*ED). It seems that MRSA isolates comprising *Luk*ED and PVL genes are more important in the disease process and associated with severe skin diseases with high morbidity and mortality.

However, the vast majority of cases of *S. aureus* disease cannot be explained by the action of a single virulence determinant and it is likely that a number of factors act in combination during the infective process. In Iraq, so far no studies are available relating to the presence of *luk*ED gene in MRSA isolates. The prevalence rate of *luk*ED gene in MRSA isolates is varied dramatically worldwide. Gravet et al. [42] have reported that *Luk*ED gene is present in 78% cases of skin infections which is consistent with the present study result. Similarly Havaei et al. [38] found that *luk*ED gene was detected from different MRSA infections with a prevalence rate of 73.8%. Khosravi et al. [41] found that the prevalence of *Luk*ED gene in MRSA strains isolated from burn patients was 66.26%.

4. CONCLUSION

The majority of MRSA isolates belonged to SCC*mec* IV suggesting that new strains were likely imported from the expanding community. High prevalence of MRSA isolates carrying a community acquired genotype enabled to test the adequacy of current hospital hygiene measures.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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