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Detection of *mecA* gene-associated methicillin-resistance coagulase-negative staphylococci (MRCoNS) from bovine mastitis in Gujarat

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ABSTRACT

Exploration of pathogenic potential of Coagulase-Negative Staphylococci (CoNS), in human as well as animals, gained the importance during the past two decades. Emergence of Methicillin-Resistant Coagulase-Negative Staphylococci (MRCoNS) in bovine milk poses to be a major public health concern. Presence of the *mecA* gene is one of the most common reasons reported for the methicillin resistant bacteria. Since the scientific data concerning the presence of *mecA* gene and MRCoNS in bovine milk is very limited in India, particularly from Gujarat state, the present study was planned to detect *mecA* gene in 25 MRCoNS and study the antibiogram of 33 CoNS isolated from 185 bovine mastitis cases between January 2018 to December 2019. Staphylococci were isolated from the milk samples of bovine mastitis with a prevalence of 38.9% (72/185), out of which 39 (54.16%) and 33 (45.83%) isolates were identified as coagulase-positive Staphylococci (CoPS) and coagulase-negative Staphylococci (CoNS), respectively. Among the CoNS, *S. xylosus* was the most predominantly isolated species (9/33, 27.27%), followed by *S. epidermis* (6/33, 18.18%) and *S. haemolyticus* (4/33, 12.12%). Out of these 33 isolated CoNS, 21 (63.63%) and 25 (75.75%) were identified as MRCoNS by disc diffusion method and CHROM agar, respectively. Contrary to the likelihood, only 3 MRCoNS showed the presence of *mecA* gene using PCR method. Antibiogram revealed that most of the CoNS isolates (84.85%) were multi-drug resistant emphasizing the urgent need of restricting the indiscriminate use of antimicrobial drugs in the area of study.

Keywords: Antibiogram, Bovine mastitis, Gujarat, *mecA* resistance gene, Milk, MRCoNS

India ranks first in the world milk production by producing nearly 22% of the world's milk (FAO 2020). However, the estimated demand for milk and its milk products in India in 2030 is 266.5 million metric tonnes, which is about 1.4 times more than the present production capacity of the nation (NDDB 2019). Bovine mastitis remains one of the major barriers in achieving the demanded production targets, since the affected quarters may have 30% productivity and dairy animals may lose about 15% production (Radostisis *et al*. 2007). Mastitis is also one of the most probable reasons for the multi-drug resistance against antibiotics in dairy animals due to indiscriminate use of such drugs. Staphylococci species are among the most commonly bacteria associated with intra-mammary infections and divided into two categories based on coagulase production capability, viz. coagulase positive staphylococci (CoPS) and coagulase negative staphylococci (CoNS). Previously, CoNS were thought to be non-pathogenic or less pathogenic and reported only in sub-clinical cases of mastitis (Cain *et al*. 2011). However, in recent years, as a group, CoNS have emerged as one of the most common bacteria associated with bovine mastitis throughout the globe (Srednik *et al*. 2017, Nobrega *et al*. 2018). About 26 species of CoNS were reported from various animal species, food stuff and environment (Schleifer and Bell 2009). At present, more than 15 CoNS species have been described in the etiology of mastitis in dairy cows, with *S. chromogenes*, *S. simulans*, *S. xylosus*, *S. epidermidis*, *S. hyicus* and *S. haemolyticus* being the most commonly isolated species (Cervinkova *et al.* 2013, Alekish 2015, Vanderhaeghen *et al*. 2015, Zigo *et al.* 2019).

The antimicrobial resistance has been recognized since the earliest day of chemotherapy. CoNS are reported to be more resistant than *S. aureus* and they easily develop multi-drug resistance (Machado *et al*. 2008). Methicillin resistance is due to the acquisition of the *mecA* gene, that encodes a new protein designated PBP2a, belonging to a family of enzymes necessary in building the bacterial cell wall. PBP2a has a very low affinity for beta-lactam antibiotics and thus, confers resistance to methicillin as well as other beta-lactams (Pantosti *et al*. 2007). Methicillin-resistant staphylococci are often resistant to other classes of antibiotics (Stapleton and Taylor 2002). Further, the transmission of bovine methicillin-resistant staphylococci to human is possible, signifying its public

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health importance (Lee 2003). Looking at the increasing importance of CoNS and methicillin-resistance, and their involvement in bovine mastitis, it is necessary to trace endemic species of *Staphylococcus* in dairy animals suffering with mastitis and their association with *mecA* gene. Additionally, antibiogram trend of such pathogen would be of great help in effective mastitis therapy and management. Such vital information on Methicillin-Resistant Coagulase-Negative Staphylococci (MRCoNS) was missing from northern Gujarat, one of the prominent milk shed area of Gujarat. Hence, the present study were planned to investigate the prevalence, antibiogram and *mecA* gene association MRCoNS in bovines suffering from mastitis.

MATERIALS AND METHODS

Milk samples (n=185) from cases of mastitis in cows (n=104) and buffaloes (n=81) reared in northern part of Gujarat were collected aseptically in sterilized vials and transported to the laboratory immediately. Samples were inoculated on the plates of nutrient agar by spreading heavy inoculums of thoroughly mixed milk. The plates were incubated at 37°C for 24 h. Thereafter, colonies showing golden yellow or white pigmented colony, presumptive of *Staphylococcus* spp., were transferred to Mannitol Salt Agar (MSA) which was used as a selective medium for *Staphylococcus*. The bacterial isolates were identified as per the standard methods (Buchanan and Gibson 1974, Cowan and Steel 1974). Gram positive, spherical cells arranged in irregular clusters resembling to bunch of grapes were considered to be *Staphylococcus*. All the *Staphylococcus* isolates were subjected to catalase and oxidase test. The coagulase test was performed using rabbit coagulase plasma (0.1 g per vial, Hi-media). The isolates, negative by coagulase test, were further subjected to species identification by HiStaph™, a biochemical test kit used for an identification as well as differentiation of genus *Staphylococcus* (Hi-media Pvt. Ltd., Mumbai).

Disc diffusion using methicillin and oxacillin disc and CHROM agar (Hi-Media) was used for the identification of Methicillin-Resistant *Staphylococcus* (MRS). The luxurious growth on CHROM agar was considered to be positive, indicating MRS. The DNA of the *Staphylococcus* confirmed isolates was extracted using a DNA extraction kit (Qiagen, Germany) and were subjected to PCR for genus confirmation using *Staphylococcus*-specific 16S rDNA gene as per method described by Lovseth *et al*. (2004). Further, molecular characterisation of MRCoNS isolates was carried out by detecting *mecA* gene using PCR method as described by Strommenger *et al*. (2003). The recommended oligonucleotide primers specific for the 16S rDNA and *mecA* genes used in the PCR assay and referred amplicon sizes are given in Table 1.

All the CoNS isolates obtained during the study were subjected to *in vitro* antibiotic sensitivity test (AST) as per the method described by Bauer *et al*. (1966).

RESULTS AND DISCUSSION

In the present study, out of 185 samples, 72 *Staphylococcus* isolates were recovered yielding overall incidence of Staphylococcal mastitis as 38.91% in bovines; which is higher than reported by Singh *et al*. (2017) who reported 30.53% from 95 bovine mastitis cases belonging to the same region during 2016-17. It indicates that incidence of staphylococcal mastitis has increased over the period of 4 years. Out of 72 isolates, 33 (45.83%) isolates were negative for coagulase production which included 21 (43.75%) from cows and 12 (50%) from buffaloes. In the present study, CoNS were isolated in 17.83% (33/185) of the total milk samples. The present finding corroborates with the findings of Sukur and Esendal (2020) who reported incidence of CoNS as 14.46% in clinical mastitis cases in north Cyprus. Similar incidence of CoNS mastitis was mentioned by Singh *et al*. (2017) in the same study area. However, in another Indian study (Mahato *et al.* 2017), higher incidence (37.12%) of CoNS mastitis was reported. This difference in incidence data might be due to the fact that clinical cases of mastitis were included in the present study and generally high proportion of CoNS is observed from animals with subclinical mastitis (Pyorala and Taponen 2009). As per the scope of the present study, only CoNS isolates (n=33) were subjected for further biochemical and molecular characterization as well as antibiogram studies.

The HiStaph™ species identification kit used in the present study is able to differentiate as many as 43 different *Staphylococcus* species. Six different CoNS species were identified from the 33 isolated CoNS using the kit (Table 2). Seven isolates could not be identified to species level based on their biochemical properties as they did not match to any of the species listed in the index provided with the kit and thus were merely considered as CoNS species. A study conducted by Shome *et al.* (2011) in India also confirmed presence of *S. chromogenes, S. epidermidis, S. sciuri* and *S. haemolyticus* as the predominant CoNS in bovine milk samples using PCR technique. Mahato *et al*. (2017) revealed 10 different CoNS species, viz. *S. sciuri*, *S. haemolyticus*, *S. chromogenes*, *S. saprophyticus*, *S. xylosus*, *S. simulans*,

Table 1. Primer sequence used for detection of 16S rDNA and mecA genes

Target gene	Primer sequence (5'-3')	Amplicon size (bp)	References
16s-rDNA	F: 'GTAGGTGGCAAGCGTTATCC' R: 'CGCACATCAGCGTCAG'	228	Lovseth *et al.* 2004
mecA gene	F: 'AAAATCGATGGTAAAGGTTGGC' R: 'AGTTCTGCAGTACCGGATTTGC'	533	Strommenger *et al.* 2003

S. agnetis, *S. epidermidis*, *S. gallinarum* and *S. cohinii*. The difference in type of predominating CoNS spp. in different studies might be due to the poor accuracy of the commercial identification systems in distinguishing different species of CoNS and/ or the variability in expression of the phenotypic characters (Renneberg *et al.* 1995, Calvo *et al.* 2000).

Table 2. Identification of coagulase-negative *Staphylococcus* spp. based on biochemical profile

Species	Number	Per cent (%)
S. xylosus	9	27.27
S. epidermidis	6	18.18
S. haemolyticus	4	12.12
S. saprophyticus	3	9.09
S. equorum	2	6.06
S. sciuri	2	6.06
CoNS species	7	21.21
Total	33	100

Antibiotic sensitivity pattern of CoNS isolates was assessed on Muller Hilton media using 15 antibacterial discs. Most of the CoNS isolates (28 of 33; 84.85%) were multi-drug resistant on a criterion of being resistant to more than 3 antibacterial discs used in the study. Moreover, four isolates were resistant to all the 15 antibacterials used in the study. Antibiogram of CoNS isolates is presented in Table 3. Similarly, higher resistance of CoNS to penicillin, oxacillin, ampicillin, clindamycin, tetracycline and gentamicin was reported by other researchers in recent times (Mahato *et al.* 2017, Ahmed *et al*. 2020).

Table 3. Antibiogram of CoNS isolates (n=33) isolated from 185 mastitis milk sample

Antibiotic disc (Conc.)	Sensitive (%)	Intermediate (%)	Resistant (%)
Penicillin G (10 Units)	15.15	00.00	84.84
Oxacillin (5 mcg)	30.30	15.15	54.54
Methicillin (10 mcg)	36.36	18.18	45.45
Oxytetracycline (30 mcg)	42.42	12.12	45.45
Cefoperazone (75 mcg)	48.48	21.21	30.30
Colistin (10 mcg)	51.51	27.27	21.21
Clindamycin (2 mcg)	54.54	6.06	39.39
Ceftriaxone (30 mcg)	57.57	15.15	27.27
Amoxicillin-clavunilic acid (30 mcg)	60.60	18.18	21.21
Gentamicin (10 mcg)	60.60	12.12	27.27
Ampicillin-sulbactum (10/10 mcg)	66.66	9.09	24.24
Linezolid (30 mcg)	69.69	15.15	15.15
Enrofloxacin (10 mcg)	69.69	12.12	18.18
Vancomycin (30 mcg)	72.72	15.15	12.12
Chloramphenicol (25 mcg)	81.81	9.09	9.09

Out of 33 isolates of coagulase-negative *Staphylococcus,* 25 isolates showed different coloured colony on the CHROM agar plate i.e. greenish (17 isolates), orange (2 isolates), bluish (6 isolates) which were considered as MRCoNS. Thus, the prevalence of MRCoNS was 13.51%. In contrast to this, higher prevalence of MRCoNS isolates from bovine mastitis was observed (23.08%, Javia *et al*. 2018; 35.71%, Santos *et al.* 2013; 70.60%, Sukur and Esendal 2020). Higher incidence of MR-CoNS might be attributed to indiscriminate use of antibiotics and intra-mammary preparations used by the owners/laymen without the prescription of the veterinarians, which is also one of the major reasons for increasing incidence of resistant strains.

All the 72 isolates of *Staphylococcus* spp. were subjected to 16S rDNA gene amplification by PCR for genus confirmation. All these isolates yielded 228 bp fragments which confirmed that these isolates belonged to the *Staphylococcus* genus (Fig. 1). The same method of genotypic confirmation using the similar primers had been reported by Darwish and Asfour (2013), El-Jakee *et al.* (2013) and Suleiman *et al.* (2012). The isolates, which showed growth on CHROM agar, were further subjected to PCR for detection of *mecA* gene.

Out of 25 MRCoNS isolates, only 3 isolates were positive for methicillin-resistance targeting a 533 bp

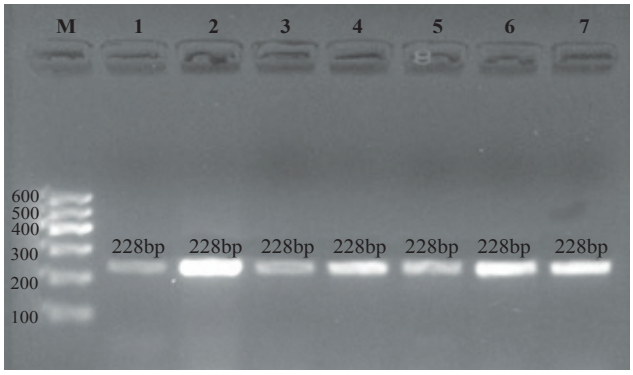

Fig. 1. Agarose gel showing PCR amplified product size 228 bp for 16S rDNA *Staphylococcus* spp. Lane M: 100 bp DNA marker; Lane 1-7: Positive for *Staphylococcus* spp.

fragment (Fig. 2). One of the CoNS isolates was *mec*A-positive but showed sensitivity to methicillin disc.

In the present study, disc diffusion test using oxacillin and methicillin discs, and CHROM agar method test were used for detection of methicillin resistant isolates. Out of 33 CoNS isolates, 21 and 25 isolates were detected as methicillin resistant by disc diffusion and CHROM agar methods, respectively. CHROM agar method is reported to be more suitable as it contains cefoxitin which is a potent inducer of the *mecA* genes. In contrast, disc diffusion test uses oxacillin which is a weak inductor of PBP2a (penicillin binding protein 2a) production (Mallick and Basak 2010, Mathews *et al.* 2010, CDC 2011).

In the present study, MRCoNS isolates confirmed phenotypically from bovine mastitis were analysed by PCR for the detection of *mecA* gene, and it was detected only in 3 out of 25 isolates. Thus, 22 CoNS isolates phenotypically resistant to methicillin in the present study did not carry *mecA* gene. Such discrepancy in correlation

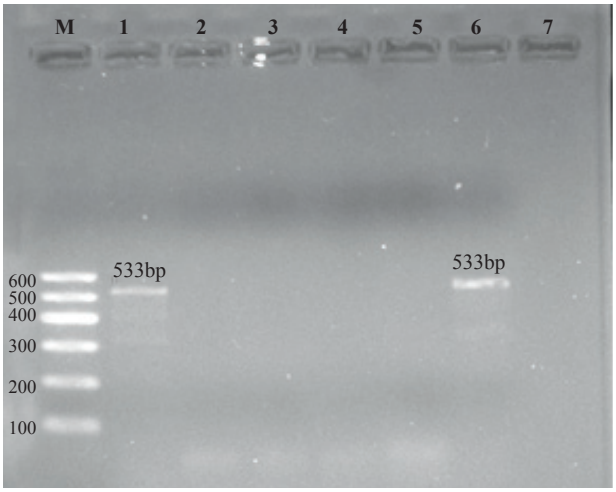

Fig. 2. Agarose gel showing PCR amplified product size 533 bp for *mecA* gene. Lane M: 100 bp DNA marker; Lane 1, 6: Positive for mecA gene; Lane 21, 3, 4, 5, 7: Negative for mecA gene.

between the *mecA* gene and phenotypically methicillin resistance is not common and had been reported by other workers (Schnellmann *et al.* 2006, Moon *et al.* 2007). Many possible reasons have been cited for this type of discrepancy including poor expression of *mecA* genes by the isolates, production of methicillinase (alteration of PBP subtypes), overproduction of β-lactamases, and variation in phenotypic expression of resistance due to different growth conditions (Moon *et al*. 2007, Turkyilmaz *et al.* 2010).

It is also remarkable to point out that there are mechanisms other than the presence of *mecA* gene responsible for beta-lactam resistance of methicillin-resistant *Staphylococcus* (MRS) species. The detection of only *mecA* may not be enough for confirmed characterization of MRS isolates (Elhassan *et al*. 2005). The absence of *mecA* gene in most of MRCoNS isolates in the present study requires further research to detect the other resistance genes related to the non-*mecA*-mediated methicillin resistance phenomena.

One of the CoNS isolates was *mec*A-positive but found sensitive to methicillin. Mahato *et al*. (2017) also reported that out of 59 *mec*A-positive isolates, 7 were oxacillin susceptible. Similarly, oxacillin-sensitive *mecA*-positive isolate of *S. haemolyticus* was reported Li *et al.* (2015) in China. 'Heterogenic expression of resistance genes' seems to be most plausible reason for existence of *mec*A-positive but methicillin-sensitive pathogen. It was stated that conventional sensitivity tests such as agar disc diffusion and broth dilution methods may not always give reliable results in detecting MRSA because of heterogenic expression of the genes (Unal *et al.* 1994). Thus, it is necessary to confirm the phenotypic results using molecular tools for detection of resistance genes.

The findings of the present study indicate the increasing prevalence of CoNS isolates in the northern part of Gujarat. High presence of multidrug-resistant MRCoNS spp. from bovine mastitis represents an alarming situation concerning antimicrobial resistance in the study area. The outcomes of study also emphasised that mere detection of *mecA* gene is not enough for designating 'methicillin-resistance' status to the isolates and correlation with phenotypic characterisation is necessary. More detailed molecular studies including more resistance genes should be carried out in the region regarding the status of MRCoNS in bovine mastitis.

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