Detection of *Staphylococcus aureus* and antibiotic sensitivity pattern in mastitic cases from Namakkal District

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Received: 15 October 2020 / Accepted: 20 February 2021 / Published online: 07 September 2021
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Abstract: Milk samples (n= 241) were collected from clinical and subclinical mastitic cows which were brought to teaching veterinary clinical complex (TVCC), Veterinary College and Research Institute (VC&RI), Namakkal, Tamil Nadu and subjected to isolation on mannitol salt agar, Gram's staining and confirmation by polymerase chain reaction (PCR) using specific primers. The prevalence of *Staphylococcus aureus* at cow level was 61.5 and 57.4 per cent in clinical and subclinical cases, respectively, and at quarter level was 58.8 and 57.4 per cent in the clinical and subclinical cases, respectively. Antibiotic sensitivity pattern revealed a high sensitivity to enrofloxacin (100.0%) and a high resistance to methicillin (100.0%) by *S. aureus* isolates, and multidrug resistance by *S. aureus* isolates was also observed.

Keywords: Antibiotic sensitivity, Culture, Clinical and sub clinical mastitis, PCR, *S. aureus*

Mastitis is one among those major reasons causing substantial loss to the dairy farmers, worldwide, resulting in loss of 15.0 per cent production per cow (Radostits et al. 2000), and loss of milk yield is estimated to range from 100 to 500 kg/cow per lactation. Subclinical mastitis is more prevalent than clinical in most countries ranging from 20.0 to 83.0 per cent in cows. The annual economic loss due to mastitis in India had been estimated to be INR 7165.51 crores, of which subclinical mastitis alone accounts for INR 4151.16 crores (57.93 per cent) and clinical mastitis

accounting for INR 3014.35 crores (Bardhan, 2013 and Ahmad Arif Reshi et al. 2015).

The major pathogens (in 95.0% cases) involved in mastitis are *Str. agalactiae*, *S. aureus*, *E.coli*, *Str. dysgalactiae* and *Str. uberis* (NAAS, 2013). Mastitis due to *S. aureus* is more difficult to eradicate than *Str. agalactiae*, which produces more damage by abscessation or gangrene in the ducts with poor cure rate and decreases milk production to 45 per cent per quarter and 15 per cent per infected cow.

Some of the potential contributing factors in the high incidence of mastitis are lack of awareness, delay in detection of sub-clinical mastitis, unhygienic milking practices, diverse production systems, inadequate treatment etc. Antimicrobial resistance, milk quality, animal welfare and human health related issues further demand proper policies for strategic control of mastitis (NAAS, 2013). India being a large and diversified country with different farming systems and agro climatic conditions, the prevalence of mastitic pathogen is likely to vary with places and herds. Hence, this study aimed at identifying the prevalence of *S. aureus* in milk samples collected from the clinical and subclinical mastitic cases and sensitivity of the isolates to commonly used antibiotics in Namakkal district of TamilNadu.

Milk samples (n= 241) were collected from clinical (n= 104) and subclinical (n=105) mastitic cows (Figure 1) which were brought to teaching veterinary clinical complex (TVCC), Veterinary College and Research Institute (VC&RI), Namakkal. The study didnot require the approval by ethical review committee. Legal and ethical requirements have been met with regards to the humane treatment of animals and the institutional guidelines have been followed in the collection of samples. All milk samples were subjected to isolation on the specific medium, mannitol salt agar (Hi-media, Mumbai) as isolation is considered the gold standard test and Gram's staining. The bacteria, *Staphylococcus aureus* was identified based on the characteristic appearance of the colonies, morphology and Grams reaction (Quinn et al.1994).

All the *S. aureus* isolates from clinical and subclinical mastitic cases were subjected to the molecular technique, polymerase chain reaction (PCR) for confirmation since molecular diagnosis

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is found to be the most appropriate technique for the species identification of mastitic pathogens that are difficult to detect by conventional methods (Mahmmud et al. 2013). The primers were custom synthesized (Bioserve, india) for amplification of *S. aureus* as recommended by Jahan et al. (2015). The nucleotide sequence of the primers (5'-3') were: Forward-GCG ATT GAT GGT GAT ACG GTD and Reverse- AGC CAA GCC TTG ACG AAC TAAAGC. The sample DNA was extracted from the isolates of clinical and subclinical mastitic cases by using polyethylene glycol (PEG) as recommended by Chomczynski et al. (2006).

The extracted DNA was amplified using the selected primers with following reaction mixture: DNA template-5µl, Master mix (red dye)- 18µl, Forward primer-1µl (10 picomoles) and Reverse primer-1µl (10 pico moles). The cycling conditions were: Initial denaturation-95°C/300 sec, denaturation-95°C/ 60 sec, annealing-55°C/45 sec, extension-72°C/90 sec and final extension-72°C/10 min. The amplicons were subjected to agarose gel electrophoresis, the gel was visualized under UV transilluminator and the bands of appropriate size were identified by comparison with the 100bp ladder.

Antibiotic sensitivity pattern using the selected antibiotic discs was identified for the *S. aureus* isolates (n=59) from 104 clinical mastitic cases to initiate early and specific antibiotic therapy. The discs namely enrofloxacin (10mcg), gentamicin (10mcg), ampicillin (10mcg), penicillin-G (10 units), ceftriaxone (30mcg), cefotaxime (30mcg) and methicillin (5mcg) (Hi Media, Mumbai) were used and the interpretation was made as per the procedure

recommended by the Kirby-Bauer disc diffusion method (Sharma et al. 2012).

Identification of large round golden yellow colonies on mannitol salt agar (Figure 2) and grape-like clusters of gram-positive cocci on Gram stained smears was suggestive of *S. aureus* (Figure 3) which is in accordance with that described by Quinn et al. (1994). The mastitic pathogen, *S. aureus* was detected in 61.5 per cent of the clinical cases and 57.4 per cent of the subclinical cases, at cow level. Out of total of 241 milk samples collected from various affected quarters which included 136 and 105 milk samples from clinical and subclinical cases, respectively, the prevalence of *S. aureus* was 58.8 per cent in clinical cases and 57.4 per cent in sub clinical cases, at quarter level.

In this study, in clinical mastitis at cow level, the prevalence of *S. aureus* is higher than that observed by Kumari and Gupta (2002), Phogat and Dahiya (2003), Chandrasekaran et al. (2014) and Lalita Sharma et al. (2015) who recorded a prevalence of 35.21, 57.0, 40.0 and 50.0 per cent, respectively in bovine clinical mastitic cases in India. In contrast, Ranjan et al. (2011) recorded a higher prevalence (75.0%) in Uttar Pradesh, India than that observed in this study. However, in all these previous studies, *S. aureus* was reported to be the predominant pathogen in clinical mastitic cases. In subclinical mastitis at cow level, the high prevalence of *S. aureus* (57.4%) recorded is in agreement with that (56.89%) observed by Sudhan and Sharma (2010). In contrast, Barkema et al. (2006) and Lalita Sharma et al. (2015) recorded a low prevalence of 18.9 and 17.0 per cent, respectively in bovine subclinical mastitic cases in India.

Table 1 Quarter- wise prevalence of *S. aureus* in bovine milk samples

Quarters	Clinical mastitis			Subclinical mastitis		
	No. of samples collected	Isolates positive	% Positivity	No. of samples collected	Isolates positive	% Positivity
LFQ	32	18	56.2	22	15	68.1
RFQ	30	21	7.0	19	9	47.3
LHQ	47	27	57.4	35	25	71.4
RHQ	27	14	51.8	29	11	37.9
Total	136	80	58.8	105	60	57.4

Table 2 Antibiotic sensitivity pattern of *S. aureus* isolates to antibiotics in clinical mastitic cases

S. no	Antibiotics	<i>S. aureus</i> (n=59)					
		S	% Cases	I	% Cases	R	% Cases
1.	Enrofloxacin	59	100.0	—	0.0	—	0.0
2.	Gentamicin	46	77.9	13	22.0	—	0.0
3.	Ampicillin	—	—	—	—	—	—
4.	Penicillin-G	21	35.5	27	45.7	11	18.6
5.	Ceftriaxone	14	23.7	24	40.7	21	35.6
6.	Cefotaxime	15	25.4	28	47.5	21	35.6
7.	Methicillin	—	0.0	—	0.0	59	100.0

S- Sensitive, I- Intermediate and R- Resistant

In clinical mastitis, the quarter-wise prevalence of *S. aureus* (Table 1) was higher in left hind (57.4%) than in other quarters. Whereas, in subclinical mastitis, the quarter-wise prevalence of *S. aureus* (Table 1), was found to be higher in left hind (71.4%) than in other quarters. This finding is in agreement with that of Mekibib et al. (2010) who reported a high prevalence in hind quarter. In contrast, Khanal and Pandit (2013) and Kavitha et al. (2009) reported a high prevalence in forequarters. In this study, the high prevalence of clinical and subclinical mastitis in hind quarters might presumably be associated with increased chance of hind quarters being soiled with urine and feces or by the tail leading to poor udder management.

All the *S. aureus* isolates (n=140) were confirmed by PCR using specific primers targeting *Nuc* genes at 279 base pairs (Figure 4), as the sensitivity of PCR also is reported to be high (Steele et al. 2015) with high correlation rates with culture (Cervinkova et al. 2013). The high prevalence of *S. aureus* in both clinical and subclinical cases is supported by Taponen and Pyorala (2009) and Awale et al. (2012) who stated that *S. aureus* had been the predominant pathogen causing clinical and subclinical mastitis in many countries including India. This could also be attributed

to its ability to survive phagocytosis inside the mammary epithelial cells and leukocytes leading to chronic or subclinical form (Pyorala, 1995), ability to resist antibiotics by releasing beta-lactamase enzyme and its transmission from quarter to quarter or cow to cow while milking (Sharma et al. 2012).

Highest sensitivity by *S. aureus* isolates was observed to enrofloxacin (100.0%) followed by gentamicin, penicillin-G, ceftriaxone and cefotaxime; This finding is agreement with that of Ranjan et al. (2011), Jeyakumar et al. (2013) and Chandrasekaran et al. (2014) who also recorded the higher sensitivity by *S. aureus* to enrofloxacin (91.67, 94.44 and 79.0%, respectively), however lower than that observed in this study, in India. In contrast, Sumathi et al. (2008) and Tufani et al. (2012) recorded the highest sensitivity by *S. aureus* to gentamicin with 90.0 and 84.21 per cent, respectively which were higher than that observed in this study, followed by enrofloxacin, penicillin, amoxicillin-cloxacillin and ampicillin-cloxacillin.

In this study, the highest resistance was observed to methicillin (100.0%) by *S. aureus* isolates. In contrast, Chandrasekaran et

Fig 1. A Jersey cow showing enlargement of udder (LFQ) and teat in per acute mastitis due to *S. aureus*

Fig 2. Yellowish colonies of *S. aureus* from mastitic milk sample on Mannito salt agar

Fig 3. Gram positive *S. aureus* from isolates of mastitic milk samples by microscopic examination (X100)

Lane 2-Ladder,
Lane 3-Positive
control, Lane 4 & 5-
Test samples, Lane
6-Negative control

1500bp
1000bp

Fig 4. PCR amplified products of *Nuc* gene of *S. aureus* in 1.5 per cent agarose gel showing bands at 279 bp

al. (2014) and Lalita Sharma et al. (2015) observed only a lower resistance (52.9% and 66.67 %, respectively) to methicillin than that observed in this study. Whereas, Jeyakumar et al. (2013) and Chandrasekaran et al. (2014) recorded a higher resistance to penicillin-G (100.0 and 63.5%, respectively) by *S. aureus* isolates in India. Multidrug resistance was observed for *S. aureus* in clinical mastitic cases and this finding is in agreement with that of Chandrasekaran et al. (2014).

The antibiotic resistance observed in this study could be attributed to an increased and indiscriminate usage of various antibiotics and intramammary preparations in combinations and increased presence of these drugs in the cow's environment, which might lead to low cure rate (Pitkala et al. 2004). Methicillin resistant *S. aureus* in this study could be a potential threat to other cattle and farm workers (Juhász-Kaszanyitzky et al. 2007).

Conclusion

A high prevalence of *S. aureus* was observed in both clinical and subclinical mastitic cases and hind quarter was found to be highly associated with the incidence of mastitis. Most of the isolates were found to be sensitive to the antibiotic enrofloxacin and all the isolates were resistant to methicillin. Periodical study is essential to identify the predominant pathogens in clinical and subclinical mastitis, development of resistant mastitic pathogens and their sensitivity to wide range of antibiotics.

Acknowledgements

The authors are thankful to the Dean, Veterinary College and Research Institute, Namakkal for providing necessary facilities to carry out the present work.

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