Journal of Animal Research: v.10 n.1, p. 127-131. February 2020

DOI: 10.30954/2277-940X.01.2020.18

Occurrence of Methicillin Resistant *Staphylococcus aureus* from Bovine Raw Milk in Chennai

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Received: 10 Dec., 2019 **Revised:** 19 Dec., 2019 **Accepted:** 29 Dec., 2019

ABSTRACT

Staphylococcus aureus and Methicillin-Resistant *Staphylococcus aureus* (MRSA) presence in milk is of significant importance as they are often involved in food borne intoxications. Effective surveillance and detection are the most important tools in outbreak investigation and control. Our study was designed to ascertain the occurrence *Staphylococcus aureus* and Methicillin-Resistant *S. aureus* (MRSA) from raw milk collected from apparently healthy cattle in Chennai city, Tamil Nadu, India. A total of 89 (89/258; 34.49%) positive isolates of *Staphylococcus aureus* obtained from 258 raw milk samples screened. Of which 24 (24/258; 9.3%) isolates positive for MRSA. Studying the antimicrobial resistance and its patterns in foods of animal origin will be of immense help to various regulatory agencies and authorities to devise and implement control measures and to note the compliance of our foods with the prescribed standards.

Keywords: MRSA, *Staphylococcus aureus,* Prevalence, Oxacillin Resistance, Raw milk

Staphylococcus aureus is an opportunistic, Gram-positive cocci and they are catalase and coagulase positive microbes implicated in various hospital acquired and food borne outbreaks. Niche of this bacterium varies from environmental samples to skin and mucosa of both animals and human (Tong *et al.*, 2015). In dairy industry, *S. aureus* is the most prevalent and economically significant important pathogen (Katsuda *et al.,* 2005). The organism enters milk either through direct contamination by infection or normal inhabitation and/or indirectly by external environmental contamination or through animal handlers (Jorgensen *et al.,* 2005). *S. aureus* contamination of milk with enterotoxigenic staphylococcal enterotoxins (SEs) production leads to intoxication and it is often associated with symptoms like vomition and diarrhoea (Veras *et al.*, 2008). The major problem with food borne *S. aureus* is that,it's a prime pathogen involved in antimicrobial resistance with the potential of transferring between animals and humans.

In 1959, methicillin was introduced as the first semisynthetic penicillin to combat penicillinase-producing *S. aureus* which were found resistant to penicillin (Livermore, 2000). The resistance to methicillin was noticed within a year of introduction and it was postulated that overproduction of PBP2a, penicillin-binding protein which has less affinity for beta-lactam antibiotics (Chambers, 1997). The methicillin-resistant *S. aureus* (MRSA) is a rampant public health issue with human health concern. MRSA harbours staphylococcal cassette chromosome *mec* (SCC*mec*) complex which aids in resistance to methicillin. According

How to cite this article: Deepak, S.J., Porteen, K., Elango, A., Kumar, TMAS., Babu, R.N., Sureshkannan, S. and Ruban, S.W. (2020). Occurrence of methicillin resistant *Staphylococcus aureus* from bovine raw milk in Chennai. *J. Anim. Res.,* **10**(1): 127-131.

to the Center for Disease Control, two out of every 100 carriers of *S. aureus* are carriers of MRSA (CDC, 2017). The *mecA* gene, a structural determinant encoding PBP2a, is therefore considered to be a useful molecular marker of putative methicillin resistance in *S. aureus*. The MRSA has clonal derivatives from healthcare associated infections (HA-MRSA) worldwide (Köck *et al.*, 2010). Similarly, community (CA-MRSA for community acquired MRSA) (David and Daum, 2010) and in livestock (LA-MRSA for livestock associated MRSA) (Nemati *et al.*, 2009). Hence, the present study was designed to envisage the occurrence of methicillin-resistant *S. aureus* (MRSA) from raw milk collected from apparently health cattle's in Chennai city, Tamil Nadu, India.

MATERIALS AND METHODS

Study design and sampling

A cross-sectional study was designed to analyse raw milk collected between October 2016 to May 2019. The milk samples were collected from various farms locations of Chennai city, Tamil Nadu, India. Purposive sampling technique was employed. A total of 258 raw milk samples were collected aseptically, labelled and transported in ice box to laboratory for bacterial isolation and characterization. Processing of samples or carried on same day or kept in refrigerator at 4°C until microbial identification.

Isolation and confirmation of *S.aureus*

The milk samples were enriched in brain heart infusion broth containing 5-7% NaCl. The sample were diluted at 1:10 dilution for enrichment in broth and incubated at 35-37°C for 18-24 h. The enriched samples plated on Mannitol Salt Agar (MSA)incubated for 24 h at 35-37°C. The characteristic appearance of golden yellow colour colonies was considered to be presumptive *S. aureus*. The organism was demonstrated by Gram's stained smears which show Gram-positive cocci that occurred in grape like clusters. Further confirmation was done by positive reaction with catalase test (3% hydrogen peroxide), slide coagulase test, latex agglutination test and confirmed by *nuc* gene based PCR assay.

DNA Preparation and PCR assay

The DNA was extracted by hot boiling and snap-chill method is used further PCR based studies. The supernatant was used as DNA template for PCR reaction mixture. The polymerase chain reaction was performed in a reaction mixture of 25 µL of final volume approximately containing 50 ng of template DNA, 12.5 µL of Taq DNA polymerase 2X Master mix (Amplicon III, Denmark), 1 µL of (10 pmol) each primer (Sigma Aldrich, India) and made up volume by nuclease free water. The PCR was performed in an A200 Gradient Thermal Cycler (LongGene®, Hangzhou, China). The gel was visualized using Imagecapture software in BioRad Gel documenter (Bio rad).

Multiplex PCR for confirmation and detection of MRSA

The *nuc* gene (thermonuclease) (Hegde *et al.* 2013) and *mecA* complex (methicillin A gene) (Ryffel, 1990) with oligonucleotide primers and cycling conditions used are given in Table 1. The multiplex PCR was designed using established primers were used for simultaneous detection of *nuc* gene and *mecA*gene from S. *aureus* isolates from test milk sample.

Phenotypic identification of MRSA

Antimicrobial susceptibility test, by disk diffusion method was performed for all *S. aureus* isolates as per the protocol of Clinical and laboratory standards institute, 2017 (CLSI, 2017). The turbidity of test isolates was adjusted to 0.5 McFarland standard units. The bacterial suspension was spread onto Muller–Hinton agar (Himedia, India) with the sterile swab and left at room temperature to dry. Following cefoxitin (CX-30) disc was placed on bacterial lawn prepared. The plates were incubated at 37°C for 24 h. The zone of inhibition around the disks were measured and classified as susceptible or resistant according to the CLSI, 2017.

RESULTS AND DISCUSSION

In the present study, a total of 89 (89/258; 34.49%) positive isolates of *Staphylococcus aureus* obtained from 258 raw milk samples screened. The isolates were

Table 1: The list of primers and cycling condition used in the present study

Sl. No	Primer Name	Product length	Oligonucleotide sequence	Annealing temperature	Reference
1	Nuc-F	188bp	GTGCTGGCATATGTATGGCAATTGT	50 °C	Hegde *et al.* 2013
	Nuc-R		TACGCCGTTATCTGTTTGTGATGC		
2	MecA1	310bp	GTAGAAATGACTGAACGTCCGATAA		Ryffel, 1990
	MecA2		CCAATTCCACATTGTTTCGGTCTAA		

confirmed by various specific biochemical tests and *nuc* gene based PCR assay. By staining the isolates exhibited clusters of grape appearance and gram-positive cocci with the ability to ferment mannitol and produce golden yellow colour colonies on Mannitol salt agar (MSA). Further biochemical characterization showed positive reaction with catalase on 3% hydrogen peroxide and slide coagulase test using rabbit coagulase plasma (Himedia, India). The molecular confirmation of *S. aureus* isolates with *nuc* gene (*Thermonuclease*) amplified a 188 bp specific amplicon product. Sudhanthiramani *et al.* (2015) reported that prevalence of 39.09% (43/110) coagulase-positive S. *aureus* from the milk samples which is in accordance with our study, whereas Bhati *et al.* (2018) reported 107/197 (54.31%) *S. aureus* isolates confirmed from mastitic milk samples, udder and milkers' hands by 23s rRNA based PCR in Rajasthan, India. Akriti *et al.* (2019) documents the prevalence of *S. aureus* as 66.66% (40/60) in the cattle milk samples collected from Vallabhnagar tehsil of Udaipur district. Sarkar *et al.* (2014) recorded 74.5% prevalence of *S. aureus* in milk samples, Haryana, India. Gundogan and Avci, (2014) reported that 56% of prevalence of *S. aureus* in raw milk and dairy product in Turkey. Variation in the occurrence of *S. aureus* may be attributed to various factors like sampling design, study location and methodology adopted.

The phenotypic method for identification of methicillin resistant (oxacillin resistant) *S. aureus* (MRSA) utilizing cefoxitin antimicrobial disc is recommended as it is an effective identification marker of *mec*A presence as per CLSI, 2017. Genotypic identification of methicillin resistant *S. aureus* (MRSA) confirmation is based on presence on *mecA* gene is recommended by various researchers (Palavecino, 2014). The cefoxitin has higher sensitivity and specificity over oxacillin test for detection of *mecA* from MRSA and MSSA strains (Elhaig and Selim, 2015). The multiplex PCR was designed for identifying both *nuc* gene based species specific *S. aureus* with an amplicon size 188 bp and *mecA* gene based MRSA with an amplicon size of 310 bpwas amplified from the genomic DNA (Fig. 1).

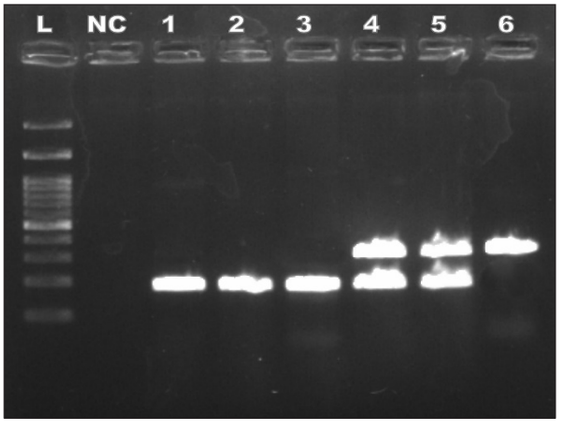

Fig. 1: The PCR products Gel image representing the products of multiplex PCR developed

L: DNA ladder 100kb; NC-Negative control; 1-3- nuc positive SA (181bp); 4-5- mecA positive MRSA (181bp & 310bp) and 6- mecA positive other *Staphlyococcus spp. (310bp*)

A total of 24 (24/258; 9.3%) isolates positive for MRSA on both phenotypic and genotypic methods respectively. Kriegeskorte *et al.* (2012) reported that phenotypic methods are accurate identification of MRSA isolates along with genotypic identification of mecA, mecC, and other Staphylococcal chromosomal cassette *mec.* Riva *et al.* (2015) found that the prevalence of 9.1% S. *aureus* in raw milk and the 20% of which are MRSA in Italy which is similar to our results. In Germany, Kreausukon *et al.* (2012) reported that 4.4% are MRSA in bulk tank milk analysed. Normanno *et al.* (2007) reported 17% S. *aureus* in milk and dairy products and 3.75% were MRSA. The presence of MRSA from milk samples of apparently

healthy animals is an important issue in dairy food production. The conditions like improper hygiene and poor management practices on before and during milking will aid in the contamination of milk with *S. aureus* which may eventually leads to emergence of antibiotic resistant strains.

CONCLUSION

In the present study we have found that a total of 89 (89/258; 34.49%) positive isolates of *Staphylococcus aureus* and 24 (24/258; 9.3%) isolates positive for MRSA on both phenotypic and genotypic methods. The multiplex PCR assay employed was identified as easy, non-ambiguous and rapid in identification of S. *aureus* and MRSA, respectively. Our study identified the presence of MRSA strains which needs due attention to curtail the spread of this potential public health risk associated antibiotic resistant strains among animals and human.

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