Occurrence of *mec*A, SCC*mec* IV, *pvl*, *luk*ED Genes in Community Acquired Methicillin Resistance *Staphylococcus aureus* (CA-MRSA) from Hilla/Iraq

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Abstract

Twenty four (24) MRSA isolates were obtained from clinical specimens and subjected to genetic study in Babylon Province in a period from July to January2011. Polymerase Chain Reaction was used for detection the genes that responsible for methicillin resistance (*mec*A, SCC*mec* type IV) and genes that responsible for toxin production (*pvl*, *luk*ED). It was found that 24 (100%) isolates have positive result for *mec*A gene. The MRSA isolates were also SCC*mec* typed to differentiate between HA-MRSA and CA-MRSA isolates by using a uniplex PCR assay, 23 (95%) were found to be carrying *SCCmec* type IV, 19 (79%) had positive result for *pvl* toxin gene, 20 (83%) had *luk*ED toxin gene.

الخلاصة

تم اخذ ٢٤ عزلة من المكورات العنقودية الذهبية المقاومة للمثيسلسن واجريت عليها دراسة جينية في محافظة بابل للفترة من تموز الى كانون الثاني. تم استخدام تقنية تفاعل انزيم البلمرة للكشف عن الجينات المقاومة للمثيسيلين وكاسيت المكورات الكروموسومي النوع الرابع والجينات المسؤولة عن انتاج السموم. وقد وجد ان ٢٤ (١٠٠%) عزلة اعطت نتيجة موجبة للجين المسؤول عن المقاومة للمثيسيلين. ان العزلات المقاومة للمثيسيلين صنفت اعتمادا على كاسيت المكورات العنقودية الكروموسومي للتمييز بين عزلات المجتمع والمستشفى با ستخدام تقنية تفاعل انزيم البلمرة الاحادي. وجد ان٢٣ (٩٥%) عزلة اعطت نتيجة موجبة لجين كاسيت المكورات العنقودية الكروموسومي باستثناء عزلة واحدة. وجد ان ١٩ (٧٩%) عزلة اعطت نتيجة موجبة لجين *pvl* و ٢٠ (٨٣%) عزلة اعطت نتيجة موجبة لجين *luk*ED.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections have been recognized for decades as hospital acquired or healthcare associated MRSA [1]. The possibility of transmission of healthcare associated MRSA (HA-MRSA) to the community was unavoidable [2]. Nowadays, MRSA is also recognized as a worldwide emerging community-acquired pathogen (CA-MRSA) [3]. It was shown, that 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. 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 [5].This is due to the production of the Panton-Valentine leukocidin toxin (PVL). Panton-Valentine leukocidin is a toxin associated with deep skin and soft tissue infections, such as furunculosis and abscesses, but necrotizing tissue infections and lethal hemorrhagic

pneumonia, even in previously healthy individuals, have recently been described as well. Most CA-MRSA strains have a common virulence factor: Panton-Valentine leucocidin (PVL). However, the prevalence rate of the PVL toxin in CA-MRSA strains varies with different studies and countries [6]. Some studies reported a prevalence of between 77% to 100% for the PVL toxin in Minnesota, USA in 2000 whilst a prevalence of less than 5% were reported in Western Europe.
The presence of PVL toxin in CA-MRSA strains can be confirmed by co-amplification of the *lukS/F-PV* genes [7].In comparison to previous detection methods such as Southern blotting and pulsed-field gel electrophoresis (PFGE), PCR assays such as multiplex-PCR (M-PCR), uniplex PCR techniques can provide a rapid amplification, detection and typing tool for MRSA strains. CA-MRSA infections spread easily by direct skin-to-skin contact. Outbreaks in closed living communities, as with jail inmates, military recruits, and gay men, have been reported in the United States [8]. The spread of PVL-positive *S. aureus* has also been described within families and among healthcare staff [9].

The aim of this study was to determine the molecular characterization of HA-MRSA and CA-MRSA isolates obtained from clinical specimens in Hilla/Iraq. The MRSA strains were also SCC*mec* typed to differentiate between HA-MRSA and CA-MRSA strains using a using a uniplex PCR assay. Furthermore, uniplex PCR method was evaluated to detect the PVL genes in MRSA isolates.

Materials and Methods

Bacterial Isolates

Twenty four (24) MRSA isolates were obtained from clinical specimens. These bacterial isolates were identified as *S. aureus* based on their morphology, Gram-staining and catalase properties. Coagulase test was performed to identify *S. aureus* isolates. Twenty four isolates were selected for genetic study, In the light of antibiotics results that mentioned in previous study [10], Out of 46 *S. aureus* isolates, 13 CA-MRSA isolates were obtained and identified as CA-MRSA. They were oxacillin and cefoxitin resistant which occupied the primacy in the resistance of antibiotics, phenotypic detection of virulence factors also detected previously [12]. other isolates (No.11) were selected from specific skin infections (abscesses, boils)for genetic study to detect other genes that considered as a marker for CA-MRSA isolates. All these 24 isolates were divided in to two groups: 9 isolates from hospital (inpatient) and 15 isolates from community (outpatient).

DNA extraction and PCR assay

Chromosomal DNA was extracted from twenty four clinical strains. One colony of each strain cultured on solid medium was inoculated into 5 mL of BHI (Broth Heart Infusion) and grown overnight at 37ºC. From these strain cultures, DNA was purified from bacterial cells using Genomic DNA Mini kit supplemented by the manufacturing company (Geneaid, UK). Chromosomal DNAs obtained were used as templates for all PCR experiments. The PCR reactions were carried out in a Thermal Cycler. Before PCR assay, DNA profiles were performed by using bacterial DNA and loading buffer without thermal cycling conditions, and according to the manufacturer.

Table 1 PCR thermocycling conditions

Monoplex Gene	Temperature (^{o}C)/ Time					Cycle No.
	Initial denaturation	Cycling condition			Final extension	
		denaturation	annealing	Extension		
Pvl	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	94/5 min	94/1 min	55/1 min	72/1.5min	72/1.5 min	35

Table 2 Primers of antibiotic resistance genes used in present study/Bioneer

Primer	Primer Sequence (5'-3')	Product size (bp)	Reference
*mec*A-F	GTG GAA TTG GCC AATACA GG	1339	18
*mec*A-R	TGA GTT CTG CAG TAC CGG AT		
SCCmec typeIV-F	TTTGAATGCCCTCCATGAATAAAAT	450	26
SCCmec typeIV-R	AGAAAAGATAGAAGTTCGAAAGA		

Table 3 Primers of Toxin genes used in present study/Bioneer.

Primer	Primer Sequence (5'-3')	Product size (bp)	Reference
*Luk-pv*l- F	ATCATTAGGTAAAATGTCTGGACATGATCCA	433	29
*Luk-pv*l- R	GCATCAASTGTATTGGATAGCAAAAGC		
*LUK*DE-F	TGAAAAAGGTTCAAAGTTGATACGAG	269	26
*LUK*DE-R	TGTATTCGATAGCAAAAGCAGTGCA		

Results and Discussion

Detection of *mec*A and SCC*mec* IV genes by PCR:

One of the most important questions in the molecular evolution of *S. aureus* and MRSA development is the possible role of horizontal gene transfer. Monoplex polymerase Chain Reaction (PCR) was used in the present study for detection of *mec*A gene. A major finding in this study was the high frequency of *mec*A gene (100%) among all *S. aureus* isolates (Figure 4). All hospital 9:9 (100%) and all community isolates 15:15 (100%) were carrying *mec*A gene (Table 4).

In the present study a total 13 out of 24 isolates were determined previously as MRSA by [10], so these isolates confirmed as MRSA by genotype. Identification of MRSA is based on phenotypic and genotypic investigations. the *mec*A gene responsible for mediating methicillin

resistance in staphylococci [11]. These results indicated that there is a large dissemination of MRSA in the community (62%) and not restricted to the hospitals as expected [12] and as reported by several authors who found that most MRSA isolates were associated with nosocomial infections such as [18] who detected *mec*A gene in MRSA isolated recovered from hospitals in Najaf and Al-Diwaniya respectively. 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 [11]. *mec*A gene carried on the Staphylococcal Cassette Chromosome mec (SCC*mec*). SCC*mec* is inserted into the *S. aureus* chromosome near the origin of replication [14]. Most of antibiotic resistance are transferred by plasmids, while methicillin resistance is chromosomal transferred by transduction.

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 [15], In Europe, MRSA strains account for less than 5% of *S. aureus* isolates in the community setting [16] and for less than 11% of isolates from SSTI patients in France. Resistance to methicillin, is independent of β-lactamase production. 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), i.e., oxacillin, nafcillin, cloxacillin, dicloxacillin, flucloxacillin, and mecillinam is necessary to ensure appropriate antimicrobial chemotherapy of staphylococcal infections, particularly those from community associated infections. Oxacillin has been the agent recommended by [17] for phenotypic tests to predict resistance to PBPs because of its stability and superior sensitivity over other PBPs for susceptibility tests. 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 [18].

Table 4 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	
Pvl	8 (42.1%)	11 (57.8%)	19	5	
*luk*ED	9 (45%)	11 (55%)	20	4	

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 [13]. Molecular techniques are often applied for the routine diagnostic MRSA detection

along with antimicrobial susceptibility testing methods [19].

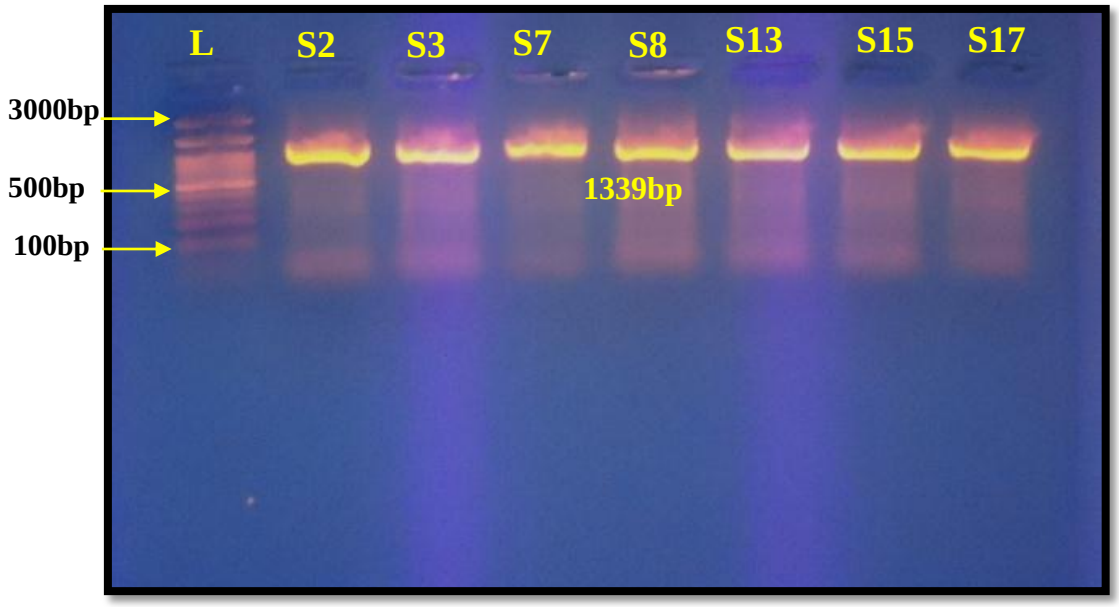

Figure 1 Gel electrophoresis of PCR of *mec*A amplicon product: Lane L: Ladder (3000-bp ladder), Lane (S2, 3, 7, 8, 13, 15, 17, 22, 23, 26, 28, 29, 31, 32) No. of isolates from skin.

SCC*mec* typing is one of the most important molecular tools available for understanding the epidemiology and strain relatedness of MRSA [20]. In the present study, One set of primers were used in an attempt to differentiate between MRSA isolates.

In the present study, Staphylococcus Cassette Chromosome type IV (SCC*mec* IV) was also detected which is a stable marker of Community Aquired MRSA (CA-MRSA) [3]. All MRSA isolates (no.24) were carrying SCC*mec* IV (95%) except one isolate, 1 (4.1%) (Figure 2). Although this SCC*mec* IV negative isolate was recovered from the community, the lack of this gene from this isolate may be due to that patient from which this isolate was recovered, was newly discharged from hospital and this isolate may be HA-MRSA, but marker genes SCC*mec* type I, II and III of HA-MRSA were not determined in the present study. 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 4). It is clear that all HA-MRSA (100%) were carrying SCC*mec* IV gene.

The high prevalence of SCC*mec* IV type in our hospitals 9:9 (100%) in the present study (which are traditionally attributed to CA-MRSA isolates and considered as a marker for CA-MRSA), this is may be due to dissemination of CA-MRSA in the hospitals of the area of the study as the SCC*mec* IV had smaller size, compared with type II and III elements, may serve as an evolutionary advantage by making them more amenable to horizontal spread among a bacterial population [21].This also interpreted as replacement of HA-MRSA with CA-MRSA. However, community acquired MRSA strains

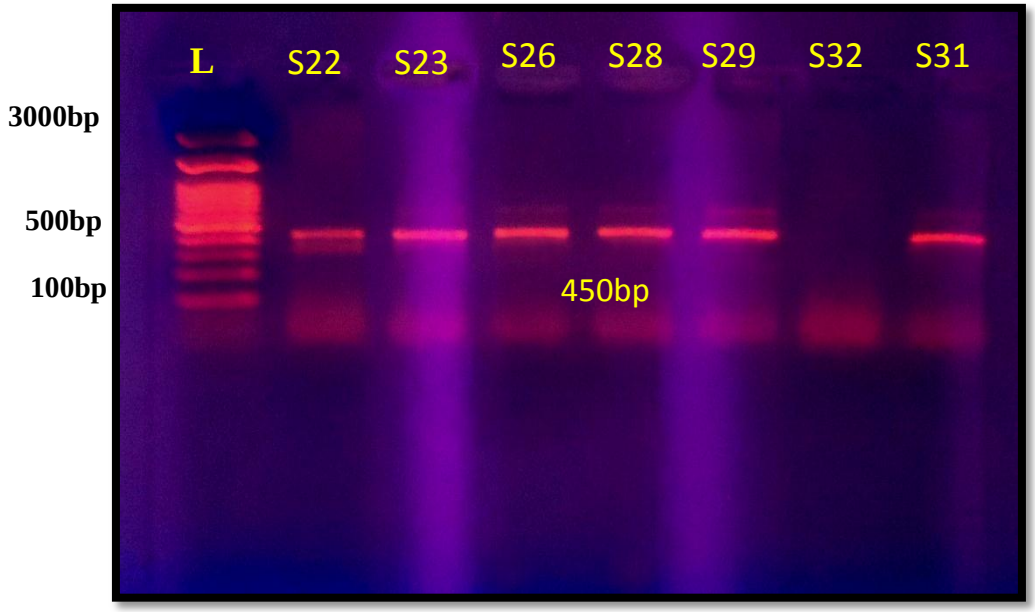

Figure 2 Gel electrophoresis of PCR of SCC*mec* IV amplicon product: Lane L: Ladder (3000-bp ladder), Lane (S2, 3, 7, 8, 13, 15, 17, 22, 23, 26, 28, 29, 31) no. of isolates from skin, Lane (S32) show negative result.

have now been found in association with nosocomial infections [22]. 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. Several studies suggest that community-acquired MRSA strains harboring the smaller SCC*mec* type IV element grow faster and achieve higher infection burdens than nosocomial MRSA strains [23] This property has been attributed to the extra metabolic burden that multi-resistant bacteria have secondary to the synthesis of extra proteins during replication, and may provide a selective advantage to community-acquired MRSA. Despite these studies it should be noted that community-acquired MRSA would likely compete mostly with MSSA strains in the outpatient setting. Such high proportions of SCC*mec* IV were found in present study have not been reported before.

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 that reported the spread of CA-MRSA SCC*mec* type IV strains in hospital settings in both Europe and the United States [24]. The carriage of *SCCmec* IV in community acquired infections is reportedly 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 . It is believed that the community strains acquired the SCC*mec* IV from a Methicillin-susceptible *S. aureus* rather than an evolution of a healthcare associated isolate that had been carried into the community [20]. Staphylococcal cassette chromosome *mec* typing is essential because it can distinguish between HA-MRSA and CA-MRSA [25]. As a result of the different antibiotic susceptibility profiles between HA-MRSA and CA-MRSA, discrimination of these two MRSA strains is important in patient

and antibiotic management [25]. 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. Molecular methods evaluated in this study proved to be useful rapid tools that can be implemented for screening and monitoring these strains to ensure outbreaks are prevented by the correct infection control principles such as isolation of infected patients.

Molecular Detection of Virulence Genes by PCR:

Polymerase chain reaction 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 (Figure 3-3). All these isolates were *mec* A gene positive and identified as MRSA by (12) and genotypic test (Figure3-1). The results also showed that *mec*A: *pvl* ratio was 24:19 *mec*A is a peptidoglycan transpeptidase, this protein is expressed at the external surface of cytoplasmic membrane, where it could interact with the extracellular protein pvl. In this case we can give the name community acquired MRSA (CA-MRSA) to our MRSA isolates, as these isolate harbored *pvl* and SCC*mec* IV genes because the two genes above considered a stable markers for CA-MRSA isolates [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 [26]. Out of these 19 positive isolates 8 (42.1%) were found in HA-MRSA while 11 (57.8%) were found in CA-MRSA isolates. Not surprisingly, horizontal transfer of virulence genes involved toxin genes, including the *pvl* genes [27]. For comparable, in Iraq, there is a little attention has been paid to the prevalence of

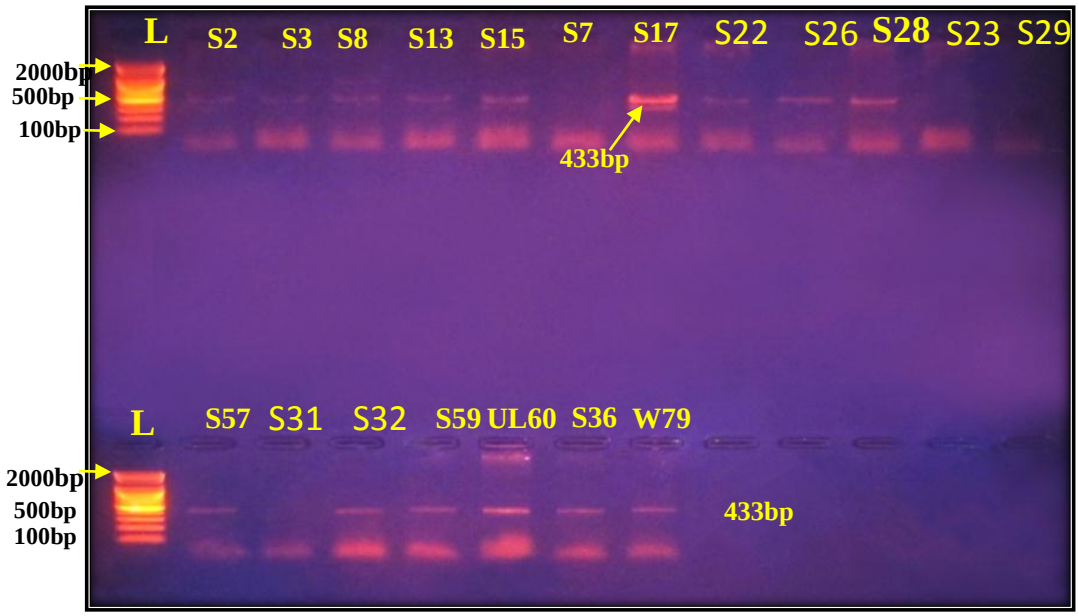

Figure 3 Gel electrophoresis of PCR of pvl amplicon product: Lane L: Ladder (3000-bp ladder), Lanes (S2, 3, 8, 13, 15, 17, 26, 28, 32, 22, 37, 41, 57, 59) no. of isolates from skin, Lane (Ul60) no. of isolate from ulcer, Lane (W79) no. of isolate from wound.

pvl gene in MRSA isolates, except some reports from Najaf city [15]. and there is also no professional center for studying or research about prevalence, distribution and purification of this toxin. The results of present study (79%) were more than that obtained by, who found that *pvl* gene were detected in 27.2% of CA-MRSA isolates. However, the high prevalence of *pvl* gene in our study was due to high numbers of selected skin samples in present study.

[26] showed an association between *pvl* genes and cutaneous infections (85%), confirming earlier findings by other workers [28]. In other studies [29], found that MRSA isolates harbored *pvl* gene were more prevalent in cutaneous MRSA isolates (83%), and he also found that 70% of *mec*A positive isolates, harbored *pvl* gene, that closely correlated with our results.

It has been suggested that the reason for this is that only a few *S. aureus* strains are susceptible to infection with PVL-converting phages. [30] showed that the temperate phage -SLT infected only 5% of clinical PVL-negative *S. aureus* strains, leading to PVL production. Different strains of *S. aureus* have been shown to harbor different PVL-carrying phages [30]. While PVL is rarely produced by *S. aureus* (less than 5% of strains), the PVL gene is detected at high rates (more than 50%) in isolates from community-acquired skin infections [26], which was in accordance with our results (57.8%). An interesting finding of our study was the presence of PVL genes in MRSA isolates separated from urine isolates, because in the previous studies, such isolates were not found [31] but in another study [32] found that MRSA isolates recovered from urine samples harbored the *pvl* gene. In recent years, the increased prevalence of CA-MRSA has become a major public health concern. In contrast to HA-MRSA, CA-MRSA strains are commonly susceptible to many non β-Lactam antibiotics. Also CA-MRSA appears to have a distinct exotoxin PVL, which has been associated with severe infections [23]. However, the result of present study showed that PVL-containing MRSA did not only exist in the community 11 (57.8%) but they were also found in hospitals 8 (42.1%) (Table 3-9). The differences were statistically not significant.

Recently it has been shown that *pvl* is not universally present in CA-MRSA isolates [33]. However, [32] found that 61.8% of HA-MRSA isolates had *pvl* genes. This is may be due to dissemination of CA-MRSA isolates in the hospitals that more virulent than hospital type and due to 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 bacteriophage, these toxin genes may be transmitted easily to other HA-MRSA strains. It is possible that the PVL- strain originated from the PVL+ strain via loss of prophage from the genome of the latter. Alternatively, the PVL+ strain may have descended from the PVL- strain by acquiring prophage [30]. Further studies are necessary to reveal the biological and evolutionary relationships between and the significance of these 2 strains Studies. MRSA isolates carrying PVL genes have also been carried out in hospitals of Florida, Germany, France, Minnesota, Latvia, Austria, Belgium, the Netherlands and Middle Tennessee [34].

Figure (3-4) showed determination of the bi-component leukotoxin gene (*luk*ED) in all 24 MRSA isolates by PCR. However, *luk*ED gene was detected in 20 (83%) of MRSA isolates recovered from all *S. aureus*

infection types, but it was not detected in 4 (16.6%) MRSA isolates. Out of 20 positive MRSA isolates, 9 (45%) and 11 (55%) isolates were detected from hospital and community respectively (Table 3-1), the differences between hospital and community were statistically not significant ($p > 0.05$).

The results also revealed that all HA-MRSA isolates (9:9,100%) were harboring *luk*ED gene. This result was in agreement with results obtained by [35], who found that *luk*ED gene was found in most HA-MRSA isolates (95%). However, *PVL* and *Luk*ED positive MRSA isolates in hospital were (42.1%, 45%) respectively (Table 3-1). From these 20 positive isolates for *luk*ED, 17 (85%) MRSA isolates shared both genes (*pvl* and *luk*ED) [35], found that 40% of MRSA isolates produced simultaneously the *PVL* and *Luk*ED from isolates with antibiotic associated diarrhea. [36] 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, also these MRSA isolates seem to be more virulent than other MRSA isolates.

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. However, in Iraq, little or 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. In retrospective study by [37] found that 78% of 131 retrospective cases with skin infections, harbored *Luk*ED gene, [50] found that *luk*ED gene was detected from different MRSA infections with a prevalence rate 73.8%, [36] found that the prevalence of *Luk*ED gene in MRSA strains isolated from burn patients in Taleghani hospital, was (66.26%). Also *Luk*ED gene was detected in 56% of *S. aureus* isolates harboring *mec*A gene [35], these isolates recovered from antibiotic associated diarrhea (AAD). In other study found that 48.9% of *Luk*ED gene harboring isolates were *mec*A positive, these isolates recovered from AAD.

Conclusions

Molecular characterization of the MRSA isolates revealed that the majority of isolates (95%) belonged to SCC*mec* IV, suggesting that new isolates, which are likely to be imported from the expanding community. High prevalence of MRSA isolates carrying a community acquired genotype enabled us to test the adequacy of current hospital hygiene measures to control outbreaks of in-hospital CA-MRSA isolates**.** Given the ability of PVL-producing MRSA isolates to cause life-threatening disease, and more prevalent in cutaneous MRSA isolates, the absence of any rapid non-molecular tests for PVL, the crucial role of awareness cannot be overemphasized and recommended that For tracking CA-MRSA rates in a systematic manner, continuing research, to detect the novel molecular markers and genotype of CA-MRSA may be beneficial. A rapid detection systems, direct tests, simple, cost-effective will contribute to reducing MRSA infections, is needed to make this a useful measurement.

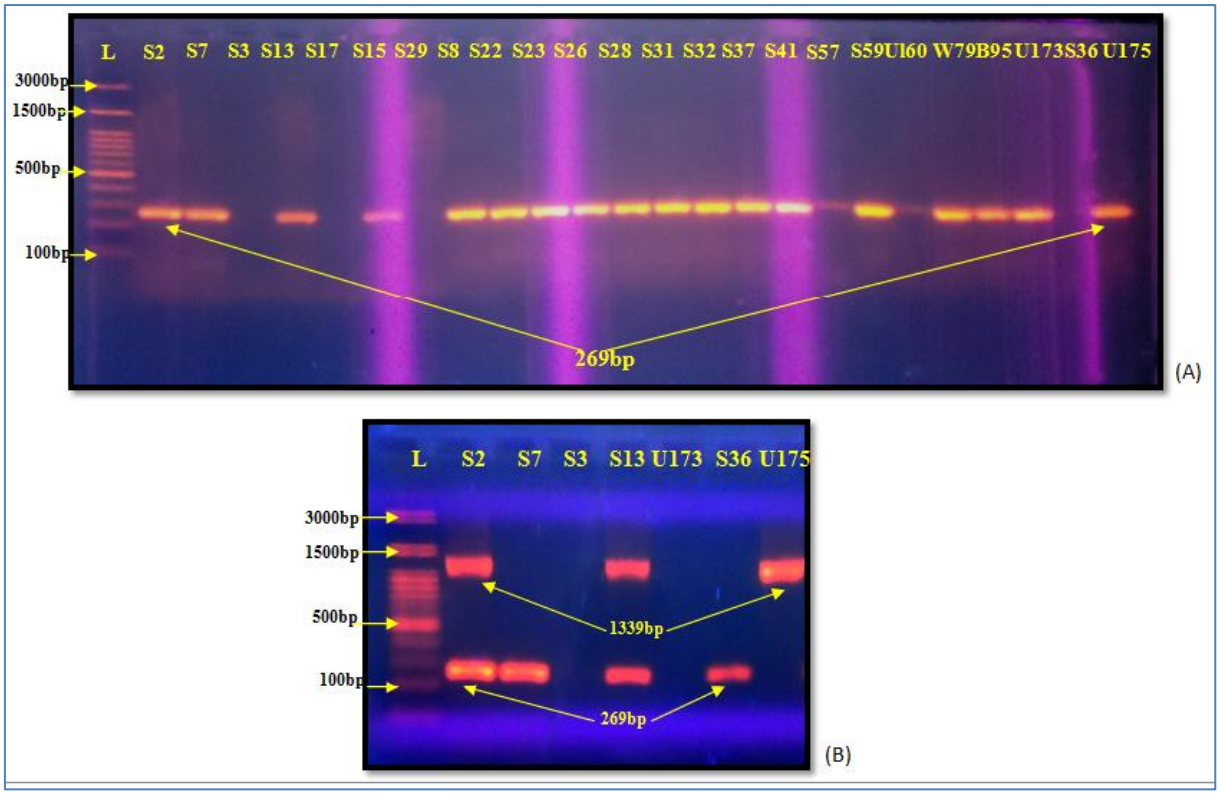

Figure 4 (A) Gel electrophoresis of PCR of lukED amplicon product. Lane (S7, 8, 13, 15, 2, 22, 23, 26, 28, 31, 32, 36, 37, 41, 57, 59, S3, 17, 29, 36), Lane (B95), Lane (Ul60) isolate from ulcer, Lane (W79) isolate from wound. (B) Gel electrophoresis of Multiplex PCR of *mec*A and lukED amplicon product. Lane (S2, 7, 3, 13, 36) no. of isolates from skin, Lane (U173, U175) no. of isolates from urine, isolate from burn,

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