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Evidence of emergence of coagulase negative staphylococcal species detected by polymerase chain reaction in sub clinical mastitis of goats in Cauvery Delta Zone (CDZ) of Tamil Nadu

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Abstract
Staphylococcus aureus has been described as one of the most important mastitis pathogens in cattle and Coagulase-negative Staphylococci (CoNS) recognized as etiologic agent associated with intramammary infections (IMI) of dairy animals in most of the developing countries. Keeping in mind the poorman's cow, this study was conducted to isolate and identify the main staphylococcal species causing caprine mastitis in 28 smaller goat farms located in Cauvery Delta Region viz., Thiruvarur, Thanjavur, Trichy and part of Pudukottai district of Tamilnadu. Out of the 54 mastitis milk samples collected, 45 staphylococcal isolates were identified (83.3%) on the basis of cultural and biochemical features as well as by genus specific PCR. Then species level identification of staphylococcal isolates was carried out using analysis of the *etb gene* at 226 bp. Overall, four different species of CoNS were identified as: 25 *Staphylococcus aureus* (55.5%), 13 *Staphylococcus chromogenes (28.8%), 5 Staphylococcus hyicus (11.1*%), 2 *Staphylococcus hominis* 4%). Although the role of *S. aureus* as an etiological agent of caprine mastitis has been elucidated previously where as studies on the relative importance of specific CoNS in caprine mastitis have not been undertaken in this Cauvery Delta Zone of Tamilnadu. This study demonstrated that *S. aureus* and *S. chromogenes* were predominant and thus be considered as emerging pathogens causing mastitis in this region and the *etb* gene PCR detection will be an useful tool for identifying staphylococcal isolates derived from subclinical goat mastitis at species level. Moreover this study will pave way for an overview on distribution of CoNS species of staphylococcus in this region.

Keywords: CoNS (Coagulase-negative Staphylococci) - Caprine mastitis- *etb* gene-PCR-*Staphylococcal spp*

1. Introduction

Sub clinical mastitis in milch goats being rare in India and often go unnoticed in rural zone where there is a practice of intense goat rearing as a part of their livelihood options. It is an economic important disease and the losses are huge in cattle as it affects the profitability of farms [2]. Due to poor management and sanitary conditions, with lack of knowledge about the disease and control measures there is more incidence of goat mastitis [3]. As the detection of bacteria by cultural and biochemical tests is cumbersome and delayed, molecular detection was aimed from goats suffering from subclinical mastitis in this region which can be adopted as a rapid method of diagnosis. Staphylococcal mastitis in goats affects the udder health and economy of the farmer in terms of lesser milk reduction or diseased milk which in turn hampers the growth of kids. Aiming the economy of the farmer in this region where they solely depend on goat rearing rather than other enterprise, the study was undertaken to identify the virulent gene of *S. aureus* in goat milk causing subclinical mastitis in smaller goat farms in this Cauvery Delta Region and for earlier detection of mastitis by molecular methods in turn early adoption of therapeutic regimen to control Staphylococcal mastitis in goats. Moreover due to inter-variant strains of Staphylococcal spp and bacterial resistance and also by identification of potential viral gene causing CONS Staphylococcal mastitis in goats paving way in development of a candidate vaccine strain against caprine mastitis.

2. Materials and Methods

Due to limitation of cultural and biochemical methods for isolation of bacteria, molecular methods using (PCR) based on the amplification of DNA coding was successfully applied as

per Riffon *et al.,* 2001. This method of identification of pathogen was in hours rather than days and also specific, sensitive, and cheap can discriminate between closely related organisms.

2.1 Milk collection

A total number of 54 individual quarter milk samples according to Arora (2003) were collected from 90 goats from 28 smaller goat farms located in Cauvery Delta Region viz., Thiruvarur, Thanjavur, Trichy and part of Pudukottai district of Tamilnadu. Using CMT, the milk samples were collected from 90 goats with sub-clinical mastitis.

2.2 Bacteriological examination

A loopful of each quarter milk sample was streaked onto Blood agar medium, Nutrient agar medium and Mannitol Salt Agar. All plates were incubated for 24 hours at 37 ^{0}C. The developed colonies were picked up and sub cultured for obtaining pure cultures of Staphylococcus spp. The purified colonies were subjected for morphological identification by Gram's stain & biochemical tests [1].

2.3 Molecular detection of *S. aureus* using gradient PCR method

The extraction of DNA was performed according to QIAamp DNA mini kit instructions Qiagen (USA). Preparation of PCR Master Mix for each of the tested genes was performed according to PCR master mix (Qiagen) and PCR grade water (Dnase free water). (Table 1 & 2). The Oligonucleotide were designed for *etb* gene as follows [5]:
Forward primer ACAAGCAAAAGAATACAGCG and
Reverse primer GTTTTTGGCTGCTTCTCTTG

Table 1: PCR components used in the PCR technique

Component	Volume/Reaction
Master mix (Emerald Amp GT PCR 2x premix)	12.5 µl
Forward (F) primer (20 pmol)	1 µl
Reverse (R) primer (20pmol)	1 µl
PCR grade De-ionized water	5.5 µl
DNA template	5 µl
Total volume	25 µl

Table 2: Cycling conditions of the primers during PCR

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
etb	94 ˚C for 5 min	94 ˚C for 30min	50 ˚C for 30sec	72 ˚C for 30 sec	35	72˚C for 20sec

3. Results

3.1 Incidence of S. aureus by bacteriological examination

Bacteriological examination of (90) goats suffering from subclinical and clinical mastitis with 54 milk samples revealed that the predominant bacterial species isolated was *S. aureus* (55.0%) along with the minor species of bacteria co occurred in isolation and are depicted in Table 3.

3.2 Molecular identification of *S. aureus*

The published primer pair for the gene (16*SrRNA*) as per Mehrotra *et al*., 2000 was applied and tested for all the *S. aureus* isolates (random 28 isolates of *S. aureus*) and the amplicon size of the examined gene (16SrRNA) was at 226 bp and the results showed that all of the tested *S. aureus* strains were positive for the 16*SrRNA* gene (100%).

3.3 Genotyping of virulence gene encoding factors by PCR

Detection of exfoliative B gene (*etb*) by agarose gel photo–documentation on PCR products showed the amplicon size of the examined gene (*etb*) at 226 bp (Fig 1). The result showed that the gene encoding the exfoliative B gene (*etb*) was found among 25% of the *S. aureus* isolates.

Table 3: Total bacterial species isolated from positive milk samples along with *Staphyloccocus spp* of both clinical and Subclinical mastitic goats

Bacteria species isolated (Sub clinical mastitis)	No.	%
Coag. Neg *Staphylococcus aureus*	25	55.5
Coag. Neg *Staphylococcus chromogenes*	13	28.8
Coag. Neg *Staphylococcus hyicus*	5	11.1
Coag. Neg *Staphylococcus hominis*	2	4.4
Total	45	
Other bacteria isolated from clinical mastitis		
Streptococcus agalactiae	3	7.0
E. coli	2	18.6
Klebsiella spp.	2	2.3
Ps. aeruginosa	2	0.0
Total	9	

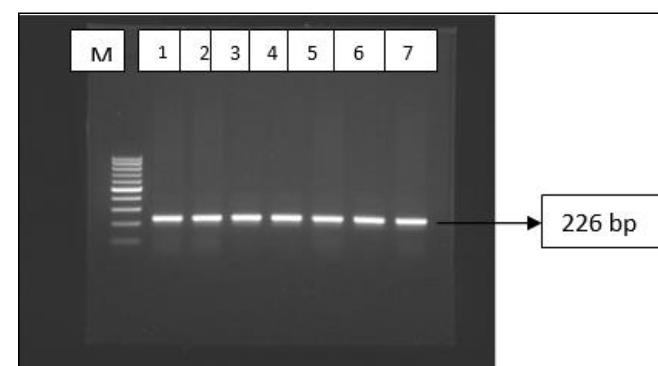

Fig 1: *Agarose gel electrophoresis of* (etb) of *S. aureus* as a genotyping identification of the isolates. Lane M: Marker encoding gene (marker (100 – 1000 bp). Lanes 1-7: positive samples for (*etb*) gene with amplicon size of 226 bp.

4. Discussion

As per the PCR technique carried out, it was found to be a rapid, sensitive and specific method for staphylococcal spp. differentiation at both strain or subspecies level in about 6-8 hours of testing and can also be used to detect staphylococcal from milk samples of antibiotics treated animals. Using *S. aureus,* specific primers that encoding *16 SrRNA* genes for testing 45 random *S. aureus* samples which were found positive initially in Californian Mastitis test and preliminary bacteriological tests employed in the laboratory concur with the findings of Riffon *et al*., (2001) [8] and Virdis *et al* (2010) [9].

The increase in CNC species of *S. aureus* and its virulence may be due to its ability to produce a large number of virulence factors in cows affected by Staphylococcal mastitis as described by Momtaz *et al*., (2011) [7] namely other genes like coagulase gene (*coa*), thermo nuclease gene (*nuc*) along with the *etb* gene concur with this research in subclinical form of mastitis of goats. Similar results of detection by PCR in mastitic milk of goats revealed CNS Staphylococcus spp in cows as reported by El-Sayed *et al* (2006) [4] for other genes

with a lesser detection of 25% for (*etb* gene). Mishra *et al* (2018) [6] reported that among the 30 bacterial isolates identified in organized farms in a region of India, predominantly it comprised 11 *Staphylococci* spp. followed by 7 Streptococci spp. And 2 Escherichia coli which is in accordance with our research in the smaller goat farms suffering from Subclinical mastitis.

5. Conclusion

In the present study, four different species of CoNS of *Staphylococci* species were identified as a causative agent of caprine mastitis as emerging pathogens and molecular basis of earlier detection of *etb* gene will be an useful tool for identifying staphylococcal isolates derived from subclinical goat mastitis at species level. This a rapid detection method which revealed the distribution of CNS Staphylococci in this agrarian region and will surely help the veterinarians for necessitating control measures to curtail Staphylococcal mastitic infections in goats in future.

6. Acknowledgement

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