

Full Length Research Paper

Rapid detection of community acquired- methicillin resistance *Staphylococcus aureus* recovered from King Saudi Arabia

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The objectives of this study were to examine methicillin-resistant *Staphylococcus aureus* (MRSA) strains recovered from major hospitals in King Saudi Arabia (KSA) to determine the percent of community acquired MRSA (CA-MRSA) phenotypically by conventional methods and genotypically by multiplex polymerase chain reaction (multiplex-PCR) for direct and simultaneous detection of *S. aureus* 16S rRNA, Pantón–valentine leucocidin (*PVL*) and staphylococcal cassette chromosome *mec* (*SCCmec*) type IVa genes. Therefore, 135 strains of *S. aureus* collected during the period of 2008 and 2009 from major hospital laboratories and public health centers, Riyadh, King Saudi Arabia were tested phenotypically by conventional methods and genotypically by multiplex-PCR. PCR enables rapid detection of all 135 bacteriologically identified *S. aureus* (100%) as well as the *mecA* gene in all strains phenotypically resistant to methicillin (100%). Moreover, it could detect the *mecA* gene in 8 strains (6%) phenotypically sensitive to methicillin. Only 18 strains (13.33%) recovered from skin and soft tissue infections were positive for *PVL* and (*SCCmec*) type IV. The results of this study indicate that the incidence of patients with CA-MRSA disease had been increased in Riyadh, KSA.

Key words: *Staphylococcus aureus*, CA-MRSA, *PVL* gene, multiplex polymerase chain reaction.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) constitutes a major problem in large medical centers. This infection is sometimes life-threatening for patients with severe underlying conditions. MRSA is a frequent cause of infections both in health care and community settings and is endemic in many U.S. hospitals (Charlebois et al., 2002; Haley et al., 1995; Herold et al., 1998; Karchmer, 2000).

MRSA emerged as a more widespread cause of community-associated infection in the late 1990 (Prevost et al., 1995; 2001). Such infections not acquired at a health care setting or institution are considered community associated MRSA (CA-MRSA), these organisms

have recently emerged as an important cause of community associated staphylococcal infections (Vandenesch et al., 2003; Begier et al., 2004).

The incidence and prevalence of CA-MRSA vary geographically, in the United States in surveillance conducted in Maryland, Georgia, and Minnesota in 2001 and 2002; the proportion of all MRSA infection that were CA-MRS infection is from 18.0 to 25.7 per 100,000 (Fridkin et al., 2005).

Pantón – valentine leucocidin (*PVL*) is a cytolytic toxin associated with *S. aureus* furuncles and necrotizing pneumonia (Gillet et al., 2002; Lina et al., 1999). *PVL* gene were detected in 98% of MRSA isolates and 42% of methicillin-sensitive *S. aureus* (MSSA) isolates of community – acquired skin and soft tissue infections (SSTIs) in multisite U.S.

PVL had been hypothesized to play a major role in the increased virulence of MRSA prevalent in the community. The presence of *PVL* genes had been associated with

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more severe local disease, greater systemic inflammatory response, and increased complications in children with *S. aureus* (MRSA and MSSA) osteomyelitis (Martinez-aguilar et al., 2004; Bocchini et al., 2006), as well as decreased survival in patients with community-acquired *S. aureus* pneumonia (Gillet et al., 2002).

One of the defining characteristics of CA-MRSA had been isolate susceptibility to most classes of antimicrobial agents other than beta-lactams and macrolides/azalides (e.g., erythromycin, azithromycin) (Fridkin et al., 2005). However, resistance to other antimicrobial agents, including fluoroquinolones, tetracyclines, and clindamycin also occurs (Fridkin et al., 2005; Maron et al., 2006).

The *mecA* gene, which confers resistance to methicillin and all currently available beta-lactam antimicrobial agents is carried on a gene complex known as the staphylococcal cassette chromosome *mec* (SCC *mec*) (Katayama et al., 2000). MRSA strains associated with community transmission typically carry SCC *mec* types IV or V, which are smaller and theoretically more easily transferable between organisms than the SCC *mec* types (I-III) characteristic of MRSA strains that predominate in healthcare settings (Boyle-Vavra and Daum, 2007).

The aim of this study was (1) characterization of the recovered MRSA from different hospitals located in Riyadh, KSA phenotypically by conventional methods and genotypically by PCR for direct detection of the *S. aureus* 16S rRNA gene (which serves as an internal control) and the *mecA* gene, and (2) using multiplex PCR reported by Moussa and Shible (2009) which could allow simultaneous detection of *S. aureus* species specific 16S rRNA (SCC*mec*) type IV and *PVL* genes to determine the incidence of CA-MRSA in Riyadh, KSA.

MATERIALS AND METHODS

Bacterial isolates

135 strains of *S. aureus* were collected during the period of 2008 and 2009 from major hospital laboratories and public health centers, Riyadh, King Saudi Arabia. They were tested phenotypically by conventional methods and genotypically by PCR for direct detection of *S. aureus* 16S rRNA and *mecA* genes of *S. aureus*, including 60 strains recovered from skin and soft tissue infection, 35 strains recovered from abscess, 15 strains recovered from surgical wound infection, 5 strains recovered from pneumonia and lung abscess and 10 strains from burn and blood stream infection, while the other 10 strains recovered from fracture bone with surgery. All isolates were identified according to colonial and microscopical morphology, catalase and coagulase production and novobiocin sensitivity. The 135 strains were tested also by multiplex PCR targeting *S. aureus* 16S rRNA, *PVL* and SCC*mec* type IV

Antimicrobial susceptibility test

Detection of oxacillin resistance by phenotypic method

Cefoxitin and oxacillin disk diffusion methods: Cefoxitin (30 µg) disk and Oxacillin (1 µg) were used. The cefoxitin disk diffusion and

oxacillin disk diffusion test was performed using the routine disk diffusion procedure, and the results were evaluated according to the interpretive criteria of CLSI, 2006.

Epsilometer test (E-test): The E-test was performed for quantitative antimicrobial susceptibility testing whereby a preformed antimicrobial gradient from a plastic-coated strip diffuses into an agar medium inoculated with the test organism.

Antimicrobial susceptibility test to non beta-lactam drugs: Antimicrobial susceptibility test to a range of antimicrobial agents was done using the following disks (chloramphenicol, tetracycline, fusidic acid, gentamicin, erythromycin, ciprofloxacin, clindamycin, Rifampicin and vancomycin) was done adopting the Kirby-Bauer disk diffusion method using Muller-Hinton broth and agar and antibiotics disks (Oxoid Limited, Hampshire, England) according to the recommendations of Clinical Laboratory Standards Institute (CLSI) formally National Committee for Clinical Laboratory Standards (NCCLS), 2006.

Detection of oxacillin resistance by genotypic method

Extraction of DNA from bacterial isolates: The bacterial isolates were re-suspended in 400 µl Tris-EDTA buffer (pH 8.0) and heated in heat block at 105°C for 25 min. They were left to cool at room temperature and centrifuged at 14,000 xg for 10 min. The supernatant was transferred to a fresh tube with double its volume absolute ethanol and 0.1 volume 3 M sodium acetate (pH 5.2) were added and the test tubes were kept at 20°C for an overnight. The DNA was pelleted by centrifugation at 14,000 xg/min for 20 min, followed by washing with 70% ethanol and re-centrifugation at 14,000 xg/min for 10 min. The DNA pellet was dried and re-suspended in 20 µl sterile distilled water.

Amplification of 16S rRNA gene specific for *S. aureus* and (*mecA*) gene: Two sets of primer pairs were used, the first one pair was (16S rRNA F: CGA TTC CCT TAG TAG CGG CG and 16S rRNA R: CCA ATC GCA CGC TTC GCC primers) *S. aureus* species specific primers, which can amplify 1267 base pair fragments and its annealing temperature is 70°C according to Riffon et al. (2001). The second pair was (*mecA* F: GTG GAA TTG GCC AAT ACA GG and *mecA* R: TAG GTT CTG CAG TAC CGG AT primers) which can amplify 1399 base pair fragments specific for *mecA* gene and its annealing temperature is 58°C according to Weller (1999). Tables 1 and 2 shows the specificity, nucleotide sequence, size of amplified fragment and annealing temperature of the primers.

All reactions were carried out separately in a final volume of 50 µl in micro-amplification tubes (PCR tubes). The reaction mixtures consisted of 5 µl of the extracted DNA template from the bacterial isolates, 5 µl 10 × PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl₂, 50 mM KCl, 20 mM (NH₄)₂SO₄), 1 µl dNTPs (40 µM), 1 µl (1U Ampli Taq DNA polymerase), 1 µl (50 pmol) from the forward and reverse primers. Each primer pair was used separately and the volume of the reaction mixture was completed to 50 µl using DDW. 40 µl paraffin oil was added and the thermal cycler was adjusted as follows: Initial denaturation at 94°C for 5 min, followed by 35 cycles of (denaturation at 94°C for 1 min, annealing at 70°C for SauF234 and SauR1501 primers and at 58°C for MR1 and MR2 primers for 1 min and extension at 72°C for 1 min). Final extension carried out at 72°C for 10 min and the PCR products were stored in the thermal cycler at 4°C until they were collected.

Table 1. The nucleotide sequences of the oligonucleotides used and its target genes.

Primer used	Nucleotide sequence	Target gene
<i>mecA</i> gene		
mecA F	5'-GTGGAATTGGCCAATACAGG-3'	<i>mec A</i> gene
mecA R	5'-TGAGTTCTGCAGTACCGGAT-3'	
16S rRNA		
F	5'-CGATTCCCTTAGTAGCGGCG-3'	16S rRNA
R	5'-ATCGCACGCTTCGCCTA-3'	
PVL gene		
Luk PV-1	5'-ATTAGGTAAAATGTCTGGACATGATCCA-3'	<i>PVL</i> gene
Luk PV-2	5'-GCATCAAGTGTATTGGATAGCAAAAGC-3'	
SCC <i>mec</i> subtype IVa		
4a1	5'-TTTAATGCCCATGAATAAAAT-3'	<i>SCCmec</i> IVa gene
4a2	5'-AGAAAAGATAGAAGTTTCGAAAAGA-3'	
16S rRNA		
Staph756F	AACTCTGTTATTAGGGAAGAAC	16S rRNA
Staph750R	CCACCTTCCTCCGGTTTTGTCACC	

Table 2. The annealing temperature, PCR conditions and the size of amplified fragments of the oligonucleotides used.

Primers used	<i>Mec A</i> gene	16S rRNA	Multiplex PCR		
			<i>PVL</i> gene	<i>SCC mec IVa</i>	16S rRNA
Annealing temperature (°C)	58	70	55	55	55
Size of amplified produced (bp)	1319	1267	433	450	756
Denaturation (°C)	94	94	94	94	94
Extent on (°C)	72	72	72	72	72

Multiplex PCR for detection of *S. aureus* species specific 16S rRNA, (*SCCmec*) type IV and PVL genes: Three sets of primer pairs were used, the first one was Staph756F and Staph750R primers which could amplify 756 base pair fragments specific for 16S rRNA of *S. aureus*; the second one was Luk-PV-1 and Luk-PV-2 primers which could amplify 433 base pair fragments specific for *lukS/F-PV* genes which encode the PVL S/F bicomponent proteins according to Jo-Ann et al. (2006). The third one was SCCmec 4a1 and SCCmec 4a2 primers which could amplify 450 base pair fragments specific for *SCCmec* subtype IVa gene according to Keiko et al. (2002).

The reaction mixtures consisted of 5 µl of the extracted DNA template of the bacterial isolates, 5 µl 10× PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM MgCl₂, 50 mM KCl, 20 mM (NH₄)₂SO₄), 1 µl dNTPs (40 µM), 1 µl (1U Ampli Taq DNA polymerase), 1 µl (50 pmol) from the forward and reverse primers. The three sets of primer pairs were used in each reaction mixture and the volume of the reaction mixture was completed to 50 µl using DDW. 40 µl paraffin oil was added and the thermal cycler was adjusted as follows: 94°C for 100 min, followed by 10 cycles of 94°C for 1 min, 55°C for 1 min., and 72°C for 1.5 min, and 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min, followed by final extension at 72°C for 1.5 min, and the PCR products were stored

in the thermal cycler at 4°C until they were collected.

Agarose gel electrophoresis

The PCR products were tested for positive amplification by agarose gel electrophoresis previously reported by Sambrook et al. (1989) using suitable molecular weight markers.

RESULTS

Antimicrobial susceptibility patterns

Susceptibility to methicillin and oxacillin

One hundred and twenty seven strains (94%) out of 135 tested strains were resistant phenotypically to methicillin and oxacillin which was confirmed by PCR using the specific primers of *mecA* resistance gene, while the other 8 strains (6%) which appeared methicillin / oxacillin

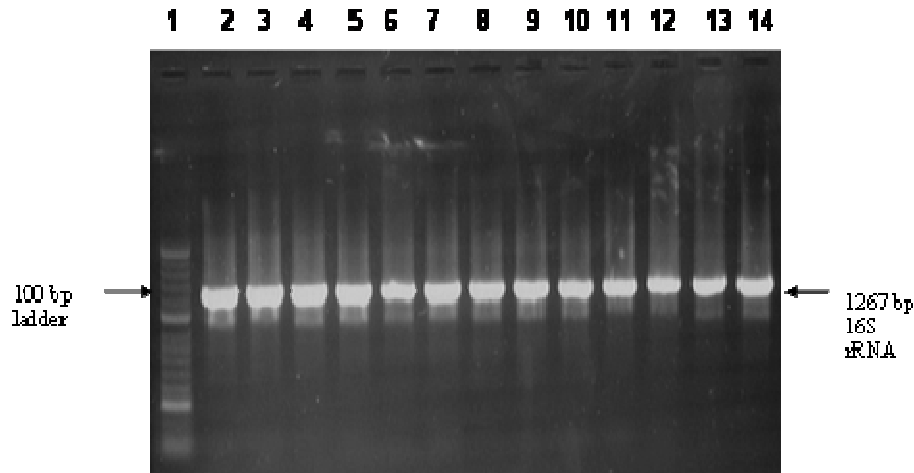


Figure 1. Agarose gel electrophoresis showing amplification of 1267 bp fragments of 16S rRNA of *S. aureus*, lanes 2....10. Lane 1 shows 100 bp ladder.

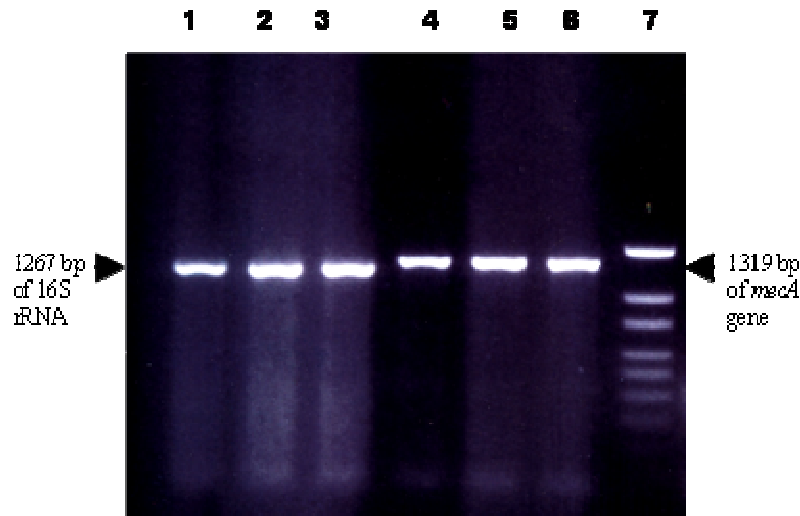


Figure 2. Agarose gel electrophoresis showing amplification of 1267 bp fragments of 16S rRNA of *S. aureus*, lanes 1, 2 and 3. Lane 4, 5, and 6 showing amplification of 1319 bp fragments of *mecA* gene. Lane 7 shows 100 bp ladder.

sensitive phenotypically were positive with PCR and harbored the *mecA* gene.

Susceptibility to other non beta-lactam drugs

Fifty four strains out of 135 tested strains (40%) were resistant to chloramphenicol, 47 strains (34.8%) were resistant to tetracycline, 40 strains (29.62) were resistant to fusidic acid, 56 strains (41.48%) were resistant to gentamicin, 45 strains (33.33%) were resistant to clindamycin and 80 strains (59.25%) were resistant to erythromycin, 54 strains (40%) were resistant to ciprofloxacin while all isolates were susceptible to vancomycin.

Detection of oxacillin resistance by genotypic method

Amplification of 1267 base pair fragments specific for 16S rRNA of *S. aureus* using *SauF234* and *SauR1501* primers

The amplification of 1267 base pair fragments specific for 16S rRNA of *S. aureus* using 16S rRNA F and 16S rRNA R primers revealed positive amplification of 1267 base pair fragments with all thirty seven isolates (100%) previously identified phenotypically as *S. aureus* with bacteriological examination specific for 16S rRNA of *S. aureus* as shown in Figures 1 and 2.

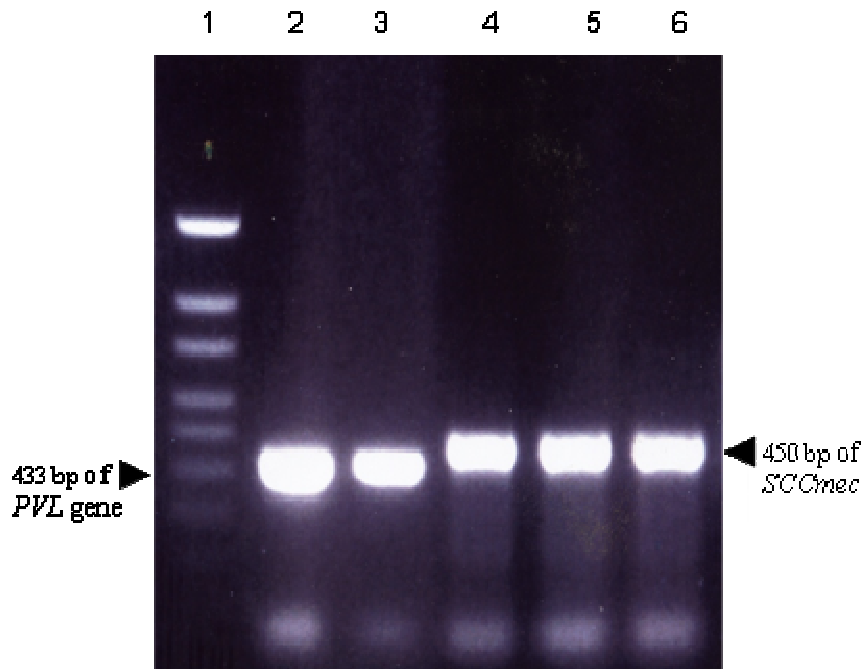


Figure 3. Agarose gel electrophoresis showing amplification of 433bp fragments of *PVL* gene, lanes 2, and 3. Lane 4, 5, and 6 showing amplification of 450 bp fragments of *SCC mec IVa* gene. Lane 1 shows 100bp ladder.

Amplification of 1339 base pair fragments specific for *mecA* gene using MR1 and MR2 primers

For amplification of 1339 base pair fragments specific for *mecA* gene using (*mecA* F and *mecA* R primers), all the 127 strains (100%) which appeared methicillin / oxacillin resistant phenotypically with antimicrobial susceptibility test were positive for amplification of 1339 base pair fragments specific for *mecA* gene. At the same time, the other 8 strains (6%) which appeared methicillin / oxacillin sensitive phenotypically were positive with PCR and harbored the *mecA* gene as shown in Figure 2.

Multiplex PCR for detection of *S. aureus* species specific 16S rRNA, (*SCCmec*) type IVa and *PVL* genes

Multiplex PCR for detection of *S. aureus* species specific 16S rRNA (*SCCmec*) type IVa and *PVL* genes were performed. All the 135 strains (100%) previously identified phenotypically as *S. aureus* with bacteriological examination were positive for amplification of 756 base fragments specific for 16S rRNA of *S. aureus* using Staph756 F and Staph750 R primers, while only 18 strains (13.33%) showed positive amplification of 433 and 450 base pair fragments specific for *lukS/F-PV* and *SCCmec* subtype IVa genes using Luk-PV-1 and Luk-PV-2 primers and *SCCmec* 4a1 and *SCCmec* 4a 2 primers respectively, as shown in Figure 3. All the eighteen strains harboring the *PVL* and *SCCmec* subtype IVa

genes were isolated from skin and soft tissue infections.

DISCUSSION

PCR based assay had been reported for the identification of staphylococcal species (Louie et al., 2002), the detection of MRSA (Huletsky et al., 2004), or the identification of specific virulence gene (Francois et al., 2004). Real time PCR and melt curve analysis based on amplification of a portion of the 16S rRNA reported to give accurate results for the most common *Staphylococcal species* (including *S. aureus* and *S. epidermidis*). Due to the high costs of equipment and reagents, use of the real time PCR technology had generally been limited to the larger microbiology laboratories. McClure et al. (2006), developed a new multiplex assay to aid with the early identification of CA-MRSA strains. This assay targets the staphylococcus genus-specific 16S rRNA gene, the *luk S/F-PV* and the *mec A* gene. This assay was shown to be 100% accurate and reliable. Moreover this assay is easily amenable to routine clinical use in any molecular biology laboratory with PCR capabilities. Therefore one of the main objectives of this study was the use of PCR for rapid and specific detection of 16S rRNA specific for *S. aureus* and the *mecA* gene specific for methicillin / oxacillin resistant. Results observed in Figures 1 and 2 revealed positive amplification of 1267 base pair fragments specific for 16S rRNA of *S. aureus* with all 135 tested strains (100%)

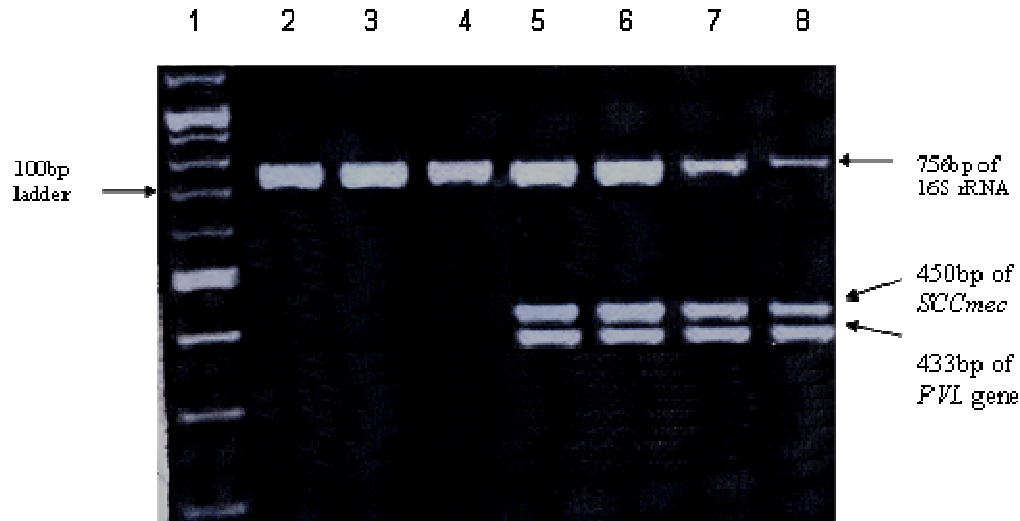


Figure 4. Agarose gel electrophoresis showing amplification of 433 and 450 bp fragments of *PVL* gene and *SCC mec IVa* gene in lanes 5, 6, 7, and 8. Lanes 2, 3, 4, 5, 6, 7 and 8 showing amplification of 756 bp fragments of 16S rRNA. Lane 1 shows 100 bp ladder.

previously identified phenotypically as *S. aureus* with bacteriological examination which indicate the higher sensitivity and specificity of PCR, in addition to the time consuming. Our results confirm the conclusion of Jo-Ann et al. (2006), Riffon et al. (2001) and Moussa and Shible (2009). PCR could detect also the *mecA* gene in all 127 strains (100%) which appeared methicillin / oxacillin resistant phenotypically with antimicrobial susceptibility test and amplifications of 1399 base pair fragments specific for *mecA* gene were observed as shown in Figure 2, which indicated the higher sensitivity of PCR as a rapid test for detection of *mecA* gene (Baddour et al., 2007; Frebourg et al., 1998; Salisbury et al., 1997, Unal et al., 1992; Moussa and Shible, 2009). At the same time, the other 8 strains which appeared methicillin / oxacillin sensitive phenotypically were positive with PCR and harbored the *mecA* gene which indicated that PCR could detect the *mecA* gene even it is not expressed phenotypically when examined with disk diffusion method (Weller, 1999), our results coincide with the results observed with Baddour et al. (2007) and Moussa and Shible (2009), they detected 39 strains harboring *mecA* gene with PCR while with disk diffusion methods only 33 strains were resistant and they concluded that the use of more than one screening method is necessary to detect all MRSA isolates in clinical settings.

The present study is also aimed to investigated the hospital acquired MRSA with multiplex PCR targeting at the same time *S. aureus* species specific 16S rRNA, (*SCCmec*) type IVa and *PVL* genes to determine the percent of CA-MRSA and *PVL* gene due to the involvement of *PVL* gene with sever skin and soft tissue infections specially necrotizing skin infection (Couppie et al., 1994; Cribier et al., 1992; Prevost et al., 1995). Results observed in Figure 4 revealed positive

amplification of 756 base fragments specific for 16S rRNA of *S. aureus* with all 135 strains (100%) but only 18 strains (13.33%) out of 135 tested strains showed positive amplification of 433 and 450 base pair fragments specific for *lukS/F-PV* and *SCCmec* subtype IVa genes, respectively, such strains could be identified as CA-MRSA as they harboring the marker genes (*PVL* and *SCCmec* type IV) of CA-MRSA as mentioned by Vandenesch et al. (2003) and Zhang et al. (2005) as shown in Figures 3 and 4. The percent of CA-MRSA obtained in this study indicated that there is an increase in the number of patient with CA-MRSA in KSA, which confirm the conclusions of Bukharie et al. (2001), they recorded that the number of patient with CA-MRSA disease increased from a single patient in 1998 to fifteen patient in the year 2000 and they suggested that MRSA is an emerging community pathogen, also confirm the conclusion of Moussa and Shible (2009) which could detect 3 CA-MRSA strains (8.1%) out of 37 tested strains and they concluded that the percent of CA-MRSA had been increased in KSA. All 18 isolates which harboring the *PVL* gene were isolated from cases of skin and soft tissue infections which indicated that most cases of CA-MRSA were recovered from skin and soft tissue infections specially necrotizing skin infection (Couppie et al., 1994, Cribier et al., 1992; Prevost et al., 1995; Moussa and Shible, 2009).

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