MULTIPLEX-PCR TO DETECT PATHOGENS AND ANALYSIS OF RELATION OF AGE AND STAGE OF LACTATION OF COWS TO SUB-CLINICAL MASTITIS

Gaddi RM[1], Isloor S[1,*], Rathnamma D[1], Avinash B[4], Veeregowda BM[1], Bhaskar R[3] and Suguna Rao[2]

[1]Department of Microbiology, Veterinary College, KVAFSU, Hebbal, Bengaluru
[2]Department of Pathology, Veterinary College, KVAFSU, Hebbal, Bengaluru
[3]Instructional Livestock Farm Complex, Veterinary College, KVAFSU, Hebbal, Bengaluru
[4]Acquity Labs Pvt Ltd, HBR Layout, Bengaluru

ABSTRACT

In this study, 225 milk samples were collected sequentially during 1st to 88th day from 25 HF cross cows in an organized farm. First five collections were obtained at a weekly interval (1,7,14,21 and 28 days) and later, fortnightly for two months (43, 58, 73 and 88 days). These milk samples were screened for Subclinical mastitis (SCM) by Somatic Cell Count (SCC). Further, multiplex-PCR for detection of *S.aureus*, *E.coli*, *S.agalactiae*, *S.dysgalactiae* and *S.uberis* was employed to detect the major bacterial pathogens. The SCM positivity was assessed based on criteria of SCC $\geq$ 500,000 cells /ml. The study revealed the high prevalence of variable SCM pattern in milking cows by SCC (73.33 %) in sequentially collected milk samples over a period of 88 days. No specific pattern of prevalence of SCM was observed during the study period. The prevalence of SCM was not influenced by the stage of lactation. In all the stages of lactation and age groups *S. aureus*, Streptococci and *E.coli* were detected with the predominance of *S. aureus*. The varied distribution of organisms in different stages of lactation did not influence the prevalence of SCM. Further, the high prevalence of SCM was noticed in aged cows. Among these, maximum number of milk samples (46 %, 52/113) revealing the presence of pathogens were obtained from cows in the age group 7-11 years. The multiplex PCR was found an easy and rapid method to detect the predominant pathogens causing SCM. The findings emphasize the need to control SCM through sequential monitoring of SCM through SCC, multiplex-PCR and proper managemental practices.

* Corresponding author

E-mail: kisloor@gmail.com (Shrikrishna Isloor)

1 Introduction

The tremendous growth of dairy industry is crippled by bovine mastitis, the most frequent and costly status as it affects the dairy herd worldwide (Halasa et al., 2007). Mastitis is the inflammation of udder parenchyma characterized by pathological changes in the mammary gland as well as physical and chemical changes of the milk. The disease continues to pose a major threat to the animal husbandry and dairy sector. Despite research for several decades, this condition still poses challenge to the researchers. The overall national economic loss in India due to mastitis was to the tune of Rs 7165.51 crores (Bansal & Gupta, 2009). It is a multifactorial disease involving three main elements viz cow, the environment and the pathogen and their complex interaction is incompletely understood (Brand et al., 1996).

Broadly bovine mastitis is of two types, viz. clinical, where the appearance of udder and milk changes and subclinical, wherein the appearance of udder as well as milk is normal. Therefore detection of clinical cases of mastitis (CM) is easy than subclinical form (SCM), which needs application of laboratory tests. The major pathogens responsible for Bovine Mastitis can be further classified as Contagious (*Staphylococcus aureus, S. agalactiae*) and environmental (*Escherichia coli, S. dysgalactiae* and *S. uberis*). Initially, clinical cases could begin as subclinical and hence controlling SCM is the best way to reduce the clinical cases indirectly (Harmon, 1994).

An early diagnosis of mastitis is utmost important to avoid fibrosis of the udder and milk loss. Conventionally, Electrical conductivity (EC), California Mastitis Test (CMT), Somatic Cell Count (SCC) is although rapid ambiguous. The conventional bacterial culturing is cumbersome, time consuming and ambiguous (Hegde, 2011; Nithinprabhu et al., 2013). Of late, the DNA based molecular tools, especially multiplex PCR has been developed and found specific and rapid in detection of major mastitis causing pathogens (Hegde, 2011; Shome et al*.,* 2011).

Bovine mastitis is highly complex disease influenced by various factors, such factors could be analyzed by prospective cohort study based on the sequentially collected data and determines the risk factors. Considering the aforementioned facts, the present study was designed with a focus on Multiplex PCR for detection of predominant pathogens at various time points within a single farm and analyzing the influence of age of cow and stage of lactation with relation to SCM detected by SCC.

2 Materials and methods

2.1 Sampling details

A temporal study was carried out to sequentially monitor the sub-clinical cases of bovine mastitis due to major bacterial pathogens such as *S.aureus*, *E.coli, S.agalactiae, S.dysgalactiae* and *S.uberis.* In this approach, conventional SCC and Multiplex-Polymerase Chain Reaction (M-PCR) were employed to sequentially monitor the SCM. Furthermore, the effect of stage of lactation and age group of milking cows on prevalence of SCM and in turn influence of SCM on milk production was also investigated.   In view of this, the milk samples were sequentially collected from 1st to 88th day. First five collections were collected at a weekly interval (1st ,7th ,14th ,21st and 28th days) and later, fortnightly for two months (43rd , 58th , 73rd and 88th days).

Table 1 Prevalence of SCM in dairy cows by SCC, at different age and stage of lactation.

Days	No. pos/No. tested, percent	Age Groups (years) No. pos/No. tested, percent			Stage of Lactation No. pos/No. tested, percent		
	$(1-88^{th}$ Day)	3-5	5-7	7-11	Early	Mid	Late
1	25/25, 100	7/7, 100	7/7, 100	11/11, 100	15/15, 100	5/5, 100	5/5, 100
7	19/25, 76	6/7, 85.7	4/7, 57.2	10/11, 90.9	13/16, 81.3	4/5, 80.	2/5, 50
14	14/25, 56	5/7, 71.4	2/7, 28.6	7/11, 63.6	7/15, 46.6	3/5, 60.	4/5, 80
21	23/25, 92	7/7, 100	6/7, 85.7	10/11, 90.9	13/14, 92.9	6/6, 100	4/5, 80
28	14/25, 56	3/7, 42.8	3/7, 42.9	6/11, 54.5	8/14, 57.1	4/6, 66.7	2/5, 40.
43	25/25, 100	7/7, 100	7/7, 100	11/11, 100	13/13, 100	7/7, 100	5/5, 100
58	12/25, 48	4/7, 57.1	2/7, 28.6	6/11, 54.5	5/11, 45.5	5/9, 55.6	3/5, 60.
73	8/25, 32	2/7, 28.6	3/7, 42.9	4/11, 36.4	3/8, 37.6	5/9, 55.6	7/7, 100
88	24/25, 96	6/7, 85.7	7/7, 100	11/11, 100	7/8, 87.5	12/12, 100	5/7, 71.4
Total	165/225, 73.3	47/63, 74.6%	42/63, 66.7%	76/99, 76.8%	84/113, 73.7%	51/64, 79.7	37/48, 77.1%

P:Positive; T:Total,

Multiplex-PCR to detect pathogens and analysis of relation of age and stage of lactation of cows to Sub-clinical mastitis
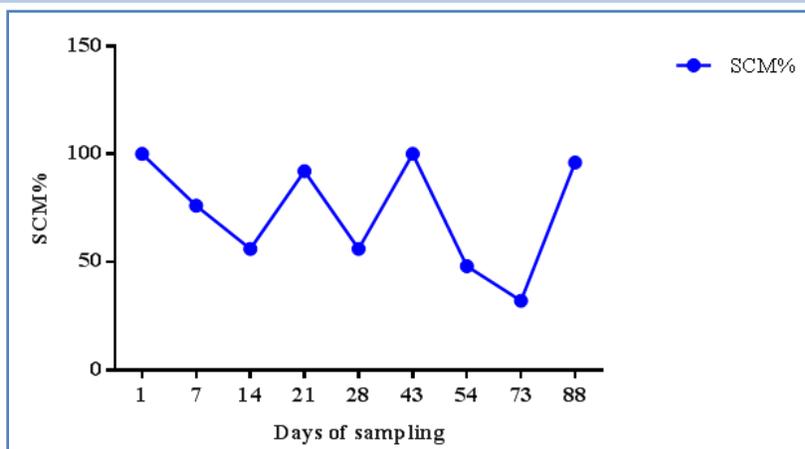
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Figure 1 Prevalence of SCM based for period of 1st to 88th days.

For the purpose of studying the sequential prevalence of SCM, 25 cows were included in the study. The milk samples were collected from these cows at a weekly interval for five collections and followed by an interval of fifteen days collection for four times. The influence of factors such as the age, stage of lactation and milk yield were studied. A total of 225 milk samples were collected as detailed above.

2.2 Somatic Cell Counting (SCC) using nucleocounter

Fresh milk samples were used for SCC estimation using Nucleocounter (Chemo Metec, Denmark) following the instructions given by the manufacturer. Initially, five hundered microlitre of milk samples was mixed with equal quantity of the lysis buffer supplied by the manufacturer. The mixture was mixed gently to lyse the cells and was aspirated into the cassette by pressing the piston. The cassette was then inserted into the Nucleocounter and the SCC values were recorded. The SCC of > 5,00,000 cells / ml of test milk sample was considered cutoff to declare the positivity (Narayana & Iya,1954).

2.3 Multiplex polymerase chain reaction (mPCR)

Multiplex polymerase chain reaction was employed for the detection of bacterial pathogens in the milk samples. The specific primers *sip, pau A, 16S rRNA dys, alr* and *nuc* were used to detect *S. agalactiae, S. uberis, S. dysgalactiae, E. coli* and *S. aureus* respectively (Hegde, 2011) .

3 Results and Discussion

In the present study, a total of 225 collections of milk samples from cows were tested by SCC. A preliminary evaluation of these samples revealed 73.33% prevalence of SCM. Sample t-test was performed on SCC with different days of sampling. No significant difference was observed between any collections in SCC (P>0.05). The prevalence of SCM was at various time points during the study period is shown in Table 1 and Figure.1.

The average SCC observed in SCM milk samples by earlier workers has exhibited variation and this could be because of primary / secondary pathogens of udder. These pathogens affected the mean SCC values depending on degree of infection (Samanta et al., 2006). Various other factors such as cytoplasmic environment, calving season and persistent contact / exposure to dung, high environmental humidity can also influence the incidence of SCM along with increased SCC in milk samples (Madsen et al., 1992).

The SCC in milk from individual cows generally is a useful tool for monitoring the probability of intramammary infection, but must be complemented with bacteriological identification and enumeration. Bacterial culture is routinely used to diagnose mastitis, and culture results are often the basis for evaluating the quality and extent of a problem at the herd level. However, bacterial culturing of milk samples is laborious and time consuming. Polymerase chain reaction based detection of various pathogens in the milk is a rapid, sensitive and reliable method of detecting mastitis causing pathogens (Khan et al*.,* 1998; Phuektes et al., 2001a; Phuektes et al., 2001 b; Phuektes et al., 2003; Shome et al., 2011; Shome et al*.,* 2012).

In the present study, a total of 225 milk samples were screened for major bacterial pathogens and 113 organisms were revealed by mPCR. Of these 113, Maximum *S.aureus* (52.21%, 59/113) followed by *S.dysgalactiae* (15.93%, 18/113), *E.coli* (15.04%, 17/113), *S.agalactiae* (12.39%, 14/113) and *S.uberis* (4.43%, 5/113) were detected. This study was in accordance with Hedge et al., 2012 wherein mPCR results showed that *S.aureus* was a predominant pathogen detected (53.77%) followed by *S.dysgalactiae* (17.92%), *E.coli* (13.12%), *S.agalactiae* (11.32%) and *S.uberis* (3.77%) (Figure. 2).In this study, *S.aureus* was found to be the predominant pathogen prevailing at 52.21%. Sequentially, the days when SCC and mPCR (*S.aureus*) were positive for SCM, the milk yield showed a negative trend as per Radostitis et al. (2000).
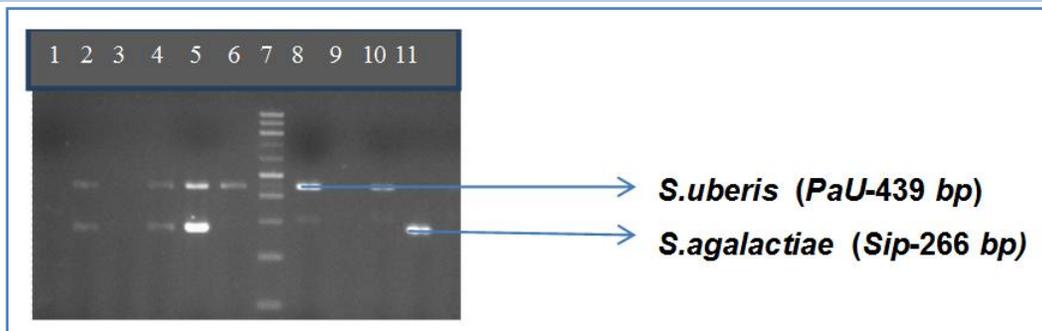
Figure 2 Two-tube Multiplex-Polymerase Chain Reaction (Tube 1) with milk samples for *S.uberis* (*PaU*-439 *bp*) and *S.agalactiae* (*Sip*-266 *bp*). [**Lane Details** : Lane 1,3,9: Negative for *S.uberis* and *S.agalactiae;* Lane 2,4,5: Positive for *S.uberis* and *S.agalactiae;* Lane 6,8,10: Positive for *S.uberis;* Lane 7: Ladder (100bp); Lane 11: Positive for *S.agalactiae]*

Only on certain days (14, 28, 54 and 73 days), there was not much influence on milk production though the organisms detected. As a predominant mastitis causing pathogen, *S.aureus* is able to survive for longer time on skin (McDonalds, 1977) and inside the neutrophils of the mammary gland (Craven & Anderson, 1979; Sandholm et al*.,* 1990) thus protecting itself from the action of antibiotics. This may be the reason that the SCC level remained elevated in our study on day 21st and 43rd due to intra cellular localization and in turn being protected from being acted upon by the antibiotics and acting as a immunogen. Boulanger et al.(2003) postulated that basal NF-kB activity is required for penetration of *S. aureus* into mammary epithelial cells, and that pharmacological NF-kB inhibitors could be used to reduce the intracellular infection of *S. aureus* (Hogan & Smith, 2003).

Yet another predominant mastitis causing pathogen detected is Streptococci. With respect to the prevalence of environmental streptococcal mastitis, a large proportion of variability in its incidence and, both between geographical locations and within a single herd, can be ascribed to a number of independent variables such as season of the year, stage of lactation, parity, and various management practices (Hogan et al., 1989; Pankey et al., 1996; Hogan & Smith, 2003). *Streptococcus agalactiae*

is one of the obligate pathogen of mammary gland in case of bovines, colonizing the teat canal (Dodd, 1983). In our study, *S.agalactiae* was prevailing at 12.39%.

The persistence of this organism is attributed to ill hygiene and general managemental factors. Further, *S dysgalactiae* and *S uberis* are other species associated with SCM. However, these species are not an obligate pathogen of mammary gland and they enter the udder by injurying the teat (Cullor & Tyler, 1996). Furthermore, Sandholm et al. (1990) reported that *S.dysgalactiae* is a predominant   pathogen associated with summer mastitis and it's frequent isolationfrom heifers and dry cows. While *S.uberis* being opportunistic could thrive and proliferate in tissues other than mammary glad. including lips, haircoat, tonsils and the rectum of cows (Bramley et al., 1979). Todhunter et al. (1994) opined that the rate of S*treptococcal* infection was high in summer season in lactating and dry cows. Further, the type of housing and associated managemental practices in the dairy farm could lead to the contamination of bedding materials and inturn expose teats to   streptococci in the environment (Smith & Hogan, 1993; Smith & Hogan,1995). However, these factors do not appear to have contributed in the present study as the farm under investigation was well organized.
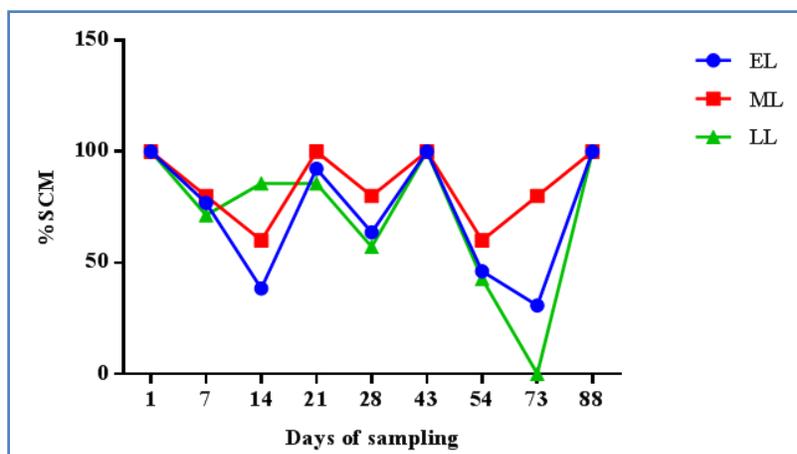

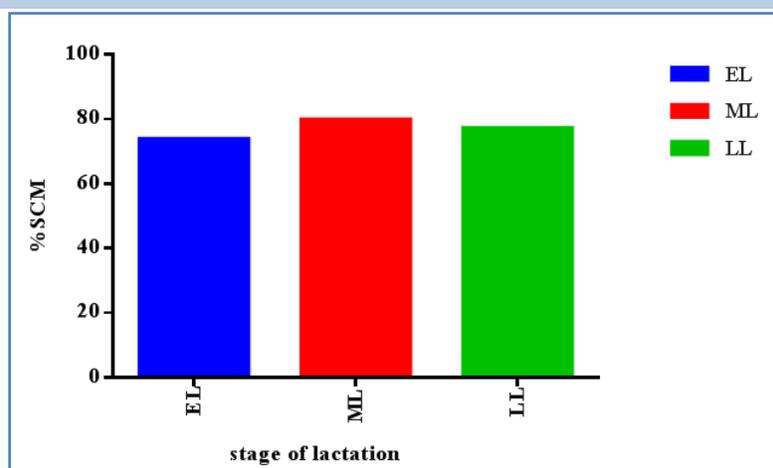Figure 3a Prevalence of SCM based on SCC in different stage of lactation.

Figure 3b Percentage prevalence of SCM based on SCC in different stage of lactation groups.

Escherichia coli is a Gram negative organism reported from the most of the bovine mastitis cases in both clinical mastitis (CM) and SCM. Although the infections due to *E.coli* are of short duration of <28 days (Todhunter et al., 1991), Many researchers reported the recurrent coliform mastitis and persistent infections due to *E.coli* in dairy animals. These studies concluded that the severity of mastitis due to *E.coli* is mainly related to host factors (Hill et al., 1979; Bradley & Green, 2001). In present study, *E.coli* was found prevalent at 15.04%, the days when SCC and mPCR were positive for SCM with the milk yield showing a negative trend. However, on certain days (28th, 54th and 73rd), there was no change in milk yield even in the presence of organism and this could be attributed to persistence stage / latent infection / carrier stage of infection / self cure, which is in agreement with Jayarao et al. (1999) who also reported that prevalence of IMI due to environmental pathogens might increase in the absence of contagious pathogens. Similarly, Schukken et al. (1989) also opined that low count of SCC due to decreased prevalence of contagious pathogens might lead to high prevalence of IMI due to environmental pathogens.

The appearance of mastitis pathogens in milk samples from a random sample of the cow population of this study revealed relationships between microbiological diagnosis and milk yield similar to those previously reported from clinical IMI. Multiplex PCR showed a similar variability as reported earlier (Hegde, 2011) and the benefits we experienced with mPCR were rapid, simple and accurate in revealing organisms. Viewed as a whole, study indicates that a positive diagnosis of *S. aureus* and *Streptococcus* species according to microbiological milk analysis of clinically normal cows correlates with production potential as opined by Reksen et al. (2007). In this study, M-PCR employed was a qualitative approach which detected the predominant species of bacteria involved in the SCM cases but not quantitative. It is necessary to estimate the bacterial load of different / various pathogens associated with SCM in order to understand the influence of the load of etiological agents on occurrence of SCM.

In the present study, the prevalence of SCM based on SCC during EL, ML and LL was 73.68%, 79.68% and 77.1% respectively (Figure.3a and 3b). The one-way ANOVA was performed on SCC of samples from the first collections to day 88, at different stage of lactation. No significant differences was observed between any stage of lactation with respect to prevalence of SCM (P>0.05). In the present study, the prevalence of SCM based on SCC was high in the third lactation (75%) which is in agreement with Islam et al. (2011) and Sripad et al. (2013) who have also reported high prevalence of SCM (47.05% and 68.89%) during the third lactation. It is well established fact that bovine immune system is less capable of battling pathogens during the periparturient period. Although exact causes for a compromised immune system are not fully understood, they are believed to be at least influenced by hormonal and metabolic changes associated with pregnancy, parturition, and onset of lactation (Burvenich et al., 2003), Although exact causes for a compromised immune system are not fully understood, they are believed to be at least influenced by hormonal and metabolic changes associated with pregnancy, parturition, and onset of lactation (Burvenich et al., 2003), Additionally, during the peripartum period a substantial reduction in the levels of trace elements, protein and energy in blood that may result in occurrence of disease (Burvenich et al., 2003). Both CM and high milk production occur more commonly in older cows and in cows early in lactation (Bartlett et al., 1990).

The present study did not reveal any association between the stage of lactation and the prevalence of pathogens. The m PCR revealed the prevalence of predominant pathogens at 35.96% (41/113) in EL; 29.68% (19/64) in ML and 33.33% (16/48) in LL Further, the application of m-PCR revealed 31.86% *S.aureus* (36/113) , 1.77% *S.agalactiae* (02/113), 8.85% *S. dysgalactiae* (10/113), 0.89% *S.uberis* (1/113), 8.85% *E.coli* (9/113) in EL; 8.85% *S.aureus* (10/113), 6.2% *S.agalactiae* (7/113), 1.77% *S.dysgalactiae* (2/113), 1.77% *S.uberis* (02/113), 1.77% *E.coli* (2/113) in ML and 11.5% *S.aureus* (13/113)*,* 1.77% *S.uberis* (02/113), 4.43% *S.agalactiae*

(5/113), 4.43% *S.dysgalactiae* (5/113) and 4.43% *E.coli* (5/113) in LL . In all the stages of lactation, *S.aureus* was found predominant. Among three stages of lactation, maximum number of milk samples (50%, 58/113) revealing the presence of pathogens were obtained from cows in the EL. However, this did not result in reduction in the milk yield during the EL. Based on this observation, it is evident that the varied distribution of organisms in different stages of lactation did not influence the prevalence of SCM and in turn affected the milk yield.

Singh & Ludri (2001) opined that the milk yield varied significantly (p<0.01) during different stages of lactation and was also negatively correlated with SCC, whereas in our study there was low correlation between milk yield and SCC (r = 0.038). Such weak correlation observed in the present work may be due to varied number of cows in different lactation stages. Furthermore, in our study, at different stages of lactation, the milk yield during the EL was at highest 3184.8 liters/cow (35.32%), with milk loss of 242.2 liters/cow (2.82%), it was followed by ML with milk production at 2074 liters/cow (22.99%) and milk loss of 792 litres / cow (8.78%). Where as in LL, the milk production was 1609.3 litres / cow (17.85%) with milk loss of 1113 litres / cow (12.34%). The milk loss was highest in LL (12.34%), followed by ML (8.78%) and least loss in EL (2.82%). The high milk yield in the EL than ML and LL in the present study is in accordance with the previous reports. Further, none of the tests employed indicated high prevalence of SCM in the EL as compared to ML and LL. Overall, the findings of the present study indicated that the stage of lactation did not influence on the prevalence of SCM.

In the present study, the animals were grouped into three age groups namely 3-5 years, 5-7 years and 7-11 years and the prevalence of SCM in these three age groups is 73.33%, 66.66% and 76.76% respectively (Figure 4a & 4b). Furthermore, the one-way ANOVA was performed on SCC with different age groups for sampling of all 9 collections.

Although, no significant difference was observed between the age groups with respect to SCC (P>0.05), and in turn the prevalence of SCM, relatively, the prevalence of SCM was higher in the age group 7-11 years. The SCC revealed the high prevalence of SCM (76.76 %) in 7-11 years as compared to 3-5 and 5-7 years age groups. The high prevalence of SCM with advancing age and in older cows draws support from the findings of earlier workers (Radostits et al., 2000; Qadri et al., 2005; Ul-Hah & Malik, 2009).

Rahman et al. (2009) also reported that the prevalence of SCM significantly increased with age in dry as well as in wet season. Islam et al. (2011) reported that the prevalence of SCM was significantly higher in the age group of animals more than 13 years at 47.61 percent. The high prevalence of SCM in older cows could be attributed to suboptimal host defensive mechanisms (Dulin et al.,1988), prior exposure to the pathogens, cumulative SCM and carrier stage (Akbar et al., 2004). The higher prevalence of SCM in older animals than in younger cows could be attributed to suboptimal defense mechanism as indicated by Dulin et al. (1988). In addition, might be the other reason for the observation of as opined by Workineh et al. (2002). The higher prevalence of SCM in the aged cross bred cows as observed in the present study was also in accordance with Samanta et al. (2006) and Mustafa et al. (2007) .

The present findings are in agreement with the general observation that the mastitis incidence and SCC levels are both higher in older cows. This paradoxical finding could be well related to the functionality of the resident milk cells where in milk PMN in primiparous cows have been found to have a higher viability and ROS production as compared to older animals (Burvenich et al.,2003; Samanta et al., 2006). Further, the findings of the present study are also supported by the observation of Hogan & Smith (2003) that the rate of IMI during the dry period was greater in multiparous cows compared with primiparous cows.
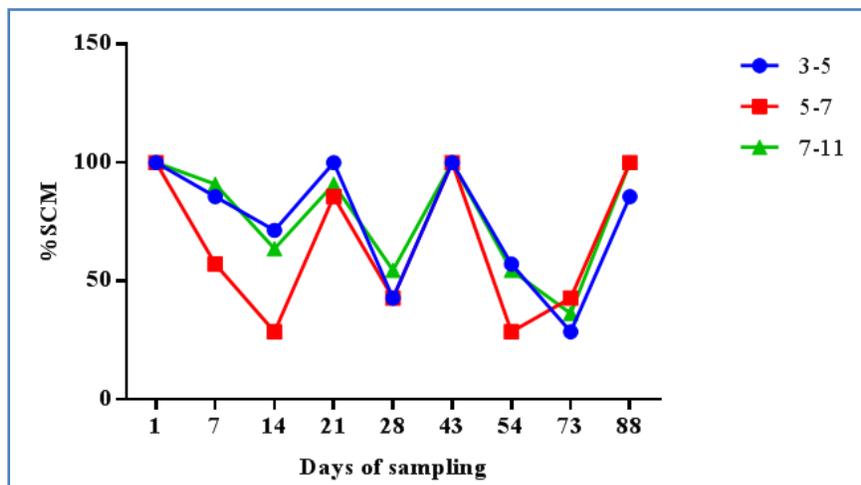

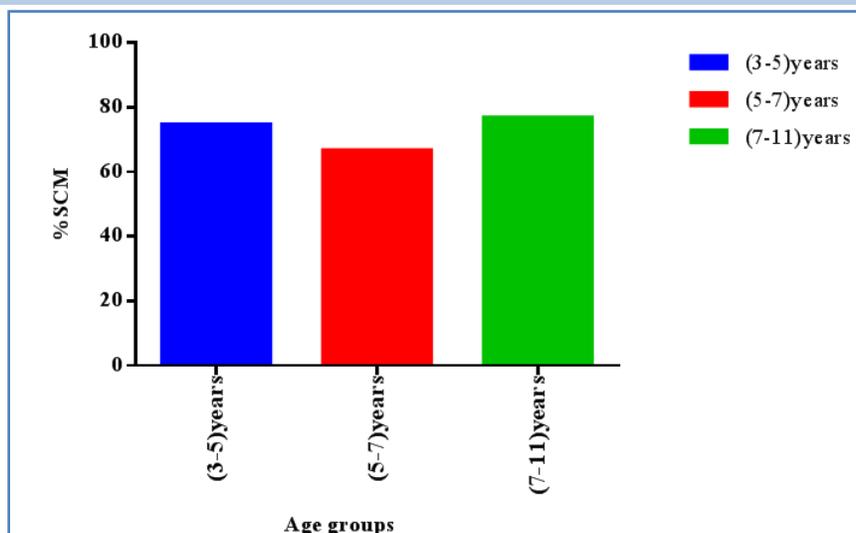Figure 4a Prevalence of SCM based on SCC in different age groups.

Figure 4b Percentage prevalence of SCM based on SCC in different age groups.

High production cows appear to be at higher risk of developing CM. Also, both CM and high milk production occur more commonly in older cows Bartlett et al. (1990). Further, high prevalence of SCM in aged cows could also be attributed to the dilatation or partial opening of teat canal in case of older cows due to repeated milking. This encourages the introduction of environment and skin–associated microorganisms into the teat canal, leading to SCM and milk production losses. In addition, cows that are multiparous / aged have poor defence mechanism. Furthermore, elevated SCC due to minor pathogens could protect the mammary gland from major pathogens (Burvenich et al., 2003). Nevertheless, the correlation between SCC and the immune response of the udder to infection is complex and unclear.

With respect to the detection of major mastitis causing bacterial pathogens in different age groups of cows, the application of mPCR revealed the prevalence of *S.aureus* at 17.77 % (20/113), *S. dysgalactiae* at 2.66 % (3/113), *E.coli* at 4.43% (5/113), *S.agalactiae* at 1.77 % (02/113), *S.uberis* at 1.77 % (2/113) in age group of 3-5 years; *S.aureus* at 11.5 % (13/113), *S.agalactiae* at 3.54 % (4/113), *S.dysgalactiae* at 5.31 % (6/113), *E.coli* at 4.43 % (5/113) in age group of 5-7 years and *S.aureus* at 23.0% (26/113)*, S.agalactiae* at 7.1 % (8/113), *S.dysgalactiae* at 7.1 % (8/113), *E.coli* at 5.31 % (6/113) and *S.uberis* at 3.54 % (4/113) in age group 7-11 years (Figure.4a and 4b). In all the age groups of cows, *S.aureus* was found predominant. Among three age groups, maximum number of milk samples (46 %, 52/113) revealing the presence of pathogens were obtained from cows in the age group 7-11 years. However, this did not affect the milk yield during the LL. In the study of Bartlett et al. (1990), pluriparous cows showed a milk loss of 2.06 times compared to that in the first lactation cows, milk loss of 1.40 times was observed in mastitic cows prior to 150 days in lactation compared to other cows and a milk loss of 1.37 times was seen in cows with mastitis during winter compared to summer season. However,

in this study, the identity of the mastitis causing agent isolated from the clinical case was not strongly associated with the drop in milk production in the 60 day following clinical onset in Based on the observations of the present study and Bartlett et al. (1990), it is evident that the varied distribution of organisms in different age groups did not influence on the milk yield.

Conclusion

In conclusion, the present study revealed the high prevalence of variable SCM pattern in milking cows by SCC (73.33 %) using sequentially collected milk samples over a period of 88 days in an organized farm. No specific pattern of prevalence of SCM was observed in the sequentially collected milk samples during the study period. The prevalence of SCM was not influenced by the stage of lactation. In all the stages of lactation, *S. aureus*, *Streptococci* and *E.coli* were detected with the predominance of *S. aureus*. The varied distribution of organisms in different stages of lactation did not influence the prevalence of SCM. Further, the high prevalence of SCM was noticed in aged cows. The M-PCR revealed the presence of *S. aureus*, *Streptococci* and *E.coli* with the predominance of *S. aureus* in all the milk samples collected from all the three age groups. Among these groups, maximum number of milk samples (46 %, 52/113) revealing the presence of pathogens were obtained from cows in the age group 7-11 years. The M-PCR assay employed in the present study was an easy and rapid method to detect the predominant pathogens causing SCM. Hence, the regular analysis of milk samples by M-PCR may be a useful tool for determining the herd status with regard to the detection of contagious and environmental mastitis pathogens. The result indicated the presence of both contagious and environmental mastitis pathogens. This emphasizes continuing need to concentrate on control both contagious pathogen such as *S. aureus* and environmental pathogen especially *E.coli* through sequential monitoring of

SCM through application of SCC, M-PCR and proper managemental practices.

Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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