Full Length Research Paper

Community-acquired methicillin-resistant Staphylococcus aureus carrying mecA and Panton-Valentine leukocidin (PVL) genes isolated from the holy shrine in Najaf, Iraq

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Two hundred and eighty six Staphylococcus aureus isolates were collected from separate places of the holy shrine in Najaf city, Iraq. Phenotypic and genotypic examination for community associated methicillin resistant S. aureus (CA-MRSA) isolates was carried out. Antibiotic and plasmid profiles of these isolates were also done. The CA-MRSA isolates were examined using polymerase chain reaction (PCR) primers for Panton Valentine leukocidin (PVL) gene. 54 (18.8%) of all of the S. aureus examined were identified as community associated methicillin resistant S. aureus, of which 11 isolates were CA-MRSA. CA-MRSA isolates were examined and subdivided into two groups according to their antibiotic profiles. Eight of the 11 MDR CA-MRSA isolates were alike in their plasmid profiles. Results of PCR revealed that 3 (27.2%) of CA-MRSA isolates carried PVL genes and 9 (72.8%) carried none. The study also revealed that CA-MRSA isolates were resistant to all ß-lactam and many of the non ß-lactam antibiotics and the frequency of resistance was higher among CA-MRSA isolates than methicillin sensitive S. aureus (MSSA), with low ratio of carrying PVL gene among CA-MRSA isolates. Surveillance and researches on CA-MRSA that carry PVL gene should continue to provide a significant insight into the prevalence and epidemiology of these important resistant pathogens.

Key words: Methicillin resistant Staphylococcus aureus, community associated methicillin resistant S. aureus (CA-MRSA), Panton Valentine leukocidin (PVL) genes, Iraq.

INTRODUCTION

Methicillin-resistant Staphylococcus aureus (MRSA) has become the most prevalent pathogen causing hospital and community infection throughout the world and MRSA incidence is still increasing in many countries (Crisostomo et al., 2001). MRSA is resistant to practically all ß-lactam antibiotics, a class of antibiotics represented by penicillins and cephalosporins (Lee, 2003). The ß-lactam resistance of MRSA is caused by the production of a novel penicillin binding protein (PBP) designated PBP 2’ (PBP 2α), which has remarkably reduced binding affinities to ß-lactam antibiotics. Despite the presence of otherwise inhibitory concentrations of ß-lactam antibiotics, MRSA can continue cell wall synthesis solely depending upon the uninhibited activity of PBP 2’ which is encoded by a mecA gene located on the chromosome of MRSA (Chambers, 2003).

Panton Valentine leukocidin (PVL) is a cytotoxin, one of the ß-pore forming toxins (Panton and Valentine, 1932), and its presence is associated with increased virulence of certain strains of S. aureus. It is present in the majority of community associated methicillin resistant S. aureus (CA-MRSA) and is the cause of necrotic flesh eating lesions, an aggressive condition that often kills patients within...
was extracted by using a standard phenol chloroform procedure (Sambrook and Russel, 2001).

### PCR analysis

Oligonucleotide primers were designed for mecA gene detection by using oligo MRS1F (5'-TAGAAATGACGTGACGTCCG-3') and oligo MRS2R (5'-TTGGCTCAATGTACCAGTACGATCCG-3') (Monterial, Alpha) (Santos et al., 1999). Amplification of mecA genes was accomplished by using LUK-PVL-1 (5'-ATCATTAGTAAAATGCTGGACATGACCA-3') and the LUK-PVL-2 (5'-GCATCAASTGTATTTGCGATCAAAGC-3') primers (Monterial, Alpha) (Scott et al., 2005). Oligonucleotide primers for standard PCR amplifications utilized PCR Master Mix (USA, Pomega) and crude cell lysates on high purity genomic DNA templates (Sambrook and Russel, 2001).

### DNA agarose gel electrophoreses

All genomic DNA and all amplified PCR products were visualized following electrophoresis in 1% agarose gels run at 70 V with ethidium bromide staining (USA, Sigma), and comparison to standard positive control (Monterial, Alpha). mecA gene and PVL gene positive strains yielded an amplification product of shining band with standard positive control (Santos et al., 1999; Scott et al., 2005).

### RESULTS AND DISCUSSION

#### Antibiotic profiles of *S. aureus*

The antibiotic profiles of all 56 *S. aureus* isolates were examined (Figure 1). All *S. aureus* isolates were susceptible to cefixime, gentamicin, chloramphenicol and vancomycin. Majority (45, 83.8%) of the isolates were highly resistant to penicillin G and resistant to ceftazidime (42, 77.7%), ampicillin-cloxacillin (29, 53.7%), amoxyclyav (26, 48.1%), azithromycin, erythromycin (20, 37%), clarithromycin (21, 38.8%), cloxacillin (17, 32.2%), ceftriaxone and trimethoprim (15, 27.7%), ciprofloxacin (9, 16.6%), and were resistant to rifampicin (4, 7.4%).

Resistance to penicillins in this study was found commonly among *S. aureus* isolates tested and is closely related to many studies (Katayama et al., 2000; Lee, 2003; Merlino et al., 2002). These results are in agreement with those results being reported by other researchers who found that production of β-Lactamase is common, under plasmid control, and makes the organisms resistant to many penicillins (penicillin G, ampicillin, ticarcillin, piperacillin, and similar drugs) (Kuehnert et al., 2006).

The resistance to penicillins produces an additional penicillin-binding protein, PBP2a or PBP2’ (Kuehnert et al., 2006), which confers resistance to all currently available β-lactam agents. This resistance is under chromosome control (Merlino et al., 2002).

Four variants of *S. aureus* β-lactamase (A, B, C, D) can be distinguished. Although the genes encoding types A, C, and D staphylococcal β-lactamases are usually located in the genomes of these staphylococcal species.
Figure 1. Antibiotic resistance patterns of *S. aureus* isolates.

**Table 1.** Numbers and percentages of CA-MRSA isolates detected by using a 30 µg cefoxitin disk and PCR analysis.

<table>
<thead>
<tr>
<th>CA-MRSA isolates carrying mecA gene</th>
<th>No. of isolates</th>
<th>No. of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mecA-positive isolates (CA-MRSA)</td>
<td>11</td>
<td>20.4</td>
</tr>
<tr>
<td>mecA-negative isolates (MSSA)</td>
<td>43</td>
<td>79.6</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>100</td>
</tr>
</tbody>
</table>

on a plasmid, the gene for type B β-lactamase is believed to reside on the chromosome of phage group II isolates. For these reasons, multiple methods should be used for detection of the different types of resistance to β-lactam antibiotics (Olsen et al., 2006).

On the other side, there are many reasons to the variations in a result for resistance to tetracyclines, erythromycins, aminoglycosides, and other drugs because the frequent plasmids are mediated in staphylococci, and these plasmids can be transferred from one strain to another by conjugation or transduction. Therefore, resistance carried by plasmids can spread rapidly amongst many different strains and genera (Black, 2002). Despite these, there are many studies describing the resistance to tetracyclines, erythromycins, aminoglycosides, carrying on the *mecA* of chromosome MRSA strains (Cauwelier et al., 2004; Skov et al., 2003).

The mechanisms responsible for resistance to erythromycin in staphylococci are target site modification and active drug efflux (Black, 2002; Berg et al., 1998). Target site modification is mediated by the presence of *erm* genes [*erm* (A), (B) and (C)] conferring resistance to Macrolide, lincosamide and streptogramin type B (MLS<sub>B</sub>) antibiotics. Phenotypic expression of MLS<sub>B</sub> resistance can be inducible or constitutive (Schmitz et al., 2000).

All isolates of *S. aureus* were susceptible to vancomycin, indicating the absence of *vanA* gene. This susceptibility to vancomycin encourages using it as the last step for treatment of hard infection with multidrug resistant *S. aureus* (Moise-Broder et al., 2004; CLSI, 2010).

Antibiotic resistance among isolates of *S. aureus* in the present study was comparable to reports from other parts of the world, which also revealed multiple drug resistance among staphylococci (Merlino et al., 2002; Santos et al., 1999; Tiemersma et al., 2004).

**CA-MRSA detection**

A total of 54 *S. aureus* isolates were investigated in this study using phenotype [cefoxitin disk diffusion (DD)], and genotypic methods PCR analysis. The phenotypic and genotypic characteristics of the *S. aureus* are shown in Table 1. PCR of the *mecA* gene confirmed the identification of all (43, 79.6%) methicillin sensitive *S. aureus* isolates and (11, 20.4%) methicillin resistant *S. aureus* isolates as previously indicated by cefoxitin disk. The results of PCR are shown in Figure 2.

Resistance to methicillin is independent of β-lactamase production. The *mecA* gene for methicillin resistance resides on the chromosome. Accurate detection of *mecA*
mediated resistance to methicillin and other penicillinase stable penicillins (PSPs) such as, oxacillin, naftilin, cloxacinil, dicloxacillin, fluclxacinil, and mecinilam, is necessary to ensure appropriate antimicrobial chemotherapy of staphylococcal infections, particularly those from community associated infections. Oxacillin has been the agent recommended by CLSI (2010) for phenotypic tests to predict resistance to PBPs because of its stability and superior sensitivity over other PBPs for susceptibility tests (Pinho et al., 2001). 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 (Skov et al., 2003).

The recent development of cefoxitin disc diffusion tests is likely to alter the recommendations for these methods as studies all suggest that tests with cefoxitin are more reliable than those with oxacillin (CLSI, 2010; Skov et al., 2003).

It is suggested that no special medium or incubation temperature is required with cefoxitin (Cauwelier et al., 2004).

In disc diffusion tests, hyper producers of penicillinase may show small methicillin or oxacillin zones of inhibition, whereas most true methicillin resistant isolates give no zone. Resistance mediated by mecA may be confirmed by PCR analysis. Some hyper producers of penicillinase give no zone, particularly with oxacillin, and will therefore be falsely reported as MRSA. Tests with cefoxitin do not appear to be affected to the same extent as oxacillin by hyper production of penicillinase. Expression of methicillin resistance in S. aureus depends on environmental factors such as temperature and osmolarity (Swenson and Tenover, 2005). As previously reported, better phenotypic expression of oxacillin resistance was observed at 30°C or after addition of NaCl (4%) to the culture medium (CLSI, 2010).

The cefoxitin DD test and PCR can be used to predict the presence of mecA gene in S. aureus with a high degree of sensitivity and specificity when compared to mecA detection using oxacillin disk. Those using disk diffusion as their routine susceptibility testing method for staphylococci should replace their 1 µg oxacillin disks with 30 µg cefoxitin disks for routine testing because the oxacillin is affected by high production of β-lactamase enzymes (Swenson and Tenover, 2005).

**Antibiotic profiles of CA-MRSA**

Eleven CA-MRSA of the S. aureus isolates underwent antibiotic susceptibility testing, and the results are shown in Figure 3. All the CA-MRSA were resistant to penicillin, cloxacinil, amoxyclav, ampicillin-cloxacinil, ceftriaxone, ceftazidime, cefixime, 8 (72.7%) were highly resistant to azithromycin erythromycin, and clarithromycin, 5 (45.4%) were resistant to ciprofloxacin, 4 (36.3%) to trimethoprim, and 15 (27.7%) to gentamicin. In addition all of the CA-MRSA isolates were susceptible to rifampicin, chloramphenicol, and vancomycin.

MRSA is practically resistant to all β-lactam antibiotics, a class of antibiotics represented by penicillins and cephalosporins (Katayama et al., 2000; CLSI, 2010). Resistance due to β-lactamases and PBPs mediated mecA gene is an emerging global problem that needs to be monitored. Resistance organisms are increasing in number and causing more severe infections because of their continuous mutation. The pharmaceutical industry makes little effort in developing new antibiotic agents because of the emergence of resistance worldwide. It takes at least 10 years to establish new agents, and after 2 years in clinical use, resistance probably emerges.
Therefore, reliable, rapid and accurate detection methods have to be developed (Pinho et al., 2001).

It was observed that the High resistance of azithromycin and erythromycin in this study is in line with many studies which reported that transposon Tn554 (Ito et al., 1999), encoding resistance to macrolides (erythromycin and spectinomycin), was located in the upstream of the mecl-mecR1-mecA gene complex that directly carries MRSA strains, while the Plasmid pUB110, encoding resistance for aminoglycosides (gentamicin, kanamycin, tobramycin and bleomycin), was inserted between two insertion sequences of IS431 (or IS257) at the left of mecA gene (Katayama et al., 2000).

The result of vancomycin resistance is expected, because the resistance is very rarely reported (CLSI, 2010; Moise-Broder et al., 2004). This pattern is most commonly found among MRSA isolates tested at the department of health public laboratories in USA, UK and most European countries, and it is closely related to the other pattern of the primary types causing community acquired infections nationwide (Schmitz et al., 2000; Tiemersma et al., 2004; Gemmell et al., 2006; Wannet, 2002).

Antibiotic profiles of MDR CA-MRSA, NMDR CA-MRSA, and MSSA

The antibiotic profiles of all 54 S. aureus isolates were examined. NMDR CA-MRSA had variable antibiotic profiles. The results are shown in Figure 4. The 54 CA-MRSA isolates examined were subdivided into two groups according to their antibiotic profiles and comprised 3 (27.3%) non multidrug resistant NMDR CA-MRSA isolates, resistant to less than two non ß-lactam antibiotics, and 8 (72.7%) multidrug resistant MDR CA-MRSA isolates, resistant to three or more non ß-lactam antibiotics.

All NMDR CA-MRSA were susceptible to azithromycin, erythromycin, clarithromycin, ciprofloxacin, chloramphenicol, rifampicin, trimethoprim, whereas all MDR CA-MRSA were resistant to azithromycin, erythromycin, clarithromycin, 5 (45.3%) of MDR CA-MRSA were resistant to ciprofloxacin, and 4 (36.3%) were resistant to trimethoprim, but they were susceptible to rifampicin, and chloramphenicol. All 54 staphylococcal isolates (NMDR CA-MRSA, MDR CA-MRSA and MSSA) were susceptible to vancomycin.

NMDR CA-MRSA isolation has been reported with increased frequency worldwide, despite universal guidelines published by the NCCLS for the susceptibility testing of S. aureus to methicillin (CLSI, 2010; Merlino et al., 2002).

Laboratories without genotypic or monoclonal based assays may have problems in detecting or confirming low level mecA phenotypic heterogeneous expression of methicillin resistance in NMDR CA-MRSA (Wannet, 2002). The degree of heterogeneity is variable in NMDR CA-MRSA strains and modification of growth conditions can increase or decrease the proportion of cells that express resistance to various degrees (Merlino et al., 2002). In this study, it was found that using PCR for the detection of mecA gene is an excellent assay, and confirms that methicillin resistance tests for these NMDR CA-MRSA shows heterogeneous resistances. The direct implementation of such assays will allow more rapid establishment of effective antibiotic therapy and should help control the spread of MRSA in hospitals and the

community (Merlino et al., 2002; Wannet, 2002). Further investigative research is in progress to elucidate factors involved in heterogeneous expression of methicillin resistance in NMDR CA-MRSA and the effects of different non β-lactam antibiotics on these strains as an alternative to vancomycin use (Merlino et al., 2002).

Because there are epidemiological and microbiological differences between community associated methicillin resistant S. aureus, these modifications could include more frequent culturing and susceptibility testing of S. aureus isolates of clinical infections, and careful selection of empirical antimicrobials when such treatment is indicated for suspected staphylococcal infections (that is, clinicians should be aware that MRSA organisms are non susceptible to β-lactam antimicrobials) (CLSI, 2010). Because most community associated MRSA isolates were susceptible to several already available antimicrobial agents, and because most patients had noninvasive infections, the treatment of community associated MRSA infections should not routinely require the use of vancomycin. However, patients with MRSA infections should be evaluated and treated appropriately and receive follow-up evaluation to ensure resolution of their infection (Merlino et al., 2002).

To improve prevention strategies, more work is needed to characterize specific risk factors for CA-MRSA infections. However, at least one study has implicated prior exposure to antimicrobial agents as an independent risk factor for CA-MRSA infection (CDC, 2007). This suggests that the judicious use of antimicrobials, particularly in outpatient settings, could help control the emergence of CA-MRSA strains and limit the acquisition of additional antimicrobial resistance genes in existing strains (CDC, 2007; Andenesch, 2003; Okuma et al., 2002). Currently, there is no Arabic or Iraqi data to suggest that decolonization protocols for MRSA patients or their families are necessary or have long term effectiveness (CDC, 2007). Resistance to antimicrobial agents used for decolonization has evolved rapidly in settings in which such strategies have been attempted (Boyle-Vavra and Daum, 2007; CDC, 2007; Skov et al., 2003).

**Plasmid profiles pattern of MDR CA-MRSA**

All eight MDR CA-MRSA were selected as β-lactam resistance, and multidrug resistant to non β-lactam antibiotics. They are use to determine their plasmid profiles patterns. Isolates in Figure 5 were alike in their plasmid profiles and there were two bands of different plasmids that appeared identical in shape and position when electrophoresis was performed. These bands must be divided depending on their molecular weight, by using restriction enzymes and ladder as marker, but in this study, plasmid profiles were used just to ensure that isolates have DNA of plasmid that encodes antibiotic resistance and many virulence characteristics. Only one study was in agreement with our results (Santos et al., 1999).
Plasmid analysis has been applied to determine the evolution and spread of antibiotic resistance among isolates with different profiles or among different species of organisms. Plasmids are not generally helpful in the differentiation between strains, because plasmids are often mobile extrachromosomal DNA fragments that can be acquired and deleted (Feil et al., 2003). Many plasmids carry antibiotic resistance determinants that are contained within mobile genetic elements (transposons) that can move in or out of plasmids and the chromosome, allowing for the DNA composition of a plasmid to potentially change rapidly (Singh et al., 2006). The analysis of plasmid content is limited to investigations in which a plasmid epidemic is responsible for the spread of a resistance trait. The selective pressure for organisms to express antibiotic resistance may cause such plasmids to spread rapidly among strains and among different species (Feil et al., 2003; Singh et al., 2006).

Several of the antibiotic resistances in *S. aureus* determinants are carried by pSK41-like plasmids (Berg et al., 1998; Firth et al., 2000). The sequence of pSK41, a prototypical multiresistance plasmid from *S. aureus*, has been determined, representing the first completely sequenced conjugative plasmid from a Gram-positive organism. The pSK41-like conjugative plasmids usually carry a Tn4001-like element encoding the bifunctional aminoglycoside resistance gene *aacA-aphD* and often contain cointegrated remnants of small plasmids encoding genes such as *aadD*, mediating resistance to the aminoglycosides neomycin and kanamycin; *smr*, conferring antiseptic and disinfectant resistance; and *dfrA*, encoding resistance to trimethoprim (Singh et al., 2006; Firth et al., 2000). In addition, members of the pSK1 plasmid family typically confers resistance to antiseptics and disinfectants, encoded by *qacA*, and may also carry Tn4001, a Tn552-like transposon, and/or a cointegrated remnant of a *dfrA*-carrying plasmid previously designated Tn4003. Analysis of the sequence has enabled the identification of the probable replication, maintenance, and transfer functions of the plasmid (Firth et al., 2000; Simpson et al., 2003).

### Detection of CA-MRSA carrying PVL gene

Using polymerase chain reaction for the determination of the presence of PVL gene, Scott et al. (2005) found that CA-MRSA isolates with PVL genes were detected in 3 (27.2%). PVL genes were not detected in 9 (72.8%) of CA-MRSA isolates. The results are found in Table 2 and

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**Table 2. Numbers and percentages of CA-MRSA isolates carrying PVL gene detected by using PCR analysis.**

<table>
<thead>
<tr>
<th>CA-MRSA isolates carrying PVL gene</th>
<th>No. of isolates</th>
<th>No. of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVL-positive isolates</td>
<td>3</td>
<td>27.2</td>
</tr>
<tr>
<td>PVL-negative isolates</td>
<td>9</td>
<td>72.8</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>100</td>
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</tbody>
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**Figure 5.** Agarose gel electrophoresis demonstrative the results of DNA content for MDR CA-MRSA.
Figure 6. For comparison, there is no study in Iraq about the PVL gene with CA-MRSA, and there is no professional center for studying or researching about it. 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 antibiotics.

Also, CA-MRSA appears to have a distinct exotoxin PVL, which has been associated with severe infections (Okuma et al., 2002; Kazakova et al., 2005). PVL is one of the many toxins associated with *S. aureus* infection. Because it can be found in virtually all CA-MRSA strains that cause soft-tissue infections, several research groups previously proposed that PVL is the key virulence factor. But new evidence strongly suggests that this is not the case (Andenesch et al., 2003; Dufour et al., 2002; Lina et al., 1999; Melles et al., 2006; Panton and Valentine, 1932).

The *S. aureus* bacterium is a growing global public health menace because of its rapid spread from hospital settings into communities of healthy people. This is an evolving pathogen that in recent decades has developed resistance to common medical treatments and is now finding new mechanisms to spread and cause severe illness. About 75% of CA-MRSA infections are localized to the skin and soft tissues and can usually be treated effectively (Boubaker, 2007; Lina et al., 1999; Voyich et al., 2007). CA-MRSA strains have enhanced virulence, meaning they can infect otherwise healthy people. One of the biggest problems with CA-MRSA skin infections is that they spread rapidly and have the potential to cause illness much more severe than traditional hospital associated MRSA infections, where PVL is less common (Boubaker, 2007; Boyle-Vavra and Daum, 2007; Lina et al., 1999, 2002; and Valentine 1932, Scott et al., 2005).

Scientists had recognized a connection between MRSA strains that contained PVL and the increased occurrence and severity of CA-MRSA disease, though no one had directly tested the role of PVL in CA-MRSA virulence (Lina et al., 1999). In striving to learn more about CA-MRSA, the RML scientists, with their colleagues at the International Center for Public Health (ICPH) in Newark, and the Université Claude Bernard in Lyon, France, decided to test the PVL virulence theory, thinking that if they could understand the role of this toxin in disease, they could more quickly diagnose serious cases and develop effective treatments (Voyich et al., 2007; Lina et al., 1999). The strong association between PVL and CA-MRSA makes the toxin an excellent marker in tracking community strains, but the assumption that it is the major virulence determinant driving this epidemic is simply not true (Voyich et al., 2007).

**Conclusion**

In summary, PVL positive CA-MRSA appears to be an increasingly prevalent pathogen worldwide. Clonal expansion of this virulent pathogen appears to be increasing in North American, Australia, Asia, and Europe (Scott et al., 2005; Wannet, 2007). Surveillance and researches on CA-MRSA that carry PVL gene should continue to provide a significant insight into the prevalence and epidemiology of these important resistant pathogens.

**REFERENCES**


Figure 6. Agarose gel electrophoresis demonstrative the results of PCR for PVL gene to CA-MRSA. Lane 1: negative PVL gene control; lane 2: positive PVL gene control; lanes 3, 4, 8, 9, 10, 11, 12, 13: CA-MRSA without PVL gene; lanes 5, 6, 7: S. carrying PVL gene.


