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Molecular Detection and Antimicrobial Resistance Pattern of *Staphylococci* Isolated from Clinical and Subclinical Bovine Mastitis

B.B. Javia*, J.H. Purohit, B.S. Mathapati, D.B. Barad, H.H. Savsani, S.N. Ghodasara, V.A. Kalariya, U. D. Patel and V.R. Nimavat

Department of Veterinary Microbiology

College of Veterinary Science and AH, Junagadh Agricultural University, Junagadh, India

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Corresponding author:

bbjavia@yahoo.com

Abstract

The present study was carried out on bovine mastitis with an objective to screen bovine milk samples around Junagadh for status of subclinical mastitis (SCM) by somatic cell count (SCC), to reveal the prevalence of staphylococcal mastitis by conventional and molecular methods and to evaluate the antimicrobial resistance pattern of the isolated *Staphylococci*. Total 390 bovine milk samples (180 from clinical mastitis and 210 from apparently healthy animals) were collected. Among 210 milk samples from healthy bovine, 72 samples showed SCC value > 5 lakhs/mL revealing 34.29% prevalence of SCM. A primary culture isolation of 252 milk samples (72 SCM and 180 clinical mastitis) showed 38.72% prevalence of *Staphylococcal* mastitis. The isolated *Staphylococci* were further characterized by biochemical tests which showed prevalence of coagulase negative *Staphylococci* and *S. aureus* 23.08% and 15.64%, respectively. The high resistance of *Staphylococci was* observed against ceftriaxone and amoxicillin-salbactum which displays antibiotic usage pattern in the region. Likewise bacterial isolates studied were highly sensitive to levofloxacin which suggest judicious use of this antibiotic in treatment of bovine mastitis. All the conventionally isolated *Staphylococci* and *S. aurues* were confirmed by polymerase chain reaction targeting *16S rRNA* and *nuc* gene respectively in shorter period of time which signifies the superiority of molecular diagnostic tools.

Introduction

Mastitis, an inflammation of the mammary gland usually occurs primarily in response to intramammary bacterial infection. Subclinical mastitis (SCM) occurs without visible changes in the appearance of the milk and/or the udder and its incidence is much higher in a dairy herd compared to the clinical one (Shaheen *et al.,* 2016).

Amongst all the aetiologies, *Staphylococcus aureus* is largely responsible for causing mastitis (Kandemir *et al.,* 2013). Somatic cell count (SCC) is the most frequently used indicator of subclinical mastitis in dairy cattle. Conventional microbiological methods have been the gold standard for identification of bacteria from milk and their antimicrobial susceptibilities for selection of appropriate therapy. But, these methods are time consuming hence, uses of DNA-based assays have become popular (Phuektes *et al.,* 2001). PCR being highly sensitive and specific, can improve the level of rapid detection of bacteria.

The assessment of antibiogram profile of the strains is the only available option for designing and implementing therapeutic regimen. The aim of this study was to reveal the prevalence of staphylococcal mastitis in bovines of Junagadh district of Gujarat, and to confirm these isolates by molecular methods and study their antimicrobial resistance patterns.

Materials and Methods

A total of 390 bovine milk samples (180 from clinical cases and 210 from apparently healthy bovine) were collected for the study. Milk samples from healthy bovine animals were subjected to measurement of somatic cell count (SCC) using Nucleocounter SCC-100™. The SCC value >5,00,000 cells/ml of milk (Hegde *et al.,* 2013) was taken as criterion to consider the milk/animal as subclinically mastitic/infected. Total 252 milk samples (72 which had SCC value >5,00,000 cells/ml and 180 clinical samples) were subjected to cultural isolation. For isolation of *Staphylococci*, mannitol salt agar was used. The pure cultures were subjected for various biochemical tests as per standard procedure (Collee *et al*., 2008). The molecular detection of *Staphylococcus* genus and *S. aureus* was carried out by PCR. Bacterial genomic DNA was extracted using the Nucleopore gDNA Fungal/Bacterial Mini Kit (Genetix Asia Biotech Pvt Ltd). *Staphylococcus* genus-specific *16S rRNA* gene and *S. aureus* species specific thermostable nuclease (*nuc*) gene were amplified by PCR. 12.5 ul of 2X PCR mastermix (Fermentas), 1 ul of each forward and reverse primers (10 pmol/L), 3 ul (1 µg) of genomic DNA template in a total reaction volume of 25 µl with addition of nuclease free water were used. The primers and the annealing conditions are shown in Table 1. All the reactions were carried out with initial denaturation 94°C for 5 min followed by 40 cycles of denaturation (94°C for 30s), annealing (52°C for 45s), extension (72°C for 45s) and final extension at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis. The reference strain of *S. aureus (*ATCC 43300) was used as positive control, while *E. coli (*MTCC 722) was used as negative control.

All the *Staphylococci* isolates obtained were subjected to *in vitro* antibiotic sensitivity test as per the Kirby-Bauer method (Bauer *et al.* 1966) with commercially available discs in market as per Table 2.

Table 1: *Staphylococcus* genus specific (*16S rRNA* gene) primer sequences

Organism	Primer sequence (5'- 3')	Target gene	Product size	Annealing temperature	Reference
***Staphylococcus* genus**	F:GGCCGTGTTGAACGTGGTCAAATCA	*16S rRNA*	370bp	52°C	Martineau *et al.* (2001)
	R:TIACCATTTCAGTACCTTCTGGTAA				
S. aureus	F:GTGCTGGCATATGTATGGCAATTGT	*nuc*	181bp	54°C	Hegde *et al.* (2013)
	R:TACGCCGTTATCTGTTTGTGATGC				

Table 2: Antibiotic resistance pattern of *Staphylococcus spp*. isolated from bovine milk samples

Name of antibiotic with its Concentration	Break point to declare resistance	*Staphylococcus* spp. (N = 151)	Antibiotic resistance (%)
Methicillin (5 µg)	9 mm	27	17.88
Amoxicillin/Salbactum(30/15µg)	31 mm	91	60.26
Ceftriaxone (30 µg)	13 mm	107	70.86
Gentamicin (10 µg)	12 mm	58	38.41
Levofloxacin (5 µg)	15 mm	34	22.52
Oxytetracycline (30 µg)	11 mm	21	13.91

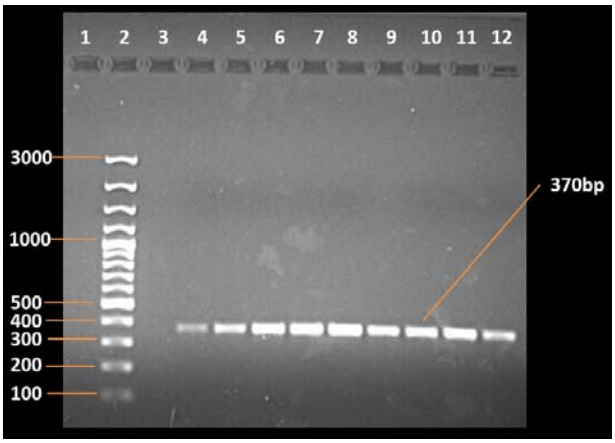
Results and Discussion

Out of 210 milk samples from apparently healthy bovine subjected to measurement of SCC, 72 (34.29 %) milk samples revealed SCC >5 lakhs cells/ml. These findings were closer to Nithinprabhu (2010) and Hegde *et al.* (2013), who reported 47 per cent and 45 per cent SCM respectively but it was in contrast to finding of Jena *et al.* (2015) who reported 74.55 per cent SCM. During mastitis, the SCC increases significantly due to an influx of somatic cells from the blood into the milk, indicating that the milk composition is adversely affected (Schukken *et al.,* 2003).

Out of 390 milk samples, 252 milk samples (72 subclinical by SCC and 180 clinical mastitis) were cultured for isolation of major *Staphylococci*. Gram positive, spherical cells arranged in irregular clusters resembling bunch of grapes in Gram staining were considered to be *Staphylococci.* Further, these isolates were subjected to biochemical tests and 151 samples were found positive for *Staphylococci*. The overall prevalence of *Staphylococci* was found to be 38.72% (151/390). These findings are in accordance with previous reports which consider *Staphylococcus* spp. as one of the most frequently isolated contagious pathogens as compared to other microflora in bovine mastitis worldwide (El-Sayed *et al.,* 2006; Moon *et al.,* 2007). These isolates were further characterized by coagulase test which revealed 90 isolates as coagulase negative *Staphylococci* (CoNS), while 61 as *S. aureus*. The prevalence of mastitis caused by CoNS and *S. aureus* was found to be 23.08% (90/390) and 15.64% (61/390), respectively. This finding indicates CoNS as an emerging mastitis pathogen. They have even become the predominant pathogens isolated from milk samples of cows with mastitis (Pitkala *et al.,* 2004).

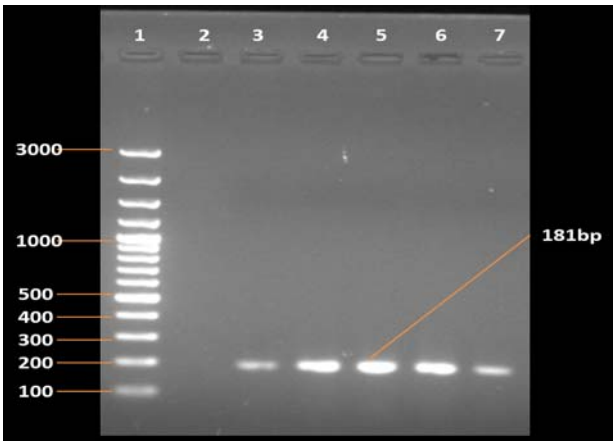
Biochemical identification of the bacteria was validated by PCR. Initially, the *16S rRNA* gene was targeted for detection of *Staphylococci* at the genus level, while *nuc* gene was targeted for detection of *S. aureus* at species level. In the study, all the biochemically identified *Staphylococci* and *S. aureus* were confirmed at molecular level with the expected amplicon sizes of 370 bp and 181 bp, respectively (Fig 1-2).

Fig.1: Genus specific PCR for detection of *Staphylococcus* spp.



Lane 1: No template control, Lane 2: DNA Ladder (100 bp Plus®), Lane 3: *E. coli* (MTCC 722) as negative control, Lane 4: *S. aureus* (ATCC 43300) as positive control, Lane 5 to 12: *Staphylococcus* spp. isolates from bovine milk samples

Fig. 2: Species specific PCR detection of *S. aureus*



Lane 1: DNA Ladder (100 bp Plus®), Lane 2: *E. coli* (MTCC722) as negative control,:, Lane 3: *S. aureus* (ATCC 43300) as positive control, Lane 4 to 7: *S. aureus* isolates from bovine milk samples

Different patterns of antibiotic-resistance were observed in the 151 isolates of *Staphylococci* (Table 2). In the present study, high resistance rates were observed for ceftriaxone (70.86%) and amoxicillin-salbactum (60.26%). Total 38.41% isolates were resistant to gentamicin. The tested isolates were least resistant to levofloxacin (22.52%), methicillin (17.88%) and oxytetracycline (13.91%) which suggests judicious use of these antibiotics in treatment of bovine mastitis. Previous studies also showed variation in the resistance

with strains of different geographical regions. Such variations in antibiotic-resistance patterns in isolates of different regions shown could be due to diverse antibiotic policies and conditions (Turutoglu *et al.,* 2009, Preethirani *et al.,* 2015).

Conclusion:

The study concludes that the *Staphylococci* are still a predominant cause of subclinical and clinical mastitis in bovine. Combination of measurement of SCC, biochemical characterization and molecular detection can be used for quick assessment and rapid detection of *Staphylococci*, S. *aureus* as well as CoNS.

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Conflict of Interest:

All authors declare no conflict of interest.

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