Molecular characterization of methicillin-resistant *Staphylococcus aureus* recovered from outpatient clinics in Riyadh, Saudi Arabia

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ABSTRACT

الأهداف : فحص العترات المعزولة مظهريا باستخدام الطرق التقليدية، وجينياً باستخدام اختبار البلمرة المتسلسل (PCR) للكشف عن جين (S. aureus 16S rRNA)الخاص بالمكور العنقودي الذهبي وجين المقاومة للميثيللين (mecA). ثانيا تقديم اختبار بلمرة متسلسل متعدد يستطيع في نفس الوقت الكشف عن (S. aureus 16S rRNA) وجين (PVL) وجين (SCC) من النوع الرابع المميزين للمكور العنقودي الذهبي المكتسب من المجتمع.

الطريقة : تم فحص 37 عتره من المكور العنقودي الذهبي مظهرياً خلال عام 2007م، من العيادات الخارجية بمستشفى الملك خالد الجامعي – الرياض – المملكة العربية السعودية. تم فحص العينات في كلية الصيدلة باستخدام الطرق التقليدية، وجينيا باستخدام اختبار البلمرة المتسلسل (PCR) للكشف عن (S. aureus 16S rRNA) الخاص بالمكور العنقودي الذهبي وجين المقاومة للميثيللين. وفي نفس الوقت تم فحص جميع العترات باستخدام اختبار البلمرة المتسلسل المتعدد (multiplex-PCR) للكشف عن (S. aureus 16S rRNA) وجين (PVL) وجين (SCCmec) النوع الرابع.

النتائج : استطاع اختبار البلمرة المتسلسل (PCR) أن يكشف جميع الـ37 عتره (100%) الايجابية للفحص البكتريولوجي والايجابية للمقاومة للميثيللين (100%)، وباستخدام تفاعل البلمرة المتسلسل المتعدد (multiplex-PCR) أظهرت النتائج وجود ثلاث حالات فقط بنسبة (8.1%) معزولة من إصابات في الجلد والأنسجة الرخوة ايجابية لكلا من جين (PVL) وجين (SCCmec) النوع الرابع المميزين للمكور العنقودي الذهبي المكتسب من المجتمع.

خاتمة : نتائج هذه الدراسة أظهرت إمكانية استخدام اختبار البلمرة المتسلسل (PCR) للكشف عن (S. aureus 16S rRNA) الخاص بالمكور العنقودي الذهبي وجين المقاومة للميثيللين، وفي نفس الوقت أظهرت الدراسة أن اختبار البلمرة المتسلسل (PCR) المتعدد المستخدم في هذه الدراسة يمكن استخدامه في سرعة الكشف عن عترات المكور العنقودي الذهبي المكتسب من المجتمع.

Objectives: To examine the recovered strains phenotypically, by conventional methods and genotypically by polymerase chain reaction (PCR), for direct detection of *Staphylococcus aureus (S. aureus)* 16S ribosomal Ribonucleic Acid (rRNA) gene (which serves as an internal control) and *mec*A gene. Secondly, introduce multiplex PCR targeting at the same time *S. aureus* 16S rRNA, Panton-Valentine Leucocidin (PVL), and staphylococcal cassette chromosome *mec* (SCC*mec*) type IV.

Methods: Thirty-seven strains of *S. aureus* collected in 2007 from outpatient clinics in King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia, were tested in the College of Pharmacy phenotypically by conventional methods and genotypically by PCR for direct detection of *S. aureus* 16S rRNA and *mec*A genes. All the 37 strains, were tested also by multiplex PCR targeting at the same time *S. aureus* 16S rRNA, PVL, and (SCC*mec*) type IV.

Results: Polymerase chain reaction detected all the 37 bacteriologically positive *S. aureus* (100%) and the *mec*A gene in all strains phenotypically resistant to methicillin (100%), at the same time it detected the *mec*A gene in 2 strains phenotypically sensitive to methicillin. Only 3 strains (8.1%) recovered from skin and soft tissue infections were positive for PVL and SCC*mec* type IV.

Conclusion: The PCR assay can be used for rapid detection of *S. aureus* and *mec*A gene. At the same time the multiplex PCR assay explained in this study is a rapid, sensitive, and reliable test for direct detection of community-acquired methicillin-resistant *S. aureus*.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been associated with nosocomial infections (hospital-acquired MRSA [HA-MRSA] strains). Despite its spread in hospitals and nursing homes, MRSA has not disseminated into the wider community until recently.[1] 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.[2-5] Panton-Valentine Leucocidin (PVL), is a highly powerful cytotoxin that affects human and rabbit mononuclear cells.[6] When injected intradermally into rabbits, it induces severe inflammatory lesions, leading to capillary dilation, chemotaxis, polymorphonuclear karyorrhexis, and skin necrosis.[7] Studies have shown that its toxic effect results from the synergistic action of 2 separate exoproteins, namely, LukS-PV and LukF-PV. These proteins are encoded by 2 contiguous and cotranscribed genes (LukS-PV and LukF-PV),[8] which are carried on temperate bacteriophages.[9] Lysogenic conversion of PVL - negative strains of *Staphylococcus aureus (S. aureus)* leading to the production of the toxin has been demonstrated.[10] Several authors suggest characterization of the PVL gene in *S. aureus* isolated from humans, due to its involvement in severe disease conditions among children and humans even with no exposure to health care establishments. The presence of PVL in *S. aureus* appears to be associated with increased disease severity, ranging from cutaneous infection requiring surgical drainage to severe chronic osteomyelitis, and deadly necrotizing pneumonia.[11-15] In the United States of America, outbreaks of severe skin infections have occurred in homosexual men, prison inmates, and schoolchildren,[16] similarly, PVL-related skin infections have been reported in the gay community in The Netherlands[17] and in schoolchildren in Switzerland.[18] More recently, cases of community-acquired pneumonia due to PVL-positive *S. aureus,* have been reported in France,[19-21] Sweden,[22] The Netherlands,[23] and the United Kingdom.[14] The PVL genes have been identified as a stable marker of community-acquired MRSA strains worldwide,[5] in addition to small mobile staphylococcal cassette chromosome *mec* (SCC*mec*) type IV or V genetic element, which harbors the methicillin resistance (*mec*A) gene, and which is more easily transferred to other strains of *S. aureus* than the large SCC*mec* types (type I to III) that are prevalent in HA-MRSA strains.[4,5,24] In the future, screening for the PVL virulence factor in *S. aureus* may become a routine laboratory procedure;[25] therefore, there are many conventional PCR methods for the detection of *S. aureus* 16S rRNA gene, PVL gene, *mec*A gene, and SCC*mec* type IV gene, which require the use of separate assays.[26-28]

The purpose of this study was, firstly, characterization of the recovered strains 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 *mec*A gene, secondly, to introduce a multiplex PCR target at the same time *S. aureus* species specific 16S rRNA, (SCC*mec*) type IV and PVL genes.

Methods. The bacterial isolates used in this study, were 37 strains of *S. aureus* that were collected in 2007 from outpatient clinics in King Khalid University Hospital, Riyadh, Kingdom of Saudi Arabia, including 20 strains recovered from skin and soft tissue infection, 12 strains recovered from abscess, and 3 strains recovered from cellulites, while the other 2 strains were recovered from infected ulcers. All isolates were identified according to colonial and microscopical morphology, catalase and coagulase production, and novobiocin sensitivity. Antimicrobial susceptibility testing to a range of antimicrobial agents including methicillin, oxacillin, chloramphenicol, tetracycline, fusidic acid, gentamicin, erythromycin, ciprofloxacin, and vancomycin was carried out 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 the Clinical Laboratory Standards Institute (CLSI) formally the National Committee for Clinical Laboratory Standards, (NCCLS), 2002. Deoxyribonucleic acid (DNA) was extracted from the bacteriologically positive strains. One milliliter of the bacterial suspension was transferred to a microcentrifuge tube with a capacity of 1.5 ml and the genomic DNA was extracted.[29] An aliquot of 5 ml of the supernatant was used as template DNA in the PCR.

For amplification of 16S rRNA gene specific for *S. aureus* and *mec*A gene, 2 sets of a primer pairs were used, (TIB, Molbiol, Berlin, Germany) the first pair was (SauF 234: CGA TTC CCT TAG TAG CGG CG and SauR 1501: 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.[30] The second pair was (MR1: GTG GAA TTG GCC AAT ACA GG and MR2 : TAG GTT CTG CAG TAC CGG AT primers), which can amplify 1399 base pair fragments specific for *mec*A gene and its annealing temperature is 58°C.[31] **Table 1** shows the specificity, nucleotide sequence, size of amplified fragments, and annealing temperature of the primers. All reactions were carried out separately in a final volume of 50μl in micro-amplification tubes (PCR tubes). The reaction mixtures consisted of 5μl of the extracted DNA template from the bacterial isolates, 5μl 10 x PCR buffer (75 mM Tris

hydroxymethyl (Tris-HCl), pH 9.0, 2 mM magnesium chloride (MgCl$_2$), 50 mM potassium chloride (KCl), 20 mM ammonium sulphate (NH$_4$)2SO$_4$, 1 µl deoxynucleotide triphosphates (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 50µl using deionized distilled water (DDW). Approximately 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 one minute, annealing at 70°C for SauF234, and SauR1501 primers, and at 58°C for MR1 and MR2 primers for one minute and extension at 72°C for one minute). Final extension was 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.

For detection of *S. aureus* species, specific 16S rRNA (SCC*mec*) type IV and PVL genes Multiplex PCR was performed as follows: 3 sets of primer pairs were used, the first was, Staph756F and Staph750R primers, which could amplify 756 base pair fragments specific for 16S rRNA of *S. aureus*, the second 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.[29] The third was, SCC*mec* 4a1 and SCC*mec* 4a2 primers, which could amplify 450 base pair fragments specific for SCC*mec* subtype IVa gene.[26] **Table 1** shows the specificity, nucleotide sequence, size of amplified fragments, and annealing temperature of the primers. The reaction mixtures as mentioned before, and the 3 sets of primer pairs were used in each reaction mixture 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 one minute, and 72°C for 1.5 min, and 25 cycles of 94°C for one minute, 50°C for one minute, 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. The PCR products were tested for positive amplification by agarose gel electrophoresis, previously reported[32] using suitable molecular weight markers (100 base pair ladder and HaeIII digest marker).

Results. The antimicrobial susceptibility patterns of 35 out of 37 tested strains, were resistant to methicillin and oxacillin, which was confirmed by the presence of the *mec*A gene using the PCR, while the other 2 strains, which appeared methicillin/oxacillin sensitive phenotypically, were positive with PCR and harbored the *mec*A gene. Seventeen strains out of 37 tested strains (45.6%) were resistant to chloramphenicol, 14 strains (37.9%) were resistant to tetracycline, 11 strains (29.8) were resistant to fusidic acid, 10 strains (27%) were resistant to gentamicin, and 8 strains (21.7%) were resistant to erythromycin. Only 2 strains (5.4%) were resistant to ciprofloxacin, while all isolates were susceptible to vancomycin. The amplification of 1267 base pair fragments specific for 16S rRNA of *S. aureus* using SauF234 and SauR1501 primers, revealed positive amplification of 1267 base fragments with all 37 isolates (100%) previously, identified phenotypically as *S. aureus* with bacteriological examination specific for 16S rRNA of *S. aureus* as shown in **Figures 1a & 1b.** For amplification of 1339 base pair fragments specific for the *mec*A gene using MR1 and MR2 primers, all the 35 strains (100%), which appeared methicillin/oxacillin resistant phenotypically with antimicrobial susceptibility test were positive for amplification of 1339 base pair fragments specific for the *mec*A gene.

Table 1 - Primer sets used in polymerase chain reaction (PCR) and multiplex PCR; specificity, nucleotide sequence, size of amplified fragments and annealing temperature.

Primer name	Target gene	Specificity	Primer sequence (5'-3')	Size of amplified fragment	Annealing temperature
SauF234	16S rRNA	*S. aureus*	CGA TTC CCT TAG TAG CGG CG	1267 base pair	70 °C
SauR1501			CCA ATC GCA CGC TTC GCC		
MR1	*mec*A	MR	GTG GAA TTG GCC AATACA GG	1339 base pair	58°C
MR2			TGA GTT CTG CAG TAC CGG AT		
Staph756F	16S rRNA	*S. aureus*	AACTCTGTTATTAGGGAAGAAC	756 base pair	55°C
Staph750R			CCACCTTCCTCCGGTTTGTCACC		
Luk-PV-1	lukS/F-PV	PVL S/F proteins	ATCATTAGGTAAAATGTCTGGACATGATCCA	433 base pair	55°C
Luk-PV-2			GCATCAAGTGTATTGGATAGCAAAAGC		
SCC*mec*4a1	SCC*mec* type IV	Type IV SCC*mec*	TTTGAATGCCCTCCATGAATAAAAT	450 base pair	55°C
SCC*mec*4a2			AGAAAAGATAGAAGTTCGAAAGA		

SauF - *Staphylococcus aureus* forward, SauR - *Staphylococcus aureus* reverse, MR - Methicillin resistant, SCC*mec* - Staphylococcal cassette chromosome methicillin, rRNA - ribosomal ribonucleic acid, PVL - Panton-valentine leucocidin

At the same time, the other 2 strains, which appeared methicillin/oxacillin sensitive phenotypically were positive with PCR and harbored the *mec*A gene as shown in **Figures 2a & 2b**. Furthermore, Multiplex PCR for detection of *S. aureus* species-specific 16S rRNA, (SCC*mec*) type Iva and PVL genes were performed. All the 37 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 Staph756F and Staph750R primers, while only 3 strains showed positive amplification of 433 and 450 base pair fragments specific for LukS/F-PV and SCC*mec* subtype

Iva genes using Luk-PV-1 and Luk-PV-2 primers and SCC*mec* 4a1 and SCC*mec* 4a2 primers as shown in **Figure 3**. The 3 isolates harboring the PVL and SCC*mec* subtype Iva genes were isolated from skin and soft tissue infections.

Discussion. The conventional methods used for identification of *S. aureus* and MRSA isolates are time consuming and the reliability of these methods have been reported to be between 80-95%, while the alternative molecular techniques, mostly based on PCR for amplification of 16S rRNA specific for *S. aureus*[29,30] and *mec*A gene specific for methicillin resistant[33-35]
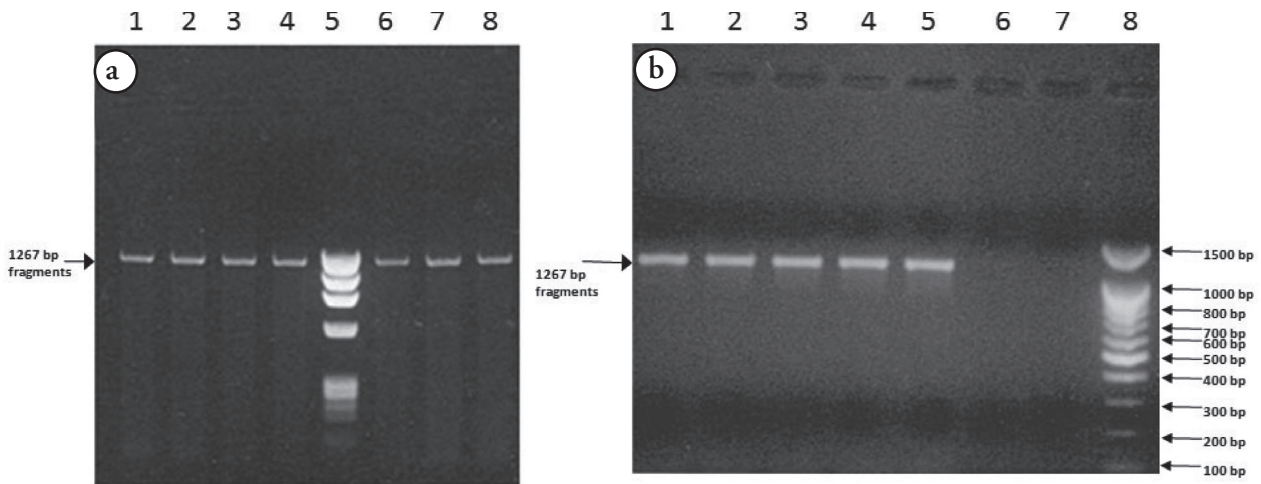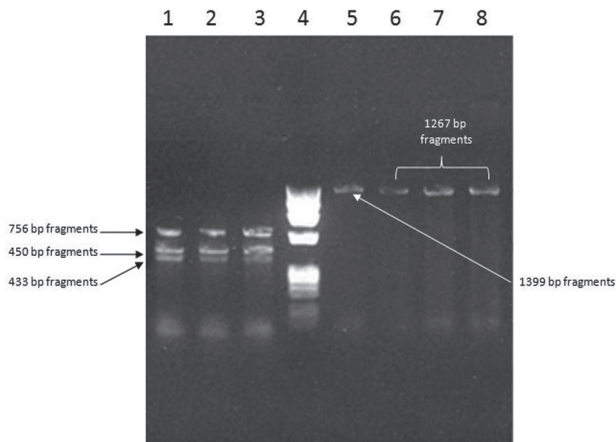

Figure 1 - Agarose gel electrophoresis showing positive amplification of 1267 base fragments specific for (rRNA of *S. aureus* using SauF234 and SauR1501 primers lanes **a)** 1, 2, 3, 4, 6, 7, and 8. Lane 5 showing Hae III digest marker. **b)** 1, 2, 3 4, and 5. Lane 6 and 7 showing negative control while lane 8 showing 100 bp ladder. rRNA - ribosomal ribonucleic acid, *S. aureus* - *Staphylococcus aureus*, SauF - *Staphylococcus aureus* forward, SauR - *Staphylococcus aureus* reverse
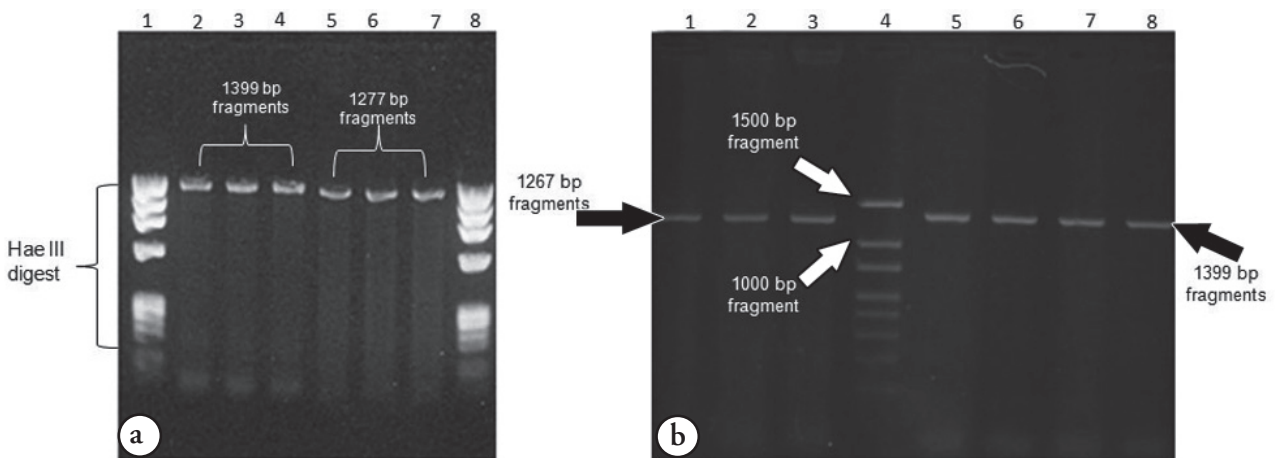
Figure 2 - Agarose gel electrophoresis showing positive amplification of 1267 base fragments specific for 16S rRNA of *S. aureus* using SauF234 and SauR1501 primers lanes 2, 3, and 4 and positive amplification of 1339 base pair fragments specific for *mec*A gene using **a)** MR1 and MR2 primers lane 5, 6, and 7. Lane 8 showing Hae III digest marker. **b)** MR1 and MR2 primers lane 5, 6, 7, and 8. Lane 4 showing 100 bp ladder. rRNA - ribosomal ribonucleic acid, *S. aureus* - *Staphylococcus aureus*, SauF - *Staphylococcus aureus* forward, SauR - *Staphylococcus aureus* reverse, MR - methicillin resistant

Figure 3 - Agarose gel electrophoresis showing positive amplification of 756 base fragments specific for 16S rRNA of *S. aureus* using Staph756F and Staph750R primers, and positive amplification of 433 and 450 base pair fragments specific for LukS/F-PV and SCC*mec* subtype IVa genes using Luk-PV-1 & Luk-PV-2 primers and SCC*mec* 4a1 and SCCmec 4a2 primers, lanes (1, 2, and 3). Lane 5 showing1399 bp fragment specific for *mec*A gene while lanes 6, 7, and 8 showing amplification of 1267 base fragments specific for 16S rRNA of *S. aureus* using SauF234 and SauR1501 primers. Lane 4 showing HaeIII digest marker. rRNA - ribosomal ribonucleic acid, *S. aureus* - *Staphylococcus aureus*, SCC*mec* - Staphylococcal cassette chromosome methicillin, SauF - *Staphylococcus aureus* forward, SauF - *Staphylococcus aureus* forward

have been reported for the rapid and specific detection and characterization of MRSA. Therefore, our study limits for 2 main objectives; firstly, the use of PCR for rapid and specific detection of 16S rRNA specific for *S. aureus* and the *mec*A gene specific for methicillin/oxacillin resistant. Secondly, the use of a multiplex PCR for detection of *S. aureus* species specific 16S rRNA, (SCC*mec*) type IV and PVL genes at the same time as a rapid method for detection of CA-MRSA and PVL gene due to the involvement of PVL gene with severe skin and soft tissue infections, especially necrotizing skin infection[8,37,38] as well as it is considered as a stable marker for CA-MRSA,[5,39-41] in addition to SCC*mec* type IV.[4,5,24] Results observed in **Figures 1a & 1b** revealed positive amplification of 1267 base pair fragments specific for 16S rRNA of *S. aureus* with all 37 strains (100%) previously identified phenotypically as *S. aureus* with bacteriological examination, which indicates the higher sensitivity and specificity of the PCR, apart from the time saving. Our results confirm the conclusion of many authors,[28,30] they stated that the main advantage of PCR method over the conventional methods is the higher sensitivity and specificity in addition to its rapid detection. The PCR can also detect the *m ec*A gene in all 35 strains (100%), which appeared methicillin/oxacillin resistant phenotypically with antimicrobial susceptibility testing and amplifications of 1399 base

pair fragments specific for *mec*A gene were observed as shown in **Figures 2a & 2b**, which indicated the higher sensitivity of PCR as a rapid test for detection of mecA gene.[33-36] At the same time, the other 2 strains, which appeared methicillin/oxacillin sensitive phenotypically were positive with PCR and harbored the *mec*A gene, which indicated that PCR could detect the *mec*A gene even it is not expressed phenotypically when examined with the disk diffusion method.[31] Our results coincide with the results observed with Baddour,[36] they detected 39 strains harboring the *mec*A 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.

Nevertheless, results observed in **Figure 3** revealed positive amplification of 756 base fragments specific for 16S rRNA of *S. aureus* with all 37 strains (100%), but only 3 strains (8.1%) out of 37 tested strains showed positive amplification of 433 and 450 base pair fragments specific for lukS/F-PV and SCC*mec* subtype Iva genes, such strains could be identified as CA-MRSA as they are harboring the marker genes (PVL and SCC*mec* type IV) of CA-MRSA5,[24] as shown in **Figure 3**. The percent of CA-MRSA obtained in this study indicated that there is an increase in the number of patients with CA-MRSA in KSA, which confirm the conclusion of Bukharied et al.[42] They recorded that the number of patient with CA-MRSA disease increased from a single patient in 1998 to 15 patients in the year 2000, and they suggested that MRSA is an emerging community pathogen. It is also noticed that the 3 isolates that harbor 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, especially necrotizing skin infection.[8,37,38] However, the previously mentioned multiplex PCR represents a new tool for simple, rapid, and early reliable testing for detection of CA-MRSA strains.

Therefore, the results of this study indicate that the PCR assay can be used for rapid detection of *S. aureus* and the *mec*A gene and at the same time, the multiplex PCR assay explained in this study is rapid, sensitive, and a reliable test for direct detection of community-acquired MRSA.

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