

IDENTIFICATION AND ANALYSIS OF MECA GENE IN MASTITIS MILK SAMPLES COLLECTED FROM FIELD CONDITIONS IN TAMIL NADU (INDIA)

G. Sathish^{1*}, Kurunchi C. Divya², E. Hemakumar², K. Murugan³, A. Stalin Vedamanickam⁴, G. Sathiyapriya⁵

ABSTRACT

Antibiotic resistance is common problem in the treatment of bovine mastitis nowadays. Detection of methicillin resistant Staphylococcus aureus (MRSA) is community associated between environment and animals. mecA gene is major one of staphylococcal cassette chromosome mec (SCCmec) elements in acquiring methicillin resistance. Here 55 mastitis milk samples collected from Tiruvallur district were screened for Staphylococcus spp. S. aureus, S. epidermidis and S. saprophyticus were confirmed by isolation and differentiation using coagulase test and novobiocin disc sensitivity. They were further confirmed by species specific PCR. Of these 31 isolates, 5 S. aureus, 2 S. epidermidis and 1 S. saprophyticus showed positive for mecA. Phylogenetic analysis of mecA gene sequences of methicillin resistant isolates formed a group with European and US isolates. Hence further research and control is necessary for extensive spread of mecA gene in environment.

Key words: mastitis milk- methicillin resistance-mecA gene-PCR-sequencing-phylogenetic analysis

INTRODUCTION

In dairy farming and production mastitis treatment is challenging because of the increasing antibiotic resistance. Staphylococcus aureus is one of the major organisms to cause mastitis. Monitoring the emergence of resistant pathogens in animal reservoirs is important particularly for

those with zoonotic potential. Methicillin-resistant Staphylococcus aureus (MRSA) has emerged as a major cause of health care-associated (HA) and community-associated (CA) infections and is also isolated from milk (livestock associated).

Methicillin resistance in S. aureus is primarily mediated by the mecA gene carried

1* Veterinary Assistant Surgeon, Panchetty, Animal Husbandry Department, Tiruvallur district, Tamil Nadu, INDIA. (Corresponding author) sg20685@gmail.com. Mobile : +918870718736

2 Department of Biotechnology, Faculty of Allied Health Sciences, Chettinad Academy of Research and Education, Chennai, Tamil Nadu – 603 103.

3 Veterinary Assistant Surgeon, Mukkarambakkam, Animal Husbandry Department, Tiruvallur district, Tamil Nadu – 601 201.

4 Veterinary Assistant Surgeon, Veterinary Clinician Centre, Cuddalore, Tamil Nadu - 607 001.

5 Veterinary Assistant Surgeon, Thiruvottiyur, Animal Husbandry Department, Tiruvallur district, Tamil Nadu – 600 019.

on a mobile genetic element, the staphylococcal cassette chromosome mec (SCCmec), and at least five types of SCCmec elements have been reported (Beck *et al.*, 1986). To date, SCC elements have been identified within *S. sciuri*, *S. hominis*, *S. epidermidis*, *S. haemolyticus* and *S. aureus*. *mecA* gene codes for the modified penicillin-binding protein 2a (PBP 2a or PBP 2'). PBP2a is located in the bacterial cell wall and has a low binding affinity for β -lactams. As such, PBP2a can continue to catalyze the transpeptidation reaction required for peptidoglycan cross linking, enabling cell wall synthesis in the presence of antibiotics. The role of inappropriate antibiotic usage, under dosage and inappropriate administration are also to be considered in acquiring antibiotic resistance.

MecA is under the control of two regulatory genes, *mecI* and *mecRI*. *mecI* is usually bound to the *mecA* promoter and functions as a repressor. In the presence of a β -lactam antibiotic, *MecRI* initiates a signal transduction cascade that leads to transcriptional activation of *mecA*. *mecA* is further controlled by two co-repressors, *BlaI* and *BlaRI*. *blaI* and *blaRI* are homologous to *mecI* and *mecRI*, respectively, and normally function as regulators of *blaZ* (β -lactamase), which is responsible for penicillin resistance. Although hyperproduction of β -lactamase has been suggested as the resistance mechanism, methicillin resistance in *S. aureus* most commonly results from the production of the novel PBP-2a. PBP-2a requires 2-10 times higher penicillin concentrations for inactivation than PBP-2, and 20 times higher than pBP-1.

At previous study of *S. aureus* from mastitic cattle in north west India reports a high prevalence of MRSA, i.e., 13% of *S. aureus* isolates (14 of 107 samples) (Kumar *et al.* 2011). *MecA*

detection by PCR is accepted as the gold standard method for identifying Methicillin resistant organisms (Buyukcangaz *et al.* 2013). The purpose of this study is to investigate the presence of *mecA* gene in mastitis cases reported from Tamilnadu (India) and its phylogenetic relationship.

MATERIALS AND METHODS

Sample collection:

The milk samples were collected from 55 mastitis affected cows (hard swollen udder with change in milk consistency) that was brought to the veterinary hospitals of Tiruvallur district. Sterile centrifuge tubes were used for collection. The consistency and colour of the milk were noted.

Bacterial identification and antimicrobial susceptibility testing

The milk samples were initially inoculated in buffered peptone water. The bacterial suspension was streaked into blood agar plates. The hemolytic colonies were grown into mannitol salt agar for *S. aureus* and coagulase test was also done for further confirmation. The Methicillin resistance was identified using oxacilin resistance (5 μ g) in Mueller Hinton Agar as per Kampf *et al.* (1998). The non-hemolytic and coagulase negative isolates were differentiated using novobiocin disc sensitivity.

Species specific PCR

The bacterial DNA was isolated from grown culture using De Freitas *et al.*, (2008) with modifications. The staphylococcus species specific PCR (16SrRNA gene) was done using reported primers.

Table 1

Species specific PCR primers (16SrRNA gene)

Species	Primer sequence	Product length	Reference
S. aureus	5' TTCGTACCAGCCAGAGGTGGA 3' 5' TCTTCAGCGCATCACCAATGCC 3'	229 bp	Pradhan et al. (2011)
S. epidermidis	5'-TCTCTTTTAAATTCATCTTCAAT-TCCATAG-3' 5'-AAACACAATTACAGTCTGGT-TATCCATATC-3'	174bp	Martineau et al. (1996)
S. saprophyticus	5'-TCAAAAAGTTTTCTAAAAAAT-TTA C-3' 5'-ACGGGCGTCCACAAAATCAATA-GG A-3'	210bp	Martineau et al. (2000a)

MecA gene detection by PCR:

Table 2

mecA gene PCR primers (designed using primer 3 software)

Primer	Sequence	Position	Product size
Forward	5'CCAGGAATGCAGAAAGACCA3'	400-1058bp (KC243783.1)	659bp
Reverse	5'TGAGGGTGGATAGCAGTACC3'		

The *mecA* gene was amplified by PCR in a 20µl reaction. The reaction conditions were as follows: Initial denaturation (94°C) -5 min, 35 cycles of denaturation (94°C) -45 sec; annealing 55°C - 45 sec; extension 72°C - 45 sec; Final extension 72°C - 5 min. The specificity of PCR primers were confirmed by negative control as *E. coli* DNA and non template control. The sensitivity was determined using 10th fold dilutions of positive DNA. The resistant isolates were tested for presence of *blaZ* gene using reported primers in PCR (Martineau, 2000b).

Sequencing and BLAST analysis

Mec A PCR products of *Staphylococcus aureus*, *S. epidermidis* and *S. saprophyticus* were sequenced using ABI PRISM 3130 Genetic

Analyzer (Applied Biosystems, USA) and the sequences were analysed for similarity search using BLASTn tool (<http://blast.ncbi.nlm.nih.gov>). The sequences have been deposited in NCBI database and Accession Numbers have been assigned from KP336393 – KP336397

Phylogenetic analysis

The similar length sequences were taken from NCBI database and phylogeny was constructed using MEGA V6 software by maximum likelihood method (Bootstrap value-1000) (<http://www.megasoftware.net/>).

RESULTS

Bacterial identification and antimicrobial susceptibility testing

Milk samples have clots in consistency and colours notified were white or yellow. *S. aureus* organisms were identified as yellow Mannitol fermenting colonies in Mannitol salt agar plate. Antibiotic resistance was confirmed by the zone size less than 20mm using antibiotic discs like oxacilin. *S. epidermidis* was differentiated using novobiocin disc sensitivity and also showed no

hemolysis in blood agar plate. Coagulase negative *S. saprophyticus* colonies were identified by novobiocin resistance.

Species specific PCR

The isolated organisms were further confirmed by species specific PCR (Fig. 5). The number of samples from which *mecA* containing organisms isolated, are listed in table 3.

Table 3:

The number of samples from which *mecA* containing organisms *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE) and *Staphylococcus saprophyticus* (MRSS) isolated

No. of milk samples collected from mastitis affected cows	No. of samples from which <i>S. aureus</i> isolated	No. of samples from which <i>S. epidermidis</i> isolated	No. of samples from which <i>S. saprophyticus</i> isolated
55	26	4	1

MecA gene detection by PCR

The sensitivity and specificity of PCR primers were confirmed (Fig. 2 and 3). The PCR results were listed in Table 4 and showed in Fig. 4.

Table 4

MecA gene PCR results

Organisms	Number of isolates	Antibiotic resistance results Oxacilin (5 µg) resistance (<15mm)	Number of MecA PCR positives (percentage)
<i>Staphylococcus aureus</i>	26	+ (5/26)	5/26 (19.23)
<i>Staphylococcus epidermidis</i>	4	+ (2/4)	2/4 (50.00)
<i>Staphylococcus saprophyticus</i>	1	+ (1/1)	1/1(100.00)

The mec A positive isolates were also showed positive for blaZ gene amplification (Fig. 5)

Phylogenetic tree construction:

Phylogenetic tree by MEGA V6.0 using maximum likelihood method (Bootstrap value-1000) was showed in Fig. 6. TN/TVR/BM/SA1/14 formed a within group and TN/TVR/BM/SA2/14 formed an out group with other isolates.

DISCUSSION

The need for rapid and reliable identification of mastitis causing organisms and investigation of local antibiotic resistant pattern of the isolates has become more important in the recent decade. Effective infection control efforts obviously depend on the performance of the laboratory to detect emerging resistant pathogens accurately and confirm their resistance patterns by additional molecular techniques (Bardiau et al. 2013). Here out of 55 mastitis milk samples, 26 samples were shown positive for *Staphylococcus aureus* (21.82%); 4 samples were shown positive for *Staphylococcus epidermidis* (7.27%); one sample for *S. saprophyticus* (1.82%) by isolation and differentiation. Organisms were also present as mixed in one sample for all three; two samples for *S.aureus* and *S. epidermidis*. In mixed type all organisms are mecA positive except only one *S. epidermidis* in dual infection. blaZ PCR was done to confirm the resistance of β -lactam antibiotics in mec A positive isolates.

Previous studies from northwest India and Chennai indicate MRSA positive percentages as 13% and 10.94% (Kumar et al. 2011). The higher percentage (19.23%) in this study clearly indicates improper antibiotic usage and poor intramammary administration of mastitis cows. The emergence of MRSA CC398 (known as livestock associated MRSA) in farm animals and human beings has shown that some *S aureus* lineages might not be strongly host-species restricted. MRSA ST398 can cause infection in people, with close animal contact being the main risk factor, suggesting that farm

animals could provide a reservoir of MRSA. Many antibiotic resistance genes are confirmed to have originated in microorganisms in the environment and have been transferred to other bacteria through mobile genetic elements, such as phages.

S. epidermidis and *S. saprophyticus* are members of coagulase negative staphylococcus (CONS), of that *S. epidermidis* is common commensal in human skin and also a contaminant in implants. *S. saprophyticus* is an opportunistic pathogen, causing cystitis and uncomplicated urinary tract infections in women. Coagulase negative *Staphylococcus* sp. might be common in subclinical mastitis also but presence of mecA in these species is significant one. Jaglic et al. (2010) reported that mecA was common in *S. epidermidis* (50%). Presence of methicilin resistance in *S. epidermidis* and *S. saprophyticus* as mixed infections implies sequel on environment. Methicilin resistant *S. saprophyticus* was isolated from dairy products due to environmental contamination. Phylogenetic tree clearly indicated that mecA of *S. aureus* isolates formed a group with other isolates. The mecA sequences of TN/TVR/BM/SE2/14 *S. epidermidis*, TN/TVR/BM/SE1/14 *S. epidermidis* and TN/TVR/BM/SS1/14 *S. saprophyticus* have 99% homology with *S. aureus* isolates. This clearly shows extensive spread of mecA in environment through mobile elements (Tsubakishita et al. 2011). So reduction of selection pressure in antibiotics for the treatment of mastitis and proper administration should be considered for antibiotic resistance control. Considering that the milking hygiene score correlated with the somatic cell count, which is increased in the presence of mastitis, it is supposable that the improvement of hygiene practices might reduce the risk for MRSA to spread on dairy farms, for example via milkers hands and milking clusters, which represent a common route of transmission for mastitis pathogens, especially *S. aureus* between cows. Prospective studies are needed to investigate MRSA transmission between animals and humans and implement preventive strategies.

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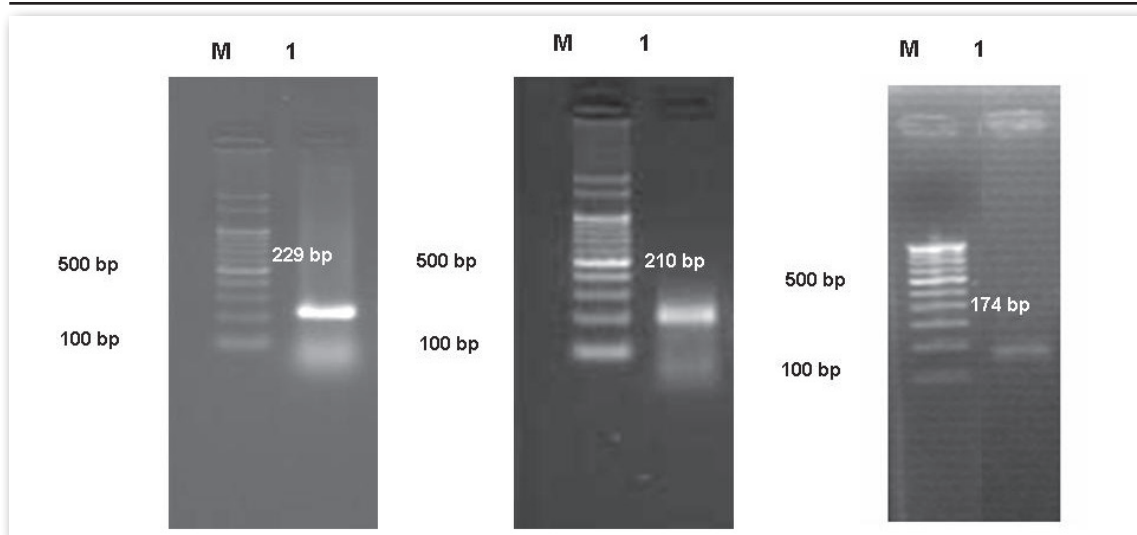


Fig. 1

Amplification of 16SrRNA gene from *S. aureus*, *S. saprophyticus* and *S. epidermidis* respectively

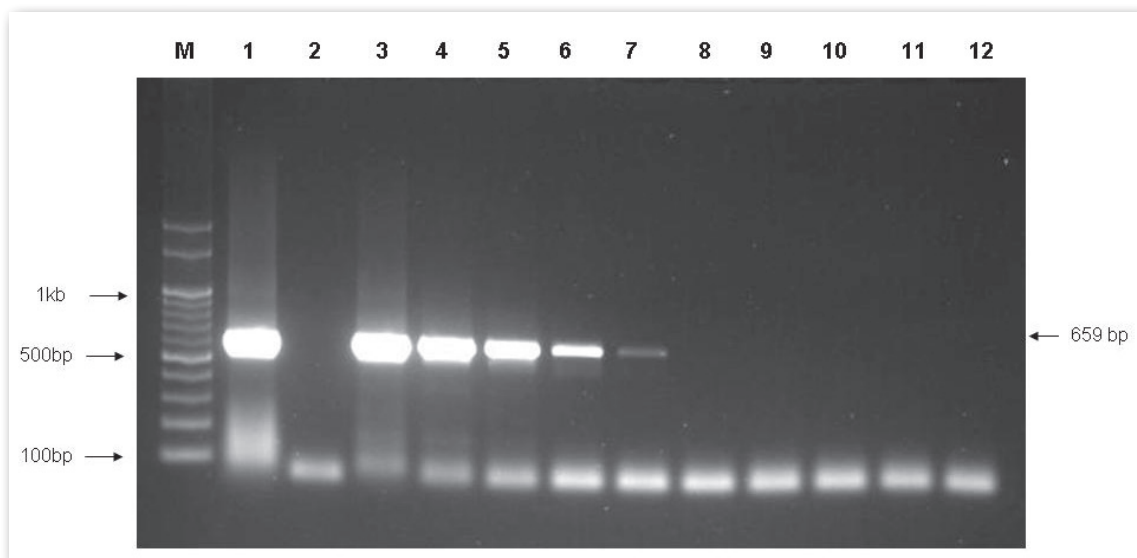


Fig. 2

Sensitivity of *mecA* gene primer

- Lane M : 100 bp DNA Ladder
- Lane 1 : *mecA* Gene amplified from MRSA DNA (887 ng/ μ l)
- Lane 2 : NTC
- Lane 3–12 : *MecA* gene amplicons (10⁻¹ to 10⁻¹⁰ dilutions of MRSA DNA)

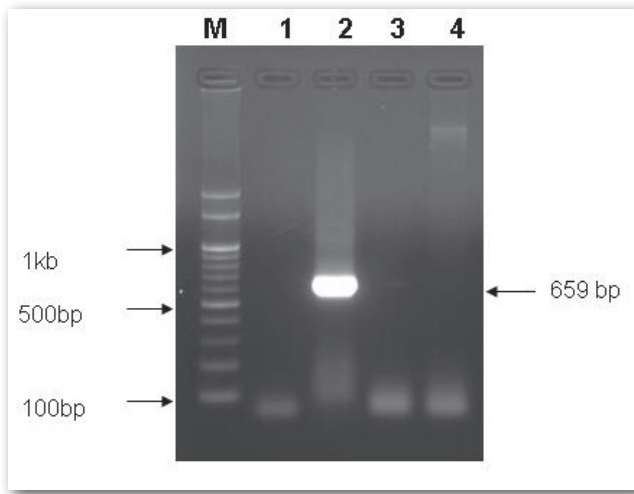


Fig. 3

pecificity of *mecA* gene primer

- Lane L : 100 bp DNA Ladder
- Lane 1 : NTC
- Lane 2 : *mecA* gene amplified from MRSA DNA (887 ng/ μ l)
- Lane 3 : No amplification from MSSA DNA
- Lane 4 : No amplification from *E.coli* DNA

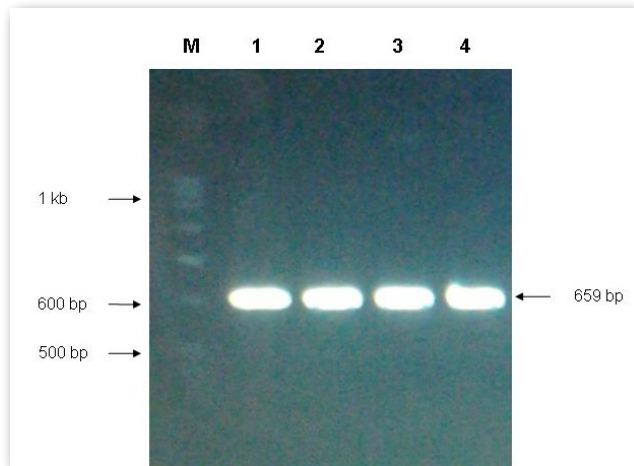


Fig. 4

Amplification of *mecA* gene

- Lane L : 100 bp DNA Ladder
- Lane 1 : *mecA* gene amplified from MR *Staphylococcus aureus* DNA
- Lane 2 : *mecA* gene amplified from MR *Staphylococcus epidermidis* DNA
- Lane 3 : *mecA* gene amplified from MR *Staphylococcus saprophyticus* DNA
- Lane 4 : *mecA* gene amplified from MR *Staphylococcus aureus* DNA

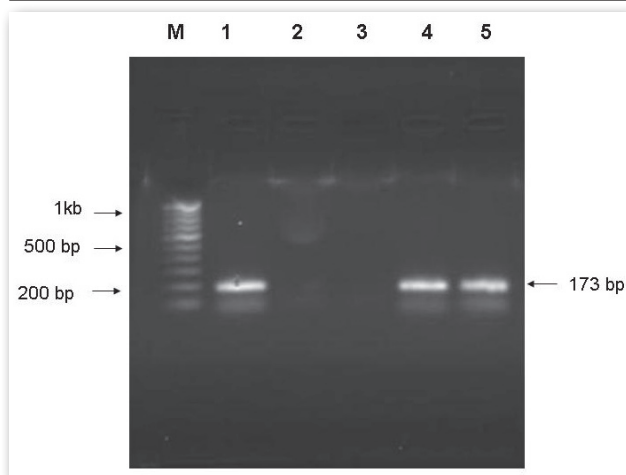


Fig . 5

Amplification of blaZ gene

- Lane L : 100 bp DNA Ladder
- Lane 1 : NTC
- Lane 2 : mecA gene amplified from MRSA DNA (887 ng/μl)
- Lane 3 : No amplification from MSSA DNA
- Lane 4 : No amplification from E.coli DNA

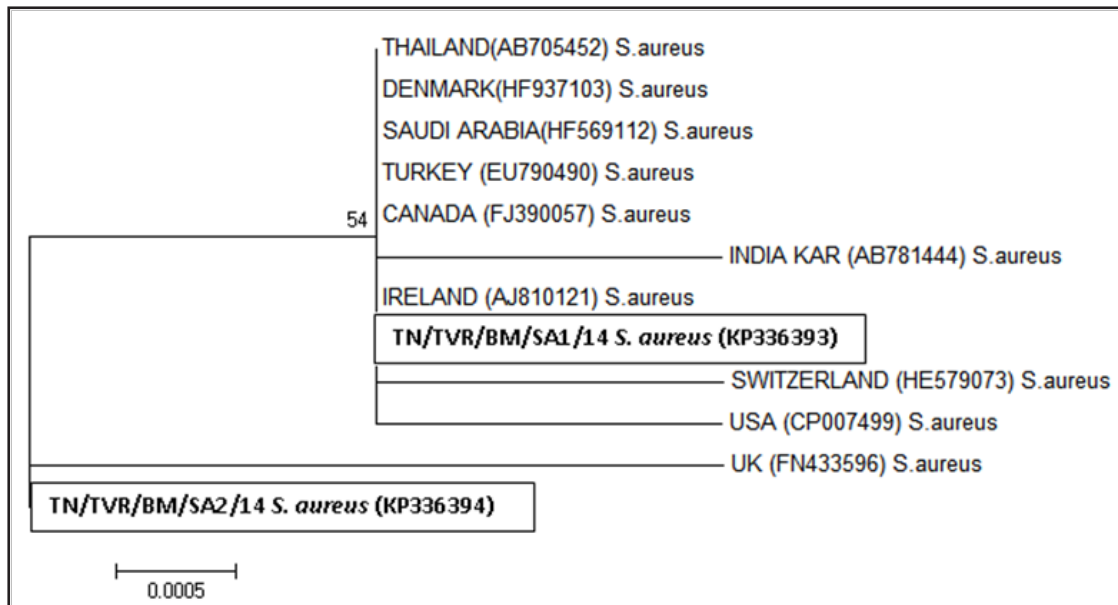


Fig. 6

Phylogenetic tree was constructed by Maximum likelihood method using MEGA V.6. Numbers at the branching points represent the percent occurrence in 1,000 random bootstrap replications