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Brief Communication

VALIDATION OF MULTIPLEX PCR STRATEGY FOR SIMULTANEOUS DETECTION AND IDENTIFICATION OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS*

S Rallapalli, S Verghese, *RS Verma

Abstract

Multiplex polymerase chain reaction (PCR) strategy is described for rapid identification of clinically relevant methicillin resistant *Staphylococcus aureus* (MRSA) that targets *mecA* and *coag*ulase genes. In this study, 150 staphylococcal clinical isolates were used that included 40 isolates of MRSA, 55 isolates of methicillin susceptible *S. aureus* (MSSA), 44 isolates of methicillin susceptible *coag*ulase negative *Staphylococcus* spp. (MS-CoNS) and 11 isolates of methicillin resistant *coag*ulase negative *Staphylococcus* spp. (MR-CoNS). Out of 55 *S. aureus* strains, three strains demonstrated *mecA* gene, which appeared to be oxacillin sensitive by disc diffusion. When (MS-CoNS) were evaluated, 10 isolates classified as oxacillin sensitive phenotypically, yielded positive results in PCR method. The results for *mecA* detection by PCR were more consistent with disk susceptibility tests in case of MRSA (100%) and MSSA (95%) isolates. In contrast to above results with MRSA and MSSA, *mecA* detection by PCR in MS-CoNS showed less correlation with disk susceptibility tests (77%). The results for *coag* detection by PCR were consistent with phenotypic tests in all isolates.

Key words: *Methicillin resistance, methicillin resistant Staphylococcus aureus, polymerase chain reaction, coag gene, mecA gene.*

Methicillin resistant staphylococci are significant pathogens causing both nosocomial and community acquired infections. High prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) in hospitals has been reported from many states of India.[1] Methicillin resistance among *S. aureus* isolates has reached phenomenal proportions in Indian hospitals, with some cities reporting 70% of the strains to be resistant to methicillin.[2] In the last few years sensitive molecular typing techniques are leading the way to track the source and transmission route of bacterial pathogens. They have also helped in establishing epidemiological investigations and comparing strains across continents. Detection of *mecA* gene by PCR has been shown to be highly discriminatory in analyzing hospital outbreaks and tracking genetic changes which occur in a relatively short time. The aim of this study was to validate the multiplex polymerase chain reaction (mPCR) technique in Indian isolates by which India specific data will be of immense benefit for the optimal application in our patients.

Beta-lactam antibiotics are the preferred drugs for serious *S. aureus* infections. Since the introduction of methicillin into clinical use, the occurrence of MRSA strains has increased steadily and nosocomial infections have become a serious problem worldwide. Indiscriminate use of multiple antibiotics, prolonged hospital stay, intravenous drug abuse and carriage of MRSA in nose are all important risk factors for MRSA acquisition.[3] In addition, MRSA infected patients require expensive and intensive isolation measures and strict hygiene. To date, the only standardized means of identifying methicillin resistance in the clinical microbiology laboratory are susceptibility tests such as disk diffusion, agar or broth dilution and agar screen methods. The performance of these tests has many drawbacks because factors such as inoculum size, incubation time and temperature, pH of the medium, salt concentration of the medium and exposure to beta lactam antibiotics influences the phenotypic expression of resistance. Rapid and accurate identification of *S. aureus* and its methicillin susceptibility pattern has important implications for therapy and management of both colonized and infected patients. Molecular diagnostic assays based on the detection of the *mecA* gene encountered difficulty in discriminating MRSA from methicillin-resistant *coag*ulase negative *Staphylococcus* species (MR-CoNS), because the *mecA* gene is widely distributed in *S. aureus* as well as in MR-CoNS.

In this study, we have developed and evaluated a mPCR method which allows the detection of MRSA by using primers specific for methicillin resistance and *coag*ulase genes. The purpose of our study was to set up a rapid and reliable identification procedure for MRSA through the amplification of specific gene determinants by PCR in order to efficiently support therapy and eradication of the pathogen. The *coag* gene was used to differentiate between

*Corresponding author: (email: <vermars@iitm.ac.in>)
Department of Biotechnology (SR,RSV), Indian Institute of Technology Madras, Chennai - 600 036, Department of Microbiology (SV), International Centre for Cardio Thoracic and Vascular Diseases, Frontier Lifeline Pvt Ltd, Chennai - 600 101, India

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S. aureus and CoNS, a gene which allows the species-specific identification of *S. aureus*. In addition, MRSA harbour the *mecA* gene encoding methicillin-resistance, which is absent in methicillin susceptible staphylococci.

Materials and Methods

Bacterial isolates

A total of 150 staphylococcal clinical isolates collected between the period 2005 to 2006 were used in this study (which includes reference strain of *S. aureus* ATCC 25923). These strains were all provided by the Department of Microbiology, International Centre for Cardio Thoracic and Vascular Diseases, Frontier Lifeline Ltd, Chennai. All the strains were clinical isolates from different specimens such as pus, blood, sputum and other body fluids. Identification of staphylococcal isolates was done by morphology, Gram stain, standard biochemical characteristics and susceptibility testing. *S. aureus* was identified by using standard tube *coag*ulase test.

Disk diffusion tests

The procedures routinely used in the clinical microbiology laboratories were employed for this study. The concentrations of oxacillin tested were 1µg/mL. Disk diffusion tests were performed with 1µg of oxacillin per disk placed on of Mueller-Hinton agar with 4% NaCl supplementation. The zone of inhibition was determined after 24 hours of incubation at 35°C. Organisms giving an inhibition zone equal to or lesser than 10 mm were interpreted as resistant to oxacillin. Organisms with a zone equal to or greater than 12 mm were interpreted as susceptible while those with an inhibition zone of 11-12 mm were interpreted as intermediate.

Bacterial genomic DNA isolation

Bacterial culture was grown overnight in nutrient broth and 2 mL of the culture was transferred into a microcentrifuge tube and spun for 2 minutes. The pellet was resuspended in 567 µL of TE buffer to which 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K were added, mixed gently and incubated for 1 hour at 37°C. Following this, 100µL of 5M NaCl was added and mixed thoroughly. After addition of 80µL of 10% CTAB-0.7M NaCl solution and the tubes were incubated for 10 minutes at 65°C. Equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed well and centrifuged at 10,000 RPM for 10 minutes. The upper aqueous phase was transferred to a new tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and centrifuged at 10,000 RPM for 10 minutes. The upper aqueous phase was transferred to a new tube and 0.8 volumes of isopropanol was added, mixed gently until the DNA was precipitated. The DNA was washed with 70% ethanol and resuspended in 50 µL TE buffer.

PCR protocol

Two sets of primers were used for the mPCR. The first pair of primers was derived from the region of the *mecA gene*, the forward primer corresponded to nucleotides 1282 to 1303(5' AAAATCGATGGTAAAGGTTGGC) and the reverse primer was complementary to nucleotides 1793 to 1814(5'AGTTCTGCAGTACCGGATTTGC). The second pair of primers was derived from the region of the *coag gene*, the forward primer was 5' CGA GAC CAAGAT TCA ACA AG and the reverse primer was 5'AAA GAA AAC CACTCA CAT CAG T.

Bacterial genomic DNA (aliquot of 1µL containing 50 ng of genomic DNA) was added to PCR mixture consisting ten fold concentrated reaction buffer (500mM KCl, 100mM Tris-HCl, pH 8·3), with final concentrations of 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 0.15 µM of each *coag* primer and 0.1 µM of each *mecA* primer. This mixture was supplemented with 2U of Taq DNA polymerase. The final reaction volume for PCR was 20 µL. DNA amplification was carried out in an automated thermocycler (MJ Research PTC-200). After an initial denaturation step for 5 minutes at 95°C, 40 cycles of amplification were performed as follows: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and DNA extension at 72°C for 90 seconds, followed by an additional cycle of 5 minutes at 72°C to complete partial polymerizations. Amplified products were analyzed using horizontal 1·5% agarose gel electrophoresis.

Results

MRSA strains were unequivocally detected within three hours using multiplex PCR with *coag* and *mecA* gene-specific oligonucleotides. For MRSA strains, two discrete DNA fragments, a 533 bp *mecA* and 810 bp *coag* specific products were obtained. The *mecA* fragment was amplified in all the methicillin resistant *Staphylococcus* isolates. Amplification product was also detected for methicillin susceptible strains. The *coag* gene fragment was observed in all *S. aureus* strains, whereas such amplification did not occur in any of the CoNS. No non-specific background amplification products were observed.

The optimization of the multiplex PCR for this protocol was done by following the general principles. [4] Each pair of primers was first tested for amplification specificity annealing temperatures between 55°C to 60°C. For the multiplex PCR, we found it necessary to decrease the annealing temperature, increase the extension time and adjust primer amounts. These alterations were tested empirically in small steps. Reliable amplification of two bands was obtained for all strains tested when the final concentrations of the different primers were adjusted to 0.15 µM *coag* and 0.1 µM of *mecA* with 40 reaction cycles (Fig. 1). Due to incomplete amplification, virtually no band or very faint band of *coag* was observed with the

Table 1: Details of samples investigated in this study

Identity	Number resistant	Phenotypic methods		mPCR results	
		Methicillin	Coagulase positive	*mec*$^+$	*coag*$^+$
MRSA	40	40	40	40	40
MSSA	55	0	55	3	55
MR-CoNS	11	11	0	11	0
MS-CoNS	44	0	0	10	0

"+" detectable genotype

lower concentrations for each of the four primers and with less number of amplification cycles (Fig. 2). On the basis of specific amplifications of the *mecA* and *coag* genes the multiplex PCR procedure allowed the specific identification of the staphylococcal species and the determination of its susceptibility to beta lactam antibiotics (Fig. 3)

The results of the multiplex PCR amplification strategy are shown in the Table 1. For 40 strains determined as MRSA by phenotypic methods, the results were 100% consistent with PCR results. Interestingly, during the validation process we found there were three isolates that were classified as methicillin susceptible by the phenotypic methods in which we were able to detect the presence of the *mecA* gene by PCR. When MS-CoNS were evaluated, similar results were observed in which 10 isolates classified as oxacillin sensitive by disk susceptibility tests yielded positive results in PCR method. All these cryptic strains should be regarded as potentially methicillin-resistant isolates bearing the *mecA* gene and should not be classified as methicillin susceptible in spite of their susceptibility to beta lactam antibiotics.

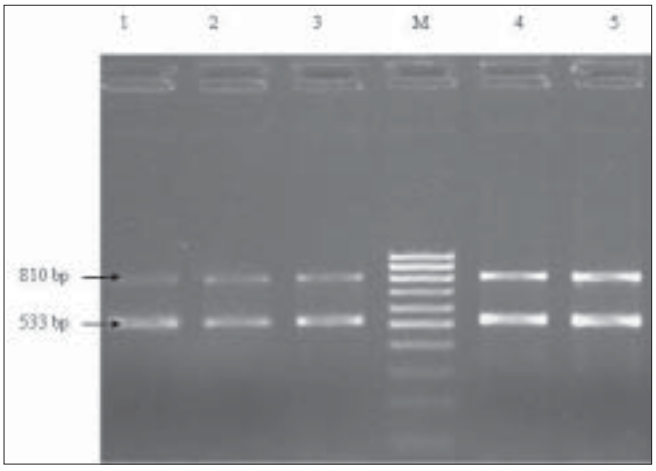

Figure 1: Agarose gel electrophoresis patterns showing PCR amplification products of different MRSA strains. Specific bands appeared around 810 base pairs for *coag* and 533 base pairs for mecA genes in polymerase chain reaction of clinically isolated MRSA strains. Lane M, molecular base pair marker (100bp - 1000bp). Lanes 1, 2, 3, 4 and 5 represent amplification products obtained from different MRSA clinical isolates

Discussion

For staphylococci, mPCR technique has been used to specifically detect MRSA.[5-7] These reports emphasize the fact that a positive result in DNA-based tests correlates well with the presence of the methicillin-resistant phenotype. PCR assays may detect microbial pathogens at concentrations below those of previously established gold standard reference methods. Distinguishing whether this result represents a false positive or false negative finding and establishing the clinical significance of these findings is a challenge. PCR assays for microbial detection may give false negative results because of relatively small sample volume permissible for PCR reactions. PCR methods in which the concentration of infectious organisms is low, the assay may yield false negative findings. To circumvent this, the methodology was modified in terms of DNA extraction, amount of DNA and primers used in mPCR amplification strategy.

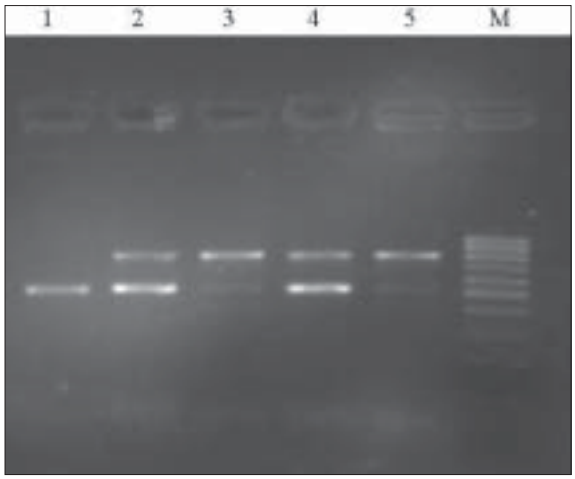

Figure 2: Agarose gel electrophoresis patterns showing PCR amplification products with different primer concentrations. Lane M, molecular base pair marker (100bp - 1000bp). Lanes 2 and 4 represents amplification products obtained when the final concentration of primers were adjusted to 0.15 µM *coag* and 0.1 µM of mecA and with 40 reaction cycles. Lanes 1, 3 and 5 shows incomplete amplification of one of the products with low primer concentration and with less number of amplification cycles

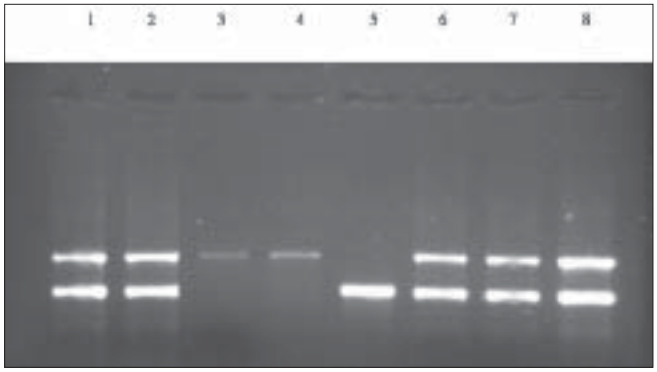

Figure 3: Agarose gel electrophoresis patterns showing mPCR specificity. Lanes1, 2, 6, 7 and 8 MRSA isolates; Lanes 3 and 4 MSSA isolates; Lane 5 methicillin-resistant CoNS isolate

Selection of primers for amplification of the *mecA* gene could have significant impact on the accuracy of test results. The position and G+C content of primers chosen for amplification of *mecA* may be critical to success when application is made to a large number of strains. In our mPCR strategy, nonspecific amplification of unrelated A+T-rich DNA regions was minimized by choosing primers containing -50% G+C and close to the variable region of the *coag*ulase gene.[8]

In earlier studies and in our present study, isolates classified as methicillin sensitive by susceptibility tests were found to harbour the *mecA* gene and accordingly should be reclassified as methicillin resistant. This could be because *mecA* gene is not consistently expressed and earlier evidence suggests that certain auxiliary genes such as femA and mecR may participate in control of its differential expression.[9] When we examined 40 isolates of MRSA, 55 isolates of MSSA, 11 MR-CoNS and 44 MS-CoNS isolates by MPCR strategy, the results for *mecA* detection by PCR were more consistent with disk susceptibility tests in case of MRSA (100%) and MSSA (95%) isolates. In contrast to above results with MRSA and MSSA, *mecA* detection by PCR in MS-CoNS showed less correlation with disk susceptibility tests (77%). Especially in case of MS-CoNS isolates, PCR results showing lesser consistency with those from susceptibility tests (60.9%) have been reported earlier.[10] This may be due to the fact that heterogeneous expression of resistance varies more for MS-CoNS compared to *S. aureus* and the subpopulation of resistant cells is smaller for MS-CoNS than for *S. aureus*.[11] These reports confirm that expression of methicillin resistance is more variable for MS-CoNS and the detection of the *mecA* gene is important in interpreting methicillin susceptible staphylococci.

Critical parameters for success of a PCR-based test are cost, reliability, speed and accuracy. We validated a more comprehensive mPCR protocol by simultaneously identifying two genetic markers that characterise the species and the antibiotic resistance mechanism. In our mPCR strategy, *coag*ulase gene was used as target which offers a better correlation between phenotypic and genotypic identification of *S. aureus*. Amplification of the *mecA* and *coag* genes in the same PCR tube allows a straightforward detection of *S. aureus* with detection of the methicillin-resistant phenotype. The absence of *coag*ulase gene product from CoNS sources demonstrates the high-level specificity of our PCR strategy. Many investigators have used PCR reaction mixtures of at least 50 µL. The small volume of the PCR reaction mixture employed by us (20 µL) reduces the consumable cost considerably.

In summary, the system evaluated here allows rapid recognition of the pathogen as well as genotypic detection of resistance to a specific drug. This information provided by a single multiplex PCR amplification can be helpful in choosing the right therapy and especially India specific data will be useful in designing larger prospective studies for preventing the spread.

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