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## Estimation of Acute Phase Proteins as Early Biomarkers of Buffalo Subclinical Mastitis

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### ABSTRACT

Mastitis is a widespread global problem of dairy animals responsible for huge economic losses. Subclinical mastitis is 15-40 times more prevalent than clinical mastitis and causes two third losses of the total milk production. Early diagnosis of subclinical mastitis is important to prevent its further progress to clinical mastitis. The aim of the present study was to compare the concentration of acute phase proteins mainly milk amyloid A and haptoglobin in milk of healthy and subclinical mastitis affected buffaloes for early diagnosis. Milk samples of apparently healthy buffaloes were screened for subclinical mastitis following International dairy federation criteria. Samples from healthy and subclinical cases were subjected to estimation of milk amyloid A and haptoglobin by commercially available kits (Phase<sup>TM</sup> Range, Tridelta, Development Ltd. Ireland). Statistical analysis and receiver operating characteristics analysis were used to determine level of significance, sensitivity and specificity. Significant increase in concentrations of milk amyloid A and haptoglobin in quarter milk samples of buffaloes having subclinical mastitis was observed as compared to healthy animals. Results of the correlation matrix revealed significantly strong positive correlations of SCC with APPs, Hp ( $r = 0.818^{**}$ ,  $p < 0.01$ ) and MAA ( $r = 0.810^{**}$ ,  $p < 0.01$ ) concentrations in milk. In conclusion, acute phase proteins can be used as rapid and sensitive biomarkers for detection of subclinical mastitis in dairy animals.

**Key words:** Subclinical mastitis, acute phase proteins, milk amyloid A, haptoglobin, somatic cell count, serum amyloid A

### INTRODUCTION

Mastitis is a multi-etiological disease with varying degrees of clinical intensity, variations in duration and residual effects. It is most prevalent disease associated with great economic losses in dairy industry due to a reduction in milk yield as well as altered compositional quality (Lakshmi *et al.*, 2014; Charaya *et al.*, 2015; Patil *et al.*, 2015). Sub-Clinical Mastitis (SCM) causes two third losses of the total milk production and is more challenging than clinical mastitis due to absence of visible changes in the udder or milk. Animals with subclinical mastitis should be considered as a constant risk of infection within and between herds (Persson *et al.*, 2011). Sub-clinical mastitis is frequently diagnosed by cultural examination, California mastitis test, electrical conductivity and by Somatic Cell Count (SCC). These tests have their own limitations.

Therefore, it is of great importance to investigate biomarkers that could be used for early and rapid detection of sub-clinical mastitis. Lai *et al.* (2009), Eckersall and Bell (2010), Guha *et al.* (2013) and Kumar *et al.* (2014) cited Acute Phase Proteins (APPs) as reliable indicators for bovine mastitis. In response to infection, mammary gland responds with initiation of acute phase response which results in rapid rise in systemic and local concentration of APPs. In present study, concentration of two APPs namely Haptoglobin (Hp) and Milk Amyloid A (MAA) were determined in milk of buffaloes to detect SCM.

## **MATERIALS AND METHODS**

**Sample collection and bacteriological examination:** Milk samples were collected aseptically from all the four quarters from apparently healthy buffaloes reared at an organized buffalo farm. Hands were properly washed with soap and water and teat apices disinfected with 70% alcohol. The first few milk stripping's were discarded and nearly 15 mL of quarter milk sample was collected separately in sterilized test tubes. Samples were transported to laboratory on ice and subjected to bacteriological examination (Quinn *et al.*, 2011) and SCC (Schalm *et al.*, 1971) for detection of subclinical mastitis following International Dairy Federation (IDF) criteria. As per this criteria, milk samples showing SCC more than 5 lac mL<sup>-1</sup> of milk with isolation of bacteria were considered positive for SCM.

**Estimation of acute phase proteins:** The concentrations of two APPs, MAA and Hp were determined by kits (Phase™ Range, Tridelata, Development Ltd., Ireland).

**Assay of amyloid (A) in milk:** The basis of assay is the solid phase sandwich enzyme linked immunosorbent assay. All the test reagents and samples were allowed to reach room temperature before use. In brief, 50 µL of diluted biotinylated monoclonal antibody was added to each well. After vortexing, milk samples were diluted 1:50 in 1x diluent buffer and added in duplicate to each well (50 µL). Following incubation for 1 h at 37°C the plate was washed four times with diluted wash buffer. After the last wash, the plates were dried on absorbent paper and 100 µL of streptavidin-HRP added to each of the wells. The plate was incubated at room temperature in the dark for 30 min. After washing the plate four times, 100 µL of TMB substrate was added. After incubation in the dark at room temperature for 30 min, 50 µL of stop solution was added to each well and absorbance was read at 450 nm. For interpretation of results mean absorbance for each sample and standards was calculated. Absorbance values of standards against the calibrator concentration were plotted. Best smooth curve was drawn through these points to construct the calibration curve. Concentrations of the test samples were determined by multiplying the interpolated value by the appropriate dilution factor. Samples that have a signal greater than the top calibrator or fall on the non linear part of curve were further diluted in 1x diluent buffer and re-analyzed.

**Assay of haptoglobin in milk:** The assay is based on the principle that haptoglobin present in the sample combines with hemoglobin and at low pH preserves the peroxidase activity of the bound hemoglobin. The preservation of peroxidase activity of hemoglobin is directly proportional to the amount of haptoglobin present in the sample (milk). Milk samples were centrifuged at 12,000 rpm for one hour and clear middle layer was taken for the test. An amount of 7.5 µL of clear milk sample and prepared calibrators (0-2.5 mg mL<sup>-1</sup>) were transferred in duplicate to the blank

micro plate. To this 100  $\mu\text{L}$  of reagent 1 was added to each microwell. The micro plate was gently tapped for proper mixing of calibrators/samples and hemoglobin. After this 140  $\mu\text{L}$  of reagent 2 was added to each well and incubated for 5 min at 25°C. Absorbance was read at 630 nm immediately. A calibration curve was generated by plotting absorbance (630) versus haptoglobin concentrations ( $\text{mg mL}^{-1}$ ). Concentration of Hp in milk samples were calculated from the standard curve.

**Statistical analysis:** Origin (version 8.5) software was used for analysis of descriptive statistics of acute phase proteins (Hp and MAA) in milk. To explore any interdependence among SCC and the studied milk variables, Pearson's bivariate correlation analysis with correlation coefficient ( $r$ ) at two-tailed significance level ( $p$ ) were applied using SPSS software package (version 16.0). In order to ascertain the magnitude of variation in APPs between two groups the data were subjected to the test of significance. Receiver Operating Characteristics (ROC) analysis was applied to determine the sensitivity and specificity of the test.

## RESULTS

**Estimation of acute phase proteins: milk amyloid A and haptoglobin:** Milk samples from 40 animals including sub clinically infected 30 and healthy buffaloes 10 were subjected to the estimation of MAA and Hp. Staphylococci and streptococci were the microbes associated with subclinical mastitis. Descriptive statistics regarding milk Hp and MAA concentration of normal and mastitis affected buffalo milk are presented in Table 1 and Fig. 1-4.

Sub clinically infected buffaloes had numerically higher variation in MAA (CV = 36% v. CV = 18%) concentration than values measured in milk from healthy buffaloes. Milk Hp concentrations exhibited similar variation in both the groups (CV = 29% v. CV = 29%). Skewness statistics revealed distribution of MAA in subclinical group to be higher, scores clustered to the right, while that of healthy group in lower range with respective negative skewness value. On the other hand reverse trend in skewness statistics was observed for Hp in both the groups. With negative kurtosis values MAA and Hp in both the groups exhibited platykurtic distributions in lower range. Lower IQR values for MAA in healthy group indicated clustering of data points around the mean value. On the other hand higher IQR values obtained for distribution of Hp in healthy and mastitic group revealed dispersion of data points. Significant increase (\* $p < 0.001$ ) in concentration of Hp and MAA in sub clinically infected buffaloes as compared to healthy ones was reported.

**Receiver Operating Characteristics (ROC) analysis:** The ROC analysis of acute phase proteins MAA and Hp at cut off value of 0.264 and 39.41  $\mu\text{g mL}^{-1}$ , respectively revealed high sensitivity (100%) and high specificity (100%) (Fig. 5). Area under curve is 1.0 for both the acute phase proteins which indicates very good test.

Table 1: Descriptive statistics of acute phase proteins in milk of healthy and mastitis buffaloes

APP	Animal health status	Min	Max	Mean	$\pm$ SD	IQR	Skewness	Kurtosis	CV %
MAA ( $\mu\text{g mL}^{-1}$ )	Subclinical	0.49	2.07	1.22 <sup>a</sup>	0.44	0.67	0.62	-0.91	36
	Healthy	0.02	0.04	0.03 <sup>b</sup>	0.01	0.01	-0.17	-2.14	18
MHP ( $\mu\text{g mL}^{-1}$ )	Subclinical	47.43	173.37	115.69 <sup>a</sup>	33.43	47.05	-0.02	-0.50	29
	Healthy	13.05	31.40	21.85 <sup>b</sup>	6.43	9.50	0.09	-1.36	29

Means in the same columns with different superscripts are significant ( $p < 0.001$ ), IQR: Interquartile range, Max: Maximum, Min: Minimum, APP: Acute phase proteins, CV: Coefficient of variation, MAA: Milk amyloid A, MHP: Milk haptoglobin

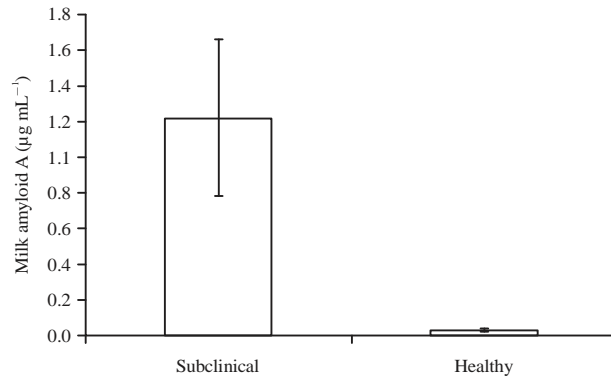


Fig. 1: Amyloid A concentrations in milk of subclinical mastitis and healthy buffaloes

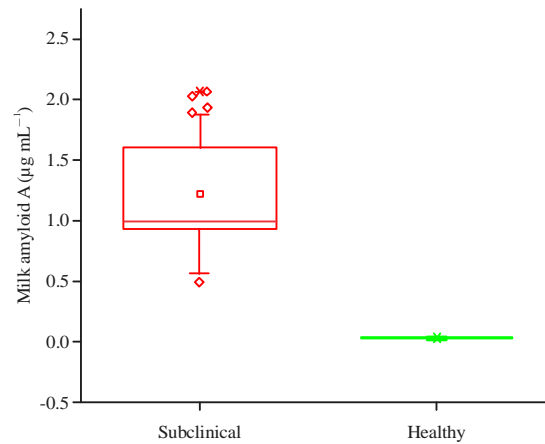


Fig. 2: Box whisker plots displaying distribution of milk amyloid A values in subclinical mastitis and healthy buffaloes

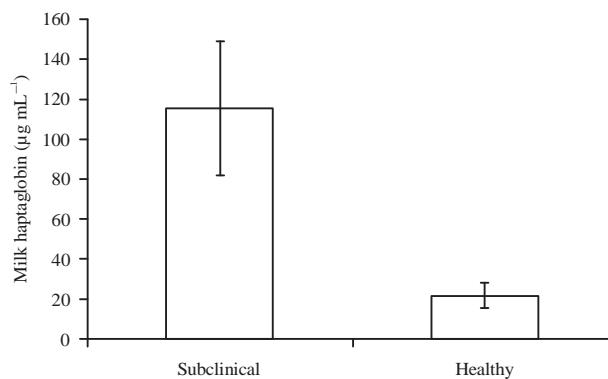


Fig. 3: Haptaglobin concentrations in milk of subclinical mastitis and healthy buffaloes

**Associations among APPs and SCC:** To explore the interdependencies among milk acute phase proteins and SCC, Pearson's correlation technique was applied to the observed values. Results of the correlation matrix revealed significantly strong positive correlations of SCC with APPs, Hp ( $r = 0.818^{**}$ ,  $p < 0.01$ ) and MAA ( $r = 0.810^{**}$ ,  $p < 0.01$ ) concentrations in milk (Table 2).

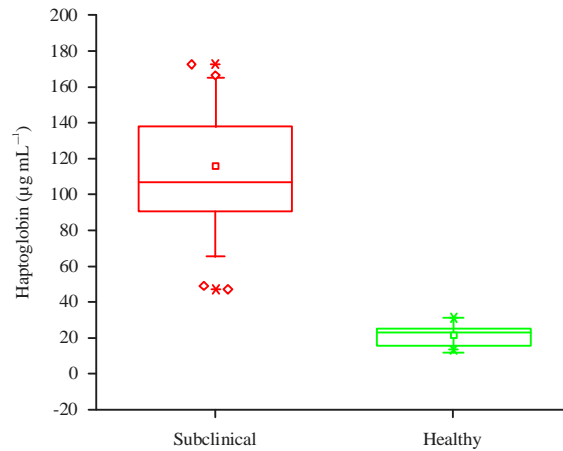


Fig. 4: Box whisker plots displaying distribution of haptoglobin values in milk of subclinical mastitis and healthy buffaloes

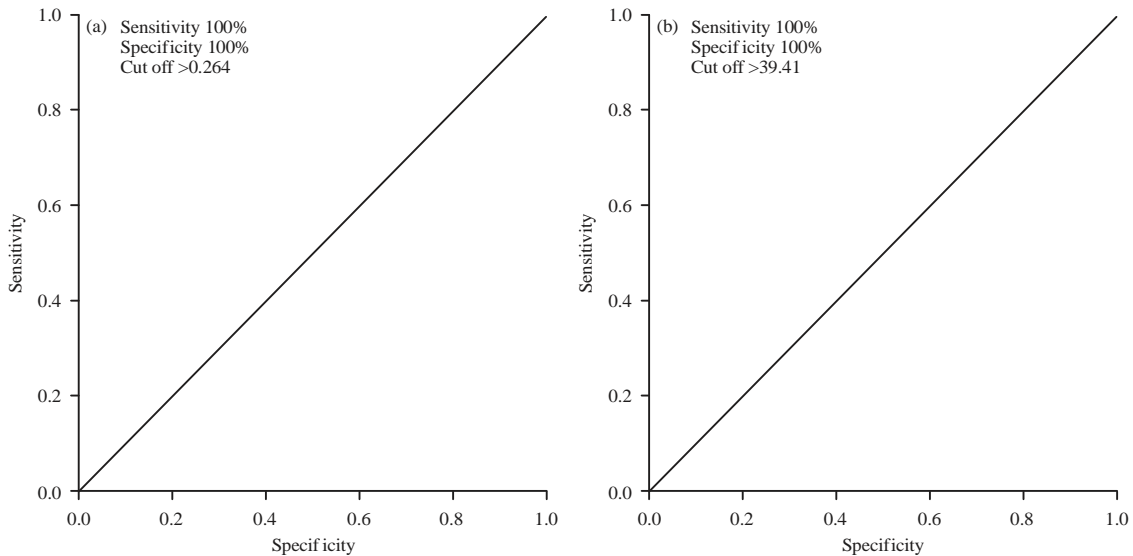


Fig. 5(a-b): Receiver operating characteristics analysis for (a) Milk amyloid A and (b) Haptoglobin

Table 2: Correlation coefficients among SCC, MAA and Hp in milk of healthy and subclinical mastitis buffaloes

Parameters	SCC	Hp	MAA
SCC	1		
Hp	0.818**	1	
MAA	0.810**	0.728**	1**

Correlation is significant at the 0.01 level (2-tailed), SCC: Somatic cell count, Hp: Hemopatology, MAA: Milk amyloid A

## DISCUSSION

Acute phase proteins are a group of proteins predominantly produced during inflammation, infection or stress conditions. The liver and mammary gland itself are the sites of synthesis of APPs; these being a group of proteins that are known to be further involved in acute phase response. In bovines, the MAA and Hp are the major APPs synthesized and secreted into milk during inflammation (Whelehan *et al.*, 2011; Ceciliani *et al.*, 2012). The use of APPs as inflammation indicators has become important over the last few years.

In the present study, MAA ranged from 0.49-2.07  $\mu\text{g mL}^{-1}$  with a mean value of  $1.22\pm 0.44$  and Hp ranged from 47.43-173.37  $\mu\text{g mL}^{-1}$  with mean of  $115.69\pm 33.43$ . Significant increase in concentrations of MAA and Hp in quarter milk samples of buffaloes having SCM was observed as compared to healthy animals. Haptoglobin and MAA in milk are specific markers of mastitis, since concentration of these APPs in milk from healthy quarters is low or undetectable (Gronlund *et al.*, 2005; Eckersall and Bell, 2010; Kumar *et al.*, 2014; Thomas, 2015). Our study is supported by various researchers (Nielsen *et al.*, 2004; Eckersall *et al.*, 2006; O'Mahony *et al.*, 2006; Kovac *et al.*, 2007; Hanaa *et al.*, 2013; Kalmus *et al.*, 2013; Kumar *et al.*, 2014; Tothova *et al.*, 2014; Ahmed *et al.*, 2015) who also reported higher concentrations of Serum Amyloid A (SAA) and hemoglobin (Hp) in serum and/or in milk of animals suffering from mastitis. The udder parenchyma has been shown to be the source of significant amounts of Hp and MAA during intramammary infection. A lack of correlation between SAA in milk and serum (Eckersall *et al.*, 2001), the expression of serum amyloid protein homologue in the mammary gland (Molenaar *et al.*, 2002) and the finding of a milk specific form of bovine SAA (McDonald *et al.*, 2001) indicated that SAA is also produced locally in the mammary gland and not just present in milk due to disruption of the blood milk barrier. Hiss *et al.* (2004) also demonstrated expression of Hp mRNA in mammary gland tissue by qPCR indicated its synthesis in udder parenchyma. Lehtolainen *et al.* (2004) reported earlier and higher peak of MAA as compared to SAA therefore suggested that acute phase responses occurred during mastitis were more accurately detected in milk rather than in serum. In an another study, Lauzon *et al.* (2006) supported these findings that Hp is synthesized locally by mammary gland and that its presence in milk is not due to leakage of the blood milk barrier alone as initially suggested by Eckersall *et al.* (2001). It is now established that the major bovine APPs (Hp and MAA) are synthesized in the mammary gland (Eckersall *et al.*, 2006; Larson *et al.*, 2006; Lai *et al.*, 2009; Thielen *et al.*, 2007; Whelehan *et al.*, 2011; Thomas, 2015) thereby improving the potentials of M-SAA3 and Hp as a biomarker for subclinical mastitis. Safi *et al.* (2009) also confirmed that measuring Hp and amyloid A in milk is more accurate than serum analysis for the diagnosis of SCM in Holstein cows. In the present study, samples were collected and processed at one point of time after confirming subclinical status of buffaloes. We could not observe changes in status of APPs as detected by Eckersall *et al.* (2006) who reported that there was a tendency for the level of MAA and milk Hp to be greater after the second infection than after first infection.

Many other workers have demonstrated the correlation of major bovine APP with other inflammatory indices (particularly SCC) during mastitis (Nielsen *et al.*, 2004; Akerstedt *et al.*, 2008; Pyorala *et al.*, 2011) and even with milk composition and protein quality (Akerstedt *et al.*, 2008, 2009) and with severity of the IMI (Pyorala *et al.*, 2011). To further emphasize the advantage of these major APP in mastitis detection, only a small variation in their levels were observed in healthy cow's milk over 42 consecutive milkings, which shows that these APP are stable and able to discriminate between healthy and inflamed tissues reliably (Akerstedt *et al.*, 2011). Milk Hp has also been evaluated by Nilgun *et al.* (2012) for its ability as a diagnostic marker for subclinical mastitis. Kalmus *et al.* (2013) noticed that MAA was not able to correctly identify inflammation by *Arcanobacterium pyogenes*, the cause of a purulent acute form of mastitis milk whereas Hp could detect inflammation correctly. It was concluded that Hp performs well as an indicator of intramammary infections than MAA.

Diagnostic sensitivity, specificity and cut off points for each test were determined via receiver-operating characteristics curve. The ROC analysis of Hp and MAA revealed 100% sensitivity and specificity. Our findings are in close agreement with that of Eckersall *et al.* (2001)

who reported high specificity (100%) and a reasonable sensitivity (86 and 93%) with measurement of MHP and SAA concentrations in milk and serum. Kumar, 2010 also reported 100% sensitivity and specificity. Haghkhah *et al.* (2010) recorded highest sensitivity and specificity (100%) using MAA as a diagnostic parameter compared to other acute phase proteins. They reported that sensitivity of evaluation of this acute phase protein is greater than other tests because acute phase proteins increase when a disease is in the development period and decrease during the recovery stage and therefore, diagnosis can occur during the early stages of the disease. Safi *et al.* (2009) compared the accuracy of APPs measured in milk and in serum with bacterial culture and MAA concentration was found to be most accurate with a sensitivity of 90.6% and specificity of 98.3% at concentrations  $>16.4 \text{ mg L}^{-1}$ .

## CONCLUSION

In conclusion, our study and previous studies provide strong evidence for production of significant amount of APPs in milk during SCM. These can be rapid and sensitive marker of inflammation. The advantage of Hp and MAA over other markers of mastitis is attributable to the fact that they are not present in the milk of healthy animals and are not influenced by factors other than mastitis. Therefore, estimation of acute phase proteins in milk is a useful diagnostic tool to detect SCM and to monitor herd health.

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