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Cow Side and Laboratory Tests for Diagnosis of Subclinical Mastitis in Cows

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A B S T R A C T

A total of 492 quarter milk samples from 123 animals were screened with CMT and among these, 366 quarter milk samples were negative for CMT reaction whereas, 40, 54 and 32 quarter milk samples showed +, ++ and +++ grades of CMT reactions with 74.39%, 8.13%, 10.98% and 6.50%, respectively. 492 milk samples were also analyzed for SCC and out of these 357, 33, 41, 18, 29 and 14 samples were showing SCC in the range of <1 lakh, 1-2 lakhs, 2-3 lakhs, 3-4 lakhs, 4-5 lakhs and 5-6 lakhs, respectively. Out of 492 quarter milk samples, 164 were found culturally positive and among these 87 (53.05%**)** positive for *Staphylococcus* spp., 22 (13.42%) for *Streptococcus* spp., 28 (17.07%) for *Escherichia coli*, 4 (2.44%) for *Klebsiella* spp., 5 (3.05%) for *Staphylococcus* spp. + *Streptococcus* spp., 5 (3.05%) for *Escherichia coli* + *Streptococcus* spp., and 13 (7.93%) for *Escherichia coli* + *Staphylococcus* spp. Comparative study of diagnostic tests was conducted between CMT, SCC and cultural examination and found animal wise incidence as 63.42, 66.67 and 73.98% respectively, whereas quarter wise incidence was 25.61, 27.44 and 33.33%, respectively. Among the CMT negative and 1+, 2+ and 3+ grades CMT positive samples, culturally positive samples were 54, 31, 48 and 31 with 14.75%, 77.50%, 88.89% and 96.86%, respectively. Whereas, culturally positive cases with regard to SCC of <1 lakh, 1-2 lakhs, 2-3 lakhs, 3-4 lakhs, 4-5 lakhs and 5-6 lakhs were 36, 28, 39, 18, 29 and 14 with 10.08, 84.85, 95.12, 100.00, 100.00 and 100.00 percent, respectively. The percent accuracy of CMT and SCC taking cultural examination as standard was 85.77 and 89.63 percent, whereas false positive are 12.70 and 11.11 percent and false negative results are 14.75 and 10.08 percent respectively. Sensitivity of CMT and SCC considering cultural examination as standard was reported as 67.07 and 76.92 percent, respectively and with specificity of 95.12 and 95.54 percent and predictive value of positive test as 87.30 and 88.89 percent, respectively.

Introduction

Mastitis is the inflammation of mammary gland and is a complex disease causing major economic loss in dairy industry throughout the world. Examination of the association between milk yield and disease in many dairy cows found that higher milk yield was not a factor for any disease except mastitis (Grohn *et a1.* 1995). The quickest and simplest way to identify the intramammary infections (IMIs) in dairy cows is using defined parameters within either SCC (Somatic Cell Count) or CMT (California Mastitis Test) scores.

The CMT is a rapid and inexpensive test to indirectly determine the somatic cell concentration in milk (Midleton *et al.,* 2004) and is a practical and easy method for demonstrating IMIs by testing milk samples on-farm (Dingwell *et al.,* 2003). Somatic cells are always present in milk and they increase due to mammary gland infections. SCC in healthy cow's milk is between 50,000 and 100,000 cells/ml. If the SCC exceeds 200,000cells/mL, it is considered unhealthy for consumers. High SCC in milk reduces the quality of both milk and dairy products, and also affects milk shelf-life and flavor (Skrzypek *et al.,* 2004). The greater the SCC increase >10000/ml increases the production loss (Halasa *et al.,* 2007). The prominent bacteria responsible for causing mastitis include both Gram-positive and Gram-negative bacteria like *Staphylococci, Streptococci, Escherichia coli and Klebsiella pneumonia* (Contreras and Rodríguez 2011).

Materials and Methods

Animals (Patients)

The study was carried out on cases of subclinical mastitis in cows from different dairy farms and individual holdings in and around the Hyderabad during the period from January 2019 to June 2019. The cows in lactation below three months after calving were taken as in their early lactation; those in between three to six months were taken as in their mid lactation and above six months were taken as in their late lactation.

The cows in mid lactation were selected for present study. The data pertaining to age, breed, lactation number, stage of lactation, method of milking was collected. The affected animals were randomly selected into two uniform groups i.e., group I and group II, each containing ten animals. A group of ten healthy animals were also examined and the milk samples were taken for detailed analysis.

California Mastitis Test (CMT)

The milk samples were subjected to CMT as per the procedure given by Schalm and Noorlander (1957) using a modified CMT reagent (Sharma and Rajani, 1969). The principle of this test is that the detergent causes rupture of somatic cells when added to a milk sample due to which DNA and other cell contents are released. Released DNA from ruptured cells unites to form a gel, the consistency of which depends upon the number of somatic cells.

Procedure

One strip of milk was drawn into a plastic paddle with four cups (designed by Glaxo-India) from each quarter. To this, 1ml of the CMT reagent was added.

The contents were then mixed by gentle stirring for 15 to 20 sec. Based on the reaction, the results were graded as per Schalm *et al.,* (1971) as negative, 1+ (there is precipitate but no gel formation), 2+ (the precipitate thickens and forms gel towards the centre of the paddle) and 3+ (distinct gel that adheres to the bottom of the paddle).

Somatic cell count (SCC)

Total somatic cell count was estimated using Eko milk scan somatic cells analyzer supplied by M/s Eon Traders, as per the manufacturer's instructions. The values obtained were expressed as $\times 10^3$ cells/ml of milk.

The milk samples containing SCC range of 0-1 lakh cells/ml were considered negative (Hadrich *et al., 2018)*, 1-6 lakh cells/ml as sub clinically infected and > 6 lakh cells/ml as positive for clinical mastitis.

Cultural examination of milk samples

The milk samples from the quarters were collected by following aseptic precautions into sterile sample collecting tubes and then subjected to bacteriological examination for isolation of etiological agents.

Preparation of glassware

All the glassware used in this study were dipped into Sulphuric acid for 24 hours, washed under running tap water, again soaked in Teepol solution for 24 hours, cleaned with brush and washed under running water.

The glassware were then rinsed in glass distilled water and dried at 50°C, carefully packed and sterilized at 160°C for one and half hours in hot air oven. The cleaned and sterilized glassware were then used for cultural and biochemical studies.

Preparation of media

All the media obtained as dehydrated powders were rehydrated as per the manufacturer's instructions by adding distilled water to a known quantity of media, mixed and boiled to dissolve. Then the media were sterilized by autoclaving at 121^oc, 15 lb. pressure for 15 minutes. The blood agar medium and Edwards's medium were prepared in laboratory as per procedure given by Quinn *et al.,* (1999).

Collection of milk

The udder and teats were cleaned with water and dried with individual paper towels. The teat orifice and the skin around the teat were wiped with cotton soaked in 70 per cent alcohol.

In sterile sample collecting tubes about 10 ml of foremilk was collected duly following aseptic precautions. Care was taken to avoid any type of contamination. The samples were brought to laboratory within 3 hours after collection in cold chain.

Isolation of bacteria

Milk samples were inoculated into the nutrient broth and incubated at 37°C for 24 hours aerobically and a loopful of broth culture was streaked on agar media plates. The plates were incubated at 37°C for 24 to 48 hours. If 5 or more colony forming units of recognized bacteria were present, then the quarter was said to be infected (Robinson *et al.*, 1988).

For isolation of fungi and yeast, milk samples were inoculated into Sabouraud dextrose broth and incubated at 37°C up to 5 days aerobically.

Identification of bacteria

Primary identification of bacteria was done based on colony morphology, type of hemolysis and Gram's staining and pure cultures were identified up to genus level as per the Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). The below tests were done on a 24-48-hour old pure culture for the identification of bacteria.

Gram's staining

For identification of gram positive and gram-negative bacteria staining of bacterial smears was done by gram's stain by mixing a 24-hour old colony with sterile saline on a microscopic slide.

The smear was then air dried, heat fixed over flame and then stained with gram's stain and examined under oil immersion.

Motility test

This was done by hanging drop method for detection of motility. A drop of 6-8-hour old broth culture was placed on a cover slip and the cover slip was then placed upside down on a microscopic slide containing a circular depression. The motility of the bacteria was examined under high power.

Bio chemical tests

For confirmation of bacteria the following biochemical tests were under taken as per standard procedure given by Cruickshank *et al.,* (1975).

Catalase activity

This was tested by placing a loopful of bacterial colony on microscopic slide and then mixed with a drop of 3 per cent hydrogen peroxide solution. Production of gas bubbles after few seconds indicated the bacteria as catalase positive.

Haemolysin test

Blood agar plates containing 5-10 per cent defibrinated sheep blood were used for this test. The bacteria were grown on these plates and the type of haemolysis was recorded after 24 hours of incubation at 37°C.

Indole test

By using 48-hour old culture in peptone water, gram negative bacteria were tested for Indole production. 0.5 ml of Kovac's Indole reagent was slowly rundown the sides of the culture tube and formation of a pink or deep red color reagent layer was taken as positive.

Methyl Red (MR) test

MR-VP medium was used for this test. The organisms were grown in this medium at 37°C for 48 hours. Then to this 48-hour old culture, about 5 drops of methyl red reagent was added. Development of red color was taken as positive reaction while development of yellow color was taken as negative reaction.

Voges -Proskauer (VP) test

This test was done to detect the acetone production by the organisms by growing in MR-VP medium at 37°C for 48 hours. To this culture, about 0.6 ml of 5 percent Alpha - Naphthol reagent was added followed by 0.2 ml of 40 per cent potassium hydroxide solution.

The tubes were kept for 30 minutes after mixing the above reagents and watched for development of a bright pink or magenta color which was considered as a positive reaction.

Citrate utilization test

The citrate utilization by bacteria was tested on Simon's citrate agar slants. A light suspension of the test organism in sterile saline was inoculated in linear streaks on citrate agar slants. The slants were then incubated at 37°C and observed for 5 days. Growth associated with a color change to Prussian blue from green color was regarded as a positive test.

Results and Discussion

California mastitis test

In present investigation out of 492 quarter milk samples from 123 animals subjected for CMT, 366 (74.39%) quarters were negative and remaining 40, 54 and 32 quarters showed +, ++ and +++ reaction with 8.13, 10.98, 6.50 percent, respectively. Similarly, Kasikci *et al.*, (2012) reported CMT +, ++ and +++ reaction in 258 (66.85%), 85 (22.02%) and 43 (11.13%) out of 386 quarter milk samples.

Somatic cell count

A total of 492 milk samples were analyzed for SCC and out of these 357, 33, 41, 18, 29 and 14 samples were showing SCC in the range of <1 lakh, 1-2 lakhs, 2-3 lakhs, 3-4 lakhs, 4-5 lakhs and 5-6 lakhs, respectively

Cultural examination

Cultural examination was conducted to know the specific etiological agent and out of 492 samples tested, 87 (53.05%) were positive for most prevalent Gram positive *Staphylococcus spp*., followed by 28 samples (17.07%) for Gram negative Coco bacillus i.e., *Escherichia coli*, 22 (13.42%) for Gram positive *Streptococcus* spp., and 4 (2.44%) were positive for *Klebsiella spp.* Combined bacterial infection were also isolated and reported 5 (3.05%) with *Staphylococcus* spp. + *Streptococcus* spp., 5 (3.05%) with *Escherichia coli* + *Streptococcus* spp., and 13 (7.93%) with *Escherichia coli* + *Staphylococcus* spp (Fig. 1–25 and Table 1–7).

Table.1 Grades of CMT reaction in subclinical mastitis

(n=492)

S. No.	CMT reaction grade	Number of quarters showing CMT reaction	Incidence (%)
1.	-	366	74.39
2.	+	40	8.13
3.	++	54	10.98
4.	+++	32	6.50
5.	Total	492	100.00

Table.2 Range of somatic cell count in subclinical mastitis

(n=492)

S.No.	Range of SCC	Number of quarters
1.	<1 lakh	357
2.	1-2 lakhs	33
3.	2-3 lakhs	41
4.	3-4 lakhs	18
5.	4-5 lakhs	29
6.	5-6 lakhs	14
7.	Total	492

Table.3 Bacteria isolated from the quarters affected with subclinical mastitis

(n=492)

S.No.	Type of bacteria	Number of quarters tested	Number of quarters positive	Percentage (%)
1.	*Staphylococcus* spp.	492	87	53.05
2.	*Streptococcus* spp.	492	22	13.42
3.	*Escherichia coli*	492	28	17.07
4.	*Klebsiella* spp.	492	4	2.44
5.	*Staphylococcus* spp. + *Streptococcus* spp.	492	5	3.05
6.	*Escherichia coli* + *Streptococcus* spp.	492	5	3.05
7.	*Escherichia coli* + Staphylococcus spp.	492	13	7.93
8.	Total	492	164	100.01

Table.4 Comparative study on incidence of sub clinical mastitis in cows by CMT, SCC and Cultural isolation

S.No.	Name of Test	No. of animals			No. of quarter		
		Tested	Positive	Incidence	Tested	Positive	Incidence
1.	CMT	123	78	63.42	492	126	25.61
2.	SCC	123	82	66.67	492	135	27.44
3.	Cultural isolation	123	91	73.98	492	164	33.33

Table.5 Grades of CMT reaction VS status of infection in SCM affected quarters

(n=492)

S.No	CMT reaction grade	Number of quarters showing CMT reaction	No. of quarters			
			Culturally positive	Per cent	Culturally negative	Per cent
1.	-	366	54	14.75	312	85.25
2.	+	40	31	77.50	9	22.50
3.	++	54	48	88.89	6	11.11
4.	+++	32	31	96.86	1	3.13
5.	Total	492	164	33.33	328	66.67

Table.6 Somatic cell count VS status of infection in SCM affected quarters

(n=492)

S.No	SCC	Number of quarters showing SCC positive	No. of quarters			
			Culturally positive	Per cent	Culturally negative	Per cent
1.	<1 lakh	357	36	10.08	321	89.92
2.	1-2 lakhs	33	28	84.85	5	15.15
3.	2-3 lakhs	41	39	95.12	2	4.88
4.	3-4 lakhs	18	18	100.00	0	0.00
5.	4-5 lakhs	29	29	100.00	0	0.00
6.	5-6 lakhs	14	14	100.00	0	0.00
7.	Total	492	164	33.33	328	66.67

Table.7 Percent accuracy of CMT and SCC considering cultural examination as standard for SCM diagnosis

S.No.	Name of the test	Total samples examined	Test positive samples	Test negative samples	Test reaction as compared to cultural examination								
					True positive	(%)	False positive	(%)	True negative	(%)	False negative	(%)	Per cent accuracy
1.	CMT	492	126	366	110	87.30	16	12.70	312	85.25	54	14.75	85.77
2.	SCC	492	135	357	120	88.89	15	11.11	321	89.92	36	10.08	89.63
3.	Cultural isolation	492	164	328	164	100.00	0	0.00	328	100.00	0	0.00	100.00

$$\% \text{ Accuracy} = \frac{\text{Number of true positive animals + Number of true negative animals} \times 100}{\text{Number of samples examined}}$$

$$\% \text{ False positive} = \frac{\text{Number of false positive samples} \times 100}{\text{Number of samples positive by test}}$$

$$\% \text{ False negative} = \frac{\text{Number of false negative samples} \times 100}{\text{Number of samples negative by test}}$$

Table.8 Sensitivity, specificity and predictive value of CMT and SCC considering cultural examination as standard for SCM diagnosis

S.No.	Name of the test	Sensitivity	Specificity	Predictive value of positive test
1.	CMT	67.07	95.12	87.30
2.	SCC	76.92	95.54	88.89
3.	Cultural isolation	100.00	100.00	100.00

Sensitivity = a/(a+c) × 100
Specificity = d/(b+d) × 100
Predictive value for +ve test = a / (a+b) × 10
a = Disease positive and test positive (true positive)
b = Disease negative but test positive (false positive)
c = Disease positive but test negative (false negative)
d = Disease negative and test negative (true negative)

Fig.1 Grades of CMT reaction in subclinical mastitis

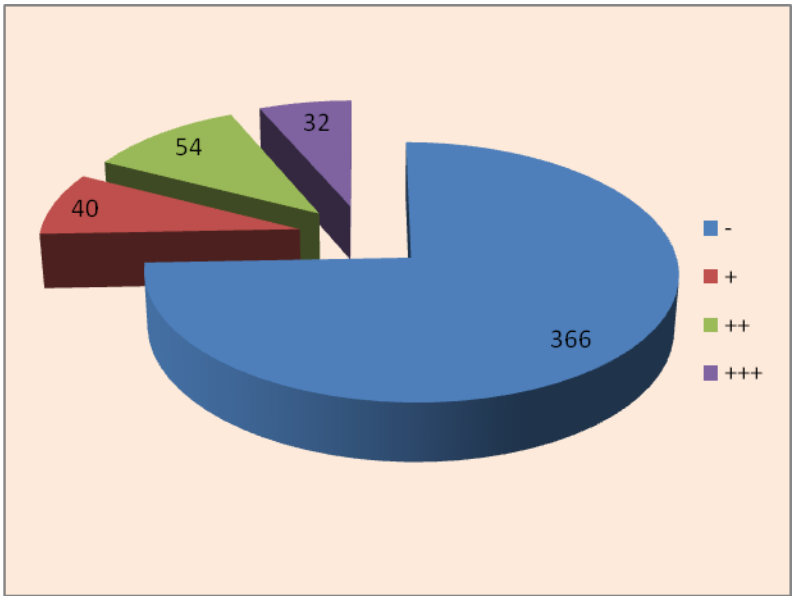


Fig.2 Milk sample with CMT positive and negative reactions

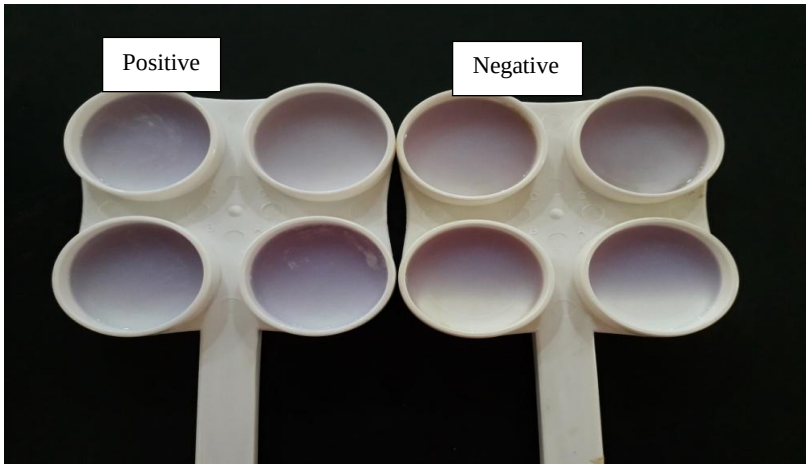


Fig.3 Range of somatic cell count in subclinical mastitis

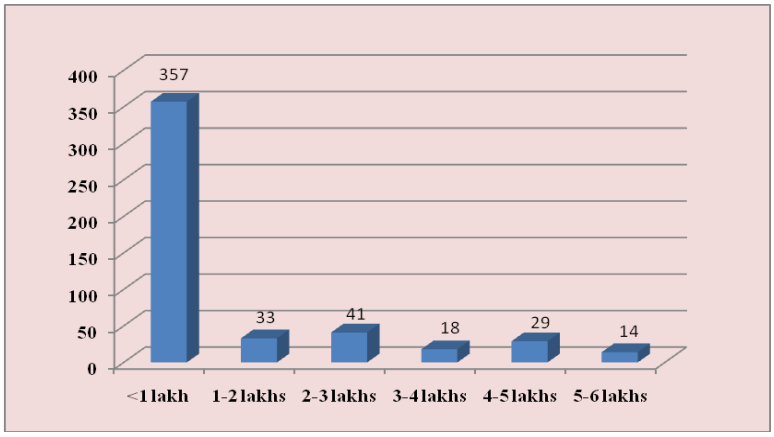


Fig.4 Bacteria isolated from the quarters affected with subclinical mastitis

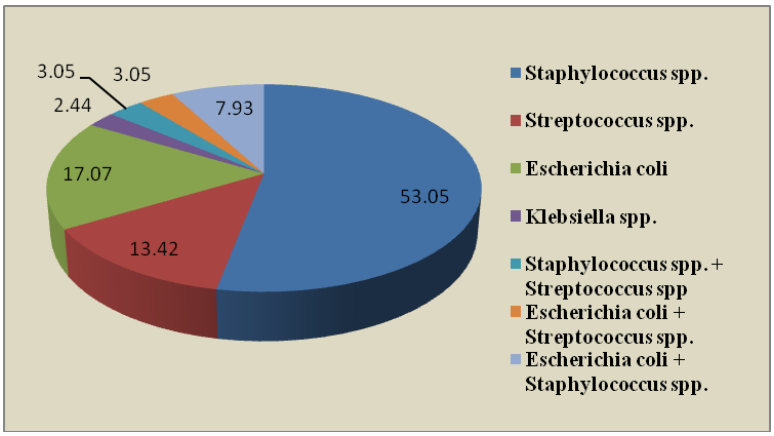


Fig.5 *Staphylococcus* spp. showing golden pigmentation on MSA

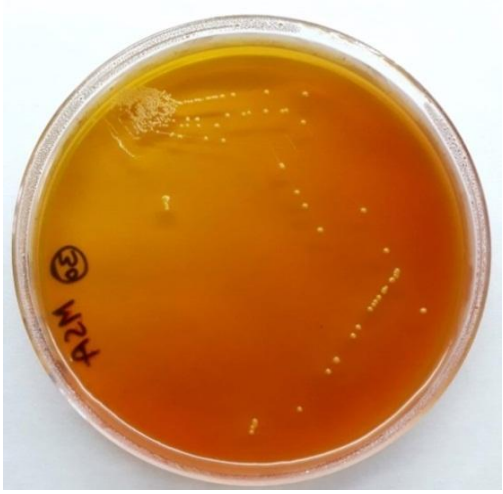


Fig.6 *Staphylococcus* spp. showing golden yellow and pink pigmentation on MSA

Fig.7 *Staphylococcus* spp. colonies on BHI agar

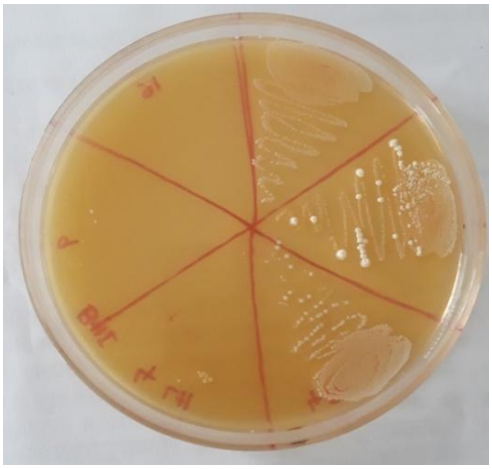


Fig.8 Catalase positive reaction of *staphylococcus* spp

Fig.9 Microscopic view of *staphylococcus* spp. with Gram's staining

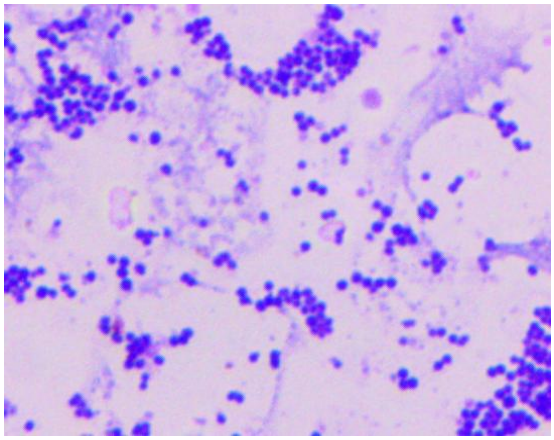


Fig.10 Small pin point transparent *Streptococcus* spp. colonies on Nutrient agar

Fig.11 Small pin point hemolytic *Streptococcus* spp. colonies on blood agar

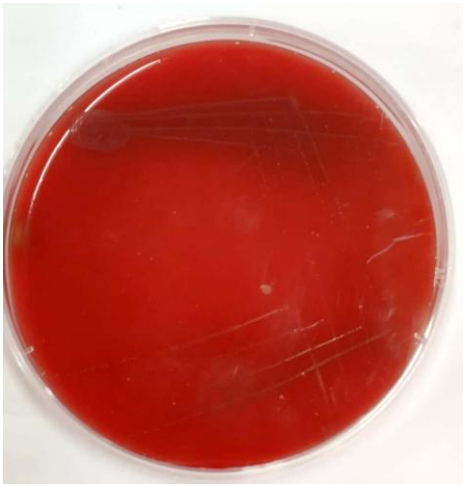


Fig.12 Small pinpoint *Streptococcus* spp. colonies on Edward's agar

Fig.13 Smear showing *Streptococcus* spp. chains with Gram's staining

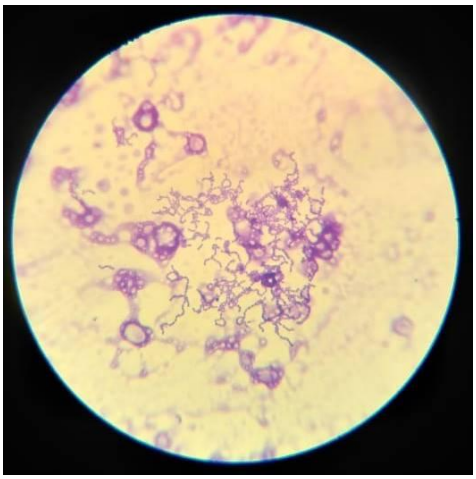


Fig.14 *E. coli* showing green metallic sheen on EMB Agar

Fig.15 Oval shaped *E. coli* organisms with Gram's staining

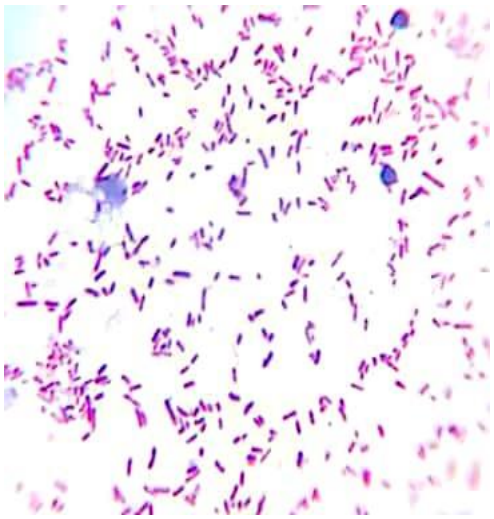


Fig.16 IMVCN reaction of *E. coli.*

Fig.17 Capsulated large and mucoid *Klebsiella spp.* colonies on MacConkey agar

Fig.18 Pink color paired rod shaped *Klebsiella spp.* organism with gram's stain

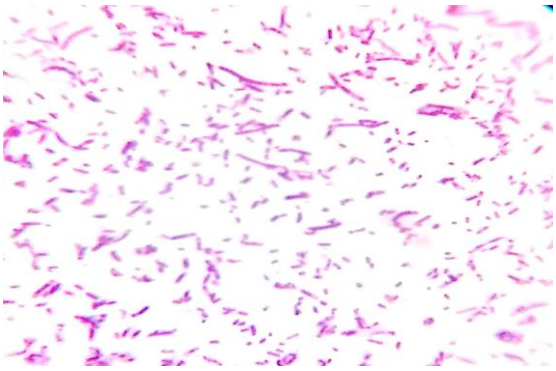


Fig.19 IMVCN reaction of *Klebsiella* spp.

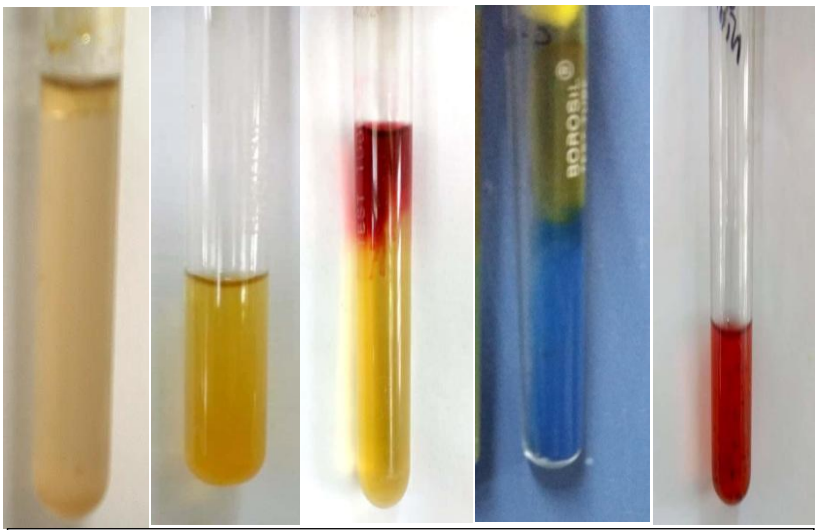


Fig.20 Animal wise Comparative study on the incidence of subclinical mastitis in crossbred cows by CMT, SCC and Cultural isolation

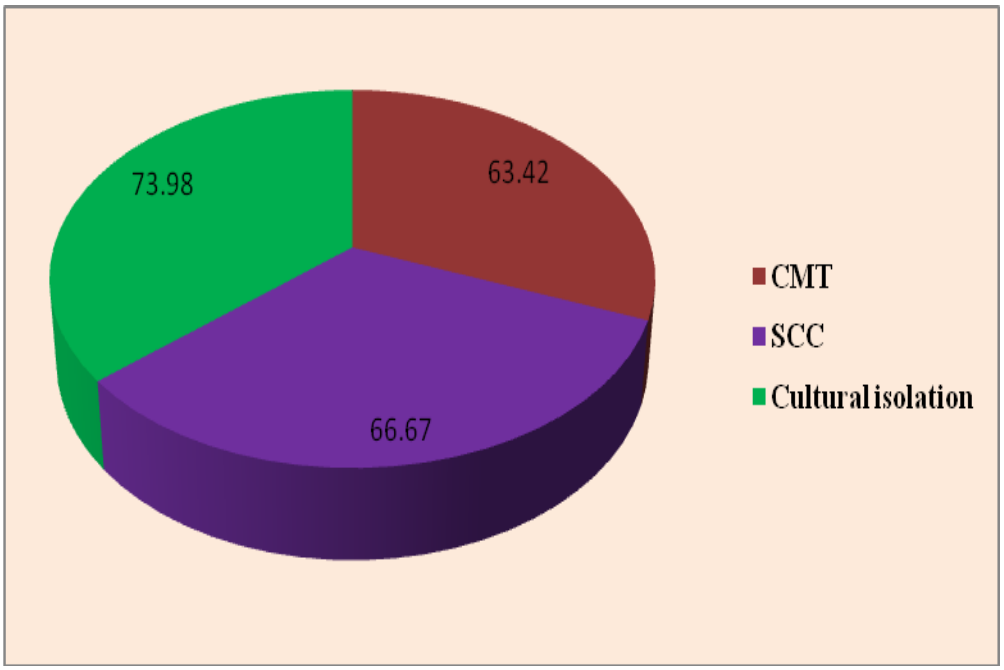


Fig.21 Quarter wise Comparative study on the incidence of subclinical mastitis in crossbred cows by CMT, SCC and Cultural isolation

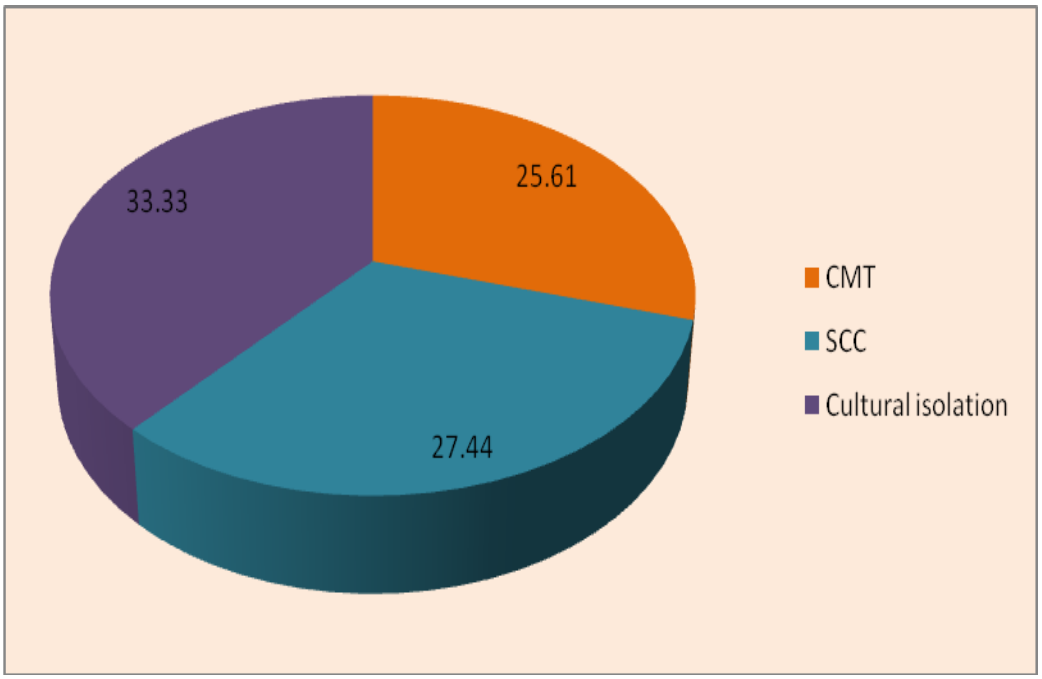


Fig.22 Grades of CMT reaction VS status of infection in SCM affected quarters

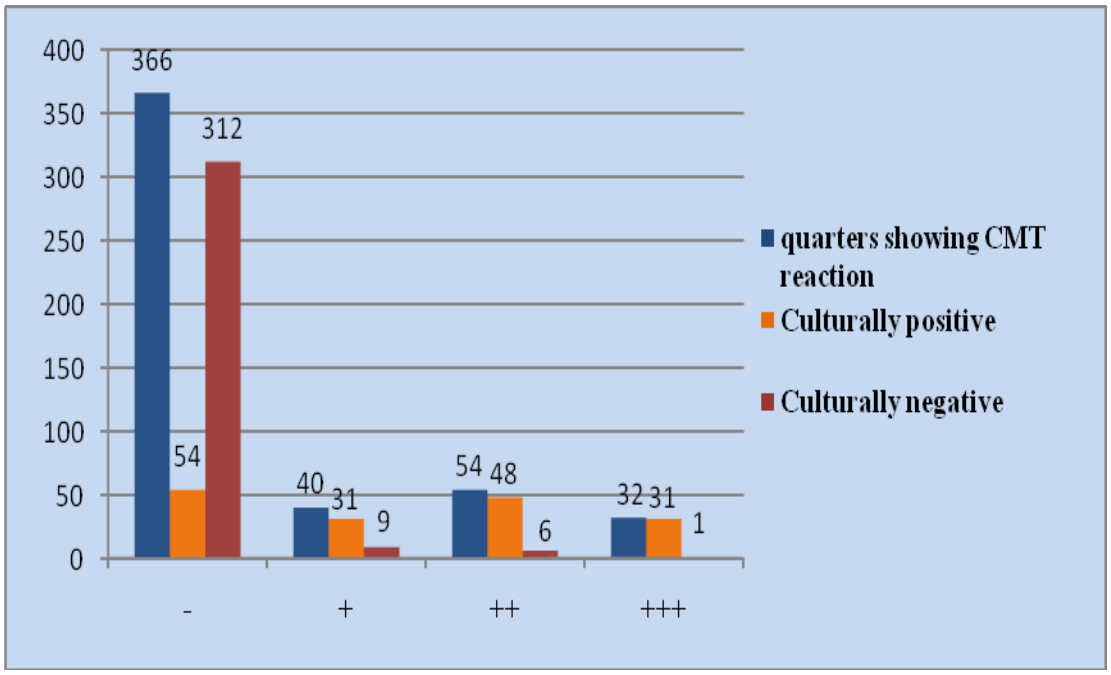


Fig.23 Somatic cell count VS status of infection in SCM affected quarters

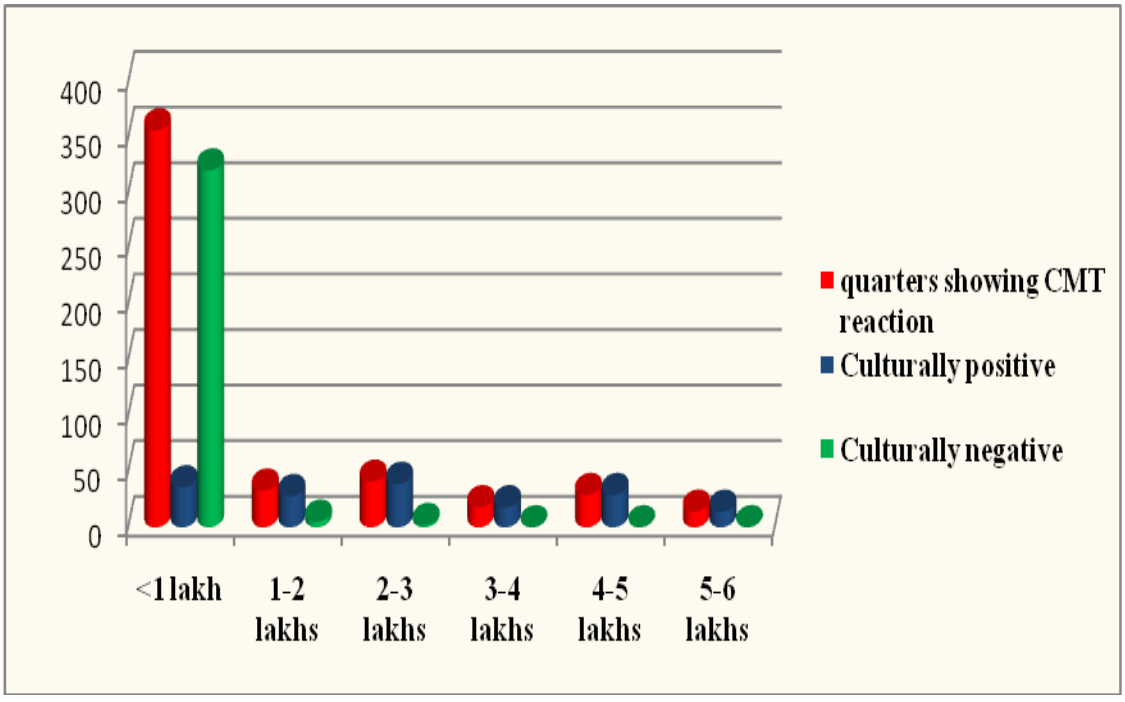


Fig.24 Percent accuracy of CMT and SCC considering cultural examination as standard for SCM diagnosis

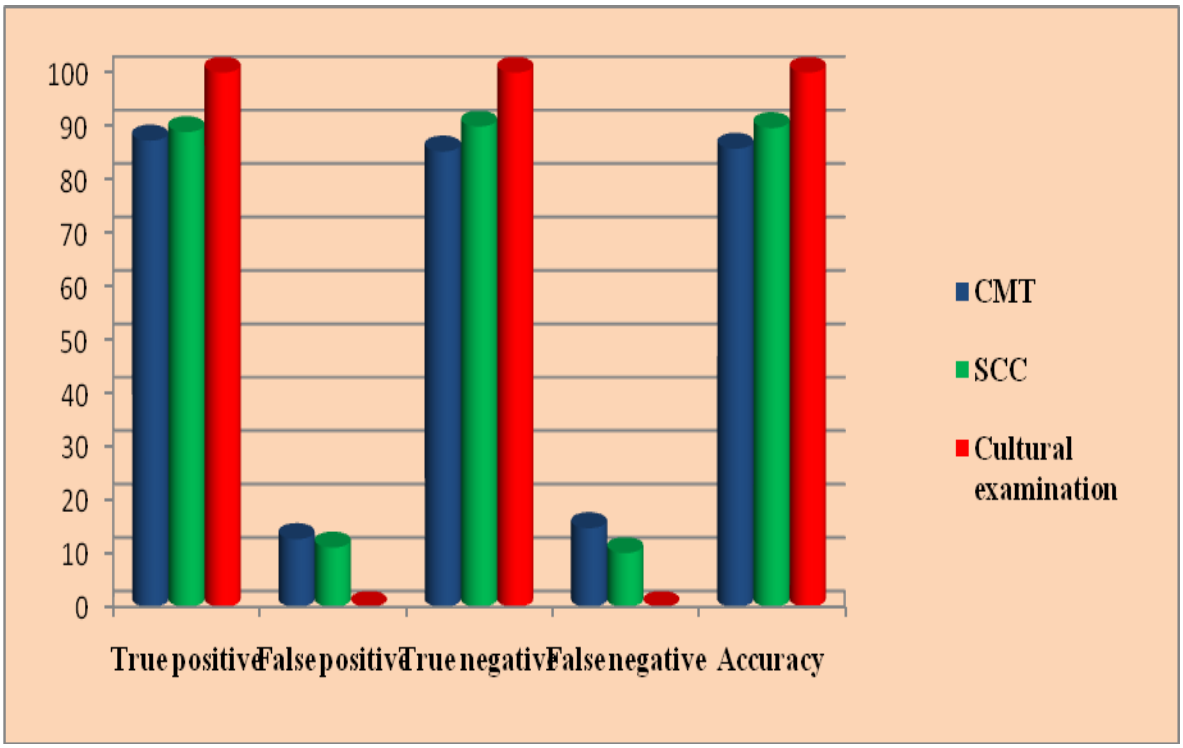
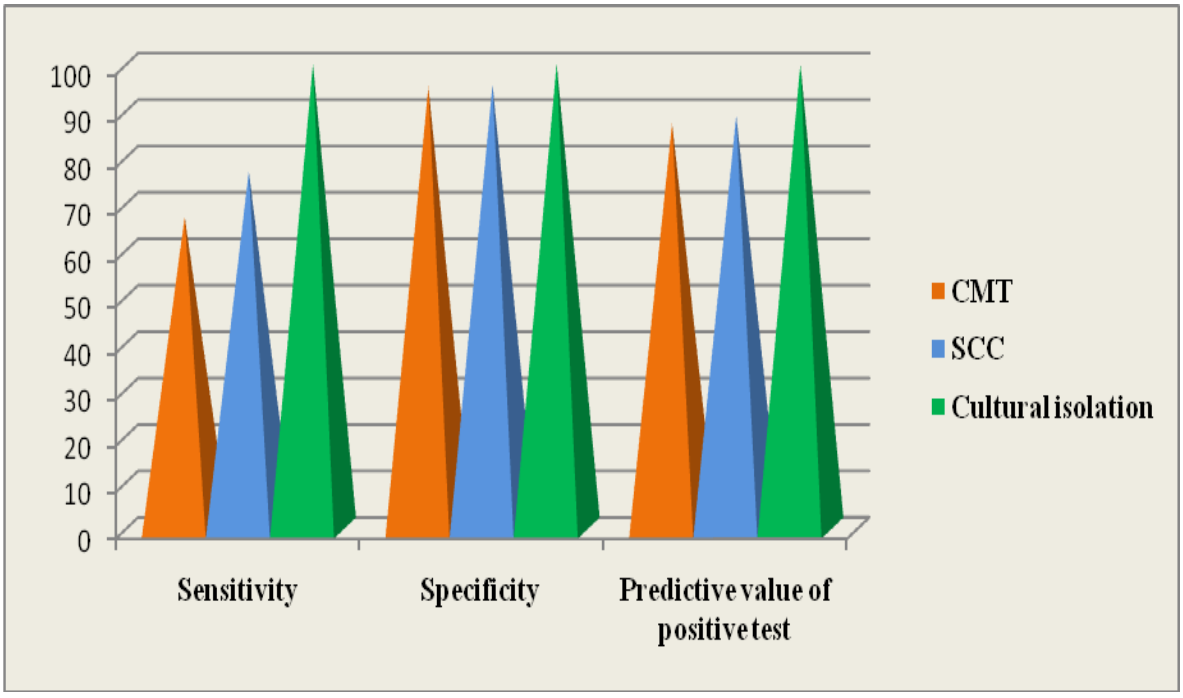


Fig.25 Sensitivity, specificity and predictive value of CMT and SCC considering cultural examination as standard for SCM diagnosis



However, Khanal and Pandit (2013) reported *Streptococcal mastitis* as the commonest (11.1%) followed by coliform (9.5%) and staphylococcal (7.9%). Whereas Saidi *et al.,* (2013) reported most prevalent organism for SCM as *Staphylococcus aureus* (40%) followed by *Streptococcus* spp. (12.5%), *Enterobacteriaceae* (2.5%), *Pseudomonas* spp. (2.5%), *Staphylococcus aureus* + *Streptococcus* spp. (12.5*%), Streptococcus* spp.+ *Escherichia coli* (7.5%), S. *aureus* + *Mycoplasma* spp.(7.5%), and S. *aureus* + *Streptococcus* spp.+ *E. coli* (5%). Krishnamoorthy *et al.,* (2017) documented 45%, 13% and 14% of prevalence of sub clinical mastitis by *Staphylococcus* sp., *Streptococcus* spp. and *Escherichia coli*, respectively. Malek *et al.,* (2011) stated that the prevalence of minor pathogens was higher (31.9%) than that of major pathogens (17.8%) in sub clinical mastitis and isolated *Corynebacterium* spp. (17.7%), *Staphylococcus aureus* (15.3%), coagulase negative Staphylococcus (CNS) (14.3%), *Streptococcus uberis* (1.9%) and *Streptococcus dysgalactiae* (0.6%). Mir *et al.,* (2014) in their investigation isolated Staphylococci (41.04%) in specific subclinical mastitis as the main organisms, while in case of latent infections Corynebacteria (36.81%) were found to be chief isolates. Highest prevalence of *E. coli* in cultural examination of present investigation could be due to improper hygienic management practices in farms leading to contamination of feed, water and milking machines which were found to be factors for high incidence of SCM by environmental pathogens.

Comparative study of CMT, SCC and cultural examination

In the present investigation, animal wise incidence with respect to CMT, SCC and cultural examination was 63.42%, 66.67% and 73.98%, respectively, whereas quarter wise incidence was 25.61%, 27.44% and 33.33%, respectively. Similar findings of animal-wise incidence of SCM were recorded as 72.07%, 66.67% by CMT and SCC, respectively and quarter-wise incidence as 59.68%, 51.80% by CMT and SCC, respectively by Badiuzzaman *et al.,* (2015). While Biswadeep *et al.,* (2015) carried out a cross sectional study on lactating cows of Jaipur district for SCM by using CMT and SCC and recorded prevalence of 67.27 and 74.55% respectively.

Out 492 quarter milk samples, 366 were negative for CMT and remaining samples of 40, 54 and 32 were found positive with 1+, 2+ and 3+ reactions, respectively. Among the CMT negative and positive with 1+, 2+ and 3+ reactions, the culturally positive samples were 54 (14.75%), 31 (77.50%), 48 (88.89%) and 31 (96.86%), respectively. Among 492 quarter milk samples 357, 33, 41, 18, 29 and 14 samples showed SCC of <1 lakh, 1-2 lakhs, 2-3 lakhs, 3-4 lakhs, 4-5 lakhs and 5-6 lakhs, respectively and among these culturally positive were 36 (10.08), 28 (84.85), 39 (95.12), 18 (100.00), 29 (100.00) and 14 (100.00), respectively. Reason for false positive cases of sub clinical mastitis by CMT and SCC during early lactation could be because of increases in somatic cell count even without intra mammary infection.

In present investigation, the percent accuracy, false positive and false negative results for SCM diagnosis with CMT and SCC considering cultural examination as standard were recorded as 85.77 and 89.63, 12.70 and 11.11 and 14.75 and 10.08 percent, respectively. Whereas, Sensitivity, specificity and predictive value of positive test of CMT and SCC for the detection of SCM considering cultural examination as standard were recorded as 67.07 and 76.92, 95.12 and 95.54 and 87.30 and 88.89 percent, respectively. Present findings are in accordance with Salvador *et al.,* (2012) who calculated the true prevalence of sub clinical mastitis with the 82.4% sensitivity and 80.6% specificity by

CMT. Present findings are in accordance with Hoque *et al.,* (2015) who screened SCM in lactating cows with CMT and SCC and reported the sensitivity, specificity, percentage accuracy and positive predictive value of 65.8 and 82.5, 76.2 and 89.4, 70.0 and 85.2 and 75.2 and 92.7 percent, respectively. Similarly, Badiuzzaman *et al.,* (2015) also recorded the sensitivity, specificity, percentage accuracy, positive predictive value and negative predictive values for CMT and SCC taking cultural examination as standard as 80.08% and 86.60%, 69.40% and 97.81%, 75.68% and 91.22%, 78.87% and 98.26% and 70.95% and 83.64%, respectively. Whereas, Sharma *et al.,* (2010) recorded the sensitivity of 86.07%, 74.63% and 88.60%, specificity of 59.70%, 17.16% and 97.76%, percentage accuracy of 75.52%, 51.64% and 91.94%, positive predictive value of 76.21%, 57.47% and 98.33% and negative predictive value of 74.07%, 31.08% and 84.52%, respectively for CMT, SLST and SCC, respectively taking cultural examination as standard.

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