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Assessment of the common methods for diagnosis of bovine subclinical mastitis

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Abstract

A total of 69 lactating Jersey crossbred cows of an organised dairy farm were examined for the presence of subclinical mastitis. The diagnosis of subclinical mastitis was done on the basis of somatic cell count (SCC) in the milk samples. The other methods of diagnosis of subclinical mastitis such as California Mastitis Test (CMT), Bromothymol Blue (BTB) test, microbial culture and milk yield evaluation were also performed. Different tests revealed variable results. It was found that although these diagnotic methods correlated linearly with SCC but not fully. CMT and BTB failed to detect the presence of subclinical mastitis in 10 and 24 percent cases, respectively; where SCC was more than 0.4 million cells/ml. However, twenty percent of cows were harboring pathogenic bacteria without any abnormal change in SCC, whereas in 41 percent cows, a high somatic cell count was observed in the absence of any bacteria. Furthermore, daily milk yield had a negative but weak correlation with SCC. No difference in the presence of various milk proteins were revealed by SDS-PAGE in the milk samples containing 0.2, 0.7, 1.3 million cells/ml, respectively. Thus, it was concluded that the correlation between the different methods of testing subclinical mastitis is tenuous and as such none of the tests may be regarded as confirmatory for the diagnosis of subclinical mastitis. This study suggests the development of alternative methods for achieving the confirmatory diagnosis of subclinical mastitis.

Key words: Subclinical mastitis; somatic cell counting; California mastitis test; bromothymol blue test; microbial culturing of milk.

Mastitis in cows is the inflammation of mammary gland that is usually caused by invading pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae*, mycoplasma, coliform, and yeast**.** It is a common health problem in lactating dairy cows; a serious animal welfare issue (Webster 1999; Broom & Fraser 2007) and causes huge economic loss to the dairy farmers due to reduction in milk production (Degraves & Fetrow 1993; Hortet & Seegers 1998; Huijps *et al,* 2008), treatment costs, and milk wastage (Halasa *et al.* 2007). In addition, the extensive use of antibiotics to treat mastitis potentially lead to increased antibiotic resistance to mastitis pathogens (Hendriksen *et al.* 2008) and increased levels of antibiotics in the secreted milk (Mitchell *et al*. 1998; Moretain and Boisseau 1989). The effective means of controlling mastitis is by prevention, however under the best prevention and control programs, mastitis is prevalent in both developing and developed countries despite stringent hygienic conditions. Therefore, diagnosis of this disease at initial stage or at subclinical level is critical failing which its management becomes more difficuilt, resulting in increased economic lossess due to reduced milk production. In fact, subclincal bovine mastitis is more prevlant than clinical mastitis (Akers 2002). However, the difficulty with the subclinical mastitis is that its detection is not easy in contrast to easily detectable clinical mastitis. Unfortunately, most infections are not detected until clinical signs are evident and by then extensive and costly irreversible damage to the udder tissue have already happened compromising the milk producing capability of even the genetically high yielders during their current and subsequent lactations (Houben *et al*. 1993; Rajala-Schultz *et*

al. 1999; Wilson *et al*. 2004). The success rate of curing mastitis is greater when infections persist for a shorter duration and therefore earlier detection and treatment is important.

In India, the estimated economic losses due to mastitis are around 526 million dollars per annum (Varshney and Naresh 2004). Various methods such as SCC, CMT, BTB, microbial culturing and milk yield evaluation are used to detect subclinical mastitis. Somatic cell count in milk is considered as the most useful method for detecting subclinical mastitis and more than 0.4 million cells/ml SCC indicates subclinical mastitis (Malinowski 2001). The present study was undertaken to evaluate different methods of diagnosis of subclinical mastitis with the objectives to ascertain a particular test to be used as confirmatory for diagnosing subclinical mastitis.

Materials and Methods

Experimental animals: The lactating Jersey crossbred cows of the Livestock Farm of Dr. G.C. Negi College of Veterinary and Animal Sciences, CSKHPKV, Palampur, India were used in this study. All the cows used were in different parities and were of mixed age groups. These were managed under loose housing system and were fed with generous allowance of mixture of green fodder, dry grass and wheat straw. In addition the concentrate feed was provided as per the routine standards. All the cows had *ad libitum* access to the clean drinking water. These animals were milked twice daily using 'Full Hand' manual milking method.

Collection of milk samples: Milk samples from individual quarter of each cow were obtained aseptically. First three or four streaks of milk were discarded and the next 20 ml milk was collected in autoclaved dry test tubes to be used for later analysis using various tests.

Somatic cell count: Somatic cell count in the milk samples was done as described by Prescott and Breed (1910). Briefly, 10 µl of milk sample was smeared on a clean glass slide in one square cm area and allowed to dry at room temperature. The slide was stained in Newman's Lampert stain (methylene blue 1g, 95% ethyl alcohol-54 ml, tetrachloroethane 40 ml & glacial acetic acid 6 ml) for two min followed by drying at room temperature. The dried slide was rinsed carefully with tap water, drained and dried again. The one square cm area of the milk-smear was divided into four equal parts. Five random microscopic fields in each part were used for counting somatic cells under 100X oil immersion using Magnus MLX-DX Olympus (India) microscope. The average number of cells per square cm area was calculated. The cells per ml of milk were calculated by multiplying the average number of cells per square cm area with the microscopic factor which was 543900 for the microscope used in this study.

California Mastitis Test (CMT): CMT was done by mixing 2 ml of milk with equal amount of CMT reagent (bromothymol blue -10 mg, sodium hydroxide- 1.5 g, teepol -15 ml & distilled water -1000 ml) in a gentle circular motion. The reaction was scored as 0 for no precipitate or color change, 1 for slight precipitate which appeared on continuous moment, 2 for distinct precipitate without gel formation, 3 for moderate gel formation with increased viscosity, and 4 for thick gel formation sticking at the centre of the cup.

Bromothymol blue (BTB) pH indicator test: Bromothymol blue indicator paper was used for conducting this test. Equal length of bromothymol blue indicator paper was impregnated with few drops of milk sample and allowed to dry. The colour of the indicator paper was scored as 0 for yellow colour, 1 for slight greenish yellow, 2 for light greenish blue, 3 for intense greenish blue, and 4 for blue colour development.

Culturing of milk: Milk samples were processed for isolation & identification of bacteria as per the standard procedures cited by Cruickshank et al. (1975) and Carter (1995). Different media *viz.* nutrient broth and agar, 5% sheep blood agar and Ayer's and Johnson's agar were used. For mixed bacterial growth, pure cultures were obtained by re-streaking the individual colony on blood agar. The bacteria were identified based on characteristic cultural, morphological & biochemical characters.

SDS-PAGE of milk and plasma: SDS-PAGE of milk and plasma from subclinical mastitic cows (n=3) (based on high somatic cell count) as well as healthy cows (control; n=3) was performed on 12% acrylamide/bis-acrylamide sodium dodecyl sulphate gels. Samples of milk and plasma were obtained from cows having somatic cell count of 0.2, 0.7 and 1.3 million cells/ml milk, respectively as well as from healthy controls. All the milk samples were used after the removal of fat by centrifugation at 1500 *g* for 20 min. The protein concentration of skimmed milk and plasma was measured using Biuret method before loading on to the gels and each loaded sample contained 60 µg proteins. Gels were viewed by staining with Coomassie Brilliant Blue dye.

Statistical analyses: The sensitivity, specificity, positive predictive value and negative predictive values of CMT and BTB test were also calculated by taking SCC as the standard method. The correlation between SCC, CMT scores, BTB scores, bacterial presence and milk yield was calculated by Pearson product moment correlation coefficient (r); a dimensionless index that ranges from -1.0 to 1.0 and reflects the extent of a linear relationship between two data sets, using standard statistical procedures (Daniel 1990).

Results and Discussion

On the basis of somatic cell count 40 per cent of cows exhibited subclinical mastitis, that is having more than 0.4 million cells/ml of milk. However, on the basis of CMT, 44 percent cows showed positive results. However, not all the cows having a higher somatic cell count had a positive CMT result. About 8 percent cows had high somatic cell count without any appreciable positive CMT result. Whereas, 7 percent cows had a high positive CMT test (scoring 3 or 4) with a normal somatic cell count.

BTB test which detects mastitis based on pH change indicated subclinical mastitis in 18.6 per cent cows only. Twenty four percent of cows having more than 0.4 million somatic cells/ ml of milk did not show a positive BTB test. And 2.9 per cent cows showed a positive BTB test (scoring 3 or 4) despite having a normal somatic cell count.

In diagnosing subclinical mastitis, CMT was 75 per cent sensitive, whereas, BTB was 60 per cent sensitive. However, BTB was more specific (95 per cent) as compared to CMT (87 per cent). Positive predictive value for CMT was 83 per cent, whereas for BTB test it was 93 per cent. Negative predictive values for CMT and BTB were 80 per cent and 67 per cent, respectively. The culturing of milk samples revealed pathogens in 54 per cent cows. *Staphylococcus* spp. were present in 79 per cent of the samples and thus was the most abundant pathogen, whereas *Bacillus* spp., *E. coli* and *Proteus* spp. were detected in 21 per cent samples. Interestingly, 20% percent of cows were harboring pathogenic bacteria without any abnormal change in SCC. On the contrary, 41 per cent cows had more than 0.4 million somatic cells/ml of milk but with no pathogenic bacterial growth in milk.

In this study (n=69), the correlation co-efficient was 0.43 between SCC and CMT, 0.52 between SCC and BTB, 0.12 between SCC and bacterial presence, 0.58 between BTB and CMT, 0.19 between BTB and bacterial presence, 0.05 between BTB and milk yield, 0.18 between CMT and bacterial presence and -0.15 between CMT and average 10 days milk yield. There was a weak negative correlation (-0.04) between SCC and average 10 days milk yield. A negative correlation of -0.17 was found between milk yield and microbial presence in the milk. SDS-PAGE of plasma or milk samples did not reveal any different protein in samples conatining 0.2, 0.7, 1.3 million cells ml, respectively as compared to the samples from healthy controls.

SCC in milk is the most widely recommended method of diagnosing onset of mastitis. This study shows that high somatic cell presence did not always associate with a peak presence of pathogenic milk bacteria. Earlier studies (Schepers *et al*. 1997) have also indicated that SCC does not always correlate with udder health status. Similar observations were seen in present study regarding the usefulness of SCC for detecting subclinical mastitis. The microbial culture of milk samples appears as an important parameter along with SCC for diagnosis of subclinical mastitis and has been the recommended method for detection and verification of subclinical infectious mastitis (Hillerton 1999). However, bacteriological sampling and examination is a time consuming and expensive process. This may be the reason that the other quick tests such as CMT and BTB are usually performed. Reduction in milk yield is also indicative of subclinical mastitis. A negative correlation between milk production and SCC in the present study also supports this observation. These results were akin with earlier studies (Bramley 1992; Harmon 1994; Hortet *et al*. 1999; Halasa *et al.* 2009), where higher SCC was associated with lower milk production. However, the negative correlation in this study indicated that a high SCC does not always coincide with reduced milk production. Similar result was found between CMT and milk yield. The correlation was positive between milk yield and BTB test scores. As CMT, BTB and SCC are linearly and positively correlated with presence of mastitis and SCC does not positively correlate with milk yield, but BTB and CMT tests correlate with milk production. In the present study, BTB and CMT tests exhibited different specificities and sensitivities with reference to SCC as standard. In addition, the findings of Dingwell *et al.* (2003) stated that the sensitivity and specificity of CMT for diagnosing intramamary infection vary with the

lactation stage of the cow, makes this test less reliable.

If microbial presence is taken as the gold standard for diagnosing subclinical mastitis, this appears to be incomplete method for diagnosis as there is a weak correlation between the bacterial presence (*Staphylococcus* spp.) in the milk and onset of inflammatory process as evident by SCC. This indicates that microbial presence does not correlate well with a concurrent increase in the SCC and the bacterial count and the SCC will not necessarily peak at the same time (Daley *et al*. 1991). In the present study the four commonly used tests to detect mastitis i.e. SCC, CMT, BTB and microbial presence detected mastitis in 40, 44, 18.6 and 54 per cent cows, respectively. There is considerable variation in the results to determine subclinical mastitis. The confirmatory diagnosis of subclinical mastitis is difficult and perhaps often inaccurate and inconclusive using these methods.

No protein variation was observed in SDS-PAGE of blood and milk samples of affected and healthy cows which indicate that milk proteome may not be a useful indicator to confirm subclinical mastitis; thus requiring identification of either specific genome based bio-markers. Despite many years of research in bovine mastitis diagnostics, there are few alternatives to SCC for identification of cows with subclinical mastitis. Many efforts have been made to find alternative biomarkers in order to replace or complement SCC, e.g. determination of antitrypsin, serum albumin, electrical conductivity, lactose and N-acetyl-β-D-glucosaminidase (NAGase) activity but so far their correlation with mastitis is limited (Mattila *et al*. 1986; Biggadike *et al*. 2002; Pyorala 2003). Further work in developing quick and efficient new biomarkers is required which can serve as confirmatory for diagnosing subclinical mastitis. It is further emphasized that identification of biomarkers for specific mastitis pathogens may contribute to an improved and faster detection of the subclinical mastitis compared to the conventional methods of diagnosis.

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Table1. Percentage of lactating cows detected with subclinical mastitis by various tests (n=69)

Somatic cell counting	CMT	BTB	Presence of *Staphylococcus* spp.	Presence of *Bacillus*, *E. coli* , *Proteus* spp.
40%	44%	I8.6%	79%	21%

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