

Original Research Article

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Incidence of Bovine Clinical Mastitis Caused by *Escherichia coli*

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ABSTRACT

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Bovine mastitis cause severe economic losses resulting from reduced milk production, treatment cost, increased labour, milk withheld following treatment and increased cost of veterinary services. *Escherichia coli* is one of the major environmental pathogen associated with clinical mastitis. Therefore the present study was done to know the incidence of clinical mastitis caused by *E. coli* in the Proddatur region of Andhra Pradesh by means of culture and confirmation by Polymerase Chain reaction (PCR). Out of 78 clinical mastitis milk samples obtained from various semi-organized private farms, a very high incidence rate of bovine clinical mastitis caused by *E. coli* has been reported which was found to be 41% as identified by isolation of gram negative bacteria on basic media, followed by selective differentiation of *E. coli* using eosine methylene blue agar and further confirmation by PCR. This study suggests an important need for the adequate milking procedures, maintenance of proper sanitation, regular screening for the early detection of sub-clinical mastitis cases, good udder health practices including pre- and post dipping for the prevention and control of mastitis due to *E. coli*.

Introduction

Mastitis is a complex, multifactorial disease and continues to be the most costly disease of dairy animals. Bovine mastitis results in a great deal of economic losses, mostly because of the reduction of milk yield, decreased milk quality, and higher production costs, medication costs, discarded milk during and shortly after treatment, loss of milking days, reduced milk price, increased labour and increased recruitment costs due to culling

(Seegers *et al.*, 2003 and Cremonesi *et al.*, 2006). As such, the incidence of bovine mastitis depends on exposure to pathogens, effectiveness of udder defence mechanisms and presence of environmental risk factors, as well as interactions between these factors (Suriyasathaporn *et al.*, 2000; Oviedo-Boyso *et al.*, 2007).

Microorganisms causing mastitis are of two types based upon their primary reservoir and mode of transmission- Environmental

pathogens which include coliform species like *E.coli*, *Klebsiella* and species of *Streptococci*. These arise from the environment in which the cow lives, when teats are exposed to mud, manure and dirty bedding materials; Contagious pathogens like *S. aureus*, *Streptococcus agalactiae* which are transmitted among cows by contact with infected milk (Cremonesi *et al.*, 2006). In Asia, major mastitis causing organisms are *Staphylococcus aureus*, *Streptococci*, *E. coli*, *Corynebacterium* Sp. and *Klebsiella* Sp. (Sharma *et al.*, 2012).

Clinical mastitis is recognized by abnormal milk (visible clots in milk, change in the colour of the milk) gland swelling and /or systemic illness. Clinical mastitis can be peracute, acute, subacute and chronic, caused by coliforms like *Escherichia coli*, *Klebsiella pneumoniae*, *K. oxytoca* etc. The reduction in milk production attributed to clinical mastitis could be 30.0 percent of the total losses (Philpot and Nickerson, 1991 and Philip *et al.*, 1993). *E. coli* is reported to be one of the commonest pathogens in environmental mastitis resulting in sudden onset of fever, loss of appetite, diarrhea, toxemia and the infected quarter shows swelling, pain with discharge of watery or bloody milk. Necrosis of the mammary epithelium occurs during severe, naturally occurring clinical *E.coli* mastitis, as well as during severe experimental *E. coli* mastitis (Bradley and Green, 2001). Early identification of the prevalence and distribution of causative pathogens is one of the important prerequisite to effectively prevent diseases and to guide treatment. So far, the incidence of *E. coli* in bovine clinical mastitis in Proddatur region of Andhra Pradesh is not known. Therefore, the present study was conducted to determine the incidence of *E. coli* in bovine clinical mastitis in Proddatur region of Andhra Pradesh by means of isolation and identification and confirmation by Polymerase Chain Reaction.

Materials and Methods

Sample collection

A total of 78 clinical mastitis milk samples (31 Jersey cross bred cows, 28 Graded Murrah buffaloes and 19 Holstein Friesian cross bred cows) were collected from various semi-organized private farms maintained in Proddatur town and villages nearby. Teats were wiped with 70% alcohol before collecting the samples. One or two streams of milk were discarded before collecting samples from the infected quarter. The collected samples were transported in ice cold condition and stored at 4°C until further processing.

Isolation and identification of bacteria

Samples were mixed well and two or three loopful of milk was streaked on to Mueller Hinton agar, incubated at 37°C for 24-48 hrs. The cultured organisms were then subjected for bacteriological analysis. The gram negative bacterial colonies from the isolates were then identified by Gram's staining technique as recommended by the manufacturer's kit (Himedia, Mumbai). The gram negative isolates were then streaked on to EMB agar for the identification of *E.coli*.

Extraction of bacterial DNA

The sample DNA was extracted from the gram negative isolates of clinical mastitic cases by using polyethylene glycol (PEG) as recommended by PioChomczynski and Michal Rymaszewski *et al.*, 2006 with slight modifications. Colonies from the isolates were transferred to 100µl of nuclease free water and then mixed with 900µl of PEG reagent. The mixture was vortexed for 2-3 min, incubated in water bath at 90°C for 15 min. and then centrifuged at 12,000 rpm for 10 min. The lysate was then stored at - 80°C

and used as the template for Polymerase Chain Reaction (PCR).

PCR for *E.coli*

PCR assay targeting an 884 bp region of *E.coli* chromosomes' *usp A* gene (Chen and Griffiths, 1998) was performed using the primer set; forward 5'-CCGATACGCTGCCAATCAGT-3' and reverse 5'-ACGCAGACCGTAGGCCAGAT-3'. A 25 µl of PCR reaction mix was prepared using 12.5 µl of Dream taq green PCR master mix (Thermo Scientific), 1 µl of forward primer, 1 µl of reverse primer, 5 µl of template and 5.5 µl of nuclease free water. Amplification was carried out as follows: an initial denaturation of 95°C for 4 minutes; 30 cycles of 95°C for 45 sec, 55°C for 45sec and 72°C for 45 sec and a final extension step at 72°C for 6 min.

Agarose gel electrophoresis

The DNA fragments were stained with ethidium bromide and were visualized using Chemi documentation system from Syngene, Biocon, U.S.A. The size of the amplified product was compared by the use of 100 bp DNA ladder, Amresco.

Results and Discussion

Isolation and identification of causative pathogens of bovine mastitis by the use of specific media for growth and culture is still considered to be the gold standard (Radostits *et al.*, 2000). However due to the appearance of false positive results and associated less sensitive and efficient results with the use of EMB agar for the detection of *E.coli* from various kinds of biological and environmental samples, use of modified media like Hicrome *E.coli* agar and further confirmation by using molecular methods like PCR has been recommended by various researchers (Antony

et al., 2016; Islam *et al.*, 2014). All the clinical bovine mastitis milk samples were used for the isolation and identification of gram negative bacteria using Mueller Hinton agar. Out of 78 clinical mastitis milk samples collected from jersey cross bred cows (31), graded Murrah buffaloes (28) and HF cross (19), 41% (32/78) of the bacterial isolates were found to be gram negative. Details are being provided in table 1. The incidence of bovine clinical mastitis caused by gram negative bacteria was found to be highest in graded Murrah buffaloes (46%), followed by Jersey cross bred cows (39%) and HF cross (37%). Among the Gram-negative bacterial etiological agents of bovine mastitis, the major group includes coliform bacteria (*Escherichia coli*, *Enterobacter*, *Klebsiella*), *Pseudomonas* and *Serratia* (Samanta 2013). Across the farms, *E. coli* tends to be the most important cause of these gram-negative infections associated with bovine mastitis. However, more precise investigation of individual farms revealed a farm-specific infection pattern where a single gram-negative bacterial species predominates. Several farms with a predominance of other gram-negative intra mammary infections (IMIs) were observed in individual dairy farm with *K. pneumoniae*, *S. marcescens*, and *Enterobacter cloacae*. Further, association of the host immune response pattern with the clinical and subclinical presentations of IMIs due to the different gram-negative organisms was identified (Schukken *et al.*, 2012).

In our study, out of the 32 gram negative clinical isolates of bovine mastitis, 41% (13/32) of the bacteria were found to be *E.coli* as identified on EMB agar with the characteristic smooth, circular, black colour colonies with green metallic sheen (Figure 1) and further confirmation by PCR (Figure 2). The highest incidence of *E.coli* in bovine mastitis was identified in jersey cross bred (50%) followed by graded Murrah buffaloes

(39%) and HF cross (29%). In contrary to the present study, a much lower prevalence rate of *E.coli* in bovine mastitis was reported by various researchers. The prevalence of *E.coli* in clinical bovine clinical mastitis was found to be 20% (Mahmoud *et al.*, 2015). The prevalence of *E.coli* in sub-clinical mastitis was found to be 4.5% (Vasquez-Garcia *et al.*, 2017). The prevalence of *E.coli* in bovine mastitis in Meerut was found to be 21.3% (Verma *et al.*, 2018). Experimental and field observations would suggest that among the gram-negative bacterial causes of mastitis, *Klebsiella* spp are causing the most severe cases, closely followed by *E. coli* and then much less clinical severity is observed in *Serratia* spp and *Enterobacter* spp cases (Schukken *et al.*, 2012).

The exotic breeds like Jersey were found to be more susceptible to bovine mastitis than indigenous breeds (Iqbal and Siddique, 1999). Dutta *et al.*, 1990 also reported that the risk ratio of developing mastitis in Jersey was approximately two times higher than indigenous breeds. Whereas, Rahman *et al.*, 2009 reported the highest prevalence of mastitis in HF followed by Jersey and the least in indigenous breed. Similarly Biffa *et al.*, 2005 reported that HF cows are affected at higher rate (56.5%) compared with local zebu (30.9%) and Jersey cows (28.9%). The difference in breed-wise prevalence of mastitis may be due to the inheritance characters, immunity of the individual breeds, and also habituation of cows to the climatic conditions.

The incidence of clinical mastitis in Holstein Friesian and jersey cows of Dharwad district of Karnataka was found to be 13.2% and 10.1% respectively. Breed of the cow and environmental conditions were found to be the most important factors associated with clinical bovine mastitis (Kurjogi and Kaliwal 2014). According to Litwinczuk and Brodziak (2015), a higher incidence of bovine mastitis was observed in Holstein Friesian breed. Polymorphism of bovine leukocyte antigen (BoLA) and lactoferrin genes were found to be associated with the susceptibility of cows to mastitis.

Therefore, marker assisted selection (MAS) of cross bred cows resistant to mastitis can be followed in the selection process of animals while purchase. Further, deficiency of selenium, vitamin A and vitamin E in the ration was found to contribute to the increase in the incidence of mastitis in dairy cows.

Studies also reported that there is increase in the trend of *E.coli* isolated from bovine mastitis to produce biofilm which inturn contributes to the aggravation and recurrence of clinical mastitis under conditions of low glucose availability (0.5-1.5%) or with either an acidic (5.5) or alkaline (8.5) pH (Costa *et al.*, 2014). Also researchers have demonstrated that sub-inhibitory concentrations of enrofloxacin (0.0125 mg/ml) were able to induce biofilm formation in *in vitro* in the *E.coli* isolated from bovine mastitis (Costa *et al.*, 2012).

Table.1 Incidence of bovine clinical mastitis caused by *E.coli*

Source	No. of samples examined	Culture and staining method		PCR for <i>E. coli</i>	
		No. of gram negative isolates	%	No. of positive	%
Jersey Cross bred cows	31	12	39	6	50
Graded Murrah buffaloes	28	13	46	5	39
HF cross	19	7	37	2	29
Total	78	32	41	13	41

Fig.1 Green metallic sheen produced by *E. coli* (bovine clinical mastitis isolate) on Eosine methylene blue agar

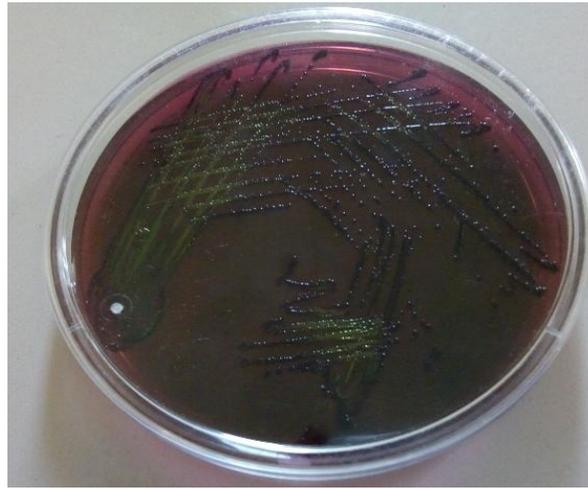
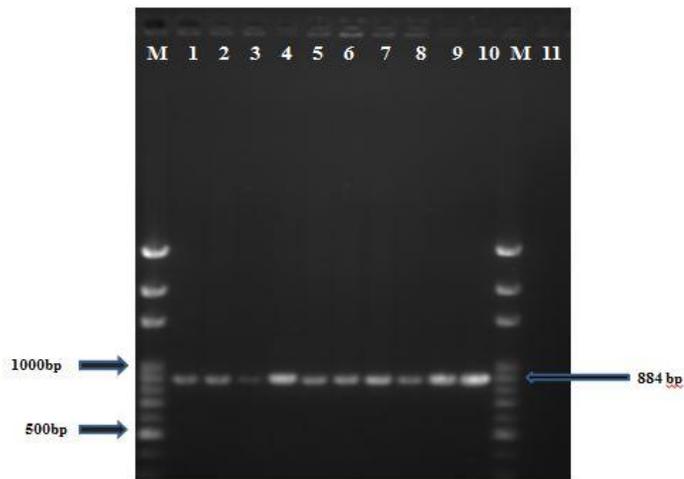


Fig.2 PCR detection of *Escherichia coli* from field clinical mastitis milk samples. Lane M: 100bp molecular weight marker; Lane 1-9: Clinical mastitis *E. coli* isolates from field samples; Lane 10: Positive control; Lane 11: Negative control



Considering the results of the current investigation and the reviewed data of the various researchers across the globe, the difference in the incidence rates of bovine mastitis caused by *E. coli* in Jersey and HF cross breeds and graded Murrah buffaloes may be due to variation in the geographical location, size of sampling, macro and micro environment in which the animal resides, size

of sampling, sanitary condition of udder, immune status of the animal, cell mediated immune response of the individual breed to *E.coli* infection (Sadashiva and Kaliwal, 2013, Shopsin *et al.*, 2000 and Sulabh *et al.*, 2019).

In this study, the incidence of bovine clinical mastitis caused by *E.coli* in various private

farms in and around Proddatur region of Andhra Pradesh as determined by specific bacteriological culture by growing the gram negative clinical isolates of bovine mastitis on EMB agar and further confirmation by PCR was found to be 41%. Therefore, it can be concluded that *E.coli* is one of the major causative agent of clinical mastitis in bovines originating from the environment of cow associated with poor sanitation, improper disposal of the farm waste, lack of udder hygiene and most importantly indiscriminate and rampant use of antibiotics ultimately leading to the severe and recurrent mastitis infections, emergence of antibiotic resistant bacteria which can even produce biofilms and persist with in the udder tissue. Hence, there is a need for the screening of dairy herds for the pathogen identification, maintenance of proper hygiene in the farm and related macro environment, nutritional supplementation to reduce the incidence of mastitis and most importantly determining the drug of choice for the treatment by the use of ABST for the successful control of coliform mastitis due to *E.coli*.

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