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 milk buffalo (Bubalus bubalis) milk is inadequate. Hence, the present study focused on comparing several APPs viz. α₁- anti trypsin, α₁- acid glycoprotein, fibrinogen and NOx as indicators of SCM in buffalo milk. These components in milk were estimated using standardised 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 ≥ 2×10⁷ cells/ml of milk. The cultural examination revealed Gram positive bacteria as the most prevalent etiological agent. It was observed that α₁- anti trypsin and NOx had a highly significant (p<0.01) increase in SCM milk, whereas, the increase of α₁- 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 ratio were calculated taking bacterial culture examination and SCC ≥ 2×10⁷ cells/ml of milk as the benchmark. Udder profile correlation coefficient was also used. Allowing for statistical and epidemiological analysis, it was concluded that α₁- anti trypsin indicates SCM irrespective of etiology, whereas α₁- 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 α₁- anti trypsin and α₁- 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 galactopoesis 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
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≥2×10⁵ 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 Log₁₀SCC.

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 cm² 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 α₁-acid glycoprotein
The α₁-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 perchloric acid. The α₁-acid
glycoprotein protein was finally precipitated with phosphotungstic acid. The tyrosine content of the precipitate was estimated by the above mentioned procedure.

Estimation of α₁-antitrypsin

The α₁-antitrypsin 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 α₁-antitrypsin 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≥2×10³ 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 formula 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 calculated 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 Log₁₀SCC. Pearson’s correlation coefficient among milk components showing substantial alternation in concentration between healthy and SCM milk samples including Log₁₀SCC 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≥2×10³ 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 (Corynebacterium 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 α₁-anti trypsin in infected milk samples irrespective of the causative agents. A significant (p<0.05) increase of α₁-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 values

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

<table>
<thead>
<tr>
<th>Genus</th>
<th>Number</th>
<th>Percentage</th>
<th>Quarters</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus spp.</td>
<td>146</td>
<td>42.07</td>
<td>210</td>
<td>42.33</td>
<td></td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>118</td>
<td>34.00</td>
<td>172</td>
<td>34.67</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>69</td>
<td>19.88</td>
<td>95</td>
<td>19.15</td>
<td></td>
</tr>
<tr>
<td>Others (Corynebacterium spp. and Bacillus spp.) + Mixed infection</td>
<td>14</td>
<td>4.05</td>
<td>19</td>
<td>3.85</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>347</td>
<td>100</td>
<td>496</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
The present study was carried out to compare the usefulness of \( \alpha_1 \)-antitrypsin, \( \alpha_1 \)-acid glycoprotein, fibrinogen and NOx in detecting SCM, with special reference to bubaline SCM.

### Table 4. Evaluation of \( \alpha_1 \)-anti trypsin, \( \alpha_1 \)-acid glycoprotein and NOx as an indicator for diagnosis of subclinical mastitis in riverine buffalo

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Positive predictive value (%)</th>
<th>Negative predictive value (%)</th>
<th>Likelihood ratio (positive)</th>
<th>Likelihood ratio (negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_1 )-Anti trypsin</td>
<td>83.00</td>
<td>94.47</td>
<td>15.28</td>
<td>0.18</td>
</tr>
<tr>
<td>( \alpha_1 )-Acid glycoprotein</td>
<td>91.70</td>
<td>94.78</td>
<td>15.28</td>
<td>0.18</td>
</tr>
<tr>
<td>NOx</td>
<td>83.00</td>
<td>94.47</td>
<td>15.28</td>
<td>0.18</td>
</tr>
</tbody>
</table>

### Discussion

The values and likelihood ratios

After calculating the percent sensitivity, specificity, accuracy, predictive values and likelihood ratios for all the significantly altered parameters, \( \alpha_1 \)-anti trypsin was most in agreement with IDF criteria for SCM (i.e. bacterial growth in culture media and SCC \( \geq 2 \times 10^5 \) cells/ml), followed by \( \alpha_1 \)-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 \( \alpha_1 \)-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 \( \alpha_1 \)-acid glycoprotein at p<0.05. NOx is correlated with \( \alpha_1 \)-antitrypsin and \( \alpha_1 \)-acid glycoprotein at p<0.01 and p<0.05, respectively.

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ( \pm ) SE of healthy milk (n = 496)</th>
<th>Mean ( \pm ) SE of subclinical mastitis milk (n = 496)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatic cell count (( \times 10^6 ) cells/ml)</td>
<td>0.935 ( \pm ) 0.007</td>
<td>2.085 ( \pm ) 0.020</td>
</tr>
<tr>
<td>( \alpha_1 )-Anti trypsin (U/L)</td>
<td>3.340.43 ( \pm ) 138.54</td>
<td>6.982.13 ( \pm ) 122.72</td>
</tr>
<tr>
<td>( \alpha_1 )-Acid glycoprotein (mg/ml)</td>
<td>0.176 ( \pm ) 0.026</td>
<td>3.08 ( \pm ) 0.02</td>
</tr>
<tr>
<td>Fibrinogen (g/dl)</td>
<td>Not detectable</td>
<td>Not detectable</td>
</tr>
<tr>
<td>NOx (Nitrate+nitrite) (( \mu )M)</td>
<td>11.09 ( \pm ) 0.19</td>
<td>18.07 ( \pm ) 0.14</td>
</tr>
</tbody>
</table>

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 \( \pm \) 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)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy milk</th>
<th>Subclinical mastitis milk</th>
<th>Cut-off points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somatic cell count (( \times 10^5 ) cells/ml)</td>
<td>0.935 ( \pm ) 0.007</td>
<td>2.055 ( \pm ) 0.056</td>
<td>-</td>
</tr>
<tr>
<td>( \alpha_1 )-Anti trypsin (U/L)</td>
<td>3.340.43 ( \pm ) 138.54</td>
<td>6.397.71 ( \pm ) 150.99</td>
<td>6.396.22</td>
</tr>
<tr>
<td>( \alpha_1 )-Acid glycoprotein (mg ml(^{-1} ))</td>
<td>0.176 ( \pm ) 0.026</td>
<td>2.925 ( \pm ) 0.032</td>
<td>2.92</td>
</tr>
<tr>
<td>Fibrinogen (g/dl)</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>-</td>
</tr>
<tr>
<td>NOx (nitrate+nitrite) (( \mu )M)</td>
<td>11.09 ( \pm ) 0.19</td>
<td>17.33 ( \pm ) 0.29</td>
<td>17.34</td>
</tr>
</tbody>
</table>

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

### Notes

NOx (Nitrate+nitrite) | 11.09 \( \pm \) 0.19 | 18.07 \( \pm \) 0.14 |

\( \alpha_1 \)-Acid glycoprotein, fibrinogen and NOx in detecting SCM, with special reference to bubaline SCM.
SCM milk samples were those that showed bacterial growth in culture media and had a SCC of ≥2×10⁵ 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 α₁-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 α₁-anti trypsin was attributed to breach in the blood milk barrier by the action of inflammatory modulators and bacterial toxins, thus, is a serum derivative (Gera and Guha, 2011).

A significant (p<0.05) increase of α₁-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 α₁-acidglycoprotein in healthy or mastitic milk of dairy animals. Mansson et al. (2006) was first to report α₁-acid glycoprotein in healthy as well as in milk showing higher SCC in cows. A weaker and negative correlation of α₁-acid glycoprotein with SCC was reported by these authors. But, in the present investigation it was observed that the concentration of α₁-acid glycoprotein had a strong positive correlation with Log₁₀ SCC. The increase could be due to excess of somatic cells in SCM. Two isoforms of α₁-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 α₁-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 α₁-anti trypsin, α₁-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 α₁-anti trypsin, followed by α₁-acid glycoprotein and NOx.

### Table 5. Evaluation of α₁-anti trypsin, α₁-acid glycoprotein and NOx as an indicator for diagnosis of subclinical mastitis in riverine buffalo caused by gram positive bacteria only

<table>
<thead>
<tr>
<th>Name of the parameter</th>
<th>Total samples examined (N)</th>
<th>Test positive buffaloes (a+b)</th>
<th>Test reaction as compared to cultural examination</th>
<th>Percent sensitivity (a/(a+b)) ×100</th>
<th>Percent specificity (c/(b+c)) ×100</th>
<th>Percent accuracy ([a+c]/N) ×100</th>
<th>Positive predictive value (%) a/(a+b)</th>
<th>Negative predictive value (%) c/(c+d)</th>
<th>Likelihood ratio (positive) sensitivity/(1-00-specificity)</th>
<th>Likelihood ratio (negative) (1-00-sensitivity)/specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁-Anti trypsin</td>
<td>1,964</td>
<td>429</td>
<td>366</td>
<td>1,489</td>
<td>46</td>
<td>88.83</td>
<td>95.94</td>
<td>94.45</td>
<td>85.31</td>
<td>97.00</td>
</tr>
<tr>
<td>α₁-Acid glycoprotein</td>
<td>1,964</td>
<td>424</td>
<td>344</td>
<td>1,472</td>
<td>68</td>
<td>83.50</td>
<td>94.82</td>
<td>92.46</td>
<td>81.13</td>
<td>95.58</td>
</tr>
<tr>
<td>NOx</td>
<td>1,964</td>
<td>454</td>
<td>329</td>
<td>1,427</td>
<td>60</td>
<td>79.85</td>
<td>91.95</td>
<td>89.41</td>
<td>72.46</td>
<td>95.97</td>
</tr>
<tr>
<td>Positive bacterial culture and SCC≥2×10⁵ cells/ml</td>
<td>1,964</td>
<td>412</td>
<td>412</td>
<td>1,552</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>95.94</td>
<td>97.00</td>
<td>21.88</td>
</tr>
</tbody>
</table>

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

|                      | Healthy milk | Infected milk | Healthy milk | Infected milk | Healthy milk | Infected milk | Healthy milk | Infected milk | Healthy milk | Infected milk |
|----------------------|--------------|---------------|--------------|---------------|--------------|---------------|--------------|---------------|--------------|---------------|---------------|
| Log₁₀ SCC            | -            | -             | 0.019        | 0.795**       | -            | -             | -            | -             | -            | -             |
| α₁-Anti trypsin      | -            | -             | -            | -             | -            | -             | -            | -             | -            | -             |
| α₁-Acid glycoprotein | -            | -             | -            | -             | -            | -             | -            | -             | -            | -             |
| 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 $\alpha_1$-anti trypsin ($83.00\%$; $15.28$) than $\alpha_1$-acid glycoprotein ($74.02\%$; $8.90$) and NOX ($69.64\%$; $7.44$) (Table 4). The percent positive predictive values and likelihood ratio (positive) when calculated in SCM milk infected with Gram positive bacteria for $\alpha_1$-anti trypsin, $\alpha_1$-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 $\alpha_1$- anti trypsin was found to be greater than 10 irrespective of the bacterial agent causing SCM. For $\alpha_1$-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 $\alpha_1$-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 $\alpha_1$-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 $\alpha_1$-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 $\alpha_1$-antitrypsin and $\alpha_1$-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. $\alpha_1$-antitrypsin and $\alpha_1$-acid glycoprotein as well as NOx was significantly higher in milk during subclinical form of the inflammatory reaction, but, only $\alpha_1$-anti trypsin was in agreement with the IDF criteria for SCM diagnosis for all kind of bacterial infections. The $\alpha_1$-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 $\alpha_1$-antitrypsin and $\alpha_1$-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**


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