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Comparison of α1-Antitrypsin, α1-Acid Glycoprotein, Fibrinogen and NOx as Indicator of Subclinical Mastitis in Riverine Buffalo (*Bubalus bubalis*)

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ABSTRACT: Mastitis set apart as clinical and sub clinical is a disease complex of dairy cattle, with sub clinical being the most important economically. Of late, laboratories showed interest in developing biochemical markers to diagnose sub clinical mastitis (SCM) in herds. Many workers reported noteworthy alternation of acute phase proteins (APPs) and nitric oxide, (measured as nitrate+nitrite = NOx) in milk due to intra-mammary inflammation. But, the literature on validation of these parameters as indicators of SCM, particularly in riverine milch buffalo (*Bubalus bubalis*) milk is inadequate. Hence, the present study focused on comparing several APPs viz. α_1- anti trypsin, α_1- acid glycoprotein, fibrinogen and NOx as indicators of SCM in buffalo milk. These components in milk were estimated using standardized analytical protocols. Somatic cell count (SCC) was done microscopically. Microbial culture was done on 5% ovine blood agar. Of the 776 buffaloes (3,096 quarters) sampled, only 347 buffaloes comprising 496 quarters were found positive for SCM i.e. milk culture showed growth in blood agar with SCC≥2×10^5 cells/ml of milk. The cultural examination revealed Gram positive bacteria as the most prevalent etiological agent. It was observed that α_1- anti trypsin and NOx had a highly significant ($p<0.01$) increase in SCM milk, whereas, the increase of α_1- acid glycoprotein in infected milk was significant ($p<0.05$). Fibrinogen was below detection level in both healthy and SCM milk. The percent sensitivity, specificity and accuracy, predictive values and likelihood ratios were calculated taking bacterial culture examination and SCC≥2×10^5 cells/ml of milk as the benchmark. Udder profile correlation coefficient was also used. Allowing for statistical and epidemiological analysis, it was concluded that α_1- anti trypsin indicates SCM irrespective of etiology, whereas α_1- acid glycoprotein better diagnosed SCM caused by gram positive bacteria. NOx did not prove to be a good indicator of SCM. It is recommended measuring both α_1- anti trypsin and α_1- acid glycoprotein in milk to diagnose SCM in buffalo irrespective of etiology. (**Key Words:** Acute Phase Proteins, Nitric Oxide, Subclinical Mastitis and Buffalo)

INTRODUCTION

Riverine buffalo milk production in the Indian sub continent has long been accepted as the backbone of the rain-fed agrarian socio-economic fabric. Sustainability of buffalo milk production even during dry spells has contributed to a lower suicide rate amongst farmers in drought stricken terrain. The quality of milk lies in its hygienic status. Milk production involves rapid physical, chemical and biological changes right from galactopoiesis to let down. Mastitis, complex multi-factorial inflammatory reaction, which often results from an intra-mammary bacterial infection entails losses due to reduced milk production, treatment costs, increased labor, milk withheld for human consumption due to residues in the form of antibiotics and micro-organisms and pre-mature culling. Consequently, an early detection at the sub clinical stage is necessary to prevent production loss and to enhance prospects of recovery (Guha et al., 2010).

Subclinical mastitis, a herd problem, affects the normal functioning of the mammary gland epithelial cells' ability to convert circulating nutrients into milk components (Gera and Guha, 2012). It often goes unnoticed due to absence of visually apparent changes in udder and milk. Detection of SCM is also difficult due to pooling of milk for sale from different milk collection points so that the source of SCM

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cannot be determined after collection (Gera et al., 2011; Guha et al., 2012). Subclincial mastitis is also a depot of micro-organisms that lead to the spread of infection to the other animals within the herd.

Acute phase proteins (APPs) are an assortment of blood hepatic glycoproteins that change in concentration due to external or internal challenges, such as infection, inflammation, surgical trauma, or stress. Quantification of APP concentration in body fluids can provide valuable diagnostic information in the detection, prognosis, and monitoring of disease in several animal species (Gonzalez et al., 2008). The recent recognition, that APPs are produced in the bovine mammary gland in response to bacterial mastitis has made it obligatory to consider them as alternative biomarkers for mastitis. An increase in concentration of APPs precedes the onset of clinical signs even in the absence of macroscopic changes in the ruminant milk (Safi et al., 2009).

Macrophages, a somatic cell fraction of milk, are the source of nitric oxide (NOx) in bovines. In intra-mammary infection (IMI), macrophages are the initially predominant cell type to travel from the peripheral circulation to the mammary gland in response to inflammatory insults and contribute to the pathophysiology of the mammary gland. NOx is produced in large amounts by inducible nitric oxide synthase (iNOS) and its derivatives, such as peroxynitrite and nitrogen dioxide, and plays a role in inflammation (De and Mukherjee, 2009).

The diagnostics based on physical and chemical changes in SCM milk is not satisfactory. A confirmatory diagnosis of SCM according to International Dairy Federation (IDF) recommendations is based on the microbiological status and inflammatory reactions i.e., somatic cell count (SCC$\geq 2\times10^5$ cells/ml of milk) of the quarter. However, the logistic and financial considerations involved with sampling all animals in a herd have precluded these techniques from being widely adopted (Guha et al., 2010). One of the principles of detecting inflammation within the mammary gland is to study the mammary epithelial integrity (Gera and Guha, 2011). For this reason alternative parameters to indicate inflammation are used to identify trends in the development of the udder health in dairy herd (Guha et al., 2010). Several superior breeds of milch buffaloes are being developed on the Indian sub-continent where buffaloes are foremost dairy animal. Thus, the present study was undertaken to investigate the effectiveness of the aforesaid APPs and NOx in detecting SCM and recognizing them as indicators for bubaline SCM for further development of kit for diagnosing SCM in herds. In the present study their concentration in healthy and SCM milk was analyzed both statistically and epidemiologically and further correlated with Log_{10}SCC.

MATERIALS AND METHODS

Collection of milk samples

Fifty ml of milk samples each were collected from 776 Murrah buffaloes over 3096 quarters under aseptic condition in sterile containers. The quarters were marked as right-fore (RF), right-hind (RH), left-fore (LF) and left-hind (LH).

Bacterial culture examination

The milk samples collected aseptically were shaken thoroughly. A 4 mm diameter platinum loop was used to streak 0.01 ml of the sample on 5% ovine blood agar plates. The plates were incubated aerobically at 37°C for 24 h. The resulting growth from the respective plates of media was purified and identified on the basis of morphology, colony characteristics and Gram's reaction (Gera and Guha, 2011).

Somatic cell count

The somatic cell count (SCC) of the milk samples was determined microscopically (Gera and Guha, 2011). Following through mixing, a 4 mm diameter platinum loop was used to evenly spread 0.01 ml of milk over four 1.0 cm^2 area template outlines. Slides were stained for 30 s in Newman-Lampert stain, with the composition as follows:

Methylene blue	1.2 gm.
95% ethyl alcohol	54 ml.
Tetrachloroethane	40 ml.
Glacial acetic acid	6 ml.

Somatic cells were stained with deep blue nuclei against a light blue background. The working factor of the microscope was calculated to be 35,400 by using a stage micrometer, calculating diameter of the microscopic field (0.012 cm) and the field per square cm (8850) for the given microscope. Total no. of cells was obtained by multiplying the total no. of cells counted in 25 fields with the working factor.

Estimation of fibrinogen

The fibrinogen was estimated by the tyrosine method as described by Varley et al. (1980). The fibrinogen was precipitated with calcium in casein free skimmed milk samples. The blue coloured complex developed due to the reduction of phosphomolybdate and phosphotungstate by tyrosine residues of polypeptide was estimated spectrophotometrically at 680 nm.

Estimation of α_1- acid glycoprotein

The α_1- acid glycoprotein protein was estimated by the tyrosine method as described by Varley et al. (1980). Casein of skimmed milk was removed by acid precipitation and the heat coagulable protein by percholoric acid. The α_1- acid

glycoprotein protein was finally precipitated with phosphotungstic acid. The tyrosine content of the precipitate was estimated by the above mentioned procedure.

Estimation of α_1- antitrypsin

The α_1-anti trypsin was measured by the Benzoyl arginine p-nitroanilide (BAPNA) method as described by Fritz et al. (1974), with little modification as described by Guha and Gera (2011). Casein and fat were removed by clearing solutions containing rennet and polyethylene glycol. The trypsin residue formed a yellow coloured complex 4 nitroaniline after reacting with BAPNA which was measure spectrophotometrically at 405 nm. The intensity of colour was inversely proportional to α_1-anti trypsin concentration.

Estimation of NOx

The NOx (nitrate+nitrite) was estimated by Griess reaction as described by Bouchard et al. (1999). Nitrate was converted to nitrite by nitrate reductase. The acidified nitrite produced nitrosating agent which reacted with sulfanilic acid to produce diazonium ions. The diazonium ions coupled with N (1-naphthyl) ethylenediamine to form choromophoric azo-dye whose intensity was measured spectrophotometrically at 550 nm.

Calculation of percent sensitivity, specificity, accuracy, predictive values and likelihood ratios

Percent sensitivity, specificity, accuracy, predictive values and likelihood ratios were found taking bacterial growth in culture media and SCC≥2×10^5 cells/ml of milk as the benchmark (Katsoulos et al., 2010; Guha et al., 2010; Gera and Guha, 2011). 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. The percent sensitivity, specificity were calculated by the formulae of Thrusfield (2005). The percent accuracy was calculated by the formula of Reddy et al. (2001). Percent positive and negative predictive values, likelihood ratios (both positive and negative) were also measured by the methods of Petrie and Watson (2008).

Statistical analysis

Analysis of variance (ANOVA) and Duncan Multiple Range Test (DMRT) were carried out to compare the milk components. Comparison of means of estimated concentration of different parameters in healthy and SCM milk, irrespective of the etiology, was done by t-test. SCC was converted to Log_{10}SCC. Pearson's correlation coefficient among milk components showing substantial alternation in concentration between healthy and SCM milk samples including Log_{10}SCC was also calculated. All statistical analysis was done with SPSS statistical software (Petrie and Watson, 2008).

RESULTS

Etio-prevalence of SCM

In the present study, on the basis of bacterial culture examination and SCC it was observed that 347 riverine buffaloes (496 quarters) were SCM positive. Milk samples showing SCC≥2×10^5 cells/ml and growth in culture media were considered positive for SCM. The SCC was observed to increase significantly ($p<0.01$) in SCM milk irrespective of the etiological agents (Tables 2 and 3). The mean SCC in SCM milk was 2.05±0.056 (Table 3). From Table 1 it is evident that the most prevalent etiological agent was *Staphylococcus* spp. followed by *Streptococcus* spp. And *Escherichia coli*. A few instances of mixed infection and (*Corynaebacterium* spp. and *Bacillus* spp.) were also encountered during the investigation (Table 1). Together the frequencies of Gram positive infections were >79%.

Effect of SCM on milk components

In the present study there was a statistically significant ($p<0.01$) increase in the concentration of α_1- anti trypsin in infected milk samples irrespective of the causative agents. A significant ($p<0.05$) increase of α_1- acid glycoprotein concentration in the SCM milk was also observed. Fibrinogen was below detection levels in both healthy and infected milk samples. NOx also showed significant increase in SCM milk (Tables 2 and 3).

Percent sensitivity, specificity, accuracy, predictive

Table 1. Prevalence of bacterial agents in subclinical mastitis milk of riverine buffalo (*Bubalus bubalis*)

Genus	Animal		Quarters	
	Number	Percentage	Number	Percentage
Staphylococcus spp.	146	42.07	210	42.33
Streptococcus spp.	118	34.00	172	34.67
Escherichia coli	69	19.88	95	19.15
Others (*Corynaebacterium* spp. and *Bacillus* spp.) +Mixed infection	14	4.05	19	3.85
Total	347	100	496	100

Table 2. Effect of different bacterial agents on acute phase proteins and NOx in healthy and subclinical mastitis milk of riverine buffalo (*Bubalus bubalis*)

Parameters	Mean±SE of healthy milk (n = 496)	Mean±SE of subclinical mastitis milk (n = 496)			
		Staphylococcus spp. (n = 210)	*Sterptococcus* spp. (n= 172)	*Escherichia coli* (n = 95)	Others+mixed infection (n = 19)
Somatic cell count ($\times10^5$ cells/ml)	0.93*±0.007	2.08**±0.020	2.05**±0.013	2.01**±0.015	2.03**±0.012
α_1-Anti trypsin (U/L)	3,340.43*±138.54	6,982.13**±122.72	6,074.22**±102.87	5,798.76**±94.78	5,998.73**±99.34
α_1-Acid glycoprotein (mg/ml)	0.176^a±0.026	3.08^b±0.02	2.99^b±0.01	2.88^b±0.08	2.89^b±0.03
Fibrinogen (g/dl)	Not detectable	Not detectable	Not detectable	Not detectable	Not detectable
NOx (Nitrate+nitrite) (μM)	11.09*±0.19	18.07**±0.14	17.09**±0.11	16.28**±0.14	16.89**±0.24

Mean having different superscripts * and ** horizontally differ significantly ($p<0.01$).
Mean having different superscripts [a] and [b] horizontally differ significantly ($p<0.05$).

Table 3. Mean±SE of SCC, acute phase proteins and NOx in milk from healthy and subclinical mastitis (irrespective of the causative agent) in riverine buffalo to decide the threshold limit (n = 496)

Parameter	Healthy milk	Subclinical mastitis milk	Cut-off points
Somatic cell count ($\times10^5$ cells/ml)	0.93*±0.007	2.05**±0.056	-
α_1-Anti trypsin (U/L)	3,340.43*±138.54	6,397.71**±150.99	6,396.22
α_1-Acid glycoprotein (mg ml^{-1})	0.176^a±0.026	2.92^b±0.032	2.92
Fibrinogen (g/dl)	Not detectable	Not detectable	-
NOx (nitrate+nitrite) (μM)	11.09*±0.19	17.33±0.29	17.34

Mean having different superscripts * and ** horizontally differ significantly ($p<0.01$).
Mean having different superscripts [a] and [b] horizontally differ significantly ($p<0.05$).

values and likelihood ratios

After calculating the percent sensitivity, specificity, accuracy, predictive values and likelihood ratios for all the significantly altered parameters, α_1- anti trypsin was most in agreement with IDF criteria for SCM (i.e. bacterial growth in culture media and SCC≥2×10^5 cells/ml), followed by α_1- acid glycoprotein. The value for NOx was at par (Table 4). However, the values for the same parameters were high when the causative agent was only Gram positive bacteria (Table 5).

Udder profile correlation coefficient

From Table 6 it can be observed that Log_{10}SCC is strongly correlated ($p<0.01$) with α_1-antitrypsin only in SCM milk. With NOx, Log_{10}SCC is correlated at ($p<0.05$) and ($p<0.01$) in healthy and SCM milk, respectively. Log_{10}SCC is correlated with α_1- acid glycoprotein at $p<0.05$. NOx is correlated with α_1- antitrypsin and α_1- acid glycoprotein at $p<0.01$ and $p<0.05$, respectively.

DISCUSSION

The present study was carried out to compare the usefulness of α_1-antitrypsin, α_1-acid glycoprotein, fibrinogen and NOx in detecting SCM, with special reference to bubaline SCM.

Table 4. Evaluation of α_1-anti trypsin, α_1-acid glycoprotein and NOx as an indicator for diagnosis of subclinical mastitis in riverine buffalo

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) ×100	Percent specificity c/(b+c) ×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)							
α_1-Anti trypsin	2,048	494	410	84	1,468	86	82.66	94.59	91.70	83.00	94.47	15.28	0.18
α_1-Acid glycoprotein	2,048	508	376	132	1,420	120	75.81	91.49	87.70	74.02	92.21	8.90	0.26
NOx	2,048	514	358	156	1,396	138	74.18	89.95	85.64	69.64	91.00	7.44	0.29
Positive bacterial culture and SCC≥2×10^5 cells/ml	2,048	496	496	-	1,552	-	100	100	100	-	-	-	-

Table 5. Evaluation of α_1-anti trypsin, α_1-acid glycoprotein and NOx as an indicator for diagnosis of subclinical mastitis in riverine buffalo caused by gram positive bacteria only

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) ×100	Percent specificity c/(b+c) ×100	Percent accuracy (a+c)/N ×100	Positive predictive value (%) a/(a+b)	Negative predictive value (%) c/(c+d)	Likelihood ratio (positive) sensitivity/ (100-specificity)	Likelihood ration (negative) (100-sensitivity) /specificity
			True positive (a)	False positive (b)	True negative (c)	False negative (d)							
α_1-Anti trypsin	1,964	429	366	63	1,489	46	88.83	95.94	94.45	85.31	97.00	21.88	0.12
α_1-Acid glycoprotein	1,964	424	344	80	1,472	68	83.50	94.85	92.46	81.13	95.58	16.21	0.17
NOx	1,964	454	329	125	1,427	60	79.85	91.95	89.41	72.46	95.97	9.92	0.22
Positive bacterial culture and SCC≥2×10^5 cells/ml	1,964	412	412	-	1,552	-	100	100	100	-	-	-	-

SCM milk samples were those that showed bacterial growth in culture media and had a SCC of ≥2×10^5 cells/ml (IDF, 2005). Gram positive bacterial agents were the most prevalent (Table 1). Similar observations were reported by Sharma et al. (2010) who attributed the contamination to the presence of organisms in the sub-continent atmosphere. The mean SCC in the SCM milk were significantly ($p<0.01$) high (Tables 2 and 3) owing to inflammatory reactions (Guha et al., 2010).

The significant increase of α_1- anti trypsin in SCM milk (Tables 2 and 3) could be due to bacterial infection. The APP showed a substantial increase in SCM milk caused by all types of organisms. The increase in the concentration of α_1-anti trypsin was attributed to breach in the blood milk barrier by the action of inflammatory modulators and bacterial toxins, thus, it is a serum derivative (Gera and Guha, 2011).

A significant ($p<0.05$) increase of α_1-acid glycoprotein concentration was also observed for all types of infections (Tables 2 and 3). Up to 2006 there was no report of the presence of this α_1-acidglycoprotein in healthy or mastitic milk of dairy animals. Mansson et al. (2006) was first to report α_1- acid glycoprotein in healthy as well as in milk showing higher SCC in cows. A weaker and negative correlation of α_1-acid glycoprotein with SCC was reported by these authors. But, in the present investigation it was observed that the concentration of α_1-acid glycoprotein had a strong positive correlation with Log_{10} SCC. The increase could be due to excess of somatic cells in SCM. Two isoforms of α_1-acid glycoprotein, a low MW group (44 kDa), produced in the mammary gland (MG-AGP), and a higher MW group (55 to 70 kDa), produced by somatic cells (SC-AGP), were isolated by Ceciliani et al. (2007). Identical SC-AGP isoforms can be found both in milk and blood polymorpho-nuclear cells. Hence, an increase in the concentration of α_1-acid glycoprotein can be attributed to increased synthesis by the somatic cells as well as by mammary gland cells as an immuno-protective measure. Gera and Guha (2011) also reported a similar observation in crossbred cow SCM milk.

In the present study, fibrinogen was not detected in either healthy or SCM milk (Table 1). Our observation agrees with Tabrazi et al. (2008) and Gera and Guha (2011); who reported fibrinogen, a mild APP, appears in the milk during acute or chronic stage as a blood clotting factor or indicator of fibrosis. Fibrinogen was not taken up for further investigation.

From Table 3 it can be observed that NOx in the infected milk samples increased significantly ($p<0.01$). A similar observation was made by Bulbul and Ylmaz (2004) and Gera and Guha (2011). They attributed the increase to increased macrophages, a fraction of SCC.

We perused percent sensitivity, specificity, accuracy, predictive values and likelihood ratios of α_1-anti trypsin, α_1-acid glycoprotein and NOx as predictors of mastitis, taking the IDF criteria as the bench mark. It was observed that % sensitivity, specificity, accuracy were better for α_1-anti trypsin, followed by α_1-acid glycoprotein and NOx

Table 6. Correlation coefficient of milk biochemical components in healthy and SCM milk of riverine buffalo (n = 496)

	Log_{10} SCC		α_1-Anti trypsin		α_1-Acid glycoprotein		NOx (nitrate+nitrite)	
	Healthy milk	Infected milk	Healthy milk	Infected milk	Healthy milk	Infected milk	Healthy milk	Infected milk
Log_{10} SCC	-	-	0.019	0.795**	0.098	0.559*	0.546*	0.845**
α_1-Anti trypsin	-	-	-	-	0.064	0.028	0.022	0.803**
α_1-Acid glycoprotein	-	-	-	-	-	-	0.056	0.644*
NOx (nitrate+nitrite)	-	-	-	-	-	-	-	-

* Indicate significant at $p<0.05$. ** Indicate significant at $p<0.01$.

(Table 4) for all kind of infections. Our observation concurs with the reports of Gera and Guha (2011) in crossbred cows. These values were more in milk samples infected with Gram positive bacteria. The predictive values and likelihood ratios for positive tests are observed to be greater for α_1-anti trypsin (83.00%; 15.28) than α_1-acid glycoprotein (74.02%; 8.90) and NOx (69.64%; 7.44) (Table 4). The percent positive predictive values and likelihhod ratio (positive) when calculated in SCM milk infected with Gram positive bacteria for α_1-anti trypsin, α_1-acid glycoprotein and NOx were found to be 85.13; 21.88, 81.13; 16.21, and 72.46; 9.92, respectively (Table 5). The variation in these values was due to the fact that the concentration of the same parameters were lesser in SCM milk infected with *E. coli* than those milk samples which were infected with Staphylococcus or Streptococcus, though the level of significance were same for all the cases when compared with healthy milk (Table 2). The elevation of the parameters in gram positive SCM samples might be due to the fact that gram positive bacteria are more pathogenic in destroying the mammary gland epithelia whereas *E. coli* are relatively less severe on mammary gland cells (Wenz et al., 2006).

Likelihood test of a positive test result >10 indicates that the test can be used to rule in the disease. Likelihood ratio of negative results describes how much more likely the animal has a negative test result when it has the disease (Petrie and Watson, 2008). The likelihood ratio for a positive test for α_1- anti trypsin was found to be greater than 10 irrespective of the bacterial agent causing SCM. For α_1-acid glycoprotein the ratio was greater than 10 when SCM causative agents were Gram positive bacteria. The likelihood ratio (positive) for NOx was lesser than 10 irrespective of the mastitogenic agents. The purpose of separately considering Gram positive bacterial agents is that they are the most prevalent mastitogenic agents in the tropical countries as discussed above. To the best of our knowledge no such studies for α_1-acid glycoprotein and NOx were conducted previously. This is the first of its type. Hence, it can be considered as a pioneer work with special reference to bubaline SCM.

To prevent any ambiguity, double statistical evaluation for each presumed indicator was done in this study by correlating with Log_{10}SCC (Gold Standard test) separately in healthy and SCM milk. From Table 6 it can be observed that α_1-anti trypsin was also found strongly correlated ($p<0.01$) with Log_{10}SCC in SCM milk (0.795), while it was insignificant in healthy milk (0.092). The α_1-acid glycoprotein had a positive correlation, significant at $p<0.05$ (0.098 vs 0.559, healthy vs infected milk) with Log_{10}SCC (Table 6). It is also evident that the correlation between Log_{10} SCC and NOx were significant at $p<0.05$ and $p<0.01$ respectively in healthy and infected milk (0.546 vs 0.845, healthy vs infected milk). This may be due to the fact that the source of NOx is macrophages, a somatic cell fraction as discussed above. Apart from Log_{10} SCC, NOx was also significantly correlated with α_1-antitrypsin and α_1-acid glycoprotein at $p<0.01$ and $p<0.05$, respectively. Similar observations were reported by Gera and Guha (2011) in cow milk.

CONCLUSION

It can be reasonably concluded that though the concentration of two APPs, viz. α_1-antitrypsin and α_1-acid glycoprotein as well as NOx was significantly higher in milk during subclinical form of the inflammatory reaction, but, only α_1-anti trypsin was in agreement with the IDF criteria for SCM diagnosis for all kind of bacterial infections. The α_1-acid glycoprotein indicates SCM when caused by Gram positive bacteria alone. NOx did not attest to be a good indicator of SCM. The threshold value for α_1-antitrypsin and α_1-acid glycoprotein is fixed at 6,396.22 U/L and 2.92 mg/ml, respectively. Standardizing easy qualitative methods for estimating these indicators is recommended to enable the development of a kit for diagnosing SCM in the field. A pathophysiological explanation of the ascertained association is also recommended for further study.

REFERENCES

Bouchard, L., S. Blais, C. Desrosiers, X. Zhao and P. Lacasse. 1999. Nitric oxide production during endotoxin induced mastitis in cows. J. Dairy Sci. 82:2574-2581.

Bulbul, A. and B. Ylmaz. 2004. Relationship between the level of nitric oxide and somatic cell count in the milk of cows with mastitis. Veteriner Bilimleri Dergisi 20:95-102.

Ceciliani, F., V. Pocacqua, C. Lecchi, R. Fortin, R. Rebucci, G. Avallone, V. Bronzo, F. Cheli and P. Sartorelli. 2007. Differential expression and secretion of alpha1-acid glycoprotein in bovine milk. J. Dairy Res. 74:374-380.

De, U. K. and R. Mukherjee. 2009. The inhibitory response of *Azadirachta indica* extract on nitric oxide production by milk leukocytes during clinical mastitis. Vety. Archiv. 79:41-50.

Fritz, H., I. Trautschold and E. Werle. 1974. Methods in Enzymatic Analysis, 2nd ed. Academic Press New York, USA, p. 234.

Gera, S. A. Guha. 2011. Assessment of acute phase proteins and nitric oxide as indicators of subclinical mastitis in Holstein× Haryana cattle. Ind. J. Anim. Sci. 81:1029-1031.

Gera, S. and A. Guha. 2012. Effect of sub clinical mastitis on milk biochemical constituents in crossbred cows. Ind. Vet. J. 89:33-34.

Gera, S., A. Guha, A. Sharma and V. Manocha. 2011. Evaluation of trace element profile as an indicator of bovine sub-clinical

mastitis, Intas Polivet 12:9-11.

Gera, S., A. Sharma, R. S. Dabur, V. K. Jain and S. L. Garg. 2006. Studies on changes in milk composition and chemotherapeutic sensitivity in camel (Camelus dromedarius) in subclinical mastitis. In: Proceedings of the Internation Scientific Conference in Camels (Vol-II pp. 937-946) Quassim University Saudi Arabia.

Gonzalez, F. D. H., F. Tecles, S. Martı´nez-Subiela, A. Tvarijonaviciute, L. Soler and J. J. Cerone. 2008. Acute phase protein response in goats. J. Vet. Diagn. Invest. 20:580-584.

Guha, A. and S. Gera. 2012. Evaluation of milk trace elements, lactate dehydrogenase, alkaline phosphatase and aspartate aminotransferase activity of subclinical mastitis milk as and indicator of subclinical mastitis in riverine buffalo (*Bubalus bubalis*). Asian Australas. J. Anim. Sci. 25:353-360.

Guha, A., S. Gera and A. Sharma. 2010. Assessment of chemical and electrolyte profile as an indicator of subclinical mastitis in riverine buffalo (*Bubalus bubalis*), Har. Vet. 49:19-21.

International Dairy Federation (IDF). 2005. Diagnostic potential of California Mastitis Test to detect subclinical mastitis 26. Maastricht, Netherlands. pp. 15-19.

Katsoulos, P. D., G. Christodoulopoulos, A. Minas, M. A. Karatzia, K. Pourliotis, K. Spyridon and S. K. Kritas. 2010. The role of lactate dehydrogenase, alkaline phosphatase and aspartate aminotransferase in the diagnosis of subclinical intramammary infections in dairy sheep and goats. J. Dairy Res. 77:107-111.

Mansson, H. L., C. Branning, G. Alden and M. Paulsson, 2006. Relationship between somatic cell count individual leukocyte populations and milk components in bovine udder quarter milk. Int. Dairy J. 16:717-727.

Petrie, A. and P. Watson. 2008. Statistics for Veterinary and Animal Science, (Blackwell Publishing, London).

Reddy, L. V., P. C. Choudhuri and P. A. Hamza. 2001. Comparative efficacy of different tests in the diagnosis of subclinical mastitis in crossbred cows. Ind. Vet. J. 78:903-905.

Safi, S., A. Khoshvaghti, S. R. Jafarzadeh, M. Bolourchi and I. Nowrouzian. 2009. Acute phase proteins in the diagnosis of bovine subclinical mastitis. Vet. Clin. Pathol. 38:471-496.

Sharma, N., V. Pandey and N. A. Sudhan. 2010. Comparison of some indirect screening tests for detection of SCM in dairy cows. Bulg. J. Vet. Med. 13:98-103.

Tabrazi, A. D., R. A. Batavani, S. A. Rezaei and M. Ahmadi. 2008. Fibrinogen and ceruloplasmin in plasma and milk from dairy cows with subclinical and clinical mastitis. Pak. J. Biol. Sci. 11: 571-576.

Thrusfield, M. 2005. Veterinary epidemiology, 3rd ed, Blackwell Science, United Kingdom., p. 158.

Varley, H., A. H. Gowenlock and M. Bell. 1980. Practical clinical biochemistry. 5th ed. William Heinemann Medical Books Ltd., London: United Kingdom. pp. 574-575.

Wenz, J. R., G. M. Barrington, F. B. Garry, R. P. Ellis and R. J. Magnuson. 2006. *Escherichia coli* isolates' serotypes, genotypes and virulence genes and clinical coliform mastitis severity. J. Dairy Sci. 89:3408-3412.