The Pharma Innovation Journal 2022; SP-11(11): 831-835

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; SP-11(11): 831-835

www.thepharmajournal.com
Received: 12-09-2022
Accepted: 15-10-2022

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Prevalenve and pathological studies of sub-clinical mastitis in Bovines

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Abstract
The research work entitled "Clinico-pathological studies of subclinical mastitis in bovines" was carried out over a period from 7th March 2019 to 15th June 2019. The research was committed to study prevalence of sub-clinical mastitis in and around Bhubaneswar, efficacy of various indirect screening tests- CMT, SFMT and WST, haematological, biochemical examination and antioxidant profile tests of some suspected cases, microbiological culture of some suspected milk samples. In the present investigation, random milk samples from 380 lactating cows and 55 buffaloes were screened for subclinical mastitis through California mastitis test at cow side and further screening by SCC for a period of 2 months from areas in and around Bhubaneswar. Out of these 380 animals, 163 cows and 5 buffaloes were found to be subclinically mastitic through SCC, accounting to 42.9% prevalence. Along with these screening test, WST and SFMT was performed to determine the efficacy of the indirect screening tests. Additional details about the animal like breed, age, parity, lactation stage and milk yield was also noted. According to these data, prevalence related to the above parameters was determined. 30 random blood samples were collected along with 6 from the apparently healthy animals. All the milk samples were subjected to cytological examination and isolation and bacterial identification. Based on the Somatic cell count, quarter milk samples were classified as normal (SCC <2 $X10^5$ /ml) and sub-clinically affected (SCC>$2X10^5$/ml) milk samples. In differential leukocyte count (DLC), the neutrophil count of the SCM affected milk samples was significantly higher than that of the normal milk samples while lymphocytes and macrophages significantly decreased in SCM. Cytological examination of the milk smear by Geimsa staining revealed leukocytes, desquamated epithelial cells, fat globules and clumps of bacteria. *Staphylococcus* spp. was determined as the predominant isolate followed by *Streptococcus* spp. and *E. coli.* Blood samples were subjected to haematological examination. Biochemical examination and antioxidant profile test was carried out from serum. Haematological analysis revealed there was significant decrease in Hb, PCV, TEC and lymphocyte count but a significant increase in TLC of blood of the sub clinically affected mastitic animals. A differential count of leukocytes revealed increase in the neutrophil % and eosinophil % but decrease in lymphocyte count. Serum biochemical analysis revealed that significant increase in in the levels of AST and TP. Recommendations for appropriate therapy was given to the farmers based on the screening tests and recovery rate was determined to be 62% by further screening after 3 weeks.

Keywords: Mastitis, bovines, California mastitis test, white side test

Introduction
Mastitis is the inflammation of the mammary gland which may be accompanied by physical, pathological and bacteriological changes in milk and glandular tissue. A break in the blood-milk barrier, along with impaired synthesis and secretary activity of udder epithelial cells due to the reduced host defence mechanism occurs. This alters the level of most components like enzymes and metabolites, both in milk and blood (Sarvesha *et al*., 2016) [16]. It has been considered as the most complex and costly disease in dairy industry, occurring throughout the world. It has been broadly classified into clinical or sub-clinical with generally subclinical infection preceding clinical manifestation (Sarvesha *et al*., 2016) [16]. If proper attention and diagnosis will not be done at the stage of subclinical infection, it will ultimately lead to clinical form which is difficult to control. Subclinical mastitis is more commonly prevalent with comparatively more economic loss caused than the clinical mastitis (Halasa *et al*., 2007) [6].
Different microorganisms, often *Staphylococcus aureus*, coagulase negative staphylococci (CNS), *Streptococcus dysgalactiae*, *Streptococcus agalactiae* and *Streptococcus uberis* are common causes of both clinical and subclinical mastitis. Other than bacteria, mycoplasmas, yeasts and algae may also cause mastitis. Predisposing factors comprise of breed type, stage of lactation, managemental practices and farmers awareness.

Viguier *et al*. (2009) [21] reported that bovine mastitis is a multifactorial disease. No effective vaccine is still available in providing cross protection against all the pathogens.

In subclinical mastitis, there are no visible changes in the animals' milk, udder or general condition unlike clinical ones, which shows appreciable changes. SCM in dairy cows is important because it is 15 to 40 times more prevalent than the clinical form, is of long duration, is difficult to detect, reduces milk production and adversely affects milk quality (Schultz *et al.,* 1978) [18]. Early and accurate diagnosis can help in effective treatment. Also prudent use of antimicrobial therapies and monitoring of antimicrobial susceptibility of bacterial flora in animals can be done to reduce the development of resistant pathogens which is a major problem now a day. There is always need of regular surveillance and monitoring of mastitis. Though there are many tests and each test is having some limitations, the choice of routine testing should have reliability and rapidity.

Materials and Methods

The study was carried out at the Department of Veterinary Pathology, C.V. Sc. & A. H., O.U.A.T. over a period from 7th March to 15th June during the year 2019. Proper sanitary measures were followed during collection of milk samples. All udders, teats and hands of the milkers were washed perfectly with soap and water. 70% ethyl alcohol was used to disinfect teats and teat orifices, just before collection of samples. The first two or three jets of milk from each quarter were discarded. The next 5-10 ml was collected separately in clean sterile vials. Samples were labelled with identification no. of affected animals, study serial no. and date of collection.

California Mastitis Test

The procedure of California mastitis test described by Schalm *et al.* (1971) [17] was being followed which is considered as an important diagnostic tool for cow-side evaluation of mastitic condition. About 2 mL of milk sample was squirted in each cup of mastitis paddle, and an equal volume (2 mL) of CMT reagent was added to the cups. The reactions were developed within 10 s in positive samples and scoring was carefully done. CMT positive cows (score≥+1) were defined as having at least one positive quarter for SCM.

White Side Test

The test is based on the increase in leukocyte count in mastitis milk. The original test was described by Schalm *et al.*, (1971) [17]. For this purpose, 4% NaOH solution was prepared by mixing 4ml NaOH with 96ml distilled water. Three ml of milk sample was mixed with 3ml of NaOH solution. The gel formation or a breaking up of milk in flakes, shreds, and viscid mass was indicative of positive reaction.

Somatic cell count

Milk total somatic cell and differential leukocyte count were estimated according to general principle described by Schalm *et al*. (1971) [17]. 1ml of milk sample was taken with the help of a micro-pippete and a smear was made in glass slide with a marked area of 1cmX 1cm. It was let dry. Then it was immersed in xylene for 10mins and transferred to 90%alcohol for 5 mins. Then it was transferred into 5%methylene blue for 7-10 mins. After this the slide was dipped in 90% alcohol 2-3 times and was let dry.

Slide was observed under 100X and cells present in this 1 ml were termed somatic cell count of milk.

Cytology of milk

To study the differential count and cytology of somatic cells in milk, Giemsa staining method was used. Thin smears were prepared and air-dried and fixed in methanol for 10 mins. The smears were then stained with Giemsa working solution (1:9 in distilled water) and left as such for 30 to 45 minutes. The stained smears were washed with distilled water for 2 mins, air dried in upright position at room temperature. Then the smear was observed under oil immersion microscope for identification of different types of cells present.

Results

Prevalence

In the above investigation, samples were collected randomly from 5 districts of Odisha. A total of 380 numbers of cows and 55 buffaloes from districts like Khurda, Cuttack, Puri, Nayagarh and Ganjam (Table 1) were screened for detection of sub-clinical mastitis in the present research programme, over a period spanning from 7th March 2019 to 15th June 2019. Cows with no visible clinical signs and producing apparently normal milk were taken into account. During the study, in total 163 numbers of cows (42.9 per cent) and only 05 buffaloes were found to be positive for subclinical mastitis. During the collection of milk samples, details regarding breed, age, parity, milk yield, lactation stage etc. was recorded and accordingly the prevalence was estimated. Prevalence rate was counted on the basis of somatic cell count method. Breed wise and age wise and prevalence is given in table 2 and 3

Table 1: Sample collected from different districts of Odisha

Districts	Indigenous	CB	Total
Cuttack	58	26	84
Ganjam	80	8	88
Khurda	63	18	81
Nayagarh	40	2	42
Puri	62	23	85
Total no. of cows	303	77	380

Table 2: Breed wise prevalence of SCM in cows

Districts	Indigenous	+ve for SCM	CB	+ve for SCM	Total +ve for SCM
Cuttack	58	22	26	18	40
Ganjam	80	37	8	5	42
Khurda	63	13	18	15	28
Nayagarh	40	8	2	2	10
Puri	62	25	23	18	43
Total no. of animals	303	105	77	58	163

Table 3: Age wise prevalence of SCM in cows

Age (yrs)	Positive SCM	%
3-5	26	15.95
5-7	77	47.24
>7	60	36.81
Total	163	

Incidence based on lactation stage

During the present study, significant effect of the stage of lactation on the prevalence of subclinical mastitis can be recorded. A higher occurrence (74.84%) of subclinical mastitis was found in cows in their early stage of lactation followed by mid lactation (17.8%) and late lactation (7.3%) (Table 4).

Table 4: Lactation stage wise prevalence of SCM in cows

Lactation stage	Positive SCM	%
Early	122	74.84
Mid	29	17.8
Late	12	7.3
Total	163	

California Mastitis Test (CMT)

In the present study, CMT was utilised as the cow side test. Out of the total 380 milk samples collected, 138 milk samples showed positive reaction towards CMT, for SCM accounting for 36.31% prevalence and 84.67% sensitivity. Milk samples were scored according to the visible gel formation as 1, 2, 3, and 4.

Somatic Cell Count

Out of the 380 milk samples screened for SCM, 163 milk samples were found to be positive by Somatic cell count. In the present investigation, a significant increase (p<0.05) in the TLC of the milk samples can be seen of the affected animals.

Based on the Somatic Cell Count, quarter milk samples were classified as normal (SCC <2*10^5 /ml) and sub-clinically affected (SCC>2*10^5/ml) milk samples (Table 5)

Table 5: Mean ±SE values of milk SCC and DLC of control and sub clinically mastitic cows

Parameters	Healthy animals	Sub-clinically mastitic animals
SCC (X10^5/ml)	1.65±0.11	9.42±0.52
Neutrophil, %	3.57±0.52	52.41±1.12
Lymphocyte, %	16.23±0.78	15.45±0.42
Macrophage, %	70.15±0.25	24.26±0.58
Others	2.50±1.23	8.25±0.69

Prevalence of SCM in dairy cows screened through three different indirect tests

The present study was conducted to probe the efficacy of the screening tests of SCM - surf field mastitis test, California mastitis test, white side test and somatic cell count (Table 6 and Fig. 1).

Table 6: Efficacy of different indirect screening tests

Name of the test	Total no of samples	CB +ve for SCM	Indigenous +ve for SCM	Positive for SCM (Total)	Prevalence	Sensitivity
Surf field mastitis test (SFMT)	380	56	73	129	33.94	79.14
California mastitis test (CMT)	380	58	80	138	36.31	84.66
White side test (WST)	380	57	78	135	35.52	82.82

Microbial culture and identification

All the 163 subclinical mastitic samples were subjected to microbial isolation and identification. The percentage of bacterial isolates and its percentage obtained by microbial cultures in milk samples is given in Table 7 and Fig. 2. The predominant pathogen was *Staphylococcus* spp. (36.86%) followed by *Streptococcus* spp. (12.56%), *Staphylococcus* + *Streptococcus* (10.13%), *Staphylococcus* +*E. coli* (9.56%) and *E. Coli* (6.14%). No isolates were detected in 15 samples. The staphylococcal isolates were catalase positive, Mannitol positive, coagulase positive but oxidase negative.

Table 7: Isolated pathogen

Sl. No.	Isolates	No. of isolates	%
1.	*Staphylococcus* spp.	60	36.86
2.	*Streptococcus* spp.	20	12.56
3.	*E. coli*	10	6.14
4.	*Staphylococcus*+ *Streptococcus*	17	10.13
5.	*Staphylococcus* +*E. coli*	15	9.56
6.	Others	26	15.75
7.	No isolates	15	9.00

Fig 1: Positive CMT

Fig 2: Microbial Colony in Nutrient Agar

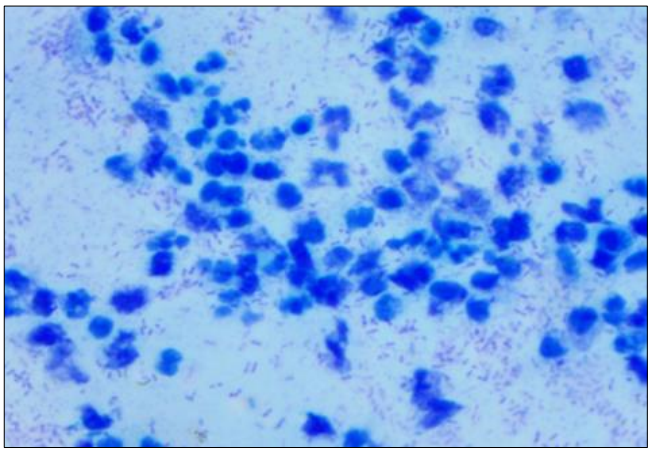

Fig 3: Methylene blue stained milk sample showing somatic cells and bacilli

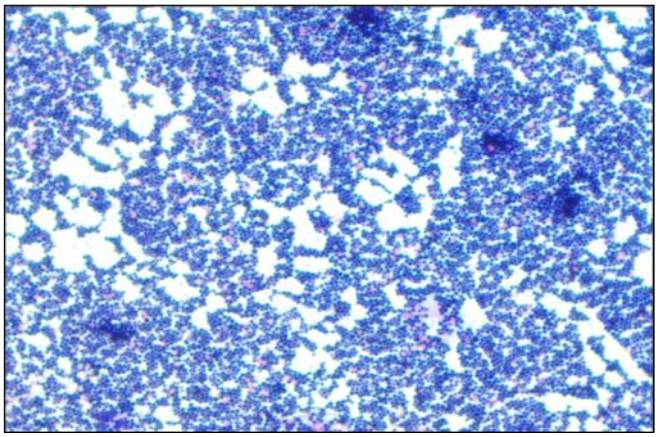

Fig 4: Photo-micrograph of gram-positive *Staphylococcus* spp.- grape bunches (Gram stain-1000X)

Follow up

After screening of milk samples for SCM, out of 163 cases which were found to be positive for SCM, were recommended for appropriate treatment and management. 62% of the cases that is 102 cases were found to be recovered and became healthy which was confirmed by laboratory test (CMT and SCC).

Discussion

Mastitis is a major endemic disease of the dairy cattle. The illness is in most respect, a very complex disease with multifactorial aetiology and predisposing factor. It mostly presents itself in a herd in subclinical form where almost no clinical signs are shown. A sudden reduction in milk production with a rise in somatic cell count in milk occurs, because of which it usually goes unnoticed. A proper diagnosis is difficult for the field veterinarians. The most common method for mastitis testing is the California mastitis test (CMT). This cow-side testing method works by disrupting the cell membranes of the somatic cells present in the milk sample and exposing the DNA of the cells to the reagent. Bacterial culture has for some time served as the gold standard for the examination of phenotypic characteristics. Phenotypic identification is based on an evaluation of morphology, growth characteristics, and ability to metabolize substrates, antimicrobial resistance, and other features that result from DNA expression

In the present investigation, out of 380 lactating dairy cows, 163 cows were found to be subclinically mastitic, accounting about 42.9% and out of 55 buffaloes, only 5 were found to be subclinically mastitic. This finding corroborates with the report of Sharma and Maity (2010) [19] according to whom bovine mastitis is 29.3-78.5% prevalent in India. Findings of the present study are corroborated by findings of Kader *et al.* (2002) who reported 46.6% SCM in Bangladesh in cows on bacteriological examination and Biswas *et al*., 2017 (51.56%). Our finding is somewhat lower than the findings of Jha *et al.* (2010) [8], Singh and Baxi (1988) [20] and Sadashiv *et al.* (2013) [13] who reported 55.0% prevalence of bovine SCM in coastal areas, 54.0% of SCM in cows in India and 61.47% prevalence respectively, but higher than the findings (20.2%) of Sarker *et al.* (2013) [15]. Species-wise highest incidence of the disease was observed in crossbred cows which implied that crossbred cows are more susceptible to the disease. Less incidence of the SCM in buffaloes might be due to the presence of thick and compact epithelium and keratin layer with thick muscle sphincter in streak canal of udder of buffaloes as compared to crossbred cows and the similar reasons were also given earlier by Saini *et al*. (1994) [14].

These differences of prevalence rates of subclinical mastitis might be due to differences of breeds of animals, geographical locations, managemental practices and the tests used for screening of the milk samples (Barua *et al*., 2014) [1].

Higher prevalence of SCM in coastal region may be associated with larger number of lactating cows in the farms, dirty floor condition, cows bathed by pouring water, dirty udder, and overall poor hygienic management which was also reported earlier by Islam *et al*. (2011) [7].

The microbial culture and identification of the 163 subclinical mastitic samples revealed that the predominant pathogen was *Staphylococcus* spp. (36.86%) followed by *Streptococcus* spp. (12.56%), *Staphylococcus* + *Streptococcus* (10.13%), *Staphylococcus* +*E. coli* (9.56%) and *E. coli* (6.14%). No isolates were detected in 15 samples. Our finding is in consonance with Rady and Sayed (2009) [11] and Das *et al*., (2018) [2]. Recently advanced studies related to genomic identification and confirmation of *S. aureus* and proteomics-based differentiation of healthy, subclinical and clinical mastitic milk caused by *S. aureus* have been conducted (Maity *et al*., 2020) [9].

The high rate of isolation of *S. aureus* may be attributed to the fact that the principal reservoirs of *S. aureus* are the skin of the mammary gland or milk of the infected mammary gland or both. Moreover, it has the capacity to penetrate into the mammary gland tissue, and produce deep-seated foci protected by a tissue barrier (Ranjan *et al.* 2010). The high frequency of staphylococcal mastitis is considered to be due to the existence of inadequate hygiene and poor animal health services. The hygiene at milking is of paramount importance in control of these infections because they are spread during the milking process (Harmon 1994 and El-Balkemy *et al*., 1997) [5]. Radositis *et al*. (2000) [12] divided the infective agents into (a) biological etiology of contagious mastitis particularly *S. agalactiae* and *S. aureus*, and (b) that of environmental mastitis e.g. *E. coli*, *Streptococcus dysgalactiae* and *Steptococcus uberis.* The 3 isolated organisms were the usual mastitis pathogens and were coincided with El-Attar *et al*. (2002) [4] and Dego and Tareke, (2003) [3] because *Staphylococci* and *Streptococci* causes around 90% of bovine mastitis (Poutrel, 1983) [10].

The failure of some pathogens to grow *in vitro* may be due to the fact that certain microorganisms (such as *Mycoplasma* spp.) require specific culture media (Ranjan *et al.*, 2010).

After screening of milk samples for SCM, out of 163 cases which were found to be positive for SCM, were recommended for appropriate treatment and management. Recovery rate was found to be 62%. This finding corroborates with the finding of Rady and Sayed (2009) [11] according to whom programs for control of subclinical mastitis may be planned around the routine examination of all lactating cows and consequently early treatment can be applied towards positive cases rapidly for preventing their conversion towards clinical form among dairy cows and for protecting the herd health, milk hygiene and consequently the consumer health. Along with this, good management practices such as milker hygiene, sanitization of milking machine and udder healthy environment as well as dry off treatment and controlling other predisposing diseases should be considered among the major prophylactic measures to minimize the occurrence of the disease.

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