

Identification of *Staphylococcus aureus* and Coagulase-negative *Staphylococcus* (CoNS) as well as Detection of Methicillin Resistance and Panton-Valentine Leucocidin by Multiplex PCR

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Methicillin-resistant *S. aureus* (MRSA) is one of the most important pathogens that cause a wide range of hospital and community-acquired infections worldwide. In the present study, the Multiplex PCR test was employed to detect the genes *16S rRNA* (*Staphylococcus* genus specific), *femA* (encoding a factor essential for methicillin resistance), *mecA* (encoding high-level methicillin resistant) and *lukS* gene (encoding Panton-Valentine leukocidin [PVL]). The results showed that all isolates harbored the 16S rRNA. Fifty-six (56 %) of these were determined as methicillin-resistant *S. aureus*, while 21 (70 %) were methicillin-resistant coagulase-negative staphylococci. 9% of *S. aureus* isolates harbored the *lukS* gene. This multiplex PCR assay represents a simple, rapid, reliable approach for the detection of methicillin-resistant staphylococci, evaluate the frequency of virulence factor in community- associated MRSA and discrimination of *S. aureus* from CoNS isolates.

Key words: coagulase-negative *Staphylococcus* (CoNS),
Panton-Valentine Leucocidin (PVL), Multiplex PCR, methicillin.

Staphylococcus aureus is a major nosocomial and community- acquired pathogen that causes a range of diseases, from mild skin infections to more serious invasive infections, including septicemia, pneumonia, endocarditis, and deep-seated abscesses¹. Coagulase-negative *Staphylococcus* is the most frequently reported

pathogen in nosocomial bloodstream infections and it is often associated with implanted foreign bodies and intravascular catheters^{2,3}.

During the past decade, increasing rates of antibiotic resistance, in particular methicillin resistance, among *S. aureus* strains and CoNS, adding complexity to the treatment of these organisms⁴.

In the early 1960s, methicillin, a semisynthetic penicillin was developed. However, soon after the introduction of methicillin, strains of *S. aureus* with methicillin resistance were reported⁵.

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Resistance to methicillin is mediated by the presence of the *mecA* gene and regulatory sequences that encode for production of a low-affinity penicillin-binding protein (PBP2A) in staphylococcal species⁶.

PCR detection of the highly conserved *mecA* gene encoding PBP2a is a useful molecular marker for identification of methicillin resistance in *S. aureus* and CoNS⁷.

Another chromosomal element, *femA*, which encodes a protein (Fem) and cooperates with *mecA* for the expression of methicillin resistance in staphylococci⁸, *femA* appears to be a unique feature of *S. aureus* and it is considered reliable molecular marker at the species level^{3,9}.

Some *Staphylococcus aureus* strains carry genes encoding Panton–Valentine leukocidin (PVL), *lukF* and *lukS*, which are harbored on a temperate bacteriophage. The presence of PVL genes are closely associated with necrotizing pneumonia and skin and soft-tissue infections, which account for the majority of community-associated methicillin-resistant *S. aureus* (CA-MRSA) infections². These isolates are referred to as CA-MRSA often differ from hospital-associated MRSA (HA-MRSA) strains by the allocation of their staphylococcal chromosomal cassette (*SCCmec*) types, IV and V. On the other hand, CA-MRSA strains are resistant to many β -lactam antibiotics^{10,11}.

In the present study, using multiplex PCR assay for the detection of *Staphylococcus* genus (16S rRNA gene), to determine the prevalence of methicillin-resistant staphylococci (*mecA* gene) and also to evaluate the frequency of virulence factor in community-associated MRSA (*lukS* gene) as well as discrimination between *S. aureus* and CoNS (*femA* gene).

MATERIALS AND METHODS

Bacterial Isolates

A total of one hundred and thirty clinical staphylococcal isolates were used in this study.

Identification of *Staphylococcus* spp. from Clinical Specimens by the Conventional Method and Susceptibility Testing

The bacterial isolates were cultured aerobically in blood and MacConky agar. The plates were incubated overnight at 37°C. The

isolates of *S. aureus* and CoNS were identified by gram staining, catalase test, mannitol fermentation and DNase test by standard laboratory procedures¹². The coagulase plasma test was done to discriminate between *S. aureus* and CoNS. Screening for methicillin resistance was done by the disk diffusion method, in which the methicillin disk 5µg (Mast Co, Merseyside, UK) was placed on Mueller-Hinton agar and incubated at 37°C for 24 h. The result was determined according to the Clinical and Laboratory Standards Institute (CLSI) recommendations¹³. pvl-positive MSSA (ATCC 25923), pvl-negative MRSA (ATCC 33591) and methicillin-susceptible *S. epidermidis* (ATCC 14990) were used as quality control strains.

Bacterial DNA Extraction

The isolated colonies from blood agar were inoculated into LB broth and incubated at 37°C for 18 h. Bacterial lysates for PCR were prepared by centrifuging the 600 µl culture at 15,700× g for 2 min; the supernatant was removed and the pellets were resuspended in 200 µl of lysis solution (25 mM TrisHCl, 10 mM EDTA, twenty units of lysostaphine [pH 8.0]) and the suspension was incubated at 37°C for 30 min. Then Phenol-chloroform extraction was performed for DNA extraction¹⁴.

Multiplex Polymerase Chain Reaction

The oligonucleotide primers used in this study have been previously described [10]: 16S rRNA-F (5'-GCAAGCGTTATCCGGATTT-3') and 16S rRNA-R (5'-CTTAATGATGGCAACTAAGC-3') for 16S rRNA, *mecA*-F (5'-ACGAGTAGATGCTCAATATAA-3') and *mecA*-R (5'-CTTAGTTC TTTAGCGATTGC-3') for *mecA*, *femA*-F (5'-CGATCCATATTTACCATATCA-3') and *femA*-R (5'-ATCACGCTCTTCGTTTAGTT-3') for *femA*, *lukS*-F (5'-CAGGAGGTAATGGTTCATTT-3') and *lukS*-R (5'-ATGTCCAGACATTTTACCTAA-3') for *lukS*. The multiplex PCR reaction mixture for the detection of the 16S rRNA, *mecA*, *femA* and *lukS* genes was consisted of 2 µl of sample containing template DNA, 5 U *Taq* DNA polymerase, 3mM MgCl₂, 1× PCR amplification buffer, 10 pmol of each *femA* primer, 20 pmol of each 16S rRNA, *mecA* and *lukS* primer and 200 µM deoxynucleotide triphosphate (dNTPS). A total of 30 cycles were used to amplify 293 bp of *mecA* gene. The PCR was performed by the thermocycler (Eppendorf Mastercycler®, MA) with one cycle of initial

denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 60°C, and extension at 72°C for 30 s, followed by an extra cycle of annealing at 60°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were stained with ethidium bromide and visualized on 1.5% agarose gel with a UV light transilluminator. Control marker with molecular mass of 100bp was used.

Nucleotide Sequencing and Submission

PCR products for *mecA* and *femA* genes were purified and sequenced using the ABI Capillary System (SEQLAB, Berlin, Germany). Sequence was analyzed using online BLAST software (<http://www.ncbi.nlm.nih.gov/BLAST/>) and submitted to the EMBL/GenBank database (www.ncbi.nlm.nih.gov).

RESULTS

Incidence of staphylococcal Species in Clinical Specimens

From the 130 *Staphylococcus* spp. isolates, 100(76.9%) strains were characterized as *S. aureus* and 30 isolates (23%) were coagulase-negative staphylococci. They were identified as *S. aureus* and CoNS by conventional phenotypic tests. *S. aureus* strains were isolated from wound, blood, tracheal aspirate, urine and all CoNS strains were isolated from blood of patients (Table 1). According to the Table 1, most of the MRSA were isolated from blood (75%) and then tracheal aspirate (42%), urine (30%), wound (29%).

Methicillin Susceptibility Testing

Of the one hundred and thirty isolates, 58(58%) of *S. aureus* and 22(73.3%) of CoNS were determined to be methicillin resistant with Kirby–Bauer disk diffusion method as previously described¹⁵.

Multiplex PCR for Identifying staphylococci, Discriminating *S. aureus* from CoNS and Other Bacteria, and Detecting Methicillin Resistance

Staphylococcus spp. isolates were analyzed by multiplex PCR in order to detect 16S rRNA, *femA*, *mecA* and *lukS* genes (Fig. 1). The molecular weight of PCR products were indicated in Table 2. The frequency of each amplified gene is summarized in Table 3. The multiplex PCR procedure allowed the specific identification of the staphylococcal species (*S. aureus* or CoNS). All isolates were positive for 16S rRNA gene. *mecA* was detected in 100% of methicillin-resistant isolates (MRSA and MR CoNS strains), in 2% of methicillin-SSA, and in 1% of methicillin-SCoNS. The frequency of *femA* in *S. aureus* were 100%, but neither of these genes were found in CoNS. Thus, there was 100% agreement between the conventional identification results and the amplification of the 450 bp fragment of the

Table 1. Methicillin resistance among different clinical isolates of *Staphylococcus aureus* isolates

Origin	Number (%) of Methicillin resistance <i>S. aureus</i>
Tracheal(15)	6(42)
Urine(28)	24(30)
Wound(27)	20(29)
Blood(30)	8(75)
Total(100)	58

Table 2. Molecular weight of PCR products

Gene	Size of Target(bp)
16S rRNA	597
<i>FemA</i>	450
<i>mecA</i>	293
<i>lukS</i>	151

Table 3. Prevalence of *femA*, *mecA*, *lukS* genes in staphylococci (n = 130)^a

Staphylococci	<i>femA</i>	<i>mecA</i>	<i>lukS</i>
<i>S. aureus</i>	100/100(100)	56/100 (56)	9/100(9)
CoNS	0/30(0)	21/30(70)	0/30(0)
Total	100/130(76.9)	77/130 (59.2)	9/130 (6)

^aPercentage in parentheses

species-specific gene, *femA* (Fig. 1). Moreover, this multiplex PCR procedure was rapid, compared with the conventional tests. The *lukS* gene was detected in 9(9%) of *S. aureus* isolates. All isolates harbored the related *lukS* gene obtained from patients with wound infections. In addition, the presence of virulence gene was associated with the *mecA* gene.

Nucleotide Sequence Accession Numbers

The partial genome sequence related to the *mecA* and *femA* genes have been deposited in NCBI GenBank under the accession no. JN712763 and JN712762.

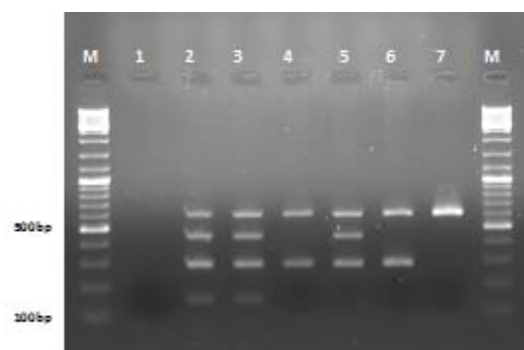


Fig. 1. Agarose gel electrophoresis patterns showing mPCR amplification products from different *S. aureus* and CoNS isolates. Lane M, 100 bp marker; Lane 1, negative control; lanes 2, Positive control for (*16S rRNA*, *femA*, *mecA*, *lukS*); lanes 3, methicillin-resistant *S. aureus* isolate (*16S rRNA*, *femA*, *mecA*, *lukS*); Lane 4 and 6, methicillin-resistant CoNS isolate (*16S rRNA*, *mecA*); lanes 5, methicillin-resistant *S. aureus* isolate (*16S rRNA*, *femA*, *mecA*); lanes 7, methicillin-sensitive CoNS (*16S rRNA*).

DISCUSSION

S. aureus and CoNS are major causes of community-acquired and nosocomial infections¹⁶. MRSA and MRCoNS have spread throughout the world and have become highly endemic in many geographical areas¹⁷. Therefore, rapid identification and discrimination of *S. aureus* and CoNS and detection of methicillin-resistant are important, helping to avoid morbidity and spread of infections. Furthermore, phenotypic techniques for methicillin resistance can be influenced by environmental factors such as temperature, pH and salt concentration. So, PCR assays have become an essential tool in laboratory programs^{10, 18}.

The present study represents a simple, rapid, and reliable approach for the simultaneous identification of the *Staphylococcus* genus from others, and allow to detect of methicillin -resistant staphylococci, and concomitant detection of PVL genes and discrimination of *S. aureus* from CoNS isolates.

S. aureus isolates were analyzed by multiplex PCR in order to detect *16S rRNA*, *femA*, *mecA* and *lukS* genes (Fig. 1). The *16S rRNA* gene has been detected in all strains. One hundred isolates out of 130 isolates were positive for *femA* gene. None of the CoNS isolates was positive for *femA* gene. In this study there was perfect correlation between the conventional phenotypic tests and molecular technique results for identification of *S. aureus*⁸.

The *mecA* gene is unique to methicillin-resistant staphylococci. Accordingly, detection of the *mecA* gene provides a useful molecular component for rapid identification of MRSA and methicillin-resistant CoNS by PCR. Comparison of results between methicillin disk diffusion method and PCR assay revealed two of the 58 MRSA and one of the MR CoNS isolates was to be *mecA*-negative according to multiplex PCR assay. The heterogeneous expression of methicillin resistance can make it difficult to determine the resistance with the disc diffusion test definitively¹⁹.

In this study PVL has been detected only in strains of methicillin -resistant *S. aureus*. In addition, PVL positive *S. aureus* was isolated from wound sample, which is in agreement with the findings of previous studies¹. That there was a significant relation between skin infections and the presence of *pvl* genes.

CONCLUSION

Multiplex PCR assay for *16S rRNA*, *mecA*, *femA* and *lukS* provides reliable and unequivocal results for identification of *Staphylococcus* spp., virulence and their resistance to methicillin. The benefit of accuracy and speed in simultaneous identification of species and methicillin susceptibility, and the determination of correct and early treatment are of great importance in hospitals of areas where MRSA is endemic.

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