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Research Article

IDENTIFICATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* BY A RAPID POLYMERASE CHAIN REACTION TECHNIQUE

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* is one of the most important causes of hospital infections worldwide. High-level resistance to methicillin is caused by the *mecA* gene, which encodes an alternative penicillin-binding protein. *S. aureus* is an important cause of bloodstream infections and a leading cause of severe health care-Associated infections. Laboratory diagnostics *S. aureus* is a Gram-positive, catalase positive aerobic or anaerobic coccus showing hemolytic and large yellow colonies. *S. aureus* strains resistant to methicillin and many other antibiotics are major causes of nosocomial infections worldwide. It is of great necessity to check for those genes and check for the potential to clone partial or major fragments into vectors, which can be a potential candidate for DNA vaccines. Novel methods are being developed for the production of antibodies to specific antigens and thus helping in the process of development of protein-based vaccines. *mec*A (methicillin-resistant) gene was isolated and ligated into pTZ57R/T cloning vector. The ligated product was then cloned into DH5α strain and allowed to propagate. The plasmids thus cloned were purified and later expressed for the gene of interest in an expression vector. The proteins specific to the gene of interest was then isolated and purified. This proteins purified can in turn be used for protein-based vaccines.

Keywords: *Staphylococcus*, Methicillin-resistant *Staphylococcus aureus*, *mecA*, Cloning.

INTRODUCTION

Staphylococcus aureus is one of the most significant human pathogens that cause both nosocomial and community-acquired infections [2]. Nosocomial infections cause a substantial burden for health and economics worldwide. Such endogenous, sporadic infections account for the majority of all nosocomial infections [18,19]. Any microbial group, bacteria, viruses, fungi, or parasites can cause a nosocomial infection, but bacteria are the most prevalent organisms. Among Gram-positive bacteria, common nosocomial agents include staphylococci, enterococci, streptococci, and clostridium difficile, and among Gram-negatives, *Escherichia coli, Klebsiella* sp., *Enterobacter, Proteus, Serratia, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Acinetobacter* spp., and *Haemophilus* spp. [4-6].

S. aureus is an important cause of bloodstream infections and a leading cause of severe health care-associated infections [10]. Infection results in significant morbidity and mortality and longer hospital stay if not treated early with effective antibiotics [6,8]. The prevalence of methicillin-resistance in *S. aureus* (MRSA) causing infection now exceeds 49% in US hospitals and continues to increase. Thus, improved methods are needed for rapid detection and differentiation of methicillin-susceptible *S. aureus* (MSSA) and MRSA bacteremia to ensure optimal treatment early in the infection [13,15]. Laboratory diagnostics *S. aureus* is a Gram-positive, catalase positive aerobic or anaerobic coccus showing hemolytic and large yellow colonies. *S. aureus* strains resistant to methicillin and many other antibiotics are major causes of nosocomial infections worldwide [7].

Resistance to methicillin is determined by the *mecA* gene, which encodes the low-affinity penicillin-binding protein (PBP2a) [3]. MRSA infections have become a global health problem particularly in the hospital setup causing simple skin infections to life-threatening infections [20]. In many developing countries, including India, the situation appears gloomy due to inadequate or poor implementation of policy on infection control, lack of political will, inadequate resources including shortage of skilled manpower, poor motivation of health care workers and researchers [1].

The *mecA* gene is a small part of the 21 to 60 kb staphylococcal chromosome cassette mec. This mobile genetic element may also contain genetic structures such as Tn *554*, pUB110, and pT181 that also encode resistance to non-β-lactam antibiotics [11]. Two hypotheses have been raised to explain the evolutionary origin of MRSA strains. The single clone hypothesis, based on early analyses of the restriction fragment length polymorphisms obtained for MRSA isolates collected worldwide by using probes for *mecA* and Tn*554*, suggests that *mecA* entered the *S. aureus* population on one occasion and resulted in the formation of a single MRSA clone which had rapidly spread around the world [11,14]. The second hypothesis, based on the detection of *mecA* in diverse *S. aureus* multilocus enzyme electrophoresis types, proposes that MRSA strains evolved a number of times by means of the horizontal transfer of *mecA* into phylogenetically distinct MSSA precursor strains [17]. By using DNA microarray technology, *mecA* has been detected in at least five divergent lineages, implying that horizontal *mecA* transfer has played a fundamental role in the evolution of MRSA [9]. The transfer of *mecA* from *S. epidermidis* to *S. aureus* was recently witnessed *in vivo*, suggesting that *mecA* may transfer more frequently to MSSA [22,21].

Owing to the many developing methods adopted by the bacteria towards the resistance to the infection, the control over the infections is of a stall [16]. Keeping in view of this, it is of great importance to isolate and characterize the genes that code for this antibiotic resistance genes. Study of this genes and characterization will give a lot of information on the mode of the genetic variation, which might help in revealing the secrets of the resistance gaining power.

It is of great necessity to check for those genes and check for the potential to clone partial or major fragments into vectors, which can be potential candidates for DNA vaccines.

In this study, we designed to amplify the partial *mecA* gene from *Staphylococcus* and clone it into a cloning vector. The vector will then be purified and cloned into an expression vector for protein production. The sequence identified from plasmid will also be sequenced to check the sequence similarity. The clones will be identified and sequenced thereafter to identify the similarity.

METHODS

Blood sample collection

Total of five clinical blood samples obtained from patients with severe wound infection were collected from various hospitals in Bangalore. The samples were collected in a clean, sterile spin win conical tube and labeled neatly (Tarsons, India). After collection, the blood sample tubes were stored at 4°C. Approximately 3 mL venous blood were collected from each patient and added to the falcon screw cap sterile tubes (50 mL) containing 50 µL of 0.5 M EDTA.

Isolation of genomic DNA from clinical specimens (blood)

Total genomic DNA from the bacteria was isolated by N- Cetyl-N, N, N-trimethyl ammonium bromide (CTAB) method. The DNA extraction from the blood samples was carried out by the phenol-chloroform-isoamyl alcohol (PCI) procedure. Briefly the procedure is as follows. To the 3mL of the EDTA-treated blood samples 45 mL of ×1 red blood cells (RBC) lysis buffer (×10 RBC Lysis buffer [pH 7.3], 89.9 g of NH_4Cl, 10 g of $KHCO_3$, and 2 mL of 0.5 M EDTA [pH:8] in liter of milliq water) was added and kept for incubation at room temperature for 1 hr. The contents are then mixed and centrifuged at 1000 rpm for 30 minutes. The pellet was resuspended in 1 mL of RBC lysis buffer. The process repeated again and the pellet obtained was washed by resuspending in 1 mL of phosphate-buffered saline. The contents then centrifuged at 10,000 rpm for 10 minutes and the obtained pellet was added with 5 µL of proteinase-K (10 mg/mL), 500 µL of DNA extraction buffer (1 M Tris HCl, 1M EDTA, 4 M NaCl), 50 µL of β-mercapto ethanol, 25 µL of 1 m dithiothreitol. The contents mixed the pellet properly was resuspended and incubated at 37°C for 30 minutes in an incubator, followed by 66°C for 30-60 minutes in the water bath. To the above mixture equal amount of PCI (25:24:1) added. The contents were mixed gently, and the tubes were centrifuged at 10,000 rpm for 15 minutes. The aqueous layer was slowly aspirated and added with an equal amount of chloroform/Isoamyl alcohol (24:1). The contents were then centrifuged at 10,000 rpm for 10 minutes and to and the aqueous layer an equal amount of Isopropanol was added and incubated for precipitation. The DNA pellet was dissolved in 30 µL of sterile milli-Q water and stored at −20°C until further use. The quantity of the isolated DNA was checked in UV-VIS spectrophotomer (Vivaspec Biophotometer, Germany).

Polymerase chain reaction (PCR) amplification

The gene selected for the primer designing was *mecA* gene with an amplicon size of 520 bp. The specific primers were designed using primer 3 plus software, and the designed oligonucleotides were synthesized in sigma corporation USA.

The PCR mixture consisted of 10 × reaction buffer with $MgCl_2$ (1.5 mM), 2 µL of dNTP mix (2.5 mM), 2 µL each of forward and reverse primers (10 picomoles/µl each primer), 0.3 µL of Taq DNA polymerase (5 U/µL), and 50 ng/µL of template DNA in a total volume of 20 µL. The PCR was performed with the following cycling profile: Initial denaturation at 94°C for 1 minutes, followed by 35 cycles of 50 seconds denaturation at 94°C, annealing at 55°C for 30 seconds, and extension at 72°C for 2 minutes. The time for the final extension step was increased to 6 minutes. The PCR products amplified were then qualitatively analyzed on 1% agarose gel. The PCR product was recovered using the QIA quick gel extraction kit, and the amplified product was then purified and used for cloning purpose.

Primer details

The *mecA* gene was amplified by PCR using purified genomic DNA as a template. Oligonucleotide primers were synthesized to amplify the intact region of *mecA* gene. The forward primer, 5' CTG CTA TCC ACC CTC AAA CAG 3' and the reverse primer, 5' CAC CTT GTC CGT AAC CTG AAT C 3', were purchased from Eurofins, Bangalore. These primers correspond to the gene *mecA* and thus the final PCR product was 525 bp (Table 1).

Cloning of the partial *mecA* gene

The gene of insert was eluted from the agarose gel using gel extraction kit (Bioline USA). Eluted gene of insert was quantified and ligated with the expression vector pET20b. The reaction mixture was incubated at 27 OC for 3-4 hrs. Vector pET20b (50 ng/µl) together with eluted gene of insert (0.52 pmole) was added to the ligation mixture and then incubated.

Table 1: The details of the forward and reverse primers designed toward amplification of the *mecA* gene

Primer	Sequences (5'-3')	GC %	Tm value	Length	Product size
FP	CTG CTA TCC ACC CTC AAA CAG	52.0	54.4°C	21	525 bp
RP	CAC CTT GTC CGT AAC CTG AAT C	50.0	54.8°C	22	

Ligated plasmid was transformed into the bacterium DH5α as described earlier in this paper and plated on LB medium containing ampicillin (50 mg/ml) to a final concentration of 50 µg/ml. The white colonies from the LB-ampicillin agar plates were inoculated in LB-ampicillin broth and incubated at 37°C for overnight and plasmid DNA was isolated by Alkaline lysis method as described earlier in this paper. The transformed bacteria were inoculated in 50 ml of LB broth containing isopropyl β-D-1-thiogalactopyranoside (0.5 mM) as an inducer for the expression of the gene. *E. coli* strain without plasmid was used as control. The inoculated culture flask was incubated at 37°C in an orbital shaker at 150 rpm for 24 hrs.

Plasmid isolation by alkaline lysis method

The transformed plates were selected for the gene of interest. The plasmids were isolated by alkaline lysis method. The cultures were cultured overnight in LB medium containing ampicillin (50 µg/ml). Following incubation the culture was centrifuged, and the bacterial pellet obtained was air dried.

The bacterial pellet was resuspended in 100 µl of ice cold solution I (50 mM glucose, 25 mM tris-Cl and 10 mM EDTA; pH 8.0) by vigorous vortexing. To the suspension 200 µl of freshly prepared solution II (0.2 N NaOH, 1% w/v sodium dodecyl sulfate) was added and mixed properly. The contents of the tube were mixed by inverting the tube rapidly for five times, and 150 µl of ice cold solution III (5 M potassium acetate, 12.5 ml glacial acetic acid and the resultant solution made upto 100 ml) was added. The tubes were then vortexed gently, and the tubes were incubated on ice for 3-5 minutes and later centrifuged at 12,000 rpm for 5 minutes at 4°C. The supernatant collected was extracted with an equal volume of chloroform and isoamyl alcohol (24:1). The plasmid DNA was precipitated with 0.6 volumes of ice cold isopropanol, and the DNA was pelleted at 12,000 rpm and the pellet was washed with 70% ethanol.

Confirmation of clones by restriction digestion

The purified plasmid was subjected to restriction digestion using restriction endonucleases (Merck, India) restriction digestion was performed in 20 µl reaction volumes with recommended units of enzyme and appropriate buffers at 37°C for 4 hrs.

The plasmid DNA (0.2 µg/µl) was double digested with restriction enzyme *Bam* HI (10 U/µl) and *Eco* RI (10 U/µl). The contents of the mixture were mixed properly by pipetting, and then the tubes were briefly spin down. The tubes were then incubated at 37°C for 2 hrs. The digested samples were resolved on 1% low melting agarose gel for confirming the release of the insert by the restriction endonucleases.

Sequencing and phylogenetic analysis

The insert in the purified plasmid was sequenced using ABI PRISM Big Dye Terminators v 1.1 cycle sequencing kit (applied biosystems Foster city, CA, USA) according to the manufacturer's instruction employing T7 or M13 primers. A comparison of the nucleotide sequences of the unique fragment with the sequences available in the gen bank database

was carried out using the NCBI basic local alignment search too (BLAST) program.

Phylogenetic tree were generated on the basis of sequences of *mec*A gene sequences using Clustal W 1.8 (Thompson *et al.,* 1994). The variable and incomplete sites at both the 5' and 3' ends of the gene sequences were excluded from the alignment. Sites presenting alignment gaps were excluded from the analysis. A rooted phylogenetic tree was constructed using the sequences reported here with diverse *S. aureus mec*A gene sequences previously deposited in the gen bank database.

RESULT AND DISCUSSION

Genomic DNA isolation and quantification

The Gram-positive bacteria were cultured in the LB broth media and genomic DNA was isolated by modified CTAB method. The isolated DNA was electrophorized in 1% agarose gel (Fig. 1 left).

PCR amplification

The result of the PCR revealed 2 isolates demonstrating both resistances to methicillin and expression of *mecA* gene. The samples showed a positive amplification to the gene of interest and showed bands approximately at about 520 bp (Fig. 1 right).

Cloning of PCR product in to T vector

PCR yielded a specific amplicon of ~520 bp in three of the samples. The fragment was separated in gel and purified the product using gel extraction kit. The purified PCR product was quantified and ligated with the cloning vector using T4 DNA ligase enzyme. The ligated plasmid was transformed into *E. coli* bacterial strain DH5α. The colonies were then screened for the blue white colonies (Fig. 2).

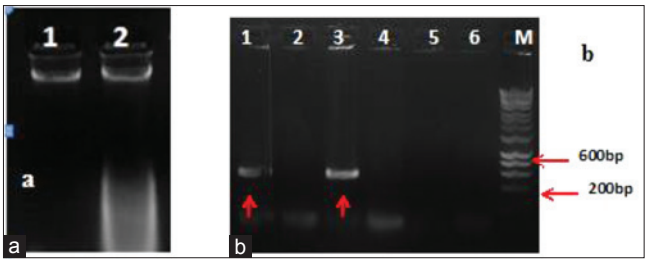

Fig. 1: Left (a): Bands showing the genomic DNA isolated from the bacteria on 1% agarose gel. Right (b): Agarose gel showing the amplified bands of the *mecA* gene. Lane 1 and 2: Amplified region of *mecA* at about 520 bp. Lane M: Marker 100 bp

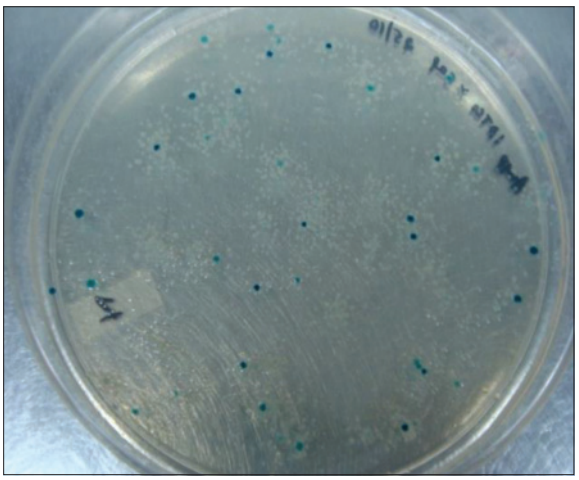

Fig. 2: Blue white selection of the transformed bacterial cells in the Xgal-isopropyl β-D-1-thiogalactopyranoside-ampicillin-LB Agar

Plasmid isolation and confirmation of clone by restriction digestion

The purified plasmid was subjected to restriction digestion using *Bam*H1 and *Eco*R1 (Merck, India). The plasmid was double digested, and the gel showed the clear cut bands at the two expected regions. The plasmid was about 3000 bp in size and the released gene product was about the same size of about 520 bp (Fig. 3).

Sequence data

The gene was identified by sequencing of plasmid. An approximately ~520 bp region of the *mec*A gene genes was sequenced at Eurofins, Bangalore. The sequence data was shown in Fig. 4. Nucleotide sequence analysis of gene was used to investigate the identity of bacterial *mecA* gene of *S. aureus.* To demonstrate the quality and accuracy of results provided from a public database, we compared sequences to their corresponding gen bank sequences. The sequence had "perfect" match (similarity, 99%) with sequences of their corresponding gene from gen bank as determined by using BLAST (version 2.7).

Sequence alignment by clustalW

The *mec*A gene sequence of *S. aureus*is also available in gen bank database, which is more similar to this sequence (99% similarity). The N-J tree with branch length was plotted using ClustalW sequence alignment showing the relationship of *mec*A gene among the closest *S. aureus* strains in the NCBI database (Fig. 5). The sequence of *S. aureus mec*A gene amplified by the specific primer is closely matching (98%) with a *S. aureus* strain. Sequence analysis of the *mec*A genes from 11 strains was also carried out to obtain new insight into the genetic differences between the methicillin-resistant gene in the population. Interestingly, we found 13 mutations within the *mec*A open reading frame of our strains compared with previously published sequences in *mec*A. This may be the main reason for the antibiotic resistance of bacteria with even in the newly emerging antibiotic drugs. It is well known that local DNA sequences containing repeat sequences (direct repeats or inverted repeats) may cause deletion by misalignment during DNA replication or recombination. However, recently published epidemiological data do not support the association of *mec*A with clinical outcome but suggest that *mec*A status reflects geographic differences in *S. aureus* strains rather than a virulence-associated trait.

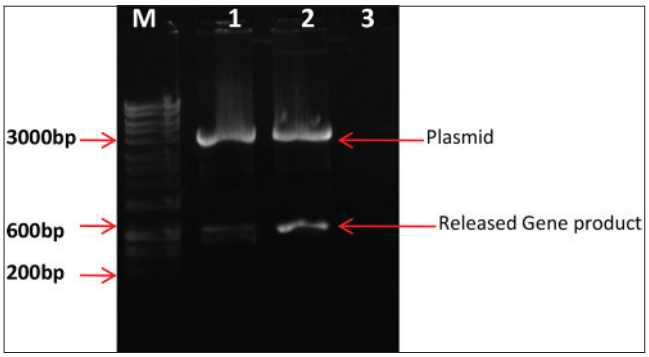

Fig. 3: Restriction digestion of ligated plasmid using *Bam* H1 and *EcoR* 1 (Lane 1, 2-restricted product, M-Marker)

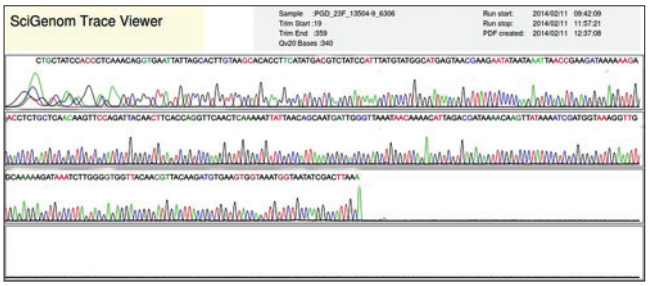

Fig. 4: Sequence of the DNA *mecA*

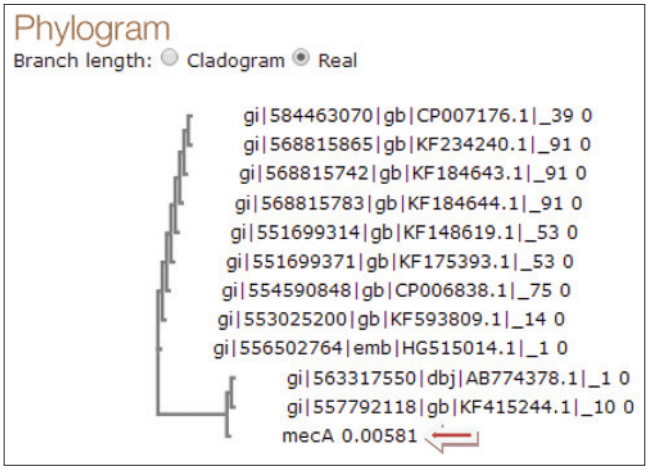

Fig. 5: A tree plot was constructed with the NJ method using 543 bp fragment of the *mecA* partialgene showing the relationship of *Staphylococcus aureus*

Further understanding of the function of the *mec*A locus and expression of its products may help resolve these discordant results.

CONCLUSION

MRSA is one of the most important causes of hospital infections worldwide. High-level resistance to methicillin is caused by the *mecA* gene, which encodes an alternative PBP2a. Molecular analysis of bacterial resistance has yielded a wealth of information during the last decade. With the aid of molecular amplification techniques, great progress has been made in our knowledge of the distribution and spread of resistance markers among the species.

The use of PCR has increased the speed and accuracy for identification of *S. aureus* and confirmation of MRSA by detection of *mec*A. In the present study, the collected blood samples are screened for the MRSA stains using culture methods and using specific primer desingned from antibiotic sensitivity (*mec*A) gene. Among seven samples studied, two of them were found to be positive. The primer was designed to amplify the antibiotic sensitivity gene *mec*A from the bacteria. The amplified product was gel purified and cloned into T vector and transformed into *E. coli* stain DH5α. The positive clones were identified, plasmid isolated and sequenced. The sequence was matching with *mec*A partial sequences of MRSA strains. The primer pair was standardized, and it can be used as a specific primer to detect the MRSA strains form the patients with nosocomial infections. This assay can be performed in <6-8 hrs, which will reduce the chance of spreading the organism in the hospital. The expenses and workload of a single PCR exceed the demands of testing one clinical specimen for the presence of MRSA. Yet, if the daily number of MRSA screening tests increases, the workload per PCR decreases and finally outweighs the expenses for molecular reagents. Although, molecular testing remains expensive relative to conventional agar based detection, there is an overall cost saving, especially if molecular testing is directed at high-risk populations.

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