Original Article

Identification of coagulase negative staphylococcal species from bovine mastitis in India

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Abstract

Background: Staphylococcal mastitis is a major cause of concern to the dairy industry in India and several countries worldwide. Though *Staphylococcus aureus* is the major cause, coagulase negative staphylococcal species (CoNS) are being increasingly reported in recent years. **Aims:** To investigate the incidence of coagulase negative staphylococcal species in bovine mastitis. **Methods:** Isolation of staphylococci was carried out from 237 milk samples of cows and She buffaloes with clinical and subclinical mastitis from different regions of Andhra Pradesh and Karnataka. CoNS isolates were identified by tube coagulase test using fresh rabbit plasma and coagulase gene PCR. We employed the biochemical test scheme published elsewhere previously for identification of the CoNS isolates up to species and subspecies levels. Seven representative isolates were identified by 16S rDNA sequencing to check the accuracy of biochemical test based identification. **Results:** The CoNS constitute the majority of the staphylococcal isolates from mastitis (80/125, 64%) in this region. Using biochemical test scheme, the CoNS isolates from bovine mastitis were identified as *S. cohnii* sub sp. *cohnii*, *S. simulans*, *S. capitis* sub sp. *capitis*, *S. cohnii* sub sp. *xylosus*, and *S. lugdunensis*. The CoNS species *S. schleiferi*, *S. haemolyticus*, *S. sciuri*, *S. xylosus*, *S. chromogenes*, and *Macrococcus epidermidis* were identified by 16S rDNA sequencing. **Conclusion:** The 16S rDNA sequencing is the appropriate method for the identification of CoNS species. This study highlighted coagulase negative staphylococcal species as possible etiological agents of mastitis.

Key words: 16S rDNA sequencing, Biochemical test scheme, Coagulase negative staphylococci, Species identification

Introduction

Mastitis is an infectious disease that affects the health of dairy cows and quality of milk, thereby causing huge economic losses. Worldwide annual losses due to mastitis have been estimated to be approximately $35 billion (Sharma *et al*., 2007). Mastitis is caused by many infectious agents. *S. aureus* is the leading cause of mastitis in dairy cows around the world. In recent years, CoNS are being most frequently isolated from cows with mastitis and are increasingly reported as emerging mastitis pathogens (Tremblay *et al*., 2013). Currently there are 49 species and 22 subspecies in the genus *Staphylococcus*. The most frequently reported CoNS species from intra mammary infections are *S. chromogenes*, *S. simulans*, *S. epidermidis*, *S. hominis*, *S. warneri*, *S. haemolyticus*, *S. xylosus*, and *S. capitis* sub sp *capitis* (Srednik *et al*., 2017). The need for differentiating and identifying CoNS to the species-level has become increasingly important but it is currently limited by the availability of appropriate, cost-effective diagnostic tests. Identification based on biochemical test profile, VITEK-2, API-STAPH system, MALDI-TOF-MS, and *16S rRNA* gene sequencing are used to identify and differentiate the staphylococcal species. The automated systems like VITEK-2 and MALDI-TOF MS are accurate but costly and cannot be used routinely in ordinary laboratories. The 16S rDNA sequencing is considered as gold standard in the identification of staphylococcal isolates (Trujillo *et al*., 2013) but it cannot be carried out on routine basis. In view of this, the present study was undertaken to investigate the incidence of coagulase negative staphylococcal species in bovine mastitis by using a simple biochemical test scheme and the results are compared with 16S rDNA sequencing of a few representative isolates.

Materials and Methods

Collection of samples

A total of 237 milk samples were collected from cows and She buffaloes with different parity, age, and

postpartum period affected with mastitis, randomly from the regions of Andhra Pradesh and Karnataka during the period from April 2019 to September 2019.

Isolation and identification of causative bacteria

The milk samples were subjected for isolation within 24 h of sampling by streaking on to Mannitol salt agar (MSA) and MacConkey agar (MAC) plates. Following incubation at 37°C for 48 h, single colonies were identified based on Gram's staining, colony morphology, and catalase test. The staphylococcal isolates were subjected to tube coagulase test using fresh rabbit plasma for differentiating coagulase negative *Staphylococcus* and confirmed by coagulase gene PCR as per the method of Akineden *et al*. (2001).

To identify specious and subspecies of the CoNS isolates, biochemical tests were employed the studies of performed by De Paulis *et al*. (2003) and Suresh Sah *et al*. (2018). As per this test scheme, all the CoNS isolates were tested for trehalose and all the trehalose non-fermenting isolates were tested for maltose, mannitol, mannose, and novobiocin (5 mcg) susceptibility. The isolates that were trehalose non fermenting, maltose fermenting, mannitol non fermenting, mannose fermenting, and novobiocin sensitive were *S. epidermidis*. All the trehalose fermenting isolates were considered non-*S. epidermidis*; this includes all other CoNS species. All non *S. epidermidis* CoNS isolates were subjected to phenotypic characterization by a panel of four biochemical tests such as ornithine decarboxylase, urease, mannose, and novobiocin (5 mcg) susceptibility on the basis of which three species *S. simulans, S. cohnii* sub sp. *cohnii* and *S. lugdunensis* can be identified. One more test maltose can identify one species namely *S. capitis* sub sp. *capitis*. To speciate the isolates to species and subspecies levels, four more tests namely acetoin, lactose, growth in anerobic condition, and xylose were performed which can identify six species/subspecies namely *S. cohnii* group (*S. xylosus* and *S. cohnii* sub sp *urealyticus*), *S. haemolyticus* group (*S. haemolyticus, S. auricularis* and *S. caseolyticus*), and *S. warneri* group (*S. warneri* and *S. hominis* sub sp *hominis*).

All the biochemical tests were performed as per the standard protocols of De Paulis *et al*. (2003), Markey *et al*. (2013), and Suresh Sah *et al*. (2018).

Sequencing of *16S rRNA* gene and phylogenetic analysis for species identification

Representative CoNS isolates that were assigned to different phenogroups/species by biochemical test scheme were chosen for *16S rRNA* gene amplification and sequencing, as per the method of Suresh Sah *et al*. (2018) with some modifications. Genomic DNA was isolated using boiling/lysis method. A 1515 bp fragment of *16S rRNA* gene of the seven representative CoNS isolates was amplified by PCR using the specific primers (F-AGA GTT TGA TCC TGG CTC AG and R-ACG GCT ACC TTG TTA CGA CTT). The PCR condition included initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, elongation at 72°C for 90 s, and final elongation at 72°C for 7 min.

After sequencing, the chromatograms of the forward and reverse strands were analyzed for chimera detection and the consensus sequences were obtained after editing the sequence errors in Bio Edit. The sequences obtained were submitted in FASTA to GenBank via Bank IT online. The nucleotide sequences obtained were analyzed and compared with reference sequences available with the GenBank by NCBI-BLAST to check for homology. The pair wise identity among the isolates was determined in mega X. Multiple alignments of the 16S rRNA sequences along with referral sequences were done by CLUSTAL-W method, using Bio Edit 7.2 software (DNA STAR). A phylogenetic tree of *16S rRNA* gene was constructed along with the aligned sequences of reference strains using MEGA X software by Neighbor joining method using the P-distance model.

Results

In this study, a total of 207 bacterial isolates were obtained from 237 milk samples from clinical and sub clinical mastitis cases together. Bacteria isolated were *Staphylococcus* species (125/207, 60.4%) and Gram-negative bacteria (82/207, 39.6%). The *Staphylococcus* isolates (125) were subjected to tube coagulase test and PCR for coagulase gene and 80 (80/125, 64%) of them were identified as coagulase negative staphylococci and these CoNS isolates were further identified by biochemical test scheme.

Initially by using five biochemical tests viz mannitol, maltose, mannose, trehalose, and novobiocin susceptibility, most of the CoNS isolates (77/80) pattern of (+ + + + S/R) were identified as belonging to phenogroup-1, one isolate with the pattern of (+ + + - S/R) to phenogroup-2, and two isolates with the pattern of (- + + + S/R) to phenogroup-3. None of the isolates from this study were observed to have the biochemical test pattern characteristic of *S. epidermidis*. The phenotypic profile of all the CoNS isolates in the study is presented in Table 1. Out of 80 CoNS isolates, 43 were found resistant to Novobiocin (5 mcg).

Table 1: Phenotypic profile for CoNS isolates

No. of isolates	Phenogroup	Mannitol	Maltose	Mannose	Trehalose	Novobiocin susceptibility
77	1	+	+	+	+	S/R
1	2	+	+	+	-	S
2	3	-	+	+	+	R

Table 2: Biochemical test results for CoNS isolates

Isolate identification No.	No. of isolates	OD	Urease	Mannose	Novobiocin susceptibility	Maltose	Species/subspecies identification (through Suresh Sah panel)	VP	Lactose	Xylose	PYR
AB-6, 7, 10, 13, 14, 15, 18; H-23; S-1, 9, 11, 14; PG-3, 7, 10, 12, 30, 31, 36, 37, 40; E-2, 4, 5, 20, 21	26	-	+	+	S	+	*S. simulans*				
MP-11; H-19, 35, 42, 55; G-2, 15, 23, 25	9	-	+	+	R		*S. xylosus*			+	
AB-2, 8, 12; P-18; S-3, 7; G-1, 7; E-8, 9, 18	11	-	-	+	S		*S. capitis* sub sp. *capitis*				
MP-3, 4, 7, 8, 9, 10, 14, 16; H-6, 11, 15, 27, 40, 41, 43, 51, 53, 54, 59; G-4, 13, 21, 29; R-4, 7, 8, 9, 15; E-3, 7, 12, 13, 23	33	-	-	+	R		*S. cohnii* sub sp. *cohnii*				
MP-5	1	+	+	+	S		*S. lugdunensis*				

OD: Ornithine decarboxylse, VP: Voges Proskauer test, and PYR: Pyrrolidonyl Aminopeptidase test

Further, the CoNS isolates were identified based on the results of a panel of four tests viz ornithine decarboxylase, urease, mannose, and novobiocin susceptibility. Based on these four tests, 33 isolates were identified as *S. cohnii* sub sp. *cohnii* based on the pattern of - - ± R), 26 isolates were identified as *S. simulans* based on the pattern of (- + ± S) and as they were also positive for maltose. Eleven isolates were identified as *S. capitis* sub sp. *capitis* based on the pattern of (- - + S). Nine isolates with the pattern of (- + + R) were placed in *S. cohnii* group, and as they were also positive for xylose, they were confirmed finally as *S. cohnii* sub sp. *xylosus*. Only one isolate was identified as *S. lugdunensis* based on the pattern of (+ + + S) and it is the only isolate from the present study which is positive for ornithine decarboxylase test. The biochemical test results of the CoNS isolates are shown in Table 2.

The CoNS isolates from bovine mastitis in the present study were identified as *S. cohnii* sub sp. *cohnii* (33), *S. simulans* (26), *S. capitis* sub sp. *capitis* (11), *S. cohnii* sub sp. *xylosus* (9), and *S. lugdunensis* (1) on the basis of biochemical tests.

Sequencing of *16S rRNA* gene and phylogenetic analysis for species identification

Seven CoNS isolates which were identified by biochemical tests as *S. simulans* (PG-10, PG-40, AB-6), *S. cohnii* sub sp. *cohnii* (MP-3), *S. xylosus* (MP-11, H-19), and *S. capitis* sub sp. *capitis* (P-18) were further characterized by *16S rRNA* gene PCR and sequencing of a 1515 bp region (Fig. 1). The sequences were submitted to the GenBank database and were given the accession numbers as MN535877, MN535878, MN535879, MN535880, MN535881, MN535882, and MN535883 for isolates AB-6, P-18, MP-3, H-19, MP-11, PG-10, and PG-40, respectively.

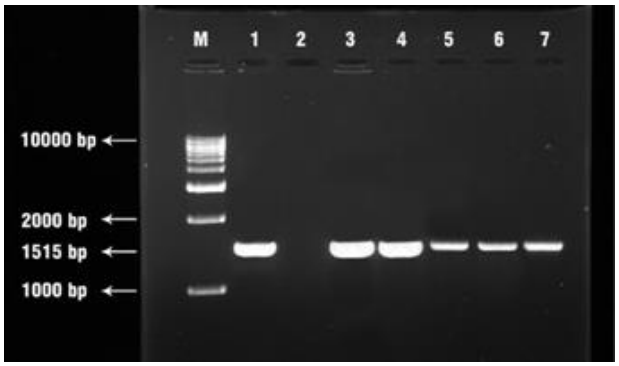

Fig. 1: PCR for *16S rRNA* gene. Lane M: Molecular weight marker (1 kb). Lane 1: Reference strain of *S. sciuri* from previous study as positive control, Lane 2: Negative control, Lanes 3-7: CoNS isolates from bovine mastitis showing 1515 bp amplicon of *16S rRNA* gene

The phylogenetic analysis of 16S rDNA sequences of seven representative isolates from each of the identified species collection identified the two CoNS isolates PG-10 and PG-40 as *S. schleiferi*, AB-6 as *S. haemolyticus*, MP-3 as *S. sciuri*, MP-11 as *Macrococcus epidermidis*, H-19 as *S. xylosus*, and P-18 as *S. chromogenes* (Fig. 2). Comparative analysis of the identification of seven isolates by biochemical test scheme and 16S rDNA sequencing is shown in Table 3.

The 16S rDNA sequencing identified the two isolates PG-10 and PG-40, which were identified as *S. simulans* by biochemical test scheme, as *S. schleiferi*. It identified another isolate AB-6 which was also identified as *S.*

Table 3: Comparative analysis of biochemical test results and 16S rDNA sequencing for CoNS species identification

S. No.	Isolate	Identification of sp/sub sp by biochemical tests	Sp/sub sp identification by 16S rDNA sequence analysis	GenBank accession No.
1	AB-6	*S. simulans*	*S. haemolyticus*	MN535877
2	P-18	*S. capitis* sub sp *capitis*	*S. chromogenes*	MN535878
3	MP-3	*S. cohnii* sub sp *cohnii*	*S. sciuri*	MN535879
4	H-19	*S. cohnii* sub sp *xylosus*	*S. xylosus*	MN535880
5	MP-11	*S. cohnii* sub sp *xylosus*	*Macrococcus epidermidis*	MN535881
6	PG-10	*S. simulans*	*S. schleiferi*	MN535882
7	PG-40	*S. simulans*	*S. schleiferi*	MN535883

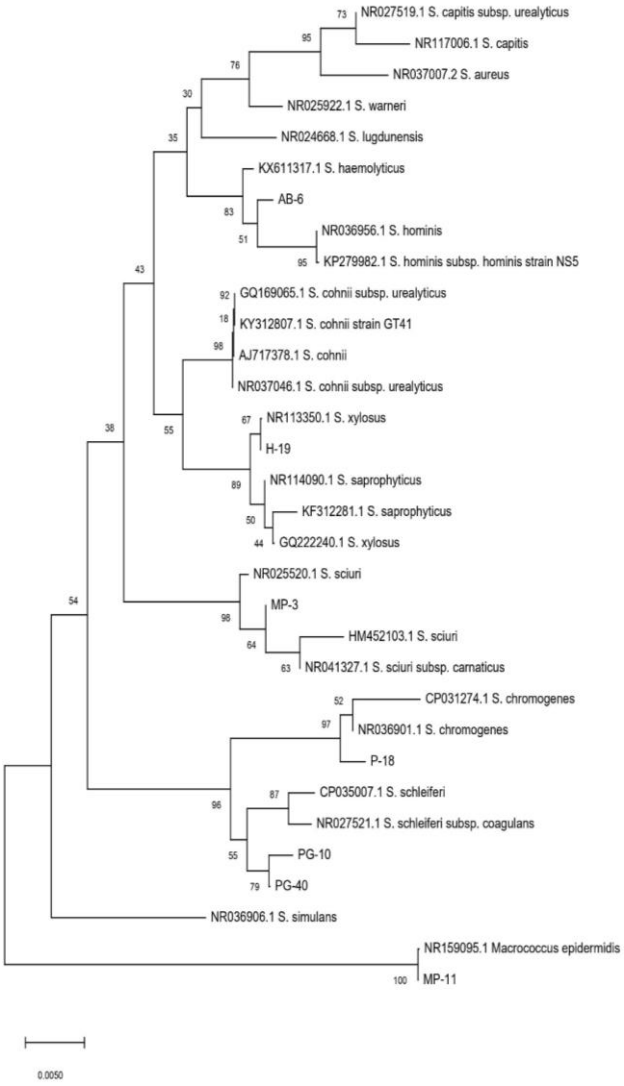

Fig. 2: Phylogenetic analysis of 16S rDNA sequences of CoNS isolates

simulans by biochemical test scheme as *S. haemolyticus.* P-18 isolate was identified as *S. capitis* sub sp. *capitis* by biochemical test scheme and as *S. chromogenes* by 16S rDNA sequencing. MP-3 isolate was identified as *S. cohnii* sub sp. *cohnii* by biochemical test scheme but as *S. sciuri* by 16S rDNA sequence analysis; similarly, MP-11 which was identified as *S. xylosus* by biochemical test scheme was confirmed as *Macrococcus epidermidis* by 16S rDNA sequencing.

Discussion

Mastitis is one of the most economically important diseases of the dairy cattle and buffaloes in India and in many countries worldwide. Management of mastitis has been a major challenge to field veterinarians and farmers. Investigations on the incidence and etiology of mastitis pathogens are desirable to choose an appropriate antibiotic for treatment and to understand the epidemiology of intramammary infections. In this study, *Staphylococcus* sp. was found as the predominant cause of mastitis in bovines in Andhra Pradesh, India. The culture results showed that the incidence of *Staphylococcus* species (60.4%) is high in bovine mastitis when compared to Gram-negative bacteria (39.6%). Several studies reported *S. aureus* as the most common bacterial cause of bovine mastitis followed by *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *E. coli* (Jey kumar *et al*., 2013). However, CoNS are being frequently isolated from bovine mastitis in many countries and are regarded as emerging mastitis pathogens (Pyorala and Taponen, 2009; Piessens *et al*., 2012; Tremblay *et al*., 2013; Srednik *et al*., 2017).

The majority of the staphylococcal isolates from the present study were found to be CoNS (64%) and only 36% of them were *S. aureus* (CPS) and this suggests the predominance of CoNS isolates in bovine mastitis in this region compared to *S. aureus*. Persson *et al*. (2011) reported that the incidence of CPS (36.36%) was high compared to CoNS (29.41%) in bovine mastitis in Sweden. Boerlin *et al*. (2003) from Switzerland reported *S. aureus* as the cause of mastitis in 58.45% of the animals. Another study by Wang *et al*. (2018) from China reported the incidence of *S. aureus* as 46.2% (90/195) from bovine mastitis.

According to Bergey's Manual of Systematic Bacteriology, 44 biochemical tests are required for CoNS identification, which is cumbersome and time-consuming to use routinely in any laboratory. Several researchers developed simple biochemical test schemes for the identification of CoNS (De Paulis *et al*., 2003; Iorio *et al*., 2007; Suresh Sah *et al*., 2018). The present study employed the simplified biochemical test scheme devised by Suresh Sah *et al*. (2018) that comprised 11 tests able to distinguish the maximum number of CoNS species (11 species and 1 subspecies).

Five CoNS species *S. cohnii* sub sp. *cohnii* (33, 41%), *S. simulans* (26, 32.5%), *S. capitis* sub sp. *capitis* (11, 14%), *S. cohnii* sub sp. *xylosus* (9, 11%), and *S. lugdunensis* (1) were identified in bovine mastitis on the basis of biochemical test scheme in the present study. However, the CoNS species *S. schleiferi*, *S. haemolyticus*, *S. sciuri*, *S. xylosus*, *S. chromogenes*, and *Macrococcus epidermidis* were identified by 16S rDNA sequencing. The biochemical test results are in disagreement with the results of 16S rDNA sequence-based identification of the six representative isolates. The biochemical test results matched with 16S rDNA sequence-based identification only in the case of one isolate, i.e. H-19, which was identified as *S. xylosus* by both methods. The disagreement of biochemical test-based identification was maybe because many diverse species can share the same biochemical phenotypes and some strains may be phenotypically aberrant (Clarridge, 2004). CoNS species like *S. epidermidis*, *S. haemolyticus*, *S. sciuri*, *S. xylosus*, *S. simulans*, *S. hyicus*, *S. warneri*, and *S. capitis* were most frequently reported from studies on mastitis (Piessens *et al*., 2012; Tremblay *et al*., 2013; Srednik *et al*., 2017). Nineteen different CoNS species were identified by MALDI from bovine

mastitis in Switzerland with the most prevalent species being *S. xylosus*, *S. chromogenes*, *S. haemolyticus*, and *S. sciuri* (Frey *et al*., 2013). Piessens *et al*. (2012) reported *S. chromogens*, *S. epidermidis*, *S. haemolyticus*, and *S. simulans* as the predominant CoNS species in intramammary infections of cows in Belgium.

Suresh Sah *et al*. (2018) also reported disagreement between the biochemical test scheme results and 16S rDNA sequencing in CoNS species identification of five representative isolates. In their study, concordance was seen only in the identification of *S. hominis*, *S. haemolyticus*, and *S. cohnii* sub sp. *urealyticus*, whereas disagreement was reported with the other two isolates. The 16S rDNA sequence is highly conserved between different species of bacteria and it is most frequently targeted in studies on bacterial evolution and ecology, phylogenetic relationships among taxa, and the exploration of bacterial diversity in the environment. The 16S rDNA sequencing is considered as the gold standard in the identification of staphylococcal isolates (Trujillo *et al*., 2013). The 16S rDNA sequencing was the accurate method for species identification of bacteria. The present study indicated that biochemical tests are non-specific and inadequate for the identification of different CoNS species. Kim *et al*. (2018) suggested that 16S rDNA sequencing though being universally adopted as a standard for bacterial identification did not effectively discriminate closely related CoNS species and suggested *sodA* gene sequencing as an alternative. This study also points to the need for fast and reliable bacterial identification systems like MALDI-TOF MS.

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Conflict of interest

The authors declare that they have no conflict of interest related to this research and authorship.

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