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PREVALENCE OF SUB-CLINICAL MASTITIS DOMINATED BY COAGULASE NEGATIVE *Staphylococci sp.* IN BOVINES

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ABSTRACT

In many cases of mastitis, an udder inflammation disease, Staphylococci sp. is reported as major pathogen along with Acinetobacter and Corynebacterium. Lack of awareness, unavailability of practical knowledge and ignorance, contributes in development of subclinical mastitis; ultimately a major cause behind preponderance of clinical mastitis. Present study was designed to understand disease prevalence and causative agents. Total number of 125 animals, out of which 45 cows and 80 buffaloes, from 3 representative sectors, in and around Jhansi, District of Bundelkhand division in India were examined for mastitis detection.Preliminary screening test used to study prevalence was CMT followed by microbial enrichment, isolation and characterization.Bacterial isolation and pure culture were done in accordance with ***Collee et al, 2008****. Overall prevalence of 56.80%. was reported. Out of 500 quarters, 25 blind teats were left, a total of 205 (43.16%) quarters were found positive for subclinical mastitis. 21 (46.66%) cows and 50 (62.50%) buffaloes were found positive for subclinical mastitis with CMT. Teat wise prevalence was found higher in cows as compare to buffalo. It was observed as 52.38 and 47.83 % respectively. Prevalence of different bacterial sp., isolated was found 14.63, 51.22, 13.41, 10.98, 3.66, and 6.10 % for S.aureus, CNS, Bacillus, Streptococci, E.coli, and Others, respectively. Among 51.22% isolates of Staphylococci sp. non-aureus.; S. epidermidis emerged as major CNS sp. with isolation frequency of 61% followed by 20, 10, 4,2,2,1% frequency of isolation for S. chromogenes S. intermedius, S. simulans, S. hyicus and S. haemolyticus, respectively. Study cocludes the emergence of Coagulase negative Staphylococci sp., specialy S. epidemidis as major contributers to subclinical mastitis.*

KEY WORDS

Subclinical Mastitis, Prevalence, Coagulase negative Staphylococci, Bundelkhand, Bovines

INTRODUCTION

Mastitis is an udder inflammation disease, which not only affects milk production but also the quality. Control of mastitis should be an integral part of clean milk production. Mastitis affects both cattle and buffalo and considered as major cause behind huge economic loss in developing and developed countries (Korhonen and Kaartinen, 1995; Bradley, 2002) and in India (Joshi and Gokhle, 2006). India is the largest producer of milk, the export of milk and milk products depends on quality, which is degraded by mastitis in very high percentage and is of value for export oriented country.. Mastitis is an infectious disease causing great economic loss due to reduction in milk yield (Oliver and Calvinho, 1995). Sub-clinical mastitis is 30-40 times more prevalent than clinical mastitis. Mastitis, one of the most prevalent

diseases in dairy animals, an endemic that happens to be the most frequent and most costly disease affecting dairy herd's worldwide (Halasa *et al.* 2007; Miller *et al.* 1993). It affects the quality of the milk through changes in milk composition and in return, affects the economy of dairy industry.

Mastitis is an infectious disease occurs in response to bacterial infection (Zhao and Laccase, 2008) and continually causing great economic loss due to reduction in milk yield. Sub-clinical mastitis is 30-40 times more prevalent than clinical mastitis. It is important to establish the major pathogens, their prevalence and correlation with disease outbreak in particular area so that proper treatment and control measures can be taken into consideration (Damien *et al.* 2005), it will also be helpful in risk calculation for severity of disease and forecasting, probabilty and occurence of clinical mastitis at herd level or in region. *Staphylococci* and *Streptococci* are the most common bacterial genera isolated from subclinical mastitis cases in previous studies. (Personn *et al.*, 2011, Botrel *et al.*, 2010, Gianeechinii *et al*. 2002). *Staphylococcus aureus* has been reported commonly in different studies on SCM, for example, in Zambia by Pandey *et al.*, 1996, in Uruguay by Gianeechinii *et al*. 2002, in Sweden by Personn *et al.*, 2011 and in India by Bhanot *et al*. 2012. *Staphylococcus sp.* is considered as dominant mastitis pathogen in both clinical and subclinical mastitis cases by Krishnamurthy *et al.* 2017 after meta-analysis of subclinical and clinical mastitis prevalence. Pyorala and Taponen, 2009 proved the common occurrence of coagulase negative *Staphylococci* in mastitis cases. Schukken *et al.* 2011 reported the occurrence of *E. coli* and *Klebsiella sp.,* as cause behind mastitis infection. Frequency of *E.coli* and *Klebsiella sp.* may vary, depending on country or on herd also Persson *et al*. 2011.

Bundelkhand is one of the most backward regions of India which is having agrarian economy. The income disparity in rural areas is a major challenging issue which is unresolved till date. Animal husbandry is the major source of income to the farmers of this region. Most of the work done here by the state & central government is to provide the basic amenities to farmers. Due to these policies farmers are more engaged now a day in animal husbandry. Cattle and Buffalo farming is the major part of farmer's economy in this region. But due to lake of awareness animal diseases like, Mastitis is prevalent in this area and continually affecting the quality and quantity of milk and milk products produced in this region. No more recent data is available on the prevalence of subclinical mastitis in Bundelkhand region. But in India It is not easy to handle the management of milk from quantitative and qualitative point of view without having a relevant data on mastitis. So the study on prevalence of mastitis pathogens is demand of time to stimulate and protect the dairy farming in a developing country like India and particularly in Bundelkhand region by to generate real time data. It will be highly beneficial in disease forecasting and its control ultimately leads towards improvement of farmer's economy in this region. In view of the above facts, the present study was designed to study the prevalence of mastitis and associated pathogens in and around Jhansi region simultaneously, to generate primary data on prevalence of subclinical mastitis (SCM), destribution and dominance of pathogens , that can assist in management of disese in proper way with respect to this particular climatic zone.

MATERIAL AND METHODS

Sample Size, Duration & Collection area

By taking strict aseptic measures, 125 cattle milk samples were collected over a period of 3 months (August to Novemebr, 2017) from 8 unorganized sectors from various geographic locations in Jhansi and surrounding areas of Bundelkhand division of Uttar Pradesh. Jhansi is located at Latitude: 25°27'31" N Longitude: 78°34'47" E with an average elevation of 248 m = 813 ft above sea level. Average annual temperature in Jhansi is recorded between 18.4 degree centigrade to 32.9 degree centigrade, with May, June as hottest, while January as coldest month on average. Jhansi also receives an annual precipitation of 891.3 with a total of 44.20 numbers of rainy days.

Milk sample collection, handling and transportation

In accordance to the procedure described by **Ayano *et al.* (2013),** Aseptic procedures were followed for collecting a composite of all quarter milk samples as described by **Harmon *et al.* (1990)**. Sample collection was done before milking. Udders and especially teats were cleaned with 70% alcohol and dried up, just before sample collection. The first 3 milking streams were discarded and thereafter approximately 10 ml of milk collected in to a sterile collecting tube. After collection, the sample was placed in an icebox and transported to

the laboratory, stored at refrigerated temperature of 4^0C for 24 Hours till the inoculation on a standard bacteriological media.

California mastitis test (CMT)

The California Mastitis Test (CMT) in accordance with National Mastitis Council, 1999 guidlines. About 2 ml of each sample collected per quarter in a plastic paddle having four shallow cups. Equal amount of CMT reagent was added to milk, and then paddle was rotated to allow proper mixing of milk and reagent. Score was noted down as 0, +1,+2 and +3 for for nrgative/trace, weak positive, distinct positive and +3 for strong positive respectively. Cows or buffaloes found positive for minimum of one quarter were declared CMT positive.

Isolation and characterization of bacterial isolates

All bacteriological tests were performed on media and reagents purchased from commercial sources (HiMedia Laboratories, Mumbai) . 100 ml of each milk sample enriched in peptone water was transfrred and spreaded thouroghly, on nutrient agar and another 100 microlitre was used for pour plate for examination of bacteriological status of milk. Further on the basis of colony morphology, and gram staing bacterial cultures were differentiated into Gram +Ve and Gram negative rods or cocci. For isolation of *Staphylococci,* initially, milk samples were enriched in brain heart infusion broth , with incubation for 6 h at 37 °C and then again incubated for additional 24 hour at 37^0C on streaked on mannitol salt agar. Colonies were re-streaked onto BHI agar for further identification procedures after recording colony morphology.

Blood agar plate incubation for 6 hours with 5% CO2 was done for isolation of Streptococci by streak plate. After recording the pattern of haemolysis and the colony morphology, the colonies were re-streaked onto blood agar plates and incubated further at 37 °C for 48 h in 5 % CO2 to obtain pure cultures. Streaking of pure cultures was again repeated for further identification procedure. Enrichment was done on tryptone phosphate broth for 18 h at 37 °C for isolation of *E.coli,* followed by MacConkey agar streaking and incubation at 37 °C for 24 h. The colonies fermenting lactose were further streaked onto Eosin Methylene Blue agar and incubated for 24 h at 37 °C. Pure cultures were subjected for various biochemical tests including Coagulase, Catalase, oxidase and carbohydrate fermentation test for trahalose, glucose, sucrose, arabinose, xylose, raffinose, lactose, malotose and mannitol, as per standard procedures (**Collee *et al.,* 2008**). Results were analysed with one way ANOVA.

RESULTS AND DISCUSSION

Prevalence of mastitis:

Mastitis is the major cause of loss to milk yield in selected area of Bundelkhand, directly responsible for losses to farmers. Subclinical mastitis was observed as neglected fact at both farmers and veterinarian level as it was not managed seriously, and generally not diagnose which is continously predispoing the serious mastitis problems in the region. Lack of primary data of mastitis prevalnce in open sources, particularly for this study area is observed as serious issuse in mastitis management. Total number of 125 animals, out of which 45 cows and 80 buffaloes, from 3 representative sectors, in and around Jhansi, District of Bundelkhand division in Uttar Pradesh were examined for mastitis detection to determine the prevalence of disease and bacterial isolates. On the basis of California mastitis test, total numbers of 71 (56.80%) animals were found positive for subclinical mastitis. Except this 2 animals with blind teats were not considered for further investigation. Out of 500 quarters, 25 blind teats were left, a total of 205 (43.16%) quarters were found positive for subclinical mastitis. Overall prevalence reported falls in range of subclinical mastitis between 19.20 and 83%, as previously established by **Sharma and Maiti (2010), Kumar *et al.* (2010) and Tuteja *et al.* (1993)**. However it is significantly higher than overall mean prevalence of 44.7% for bovine mastitis as calculated by **Nilesh *et al.* (2012)** after compiling results from more than 100 studies in India but in agreement with **Ravindra *et al*. (2013).**

Dua *et al.* (2003) reported higher susceptiblility of cows (48.7%) against buffalo (23.935) for subclinical mastitis which is also supported by **Hussain *et al.*(1984)** and **Sharma (2003).** In our study fact was found true in case of teatwise prevalence but at animal level results reported were against these studies in case of Jhansi. In present study, 21 (46.66%) cows and 50 (62.50%) buffaloes were found positive for subclinical mastitis with CMT. Teat wise prevalence was found higher in cows as compare to buffalo. It was observed as 52.38 and 47.83 % respectively. But the fact observed is in agreement with **Sharma *et al.* (2007),** who has reported 68.60 % prevalence in case of buffalo as compared to 32.40% subclinical mastitis prevalence in cows.

Total viable count:

Total viable count of milk samples was done nutrient agar, CLED Agar, Mckonkey agar and MS media to obtain the initial information for further processing and confirmation of bacterial agents. Bacterila load, obtained in samples from different source is presented in **table-01**. Our results revealed that total viable count in milk samples from animals tested positive for subclinical mastitis ranged from $6x10^3$ to $2.14x10^5$ cfu/ml on diffrent media which was found significantly higher than counts ranged from $1.9x10^3$ to $8.22x10^4$ in non mastitic milk samples($p<0.05$) which is in agreement with **Hassan *et al.* (2015)**. Higher bacterial laod in mastitis milk samples is alos observed by **Joao *et al.* (2012)**. whi find total viable count from $1.3x10^6$ to $7.4x10^5$. Significantly higher bacterial count was observed in buffalo milk than cow milk in both mastic and non mastitic milk samples ($p<0.05$) indicating the higher susceptibility of buffalo in compraison to cow. Range of bacterial count obtained in our study is also in agreemnet with **Devi and Sowmy, (2012)** and **Perku, (2011)**. Highest bacterial count obtained on nutrient agar plates while lowest on mckonkey agar plates which indicate the lower rate but contamination with environmentol *Enterobacteriaceae* members of 3 samples but with no significat difference between mastitic and non mastitic milk samples from both buffalo and cow. No significant difference was observed in bacterial count on MS agar from normal and mastitic milk samples from buffalo milk, may indicating the higher prevalence of non mastitis causing Coagulase negative *Staphylococci*.

Table-01: Total Bacterial count on different media in cfu/ml.

Disease Status	Source	Nutrient agar	CLED Agra	McKonkey Agar	MS Agar
SCM +Ve	Buffalo	$2.14x10^5$	$9.55x10^4$	$8.4x10^3$	$1.65x10^4$
	Cow	$1.24x10^5$	$7.57x10^4$	$6.0x10^3$	$1.14x10^4$
SCM -Ve	Buffalo	$7.4x10^4$	$8.22x10^4$	$8.6x10^3$	$1.65x10^4$
	Cow	$6.5x10^4$	$5.25x10^4$	$1.9x10^3$	$4.8x10^3$

Prevalence of mastitis pathogens: Milk samples found positive for Subclinical mastitis (n=71) were further used to isolate bacterial pathogens. Results for species wise destribution with respect to animals and prevalence with respect to total isolates (82) is represented in **Table-02.** Among *Staphylococci sp*. other than *aureus*; *S. epidermidis* (20%) was most prevalent followed by *S. chromogenes, S. intermedius, S. simulans, S. hycus* and *S. haemolyticus*, with isolation rate of 10, 4,2,2,1% respectively . Higher prevalence of *S.epidermidis* and *S. chromogenes* among CNS sp. is in agreement with **Kudinnaha and Simango (2002), Waller *et al.* (2011)** later prooved by **Sandra *et al.* (2014).**

Table-02: Prevalence of bacterial agents isolated from CMT subclinical mastitis positive milk samples. (n=71)

S.No.	Bacterial isolates	No. of sample positive	Prevalence (%)
1.	*S.aureus*	12	14.63
2.	*CNS**	42	51.22
3.	*Bacillus*	11	13.41
4.	*Streptococci*	09	10.98
5.	*E.coli*	03	3.66
6.	*Others*	05	6.10

CNS=Coagulase Negative *Staphylococci; *CNS=Coagulase Negative Staphylococci *Percentage given out of total isolates=82

Coagulase negative *Staphylococci* was observed as most prevalent bacteria isolated with rate of 51.02% followed by *Staphylococcus aureaus, Streptococci sp., E. Coli* and other *Enterococci*. Prevalence of coagulase negative *Staphylococci sp.* is in agreement with **D. Cervikova *et al.* (2013)** who had been reported higher prevalence (53.5%) in samples from asymptomatic cows. Dominance of Coagulase negative *Staphylococci* followed by *S. aureus* is also established by **S.A. Makonenn *et al.* (2017)**. Frequency of isolation for *S. epidermidis* was found to be 61% followed by 20, 10, 4,2,2,1% frequency of isolation for *S. chromogenes S. intermedius, S. simulans, S. hyicus* and *S. haemolyticus*, respectively, which proved its dominance over other Staphylococci, which is also reported by **Sandra *et al.* (2014)**. CNS sp. as a whole were alos reported as

dominant mastis pathogens by in studies from Uganda by **Byarugaba *et al.* (2008)** and **Abrahmsen *et al.* (2014)**. **Bochniarz *et al.* (2013)** reported higher prevalence of *S.chromogenes* simultaneously with *S.epidermidis* while **Trinidad *et al.* (1990)** and **Mathews *et al.* (1992)** reported *S. chromogenes* and *S. simulans* as common mastic pathogens among CNS sp. These findings also support our finding if considered in combination.

Prevalence of *Streptococci* is in agreement with **Schawrz *et al.* (2010) and Botrel *et al.* (2010)** who get its prevalence between 10-20% but more close to **Pitkala *et al.* (2004)**. *Bacillus sp.* isolated from samples with prevalece rate of 13.41% indicating the unhygienic practices in management of milking animals. *Corynebacterium sp.* and *Klebsiella sp*. were found dominant bacteria among others with overall prevalence rate of 2.6% and 1.4 % respectively,which is supported by thhe study done by **Sarvanan *et al.* (2000).** Presence of *Corynebacerium sp.* may indicate the latent infections in animals as evidenced by **Mir *et al. (*2014),** can be correlated with latent mastitis pathogens in study population. Low prevalence of *E. Coli* (3.66%) is in agreement with **Kivaria *et al.* (2007)** who reported 4.1% isolates of *Coliforms* in Tanzania and may be correlated with temeperature and dry climatic conditions during most of the months in this zone, as indicated by **Hogan and Smith (2003).** *Staphylococci sp.* and *Corynebacterium sp.* were also considered as abundant in milk samples from asymptomatic cows by **Erica *et al.* (2017).**

CONCLUSION

Animals, positive for CNS sp. were found more prevalent towards development of mastitis in comparison to animals infected with other nonpathogenic bacterial sp. Study recommends the development of managerial and clinical practices based on regular monitoring of subclinical mastitis to prevent the bigger loss in live stocks. Higher frequency of CNS sp. like *S. epidermidis* as reported in higher frequency among all may also create a public health problem, if not controlled. Study recommends further observations and preparation of detailed report on association of specific pathogens responsible for subclinical mastitis in this climatiz zone.

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