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Isolation and identification of some most important bacterial pathogens from sub clinical mastitis of bovine in Sirohi, Rajasthan

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Abstract
The major Objective of this study was focused on using of rapid & efficient methods for detection of Subclinical Mastitis and prevalence of Some Most Important Bacterial Pathogens in Sirohi District of Rajasthan. A total of 200 milk samples from Bovine i.e., Cow and Buffaloes were collected from period of July 2020 to December 2020 to detect and determine pathogens responsible for subclinical mastitis in Bovine. Screening Subclinical Mastitis (SCM) were done using modified California mastitis Test (MCMT) & and Somatic Cell Count (SCC). The prevalence percentage of SCM in California mastitis and Somatic Cell Count in age group of 5 to 8 years Bovines was (74% and 70%) respectively. Identification of the isolates was achieved using Gram's staining, hemolytic pattern, colony morphology, Catalase, Coagulase test, IMVIC test and confirmation of bacteria species was done by growth on specific agar medium. Bacteriological examination of all milk samples found the presence of (48.00%) isolates where *Staphylococcus* was predominant species (27.00%) followed by *Streptococcus agalactia* (10.5%), other streptococci species (4.5%), while environmental pathogen represented *E. coli* third common pathogen was found in least presence (6.5%). Subclinical mastitis seems to be, as deduced from the high prevalence observed in this study, an important health problem for milking dairy cows and buffaloes in Sirohi. The Identification and biochemical identification is still the important aspect and could be practiced at selected samples from time to time to confirm identification of causative organisms.

Keywords: isolation, identification, bacterial, pathogens, sub clinical mastitis

Introduction
The subclinical mastitis (SCM) is a much serious problem and responsible for much greater monetary loss to the livestock holders. Greater than three times losses due to SCM, as compared to clinical mastitis is estimated. In this form of mastitis as milk appears normal and visible abnormalities such as udder swelling, hardness of the affected quarter, pain, and watery milk remains not visible, which can be identified only in a laboratory or field test, and mostly remains unnoticed by the farmer but physical and chemical changes occur in the milk. Seasonality influenced milk composition both in mammary quarters and composite milk samples. Somatic cell count levels affected milk composition in both mammary quarters as well as composite samples of milk (Dos Reis *et al.*, 2016).

Depending upon the climatic condition, animal species and disease management practices, etiological agents may vary place to place and case to case. Thus, the control and prevention of sub clinical mastitis is a challenge and despite of the continuous efforts, is still causing severe economic losses to dairy farmers and thus industry. Earliest detection of mastitis with low cost and rapid screenings in field level, hygienic farm management, biosecurity and awareness building among farmers will be asset to control the clinical and SCM of dairy cows. (Kabir, *et al.* 2017) [24].

Sub-clinical mastitis remains to be an obscure and latent form of this disease that poses more serious economic concern to the dairy livestock sector, as the incidence is much higher in a dairy herd than the clinical one (Shaheen *et al.*, 2016) [42]. The cost of subclinical mastitis is exceedingly difficult to quantify, but most experts agree that subclinical mastitis costs the average dairy farmer more than does clinical mastitis. The animal wise (6.67 per cent) and quarter wise (3.67 per cent) incidence was found more in cows as compared to buffaloes (Sawmi *et al.*, (2017) [45].

Mastitis, an inflammation of the mammary gland that occurs primarily in response to intra mammary infections of mostly bacteria, mycoplasma, fungi, virus, or algal.

Beside this mechanical trauma, chemical, and thermal trauma are also predisposing factor of intra mammary infection (IMI). Occurrence of mastitis depends on the interaction of host, agent, and environmental factors etc. It is much important to identify the mastitis causing pathogens to find accurate and selective treatment with suitable antibiotics, understand their route of spread and evaluate the contagiousness of the case, estimate their public health impact, to evaluate the prognosis of the affected quarter/animal considering early culling decision, select the suitable hygienic and preventive measures (El-Sayed, *et al.,* 2017) [17]. The major mastitis producing organisms are *Staphylococcus aureus, Streptococcus agalactiae*, and *Streptococcus dysgalactiae, Streptococcus uberis* and *Escherichia coli.* (Levison *et al.*,2018). Due to the multifactorial etiology and the risk of antibiotic resistance, the best method of mastitis treatment is to accurately identify the causative pathogen, which typically has been carried out by microbiological culture, still a standard diagnostic tool. However, because cultures of mastitis milk samples may not always result in bacterial growth, an increasing number of studies have shown the potential of molecular techniques to improve the diagnosis of mastitis, with high sensitivity and specificity (Lima *et al.* 2018) [32].

Staphylococci are the most isolated bacteria from milk of dairy cows (Wald *et al.,* 2019). In routine mastitis diagnosis, staphylococci are usually divided either into coagulase-negative (CNS)/non-aureus staphylococci (NAS) and coagulase-positive staphylococci/*Staphylococcus aureus*. CNS is a heterogeneous group with more than 15 species having been isolated in association with bovine mastitis. (Parth *et al.,* 2016) [37] suggested that phenotypic and genotypic properties of organisms might help to understand the distribution of prevalent *S. aureus* clone among bovine mastitis isolates to control *Staphylococcus aureus* infections in dairy herds. The prevalence of *Staphylococcus* species might be due to the incomplete milking, painful lesions, or any wounds on the outer surface of the udder. There are three major streptococci producing mastitis: *Strept. agalactiae*, *Strept. dysgalactiae* and *Strept. Uberis. Streptococcus agalactiae* and *Streptococcus dysgalactiae* are strict udder pathogens and contagious. Environmental mastitis is caused by bacteria that are transferred from the environment to the animal, rather than from other infected quarters. *Escherichia coli (E. coli)* and *Klebsiella* spp are coliforms that can cause mastitis. *Escherichia coli* are described as one of the most frequently isolated causative agents associated with bovine clinical mastitis. (Bradley *et al.,* 2007) [12]. Because *Escherichia coli* is ubiquitous in the environment, there are multiple opportunities for it to invade the udders via the teat canal. *E. coli* most frequently induce acute CM, often of serious character with a rapid progress and sometimes with a fatal outcome.

Identification of a bacterial pathogen in milk from a cow with mastitis is regarded as the definitive diagnosis of an Intra-mammary (IM) infection. Identification of mastitis pathogens is generally performed by traditional culture method followed by the biochemical tests on bacterial isolates (Oliver *et al.,* 2004) [35].

Material and Method

Sampling and general microbiological analysis

About two hundred pooled random milk samples were collected under aseptic conditions from domesticated dairy Cattle and buffaloes (5–8-year age group) from organized and unorganized dairy farms of four different tehsil of Sirohi district of Southern Rajasthan. The screening for SCM will be conducted by modified California mastitis test (Kandeel *et al.,* 2018) [25] and Somatic cell count (Lactoscan milk analyzer Belgium). Details of the milk samples collected are presented in Table 1.

Table 1: Details of the milk samples collected

Area	Sub clinical mastitis		
	No. of Samples	Cow	Buffalo
Sirohi	50	25	25
Revdar	50	25	25
Sheoganj	50	25	25
Pindwara	50	25	25
Total	200	100	100

California Mastitis Test (CMT)

The CMT was performed and interpreted as described by Kandeel *et al.,* (2018) [25]. Briefy, 2 mL of fresh foremilk sample from each quarter was placed in the appropriate chamber of the CMT plastic paddle and mixed with 2 mL of CMT reagent at ambient temperature by gently moving the paddle in a circular motion. A change in viscosity indicated an increase in quarter SCC, with the CMT reaction being visually scored at 45 seconds after adding the reagent. A 5-point scale was used to measure the score of viscosity as follows: negative, mixture remains liquid with no evidence of formation of precipitate; trace, a slight precipitate evident which tends to disappear with continued movement of the paddle; CMT +, a distinct precipitate but no tendency toward gel formation; CMT ++, the mixture thickens immediately with some gel formation, and with motion, the mixtures tend to move in toward the centre leaving the bottom of the outer edge of the cup exposed, and out again covering the bottom of the cup if the motion stopped; CMT +++, a distinct gel forms which tends to adhere to the bottom of the paddle and a distinct central peak forms during swirling.

Screening for Subclinical Mastitis by Estimation of Somatic Cell Count

The udders were tested for subclinical mastitis using Modified California Mastitis Test (MCMT) and only those samples found positive for sub clinical mastitis were used in the further study. Somatic cell count was measured by Lactoscan milk analyzer (Belgium) as per the technique prescribed by Manufacturer brochure. In order to count the somatic cell with Lactoscan SCC, the milk sample is mixed with the dying reagent, containing fluorescent dye Sofia Green. Only 12 µL from the dyed sample is pipetted on the measuring chamber of disposable LACTOCHIP. The chip is loaded into the device and for a period between few seconds and 2 minutes, depending on the measuring mode, the analysis is done. Lactoscan SCC system focuses automatically on the chip and the dyed cells are captured by the sensitive CCD camera. The analysis algorithm of digital images determines the number and dimension of the fluorescent cells and counts their concentration. The SCC value >5,00,000 cells/mL (Hegde *et al.,* 2013) [21] of milk was taken as criteria to declare the milk / animal as subclinically mastitic/infected and such milk samples were subjected to cultural isolation.

Isolation and biochemical characterization

A total of 74 milk samples based on CMT and SCC were

subjected for bacteriological examination. All the milk samples having SCC more than 5, 00,000 cells / mL were subjected for isolation.

For isolation of Staphylococci, 0.1 mL of milk sample was initially enriched in Brain heart infusion broth for 6 hrs. at 37 ºC and then streaked onto Mannitol salt agar and incubated at 370 C for 24 hr. After reading the colony morphology, the colonies were further streaked onto BHI agar for further identification procedures.

For isolation of Streptococci, 0.1 mL of milk sample was initially enriched in *Streptococcus* Selection Broth, with 5-10 per cent CO2 tension for 6 hours (hr.) at 37 ºC and then streaked onto Blood agar plates, incubated at 37 ºC for 48 hr. After reading the hemolysis pattern and colony morphology, the colonies were again streaked onto blood agar plates and incubated further at 37 ºC for 48 hr to obtain pure culture. These pure cultures were then streaked onto BHI agar for further identification procedures.

For isolation of *Escherichia coli*, 0.1 mL of milk sample was initially enriched in Tryptone phosphate broth for 18 hr. at 37 ºC and then streaked onto MacConkey agar and incubated at 37 ºC for 24 hr. The lactose fermenting colonies were further streaked onto EMB agar and incubated at 37 ºC for 24 hr. The metallic sheen colonies were streaked onto BHI agar for further identification procedures.

Identification of Bacterial isolates

Staphylococci

Pure cultures of isolates were subjected for Gram staining and further by catalase test. The catalase positive cultures were streaked onto nutrient agar slants and preserved at 4ºC. From these slants the pure cultures were subjected for various biochemical tests as per standard procedures (Barrow and Feltham, 1993; FDA bacteriological analytical manual, 2001; Collee *et al*., 2008) [8, 18, 13].

Gram staining

Gram's staining was performed as per procedures described by Hucker and Cohn (1923) [22] to determine the size, shape and arrangement of bacteria. A loop full of an overnight culture was air-dried and heat fixed on a glass slide. Crystal violet stain (0.3% w/v) was added and allowed to stand for one minute. Excess stain was washed off with a gentle stream of water. Then Grams iodine (0.4% w/v) was added and allowed to stand for 30 sec. before being rinsed off. The smear was drained with ethanol (95% v/v) and then stained with the secondary stain, safranin (0.4% v/v), for one minute. This was then washed with water for 5 sec. The smear was examined for the gram reaction of organisms and then for size, shape and arrangement. The stained slide was examined under a microscope and organisms were classified into two groups as Gram positive and negative. The Gram-positive cocci in chain morphology were presumptively considered as *Staphylococcus* and were subjected to biochemical tests.

Biochemical tests for *Staphylococcus aureus*

Catalase test

A drop of 3% (v/v) hydrogen peroxide was mixed with a loop of pure colony on a slide. Presence of effervescence, caused by the liberation of free oxygen as gas bubbles, indicated the presence of catalase and was considered positive test (AOAC, 1995) [6].

Oxidase test

A loop full of bacterial growth with the help of sterile platinum loop was rubbed on a moistened oxidase disc (Hi Media Lab. Pvt. Ltd., Mumbai). Development of deep purple blue or mauve color within 10 seconds was considered as positive and no change in color was taken as negative reaction.

Coagulase production test

The coagulase production by *Staphylococcus* species was detected using tube coagulase test. All the isolates to be tested for coagulase production were incubated overnight in BHI broth at 37 °C. The tube coagulase test was performed by adding 0.1 ml of the overnight BHI broth culture to 0.5 ml of 1: 5 diluted rabbit plasma in a small test tube. After gentle mixing, the tubes were incubated at 37 °C and examined after 1, 3, 6 and 24 hours. Simultaneously negative control was maintained to interpret the results. The test was considered positive showing any degree of clot formation, often the plasma converted into stiff gel that remained in place when tube was tilted or inverted but sometimes clots were also seen floating in the field. Whereas plasma remained wholly liquid or showing only a flocculent or ropy precipitate was considered as negative.

Oxidation-Fermentation (O-F) test

The bacterial isolates were stab Inoculated in a pair of test tubes containing Hugh and Leifson O-F media one of which was sealed with 1-2 mm thick layer of sterilized paraffin wax to provide anaerobic condition and the other was left unsealed. The tubes were inoculated and incubated at 37 °C for 24 hrs. if both the inoculated tubes had changed from bluish green to yellow, the bacterial isolate was considered as fermentative, if only unsealed tube turned yellow, the bacterial isolate was considered as oxidative.

Mannitol Salt Agar

Mannitol salt agar plates were streaked with the test culture and incubated for 24-48 hours at 37 °C. The mannitol fermenting the organisms changed the colour of medium to yellow, whereas, non-fermenting organisms did not change the colour of medium.

Streptococci

Pure cultures of isolates were subjected for catalase test. Further, catalase negative cultures were streaked onto nutrient agar slants and preserved at 4 ºC. From these slants, the pure cultures were subjected for various biochemical tests as per standard procedures (Barrow and Feltham, 1993; FDA bacteriological analytical manual, 2001; Collee *et al*., 2008) [8, 18, 13].

Hemolysis pattern

On 5 per cent sheep blood agar plates, a greenish discoloration around the colonies was considered as α hemolysis and a complete zone of clearance was considered as β hemolysis.

Gram staining

Gram's staining was performed as per procedures described by Hucker and Cohn (1923) [22] to determine the size, shape and arrangement of bacteria. A loop full of an overnight culture was air-dried and heat fixed on a glass slide. Crystal violet stain (0.3% w/v) was added and allowed to stand for one minute. Excess stain was washed off with a gentle stream of water. Then Grams iodine (0.4% w/v) was added and

allowed to stand for 30 sec. before being rinsed off. The smear was drained with ethanol (95% v/v) and then stained with the secondary stain, safranin (0.4% v/v), for one minute. This was then washed with water for 5 sec. The smear was examined for the gram reaction of organisms and then for size, shape and arrangement. The stained slide was examined under a microscope and organisms were classified into two groups as Gram positive and negative. The Gram-positive cocci in chain morphology were presumptively considered as considered as *Streptococcus* species and were subjected to biochemical tests.

Biochemical tests for *Streptococcus*

Catalase test

A drop of 3% (v/v) hydrogen peroxide was mixed with a loop of pure colony on a slide. Presence of effervescence, caused by the liberation of free oxygen as gas bubbles, indicated the presence of catalase and was considered positive test (AOAC, 1995) [6].

Oxidase test

A loop full of bacterial growth with the help of sterile platinum loop was rubbed on a moistened oxidase disc (HiMedia Lab. Pvt. Ltd., Mumbai). Development of deep purple blue or mauve color within 10 seconds was considered as positive and no change in color was taken as negative reaction.

Esculin Hydrolysis test

The cultures were inoculated onto modified bile esculin azide agar plates and incubated for 24 hr. at 37 ºC. The development of black color around the colonies considered positive. No change in color was considered negative.

Christie, Atkins, Munch-Petersen (CAMP) test

All the presumptive streptococci isolates were tested by CAMP test as per the method of Sandholm *et al.*, (1995) [41] with some modifications. Briefly, the standard (ATCC-43300) strain of *S. aureus* was grown overnight on 5 per cent sheep blood agar (SBA) plates at 37 ºC and their colonies were again streaked onto freshly prepared SBA plates containing 5 per cent sheep blood. β haemolysin producing S. aureus was inoculated onto an SBA plate by making a narrow streak down the center of the plate with a platinum loop. The streptococci isolates were streaked at 90o angle and 3 mm apart not touching the S. aureus streak, before incubating the plate at 37 ºC for 24 h. A positive result was indicated by an "arrowhead"-shaped enhanced zone of β -hemolysis.

Growth on Edward's Media

The cultures were directly inoculated on the surface of the medium plate.

Escherichia coli

Pure cultures of isolates were streaked onto nutrient agar slants and preserved at 4oC. From these slants, the pure cultures were subjected for various biochemical tests as per standard procedures (Barrow and Feltham, 1993; FDA bacteriological analytical manual, 2001; Collee *et al*., 2008) [8, 18, 13].

Gram staining

Gram's staining was performed as per procedures described by Hucker and Cohn (1923) [22] to determine the size, shape and arrangement of bacteria. A loop full of an overnight culture was air-dried and heat fixed on a glass slide. Crystal violet stain (0.3% w/v) was added and allowed to stand for one minute. Excess stain was washed off with a gentle stream of water. Then Grams iodine (0.4% w/v) was added and allowed to stand for 30 sec. before being rinsed off. The smear was drained with ethanol (95% v/v) and then stained with the secondary stain, safranin (0.4% v/v), for one minute. This was then washed with water for 5 sec. The smear was examined for the gram reaction of organisms and then for size, shape and arrangement. The stained slide was examined under a microscope and organisms were classified into two groups as Gram positive and negative. The gram-negative pink color bacilli were presumptively considered as *E. coli* and subjected to further testing.

Biochemical tests for *Escherichia coli*

Indole test

Few drops of xylene were added into two-day old growth of the test isolates grown in two ml of tryptone water. It was than mixed thoroughly to dissolve indole. About 0.2 ml of Kovac's reagent was added from the side of the tube. Development of pink layer of xylene was considered as indole positive reaction.

Methyl red (MR) test

Five to six drops of MR reagent were added to a two-day old growth of the isolate in five ml of glucose-phosphate peptone water (GPW). Development of a pink or bright red colour was considered to be positive.

Voges Proskauer test

Three ml of five per cent solution of α- naphthol in absolute ethanol and one ml of 40 per cent KOH were added to the growth of the test isolates in five ml of GPW. Development of a pink colour and later crimson red in the mixture was indicative of a positive test. If the colour remained yellow, then it was considered as negative.

Citrate test

Slant of Simmon's citrate agar (Hi media Lab.) was inoculated with each test culture and incubated at 37 °C for 2 days. Growth with a development of blue color of the medium was considered as a positive reaction.

Triple sugar iron (TSI) test

TSI medium was prepared, and the test culture was inoculated initially in the butt and then on the slant with the use of a straight wire. It was incubated at 37oC for overnight and the color of butt and slant was recorded along with presence or absence of gas and H2S to interpret the result.

Results & Discussion

The present study was carried out with an objective to find out prevalence of bovine subclinical mastitis, isolate most important bacterial pathogens (*Staphylococcus aureus*, streptococci species and *Escherichia coli*). The results obtained during the period of study are documented under the following subheadings. A total of 200 bovine milk samples were collected from four different tehsil of Sirohi district of Southern Rajasthan for evaluation of status for subclinical mastitis from apparently healthy lactating bovines.

Screening of the milk samples by CMT

A total of 200 milk samples collected from cows and buffaloes from Sirohi district of Southern Rajasthan were subjected to California Mastitis Test (CMT) for screening for subclinical bovine mastitis. Among the 200-milk sample, CMT was found to be positive in 45% (n=90/200) samples. (Birhanu *et al.*, 2017) [10] examined 262 cows and found 105 (40.1%) positive for sub-clinical mastitis using CMT.

Table 3: Distribution of CMT scores of milk samples collected

S. No.	Observation scale	No of sample (Buffalo)	No of sample (Cow)	Overall sample
1	Negative	40	37	77
2	Trace	21	12	33
3	CMT +	10	08	18
4	CMT ++	11	09	20
5	CMT +++	18	34	52
6	Total	100	100	200

Table 4: Prevalence of sub clinical mastitis by California Mastitis Test (CMT)

S. No	Animal	No of positive sample	Prevalence	
1	Buffalo	39	39/100	(39%)
2	Cow	51	51/100	(51%)
3	Overall	90	90/200	(45%)

Screening of milk samples by SCC and incidence of sub clinical mastitis

Somatic cells are indicators of both resistance and susceptibility of animal to mastitis and can be used to monitor the level or occurrence of subclinical mastitis in herds or individual animal. To study the status and incidence of sub clinical mastitis Hamann (2002) [19] reported cytological examination i.e., milk somatic cell count (SCC) as gold standard to measure inflammation. Hegde *et al.*, (2013) [21] studied the incidence of subclinical mastitis and prevalence of major mastitis pathogens in organized farms and unorganized sectors in Karnataka state of India. Javia *et al., (2018)* [23] studied the bacteriological studies and molecular detection of major pathogens from subclinical and clinical bovine mastitis. They considered more than 5×10^5/mL somatic cell count as positive case for subclinical mastitis. The same criteria were considered during these present investigations and a total of 200 milk samples were collected from apparently healthy lactating bovines. Out of these 200 milk samples, 100 milk samples were collected from cows and 100 milk samples were collected from buffaloes. The collected milk samples were subjected to measurement of SCC for evaluation of subclinical mastitis.

According to the result of SCC, milk samples were grouped into six different groups viz., 0-1 lakh, 1-2 lakh, 2-3 lakh, 3-4 lakh, 4-5 lakh and >5 lakh cells/mL Overall 20 milk samples showed SCC value of < 1 lakh cells/mL, 22 showed 1-2 lakhs cells/mL, 35 showed 2-3 lakhs cells/mL, 28 showed 3-4 lakhs cells/mL, 21 showed 4-5 lakhs cells/mL and 74 milks samples showed SCC > 5 lakhs cells/mL SCC values of cows and buffaloes milks samples given separately. A result of the SCC of 200 milk samples indicated SCM at 37% since, 74 out of 200 samples were positive for SCM.

These findings are in agreement with the Hegde *et. al.*, (2013) [21] and Nithinprabhu (2010) [34] who reported 45 per cent and 47 percent SCM respectively in bovine. The similar results were also observed by the Javia *et. al.*, (2018) [23] in this study 34.29% prevalence of SCM was observed in bovine by measurement of SCC. Sharma *et al.*, (2012) [43] also evaluated the prevalence of SCM by somatic cell count with criteria of 5, 00,000 per mL of milk and observed only 15.38% of cow's positive for SCM which is quite lower than the present finding. Das *et al.*, (2018) [14] also studied the status of mastitis with criteria of SCC of > 2 lakhs cells/mL and reported 46.63% (485/1040) of milk samples were having but no visible clinical signs of mastitis.In present study, the overall prevalence of SCM was lower in buffaloes as compared to the cows. As (48/100) 48% cow milk sample and (26/100) 26% buffalo milk sample were positive for subclinical mastitis. This observation agreed with the findings of Swami *et al.*, (2017) [45] which showed that 35% cows, and 28.33% buffaloes were suffering from subclinical mastitis and pointed that the reason behind this lower prevalence in buffalo as compared to cow might be attributed to the tighter teat sphincter of buffaloes as compared to that of cow.

It was evident from present study that (samples have no changes in physical quality) milk samples have higher incidence of subclinical mastitis based on somatic cell count as 74 out of 200 milk samples (37%) showed SCC more than > 5 lakhs cells/mL .This elevated somatic cell count in subclinical mastitis milk samples was in agreement with the findings of *Hegde et al.*, (2013) [21] and Javia *et al.*, (2018) [23]. High SCM prevalence in dairy herds might be attributed to poor housing and bedding materials, poor hygienic condition, previous history of mastitis, bad milking practice and contaminated milking machines (Rahulraj *et al.*, 2018, Ahmed *et al.*, 2018, Abebe *et al*., 2016, Zecconi *et al.*, 2003,) [3, 1, 48] Therefore, the whole farming and housing systems and udder health management practices inside dairy farms should be improved to minimize the burden of SCM.

Table 5: showing the value of SCC and incidence of subclinical mastitis in bovine.

SCC Value	Buffalo		Cow		Overall	
	No. of Samples (n)	Percentage%	No. of Samples (n)	Percentage%	No. of Samples (n)	Percentage%
0 - 1 Lakh cell/mL	08	08%	12	12%	20	10%
1 - 2 Lakh cell/mL	14	14%	08	08%	22	11%
2 - 3 Lakh cell/mL	21	21%	14	14%	35	17.5%
3 - 4 Lakh cell/mL	18	18%	10	10%	28	14%
4 - 5 Lakh cell/mL	13	13%	08	08%	21	10.5%
> 5 Lakh cell/mL	26	26%	48	48%	74	37%
Total Sample	100		100		200	

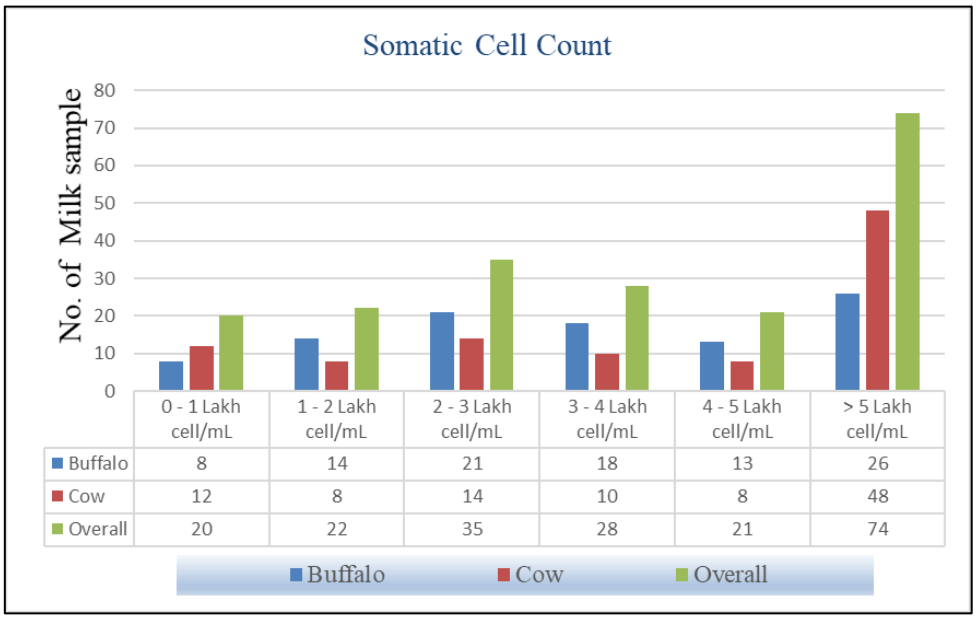

Fig 1: Distribution of milk samples according to SCC value of apparently healthy bovine milk samples

Isolation and Characterization of Most Prevalent Microorganism in Sub Clinical Mastitis

The current study was carried out as per methodologies described for isolation, identification, and biochemical characterization of microorganism previously and showed agreement with all previous studies especially with relation to intra mammary infection caused by major bacterial pathogens. In the present study out of 200 milk samples 74 milk samples which showed SCC value > 5 lakh cells / mL were cultured for primary isolation of predominant *Staphylococcus aureus*, Streptococci, and *E. coli* found positive for presence of bacteria. Out of these 74 positive samples for SCC, 72 samples had bacterial growth and while in 02 samples there was absence of bacterial growth. Out of the 72 samples that showed bacterial colonies, only 40 had single bacterial growth whereas rest of the 32 samples had mixed growth. A total of 97 isolates were recovered from these milk samples. The prevalence of mastitis caused by *Staphylococcus aureus*, (54/200, 27%), *Streptococcus* spp. (30/200, 15%), and *E. coli* (13/200, 6.5%) respectively either as single and or as mixed infections. A total of 97 isolates were recovered from these milk samples. The similar results were also reported by Lakshmi & Jayavardhanan (2016) [30] which found 36% *Staphylococcus aureus*, and 27% *E. coli*. Omar, & Mat-Kamir, (2018) [36] find *Staphylococcus* spp. (73.2%). Coagulase negative staphylococci encompassing 68.3% of the isolates, whereas 4.9% was coagulase positive staphylococci. Similarly, Sztachanska *et al.*, (2016) [46] reporting 31.6% Coagulase negative staphylococci, 15.6% *Streptococcus (Str.) agalactiae*, 12.1% *Staphylococcus aureus* from subclinical mastitis.

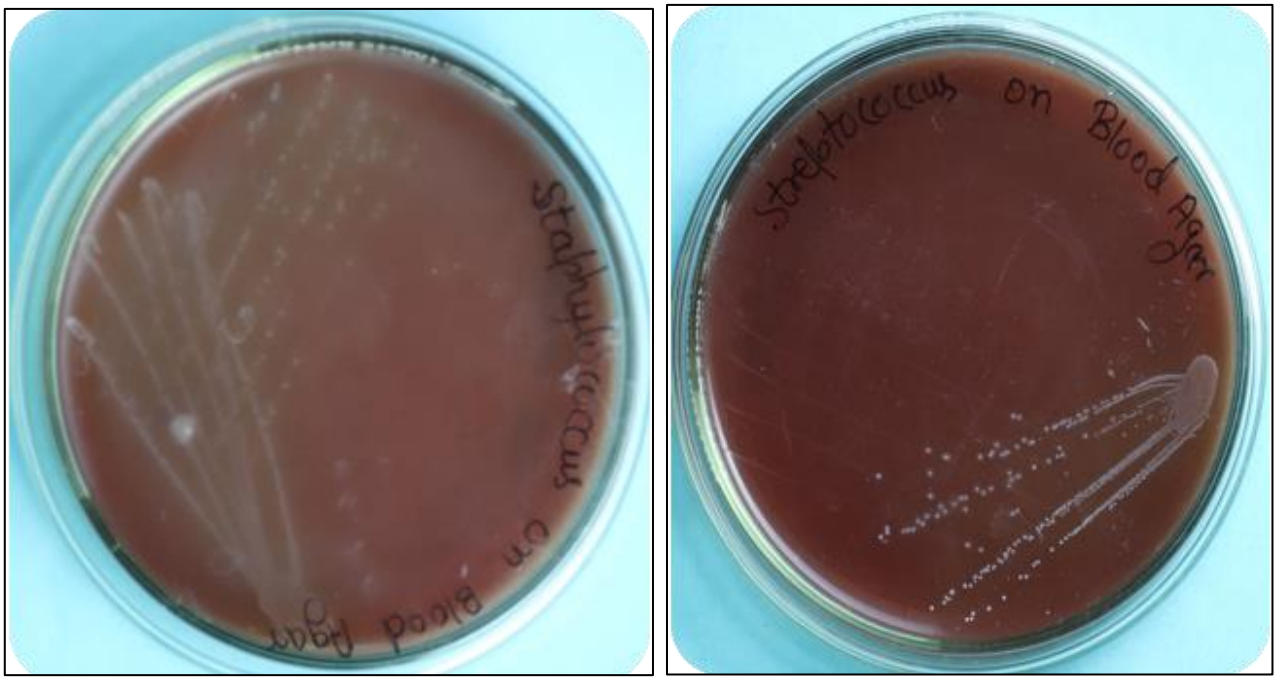

Plate 1: Blood Agar Plate of 1. *Staphylococcus aureus* and 2. *Streptococcus*

Plate 2: Mannitol Salt Agar 1. *Staphylococcus aureus* and EMB agr 2. *E. coli*

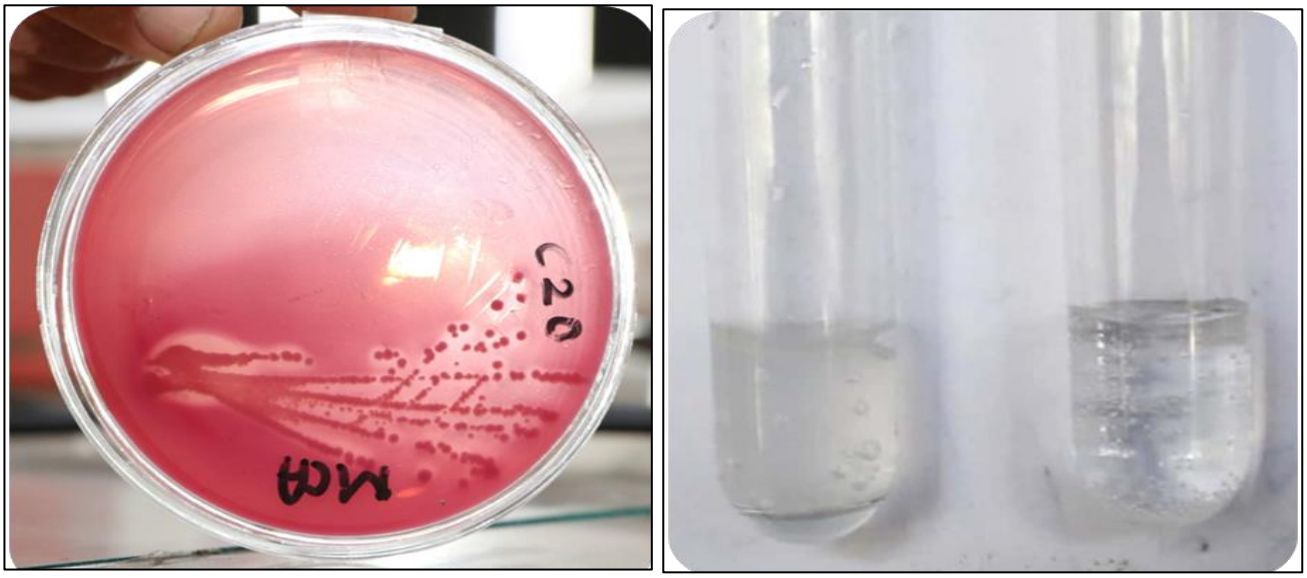

Plate 3: MacConKey agar 1. *E. coli* and Coagulase Test 2. *Staphylococcus aureus*

Plate 4: O-F Test - *Staphylococcus aureus* and IMVic Test -2 *E. coli*

Table 6: Details of the bacterial species isolated from subclinical mastitis

S. No.	Bacterial species	Total no of milk samples screen	No. of isolates	Prevalence (%)
1	*Staphylococcus aureus*	200	54	27%
2	*Streptococcus agalactiae*		21	10.5%
3	Other *Streptococcus* spp.		09	4.5%
4	*E. coli*		13	6.5%

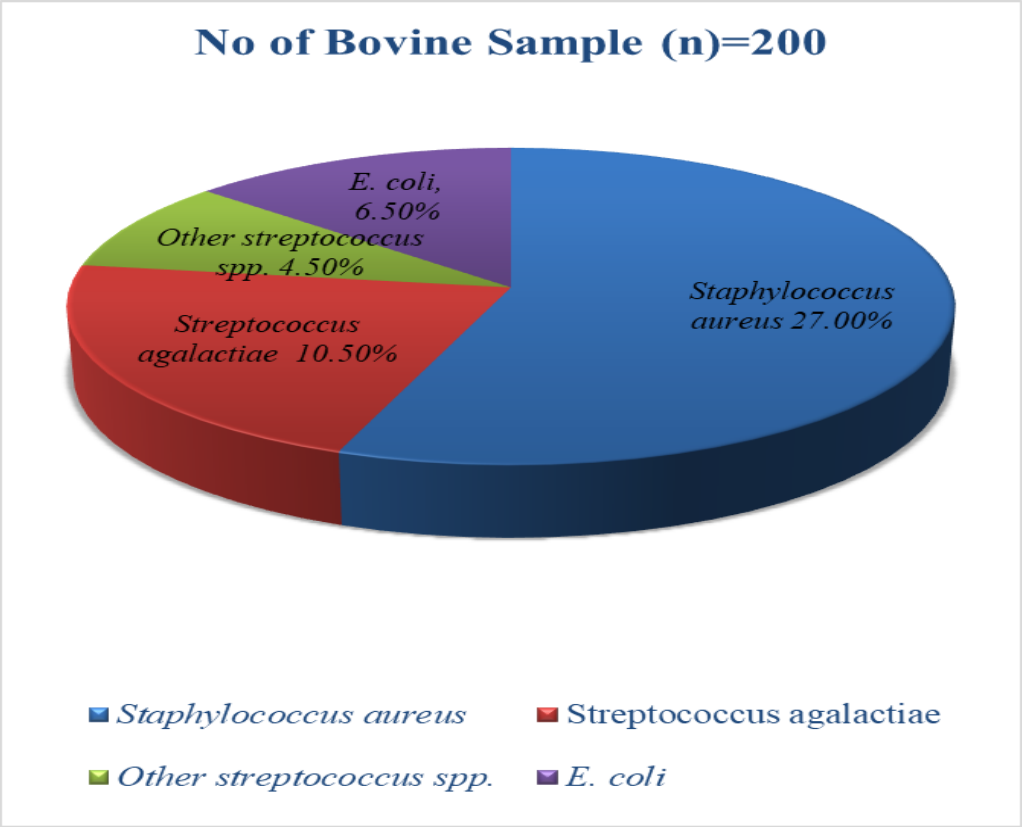

Fig 2: Major bacterial etiological prevalence of bovine sub clinical mastitis

The high prevalence of *Staphylococcus aureus.* in the bovine milk sample can be attributed to the ubiquitous nature of this organism and might be due to transmission through the use of contaminated milking machines and utensils and contaminated milkers' hands. (Algammal *et al.*, 2020, Bihon *et al.*, 2019, Abed *et al.*, 2018) [5, 9, 2]. *S. aureus* also can evade and influence the bovine immune system through the production of various enzymes and toxins that cause damage to the mammary tissue and allow more tissue invasion (Abed *et al.*, 2018) [2] Furthermore, *S. aureus* can survive on the skin and keratin layer of the teat canal of healthy cows, and can confront phagocytosis (Alekish *et al.*, 2013) [4].

This highlights the importance of hygiene and managemental practices inside dairy farms or farmers. Moreover, it would be a serious hazard for public health because that mastitic milk is usually further added into a bulk milk tank, especially in populations where some people could consume raw milk or non-heat-treated dairy products like yogurt or cheese (Awad *et al.*, 2017) [7].

The prevalence of mastitis caused by *S. agalactiae*, and other *Streptococcus* spp was found (10.50%) and (4.50%) respectively. Our results showed a clear overall predominance of *S. agalactiae* among *Streptococcus* species. This finding endorse the results reported by Klimiene *et al.*, (2005) [28] and Kivaria and Noordhuizen (2007) [27] who isolated *S. agalactiae* with an incidence of 15.1% and 15.4%, respectively. The high prevalence of *S. agalactiae* may indicate the poor management for the investigated cows. While higher incidences of *S. agalactiae* isolated from mastitic cows were recovered by Kuzma and Malinowski (2001) [29], Khan and Mohammad (2005) [26], Borkowoska *et al.*, (2006) [11], Momtaz *et al.*, (2012) [33] and El-Jakee *et al.*, (2013) [16] with isolation rate of 41.2%, 30%, 84.8%, 16%, 19.3%, respectively.

These studies in general indicated the high prevalence of *S. agalactiae* among Streptococci isolated from bovine mastitis cases based on their biochemical properties which is in line with the finding of present study. Furthermore, *S. agalactiae* were the most frequent isolates for bovine SCM among *Streptococcus* species, and many previous reports supported this study (Alekish *et al.*,2013, Sztachanska *et al.*,2016) [4, 46]. *S. agalactiae* is a highly contagious pathogen causing bovine SCM that can survive for a long period within the udder of cows and can be transmitted to healthy cows via poor milking hygiene, contaminated milking machine, utensils and contaminated milkers' hands (Hande *et al.*,2015) [20]. Therefore, the hygiene of the dairy farms should be improved to prevent and control SCM. On the other hand, *S. uberis* and *S. dysgalactiae* were considered environmental pathogen, and their main source is the bedding material (Hande *et al.*, 2015) [20]. Therefore, clean pastures and dry environments, dry milking machines and utensils, and optimum hygiene should be maintained inside dairy farms to decrease such pathogens' persistence.

The presence of coliform bacteria, such as *E. coli* is a common indicator of fecal contamination. Environmental

pathogens especially *E. coli* may range in severity from fatal per acute cases to chronic and subclinical infections. *E. coli* is the most prevalent organisms involved in coliform mastitis. These microorganisms are widely disseminated in the environment of the dairy cow especially in bedding material, manure, and water. It is a multifactorial disease for which no program of prevention or control has proved to be entirely successful. The prevalence of *E. coli* was found (13/ 97), 13.40% in present study. The prevalence of *E. coli* as a major pathogen along with *Streptococcus* and *Staphylococcus aureus* has been reported by several researchers (Rajeev, 2006) [40], (Botrel *et al.,* 2010), (Patnaik *et al.,* 2014) [38], (Singh *et al.,* 2016) [44]. The prevalence reported by earlier work ranged from 13 to 25%.

Conclusion

Staphylococcus aureus is predominant followed by *Streptococcus aglactiae* and *E. coli* which are identified as major and most important SCM causing pathogens. The mastitis causing pathogens are *staph. aureus, Streptococcus aglactiae, E. coli*. The MCMT and SCC is still the most reliable cow side test for preliminary identification of sub clinical mastitis. Sirohi District being mostly the tribal area needs awareness among farmers about the disease and loss encumber to them.

Conflict of Interest

There is no conflict of interest

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