



Assessment of acute phase proteins and nitric oxide as indicator of subclinical mastitis in Holstein × Haryana cattle

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Dairy income is cushion against crop failures (Bharadwaj *et al.* 2006). Mastitis, one of the most costly and knotty diseases of dairy animals, is a complex multi-factorial inflammatory reaction (Grohn *et al.* 2004). Subclinical mastitis (SCM) usually goes unseen. Timely detection with suitable test and treatment is essential (Gera *et al.* 2006). Somatic cell count (SCC), the gold standard test, increases sometime without any inflammatory reaction (Wanger *et al.* 1976) or latent infection (Nickerson 1986). The tests are cumbersome hence, there is need to explore for easy, effective and economical screening of SCM in the field.

Quantification of acute phase proteins (APPs) concentration in body fluids can provide valuable diagnostic information in the detection of the disease (Gonzalez *et al.* 2008). Its increase in concentration in the absence of macroscopic changes in ruminant milk may precede onset of clinical signs (Safi *et al.* 2009). Clinical relevance of NO and its derivatives is through a role in inflammation (De and Mukherjee 2009). One of the principles of detecting inflammation within the mammary gland is to study the mammary epithelial integrity (Mattila *et al.* 1985). Thus, keeping in view the importance of early diagnosis of SCM, the present study was undertaken to investigate whether the aforesaid APPs and NOx can be used as an indicator of SCM in Holstein × Haryana cows.

Milk samples collected aseptically from 212 (836 quarters) apparently healthy Holstein × Haryana cows (F₁ generation) with no history of mastitis were cultured in 5% ovine blood agar. The resulting growth was identified on the basis of morphology, colony characteristics and Gram's reaction, SCC was performed microscopically (Tuteja *et al.* 2001). α_1 , anti trypsin was estimated by Benzaldehyde arginine p-nitroanilide (BAPNA) method (Fritz *et al.* 1974)

after removal of casein and fat (Sandholm *et al.* 1984). α_1 - acid glycoprotein and fibrinogen were estimated by tyrosine method (Varley *et al.* 1980). The NOx was estimated by the reaction of griess (Bouchard *et al.* 1999).

The % sensitivity, specificity were calculated by the formulae of Thrusfield (2005). The percent accuracy was calculated by the method described by Reddy *et al.* (2001). The formulae are as follows:

$$\% \text{ sensitivity} = a / (a+d) \times 100.$$

$$\% \text{ specificity} = c / (b+c) \times 100.$$

$$\% \text{ accuracy} = (a+c) / N \times 100.$$

where a, true positive; b, false positive; c, true negative; d, false negative.

Mean comparison was done by t-test. Arithmetic means of each component were considered as cut off points. SCC values were transformed to Log₁₀. Pearson's correlation coefficients were used to investigate the relationship between the investigated APPs with each other and with Log₁₀SCC. All statistical analysis was done with SPSS statistical software (Petrie and Watson 2008).

Cows (158) comprising 502 quarters from 212 cows (836 quarters) sampled were found SCM positive. SCM was diagnosed based on 2 criteria, bacterial growth in culture media and substantial increase in SCC. *Staphylococcus* spp. (52%) followed by *Streptococcus* spp. (37%) was mostly isolated bacterial agent. The number of somatic cells, significantly ($P < 0.01$) higher in infected milk, was found to be 2.08 ± 0.20 (Table 1). $SCC \geq 2$ lakh cells/ml was taken as threshold limit for SCM positive (Apparao *et al.* 2009).

In the present study α_1 anti trypsin increased significantly ($P < 0.01$) in infected milk samples (Table 1). This can be on account of bacterial infection. α_1 anti trypsin was strongly correlated ($P < 0.01$) with Log₁₀SCC in SCM milk (0.257), which was insignificant (Table 2) in normal milk (0.092). 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 toxin, thus, it is serum derived (Sandholm *et al.* 1984).

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Table 1. Mean $\pm$ SE of SCC, acute phase proteins and NOx in milk from normal and sub clinical mastitis cases

Parameter	Normal milk	Infected milk
Somatic cell count ($\times 10^5$ cells ml ⁻¹)	0.61 ** $\pm$ 0.12	2.08 ** $\pm$ 0.2
α_1 anti trypsin (UL ⁻¹)	3338.43 ** $\pm$ 91.22	6976.13 ** $\pm$ 123.81
α_1 acid glycoprotein (mg ml ⁻¹)	0.156 * $\pm$ 0.012	0.197 * $\pm$ 0.017
Fibrinogen (g dl ⁻¹)	Not detectable	Not detectable
NOx (nitrate + nitrite) (μ M)	11.07 ** $\pm$ 0.19	17.97 ** $\pm$ 0.42

Mean having different superscripts * and ** horizontally differ significantly at P<0.05 and P<0.01, respectively.

Significant (P<0.05) increase of α_1 acid glycoprotein concentration (Table 1) with positive correlation, significant at P<0.05 (0.094 vs 0.173 : : normal vs infected milk) with Log₁₀SCC (Table 2) was observed. Mansson *et al.* (2006) was first to observe its presence in cow milk, reported weaker negative correlation with Log₁₀SCC. But, in the present investigation it was observed that the concentration of α_1 acid glycoprotein had positive correlation with Log₁₀SCC. The increase might be due to excess production of somatic cell and mammary gland isoforms of α_1 acid glycoprotein (Cecilian *et al.* 2007) as an immuno-protective measure.

In the present study fibrinogen was not detected in both

normal and SCM milk (Table 1). Present results agree with Tabrazi *et al.* (2008), who reported fibrinogen, a mild APP appears in the milk during acute or chronic stage as a blood clotting factor or fibrosis, respectively. Fibrinogen was not taken up for further investigation.

NOx in the infected milk samples increased (Table 1) significantly (P<0.01). Similar observation was made by Bulbul and Ylmaz (2004). They attributed the augment to increased macrophages. The correlation between Log₁₀ SCC and NOx became strong in infected milk (0.152 vs 0.216 and normal vs infected milk), significant at P<0.01 (Table 2).

Once we peruse the % sensitivity, specificity and accuracy of α_1 - anti trypsin, α_1 acid glycoprotein and NOx taking bacterial culture examination as the bench mark (Table 3), It was found that α_1 anti trypsin were most sensitive, specific and accurate, followed by α_1 acid glycoprotein. SCC although being a Gold Standard was not taken as the bench mark in present study as it shows variation due to non-specific and latent infections. Similar results for α_1 anti trypsin were reported by Mattila *et al.* (1985).

To prevent any unambiguity double statistical evaluation for each presumed indicators was done in this study. First compared with SCC and then for better assessment weighed against bacterial culture examination. Log₁₀SCC had positive correlation with α_1 anti trypsin (P<0.01) and α_1 acid glycoprotein (P<0.05) in infected milk samples only (Tables 2 and 3) and both are in agreement with bacterial culture examination, (α_1 anti trypsin being better), it can be said

Table 2. Correlation coefficient amongst milk components in normal and subclinical mastitis milk (n,502)

	Log ₁₀ SCC		α_1 anti trypsin		α_1 acid glycoprotein		NOx (nitrate + nitrite)	
	Normal milk	Infected milk	Normal milk	Infected milk	Normal milk	Infected milk	Normal milk	Infected milk
Log ₁₀ SCC			0.092	0.257**	0.094	0.173*	0.152*	0.216**
α_1 -anti trypsin					0.008	0.018	0.021	0.245**
α_1 -acid glycoprotein							0.034	0.162*
NOx (nitrate + nitrite)								

*and ** indicate significant at P<0.05 and P<0.01 respectively.

Table 3. Evaluation of α_1 anti trypsin, α_1 acid glycoprotein and NOx (nitrate + nitrite) as an indicator of subclinical mastitis in Holstein $\times$ Haryana cows

Milk component	Total samples examined	Test positive samples	Test reaction as compared to cultural examination				Sensitivity %	Specificity %	Accuracy %
			True positive (a)	False positive (b)	True negative (c)	False negative (d)			
α_1 anti trypsin	836	490	412	78	256	90	82.07	76.65	79.90
α_1 acid glycoprotein	836	484	397	87	247	105	79.08	73.95	77.03
NOx (nitrate + nitrite)	836	459	355	104	230	147	70.71	68.86	69.98
Bacterial culture examination	836	502	502		334		100	100	100

that both can detect bovine mastitis at an early stage. NOx was not considered, though it showed appreciable agreement with bacterial culture examination as it is significantly related with Log₁₀SCC in normal milk.

It can be concluded that, the presence of both these 2 APPs, α_1 anti trypsin and α_1 acid glycoprotein, in milk during inflammatory reaction is evident in the subclinical state of infection. Of the two, the former showed strong statistical significance with SCC in SCM milk and confirmed maximum agreement with bacterial culture examination. Hence, the present study revealed that α_1 anti trypsin and α_1 acid glycoprotein as the indicators of SCM former being safer.

SUMMARY

The present investigation was conducted to evaluate acute phase proteins (α_1 anti trypsin, α_1 acid glycoprotein and fibrinogen) and NOx as indicators of subclinical mastitis (SCM) in 212 Holstein $\times$ Haryana (F₁) cow milk. The assay revealed that fibrinogen was below detection level in both healthy and diseased milk. Somatic cell count (SCC), α_1 anti trypsin and NOx increased significantly high in SCM milk, whereas, the increase of α_1 acid glycoprotein was noted to be significant. From the udder profile correlation coefficient it was observed that log₁₀SCC vs α_1 anti trypsin and NOx was significant (0.257 and 0.216, respectively) and α_1 acid glycoprotein was significant (0.173) in SCM milk only. Normal milk log₁₀SCC was significantly correlated to NOx (0.152). Highly significant correlation was observed between α_1 -anti trypsin and NOx (0.245) in SCM milk only. Correlation between α_1 acid glycoprotein and NOx was significant (0.162). This illustrates that these components are linked with each other in SCM milk. α_1 anti trypsin followed by α_1 acid glycoprotein were most sensitive, specific and accurate of the three investigated component. Though the magnitude of percent specificity for each parameter is less but their sensitivity is very high, which implies that they can detect SCM at an early stage. Thus, it can be concluded that α_1 -anti trypsin and α_1 acid glycoprotein are indicators of bovine SCM, the former being the better.

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