

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.707.397>

Detection of *Staphylococcus aureus* and Methicillin Resistant *Staphylococcus aureus* (MRSA) from Bovine Raw Milk by PCR

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ABSTRACT

Staphylococcus aureus and Methicillin-resistant *Staphylococcus aureus* (MRSA) has been considered to be a major cause of healthcare infections worldwide and poses a major threat to public health. It is also one of the main etiological agents which is responsible for clinical and subclinical mastitis in dairy herds. This study was designed to investigate the occurrence of *S. aureus* and Methicillin resistant *Staphylococcus aureus* (MRSA) from bovine raw milk by Polymerase chain reaction (PCR) targeting thermonuclease (*nuc*) and *mecA* gene. A total of 115 bovine raw milk samples were collected and screened for the presence of *S. aureus* and MRSA. The samples were processed by standard conventional procedures for isolation of the *S. aureus* organism. Conventional culture method which include, Brain heart infusion broth with 10% sodium chloride followed by direct plating on Baird-Parker agar (BP) at 37° C for 24-48 hours. Molecular characterization of the isolates was done by PCR targeting thermonuclease (*nuc*) gene for *S. aureus* and for Methicillin Resistant *Staphylococcus aureus* (*mecA*) gene was used. Out of 115 milk samples 52/115 (45.22%) samples were found positive for *S. aureus* by conventional culture method. DNA was extracted from all the presumptive positive isolates. PCR targeting *nuc* gene for *S. aureus* and *mecA* gene for MRSA was carried out and the results showed that out of 48/52 (92.31%) for *nuc* gene and 39/52 (75%) for *mecA* gene were positive. This study showed that the prevalence of *S. aureus* and MRSA in bovine milk can play a role in zoonotic transmission, and PCR can be used as one of the rapidly and highly sensitive tests for detection and classification of *S. aureus* and MRSA by targeting *nuc* and *mecA* gene.

Keywords

Staphylococcus aureus, Methicillin-resistant *Staphylococcus aureus* (MRSA), Polymerase chain reaction, Conventional

Article Info

Accepted:
24 June 2018
Available Online:
10 July 2018

Introduction

Staphylococci is the major cause of both nosocomial and community-acquired infections (Diederer and Kluytmans, 2006) and out of all the *Staphylococcus* species the most important is the *S. aureus*. It is a

ubiquitous Gram-positive microorganism as well as an important opportunistic pathogen in human and also the main etiological agent of clinical and subclinical mastitis in dairy herds (Gilbert *et al.*, 2006). Mastitis is one of the major causes of economic losses in dairy industry worldwide. Its presence in raw milk

is a major concern for the safety and the quality of traditionally dairy products (Elbes *et al.*, 2006).

The emergence of multi-drug resistance in *Staphylococcus aureus* bacteria has become a major healthcare problem. Today greater than 95% of all *S. aureus* isolates possess resistance to penicillin and 40–60% of clinical isolates in the United States of America and the United Kingdom express methicillin resistance (Levy and Marshall, 2004; Neu, 1992).

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognised as major cause of healthcare-associated infections worldwide. MRSA is a pathogen emerging in hospitals as well as in community and livestock. MRSA strains appear to have been transferred from health care settings into the community and have emerged as particularly associated with community-associated infections in humans (Scientific Report of EFSA and ECDC, 2015).

In recent years, MRSA has been identified as an emerging pathogen in livestock and companion animals, as well as some other farm animal species (Antoci *et al.*, 2013). Resistance to methicillin is conferred by the *mecA* gene which encodes a modified penicillin-binding protein (PBP2a or PBP2), that has low affinity for almost all β -lactam antibiotics (penicillins, cephalosporins, carbapenems).

The *mecA* locus is a highly conserved gene that encodes PBP2a in resistant strains but is absent from susceptible ones making it a useful molecular marker of β -lactam resistance (Pinho *et al.*, 2001). Also, MRSA strains are often resistant to antimicrobials other than β -lactams of which many members are widely used in both human and veterinary medicine (Lowy, 2003; Pinho *et al.*, 2001). Thus the detection of the *mecA* gene using

polymerase chain reaction (PCR) can be used to identify MRSA.

The aim of this study was to isolate and molecular characterization of *S. aureus* and Methicillin resistant *Staphylococcus aureus* (MRSA) from bovine raw milk by Polymerase chain reaction (PCR) targeting thermonuclease (*nuc*) and *mecA* gene.

Materials and Methods

Ethical approval

Milk samples were collected as per standard method without any harm to the animals so approval from Institutional Animal Ethics Committee to carry out this study is not required.

Sample collection

A total of 115 raw milk samples were randomly collected from cows brought to Madras Veterinary College (MVC) Teaching Hospital, Chennai. The milk samples were collected aseptically in sterilized screwcork tubes and transported in an icebox to laboratory of the Department of Veterinary Public Health and Epidemiology, MVC, Chennai for further processing and microbiological analysis.

Isolation of *Staphylococcus* species

Isolation of *Staphylococcus spp.* from milk samples was done by enriching into sterile Brain heart infusion broth supplemented with 10% sodium chloride and incubated at 37° C for overnight and selective plating was done by transferring loopful of inoculum on Baird-Parker (BP) agar medium supplemented with (5% Egg yolk tellurite and 3.5% potassium tellurite) and incubated at 37° C for 24-48 hours.

Bacterial identification based on cultural and morphological characteristics

Cultural characteristics

Appearance of circular, smooth, moist, jet gray black to jet black colonies surrounded by a clear halo zone were considered to be presumptive for *S. aureus* (Fig. 1).

Morphological characteristics

Microscopic examination of the smear slide stained with Gram's stain revealed Gram positive, spherical cells arranged in irregular clusters resembling to bunch of grapes (Fig. 2).

Polymerase Chain Reaction (PCR) for detection of *nuc* and *mecA* gene

DNA extraction

Presumptive *Staphylococcus aureus* isolates were used for extraction of DNA by Alkanine lysis Polyethylene glycol (AL-PEG) method as outlined by Chomczynski and Rymaszewski (2006). DNA was extracted by taking a loopful of presumptive colonies and dissolved in 100 µl of distilled water and 500 µl of AL-PEG reagent which constitute of (60g PEG + 0.93ml 2M KOH + 39 ml water) for 100ml AL-PEG reagent) was added and incubated in water bath at 60° C for 10 minutes. Supernatant was collected in separate eppendorf and from this 2-3 µl of supernatant was used as a template for PCR.

Primers used for PCR

Molecular characterization of the isolates was done by targeting the thermonuclease (*nuc*) gene for *S. aureus* and Methicillin resistant *Staphylococcus aureus* (MRSA) by using *mecA* gene. Details of the primers and cycling condition are given in Table 1 and 2.

PCR reaction

PCR was performed in a 25 µl reaction mixture which includes 12.5 µl master mix (Ampliqon), 10pM concentration of each primer and 2.5 µl of DNA template and remaining volume was adjusted using nuclease free water.

Electrophoresis and gel documentation

PCR products (amplicon) were subjected to gel electrophoresis (1.2 % agarose gel with 0.8µg/ml ethidium bromide) at 100V for 30 min was performed. Gels were visualized under UV transilluminator and the results were documented using gel documentation system (Biorad).

Results and Discussion

S. aureus causes a variety of diseases in human and animals. Infections vary from a mild skin infection to severe pneumonia and septicemia (Lowy *et al.*, 1998). *Staphylococcus aureus* is associated with subclinical mastitis in dairy cattle and may be present in milk and other dairy products (Capurro *et al.*, 2010). The emergence of multi-drug resistance in *Staphylococcus aureus* bacteria has become a major healthcare problem in recent years. MRSA is a significant public health concern given its ability to contaminate food of animal origin and to colonize and infect humans and animals (Petinaki and Spiliopoulou, 2012). In this study a total of 115 bovine milk samples was collected randomly out of which 52/115 (45.22%) samples were found positive for *S. aureus* by conventional culture method. DNA was extracted from all the presumptive positive isolates for *S. aureus* by culture. PCR targeting *nuc* gene for *S. aureus* and *mecA* gene for MRSA was carried out and the results showed that out of 48/52 (92.31%) for *nuc* gene and 39/52 (75%) for *mecA* gene were positive (Fig. 3 and 4).

Table.1 Details of the primers

Target	Primer Sequence 5'-3'	Amplicon size (bp)	Reference
<i>nuc</i> -F	GTGCTGGCATATGTATCGCAATTGT	181	Hedge, 2013
<i>nuc</i> -R	TACGCCCTTATCTGTTTGTGATGC		
<i>mecA</i> -F	GAAATGACTGAACGTCCGATAA	310	Kobayashi <i>et al.</i> , 1994
<i>mecA</i> -R	CCAATTCCACATTGTTTCGGTCTAA		

Table.2 Primers cycling conditions

Sl.No	Cycling condition	<i>nuc</i> gene			<i>mecA</i> gene		
		Temperature and Time	Cycles		Temperature and Time	Cycles	
1.	Initial Denaturation	94° C 5 minutes	1		94° C 5 minutes	1	
2.	Denaturation	94° C 30 seconds	30		94° C 30 seconds	25	
3.	Annealing	54° C 30 seconds			50° C 40 seconds		
4.	Extension	72° C 30 seconds			72° C 1 minute		
5.	Final extension	72° C 10 minutes	1		72° C 5 minutes	1	

Fig.1 Characteristic colonies in BP agar medium (circular, smooth, moist, gray black to jet black colonies, surrounded by a clear halo zone were considered to be presumptive for *S. aureus* zone

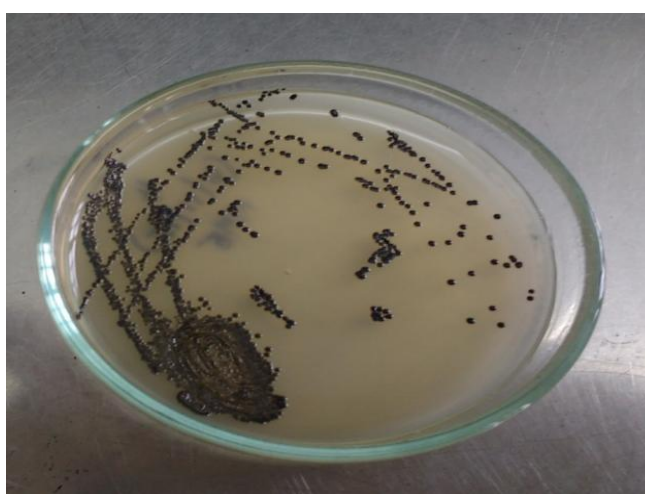


Fig.2 Gram's staining showing spherical cells arranged in irregular clusters resembling to bunch of grapes

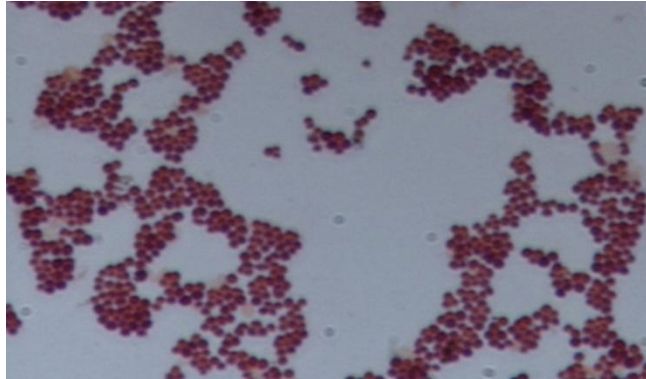
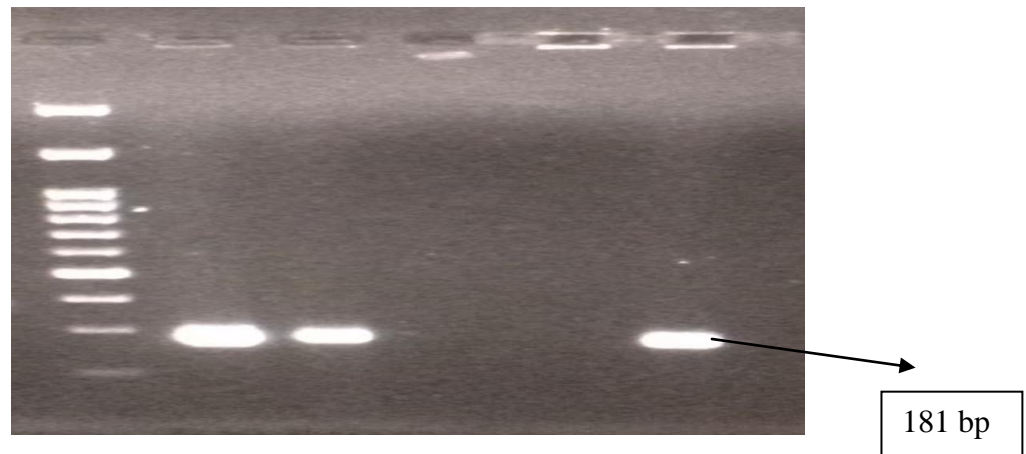
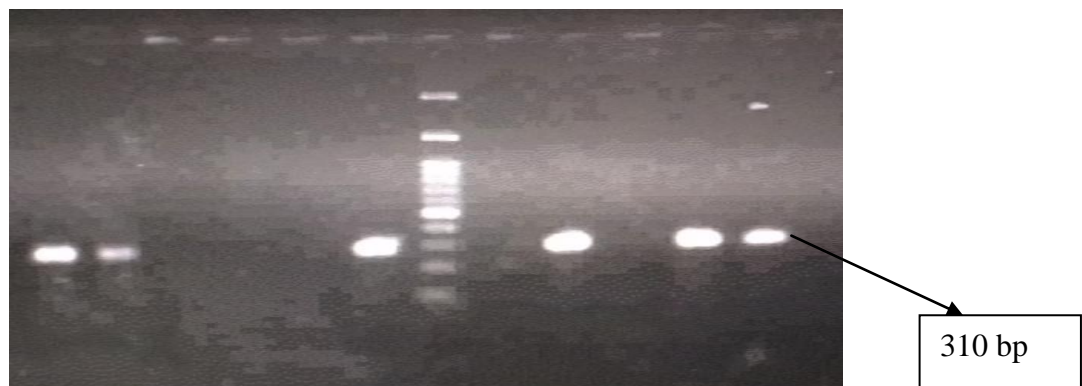


Fig.3 Agarose gel electrophoresis of PCR product amplified from *nuc* gene (181 bp)



Lane 1 - 100 bp DNA Ladder
Lane 2 - Positive control
Lane 3, 6 – positive samples
Lane 4, 5- negative samples

Fig.4 Agarose gel electrophoresis of PCR product amplified from *mecA* gene (310 bp)



Lane 7 – 100bp DNA ladder
Lane 6 – *mecA* positive control
Lane 3,4,5,8,10- Negative samples
Lane 1, 2, 9,11,12 – Positive samples

The primary objective of this study was to isolate and identify *S. aureus* and MRSA from bovine raw milk. In this study the overall presence of *S. aureus* by conventional culture method was 45.22% (52/115). Further confirmation was done by doing PCR targeting *nuc* gene for *S. aureus* and *mecA* gene for MRSA and the results showed that 92.31% (48/52) and 75% (39/52) samples were positive for *S. aureus* and MRSA.

Isolation rates of *S. aureus* observed in our study is consistent with the findings of other studies such as 53.3% by Gundogan *et al.*, (2005), 57.3% by Ertas *et al.*, (2010) and 52.4% by Gucukoglu *et al.*, (2012). In another study (Orges *et al.*, 2008) observed *S. aureus* in 67% of isolates from raw milk. Worldwide several studies suggest that *S. aureus* isolation rates in milk can vary from (13.5%) to (64.7%) (Umathi *et al.*, 2008; Nakal and Kaliwal, 2010). In Morocco, Bendahou *et al.*, (2008) studied 27 samples and found 40% of the milk samples were containing *S. aureus*, Lingathurai and Vellathurai, (2010) 61.7% of the raw milk samples were found positive out of 60 samples studied

The *nuc* gene is widely employed as the target gene for specific detection of *S. aureus* (Wilson *et al.*, 1991; Kim *et al.*, 2001; Ramesh *et al.*, 2002). Akindolire *et al.*, (2015) also used PCR targeting *nuc* gene for identification of *S. aureus* and the proportion of *S. aureus* was higher (75%) in raw milk than in pasteurised milk (29%).

Our results are in accordance with the results of various authors who has also reported the presence of MRSA by using PCR based *mecA* gene amplification which confirmed more than 99% of MRSA isolates (Hata *et al.*, 2010). Chandrasekaran *et al.*, (2014) reported 49.36% samples positive for *S. aureus* of which 10.34% were MRSA from clinical mastitis milk samples. Riva *et al.*, (2015) found that the prevalence of *S. aureus* was

9.1% in raw milk and the 20% were MRSA. Out of the total 160 milk samples, 36 (22.5%) samples yielded *S. aureus* by using *nuc* gene and 23SrRNA gene. Out of the total 36 confirmed *S. aureus* isolates, 6 (16.6%) isolates were confirmed to be MRSA when subjected to PCR amplification using specific primers for *mecA* gene (Hamid *et al.*, 2017).

Variation in the prevalence percentage of *S. aureus* in comparison to other workers might be due to sample size, antibiotic use in animal husbandry and hygiene practices among the dairy cows. The source of acquisition of MRSA may be due to contact with human or animal carriers. MRSA infected cattle acts as a reservoir and later transmit the infections to other animals and humans (Spoor *et al.*, 2013).

MRSA colonization in cattle may be an occupational risk to the people in close contact with MRSA infected cattle viz. veterinarians, farmers, milkers and people working at slaughterhouses (Paterson *et al.*, 2012; Juhasz- Kaszanyitzky *et al.*, 2007). High incidence of *S. aureus* is indicative of poor hygienic measures during production, handling and distribution, (Zakary *et al.*, 2011).

The presence of *S.aureus* and MRSA in milk is a matter of concern and it needs strict farm management practices as well as proper sanitary procedures such as storage, handling and transportation plays major factor in *S. aureus* contamination. As well as proper heat treatment followed by the refrigeration can minimize the chance of contamination with *S. aureus* and monitoring of food-producing animals and improving hygiene in food practices in order to limit the spread of the microorganism and reduce the microbiological risk to minimum.

In conclusion, irrational use of antibiotics in the treatment of human diseases and non-

therapeutic use of antibiotics in animals have played a significant role in the emergence of resistant clones due to selection pressure. Such resistance may pose a great impact on public health if animal associated strains enter into the community and health care settings.

This study was intended for isolation and identification of *S. aureus* and MRSA from bovine raw milk. The data obtained from this study showed the prevalence of *S. aureus* and MRSA from milk and PCR was a very useful tool in investigating this by targeting *nuc* and *mecA* gene.

Further studies should be conducted to monitor the presence and evolution of these pathogens. Strict regulations on the use of antibiotics in human medicine as well as in animal food production, strengthening surveillance and screening of animal population are required for effective infection control programme to limit the spread of drug resistant clones of *S. aureus*.

Authors' contributions

SB and MS designed the study. Laboratory work was done by SB and GS. SB and GS prepared the manuscript and analyzed the data. SB and GS done collection of sample and isolation. All authors read and approved the final manuscript.

Acknowledgments

The authors wish to acknowledge the Faculty of Department of Veterinary Public Health and Epidemiology, Madras Veterinary College, Chennai (India) for providing the necessary facility to carry out the research.

Competing interests

The authors declare that they have no competing interests.

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How to cite this article:

Sonuwara Begum, G. Suganya and Sekar, M. 2018. Detection of *Staphylococcus aureus* and Methicillin Resistant *Staphylococcus aureus* (MRSA) from Bovine Raw Milk by PCR. *Int.J.Curr.Microbiol.App.Sci.* 7(07): 3419-3427. doi: <https://doi.org/10.20546/ijcmas.2018.707.397>