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Cultural and biochemical studies of sub-clinical mastitis in cows "in and around Hyderabad''

Anusha Dasohari, Ayodhya Somasani and Nagaraj P

Abstract
The present study "Cultural and Biochemical studies of subclinical mastitis in cows " in and around Hyderabad" was undertaken to diagnose the subclinical mastitis in cows, was carried out during a period of 7 months *i.e.,* February to August, 2015. To diagnose subclinical mastitis (SCM), a total of 534 quarter milk samples from 136 apparently healthy cows of three local dairy farms and individual holdings were collected and subjected to cultural and biochemical (IMViC) test, for isolation of etiological agents. According to cultural and biochemical (IMViC) test *Staphylococcus* spp., were most prevalent followed by *Streptococcus* spp., and *Escherichia coli.* Among the *Staphylococcus* spp. isolated, coagulase positive organisms were more prevalent (37.31%) compared to coagulase negative *Staphylococcus* spp. (CoNS) (19.4%)

Keywords: Subclinical mastitis, cultural, (IMViC) test, Staphylococcus, Streptococcus, *Escherichia coli*

1. Introduction
Subclinical mastitis is bereft of any obvious manifestation of inflammation and is characterized by having no visible signs either in the udder or in the milk, but the milk production decreases and there is change in milk composition [5]. Subclinical mastitis is 3–40 times more common than clinical mastitis and causes the greatest overall losses in most dairy herds [1]. Besides causing huge losses to milk production, the sub clinically affected animals remain a continuous source of infection to other herd mates [7]. The subclinical form of mastitis in dairy cow s is important because it is 15 to 40 times more prevalent than the clinical form and is difficult to detect, reduces milk production and adversely affects milk quality [12]. The diagnosis of mastitis according to the International Dairy Federation (IDF) recommendations is based on the somatic cell counts (SCC) and microbiological status of the quarter. Though bacteriological culture of milk samples is the standard method for identifying mastitis, the logistic and financial considerations involved with sampling all fresh cows have precluded this technique from being widely adopted [14].
The present study "Cultural and Biochemical Studies of Sub-Clinical Mastitis in Cows "in and around Hyderabad" was undertaken to study therapeutic efficacy of two different antibiotics for the treatment of subclinical mastitis in cows.

2. Material & Method
The study was carried out on cases of subclinical mastitis from three different dairy farms and individual holdings during the period from February 2015 to August 2015.

Screening of Animals for Subclinical Mastitis: Total of 534 quarter milk samples from 136 apparently healthy cows were collected and subjected to California mastitis test (CMT), White side test (WST) and Surf field mastitis test (SFMT) and Somatic Cell Count (SCC) to differentiate subclinical mastitis from clinical mastitis. Based on the above test results the milk samples were collected by aseptic precautions into sterile vials and then subjected to bacteriological examination for isolation of etiological agents.
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 [4]. Gram staining, Motility test, Catalase activity, coagulase test, sugar fermentation test, Haemolysin test, Indole test, Methyl Red test, Voges – Proskauer test, Citrate utilisation tests were done on a 24-48 hour old pure culture for the identification of bacteria.

3. Result

The 115 quarter milk samples collected from 30 animals which were subjected to all the diagnostic tests were only cultured. Out of 115 quarter milk samples, 59 were culturally positive (51.30%). Examination of gram stained milk smears revealed gram positive cocci in 49/59 (83.05%) samples. Hence, for further isolation, they were streaked on blood agar, nutrient agar, Edward's medium and mannitol salt agar plates. The gram positive cocci in 38/49 quarter samples produced hemolysis after 24 hours of incubation at 37°C when streaked on blood agar plates (fig. 1). Small, round, cream coloured colonies appeared on nutrient agar after incubation for 24 hours. The cocci from 38 quarter samples fermented mannitol that was present in the medium and turned the color of the medium to yellow. Colonies appeared on MSA plates were round, smooth and glistening and had a golden-yellow pigment (fig. 2). Hence, they were considered as *Staphylococcus* and tested further biochemically.

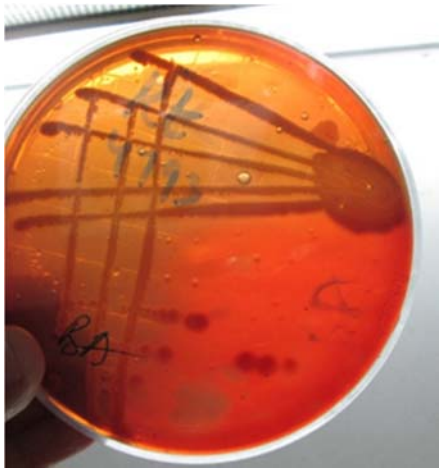


Fig 1: Haemolytic colonies of Staphylococcus on blood agar

Fig 2: Staphylococcus showing golden yellow pigment on MSA on blood agar

When the isolates were subjected to coagulase test, the isolates from 25 quarters yielded a coagulase positive reaction (fig. 3) and isolates of 13 quarter samples yielded a coagulase negative reaction (fig. 3). The bacteria isolated were also positive for catalase activity (as they had produced bubbles immediately after mixing of the colony with 3 per cent hydrogen peroxide on a glass slide). When incubated anaerobically, the bacteria fermented both d-glucose and d-mannitol which were incorporated in the *Staphylococci* and *Micrococci* differentiation media and turned the medium to yellow colour by acid production associated with growth by which the possibility of presence of *Micrococcus* spp. was ruled out. Hence, the 38 isolates tested were confirmed as *Staphylococcus* spp. (fig. 4).

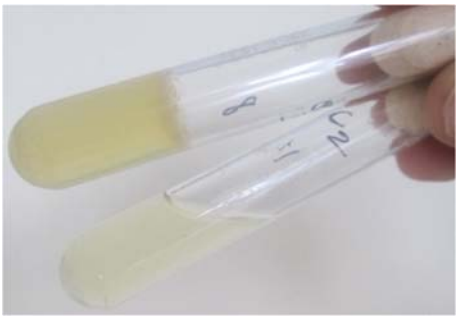


Fig 3: Staph. showing coagulase +ve reaction (A) & coagulase -ve reaction (B)

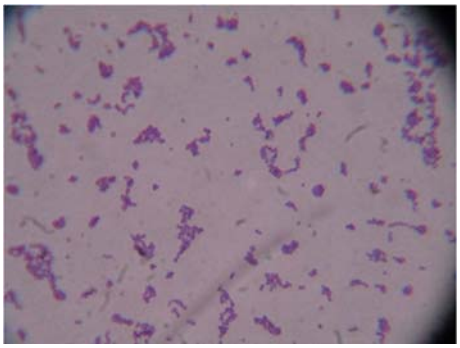


Fig 4: Coagulase +ve Staphylococcus from a colony on nutrient agar Gram's stain)

The remaining 11 isolates of gram positive cocci out of 49 isolates (22.45%) appeared as chains of different lengths when seen under oil immersion after staining the milk smear with grams stain. Twenty four hours broth culture also revealed chains of cocci. When streaked on blood agar the isolate produced small, round, non hemolytic colonies. A colony was tested for catalase and coagulase activity which showed negative reaction for both. On incubation of Edwards medium plates (fig. 5) at 37°C for 24 hrs after streaking the broth cuture on them, the bacteria produced small, round colonies and did not show aesculin hydrolysis. According to these observations, the isolate was considered as non-hemolytic *Streptococcus* spp. (fig. 6). Out of 59 quarter samples examined, 38/59 (64.41%) were *Staphylococcus* spp. (25/38 (65.79%) were coagulase positive and 13/38 (34.21%) were coagulase negative) and 11 of 59 samples (18.64%) were non hemolytic *Streptococcus* spp.

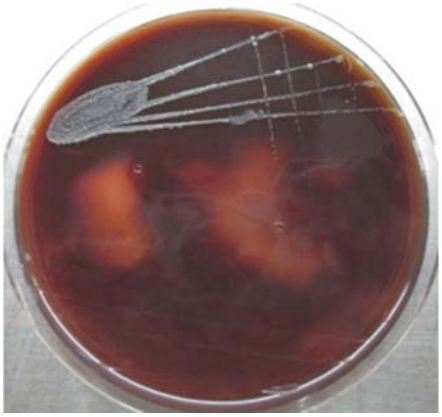


Fig. 5: Small pinpoint round colonies of *Streptococcus* on Edward's agar

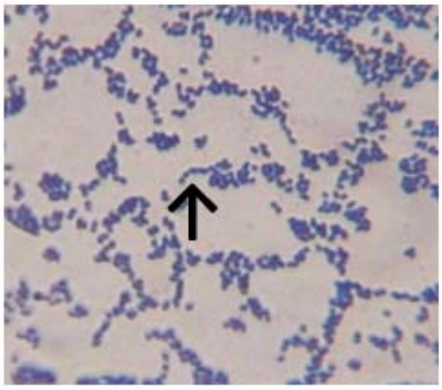


Fig 6: Smear showing *Streptococcus* (↑) spp. in chains (gram's stain)

Gram stained milk smears of broth culture revealed the gram negative, medium sized rods in 15/59 quarters (25.42%). Hence, the culture was streaked on nutrient agar, Mac Conkey and EMB agars. On nutrient agar the colonies appeared as greyish in color which were round and shiny. Round, shiny and bright pink color colonies appeared in 13/15 samples (86.67%) on Mac Conkey agar plates incubated at 37°C for 24 hours (fig. 7). On EMB agar plates, the bacteria produced metallic sheen (fig. 8). When the isolates were subjected IMViC test, the bacteria showed a positive reaction for Indole production as well as for Methyl Red (MR) test and was negative to Voges-Proskauer (VP) test and citrate utilization (fig. 9). Hence, they were considered as *E.coli* (fig. 10).

Fig 7: Pink colonies of *E. coli* on Mac Conkey agar

Fig 8: *E.coli* showing metallic sheen on EMB agar

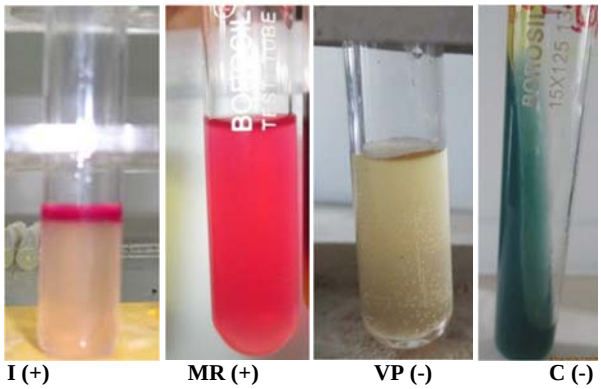


Fig 9: IMViC reaction of *E.coli*
I- Indole
MR-Methyl Red
VP- Voges-Proskauer
C- Citrate

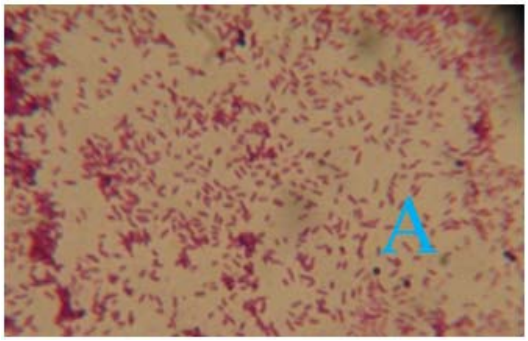


Fig 10: *E. coli* from a colony on nutrient agar (A)(gram's stain)

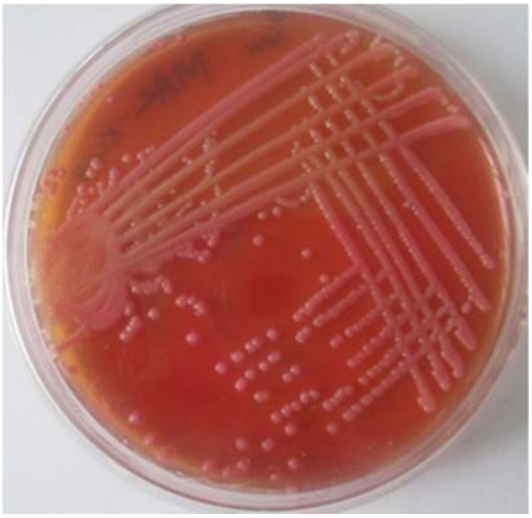
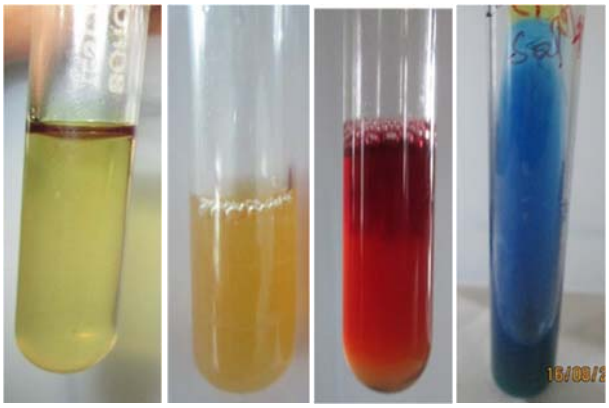


Fig 11: Large, mucoid pink coloured colonies of *Klebsiella* on Mac conkey agar

Two out of fifteen (13.33%) gram negative isolates produced capsulated, large and highly mucoid colonies on Mac Conkey agar plates when incubated for 24 hours at 37°C (fig. 11). When the isolate was subjected to IMViC test, isolates were negative for Indole production and Methyl Red (MR) test and positive for Voges-Proskauer (VP) test and citrate utilization (fig. 12). Hence it was considered as *Klebsiella* spp. Out of the 59 quarter samples examined, 13/59 (22.03%) and 2/59 (3.39%) were *E.coli* and *Klebsiella* spp., respectively.



I (-) MR (-ve) VP (+) C (+)

Fig 12: IMViC reaction of *Klebsiella*

I - Indole
MR- Methyl Red
VP- Voges-Proskauer
C- Citrate

Table 1: Bacteria isolated from quarters affected with subclinical mastitis (n=59)

S. No	Type of bacteria	Number of quarters		
		Tested	Positive	Percent (%)
1.	*SStaphylococcus* spp.	59	32	54.24
2.	*Streptococcus* spp.	59	9	15.25
3.	*Escherichia coli*	59	8	13.56
4.	*Klebsiella* spp.	59	2	3.39
5.	*Escherichia coli* + *Streptococcus* spp.	59	2	3.39
6.	*Staphylococcus* spp. + *Streptococcus* spp.	59	3	5.08
7.	*Escherichia coli* + *Staphylococcus* spp.	59	3	5.08
	TOTAL		59	100.00

From the table 1 it is evident that monomicrobial infection was prevalent in 51/59 (86.44%) samples than mixed infections 8/59 (13.55%). The per cent of various bacterial isolates associated with SCM caused by *Staphylococcus* spp., *Streptococcus* spp., *Escherichia coli*, *Klebsiella* spp., mixed infections of *Escherichia coli* and *Streptococcus* spp., *Staphylococcus* spp. and *Streptococcus* spp., *Escherichia coli* and *Staphylococcus* spp. were 54.24, 15.25, 13.56, 3.39, 3.39, 5.08 and 5.08 per cent, respectively. Out of 59 culturally positive samples, 67 isolates were obtained. *Staphylococcus* spp. were predominant (38/67= 56.71%) out of which 37.31% (25/67) constituted coagulase positive *Staphylococcus* spp. and 19.40% (13/67) constituted coagulase negative *Staphylococcus* spp., followed by *Streptococcus* spp. (14/67= 20.90%), *Escherichia coli* (13/67= 19.40%) and least *Klebsiella* spp. (2/67= 2.99%).

4. Discussion

There are several direct and indirect tests with varying efficacies for detection of subclinical mastitis viz. culture, isolation and identification of causal agents, somatic cell count, California mastitis test, modified white side test (WST), bromothymol blue card test, electrical conductivity of milk, Cl^- estimation in milk, Modified Aulendorfer Mastitis Probe (MAMP) test, N-Acetyl-β-D-Glucosaminidase (NAGase) enzyme activity and ELISA etc., among these tests, bacterial culture from the milk has been considered as standard method for confirming subclinical udder infections in dairy cows [15].

Cultural test is proved to be more efficacious and superior to other indirect tests of mastitis. Although, the cultural examination of milk is most accurate, it is time consuming, requires a good lab, and costly, furthermore it may give false negative results where the shredding of the bacteria from the udder is not constant and if the inflammation is due to injury. Cultural characterization of bacteria in the present study was limited only to isolation and identification of bacterial isolates upto genus level. Out of 115 quarter milk samples, 59 were culturally positive (51.30%) and it is evident that monomicrobial infection was prevalent in 51 (86.44%) out of 59 culturally positive samples than mixed infections 8/59 (13.55%). 67 isolates were obtained from sub clinically infected quarters where *Staphylococcus* spp. constituted 56.71% (38) of the isolates out of which 25 (37.31%) belonged to coagulase positive *Staphylococci* and 13 (19.40%) belonged to coagulase negative *Staphylococci*. The minor pathogens included *Streptococcus* spp. (20.90%), *E.coli* (19.40%) and *Klebsiella* spp. (2.99%).

Among different mastogenic agents isolated, *Staphylococcus* spp., were most prevalent followed by *Streptococcus* spp., and *Escherichia* coli. Almost similar pattern was noticed by Patnaik *et al*.[9] who reported *Staphylococcus* spp. (53.33%) as predominant isolates, out of which 28.00% belonged to coagulate positive *Staphylococcus* spp. and 25.33% belonged to coagulase negative *Staphylococcus* spp., *Streptococcus* spp. constituted 17.3% and *E. coli* constituted 14.6% of the total isolates. Findings of Harini and Sumathi [6] revealed *Staphylococcus aureus* (58%) and *Escherichia coli* (23.5%) followed by *Staphylococcus epidermidis* (8%), *Streptococcus* spp. (5.5%), *Klebsiella* spp. (3%) and *Bacillus* spp. (2%). Mir *et al* [8]. reported *Staphylococcus* spp. (41.04%), *Corynebacterium* (30.60%), *Streptococcus* spp. (21.27%), *E. coli* and others (7.09%). The higher incidence of Staphylococci indicates unhygienic milking practices as this pathogen is mainly spread during milking via milkers' hands [3]. However, Zeedan *et al* [18]. reported that the major bacterial isolates were *E. coli* (22.16%), *S. aureus* (20.19%), *Streptococcus* spp. (13.30%), *Pasteurella* spp. (2.45%), *Klebsiella* spp. (1.47%) and *Pseudomonas* spp. (0.5%). The variation in the involvement of a wide range of pathogens and their proportion in different cases of bovine subclinical mastitis could be the outcome of the interaction of the pathogens with the host and its environment and also due to the use of different antibiotic preparations for the treatment of mastitis.

In the current study, among the *Staphylococcus* spp. isolated, coagulase positive organisms were more prevalent (37.31%) compared to CoNS (19.4%) which were in agreement with Saini *et al* [11]. Sharma *et al*. (2012a) [13], Saidi *et al* [10]. Harini and Sumathi [6] and Patnaik *et al* [9]. On contrary, Tumlam *et al* [17], Bjork *et al* [2] and Thorberg *et al* [16] reported CoNS to be the major pathogens among the *Staphylococcus* spp. isolated.

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