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Cost Effective Screening and Characterization of *Staphylococcus aureus* and Methicillin-Resistant *Staphylococcus aureus* from Bovine Mastitis in India

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ABSTRACT

The present study was conducted to characterize *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) in mastitic milk of cow using rapid and cost effective genotypic as well as phenotypic method. Different phenotypic and genotypic methods were compared in detection of *S. aureus* and MRSA. Present study also optimized and standardized the boiling and snap chilling method of genomic DNA extraction for rapid screening of *S. aureus* and MRSA in pure culture. Detection of species specific thermo nuclease (*nuc*) gene was found more sensitive than phenotypic test in identification of *S. aureus* whereas both rapid cultural methods such as cefoxitin disc diffusion test and use of chromogenic agar was found comparable to *mecA* gene based PCR in characterization of MRSA. Furthermore, despite less purity and minute concentration of DNA obtained from boiling and snap chilling method, there was no non-specific and cross reaction in PCR assay. Therefore the boiling and snap chilling method could be recommended as an alternative and economical method of genomic DNA extraction for rapid screening of *S. aureus* and MRSA particularly in developing countries like India.

HIGHLIGHTS

- Boiling and snap chilling was found economic method of genomic DNA extraction for screening of *Staphylococcus aureus* and MRSA associated genes.
- Cefoxitin disc diffusion test and chromogenic MeReSa agar were observed as good as *mecA* gene based PCR for screening of MRSA.

Keywords: *Staphylococcus aureus*, *nuc* gene, MRSA, *mecA* gene, boiling and snap chilling method.

Emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) is an upcoming threat to animal and public health. MRSA is usually associated with skin and soft tissue infections in pets and humans. Now it becomes one of the most infectious pathogenic bacteria that cause bovine mastitis and significantly affects the economy of the dairy industry (Sartori *et al*., 2018). Most of the cattle in India are reared in a backyard system that makes direct contact with human and therefore the possibility of cross-transmission cannot be ruled out. The chronicity of multidrug resistant *Staphylococcus aureus* infection is reported to be responsible for more than 80 % of subclinical bovine mastitis with associated pecuniary losses of USD 2 billion per year in USA per year (Kuehn *et al*., 2013) and has enforced to develop an efficient and cost effective method for its screening and characterization.

Methicillin resistance in MRSA is mediated by 78-kDa penicillin binding protein PBP2′ (or PBP2a) which has a very low affinity for beta-lactam antibiotics. PBP2a is encoded by the *mecA* gene located on a mobile genetic

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element called staphylococcal cassette chromosome *mec* (SCC*mec*) elements (Hiramatsu *et al*., 2013). The screening, characterization and confirmation of MRSA are based on conventional cultural and morphological characteristics of the pathogen in routine laboratory practices. Identification of *S. aureus* from milk samples always remains prerequisite before characterization of MRSA. Several phenotypic and genotypic methods with varying specificity and sensitivity are available to isolate and identify *S. aureus*. Likewise, MRSA screening can be accomplished using quick and cost effective cultural methods such as cefoxitin disc diffusion and the use of chromogenic agar media (Koupahi *et al*., 2016). However, detection of *nuc* gene (thermonuclease gene) and *mecA* gene in pure culture by PCR is considered as the gold standard for detection of the *S. aureus* and MRSA, respectively (Sahebnasagh *et al.*, 2014). Genomic DNA extraction from organisms is the first step in PCR that uses a DNA isolation kit which makes the process costly and less feasible particularly in developing countries like India. It is therefore necessary to improve or optimize the pre-existing fast and efficient yet cost-effective boiling and snap chilling method for the genomic DNA extraction from organisms in pure culture. The boiling and snap chilling method of genomic DNA extraction however limited to a certain extent in Gram negative bacteria (Martins *et al*., 2015) and difficulties are encountered when extracting DNA from Gram-positive bacteria like *S. aureus* due to its thicker cell wall and rich peptidoglycan content.

The present investigation was therefore undertaken to compare and evaluate a rapid and cost effective method for screening of *S. aureus* and MRSA from bovine mastitis in Chhattisgarh, a major cattle rearing state in India.

MATERIALS AND METHODS

Staphylococcus aureus reference control

One of the suspected *Staphylococcus aureus* isolate was characterized by MALDI-TOF MS (Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry) analysis and 16S rRNA sequencing at the National Center for Microbial Resource in Pune, India and was used as reference control.

Isolation of *Staphylococcus aureus*

Staphylococcus aureus was isolated using a standard protocol from milk samples collected from mastitic cows that were resistant to conventional antimicrobial therapy. To summarize, samples were grown in nutrient broth containing 6.5 percent NaCl. Broth culture was plated on nutrient agar and incubated for 18 h at 37°C. Gram's staining technique was used to study the morphology of organisms. Isolates were maintained at 4°C by routine subculture in nutrient agar slant.

Identification of *Staphylococcus aureus* by *nuc* gene based PCR

Suspected *S. aureus* isolates were identified through detection of *S. aureus* specific *nuc* gene by PCR (Ali *et al*., 2014). Genomic DNA was extracted by boiling and snap chilling method. *S. aureus* isolates were cultured at 37ºC for 18 h in tryptone soya broth. 1.5 ml broth culture was transferred into 2 ml eppendorf tube and centrifuged at 10,000 g at 4ºC for 2 min. The supernatant was decanted from each tube, and the pellet was washed three times with 1.5 ml of sterile PBS (pH 7.4). Finally, the pellet was eluted with 300 µl of nuclease free water by vortexing and then kept in the water bath at boiling temperature (100ºC) for 15 min, followed by immediate cooling at-10ºC for 20 min. Lysed cells were then detached at room temperature and centrifuged at 10000 g for 2 min at 4ºC. The supernatant was transferred from each tube to the new microfuge tube and was used as a DNA template. The DNA template was stored at -20 °C.

Determination of concentration and purity of DNA

DNA was quantified spectrophotometrically with NanoDrop and expressed as ng / µl. The DNA purity was determined by the absorbance ratio of 260 and 280 nm. The ratio of A260/280 between 1.8 and 2.0 is generally considered pure (Lucena-Aguilar *et al*., 2016).

Polymerase chain reaction (PCR)

Present study optimized and standardized PCR protocol for extracted genomic DNA using *S. aureus* specific *nuc* gene primers viz. Forward- 5' GCGATTGATGGTGATACGGTT 3'and Reverse- 5'

AGCCAAGCCTTGACGAACTAAAGC 3'. PCR reactions were performed with a total volume of 20 µl containing 10 µl PCR master mix (Ready mix, Sigma), 1.25 µl of 10 pmol each gene-specific forward and reverse primer, 1.5µl of Template DNA and 6 µl nuclease free water. Initially, two different annealing temperatures (55°C and 60°C) were used for the standardization of PCR amplification of *nuc* gene for the positive control in gradient thermocycler (ProFlex PCR System, applied biosystems, Thermo Fisher Scientific). Subsequently, PCR amplification was performed for other samples with optimized annealing temperature (Table 1). Amplified products were electrophoresed on 1.5 % agarose gel stained with SYBR safe (1µl/ 10ml) in 1X TAE buffer initially at 100 V for 5 min followed by 80 V for 30-45 min. Stained DNA bands were visualized and digitized using a Gel Documentation System (Bio-print, VILBER). The sample DNA band size was compared to the known 100 bp DNA ladder (Bangalore Genei, India).

Table 1: PCR cyclic conditions

Steps	*nuc* gene	*mecA* gene
Denaturation	94 °C for 3 min	94 °C for 3 min
Denaturation	94 °C for 30 sec	94 °C for 30 sec
Annealing	60°C for 30 sec	60°C for 30 sec
Extension	72°C for 30 sec	72°C for 40 sec
Final extension	72°C for 5 min	72°C for 7 min
Hold	4°C	4°C

Phenotypic characterization of *S. aureus* isolates

All the suspected *S. aureus* isolates were further characterized phenotypically for mannitol fermentation. To determine mannitol fermentation, bacterial isolates were subcultured on mannitol salt agar and incubated at 37°C for 24 h.

Screening of MRSA by *mecA* gene amplification in PCR

All *nuc* gene positive isolates and *S. aureus* reference control were screened for methicillin resistance by detection of MRSA specific *mecA* gene (*mecA* gene primers viz. Forward- 5' AAAATCGATGGTAAAGGTTGGC 3'and Reverse- 5' AGTTCTGCAGTACCGGATTTGC 3') by PCR (Rajabiani *et al*., 2014) as per protocol mentioned for detection of *nuc* gene.

Screening of MRSA by rapid cultural method

All *mecA* gene positive isolates were further screened phenotypically for methicillin resistance by cefoxitin disc diffusion method and using selective chromogenic MeReSa agar.

Cefoxitin disc diffusion method

Antibiotic sensitivity testing (AST) was performed on *S. aureus* isolates using the disc diffusion method according to guidelines of CLSI (2019). During the current study, antibiotic markers of MRSA such as methicillin (10 g; cat. No. SD136 HiMedia), cefoxitin disc (30 g; cat. No. SD041 HiMedia), and penicillin discs (2IU; SD028 HiMedia) were used in AST. To perform this test, a loopful of the culture was inoculated in 5 ml of nutrient broth and incubated at 37°C for 2-4 h until the opacity of broth culture matched Mc-Farland's turbidity standard 0.5 (cat no. R092 Himedia). Using cotton swabs, each broth culture was then evenly spread on Mueller Hinton Agar Petri-plates (90 mm diameter with uniform bottom surface; cat. No. M173 Himedia). The inoculated Petri-plates were kept at room temperature for 15-20 min to allow the inoculums to be adsorbed on the surface. Each antimicrobial disc was placed on the plates at an equal distance of 25 mm using flame sterilized forceps. The plates were then incubated at 37°C for 18-24 h. CLSI (2019) guidelines were used to interpret the results as sensitive, intermediate sensitive, and resistant.

Growth on selective chromogenic MeReSa agar

One loopful nutrient broth culture of *S. aureus* was streaked on selective chromogenic MeReSa agar (cat. no. M1674 Himedia) supplemented with cefoxitin (cat. no. FD259 Himedia) and incubated at 37 °C. After 24-48 h, the cultural characteristics of the isolates were recorded.

RESULTS AND DISCUSSION

Isolation of *Staphylococcus aureus*

A total of 15 bacterial isolates showing golden yellow pigmented colonies on nutrient agar (Fig. 1) and large

irregular clusters of Gram positive cocci on Gram's staining were presumptively identified as *S. aureus* (Nazir *et al*., 2017).

Staphylococcus aureus reference control

MALDI-TOF MS biotyper database yielded a score of 2.292 for the tested representative isolate, indicating the best match with *S. aureus*. *Staphylococcus aureus* was the closest neighbor recorded in the database using NCBI blast of obtained nucleotide sequence. 16S rRNA gene and MALDI-TOF MS were employed earlier also for identification of *Staphylococcus* isolates up to species level (Achek *et al*., 2020).

Comparison of genotypic and phenotypic test for screening of *S. aureus*

All the isolates showed mannitol fermentation (Fig. 1) whereas only 13 isolates were reported coagulase positive both by slide coagulase and tube coagulase test which indicated discrepancy in result with detection of *nuc* gene by PCR. Presence of coagulase negative variant of *S. aureus* during present study suggests that phenotypic test alone is not adequate in identification of *Staphylococcus aureus* and it requires further confirmation by molecular technique. Likewise, present finding Hosseinzadeh and Saei (2014) also reported coagulase negative variants of *Staphylococcus aureus* in bovine mastitis milk. Such inconsistent and incoherent findings of coagulase and other phenotypic tests suggest not considering conventional

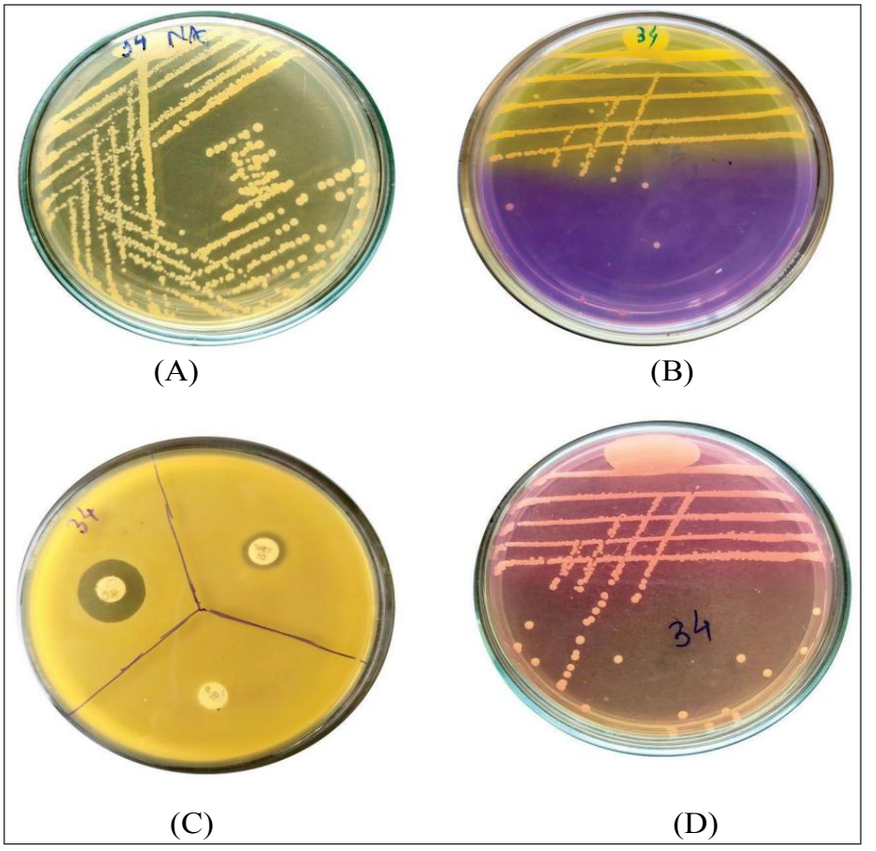

Fig. 1: Conventional cultural method for characterization of *S. aureus* and MRSA **(A)** Golden yellow pigmented colonies of *S. aureus* on nutrient agar **(B)** Yellow colonies showing mannitol fermentation by *S. aureus* on mannitol salt agar **(C)** *S. aureus* showing cefoxitin, methicillin and penicillin resistance by disc diffusion test **(D)** Pink colonies produced by MRSA on selective chromogenic MeReSa agar

culture methods for speciation of *S. aureus*. It therefore demands for rapid molecular techniques for detection of *S. aureus*. Use of nucleic acid targets has high sensitivity and specificity and hence they are the only technique for the accurate identification and classification of *Staphylococcus* species.

Comparison of genotypic and phenotypic test for screening of MRSA

mecA gene (533bp) was detected in 13 out of 15 isolates (Fig. 2) which was cent percent comparable result shown by cefoxitin disc diffusion test and growth on selective chromogenic MeReSa agar (Fig. 1). Therefore both the phenotypic methods were found in concordance to PCR for detection of methicillin resistance in *S. aureus* isolates. Detection of *S. aureus* specific thermonuclease (*nuc*) gene and MRSA specific *mecA* gene remained more specific and sensitive than any of the phenotypic test for confirmation of identity of *Staphylococcus aureus* and MRSA, respectively (Sahebnasagh *et al.*, 2014; Koupahi *et al*., 2016).

Efficacy of boiling and snap chilling method

Boiling and snap chilling method of DNA extraction from *Staphylococcus aureus* isolates yielded 21.6 – 82.7 (51.1±6.49) ng/µl DNA with the 260/280 absorbance ratio of 1.33-2.52 (2.09±0.08) (Fig. 2). PCR amplification of the *nuc* and *mecA* gene was successfully done at both annealing temperatures viz. 55 °C and 60 °C (Fig. 2). All suspected *S. aureus* isolates (n=15) were detected positive for *nuc* gene (270 bp) by PCR. Boiling and snap chilling method is proved to be a rapid and economical method of extraction of genomic DNA for detection of *Staphylococcus aureus* and MRSA during present study. Despite less concentration and purity of DNA in present study, PCR assay yielded desired amplicons for the *Staphylococcus aureus* specific thermonuclease (*nuc*) gene and MRSA specific *mecA* gene and did not show any nonspecific and primer-dimer reactions. Boiling and snap chilling method of genomic DNA extraction was found to be rapid, cost effective as well as economical and was used earlier also for amplification of *S. aureus* specific thermonuclease (*nuc*) gene (Nazir *et al*., 2017).

mecA gene (533bp) was detected in 13 out of 15 isolates (Fig. 2) which was cent percent comparable result shown by cefoxitin disc diffusion test and growth on selective chromogenic MeReSa agar (Fig. 1). Therefore, both the phenotypic methods were found in concordance to PCR for detection of methicillin resistance in *S. aureus*

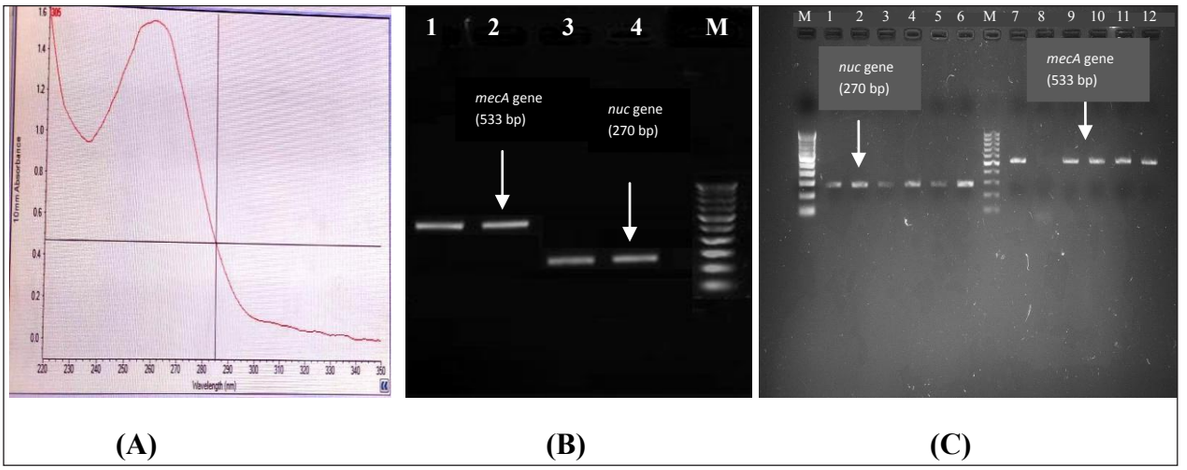

Fig. 2: Genomic characterization of *S. aureus* and MRSA **(A)** gDNA absorbance spectrums and purity evaluation of DNA isolated from *S. aureus* **(B)** Optimization of annealing temperature for amplification of *nuc* (270 bp) and *mecA* (533 bp) genes. Lane 1 and 3 are showing amplification of *mecA* and *nuc* genes, respectively at annealing temperature of 55°C; and Lane 2 and 4 at annealing temperature of 60°C, Lane M: 100 bp DNA Marker **(C)** Agarose gel image for amplification of *nuc* and *mecA* genes. Lane M: 100 bp DNA Marker, Lane 1-6: *S. aureus* isolates positive for *nuc* gene (amplicon size 270 bp), Lane 7 and 9-12: *S. aureus* isolates positive for *mecA* gene (amplicon size 533 bp).

isolates. Detection of *S. aureus* specific thermonuclease (*nuc*) gene and MRSA specific *mecA* gene remained more specific and sensitive than any of the phenotypic test for confirmation of identity of *Staphylococcus aureus* and MRSA, respectively (Sahebnasagh *et al.*, 2014).

mecA gene encodes PBP2a protein which has a very low affinity for beta-lactam antibiotics and thus induces resistance towards methicillin and other beta-lactams. Therefore, detection of *mecA* gene by PCR is considered as gold standard for identification of the methicillin resistance in *S. aureus* and was used earlier for studying prevalence of MRSA in human and animals (Rajabiani *et al*., 2014; Shah *et al.* 2019). Although *mecA* gene based PCR is more accurate and precise but is not economical. Therefore, conventional methods such as cefoxitin disc diffusion tests and growth on chromogenic agar media are becoming alternatives to PCR as they provide cost effective, rapid and easy screening of MRSA. Cefoxitin and oxacillin are considered as markers of methicillin resistance as they induce the *mecA* gene of MRSA. However, cefoxitin disk test and chromogenic MRSA agar is preferred over oxacillin disc test and is found comparable to PCR (Sharma *et al*., 2017). Detection of cefoxitin resistance in MRSA is therefore a widely employed method for screening of MRSA in routine laboratory practices. On contrary Hamid *et al.* (2017) reported variable results between PCR and cefoxitin disc test on screening of MRSA. Therefore, detection of the *mecA* gene is considered the most sensitive way to detect methicillin resistance in *S. aureus*.

CONCLUSION

It has been inferred from the present investigation that inconsistent and incoherent findings of phenotypic tests suggest to use molecular assay for accurate identification of *S.aureus.* However phenotypic tests were found in accordance with PCR for identification of methicillin resistance in *S. aureus*. Use of boiling and snap chilling method for genomic extraction of DNA not only reduces the cost of molecular assay but is employed as a quick and efficient method for screening of *S. aureus* and MRSA. Therefore, genomic DNA extraction from *S. aureus* and MRSA by boiling and snap chilling methods can be used as the best alternative to other standard protocols preferably in developing countries.

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