

Identification of Methicillin Resistant *Staphylococcus aureus* in Raw Cow Milk through Amplification of *mecA*

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Conventional detection of *Staphylococcus aureus* in farm animals has a greater importance as they are not only reservoirs of drug resistant *S. aureus* but also there is a possible chance of transmission between hosts. Thus in the current study 30 milk samples from dairy herds and milk venders of different places were examined. On the basis of black shiny colonies with clear zone on Baird parker plate, Gram staining followed by catalase and coagulase tests; 12 of 30 samples (40%) were morphologically identified as *S. aureus*. Further growth in *aureusAlert*TM screening kit and amplification of 1.5kb 16S rRNA confirms screened isolates were *S. aureus*. The antibiotic susceptibility profile showed all the 12 isolates were resistant to Ampicillin and Pencillin where as 3 isolates (25%) LMV3, LMV4 and LMV5 exhibited additional resistance to Oxacillin with MIC of 14 ± 1.5 to $16 \pm 0.75 \mu\text{g/ml}$ indicating methicillin resistance. Colour change in *aureusAlert*TM/S confirmation kit and presence of *mecA* gene (304 bp PCR product) on the chromosome of 3 oxacillin resistance isolates confirms them genetically as methicillin resistant *S. aureus* (MRSA) strains. Therefore results of the present study corroborate the gold standard methods for detecting MRSA in the raw milk.

Key words: *Staphylococcus aureus*, 16S rRNA, *mecA*, *aureusAlert*TM, MRSA, raw milk.

Staphylococcus aureus is an endogenous commensal pathogen of both animals and humans. It secretes numerous toxins, cell surface proteins and virulence factors which promotes evasion of the host immune system and causes most of the nosocomial and community acquired infections which are very difficult to treat¹. Routine investigations have reported that *S. aureus* affects the milk producing gland of farm animals², becoming shedders of drug resistant *S.*

aureus; that has been increasing in many countries throughout world and has become most prevalent pathogen causing high morbidity and mortality³. To maintain the population at sanitary level microbiological analysis of milk is very important. Conventional examination of milk from veterinary public health has detected the incidence of *S. aureus* in cattle associated with mastitis; contaminates the raw milk and infects host tissues due to the characteristic feature of *S. aureus* to coagulate plasma and production of entero toxins⁴. As milk and its derivatives are important sources for transmission of infections between species⁵⁻⁷; there is a greater risk of community acquired and nosocomial infections⁸. Excessive usage of antibiotics to treat infections has resulted in evolving multidrug resistant *S. aureus* which has

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become a major public health burden⁹. Some drug resistant strains of *S. aureus* have been designated as epidemic strains associated with high incidence of infections⁷ and the risk of infections are very high among diabetic patients, intravenous drug using patients and patients undergone to surgeries when compared to normal^{10,11}. One of such dangerous drug resistant strain is methicillin resistant *Staphylococcus aureus* (MRSA)

S. aureus acquire resistance to most of the β -lactams evolving MRSA and Vancomycin resistant enterococci (VRE). One of the factors responsible for evolving MRSA is acquisition of *mecA* operon which is involved in synthesis of Penicillinase binding protein (PBP2a) that reduces the affinity for binding to β -lactam antibiotics ensuing β -lactam resistance¹². This *mecA* gene is carried by *Staphylococcal* cassette chromosome *mec* which is integrated at 3' end of the *orfX* on the chromosomes of these MRSA strains¹³. The *mec* operon span around 30-50 Kb in size¹¹ that primarily consists of one structural gene, two regulatory genes and *mec* associated gene. The structural gene in *mec* operon is *mecA* which is indispensable for intrinsic methicillin resistance and the regulatory genes are *mecI* and *mecR*¹³. The regulatory protein *mecI* lowers the *mecA* activity by acting as a repressor; whereas *mecR* is a transmembrane sensor-transducer protein that can sense the presence of β -lactam antibiotics in the extracellular environment¹⁴. Thus *S. aureus* acquire resistance to methicillin through *mecA* gene which has become a widespread problem across the globe¹⁵. Therefore the series of cattle infections due to *S. aureus* has prompted us to examine the raw milk, as milk and its products are highly consumable as routine diet throughout world. Thus present study deals identification of MRSA in raw milk through PCR amplification of *mecA* which was responsible for methicillin resistance.

MATERIALS AND METHODS

In the present study all the chemicals, reagents, enzymes, plastic and glass ware and other consumable items were procured from Sisco Research Laboratories Pvt. Ltd., India, Hi-Media Laboratories Pvt. Ltd., India, Sigma-Aldrich, USA, New England Biolabs, USA and QIAGEN Inc., Valencia, CA.

Sample collection

The routine bacteriological investigations of milk samples from veterinary public health of Sri Venkateswara Veterinary University Tirupati, dairy herds and local milk vendors was detected *S. aureus* infections. From this investigation 30 milk samples were screened for detecting bacterial isolates. Thus the samples from local milk vendors regarded as LMV and dairy cows regarded as D were streaked on blood agar, MacConkey agar plates (Himedia) following incubation at 37°C for 48-72hrs^{13,16}.

Screening tests for *S. aureus*

The colonies showing phenotypic characters of *Staphylococci* were further sub cultured on Baird-parker (BP) agar containing 5% egg yolk and 1% potassium tellurite¹⁷ at 37°C for 15-24h. The colonies thus, appeared on the BP plates were further characterized using classic tests of Gram staining, catalase, coagulase and PCR amplification of unique genes. Gram staining was performed for morphological identification of the bacteria¹⁸. Catalase test was performed by adding few drops of 3% H₂O₂ on the vicinity of fully grown colonies present on the BP plate.

*Aureus Alert*TM screening kit

The strains showing catalase positive were further tested for coagulase activity to distinguish *aureus* from other species of *Staphylococci* using *aureusAlert*TM screening kit as per manufactures protocols (Cat K053A-Himedia). Media present in the *aureusAlert*TM screening kit was rehydrated with the suspension fluid provided in the kit. Then a single colony from each strain on BP agar plate was picked and inoculated into the *aureusAlert*TM screening kits and were incubated at 37°C for 15hrs.

Antimicrobial susceptibility screening

The susceptibility of all positive *S. aureus* strains to different antibiotics were performed according to the guidelines of Clinical Laboratory Standard Institute (CLSI) using disk diffusion method (Bauer et al, 1966, CLSI-2007). For this standardized suspension of inoculum was prepared to a concentration of 10⁶CFU/ml and 0.1ml of this inoculum was spreaded on Mueller Hinton (MH) agar plates using a sterile cotton swab and incubated for few minutes at room temperature for moisture drying. Then commercially available selected antibiotic disks (listed in Table1) were

placed at equal distance from each other and incubated for 24-30hrs at 37°C. Then susceptibility, intermediate resistance and resistance were discriminated by measuring the inhibitory zone around the disks using Himedia zone measure scale and expressed according to the ^{19, 20} methods. *S. aureus* ATCC12600 was used as standard.

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) was also examined for Oxacillin resistant isolates according to NCCLS recommendations by an agar dilution method using an inoculum of 10⁶ CFU/ml on MH agar plates containing Ampicillin, oxacillin and pencillin ranging from 25 to 100 µg/ml, 0.5 to 25 µg/ml and 25- 75 µg/ml respectively.

Screening of MRSA with *aureusAlert R/S™* confirmation kit

After MIC determination Oxacillin resistant isolates were further cross checked to confirm methicillin resistance using *aureusAlert™ R/S confirmation kit* (Cat K053B-Himedia) which was highly specific to MRSA. For this the media present in the MRSA confirmation kit was rehydrated with suspension fluid and Oxacillin resistant isolates were inoculated into the kit with antibiotic (Tube 1) and without antibiotic (Tube 2) followed by 15hrs of incubation at 37°C. *S. aureus* ATCC12600 was taken as control strain.

Genetic analysis of milk isolates through Polymerase chain reaction

In order to screen the isolates at genetic level we have gone through polymerase chain reaction (PCR). For this genomic DNA was extracted from all *S. aureus* positive isolates according to the method of ^{21, 22}. Then PCR was performed for each strain using the extracted genomic DNA as template for amplification of *16S rRNA* and *mecA* genes.

Amplification of *16S rRNA*

All the isolates were tested for the presence of *16S rRNA* to confirm the strains at genetic level. The primer sequences used for *16S rRNA* were Forward 5'-AGTTTGATCCTGGCTCAG-3' and Reverse 5'-AGGCCCGGAACGTATTCAC-3'²³. The reaction mixture for amplification of *16S rRNA* consists of 1X Taq DNA buffer (2.5 mM MgCl₂ and 15mM Tris Hcl), 200 µM dNTP mix, 20 picomole of each primer, 1U of Taq DNA polymerase and 1 µg of template DNA to a final volume of 50 µl with filter sterilized

Milli-Q water. The PCR protocol was programmed for 40 cycles of initial denaturation at 94°C for 10 min, denaturation at 94°C for 50 seconds, 54°C for 60 seconds of annealing, 72°C for 80 seconds of extension and 72°C for 10 min of final elongation in a thermo cycler. The amplified product was electrophoresed on a 1.2% Agarose gel electrophoresis using 1X-TAE (Tris -Acetate-EDTA) buffer and stained with Ethidium bromide and viewed under UV trans illuminator. The results were recorded using vilbour loulmart gel documentation system (France).

PCR amplification of *mecA*

Presence of *mecA* gene was examined in all the *S. aureus* isolates using polymerase chain reaction (PCR), with Forward 5'-TGGCTATCGTGTCAACAATCG-3' and Reverse 5'-CTGGAACCTTGTTGAGCAGAG-3' primers according to ²³. The cocktail mixture consists of 1X Taq DNA buffer (2.5 mM MgCl₂ and 15mM Tris HCl), 200 µM dNTP mix, 20p of each primer, 1U of Taq DNA polymerase and 1 µg of template DNA to a final volume of 50 µl with Milli-Q water. The thermocycler was programmed for 40 cycles in Eppendorf (Master gradient) with following conditions ; initial denaturation at 94°C for 5 min, denaturation at 94°C for 50 seconds, 52°C for 40 seconds of annealing, 72°C for 45 seconds of extension and 72°C for 5 min of final elongation. Amplified product was electrophoresed and viewed as described in earlier.

RESULTS AND DISCUSSION

S. aureus colonizes on unhygienic surfaces and possesses relentless threat causing heavy morbidity and mortality resulting in high economic loss³. Regular usage of antibiotics to treat *S. aureus* infections leads to emergence of MRSA that has become most prevalent pathogen in animals². Hence in the present study the suspected cows with *S. aureus* infection were investigated and 30 raw milk samples from different cows were streaked on blood and Mac Conkey agar plates¹³ showed distinct colonies of *Staphylococci* morphology which were identified upon Gram staining. Presence of dark clear black, shiny, convex shaped colonies surrounded by clear zone observed within 24 hrs on modified BP agar plates indicated the presence of *S. aureus* in

40% of the milk samples (Fig 1A) ¹⁷. Presence of egg yolk and anaerobic conditions makes *S. aureus* to express large amounts of coagulase enzyme which coagulates the egg yolk and form clear zone while tellurite in the media gives black shiny nature¹⁷. Clear effervescences appeared from the colony upon adding 3% H₂O₂ in the vicinity of the colony

indicates that the strains are catalase positive; further, clotting of the *S. aureus* inoculated media in *aureusAlert*TM screening kit represented the coagulase positive nature of *Staphylococcus aureus* thus 12 samples (40%) were morphologically identified as *S. aureus* (Fig 1B).

Antibiotic susceptibility profile indicated

Table 1. Antibiotic susceptibility profile of *S. aureus* isolates from milk samples

S. No	Milk isolates	Zone diameter of different antibiotics in (mm)			
		Ampicillin	Oxacillin	Pencillin	Vancomycin
1	LMV 1	15 ± 1	21 ± 1	No zone	18 ± 1
2	LMV 2	12 ± 2	20 ± 2	No zone	17 ± 2
3	LMV 3	10 ± 1	8 ± 1	No zone	19 ± 1
4	LMV 4	11 ± 1	9 ± 1	No zone	19 ± 2
5	LMV 5	10 ± 2	8 ± 1	No zone	18 ± 1
6	LMV 6	22 ± 1	25 ± 1	No zone	18 ± 1
7	LMV 7	No zone	27 ± 1	No zone	17 ± 2
8	LMV 8	21 ± 2	22 ± 2	No zone	18 ± 1
9	D 1	No zone	20 ± 2	No zone	18 ± 2
10	D 2	No zone	23 ± 1	No zone	19 ± 1
11	D 3	No zone	23 ± 1	No zone	20 ± 1
12	D 4	No zone	21 ± 2	No zone	20 ± 1

± SD values obtained from three determinations

The *S. aureus* isolates screened from milk sample are showing different zone diameter. Isolates from dairy herds are showing no zone to Ampicillin and Pencillin. Isolates from milk vendors are showing no zone to Pencillin. All isolates are showing 17±2 to 20±1 mm zone to vancomycin.

Table 2. Resistance pattern of *S. aureus* isolates from milk samples

S.No	Strains	Resistant Against Antibiotics
1	LMV 1, LMV2, LMV6 , LMV7, LMV 8	Ampicillin, Pencillin
2	LMV 3, LMV4, LMV 5	Ampicillin, Oxacillin, Pencillin
3	D1, D2, D3, D4	Ampicillin, Pencillin

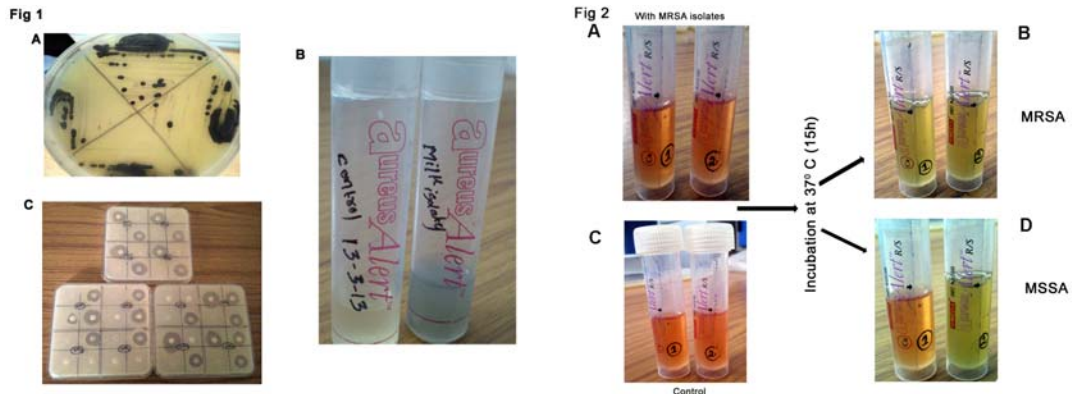
Resistance pattern of milk isolates indicated that the isolates from milk samples are showing different antibiotic susceptibility. 12 isolates are showing resistance to Pencillin and Ampicillin. Three isolates LMV 3, LMV 4 and LMV 5 are showing resistance to Oxacillin. All isolates are sensitive to vancomycin.

Table 3. MIC of MRSA strains against different antibiotics (µg/ml)

S.No	MRSA strains	Ampicillin	Oxacillin	Pencillin
1	LMV 3	50 ± 5	14 ± 1.5	27 ± 0.5
2	LMV 4	55 ± 3	16 ± 0.5	30 ± 0.75
3	LMV 5	53 ± 3	16 ± 0.75	29 ± 0.5

±SD values obtained from three determinations

Three milk isolates were showing different MIC values against 1µg of Oxacillin.

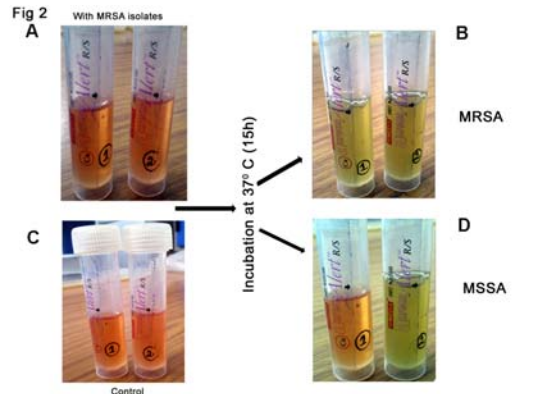


1A: BP agar plate showing black, shiny colony surrounded clear zone indicating coagulase positive *Staphylococcus aureus* nature of the milk isolates.

1B: *aureusAlert*TM screening kit (Himedia) confirms the coagulase activity by clotting the milk isolates inoculated media. *E. coli* was used as control strain.

1C: Disk diffusion test results of milk isolates showing different zone diameter.

Fig. 1. Screening results of milk isolates



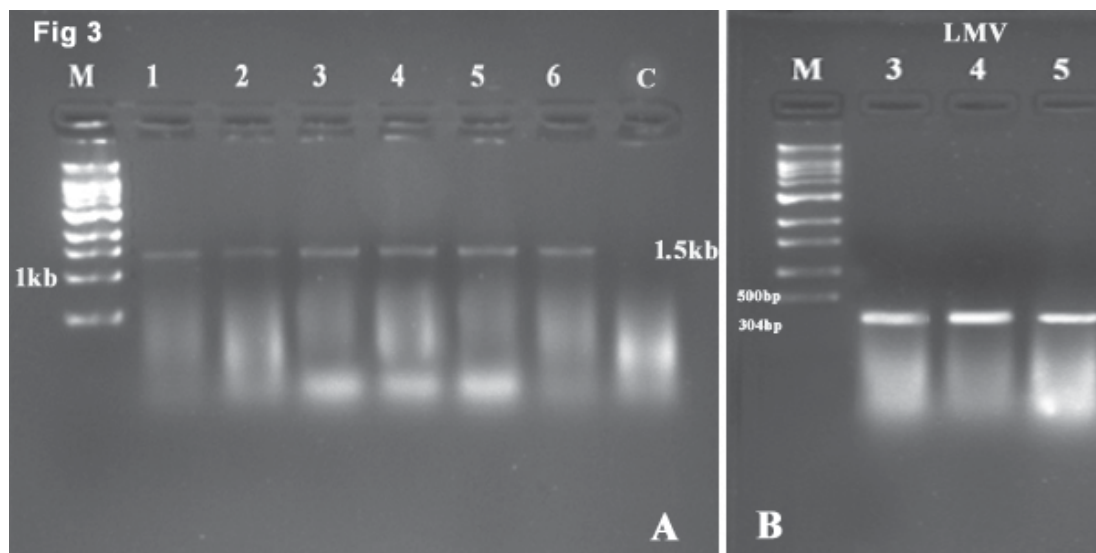
A: cultures of three milk isolates (LMV 3, LMV 4 and LMV 5) were inoculated in to the media containing Oxacillin (Tube 1) and without Oxacillin (Tube 2).

B: after incubation of cultures at 37°C for 15hrs Yellow colour appearance in Tube 1 and 2 (with and without oxacillin) indicates three isolates LMV3, LMV 4 and LMV 5 are MRSA positive.

C: culture of *S. aureus* ATCC 12600 (control) was inoculated in to the media containing Oxacillin (Tube 1) and without Oxacillin (Tube 2).

D: Colour change in Tube 2 and no colour change in Tube 1 (Tube with Oxacillin) indicate *S. aureus* ATCC 12600 is sensitive to methicillin.

Fig. 2. MRSA screening results of milk isolates (Oxacillin resistance)



Lane M: Molecular size markers obtained from Bangalore Genei Pvt Ltd,
Lanes L1 to L6: PCR amplification of 1.5 kb *16S rRNA* gene obtained from milk isolates.
Lane C: control

Fig. 3(a). The electrophoretogram showing PCR amplification of *16S rRNA* from milk isolates

Lane M: Molecular size markers obtained from Bangalore Genei Pvt Ltd,
Lanes L3 to L5: PCR amplification of 304 bp *mecA* gene obtained from milk isolates of LMV3, LMV 4 and LMV5
Fig. 3(b). Electrophoretogram showing the PCR amplification of *mecA* from milk isolates

that all isolates were found to be resistant to both Ampicillin and Pencillin whereas; highly sensitive to Vancomycin showing 17 ± 2 to 20 ± 1 mm zone (Fig 1C). However; 25% of samples LMV 3, LMV 4, LMV 5 showed additional resistance to oxacillin with MIC in the range of 14 ± 1.5 to 16 ± 0.75 $\mu\text{g/ml}$ (Tables 1, 2 and 3)¹¹. Further MRSA validation was performed using *aureusAlertR/S™ confirmation kit* which was highly specific to MRSA that do not misclassify MRSA as MSSA. orange to yellow colour change in both tubes of 3 Oxacillin resistant isolates indicated the presence of MRSA compared with controls Tube 1 and 2 whereas, no colour change was observed in rest of the 30% isolates when tested with same kit indicating these are MSSA strains (Fig 2)¹¹. Genotypical characterization of milk isolates showed 1.5kb *16S rRNA* gene product specific to *S. aureus* in 40% of the isolates confirming as *Staphylococcus aureus* (Fig 3A). However; only 25% of the isolates LMV 3, LMV 4 and LMV 5 showed 304 bp *mecA* amplicon in addition to *16SrRNA* (Fig 3B) demonstrating that oxacillin/methicillin resistance was due to expression of *mecA*¹⁵.

The result of the present study indicated that in total 40% of the isolates from milk were identified as *S. aureus* morphologically on culture, Gram staining, traditional biochemical tests and using highly specific *aureusAlertR/S™* kit. Antibiotic susceptibility profile followed by MIC indicated that only 3 (25%) isolates from raw milk were showing resistance to oxacillin which was further confirmed through MRSA confirmation kit. According to PCR results 25% (3 of 12) of the *S. aureus* isolates are *mecA* positive (*mecA*⁺); whereas 30% of the milk isolates are *S. aureus* positive but *mecA* negative (*mecA*⁻). Moreover a common feature represented by *mecA*⁺ and *mecA*⁻ isolates is sensitivity towards Vancomycin. Expression of methicillin resistance depends on genetic and environmental factors. The genetic factors *mecI* and *mecR1* repress the *mecA* activity whereas interaction of 2-lactams with *mecR1* through signalling pathway induces *mecA* activity¹¹. Therefore from the present study it was very clear that divergent reports of presence of MRSA in milk isolates was due to differential distribution of *mecA* gene which was responsible for methicillin resistance²⁴. Although high methicillin resistance was not found in all isolates (25% of the isolates

are MRSA) current study indicates that emerging profile of MRSA is a dangerous sign of both community acquired and nosocomial infections as milk and their products are highly consumed as routine diet through worldwide¹¹. Therefore current study gains a novel importance by creating awareness in society about MRSA infections from raw milk suggesting effective food processing methods are required for proper public health protection.

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