

PREVALENCE OF COAGULASE GENE POSITIVE *Staphylococcus aureus* BOVINE
MASTITIS IN THREE DISTINCT GEOCLIMATIC REGIONS OF INDIA

B.D. Sahu¹, R. Mukherjee^{1,*}, Ajay Kumar², Amit Kumar³ and Jyoti Sahu⁴

ABSTRACT

A two-year retrospective study was performed to determine the bacteriological aspects of coagulase positive Staphylococcal mastitis by bacterial culture and by polymerase chain reaction using specific primers in cattle and buffalo farms from three distinct geoclimatic zones of India. A total of 3934 quarters from 1022 cattle and buffaloes were screened from the Bareilly, Kumaon and Rajnandgoan regions of India. Mastitis cases were detected from 754 quarter milk samples on the basis of CMT positive, SCC > 7 x 10⁵ cells /ml and milk positive on bacterial culture. The overall prevalence of Staphylococcal mastitis (BSM) was 22.02% on cow basis and 8.64% on quarter basis in 1022 lactating cows with 3934 functional quarters. The intra-mammary infection (IMI) was higher to the extent of 45.54% in the Bareilly and 56.31% in the Rajnandgoan regions as compared to the Mukteshwar region. The coagulase gene of *S. aureus* was amplified with a pair of primers using the polymerase chain reaction (PCR). PCR revealed the *coa* gene from 14.73%, 10.53% and 13.24% more mastitic milk samples as compared

to the bacterial culture method. Amplification of the *coa* gene by PCR is an important technique for quick diagnosis of prevalent bacterial pathogens of a particular region. With the help of PCR, a large number of lactating dairy animals can be screened with accuracy in less time in the study area for implementing therapeutic and preventive measures.

Keywords: bovine mastitis, coagulase gene, geoclimatic zones, *Staphylococcus aureus*, polymerase chain reaction

INTRODUCTION

Mastitis is generally associated with intensive dairy farming systems. It causes great economic losses due to lower and poor quality of milk production and treatment cost (Ruegg, 2011). *Staphylococcus aureus* is the major contagious pathogen. It spreads rapidly in dairy herds during milking and generally leads to subclinical (SCM), chronic and sometimes clinical mastitis (Ott, 1999). The implementation of proper therapeutic and

¹Division of Medicine, Indian Veterinary Research Institute, Izatnagar, UP, 243 122, India, *E-mail: reenam1992@gmail.com

²Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar, UP, 243 122, India

³Division of Animal Breeding and Genetics, Indian Veterinary Research Institute, Izatnagar, UP, 243 122, India

⁴Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Anjora, Durg, Chhattisgarh, 491 001, India

preventive measures depends on the appropriate knowledge of clinical, epidemiological and bacteriological aspects of mastitis (Shpigel, 1998). Raw milk is a potential source of *S. aureus*. The gene encoding coagulase (*coa*) is an important virulence factor responsible for invasion of *S. aureus* into the mammary epithelium. *Coa* gene polymorphism is found suitable for epidemiological investigation of bovine *S. aureus* mastitis (Zecconi *et al.*, 2006). The bacterial culturing of the milk samples is the standard procedure for mastitis testing, but the method is time consuming. About 25% of the mastitic milk samples do not yield bacterial growth with the conventional culturing method, and therefore a proper control program cannot be implemented on a particular farm (Makovec and Ruegg, 2003). The polymerase chain reaction (PCR) can identify the pathogen in the milk if present in traces (Bradley *et al.*, 2007). The technique is simple and fast. In the present study, we collected milk samples from three distinct geoclimatic zone of India for detection of coagulase positive *S. aureus* by bacterial culture and PCR directly from the raw milk to note the sensitivity of these two techniques.

MATERIALS AND METHODS

A total of 1022 cattle and buffaloes were

screened for udder health status in the Bareilly, Kumaoun and Rajnandgoan regions. Udder health status was screened on the basis of CMT reaction, somatic cell count (SCC) and bacterial isolation. Milk samples positive for CMT and SCC > 0.6 to 3.2 million cells / ml of milk and positive for bacterial isolate were diagnosed as subclinical and clinical mastitis. Ten ml milk was collected from each teat, after cleaning the teat end with cotton soaked in 70% alcohol and after discarding 3-4 milk squirts. SCC of the milk samples were done as per the standard method (Schalm *et al.*, 1971). Milk samples were collected for bacterial isolation and identification from 387, 95 and 272 quarters, found positive on CMT. The identification and biochemical characterization of causative organism in collected milk samples were carried as per (Balows *et al.*, 1991).

Amplification of the coagulase (*coa*) gene from raw milk samples

DNA extraction was carried out as per the method described by Ahmadi *et al.* (2010). The DNA pellet was quantified in NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). The coagulase gene of *Staphylococcus aureus* from milk samples was amplified using the primer pair (Eurofins Genomics India Pvt Ltd, Bangalore, India) listed as below:

Table 1. Primer pair used for amplification of in the study *coa* gene.

Gene	Primer	Oligonucleotide sequence	Gene bank accession no.	Amplicon size (bp)
Coagulase (<i>coa</i>)	Forward	TGGTTTATGCCGGCTTTGATC	AB436983.1	807
	Reverse	GCAGCAGCTTTCAATGACGGA		

For PCR, each reaction mixture contained 2 µl of target DNA (approximately 260 ng/µl), 1 µl each of primers (20 pmol), 0.5 µl of a mix of deoxynucleotide triphosphate (200 µM each), 0.3 µl of *Taq* polymerase (5U) and 3 µl of PCR 10X buffer (Tris-HCl 100 mM; KCl 500 mM; Triton X-100 1% MgCl₂; pH 8.4). The volume was adjusted to 30 µl with nuclease free water. All the reactants were thoroughly mixed and flash spun in microcentrifuge (St.Louis, MO, USA). Amplification was carried out in a thermal cycler (MJ Mini Cycler Bio-Rad Laboratories, Hercules, California, USA) as follows: initial denaturation at 94°C for 4 minutes, 30 cycles of amplification (denaturation at 94°C for 1 minute, annealing at 56 °C for 1 minute and extension at 72°C for 1 minute) and final extension at 72°C for 5 minutes. One positive control containing *S. aureus* reference strain MTCC No.96 (Microbial Type Culture Collection, Chandigarh, India) was included in each reaction. The PCR product was visualized by electrophoresis in 1.2% agarose gel containing 0.5 µg/ml ethidium bromide (Fermentas, Germany). The size of PCR product was determined by comparing with a standard molecular weight marker, and was photographed by gel documentation system (Alpha Imager™ 1220, Documentation and Analysis, Alpha Innotech Corporation, USA). The chi-square Test was used to assess association between the probability of developing Staphylococcal mastitis and region, CMT, SCC and bacterial examination.

RESULTS AND DISCUSSION

The prevalence of bovine Staphylococcal (*coa* gene positive) mastitis (BSM) was studied in three distinct geo-climatic regions of India i.e., the sub-tropical humid climate zone of the

Bareilly region (Uttar Pradesh), the temperate and mountainous climatic zone of the Uttarakhand (Kumaoun) region and the tropical wet and dry climatic zone of the Rajnandgaon region (Chattisgarh). The prevalence of mastitis varied in these three distinct regions. The climatic conditions of Bareilly (U.P), Uttarakhand (Kumaon) and Rajnandgaon (Chattisgarh) are detailed in Table 2. One thousand twenty two (3934 functional quarters) cattle and buffaloes were screened for udder health status. In the present study, the prevalence of Staphylococcal mastitis (BSM) was 22.02% on animal basis and 8.64% on quarter basis in 1022 lactating dairy animals with 3934 functional quarters in three geo-climatic regions of India. The animal wise prevalence of BSM in Bareilly, Kumaoun and Rajnandgoan was 23.05%, 15.84% and 24.76% respectively. The prevalence of Staphylococcal intra-mammary infection (IMI) was higher to an extent of 45.54% in Bareilly and 56.31% in Rajnandgoan compared to the Kumaoun region.

Bacterial isolation and biochemical characterization in milk samples collected from Bareilly, Kumaoun and Rajnandgoan region of India

Out of 387 milk samples collected from mastitic quarters from Bareilly, 36 (9.30%) isolates were found coagulase positive Staphylococcus sp. (CPS) (Figure 1) and 110 (28.42%) isolates were coagulase negative Staphylococci (CNS). Whereas 6 (6.32%) and 22 (8.09%) milk samples from the Kumaoun and Rajnandgoa regions identified as CPS, while 38 (40.0%) and 89 (32.72%) were identified as CNS. The prevalence of CPS was 47.15% and 28.01% higher in the Bareilly and Rajnandgoan regions as compared to the Kumaoun region. The lowest prevalence (6.32%) of CPS was

recorded from the Kumaoun region.

Prevalence of Coagulase Positive Staphylococci in quarter milk samples from bovine mastitis in three geo-climatic regions of India

Amplification of the coagulase gene yielded a single PCR product of 807 bp in raw milk samples (Figure 2). The prevalence of CPS was higher to an extent of 39.89% and 25.74% in the Bareilly and the Rajnandgoan regions as compared to the Kumaoun region. The prevalence rate of CPS in quarter milk samples from mastitic cows and buffaloes varied significantly with the two different methods used for identification of the causative organism i.e. by bacterial culture and PCR amplification of the *coa* gene. Mastitic milk samples revealed growth in 9.3%, 6.32% and 8.09%, positive CPS by conventional culturing method from the Bareilly, Kumaoun and Rajnandgoan regions, respectively. Whereas *S. aureus* by PCR technique yielded the *coa* gene in 14.73%, 10.53% and 13.24% positive milk samples from the Bareilly, Kumaoun and Rajnandgoan regions, respectively (Table 3). The prevalence of coagulase positive *S. aureus* by the PCR technique was found to be higher to an extent of 58.39%, 66.61% and 63.66% as compared to bacteriological examination in the Bareilly, Kumaoun and Rajnandgoan regions, respectively.

S. aureus is the most common cause of contagious and sub-clinical mastitis in dairy herds (Mukherjee and Dash, 2003). For implementation of preventive and control measures for bovine mastitis on a dairy farm, somatic cell count and bacteriological aspects must be assessed at frequent time intervals. The coagulase gene is one of the virulence factors of *S. aureus*; it determines the ability to coagulate mammalian plasma. *Coa* gene polymorphism is frequently applied for epidemiological investigations of bovine *S. aureus*

mastitis (Zecconi *et al.*, 2006). Mukherjee *et al.*, (2004, 2010) recorded 14.28% and 36% positive *S. aureus* mastitis in organized and unorganized dairy farms by bacterial and biochemical characterization. Moreover, the prevalence of CPS positive mastitic cases by isolating marker gene was reported from many countries (Su *et al.*, 1999). Sindhu *et al.* (2010) reported 56% of quarter milk samples from buffaloes were positive for *S. aureus* based on detection of the *coa* gene in Haryana. Similarly, Ahmadi *et al.* (2010) reported 21% of the milk samples from cow were positive for *S. aureus* using amplification of the *coa* gene in industrial dairy herds in Iran. In conclusion, case occurrence of Staphylococcal mastitis varied with the geo-climatic conditions of the area, with the greatest number of Staphylococcal intramammary infections in the tropical, dry climate of Rajnandgaon followed by subtropical humid climatic zone of Bareilly and the fewest number of cases in the temperate Kumaoun zone. Amplification of the *coa* gene by PCR is an important technique for quick diagnosis of prevalent bacterial pathogens of a particular region. With the help of PCR, large numbers of lactating dairy animals can be screened with accuracy in less time in the study area.

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Table 2. Geographical and meteorological parameters of three geo-climatic regions of India.

Region	Specifications	Latitude	Longitude	Air temp (degree celcius)	Average annual rainfall (mm)
Bareilly (Tropical & humid climate)	Hot & humid summer and cold winters, average three winter month	28°10' N	78°23' E	4.5-45.8	1032.0
Kumaoun (Temperate climate)	moderate summer and very cold winters, average six winter months	29°29' N	79°39' E	Summer-17-26 Winter-0 -14	1494.1
Rajnandgaon (Tropical climate and dry)	Hot and dry summer and moderate winters, average one winter months	20°70' & 22°29' N	81°29' & 88°29' E	8.4-46.6	1275.0

Table 3. Prevalence of coagulase positive staphylococci (CPS) (based on bacteriological and PCR method of identification) in quarter milk samples from bovine mastitis in three geo-climatic regions of India.

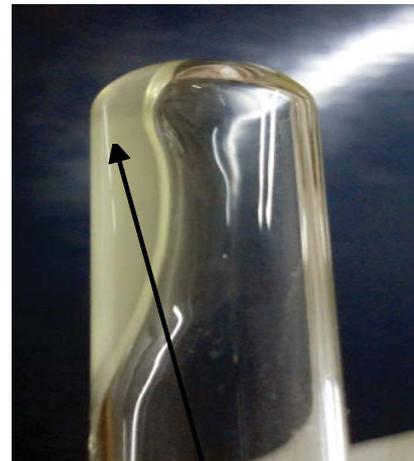
Geo-climatic regions	Quarters screened	Positive for mastitis	Coagulase positive mastitis on the basis of bacterial culture	Coagulase positive mastitis on the basis of PCR
Bareilly	1872	387	36 (9.3 %)	57 (14.73 %)
Kumaoun	856	95	6 (6.32 %)	10 (10.53%)
Rajnandgoan	1206	272	22 (8.09%)	36 (13.24 %)

Wald Chi-square (P value) = <0.0001, Intercept (P value) = <0.0001.

Paired T Test Results: DF = 2, t value = 13.36; Pr>|t| = 0.006.



Coagulase negative sample



Coagulation of plasma
Coagulase positive sample

Figure 1. Coagulation of rabbit plasma by pathogenic *Staphylococcus aureus* isolated from mastitic milk samples.

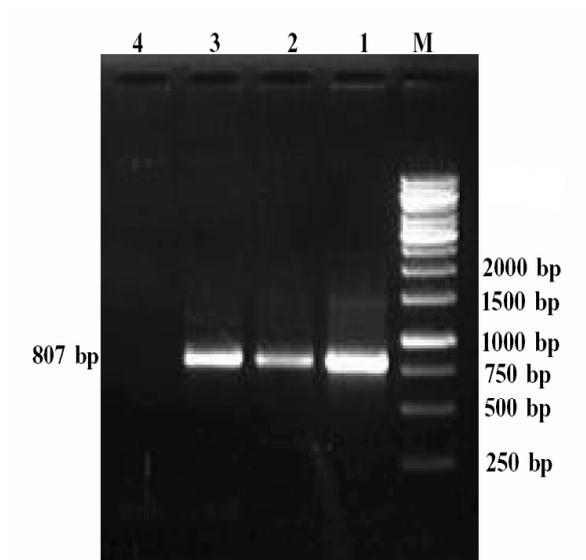


Figure 2. Amplification of Coagulase gene from mastitic milk samples.

- Lane M – 1kbp molecular weight marker
- Lane 1 - *S. aureus* reference strain MTCC 96
- Lane 2 and 3 - *S. aureus* positive samples

- in milk by PCR. *Comp. Clin. Pathol.*, **19**: 91-94.
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