African Journal of Agricultural Research Vol. 7(41), pp.5578-5584, 29 October, 2012
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR12.922
ISSN 1991-637X ©2012 Academic Journals

Full Length Research Paper

Comparison of somatic cell count, California mastitis test, chloride test and rennet coagulation time with bacterial culture examination to detect subclinical mastitis in riverine buffalo (*Bubalus bubalis*)

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Accepted 17 July, 2012

The study investigates the etio-prevalence of bubaline subclinical mastitis (SCM) with reference to relationship between somatic cell counts (SCC), California mastitis test (CMT), rennet coagulation time (RCT) and chloride test with bacterial cultural examination. Each test was conducted by standardized protocol. The prevalence of subclinical mastitis (SCM), latent infected quarters and non-specific infected quarters were found to be 17.83, 2.89 and 3.06%, respectively, when partitioned on the basis of International Dairy Federation criteria for SCC. S*taphylococcus* spp. (44.70%) and *Streptococcus* spp. (34.20%) were the most prevalent etiological agents isolated in this study. The mean values of SCC and RCT were compared separately in healthy and SCM milk infected by different etiological agents by Duncan multiple range test. Mean comparison for these values for healthy and SCM milk was done by t-test. The elevation recorded for SCC and RCT was highly significant ($p<0.01$) irrespective of the bacterial agents causing SCM. After calculating the percent sensitivity, specificity and accuracy, predictive values and likelihood ratios, it can be accomplished that SCC was mostly in agreement with bacterial culture examination. It is recommended that it is better to cross match the result of SCC with RCT and CMT. Chloride test is not suggested to diagnose SCM in riverine buffaloes.

Key words: Bacterial culture, buffalo, California mastitis test, chloride test, rennet coagulation time, somatic cell count, subclinical mastitis.

INTRODUCTION

Buffalo farming guarantees huge pecuniary gain to the national financial system of many countries in the world (Atasever et al., 2011). India is not an exception where buffalo production has long been recognized as the backbone of rain-fed agrarian socio-economic fabric and has been recently recognized as the main reason behind absence of suicides in buffalo tract when rain fails but a farmer falling to tragic ends, when draught hits the Bidharba, central or eastern India. The sordid turn of events towards the end of 2008 world wide on economic and industrial front has left all special and sundry aghast. India is no exception where gains in animal husbandry, horticulture, and rice overwhelmed the poor harvest of sugarcane, cotton, pulses and oilseeds and brought the nation to second best growth rate (5.3%) worldwide next to China.

Milk has emerged as the second best agricultural

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commodity next to rice production in India. The National Commission on Agriculture had put dairy next to agriculture. Dairy is emerging as a better alternative for diversification in agriculture, especially, under recession containing tools recommended as umbrella action modalities. It can provide cushion against crop failures (Guha and Gera, 2012b).

Mastitis namely, clinical and subclinical, is an economically damaging disease of the dairy industry, which causes physical, chemical and bacteriological alternation in the milk and blood along with morpho-pathological changes in the mammary gland (Guha et al., 2012). The causes of mastitis involve a complex relationship of three major factors, that is, host resistance, bacterial agents and the environmental factors (Gera and Guha, 2011b). Of the two, subclinical mastitis causes more damage to the dairy industry as it is often precluded owing to absence of visible changes in the udder and milk. In addition, the bacterial contamination of milk from affected cows render it unfit for human consumption and provide a mechanism of spread of diseases like tuberculosis, sore-throat, Q-fever, brucellosis and leptospirosis etc and has zoonotic importance (Sharma et al., 2011). Hence, timely detection with suitable test and treatment is essential (Gera and Guha, 2011a).

As a common perception, the inflammation of udder strikingly augments the somatic cell count (SCC) in milk, leading to low-grade processing characteristics and reduced acceptance of dairy products due to changes in components and properties of raw milk. Indeed, rise in the leucocyte number in milk and in the mammary gland, as a response to the assaulting pathogens or to their metabolites leads to an increase in SCC (Atasever, 2012). For this reason, the most reliable index next to bacterial culture examination, subclinical mastitis (SCM) detection is by somatic cell count (SCC) (Guha et al., 2012). According to International Dairy Federation (IDF, 2005) threshold limit of 200×10^3 cells/ml of milk had been recommended for SCM. In EU countries, according to the directive 92/46/EEC, bulk milk samples with SCC greater than 400×10^3 cells/ml of milk is advised not to be used for human consumption (Atasever et al., 2010).

Quality milk production is a major problem for milk sector in developing nations like India, where, milk may be a detrimental product for people, when efficient techniques related to hygiene, storage or control has not been performed. That is the most important reason, why, in many parts, a payment system based on quality milk production has been in progress. Thus, early diagnosis and prevention of mastitis must be a priority for each dairy holder because of the acquisition of good quality milk and the prevention of economical losses (Atasever and Erdem, 2010).

SCM is a herd problem, acts as a repository of micro-organisms that leads to the spread of infection to the other animals undetectable to naked eyes. Detection of mastitis at subclinical form is also ignored owing to mixing of milk from different sources without proper checking at milk collection point in the villages. Also, the accuracy of indirect tests to detect mastitis in field varies from place to place. Thus, their sensitivity, specificity, accuracy, predictive values and likelihood ratios for diagnosing SCM need to be evaluated from time to time under different environmental conditions.

The objective of the present study was to investigate the etio-prevalence, the efficacy of SCC, California mastitis test (CMT), chloride test and rennet coagulation time (RCT) taking bacterial examination as the benchmark to diagnose SCM.

MATERIALS AND METHODS

Milk samples from 615 riverine buffaloes comprising 2452 quarters from the local herds of Hisar city, Haryana, India were collected aseptically in sterile containers as Right fore (RF), Left fore (LF), Right hind (RH) and Left hind (LH) to find the prevalence of SCM on the basis of quarter. Milk samples were collected according to the National Mastitis Council (1990). After a quarter had been cleaned up by removing any possible dirt and washed with tap water, the teat end was dried and swabbed with cotton soaked in 70% ethyl alcohol.

California mastitis test was conducted on the milk samples following the methods as described by Dhakal (2006). Briefly, mastitis reagent was prepared by dissolving 3 g sodium lauryl sulfate in 100 ml warm distilled water. The suspension was kept for an hour and then mixed gently to avoid any frothing to make a clear solution. The pH of the solution was adjusted to 8.0. Bromocresol purple at the ratio 1:10,000 was added to the solution. The test required a plastic paddle having four chambers. The testing reagent was dispensed in plastic bottle provided with a fine nozzle. Equal volume milk and reagent was put in each cup and gently rotated by movement of the paddle in a horizontal plane. The reaction was observed immediately. The grading and interpretation was done as shown in Table 1. Score of 1 or more was considered positive for the test.

Chloride test (CT) was performed as described in the laboratory manual of milk industry foundation called “Analysis of milk and its product (2005)”. Briefly, to 5 ml of 0.1341% $AgNO_3$ solution, two drops of 10% K_2CrO_4 was added. This gives red color. To this mixture, 1 ml of milk was added. The milk containing abnormally high percentage of chloride (> 0.14%), yellow color appeared, indicating a positive sample. (The yellow color is due K_2CrO_4). Persistent red color (due to formation of silver chromate) indicated a negative sample.

The milk samples were simultaneously cultured, isolated and identified by the method as described by Guha et al. (2010). Briefly, One hundred microlitre of milk from each quarter was streaked on to MacConkey agar and 5% ovine blood agar plates for bacterial culture and isolation. The colonies were counted after 48 h incubation at 37°C. The resulting growth was identified on the basis of morphology, colony characteristics and Gram’s reaction. Animal culturally positive for at least a single quarter was considered SCM positive.

The rennet coagulation time (RCT) of the milk was estimated as per the method described by Klandar et al. (2007), using 0.2% Rennet solution. Somatic cell counting was done microscopically by the method as described by Guha et al. (2012). Briefly, following thorough mixing of milk samples and with a 4 mm diameter platinum loop, 0.01 ml of milk was spread evenly over four 1.0 cm^2 area template outlines. Slides were stained for 30 s in Newman-

Table 1. CMT scoring of milk samples from clinically normal buffaloes.

Symbol	Suggested meaning	Description of visible reaction
-	Negative	Mixture remains liquid with no evidence of formation of precipitate.
T	Trace	A slight slime with no tendency towards gel formation.
1	Weak	A distinct slime with no tendency towards gel formation
2	Distinct positive	Mixture thickens immediately with gel formation. On continued swirling, mass moves around the periphery leaving the bottom of the cup exposed.
3	Strong positive	A distinct gel forms which tends to adhere to the bottom of the paddle and during swirling a distinct central peak is formed.

Table 2. Frequency of isolation of different bacteria from culturally positive quarters of riverine buffaloes.

Genus	Animal		Quarters	
	n	%	n	%
Staphylococcus spp.	66	43.42	228	44.7
Streptococcus spp.	51	33.55	174	34.2
Escherichia coli	30	19.74	94	18.5
Others (*Corynebacterium* spp. and *Bacillus* spp.) +mixed infection	5	3.29	12	2.6
Total	152	100.00	508	100.00

Lampert stain is 1.2 g Methylene blue, 54 ml 95% ethyl alcohol, 40 ml tetrachloroethane and 6 ml glacial acetic acid.

Somatic cells were stained with deep blue nuclei against light blue background. The working factor of the microscope was calculated to be 35400 by using stage micrometer, calculating diameter of the microscopic field (0.012 cm) and field per square cm (8850) for the given microscope. Total number of cells was obtained by multiplying the total number of cells counted in 25 fields with the working factor.

The percent sensitivity, specificity, accuracy, positive and negative predictive values and likelihood ratios (both positive and negative) were calculated by the formulae of Petrie and Watson (2008) taking bacterial growth in culture media as benchmark (Gera and Guha, 2011b). The cut-off values for each significantly altered parameter were obtained from Receiver Operator Characteristic (ROC) analysis curve with the aid of the MedCalc software (Petrie and Watson, 2008). Mean of SCC and RCT in healthy and SCM milk as a whole and separately for each causative agent was done by t-test and Duncan multiple range test (DMRT), respectively. Mean of SCC and RCT between negative and positive CMT and chloride test were also compared with Duncan Multiple Range Test (DMRT). Analyses were performed using “SPSS (1999)”, the same recommended by Petrie and Watson (2008).

RESULTS AND DISCUSSION

Out of 2452 quarter milk samples culturally examined, 508 milk samples comprising 152 animals, showed bacterial growth in blood agar media. Based on the result of culture examination, the prevalence of subclinical mastitis in riverine buffalo (on animal basis) and 20.72% (on quarter basis) in the present study was 24.72% (Table 5), which was higher than the report of Saini et al. (1994) and lower than the observation of Chavan et al. (2007). The dissimilarity might be due to the differences in the managemental practices, genetic divergence and climatic conditions (Ramprabhu and Rajeswar, 2007).

From Table 2, it is evident that the most prevalent etiological agent (on quarter basis) was *Staphylococcus* sp. (44.70%) followed by *Streptococcus* sp. (34.20%) and *Escherichia coli* (18.5%). Twelve samples with mixed infection were detected in the study, of which, 4 *Staphlococcus* sp. and *Streptococcus* sp., 3 *Staphlococcus* sp., *Streptococcus* sp., *E. coli and Bacillus* sp. and 5 *Staphlococcus* sp. and *Corynebacterium* sp. Similar observations were found by Chavan et al. (2007) which was attributed to abundance of the organisms in the atmosphere.

The pie-chart representation of the percentage of quarters positive for SCM is shown in Figure 1. The prevalence of SCM was observed more for hind quarters than the fore quarters. Similar observation was reported by Sharma et al. (2007). The hind quarters are closer to the legs and thus more exposed to dung and urine, that is, unhygienic condition than the front quarters. In addition, more turn over of milk in the hind quarters make them more susceptible to wear and tear, hence, prone to inflammatory reactions (Ramprabhu and Rajeswar, 2007).

The rennet coagulation time (RCT) in the present study showed significant ($P<0.01$) increase in milk samples from infected quarters (Table 3) irrespective of the etiological agent. This finding is in agreement with the report of Tripaldi et al. (2003). The reason put forth by previous worker was increased alkalinity in mastitis milk samples or decreased level of calcium, augmenting

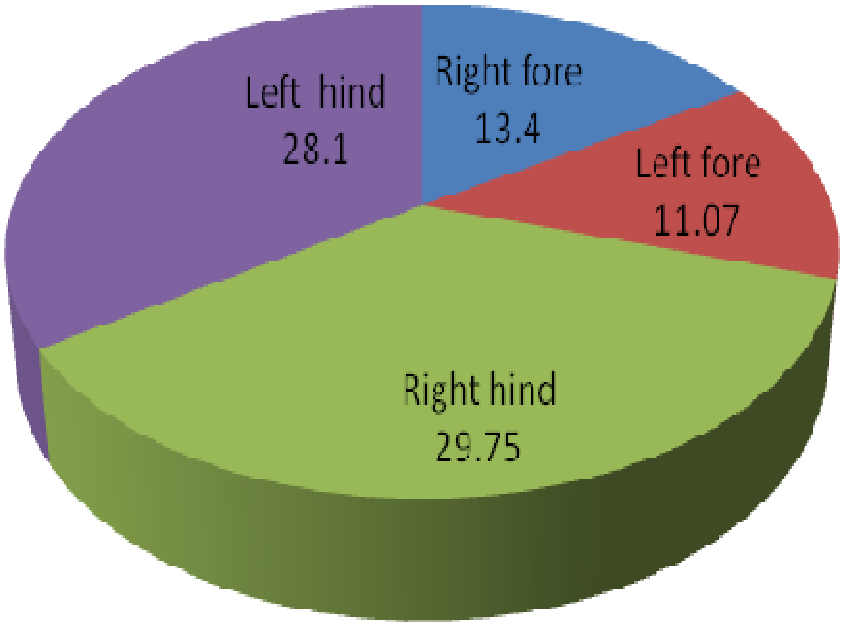

Figure 1. Pie chart representing the prevalence of subclinical mastitis in buffaloes in different quarters.

rennet gel formation (Wang et al., 2007). The cut-off value calculated in the present study was 278.65 (Table 4). To the best of the knowledge of all the authors, no literature exists to support our calculated cut-off value.

From Table 3, it is also evident that SCC is significantly higher ($P<0.01$) in SCM milk samples caused by different bacterial agents. The National Mastitis Council defines subclinical mastitis in cows as a quarter with a SCC of 200×10^3 cells / ml of milk or more, with a normal quarter having counts around 100×10^3 cells / ml of milk (Dhakal, 2006). In the present study, the cut-off value of SCC of SCM milk was 215 × 103 cells /ml or Log10 5.34 cells/ml of milk which is marginally higher (Table 4) than the observation of Dhakal (2006). The possible reason may be due to compositional changes of the milk, environmental stress and quarter udder capacity of the buffaloes studied (Guha et al., 2012). This threshold for the SCC may be useful for detection of subclinical mastitis in buffaloes in the future. But in the present study, the IDF criteria that is, 200 × 103 cells / ml of milk was taken as the threshold limit for SCM milk.

Taking into account the IDF (2005) criteria for SCC alone (Table 5), 32.20% of the buffaloes and 20.89% of the quarters were found to have SCC ≥ 200 × 103 cells /ml of milk. Guha and Gera (2012a) reported similar figures in crossbred cow milk. It is also observed that though elevation of SCC reflects inflammatory reactions in the udder, yet, with SCC, the infected milk samples detected were less compared to bacterial growth in culture media. The possible reason for lesser infection diagnosed by SCC than cultural examination might be due to recent latent infections (2.89%), which might be due to colonization of teat canals by mastitogenic agents (Guha and Gera, 2012a).

In the present study, 3.06% quarters was observed (Table 5) having 'non-specific' infection. Failure to detect pathogens in such cases might be due to spontaneous recovery (Guha and Gera, 2012b). Seasonal effects, diurnal variations, physiological stress and environmental heat stress were reported to increase SCC without any inflammatory reaction (Guha et al., 2012).

The result of degree of gel formation during CMT of milk samples from clinically healthy buffaloes are grouped on the basis of Table 1. From Table 6, it is clear that a total of (234 + 396 + 98 =) 728 milk samples showed weak to strong gel formation considered as CMT positive milk samples. Our observations concur with the reports of Guha and Gera (2012a), Barlowska et al. (2009) and Leitner et al. (2004) in species other than buffalo. The scores of CMT was further compared with the result of bacterial culture examination (Table 6), where we found that E. coli was isolated maximum in CMT scores with trace gel formation. The positive reaction of CMT is due to alkalinity owing to the increase of inflammatory cells that is, SCC. From Table 3, it is evident that the SCC was least for *E. coli.* This might be attributed to the fact that gram negative bacteria like *E. coli* are less pathogenic in the destruction of the mammary gland epithelia and lesser generation of inflammatory reaction, hence, less inflammatory cells (Guha et al., 2012).

Comparison of positive and negative CMT and chloride test with SCC and RCT (Table 7) revealed that SCC and RCT showed highly significant ($P<0.01$) increase in CMT

Table 3. Effect of different bacterial agents on somatic cell count in healthy and subclinical mastitis milk of riverine buffalo.

Parameter	Mean ± S. E. of healthy milk (n=508)	Mean ± S.E of subclinical mastitis milk (n=508)			
		Staphylococcus spp. (n = 228)	*Sterptococcus* spp. (n=172)	*Escherichia coli* (n=95)	Others + mixed infection (n=19)
Somatic cell count (x 10^3 cells ml^{-1})	94* ± 0.61	218** ± 2.45	215** ± 3.52	205** ± 1.92	211** ± 3.37
Somatic cell count (Log_{10} cells ml^{-1})	4.973* ± 2.79	5.35** ± 3.38	5.33** ± 3.54	5.31** ± 3.28	5.32** ± 3.52
Rennet coagulation time (s)	128.70^a ± 3.80	285.27** ± 6.98	283.95** ± 5.47	275.18** ± 4.06	278.96** ± 3.39

Mean having different superscripts * and ** horizontally differ significantly (P<0.01).

Table 4. Mean ± S.E of somatic cell count and rennet coagulation time in healthy and subclinical mastitis (irrespective of the causative agent) in riverine buffalo to decide the threshold limit (n=508).

Parameter	Healthy milk	Subclinical mastitis milk	Cut-off points
Somatic cell count (x 10^3 cells ml^{-1})	94* ± 0.61	217** ± 2.83	215
Somatic cell count (Log_{10} cells ml^{-1})	4.973* ± 2.79	5.37** ± 3.45	5.34
Rennet coagulation time (seconds)	128.70* ± 3.80	286.07** ± 12.87	278.65

Mean having different superscripts * and ** horizontally differ significantly (P<0.01).

Table 5. Prevalence of subclinical mastitis on the basis of bacteriological examination and somatic cell count in milk samples from riverine buffaloes.

Buffaloes culturally positive	Quarters culturally positive	Buffaloes showing SCC > 200 × 10^3 cells/ml	Quarters showing SCC > 200 × 10^3 cells/ml	Quarters showing SCC > 200 × 10^3 cells/ml and culturally positive (SCM)	Quarters showing SCC < 200 × 10^3 cells/ml and culturally positive (latent)	Quarters showing SCC > 200 × 10^3 cells/ml and culturally negative (non-specific)
152/615(24.72)	508/2452(20.72)	198/615(32.20)	512/2452(20.89)	437/2452(17.83)	71/2452(2.89)	75/2452(3.06)

Figures in parentheses indicate percentages.

Table 6. Comparison between California mastitis test scores with bacterial culture examination.

California mastitis test scores	No. of milk samples showing bacterial growth				No. of milk samples showing absence of bacterial growth
	Staphylococcus spp.	*Streptococcus* spp.	*Escherichia coli*	Mixed infection	
N = Negative (1527)	0	1	18	1	1507
T = Trace (197)	1	2	69	2	123
1 = Weak (234)	54	34	4	4	138

Table 6. Contd.

2 = Distinct positive (396)	125	103	2	2	164
3 = Strong positive (98)	48	34	1	3	12
Total	228	174	94	12	1944

Figures in parenthesis indicate number of milk samples.

Table 7. Means ± S.E of somatic cell count (SCC) and rennet coagulation time (RCT) of negative and positive California mastitis test (CMT) and chloride test in milk samples from clinically normal riverine buffaloes.

Parameter	No. of milk samples	SCC × 10^3 cells / ml of milk	Log_{10} SCC	Rennet coagulation time (s)
CMT negative	1724	165* ± 9.87	5.22* ± 3.99	197 ± 12.98*
CMT positive	728	214** ± 6.54	5.33** ± 3.82	273 ± 19.09**
Chloride test negative	1675	183* ± 6.17	5.26* ± 3.79	209 ±22.34*#
Chloride test positive	777	204** ± 7.35	5.31** ± 3.86	252 ± 27.98##

Mean having different superscripts * and **, # and ## vertically differ significantly at P<0.01 and P<0.05, respectively. CMT score of above 1 was considered CMT positive milk samples.

positive milk samples. SCC was also significantly high (P<0.01) in chloride test positive milk samples than chloride test negative milk samples. But, increase of RCT in chloride test positive samples was significant at P<0.05. The SCC and RCT of CMT and chloride test negative was P<0.05. The SCC and RCT of CMT and chloride test negative was insignificant, which was also true when compared with positive CMT and chloride test. Similar observation of SCC status in CMT negative quarters in clinically normal buffaloes was reported by Dhakal (2006).

Once we peruse percent sensitivity, specificity, accuracy, predictive values and likelihood ratios, it is obvious that SCC is more in agreement with the bacterial culture test followed by RCT, CMT and chloride test (Table 8). In the present study, CMT was found to be more sensitive, specific and accurate than chloride test in diagnosing SCM in the field. This might be due to quick immune response to foreign agents by the defense cell than alternation in the chloride concentration in the milk (Chander and Baxi, 1975).

To assess the exactness of the tests, the predictive values and likelihood ratios were calculated (Table 8). A positive predictive value indicates the proportions of animals with positive tests which are really diseased.

The likelihood ratio of a positive test result expresses how much more likely the animal is to have a positive test result when actually diseased than if disease free (that is, it is the ratio of the likelihoods of having and not having the disease). Likelihood test of a positive test result >10 indicates that the test can be used to rule the disease (Petrie and Watson, 2008). From Table 8, it is obvious that out of all the tests conducted; only the likelihood ratio for positive test for SCC was found to be greater than 10 irrespective of the bacterial agent causing SCM. To the best of our knowledge, this type of statistical model was not reported till date to support our findings. Although, the likelihood ratios of RCT and CMT is less than 10, yet, their percent sensitivity, specificity and accuracy values are not undersized to overlook them to be used in SCM screening programs. The same cannot be said for chloride test.

Conclusion

Though SCC is the only test to have likelihood ratio > 10, yet, SCC alone is diagnostically insufficient sometimes due to latent and non-specific infections. Thus, it can be concluded that in order to substitute cultural examination, the results of RCT and CMT supported by SCC is better for pinpoint diagnosis of SCM in riverine buffaloes, than alone. Chloride test was not suggested for diagnosing SCM.

Table 8. Evaluation of indirect test namely, California mastitis test (CMT), chloride test, somatic cell count (SCC) and rennet coagulation time (RCT) for diagnosis of subclinical mastitis in riverine buffaloes.

Name of the parameter	Total samples examined (N)	Test positive buffaloes (a + b)	Test reaction as compared to cultural examination				Percent sensitivity a / (a + d) x100	Percent specificity c / (b + c) x 100	Percent accuracy (a + c) / N ×100	Positive predictive value (%) a / (a+b) ×100	Negative predictive value (%) c / (c+d) ×100	Likelihood ratio (positive) sensitivity / (100 - specificity)	Likelihood ration (negative) (100 – sensitivity) / specificity
			True positive (a)	False positive (b)	True negative (c)	False negative (d)							
CMT	2452	728	414	314	1630	94	81.50	83.85	83.36	56.87	94.55	5.05	0.22
Chloride test	2452	777	337	440	1504	171	66.34	77.37	75.08	43.37	89.79	2.93	0.43
SCC	2452	512	437	75	1869	71	86.02	96.14	94.05	85.35	96.14	22.28	0.15
RCT	2452	696	406	290	1654	102	79.92	85.08	84.01	58.33	94.19	5.35	0.24
Bacterial cultural examination	2452	508	508	-	1944	-	100	100	100	-	-	-	-

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