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RESEARCH ARTICLE

A GENETIC STUDY TO DIFFERENTIAL HA/CA MRSA ISOLATED FROM CLINICAL CASES IN IRAQ HOSPITALS.

ISRAA MOHAMED SAFI AL- KADMY

Department of Biology College of science /Al –Mustansiriyah university .Email: esraa_al_kadmy@yahoo.com

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ABSTRACT

Methicillin-resistant Staphylococcus aureus (MRSA) has been a major cause of nosocomial infections since the 1960s . Currently, MRSA is divided into two subgroups: the healthcare associated MRSA (HA-MRSA) and community associated MRSA(CA-MRSA), CA-MRSA infections have been increasing. The most common of these infections present in soft skin. The aim of this study to different between CA and HA MRSA in clinical isolates of Baghdad hospitals.

Methods: clinical isolates were collected from patients with different infections, Simple laboratory testing followed by the complementary API Staph, followed by antibiotic sensitivity and D-test, and finally by using PCR technique, detection of this genes : *mecA*, *PVL*, *SCCmec IV* and *V* .

Results: A total of 105 *S.aureus* found 104 methicillin-resistant Staphylococcus aureus (MRSA) strains, after a D-test , *S.aureus* divided to two group: CA and HA –MRSA, where the ratio of CA 18(17.1%) out of 105 isolates, while HA reached 87(82.8%). MRSA was characterized by PCR amplification mecA gene, 104(99.04%) isolates out of 105 gave positive result, all isolates of HA carry mecA gene, while 17 out of 18 isolates of CA carry mecA gene which was CA-MRSA and one isolates was CA-MSSA . All isolates 18(100%) of CA gave positive result in risk factors PVL gene, while for detection of *SCCmec IV* 17 (94.4%) out of 18 isolates of CA gave positive result, and finally two isolates of CA-MRSA gave positive result in *SCCmec V* gene .

Conclusions: This is the first report in Iraq for the emergence of CA isolates especially CA-MRSA which is responsible for the majority of infection in soft tissue and skin abscesses , are likely to be sensitive to clindamycin..

Key words: MRSA, MSSA, D- test, CA-MRSA, HA-MRSA, PVL, *mecA*, *SCCmec IV*.

INTRODUCTION

Staphylococcus aureus is a facultatively anaerobic, Gram (+) bacterium that causes diseases ranging from common skin infections to life threatening septicemia and is one of the most virulent microbial pathogens to cause nosocomial and community acquired infections. It is the most prevalent pathogen causing hospital infection throughout the world, and the incidence is still increasing [1]. The drugs of choice for treatment of staphylococcal infections are the b-lactam antibiotics, such as penicillins, cephalosporins or cephems, monobactam and carbapenems. However, through the years, the bacterium has evolved several mechanisms that render it to be resistant to the antimicrobials. The most common mechanism is the production of β-lactamase that inactivates many of the β-lactam antibiotics [2] .Methicillin-resistant *Staphylococcus aureus* (MRSA) has been a major cause of nosocomial infections since the 1960s [3]. Currently, MRSA is divided into two subgroups: the healthcare associated MRSA (HA-MRSA) and community associated MRSA(CA-MRSA), CA-MRSA strains are genetically different from HA-MRSA strains [4]. These divisions were originally based on epidemiological features and microbiological characteristics .Later it become an important character for molecular typing, antimicrobial susceptibility testing, and identification of methicillin resistance beside presence of special toxin genes [5] . HA-MRSA is the major problem in nosocomial infections for instance, patients in hospital with open wounds, invasive devices or under immune compromise conditions are at much higher risk of getting HA-MRSA infection [6]. Community-acquired methicillin-resistant *S. aureus* (CA-MRSA) was initially defined as an infection with Methicillin-resistant *S. aureus* (MRSA) in an outpatient or inpatient that manifested infection within 48 hours of hospital admission [7] . From the other hand, CA-MRSA has recently risen as a major public health concern. Although the border between HA-MRSA and CA-MRSA are not clearly distinguishable, CA-MRSA infections are generally differ from the HA-MRSA and both of them differ phenotypically and genotypically [8] . CA-MRSA is usually resistant to the β-lactam antibiotics and usually susceptible in vitro to Fluoroquinolones, Trimethoprim/ sulfamethoxazole , Clindamycin, and Chloramphenicol. This is in contradistinction to HA-MRSA, which is usually resistant to Fluoroquinolones, Clindamycin, and Chloremphenicol, and is less sensitive to Trimethoprim/sulfamethoxazole. While HA-MRSA isolates are typically multi-drug-resistant, CA-MRSA isolates are susceptible to more classes of antibiotics [7,9] . In addition, the Panton-Valentine leukocidin (PVL) gene encodes a pore-forming cytotoxin that acts preferentially against leukocytes and erythrocytes is commonly found in CA-MRSA infections and only rarely in HA-MRSA [10]. CA-MRSA differs in several ways from HA-MRSA and these differences are summarized in table (1).

Table 1: Characters that are used to distinguish between HA-MRSA and CA-MRSA [11].

Factor	HA-MRSA	CA-MRSA
Risk factors and at-risk populations	Previous contact with healthcare settings	Team-sport participants, incarcerated persons, military, and children
SCC type	Type I, II, III	Type IV, V
Toxins	Fewer	More
PVL	Rare	Common (almost 100%)
Antibiotic resistance pattern	Multiply resistant	Sensitive to many except β-lactams
Associated clinical syndromes	Bacteremia, pneumonia	Skin and soft tissue infections
Mean age at infection	Older	Younger

***Abbreviations*: CA-MRSA, community-acquired methicillin-resistant *Staphylococcus aureus;* HA-MRSA, healthcare-associated methicillin-resistant *Staphylococcus aureus*; PVL, Panton-Valentine leukocidin; SCC, staphylococcal cassette chromosome**.

Risk factors associated with CA-MRSA infections

The following risk factors should increase suspicion for CA-MRSA in patients presenting with compatible signs and symptoms: History of MRSA infection or colonization in patient or close contact, high prevalence of CA-MRSA in local community or patient population, recurrent skin disease, crowded living

conditions (e.g. homeless shelters, military barracks), participation in contact sports, skin or soft tissue infection with poor response to B-lactam antibiotics, child under 2 years of age, male with history of having sex with men, shaving of body hair [12] .

Aim of this study was to differentiate between HA-MRSA and CA-MRSA phenotypic by D-test and genotypically by detection of SCC*mec* and determination the prevalence of PVL gene among Iraqi MRSA isolates .

MATERIAL & METHODS

Collection and diagnosis of Bacterial isolates

One - Hundred and five isolate of *S. aureus* were isolated from patients with different infections at Al-Kindey teaching hospital, Al-Kadhymia teaching hospital, Ibn-Albalady hospital , Medical City Hospital, Al-habybya Hospital and Alemam-Ali hospital in Baghdad . They were obtained from midstream urine from patients suffering from urinary tract infections (20 isolates), ear from patients suffering from middle ear infection (11 isolates), wounds infections (9 isolates), from bacteremia (14 isolates), from nasal (22 isolates), from boils (21 isolates) and from tonsillitis (8) isolates. Bacterial diagnosis including morphological and biochemical tests were done according to Atlas *et al.* (13) followed by the complementary API *Staph* test. While Coagulase test It was done according to Atlas *et al.* (13) by Slide method .

Antibiotic susceptibility tests:

All isolates were tested for antimicrobial susceptibility by disk diffusion method according to the CLSI (14) using Methicillin, Oxacillin, chloramphenicol and cefotaxime. The diameter of inhibition zone were measured after 18 hrs and were compared with the control strains; *S.aureus* ATCC 25923 .

Differential between HA-MRSA and CA-MRSA phenotypically

To differential between them use simple test called D- test , this test is performed for the detection of inducible clindamycin resistance that means that some isolates of MRSA are in vitro resistance to erythromycin and susceptible to clindamycin. Erythromycin disk is placed in close proximity (20 mm) to a clindamycin disk. These isolates should be reported as resistant to clindamycin. The diameter of inhibition zone for clindamycin appear like D- shape letter from the side of erythromycin disk in blunt site [15], this is clindamycin susceptible and it is CA-MRSA and resist to erythromycin, while HA-MRSA no appear inhibition zone in clindamycin [15] .

Differential between HA-MRSA and CA-MRSA genetically by using PCR

The primers sequences used in the determination of the CA-MRSA and detection of the different target genes and the expected sizes of the PCR products are found in Table (2). The reaction mixture was prepared according to the procedure that suggested by the manufacture company (KAPA, south Afriqa) PCR products were electrophoreses in 1.5% agarose gels and visualized under UV light according to Sambrook and Russell (18).

Table 2: Primers used for detection specific genes.

Primer	primers sequence 5'--------→3'	Product size(bp)	Tm	Reference
mecA	F- GTAGAAATGACTGAACGTCCGATAA R- CCAATTCCACATTGTT TCGGTCTAA	310	54	Cabrera *et al.*, 16
PVL	F-ATCATTAGGTAAAATGTCTGGACATGATCCA R- GCATCAASTGTATTGGATAGCAAAAGC	433	54	Cabrera *et al*., 16
SCC*mec IV*	F- GCCTTATTCGAAGAAACCG R- CTACTCTTCTGAAAAGCGTCG	776	57	Japoni *et al*., 17
SCC*mec V*	F- GAACATTGTTACTTAAATGAGCG R- TGAAAGTTGTACCCTTGACACC	325	64	Japoni *et al*., 17

RESULTS AND DISCUSSION

A total of 105 isolate were recovered from patients with different infection. They were obtained from midstream urine from patients suffering from urinary tract infections 20(19.04%) isolates, ear from patients suffering from middle ear infection 11(10.4%) isolates, wounds infections 9 (8.5%) isolates, from bacteremia 14(13.3%) isolates, from nasal 22(20.9%) isolates, from boils 21(20%) isolates and from tonsillitis 8(7.6%) isolates Which show in figure (1). Coagulase test was performed to identify the ability to coagulate the plasma. From the total 105 ,only 100(95.2%) isolates showed Coagulase-positive result(COPS), while 5(4.7%)were Coagulase-negative (CONS).

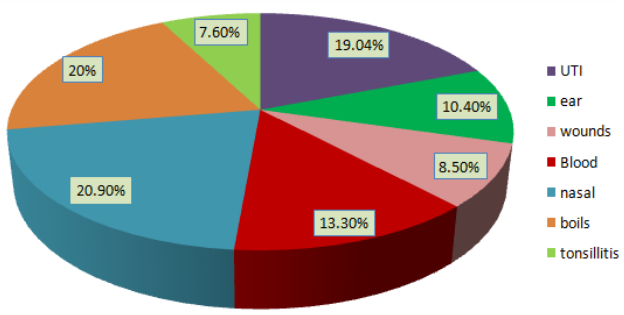

Figure 1: Distribution of *S.aureus* from different infection.

Detection of methicillin resistance by Disc diffusion method: out of 105 S. aureus strains; 100 (95. 2%) were resistant to methicillin, 64(60. 9%) isolates to cefotaxime disc, 79(75.2%) to chloramphenicol , while for Oxacillin the percentage of resistant reached (97.1%)102 out 105, and finally this percentage reached 75(71.4%) to erythromycin and 80(76.1%) to Clindamycin.

D- test : Inducible resistance to Clindamycin was defined as blunting of the clear circular area of no growth around the Clindamycin disk on the side adjacent to the Erythromycin disk and was designated D - test positive and colonized with community acquired MRSA (CA-MRSA). Absence of a blunted zone of inhibition was designated D - test negative, which colonized HA-MRSA[19] . Out of 105 MRSA 18(17.1%) appear D-shape in this test and colonized with (CA-S.aureus) while 81(77.1%) were HA- *S.aureus*.

As erythromycin would act as an inducing agent isolates expressing *erm* gene will grow in resulting in a D-shaped zone around the clindamycin disk, figure (2) appear D- shape inhibition zone in community acquired *S.aureus* . Clindamycin cannot be an effective inducer. Clindamycin is a unique antibiotic because isolates can be sensitive when tested in vitro, but some strains will become resistant when clindamycin is used in treating the infected patient. Every MRSA strain that is erythromycin resistant and clindamycin sensitive should be followed with a D test. A positive D-test indicates the ability of MRSA strains to become resistant to clindamycin during antibiotic therapy. A negative D-test indicates the effectiveness of clindamycin in treating patients with MRSA.

The mechanism of clindamycin resistance is considered to be that in the absence of an erythromycin inducer, a ribosome-binding (SD) sequence of clindamycin resistance mRNA is hidden due to the mRNA secondary structure, and thus clindamycin resistance mRNA cannot be translated, while with the presence of the

inducer, this secondary structure of mRNA is disrupted resulting in translation of clindamycin resistance mRNA [20].

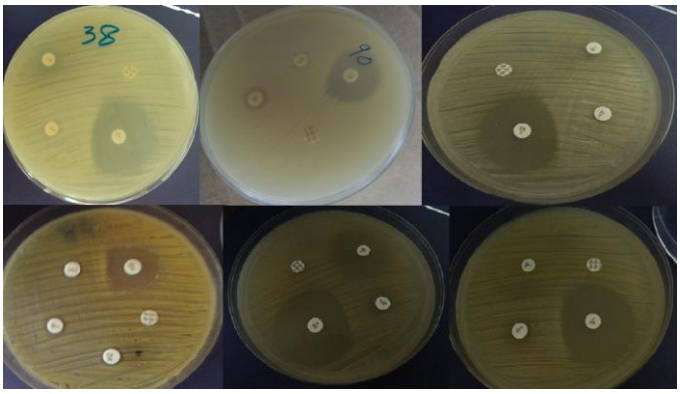

Figure 2: Disc diffusion test Erythromycin resistant and clindamycin sensitive Staphylococcal isolate giving D shaped zone of inhibition around clindamycin with flattening towards erythromycin disc suggestive of inducible CA-*S. aureus* phenotype.

All isolates of HA-MRSA 87 out 87(100%) were resist to methicillin while 17 out 18(94.4%) of CA-MRSA were resist to methicillin. For Oxacillin, 15 of 18(83.3%) of CA-MRSA and 78 out 87 (100%) of HA-MRSA, while for cefotaxime, rate of resistance for CA-MRSA were 13 out 18(72.2%) and for HA-MRSA were 51 out 87(58.6%). Of these, 79 out 87(90.8%) were resist to chloramphenicol, while all isolates of CA-*S.aureus* were sensitive to chloramphenicol and clindamycin, but 80 out 87(91.9%) were resist to clindamycin of HA-MRSA. Finally for erythromycin, all CA-S. aureus 100% were resist and 57 out 87 (65.5%) were resist of HA-MRSA. This result show in figure (3). While for Coagulase test, 2 isolate out of 17 (11.1) gave negative result of CA-S. aureus and 3 isolate out of 81(3.7%) gave negative result in HA-MRSA.

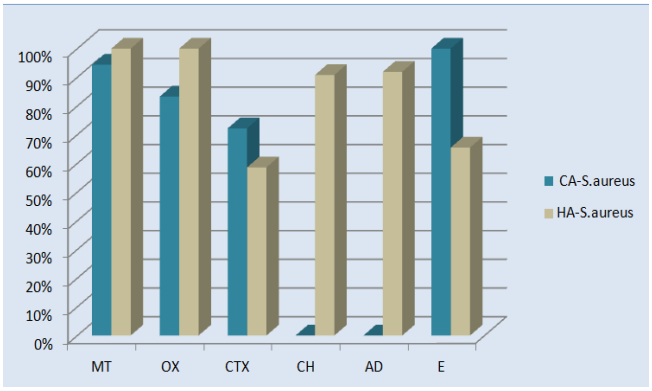

Figure 3: The percentage of resistance for CA-*S.aureus* and HA- *S.aureus* isolates against many antibiotics.

MT:Methicillin, OX: Oxacillin, CTX: cefotaxime, CH: chloramphenicol, AD: clindamycin,E: erythromycin.

Methicillin-resistant Staphylococcus aureus (MRSA) is a formidable bacterial pathogen responsible for a variety of infections commonly seen in patients of all ages[2] . Al-Oubaidy [21] illustrated that the rate of percentage for methicillin reached (%96.5), thus it agreed with this study which reached (99.04%) for . Kaddora [22] reported that Fifteen isolates out of 35(42.8%) were identified as HA-MRSA, and 20 out 35 (57.1%) isolates were determined to be CA-MRSA, these values are much lower than the result of this study , while in India in 2011 Vivek [23] reported higher values of 36 MRSA 9(25%) were CA-MRSA and 27 (75%) were HA-MRSA which is convenient with the current study. The patterns of resistance that MRSA isolates display can play a major role in differentiating between hospital-acquired and community-acquired *S.aureus* strains.

Community-acquired *S.aureus* infections are almost universally sensitive to a wider range of antibiotics than typical hospital-acquired MRSA infections. In general, hospital-acquired infections are sensitive only to vancomycin and resistant to most commonly used regimens [4,6] . While resistant to the β-lactams, CA-MRSA and CA-MSSA are often sensitive to most other commonly used antibiotics such as cefotaxime, doxycycline/minocycline, rifampin, and usually susceptible to clindamycin , chloramphenicol, Oxacillin, tetracyclines, and sometimes fluoroquinolones [7,10] .

From noticing the net result obtained for the locally isolates, shows a low prevalence of community-acquired in S. aureus isolates in Baghdad hospitals which reached 18 out 105 (17.1%), while in Egypt in 2011, Kader et al [24] recorded high rate of CA-MRSA which reach 18(52.9%) CA-MRSA out of 34 MRSA and 16(47%) HA-MRSA. Both CA-MRSA and HA-MRSA are resistant to traditional anti-staphylococcal β-lactam antibiotics. However, CA-MRSA isolates tend to be more susceptible to other antibiotics (including to clindamycin, chloramphenicol, tetracyclines) than are HA-MRSA[17], and their narrow spectrum of resistance is solely due to determinants harbored on genetic elements present on the *SCCmec* [25] . In this study, all of the 18 CA-S. aureus isolates were susceptible clindamycin, chloramphenicol antibiotics than HA-MRSA, and agree with Kaplan[26] which noted that most of the CA-S. aureus isolates are susceptible to most antibiotics. This results are illustrated in figure (3).

Detection of mec A gene : all isolates of *S.aureus* show *mecA* gene positive result in PCR except one isolate from the isolates which appear D-zone don't contain *mecA* gene it was CA-MSSA no.(39), so the percentage of CA-MRSA reached 94.4% while the ratio of CA-MSSA reached 5.5% and this result agree with Davis[27] which found the ratio of CA-MSSA reached 3%, CA-MRSA infections were associated with a more adverse impact on outcome than CA-MSSA infections. The presence of mecA gene detected by PCR was considered to be the gold standard in evaluating the disc diffusion methods to identify MRSA, figure (4) which show agarose gel electrophoresis of mecA gene and product size (310bp), and it was detected in 104 out of 105(99.04%) isolates of *S.aureus*. In conclusion, molecular techniques remain the most sensitive method in detecting S. aureus genus and species level and with 100% accuracy in detecting MRSA, as compared with the classical identification method, MRSA has emerged as a serious public health problem in Iraq and other regions of the world. Because of the ability of staphylococci to acquire antimicrobial resistance over time, MRSA will continue to be a problem in the future.

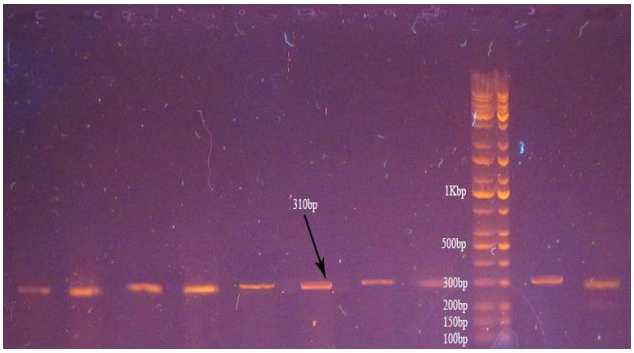

Figure 4: Agarose gel electrophoresis (1% agarose, 75 v/cm) for *mecA* gene (amplified size 310bp as compared with 100bp DNA ladder), all line represents positive results.

Differential between HA-MRSA and CA-MRSA genetically

Depend on Presence of *SCCmec IV* (rarely *SCCmec V*) gene, and the important Panton-Valentine leukocidin (*PVL*) toxin gene in the CA-MRSA and CA-MSSA isolates, all 18 community acquired gave positive result of PVL, while 17 out of 18(94.4%) carry *SCCmec IV* gene, and only 2(11.1%) isolates appear *SCCmec V* gene, all this (18) isolates resist to erythromycin , 16 isolates of community acquired S. aureus were Coagulase positive and only (2) isolates were Coagulase negative. The source of this (18) community acquired S. aureus distributed between wound, boils, ear, nasal and blood. figure (5) show (PVL) toxin gene, it was detected in all CA-MRSA and size product 433bp, while figure (6) appear *SCCmec IV* gene which was detected in all CA-MRSA with size product 776bp, and finally

figure (7) illustrate *SCCmec V* gene was detected only two isolates of CA-MRSA with size product 325bp. While HA-MRSA isolates don't appear any positive result of PVL, *SCCmec IV* and *SCCmec V* gene, table (3) illustrate the genetic content between CA-MRSA and CA-MSSA . PVL makes community acquired S. aureus more virulent by creating pores in the membrane of infected cell host. Four major types of *SCCmec* elements have been defined based on the mec gene complex, which encodes methicillin resistance, and the *ccr* gene complex, which encodes the genetic recombination enzymes responsible for gene mobility [28], *SCCmec* carries a set of antibiotic resistance genes besides the mecA gene that is responsible for resistance to methicillin. This study show CA-MRSA and CA-MSSA carry PVL gene which responsible for cytotoxin Panton-Valentine Leukocidin, while CA-MRSA carry *SCCmec* type IV or V gene. The *SCCmec* type IV of the *mecA* gene found in 94.4% of the community acquired isolates tested, and the presence of the PVL gene in 100% of these community acquired *S.aureus* isolates, are consistent with the characteristics of CA-MRSA and CA-MSSA reported in other studies (Salaam-Dreyer [29] and Costello [30]). These two characteristics of CA-MRSA and CA-MSSA render it a cause for public health concern. The results of the current study extend the findings of earlier studies and show that patients with CA-MRSA infections are similar to patients with CA-MSSA infections in respect to their epidemiologic characteristics, also firmly established the high prevalence with the very mobile *SCCmec* type IV and the PVL virulence genes among CA-MRSA. The first and the most effective way to control community acquired is good hand hygiene to reduce nosocomial rates of infection, along with environmental cleaning between patients.

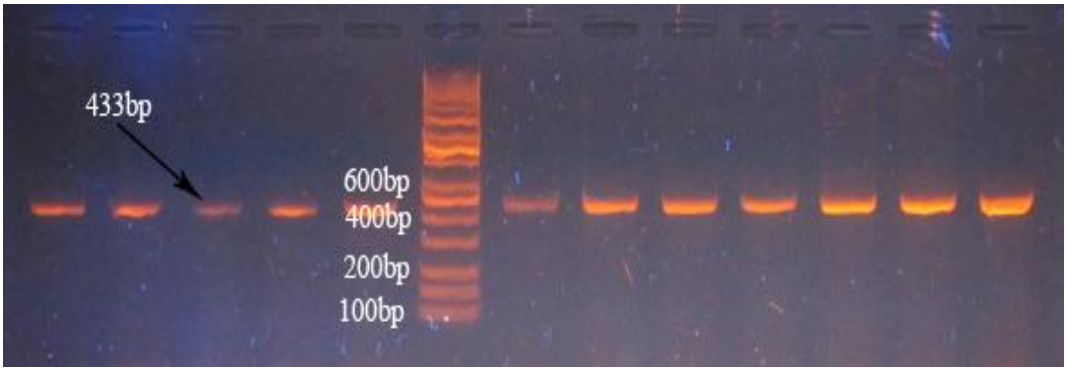

Figure 6: Agarose gel electrophoresis (1% agarose, 75 v/cm) for *PVL* gene (amplified size 433bp as compared with 100bp DNA ladder), all lines represents positive results.

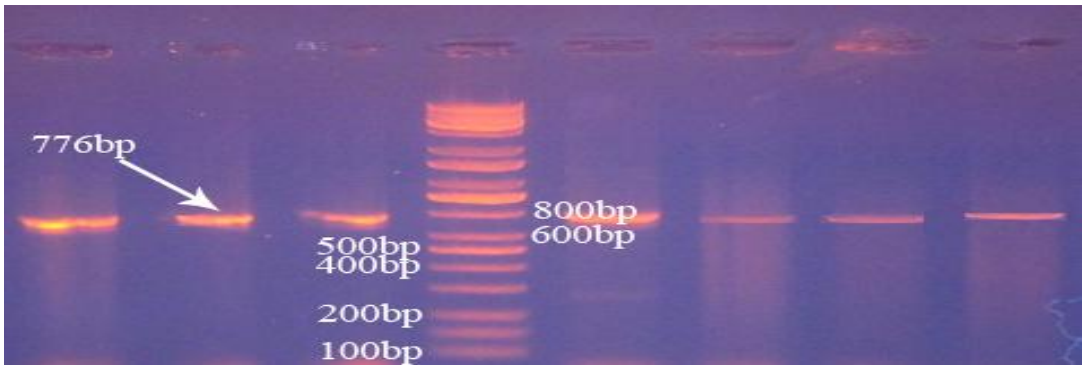

Figure 7:Agarose gel electrophoresis (1% agarose, 75 v/cm) for SCC*mec IV* gene (amplified size 776bp as compared with 100bp DNA ladder), all lines represents positive results.

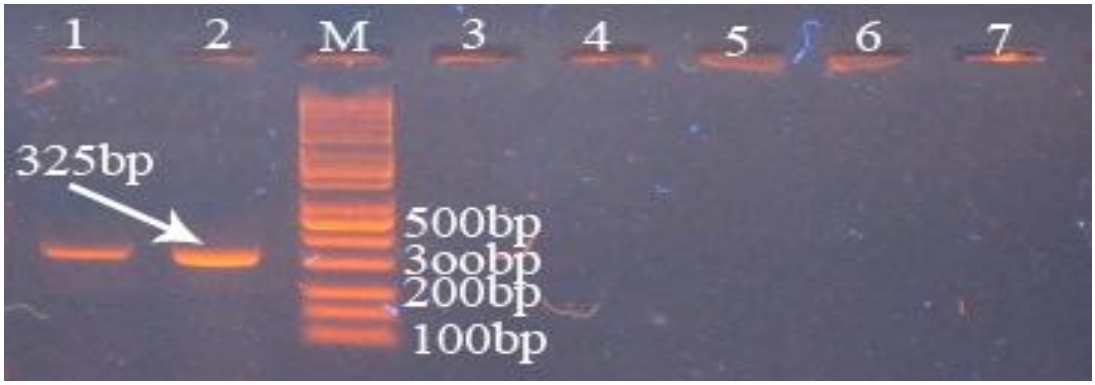

Figure 8:Agarose gel electrophoresis (1% agarose, 75 v/cm) for SCC*mec V* gene (amplified size 325bp as compared with 100bp DNA ladder), lines (1,2) represents positive results, while line (3,4,5,6,7) give negative result.

In conclusion, *S.aureus* is a common pathogen that causes a variety of infection including soft tissue, septicemia and scalded skin syndrome . Traditionally, *S.aureus* including MRSA was considered an infection that was acquired from the hospital environment . MRSA infection in the community setting are increasing in frequency. Recently one of the important particular virulence factor has emerged as a possible driving force in the CA-MRSA and CA-MSSA, the *PVL* gene . At present, MRSA infections are treatable, but there is a need to prevent the spread of MRSA in community and hospital settings. Hand hygiene and screening health care takers and workers for the presence of these organisms will help in preventing the spread of pathogens..

Table 3: different between CA-MRSA and CA-MSSA.

No. isolates	Source	D-test	Presence of *PVL* gene	Presence of *SCCmec* IV	Presence of *SCCmec* IV	Presence of *mecA* gene	Coagulase test
21	nasal	+	+	+	-	+	+
23	wound	+	+	+	-	+	+
27	nasal	+	+	+	-	+	+
28	nasal	+	+	+	-	+	+
35	boils	+	+	+	-	+	+
38	boils	+	+	+	-	+	+
39	blood	+	+	-	-	-	+
50	wound	+	+	+	-	+	+
54	boils	+	+	+	-	+	+
60	wound	+	+	+	+	+	+
63	boils	+	+	+	-	+	+
78	ear	+	+	+	-	+	+
90	wound	+	+	+	-	+	+
91	boils	+	+	+	+	+	+
92	wound	+	+	+	-	+	+
93	blood	+	+	+	-	+	-
101	wound	+	+	+	-	+	+
102	blood	+	+	+	-	+	-

(+) Positive; (-) Negative

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