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A CASE OF MESOTHELIOMA IN A SHE-BUFFALO

V. Rama Devi, P. Annapurna and N. Sasidhar Babu

ABSTRACT

Mesothelioma is a tumour arising from the mesothelial lining cells of serous cavities, especially peritoneum and pleura. These mesotheliomas are very rare among animals and have been reported in the ox, horse and dog. It is found most commonly as a congenital tumour in calves (Moulton, 1978) and Magnusson and Veit (1987) reported a mesothelioma in a calf. Singh and Singh (1984) noticed mesothelioma on the parietal and visceral pleura as well as on the pericardium in a buffalo heifer. It was also reported in an Ongole bullock by Bhaskar Singh and Christopher (1987) and in cow by Prasad et al. (1993). The present paper deals with a case of mesothelioma in a she buffalo.

HISTORY AND OBSERVATIONS

Tissue pieces collected from lung, heart and intestines of a she buffalo were sent in 10 percent formalin for histopathological examination to NTR College of Veterinary Science, Gannavaram from State Institute of Animal Health, Tanuku, West Godavari (Dt.). History revealed that the animal had a complaint of dyspnoea when alive, and on post mortem examination, small nodular growths were found on the lungs and on the serosa of intestines. The tissues were processed routinely by paraffin embedding and sections were stained with haematoxylin and eosin.

RESULTS AND DISCUSSION

Histological examination of tissue sections of nodules of lung and intestines revealed solid masses made up of single to multiple layers of large cuboidal cells over a fibrocellular stroma. At places, the cells formed papillary projections with fibrous tissue cores. The nodule was interspersed out throughout by the fibrous tissue septa. The cells had large vesicular nuclei and prominent nucleoli with rare mitotic figures. At some places, the cells also formed tubules or gland-like spaces or pseudo acini. The tumour cells lining the pseudo acini were flat mostly, and only few cells were cuboidal. In some areas, the cells were necrotic, and the tumour cells replaced the lung tissue. The nodules on the serosa of the intestines were fairly encapsulated and did not invade the musculature of the intestines. The tissue sections prepared from heart revealed degenerative changes and infiltration of mononuclear cells in the myocardium and the presence of a few inflammatory cells in the pericardium. The histological findings observed in the nodules of lung and intestines were consistent with mesothelioma, noticed by previous authors (Singh and Singh, 1984; Bhaskar Singh and Christopher, 1987; Magnusson and Veit, 1987 and Jubb et al., 1993). Interest in mesotheliomas has increased since the association between asbestos fiber and mesothelioma, was discovered in humans. This association has not been confirmed in animals, though ferruginous bodies, suggestive of asbestos exposure, have been found in the lungs of some urban dogs with mesothelioma, and an association has been made between mesothelioma in dogs and exposure of owners to asbestos (Jubb et al., 1993).

*Continued on page 184*
INTRODUCTION

The glucose-6-phosphate dehydrogenase (G-6-PD) enzyme plays a very important role in the glucose metabolism in the body and is the first line of defense of the body (Ganong, 1999). Deficiency of this enzyme leads to acute hemolytic anemia in affected individuals (Yoshida, 1973). More than 250 variants of G-6-PD enzyme in blood have been reported in man (McKusick, 1982). It has been reported that the incidence of cancer is inversely proportional to the G-6-PD deficiency in American Negroes. (Naik and Anderson, 1970). G-6-PD deficiency also gives protection against malarial infection in man (Motulsky et al., 1966). Erythocytic G-6-PD enzyme have been studied in different species of animals either by screening or electrophoretic methods, but studies in buffaloes are very few. According to Singari et al. (1991) decreased erythrocytic glucose-6-phosphate dehydrogenase (G-6-PD) activity in hemoglobinuric buffaloes may be partially responsible for a decrease in reduced glutathione, thereby causing oxidative stress to erythrocytes, which results in haemolytic syndrome.

MATERIALS AND METHODS

Blood samples were collected from 1527 buffaloes belongs to six different breeds, viz., Murrah, Jaffarabadi, Bhadawari, Surti, Nili-Ravi and non-descript, different age groups: adult, young and calves, different health statuses, and sexes. All the blood samples were studied by screening

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methods originally described by Bernstein (1962) and modified by Singh (1992) for cattle blood samples and with a further modification for buffalo blood samples for present study for different G-6-PD phenotypes by using 2,6-dichlorophenol indophenol. On the basis of the screening test, three types of G-6-PD phenotype were classified: normal, heterozygote and deficient. The G-6-PD activity levels were estimated by colorimetric methods using the technique described by Ells and Korkman (1961) for all blood samples. The statistical analysis of data was done as per Snedecor and Cochran (1989).

Glucose-6-phosphate dehydrogenase in red blood cell reacts with glucose-6-phosphate and NADP+ forming 6-phospho gluconolactone and NADPH. The latter in the presence of phenozine metho sulphate (PMS) reduces a blue dye (2,6-dichlorophenol indophenols) to a colorless state. The rate of dye disappearance is proportional to the G-6-PD activity.

RESULTS AND DISCUSSION

All the blood samples were screened for the three G-6-PD phenotypes, namely- normal, heterozygote and deficient. The data were analyzed according to G-6-PD type, breed, age group, health status and sex. The mean observed and expected values of normal, heterozygote and deficient types were statistically significant. In heterozygote G-6-PD types the observed values were found significantly higher than expected values, show a heterozygote advantage. These heterozygote advantages were found in all six breeds of buffaloes and in all age groups, health statuses and sexes.

The gene frequencies of G-6-PD normal and deficient (d) genes were calculated, and it was found that G-6-PD deficient gene frequency was highest in Jaffarabadi (0.47) followed by non-descript (0.46), Surti (0.40), Nili-Ravi (0.30), Bhadawari (0.27) and lowest in Murrah (0.26). On the basis of gene frequency and genetic distance, it appears that these breeds belong to three distinct groups. Group 1 consists of Jaffarabadi, non-descript and Surti, Gourp 2 consists of Nili-Ravi and Group 3 consists of Murrah and Bhadawari. The gene frequency of breeds of Group 1 and Group 3 were significantly different from each other, but they did not differ significantly within the group, whereas breeds of Group 3 did not differ significantly either from Group 1 or Group 2. The percentage of G-6-PD deficient animals were found highest in non-descript (10.87%) followed by Jaffarabadi (6.61%), Surti (3.23%), Murrah (0.96%) and lowest in Bhadawari (0.81%). The data were further analyzed according to age group, health status and sex. With respect to age groups, adults were found to have a higher percentage of G-6-PD deficient phenotype. The frequency of the G-6-PD deficient gene was found also highest in the adult age group in all breeds of buffaloes. On the contrary, in Nili-Ravi breed gene frequency of G-6-PD deficient gene was found higher in the young age group and no deficient animals were found in this breed, although the gene frequency of deficient gene was found to be 0.30±0.06. With respect to health status, percentages of G-6-PD deficient phenotypes were found higher in emaciated animals than in healthy animals. In males and females, the G-6-PD deficient percentages were found almost same.

The G-6-PD enzyme activity has calculated according to G-6-PD phenotype in all the six breeds, all age groups, health statuses and sexes, in buffaloes. The G-6-PD activity was found highest in the normal phenotype at 915.55±6.61 miu. and lowest in the deficient phenotype at 260.46±0.76 miu. Whereas in heterozygote phenotypes, it was found to be intermediate at 592.66±9.44 miu. The result of analysis of variance shows a highly significant (p<0.01) effect of G-6-PD types on G-6-PD enzyme activity, which is due to significant difference in G-6-PD enzyme activity level among three G-6-PD phenotypes. The effect of breed, age group, and sex was found non-significant on G-6-PD activity, but the activity was found higher in Murrah and Nili-Ravi breed and lower in Jaffarabadi and non-descript breed. Again the activity was recorded as higher in young animals than in adults and calves. The effect of health status was found significant on G-6-PD activity, which may be due to higher activity level in healthy animals than in emaciated animals. Glucose-6-phosphate dehydrogenase also had a correlation
Table 1. Distribution of G-6-PD gene frequency, observed and expected numbers of phenotypes in different age groups in different breeds of buffaloes.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Age group</th>
<th>Normal (Observed)</th>
<th>Heterozygote (Observed)</th>
<th>Deficient (Observed)</th>
<th>X Value at 1. d.f</th>
<th>Gene frequency</th>
<th>N</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(Expected)</td>
<td>(Expected)</td>
<td>(Expected)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MURRAH</td>
<td>Adult (88)</td>
<td>42 (46)</td>
<td>44 (35)</td>
<td>2 (6)</td>
<td>5.32*</td>
<td>0.73±0.03</td>
<td>0.27±0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young(93)</td>
<td>55 (58)</td>
<td>38 (31)</td>
<td>- (4)</td>
<td>6.13*</td>
<td>0.79±0.02</td>
<td>0.21±0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calves(28)</td>
<td>7 (11)</td>
<td>21 (13)</td>
<td>- (4)</td>
<td>10.08**</td>
<td>0.63±0.06</td>
<td>0.37±0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall(209)</td>
<td>104 (115)</td>
<td>103 (80)</td>
<td>2 (14)</td>
<td>18.04**</td>
<td>0.74±0.02</td>
<td>0.26±0.02</td>
<td></td>
</tr>
<tr>
<td>JAFFARABI</td>
<td>Adult (71)</td>
<td>5 (16)</td>
<td>59 (35)</td>
<td>7 (18)</td>
<td>31.23**</td>
<td>0.49±0.04</td>
<td>0.51±0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young(50)</td>
<td>9 (17)</td>
<td>40 (24)</td>
<td>1 (9)</td>
<td>20.61**</td>
<td>0.58±0.04</td>
<td>0.42±0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall(121)</td>
<td>14 (33)</td>
<td>99 (60)</td>
<td>8 (27)</td>
<td>49.62**</td>
<td>0.53±0.47</td>
<td>0.47±0.03</td>
<td></td>
</tr>
<tr>
<td>BHADAWARI</td>
<td>Adult (58)</td>
<td>21 (26)</td>
<td>36 (25)</td>
<td>1 (6)</td>
<td>9.69**</td>
<td>0.67±0.04</td>
<td>0.33±0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young(65)</td>
<td>36 (39)</td>
<td>29 (22)</td>
<td>- (3)</td>
<td>5.35*</td>
<td>0.77±0.03</td>
<td>0.23±0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall(123)</td>
<td>57 (65)</td>
<td>65 (48)</td>
<td>1 (9)</td>
<td>13.66**</td>
<td>0.73±0.02</td>
<td>0.27±0.02</td>
<td></td>
</tr>
<tr>
<td>SURTI</td>
<td>Adult (41)</td>
<td>6 (12)</td>
<td>32 (20)</td>
<td>3 (8)</td>
<td>13.29**</td>
<td>0.54±0.05</td>
<td>0.46±0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young(52)</td>
<td>17 (22)</td>
<td>35 (23)</td>
<td>- (6)</td>
<td>13.37**</td>
<td>0.66±0.04</td>
<td>0.34±0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall(93)</td>
<td>23 (34)</td>
<td>67 (44)</td>
<td>3 (14)</td>
<td>24.25**</td>
<td>0.60±0.03</td>
<td>0.40±0.03</td>
<td></td>
</tr>
<tr>
<td>NILI-RAVI</td>
<td>Adult (8)</td>
<td>4 (4)</td>
<td>4 (3)</td>
<td>- (1)</td>
<td>0.88</td>
<td>0.75±0.10</td>
<td>0.25±0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young(16)</td>
<td>6 (8)</td>
<td>10 (7)</td>
<td>- (1)</td>
<td>3.30</td>
<td>0.69±0.08</td>
<td>0.31±0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall(24)</td>
<td>10 (12)</td>
<td>14 (10)</td>
<td>- (2)</td>
<td>4.06*</td>
<td>0.70±0.06</td>
<td>0.30±0.06</td>
<td></td>
</tr>
<tr>
<td>NON- DESCRIPT</td>
<td>Adult (509)</td>
<td>79 (131)</td>
<td>359 (254)</td>
<td>71 (123)</td>
<td>85.96**</td>
<td>0.50±0.01</td>
<td>0.50±0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Young(422)</td>
<td>109 (147)</td>
<td>281 (204)</td>
<td>32 (70)</td>
<td>60.17**</td>
<td>0.59±0.01</td>
<td>0.41±0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calves(26)</td>
<td>1 (6)</td>
<td>24 (13)</td>
<td>1 (6)</td>
<td>18.61**</td>
<td>0.50±0.01</td>
<td>0.50±0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Overall(957)</td>
<td>189 (284)</td>
<td>664 (475)</td>
<td>104 (198)</td>
<td>152.12**</td>
<td>0.54±0.01</td>
<td>0.46±0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total overall (1527)</td>
<td>397 (533)</td>
<td>1012 (738)</td>
<td>118 (254)</td>
<td>210.46**</td>
<td>0.59±0.008</td>
<td>0.04±0.008</td>
<td></td>
</tr>
</tbody>
</table>

** Significant at p<0.01    * Significant at p<0.05    Values in the parenthesis are the number of animals.
REFERENCES


*Continued from page 180*
PHYTOBEZOAR IN A SHE-BUFFALO-A CASE REPORT

G. Veeraiah¹, Manda Srinivas² and N. Lakshmi Rani³

ABSTRACT

The present communication reports complications due to a phytobezoar obstructing the reticuloomasal orifice causing ruminitis, regurgitation, aspiration pneumonia and death in a she-buffalo.

Keywords: phytobezoar, reticuloomasal orifice, rumenotomy, regurgitation, complications

INTRODUCTION

Phytobezoars of varied origin have been reported in bullocks, small ruminants and wild animals, and these could be fatal. But reports on phytobezoars in buffaloes are few. The present communication places on record the fatal complications of phytobezoar in the rumen of a she-buffalo.

CASE HISTORY AND CLINICAL FINDINGS

A graded Murrah she-buffalo aged about 8 years was referred to the Veterinary Dispensary with history of no defecation and anorexia for the past 12 days and regurgitation since 5 days. The she-buffalo was treated with purgatives, rumenotorics, B-complex vitamins and dextrose salines by the local veterinarian with no improvement. On clinical examination, the she-buffalo revealed mild respiratory distress, depression, normal rectal temperature and ruminal fluid was observed at both the nostrils. Per rectal examination ruled out intestinal obstruction, hence exploratory rumenotomy was attempted.

SURGICAL TREATMENT AND DISCUSSION

Rumenotomy was performed as per the procedure described by Tyagi and Singh (1993). Through examination of the rumen and reticulum revealed ruminitis and a bezoar obstructing the reticuloomasal orifice, which was removed. The phytobezoar measured 12 cm X 6 cm and weighed 185 grams. It consisted of plant materials, polythens and mineral deposits (Figure 1.). The phytobezoar consisted of 8.22% moisture, 5.58% crude protein, 5.80% crude fiber and 80.40 % total ash on chemical analysis. Post operative care included Inj Streptopenicillin @ 2.5 gm, Inj Meloxicam @ 15 ml and Inj Chlorpheneramine maleate @ 10 ml intramuscularly and fluid therapy. Following surgery the buffalo developed sign of dyspnea, coughing, fever. Auscultation of the lung area revealed crackles and wheezes. The she-buffalo died on the second day following surgery. Postmortem examination revealed rumenitis, congested and empty intestines and tracheitis due to regurgitated ruminal contents into the trachea. Plant fibers and polythens ingested through the feed might have acted as a nucleus for phytobezoar formation around which salts and

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mucous secretions were deposited. The size, shape, composition and formation of phytobezoars in the present case are in agreement to those reported by Sharma and Chauhan (1997) and Bath et al., (1992).

In the present case the phytobezoar was fatal as it has obstructed the reticuloomasal orifice leading to excessive ruminal contractions, regurgitation, aspiration pneumonia and death. These are often revealed at postmortem examination as reported by Sastry (1983).

REFERENCES


Figure 1. Phytobezoar recovered from the reticuloomasal orifice.
MICROBIOLOGICAL EXAMINATION OF GROSS CASES OF PYOSALPINX IN BUFFALOES DIAGNOSED AT POST MORTEM

O.I. Azawi1, A.J. Ali1 and H.F. Al-Abidy2

ABSTRACT

The objectives of the present study were to establish the bacterial isolate variations in pyosalpinx of buffalo cows and to investigate the correlation between bacterial infection of the uterus and pyosalpinx. Buffalo cow reproductive tracts were collected from a Mosul abattoir. A total 385 uterine samples were examined, of which 12 had pyosalpinx. Swabs for bacteriology, fluid for cytology and biopsies for histopathology were collected from the pyosalpinx and the uterus from each sample included in the present study. The results of the present study indicated a prevalence of pyosalpinx of 3.1%, comprising unilateral (n = 11; 91.7%) and bilateral (n = 1; 8.3%) pyosalpinx. All samples (100%) of the pyosalpinx lesions had bacterial growth, the most prevalent bacteria were *Escherichia coli*, *Archanobacterium pyogenes* and *Staphylococcus aureus*, 33.3%, 26.7% and 16.7%, respectively. The most prevalent bacteria in the uterus were *Archanobacterium pyogenes* (23.7%), *Escherichia coli* (18.4%), *Bacillus licheniformis* (10.5%) and *Streptococcus ubris* (7.9%). However, *Escherichia coli*, *Archanobacterium pyogenes* and *Staphylococcus aureus* isolated from the pyosalpingeal fluid were frequently isolated from uterine cavity of buffaloes with pyosalpinx lesion. Higher rates of leukocyte infiltration were observed in the uterine discharge and pyosalpinx. A significant (P<0.01) increase in lymphocytes were found in pyosalpinx of samples included in this study. It could be concluded that there is a high correlation between bacteria isolated from uterus and pyosalpinx. The occurrence of pyosalpinx is mainly due to bacterial infection. Inflammation of the uterine tissue could be extended to utero-tubal junction producing local inflammation resulted in salpingitis and tubal obstruction.

Keywords: pyosalpinx, bacteria, leukocytes, buffalo cow

INTRODUCTION

Genital organ disorders are important causes of infertility and sterility in buffalo cows and result in high economic losses (Azawi, 2006). Pyosalpinx is a Greek word that means a uterine tube filled with puss. Pyosalpinx can be defined as a blocked dilated puss-filled uterine tube leading to reduced infertility or even sterility. Although many papers deal with the isolation of particular pathogens or potential pathogens from the uterus (Baishya et al., 1998; Azawi and Taha, 2002; Jadon et al., 2005; Azawi et al., 2007a and 2007b), there has been no attempt to isolate bacteria from pyosalpinx in buffaloes. Pyosalpingeal fluid provides a physical barrier or mechanical hindrance to fertilization resulting in infertility or even sterility. This is the first report that describes pyosalpinx in buffaloes.

This paper describes the results of an investigation of the bacteria accompanying pyosalpinx of the buffalo cow. The principal purpose of this study was to establish the bacterial isolate variations in the presence of pyosalpinx of the buffalo cow genital tract. In addition, the study was designed to investigate the correlation between bacterial infection of uterus and pyosalpinx in buffaloes.
MATERIALS AND METHODS

Genital tract specimens
Buffalo cow reproductive tracts were collected at random intervals from animals slaughtered at a Mosul abattoir from January 2006 to June 2007. The specimens were transported in a cool box provided with ice within 2 h to the College of Veterinary Medicine, University of Mosul. Each specimen was grossly examined in the laboratory in order to determine the nature of the reproductive abnormality and its location in the tract. A total of 385 uterine samples were examined, of which 12 were included in this study having unilateral or bilateral pyosalpinx. The pyosalpinx was diagnosed and evaluated by measurement using ruler and caliper and an aliquot of fluid was aspirated by a syringe to identify the presence of pus and then photographed.

Bacterial isolation
Bacteriological examinations were performed on selected gross lesions. Gross lesions were classified into bilateral and unilateral pyosalpinx. In specimens with pyosalpinx, an aliquot of fluid (1 ml) was aspirated under aseptic conditions using a sterile 2 ml disposable syringe with 18 G needle. In addition, swabs were taken from the body of the uterus and the two uterine horns, after an incision was made aseptically in the uterine wall and a swab was gently moved in the uterine lumen. Swabs were transferred into sterile tubes containing thioglycolate broth as a transport medium, transported to the laboratory at 40°C, and immediately processed for bacteriological examination. Swabs were cultured on sheep blood agar, MacConkey agar and nutrient agar. After 24 h incubation at 37°C, identification of bacteria was based on the characteristic of colony, hemolysis, gram stain, morphology, catalase, coagulase, oxidase, indole production, methyl red, Voges-Proskauer, citrate production tests and sugar utilization as the methods described by (Koneman et al., 1997; Baron, 2004; Carter and Wise, 2004).

Leukocyte concentration
A fluid aliquot (1 ml) was recovered from the uterus and pyosalpinx, put into sterile tubes, and diluted with PBS into 1:10. The concentration of leukocytes in the recovered fluid was counted in the sample using a hemocytometer and expressed in million of cells per ml of fluid. The sample was then centrifuged at 700 g for 10 minutes. The supernatant was removed, and the pellet was used to prepare a smear for cytological evaluation. Smears were fixed with absolute methyl alcohol and then stained with Wright’s-Giemsa stain. Slides were assessed twice by the investigator and once by a technician who was blinded regarding sampling and were counted and classified as neutrophils, lymphocytes, macrophages, eosinophils and basophiles. The concentration of nucleated cells in the recovered fluid was counted in the sample and expressed as number of cells per ml of fluid. The pellet was resuspended in 1 ml of PBS and leukocytes were counted by hemocytometer (25 µl sample + 475 µl of 1% acetic acid). The differential count was used to calculate the concentration of leukocytes in the fluid. Calculations were done as follows:

\[
\text{Conc. of leukocytes / ml} = \frac{\text{conc. of nucleated cells / ml}}{\text{percentage of leukocytes}}
\]

Statistical analysis
Statistical analyses were performed with the software (Sigma sat, Jandel Scientific Software V2.0, Richmond, CA, 2004). The differential leukocyte concentration was tested by the analysis of variance (ANOVA) and least significance differences (LSD). The chi square test was performed for the difference between the percentages.

RESULTS AND DISCUSSION

Twelve of the 385 buffalo genital tracts examined (3.1%) in this study had pyosalpinx. The results of the present study indicated a higher prevalence (P<0.01) of unilateral pyosalpinx (n = 11; 91.7%) than bilateral pyosalpinx (n = 1; 8.3%) in the buffalo cow genital tracts. The examination of the uterine tubes affected with pyosalpinx revealed that the obstruction in these tubes were mostly near the utero-tubal junction or in the end part of the isthmus. Macroscopic examination of the uterine endometrium of the buffaloes affected
with pyosalpinx showed multiple hemorrhagic foci with nodules in the endometrium with mucopurulent exudates (n = 2; 16.7%), diffuse hemorrhage in the entire surface of the endometrium with purulent exudates (n = 8; 66.7%), ovarian-bursal adhesions (n = 1; 8.3%) and complete adhesions of the uterus and the adjacent organs (n = 1; 8.3%).

Table 1 summarizes the bacteriological findings of the pyosalpinx and uterus. Although all samples (100%) of the pyosalpinx lesions had a mixed bacterial growth, the most prevalent bacteria recovered from pyosalpinx were Escherichia coli, Archanobacterium pyogenes and Staphylococcus aureus, 33.3%, 26.7% and 16.7%, respectively. The most prevalent bacteria in the uterus were Archanobacterium pyogenes (23.7%), Escherichia coli (18.4%), Bacillus licheniformis (10.5%) and Streptococcus ubris (7.9%). However, Escherichia coli, Archanobacterium pyogenes and Staphylococcus aureus isolated from the pyosalpingeal fluid were frequently isolated from uterine cavity of buffaloes with pyosalpinx lesion. In addition, Archanobacterium pyogenes and Escherichia coli had a higher prevalence of isolation in the uterus and pyosalpinx. When the bacteria were considered in relation to the degree of uterine infection and pyosalpinx, marked accordance of incidence was noticed. A correlation was noticed between bacteria isolated from the uterus and pyosalpinx. Results of cytological examinations of the uterine discharge and pyosalpingeal fluid showed higher rates of leukocyte infiltration observed in the uterine discharge and pyosalpinx. A significant (P<0.01) increase in lymphocytes compared to other kinds of leukocytes in pyosalpinx and uterine samples included in this study.

Microscopic examination of the uterine tubes with pyosalpinx showed mucosal atrophy and dilatation of uterine tube lumen with signs of severe inflammation including higher infiltration of lymphocytes and sloughing of the mucosa epithelial layer lining uterine tubes. Histopathological studies of the uterus revealed a high prevalence of chronic endometritis (n = 8; 66.7%) and acute endometritis (n = 4; 33.3%). The chronic endometritis observed in these genital tracts was characterized by predominately lymphocytic infiltration, with the presence of plasma cells and macrophages and by irreversible changes including atrophy of endometrial glands and fibrosis, reduced endometrial gland diameter and secretory activity and pyknotic nuclei in the glandular epithelial cells. These degenerative changes were mainly because of the presence of high perivascular fibrosis.

Bacteriological studies of pyosalpinx and uterus demonstrated that there was a correlation between the bacterial contents in the uterus and pyosalpinx. From cytological and histopathological studies of pyosalpinx, it could be possible to conclude that the occurrence of pyosalpinx is due to bacterial infection. Bacterial isolates from pyosalpinx might be related to ascending infection from the uterus. No studies were available concerning pyosalpinx either in buffaloes or in cows with which to compare the results of the present study. Studies on pyosalpinx in humans indicated that the most prevalent bacteria were E. coli, Chlamydia trachomatis and Neisseria gonorrhoeae (Kihaile et al., 2003). The strikingly high incidence of bacterial isolates in pyosalpinx with very high cytological evidence of an inflammatory reaction was associated, on histopathological studies, with a severe histopathological changes in uterine tubes extended with puss. In particular, higher incidence of Archanobacterium pyogenes, Escherichia coli, Staphylococcus aureus, Bacillus licheniformis, Streptococcus ubris and Pseudomonas aeruginosa in the uterus, which were considered as highly pathogenic bacteria of the buffalo uterus (Azawi and Taha, 2002; Azawi et al., 2007a and 2007b), produce severe irreversible histopathological changes in the uterine tissue. This severe inflammation of the uterine tissue could be extended to the utero-tubal junction or the end part of the isthmus producing local infection and inflammatory changes resulting in salpingitis and tubal obstruction and then accumulation of puss. The obstruction in the lumen of the uterine tubes resulted in accumulation of puss. It is tempting to attach some special significance to the association of these organisms with the occurrence of pyosalpinx, and to suggest some contributing role in the production of severe inflammation in the endometrium.
extended to the utero-tubal junction. It is still not clear whether these organisms play a significant pathogenic role in pyosalpinx. This theory could be confirmed by the results of the present study as all obstructions of the uterine tubes examined were near the utero-tubal junction or in the end part of the isthmus. These observations are in agreement with Nikolic et al. (2004) who claimed that pyosalpinx is a sequel to salpingitis in humans. In addition, Mastroianni (1999) suggested that the principal causes of tubal diseases resulting in infertility are the result of some inflammatory process in or around the uterine tubes. However, further studies are required to understand the interactions between pyosalpinx and endometrium.

CONCLUSION

It could be concluded that there is a high correlation between bacteria isolated from uterus and pyosalpinx. Higher rates of leukocyte infiltrations were observed in the uterine discharge and pyosalpinx. The occurrence of pyosalpinx is mainly due to bacterial infection. Inflammation of the uterine tissue could be extended to utero-tubal junction producing local inflammation resulting in salpingitis and tubal obstruction. The obstruction in the lumen of the uterine tubes resulted in accumulation of puss.

![Figure 1. Concentration of leukocytes in pyosalpinx and uterus of buffalo cows.](image-url)
Table 1. Analysis of bacteria of the Pyosalpinx and uterus of buffalo cows.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Pyosalpinx</th>
<th>Uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Percent</td>
</tr>
<tr>
<td>Actinomyces bovis</td>
<td>2</td>
<td>6.7</td>
</tr>
<tr>
<td>Archanobacterium pyogenes</td>
<td>8</td>
<td>26.7</td>
</tr>
<tr>
<td>Bacillus licheniformis</td>
<td>2</td>
<td>6.7</td>
</tr>
<tr>
<td>Corynebacterium bovis</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Corynebacterium hemolyticum</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>10</td>
<td>33.3</td>
</tr>
<tr>
<td>Klebsiella pneomoniae</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Morganella morganii</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>5</td>
<td>16.7</td>
</tr>
<tr>
<td>Staphylococcus intermedius</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Staphylococcus saprophyticus</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus ubris</td>
<td>2</td>
<td>6.7</td>
</tr>
<tr>
<td>Streptococcus zooepidimicus</td>
<td>1</td>
<td>3.3</td>
</tr>
<tr>
<td>Total Number of isolates</td>
<td>30</td>
<td>100</td>
</tr>
</tbody>
</table>

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ABSTRACT

The present study was conducted to measure various biometric parameters of the Murrah buffalo bulls through computer image analysis. Spermatozoa with intact acrosomes were selected and assessed using an immersion lens (1,000×) and standard illumination. The software made it possible to take linear measurements of each spermatozoon: head length, head width, head base, tail length, acrosomal cap length and acrosomal cap width. The results of the morphometric study did not demonstrate the existence of sperm subpopulations. Mean head length, mean head width, mean head base, mean acrosomal cap length and mean acrosomal cap width were 7.59±0.01 µm, 4.91±0.01 µm, 2.47±0.01 µm, 3.46±0.01 µm and 4.51±0.01 µm, respectively. The mean tail length was 56.14±0.01 µm.

Keywords: buffalo, biometry, sperm head, acrosomal cap, sperm morphometry

INTRODUCTION

Associations of abnormal spermatozoa with bull fertility have yielded varying results. Manual methods of analysis are subjective and highly variable within and between technicians, and this may account for these differences. Much time and effort are also spent subjectively evaluating sperm morphology to determine semen quality. For conventional morphology evaluation, sperm are fixed and classified by observers as either normal or abnormal (Barth and Oko, 1989). The percent of total abnormalities or the percent of specific abnormalities are then used as criteria for assessing semen quality. It has been observed that individuals can classify the same sperm differently depending on their interpretation of normal and abnormal morphology (Neuwinger et al., 1990). In fact, the same observer may even classify the same sperm differently on successive occasions. Thus, this approach is subjective, is not very repeatable or sensitive, and because of the inclusion the morphologies of dead sperm, may incorporate information from cells which cannot participate in fertilization. Other approaches using computer aided image analysis have been proposed to address subjectivity, improve repeatability and enhance sensitivity. To address differences in the shapes of the sperm heads, several investigators have used computer aided image analysis to measure the area, perimeter, length, width and obtained several other measures based on these from sperm heads (Auger and Dadoune, 1993; Gravance et al., 1996; Park et al., 1997; Aziz et al., 1998). Keeping on this view the following study was designed to evaluate the Murrah buffalo sperm biometry through computer image analysis.

MATERIALS AND METHODS

Twelve healthy, sexually mature and clinically normal Murrah buffalo (MU) bulls of almost similar body weight age group (nearly 3.0 to 6.5 years) were selected for the study. Semen from the 12 bulls was diluted to 200 X 10^6 sperm/...
ml. To avoid individual technician variation, one person measured all the parameters from the captured image. Dual staining procedure initially developed by Sidhu et al. (1992), which had been used with some modification to identify the clear acrosome structure of buffalo spermatozoa. One hundred microliters of semen were mixed with 0.2 percent trypan blue (in TALP medium without BSA) and incubated for 10 minutes on a clean glass slide at 37°C. After the incubation period, smears of the semen were prepared gently on the glass slides and allowed to dry for 15 minutes at room temperature. A 0.72% (W/V) Giemsa stock solution was prepared by dissolving 1 g of Giemsa dye in a glycerol-methanol mixture (54:84). One gram of Giemsa was diluted five times with distilled water (final concentration of Giemsa working solution was approximately 0.15%). The smears of spermatozoa previously stained with trypan blue were then stained with Giemsa for 1 h at room temperature to evaluate the acrosomal status of the spermatozoa. Smears were dried between the folds of filter paper and stored. The dried smears were studied at 1000X under a light microscope using oil immersion without cover-glass. The slides were used for measurement within a week of preparation. A total of 1100 spermatozoa were measured for the experiment.

Image Analysis Measurements

Images were randomly selected from each slide by using in Nikon Eclipse E600 (Tokyo, Japan) microscope attached to an Nikon camera, interfaced to a PC computer and ACT1 software. The images were obtained by using 100X objectives (oil immersion) in standard light transmission mode (transillumination). Only fresh images were used for the measurements. One speciality of this programme is that stored images cannot be used for measurements. The software was standardizing against a decimal scale. One hundred normal sperm were obtained for each bull from different days of semen sample to avoid any day-to-day variation.

Sperm morphology was quantified in terms of the following morphological features: head length (L), width (W), base (B), head area (A), perimeter (P), acrosomal cap length and width, tail length, ellipticity (e), shape factor (SF) (Ostermeier et al., 2001). The units for measurement variables are micrometers (µm); the ratios are without units. The head area, ellipticity and shape factor are defined in Equations (1), (2) and (3), respectively.

\[ A = 1.05 - 0.081 \times B^2 + 0.64 \times W \times L \]  
Van Duijn (1960)  
(1)

\[ e = \frac{L - W}{L - W} \]  
(2)

\[ SF = (1 - e) \times \frac{p^2}{4\pi A} \]  
(3)

The head shape was calculated as the ratio of head length and head width (Beatty and Napier, 1960). The width base is defined as the distance between the vertices of the base of the sperm head. Sperm head roundness was calculated as convex perimeter (Hunt et al., 1992).

Descriptive statistics (Systat 11.0) were performed on the data to determine normality. Statistical analysis was performed as per standard statistical methods (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSIONS

The measurements of spermatozoa are presented in Table 2. Individual bull variation was not found for the head length, width, base, head area and shape (width:length), acrosomal cap length and width, tail length, perimeter, ellipticity and shape factor.

Sperm morphometry, in combination with other objective traits, can be useful for developing a fertility index. Associations of abnormal spermatozoa with bull fertility have yielded varying results. Abnormal bull sperm morphology has been correlated with reduced fertility (Sekoni and Gustafsson, 1987; Correa et al., 1997). In particular, the occurrence of abnormal sperm head morphology is associated with lower fertility in the bull (Saacke and White, 1972; Sekoni and Gustafsson, 1987). However, a number of other studies have shown no correlation between sperm morphology and fertility (Bratton et al., 1956; Linford et al., 1976) with clear associations between normal bull sperm
morphology and fertility continuing to remain elusive (Johnson, 1997). Any correlation which have found between sperm morphology and bull fertility have been based on subjectively performed analyses. Barth (1992) has suggested that these varying results may be due to experimental and classification errors. In the bull, metric criteria for normal sperm head measurements have not been readily applied to fertility assessment; however, large variability in assessing primary sperm abnormalities, including sperm heads, has been found between laboratories (Bishop et al., 1954). While manual assessment of bull sperm head morphometry has been associated with fertility (Williams and Savage, 1925) and chromatin structure (Sailer et al., 1996), the visual measurement methods employed in these limited studies were extremely laborious or supplied a limited amount of information regarding the overall shape of the sperm head. The wide variation in these sperm head measuring methods makes accurate interpretation of the resulting data difficult; hence, various studies reveal contrasting results. Manual methods of analysis are subjective and highly variable within and between technicians, which may account for these differences. Computer-aided sperm morphometry appears to be a precise method of assessing sperm head dimensions. In the present study, the measurements were taken using Nikon ACT1 software. Our results support the previous work conducted by Aggarwal et al. (2007) on Murrah buffalo bulls.

**CONCLUSION**

In the present study, computer aided image analysis software was used to measure the area, perimeter, length and width and obtain several other measures based on these of sperm heads. Manual methods of analysis are subjective and highly variable within and between technicians. Computer-aided sperm morphometry appears a precise method of assessing sperm head dimensions. The result of the present study gives an idea about the normal sperm morphometry and it will be helpful in judging abnormal morphometry.

**ACKNOWLEDGEMENT**

The authors would like to thank the Director of National Dairy Research Institute for providing financial support during the period of research work.

Table 1. Various sperm morphometric indices\(^a\) of Murrah bulls.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pooled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head length (µm)</td>
<td>7.59±0.01</td>
</tr>
<tr>
<td>Head width (µm)</td>
<td>4.91±0.01</td>
</tr>
<tr>
<td>Head base (µm)</td>
<td>2.47±0.01</td>
</tr>
<tr>
<td>Acrosomal cap length (µm)</td>
<td>3.64±0.01</td>
</tr>
<tr>
<td>Acrosome cap width (µm)</td>
<td>4.51±0.01</td>
</tr>
<tr>
<td>Tail length (µm)</td>
<td>56.14±0.18</td>
</tr>
<tr>
<td>Head shape (width:length)</td>
<td>0.65±0.00</td>
</tr>
<tr>
<td>Ellipticity (e)</td>
<td>0.21±0.00</td>
</tr>
<tr>
<td>Head area (µm(^2))</td>
<td>24.41±0.05</td>
</tr>
<tr>
<td>Perimeter (µm)</td>
<td>19.65±0.02</td>
</tr>
<tr>
<td>Shape factor</td>
<td>0.99±0.00</td>
</tr>
</tbody>
</table>

\(^a\)Mean±SE
REFERENCES


*Continue on page 201
LACTOGENIC EFFECTS OF GROWTH HORMONE IN POST-PARTUM LACTATING RIVERINE BUFFALOES (BUBALUS BUBALIS)

A. Mishra, P.K. Pankaj and B.S. Prakash

ABSTRACT

Twenty two post-partum riverine buffaloes (Murrah breed) were selected from National Dairy Research Institute (NDRI) herd of first or second lactation to determine the length of the post-partum period for cyclicity commencement by Growth Hormone (GH). Blood samples (5-10 ml) were collected twice a week until commencement of cyclicity as determined by progesterone analysis or for period of 3 months (whichever was earlier). Out of these twenty two animals, ten were found to be cyclic as determined through progesterone analysis. Blood samples were collected twice a week at 3-4 day intervals from all animals by means of jugular vein puncture from 5 days post-partum. To fulfill the objectives of the present study, plasma GH was assayed by enzyme immunoassays (EIA) techniques and plasma progesterone was analyzed by a direct radioimmunoassay (RIA) method. The plasma GH profiles in the two groups of animals were not significantly different (P>0.05). In high yielders, a significant (P<0.01) positive correlation was found between GH and milk yield.

Keywords: growth hormone, lactogenic, milk yield, post-partum, buffalo

INTRODUCTION

The buffalo is the mainstay of the milk production system in India. Riverine buffaloes are the major milk producing animal along with their remarkable contribution to draft power as well as meat production in India. The relationship of GH in galactopoesis and lactogenesis has been well documented for more than 50 years, beginning with a Russian scientist’s use of crude pituitary extract to increase milk production by the use of GH, but in the last two decades, the main emphasis has been given to study of the effect of exogenous GH/rbST use on lactation (Etherton and Bauman, 1998).

An earlier hypothesis regarding the mechanism of action of bovine GH was that it enhances mammary alveolar-lobular development (Asimov and Kronze, 1937) but later it was proposed that GH exerted a long lasting homeorhetic control that regulates utilization of absorbed nutrients (Bauman and Vernon, 1993). They proposed that two main cell types, i.e. adipocytes and hepatocytes, are the major targets of somatotropin. Numerous studies confirmed that exogenous bGH could increase milk yield by 6-35% (Ludri et al., 1989). GH concentrations appear to be related to glucose availability in lactating dairy cows (Bines et al., 1980). In high producing dairy cows, glucose is derived predominantly via hepatic gluconeogenesis, and about 60-85% of the glucose is used for milk synthesis.

Bauman et al. (1985) originally demonstrated the significant increase (23-41%) in milk yield from dairy cows which were treated with varying doses of rbST. In the majority of the trials, it has been seen rbST administration increases milk yield within 2-3 days of treatment and prolongs the persistency of lactation (Mc Bride et al., 1990). The average gain in the milk yield of 3-5 kg per day is reported for dairy cows of different breeds, parities

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and genetic potential (Muller, 1992). Recombinant bST administration also increased milk production in buffaloes (Ludri et al., 1989). In the bovine, a positive correlation was observed between serum GH concentration and milk yield (Sartin et al., 1988).

In the present article, we discuss our studies on the endocrinology of the riverine buffaloes (Murrah breed) with emphasis on the endocrine causes for lactogenic ability of the GH in lactating buffaloes.

**MATERIALS AND METHODS**

**Hormone analysis**

To fulfill the objectives of the present study, plasma GH was assayed by enzymeimmunoassays (EIA) techniques, and plasma progesterone was analyzed by a direct RIA method developed in our laboratory.

**Radioimmunoassay (RIA) for progesterone quantification**

Progesterone was estimated by a direct RIA procedure used routinely in our laboratory (Kamboj and Prakash, 1993) with some modifications. Plasma samples and progesterone standards (containing progesterone concentrations ranging from 0 to 250 pg/20 µl) prepared in plasma (20 µl each) were pipetted out in duplicate in 12x75 mm tubes. RIA assay buffer (300 µl) was then added to each tube. Subsequently, 100 µl of anti-progesterone serum (diluted 1:16,000 in the RIA assay buffer) and 100 µl of tracer (≈ 10,000 dpm/100 µl) were added to each tube. The resulting mixture was vortexed and incubated at 4°C in a refrigerator overnight. The free and antiserum-bound hormone were separated by the addition of 0.5 ml of freshly prepared cold (4°C) charcoal-dextran suspension (0.625% activated charcoal + 0.0625% dextran in RIA assay buffer without-gelatin) under constant stirring at 4°C. The tubes were then stirred, incubated at 0°C in an ice-water bath for 10 minutes, and then centrifuged at 3,000 rpm at 4°C for 15 minutes. The supernatant containing the bound progesterone was decanted into scintillation vials. Scintillation fluid (5 ml) was then added to each vial after which the vials were counted after being kept overnight at room temperature. In addition to the above, three sets of tubes were also run. Blank tubes, in duplicate, containing 400 µl RIA assay buffer, 20 µl progesterone-free buffalo plasma and 100 µl tracer, for the calculation of non-specific binding for the charcoal separation procedure. Four tubes containing 300 µl RIA assay buffer, 20 µl progesterone-free buffalo plasma, 100 µl anti-progesterone serum and 100 µl tracer for the calculation of the maximum binding of tracer by the antibody. Total count tubes, containing 100 µl tracer and 920 µl RIA assay buffer, were prepared in duplicate for obtaining total counts of tracer added. This mixture was decanted directly into scintillation vials.

**Enzyme Immunoassay (EIA) procedure for plasma GH**

A highly sensitive enzyme immunoassay procedure using the second antibody coating technique developed in the laboratory (Prakash et al., 2003) was carried out to estimate plasma GH. The bovine GH antibody (Rabbit 3-anti-bGH, Pool 7-12) used in the present investigation was specific for estimation of bovine GH. The details of the specificity of the antiserum were given by Hennies and Holtz (1993).

**First coating**

The first coating was performed by adding 0.63 µg of goat IgG anti-rabbit IgG dissolved in 100 µl of coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) per well of the microtitre plate, (Linbro, Flow Laboratories, Scotland). These plates were subsequently incubated overnight under refrigerated conditions.

**Second coating**

For saturating the remaining binding sites, 300 µl of PBS containing 1 percent BSA was added to all the wells and incubated for 40 to 50 minutes at room temperature under constant shaking on a microtiter plate shaker (Titertek, Flow Laboratories, Germany).

**Washing**

The coated plates were washed twice with 350 µl of washing solution (0.05% Tween-20, 10% PBS in distilled water, Sigma, Germany) per well using an automated microtiter plate washer (Model: EL 50 x 8MS, USA).
Assay protocol

Duplicates of 100 µl of unknown plasma or bovine GH standards prepared in assay buffer ranging from 40 pg/100 µl/well to 10,000 pg/100 µl/well were simultaneously pipetted into respective wells along with 100 µl of charcoal treated plasma (C.T.P). GH antibody diluted 1: 40,000 in assay buffer (50 mM NaPO₄, 0.15 M NaCl, 0.02% Thiomersal, pH 7.4) were added with the aid of a dilutor dispenser (Microlab 500 series, Hamilton, Switzerland). Thereafter, the plates were incubated overnight at room temperature after 30 minutes constant agitation. The next day plates were decanted and washed two times with washing solution before addition of 100 µl of biotinyl-GH conjugate diluted 1:3000 in assay buffer. The plates were further incubated for 30 minutes with constant agitation, decanted and washed four times with washing solution. Then 20 ng streptavidin-peroxidase (Sigma, Germany) in 100 µl of assay buffer was added to all the wells using a digital multichannel pipette (Flow Titertek, Finland) and the plates were wrapped in aluminum foil and incubated for 30 minutes further under constant agitation. All steps were performed at room temperature.

Substrate reaction

The plates were then washed four times with washing solution and incubated further in the dark for 40 minutes after addition of 150 µl of substrate solution per well [Substrate buffer: 0.05 M citric acid, 0.11 M Na₂HPO₄, 0.05% urea peroxide, pH 4.0 with 5 N HCl, substrate solution: 17 ml of substrate buffer plus 340 µl 3,3',5,5'-tetramethyl benzidine; 12.5 mg/ml dimethyl-sulfoxide (Sigma, Germany)]. The reaction was stopped by the addition of 50 µl 4 N H₂SO₄ and the colour produced was measured at 450 nm with a 12-channel microtitre plate reader (ECIL, Microscan, India). GH concentration in buffalo plasma samples was calculated from the standard curve plotted against absorbance at 450 nm by using a Graphpad PRISM 3.0, software package. The quality control of the assay was carried out by performing the following:

Assay sensitivity

The lowest GH detection limit significantly different from zero concentration, was 40 pg/100 µl plasma/well, which corresponds to 0.4 ng/ml in plasma and the 50 percent relative binding was seen at 900 pg/100 µl/well. Intra-and inter-assay coefficient of variations were determined using pooled plasma containing 2.0 ng/ml and were found to be 3.68 and 6.89 percent respectively from eight assays. The specificity of the antiserum used was determined by Hennies and Holtz (1993). The antiserum showed high specificity for GH.

Statistical analysis: The data obtained were analyzed by using Microsoft Excel 2000 and a Graphpad Prism software package, 1995. Paired t-test was employed to test the difference between plasma GH and PRL amongst high yielders and low yielders. To test the metabolic and hormonal parameters, analysis of variance technique was used in the following statistical model:

\[ Y_{ijk} = \mu + \alpha_I + \beta_j + e_{ijk} \]

where, \( Y_{ijk} \) = the dependent variable
\( \mu \) = overall populations mean
\( \alpha_I \) = the effect of \( i^{th} \) treatment,
\( \beta_j \) = the effect of \( j^{th} \) week of experimental period and

\( E_{ijk} \) = random error associated with \( k^{th} \) individual, normally and independently distributed with mean zero, and variance \( \sigma^2 \).

The correlations among these traits were calculated within each group, and the significance was tested using standard statistical methods as described by Snedecor and Cochran (1968).

RESULTS AND DISCUSSION

Cyclicity commencement

Commencement of cyclicity was determined by progesterone analysis at regular 3-4 day intervals. Continuous monitoring of progesterone levels (from 5 days post-partum up to 3 months) was carried out on 12 animals which incidentally had also calved recently. On the basis of progesterone profiles, six had commenced cyclicity during the course of 3 months, while six animals had not commenced cyclicity even up to 90 days post-partum.

Milk production performance

On the basis of milk yield data obtained from them, the animals were also divided into two groups, viz. low yielders (n=6) and high yielders (n=6). The high yielders gave 9.0 to 13.0 litres milk/
day whereas the low yielders produced between 5.0 to 8.5 litres milk/day. The overall milk yield ± SEM for low yielders was 6.58 ± 0.469 kg/day as against 10.92 ± 0.608 kg/day for the high yielders, which was a significant (P<0.01) difference.

**Endocrine parameters**

**Plasma growth hormone in high and low yielders**

There was no significant (P>0.05) difference between mean growth hormone concentrations post-partum (n=6), which was 14.91±2.315 ng/ml and 10.51±1.646 ng/ml for high and low yielders respectively.

While no reports are available for comparing our data on growth hormone in high and low yielding buffaloes (or even cows), the magnitude of growth hormone levels were similar to those reported by Sartin *et al.* (1988) who recorded hormone levels of 19.3, 16.4 and 13.6 ng/ml at 1 to 20, 21 to 40 and 41 to 56 days lactation in cows, respectively. In lactating Murrah buffaloes, the level of growth hormone has been reported to be 2.48 ng/ml (Jindal and Ludri, 1990) but in contrast to this, according to Patel (2001), the level was 14 to 20 ng/ml and decreased in advanced lactation. The normal concentration of growth hormone in the plasma of cattle ranges between 3 to 30 ng/ml depending upon age, sex and stage of lactation (Schams *et al.*, 1989).

The variation in growth hormones levels in different studies can also be attributed to the different sources of purified growth hormone used in different studies as well as differences in antisera specificities. No significant trend in growth hormone profile was recorded for either high or low yielders (Figure 1) over 90 days post-partum. Vasilatos and Wangsness (1981) reported that in cattle during early lactation (30 days post-partum) the plasma growth hormone concentration was elevated (13.2 ng/ml) compared to that in later lactation (90 days post-partum) 9.8 ng/ml. However, they stated that the increased growth hormone status in early lactation was due to greater magnitude of individual secretory spikes rather than a difference in frequency of spikes or baseline plasma levels of GH.

**GH and milk yield**

In high yielders a significant (P<0.01) positive correlation was found between GH and milk yield, whereas in low yielders a positive correlation of 0.1832 (p<0.05) was recorded between plasma GH and milk yield. Increased milk production by GH was found by Bines *et al.* (1980) and Peel and Bauman (1987). Also a high correlation between GH and milk production might be expected; however, Koprowski and Tucker (1973) found no such correlation in cows. Contradictory results were reported by Herbein *et al.* (1985) who found no-significant relationship between GH concentration and milk production.

Positive correlation between GH and milk yield has also been recorded by Sartin *et al.* (1988) in cows. Bines *et al.* (1980) and Peel *et al.* (1981) have reported no significant correlation between the two parameters. No studies attempting to correlate GH with milk yield have been carried out in buffaloes. Present observations are also supported by the earlier reports by Bines *et al.* (1980) and Peel *et al.* (1981) in cattle and Ludri *et al.* (1989) in buffaloes, which have shown an increase in milk yield in response to exogenous GH injections. These observations imply that a high correlation between GH and milk yield as in the present study could be expected. During normal lactation increasing milk yield is associated with elevated circulating GH (Ingalls *et al.*, 1973; Hart *et al.*, 1979; Vasilatos and Wangsness, 1981; Bines and Hart, 1982 and Sartin *et al.*, 1988), and the level declines with decrease in lactation (Koprowski and Tucker, 1973). High yielding cows have higher serum growth hormone than low yielding cows during lactation (Hart *et al.*, 1978; Sartin *et al.*, 1988 and Collier *et al.*, 1989).

**CONCLUSION**

The endocrine profiles associated with the lactogenic property of the hormone principle was studied with respect to plasma GH and progesterone using sensitive EIA and RIA procedures standardized in our laboratory. The overall milk yield ± SEM for the low yielders was 6.58±0.469 kg/day as against 10.92±0.608 kg/day for the high yielders, which were significantly different (P<0.01). In high yielders, a significant (P<0.01) positive correlation of was found between GH and milk yield. In low yielders, a positive correlation of (P<0.05) was recorded between plasma GH and
milk yield. The plasma GH profiles in the two groups of animals were not significantly different (P>0.05). The positive correlation of GH with milk yield indicates that the hormone can be used as an index of production performance in riverine buffaloes.

ACKNOWLEDGEMENT

The authors would like to thank the Director of NDRI for providing financial support during the period of research work.

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yielding cattle at various stages of lactation. *J. Endocrinol.*, **77**: 333.


*Continued from page 195


DETECTION OF BOVINE HERPESVIRUS 1 (BHV-1) INFECTION IN SEMEN OF BREEDING BULLS OF GUJARAT BY A DIRECT FLUORESCENCE TEST


ABSTRACT

Bovine herpesvirus 1 (BHV-1), the causative agent of infectious bovine rhinotracheitis (IBR), is considered to be the most common viral pathogen found in bovine semen. BHV-1 is associated with several clinical conditions including infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, balanoposthitis, conjunctivitis and generalized disease in newborn calves causing great economic loss to the livestock industry. The present study was undertaken to detect the presence of viral antigen by direct immunofluorescence in semen of breeding bulls of five different semen collection centres of Gujarat. A total of 101 semen samples from cattle and buffalo breeding bulls were tested for BHV-1 antigen using a direct immuno-fluorescence kit made available by VMRD, Inc. Pullman, USA., and green fluorescence was observed in 33 (32.67%) samples. Out of 49 cattle bulls and 52 buffalo bulls, 16 and 17 bulls, respectively, were found to be positive. This shows an equal distribution of BHV-1 antigen in both the species. Finally, the study revealed presence of BHV-1 in the semen of breeding bulls of Gujarat. Thus, under the Sexual Health Control Programme, proper measures must be taken at the State level for controlling BHV-1 infection. All breeding bulls must be tested periodically for detection of both BHV-1 antibody in serum and the presence of BHV-1 in semen. The bulls must be free from BHV-1 infection prior to use.

Keywords: BHV-1, semen, direct FAT, breeding bulls

INTRODUCTION

Bovine herpesvirus type 1 (BHV-1) is a member of the Alphaherpesvirinae. BHV-1 infects the respiratory and genital tracts of cattle and buffaloes, causing various diseases such as infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, and infectious pustular balanoposthitis, abortion, mastitis, infertility, tracheitis, conjunctivitis-keratoconjunctivitis, encephalitis and fatal disease in newborn calves and thus causing great economic losses to the livestock industry (Gibbs and Rweyemamu, 1977).

Bovine herpesvirus-1 infection was first reported in India by Mehrotra et al., 1976 and various workers have since reported the widespread prevalence of the disease in various parts of the country (Samal et al., 1981; Renukaradhya et al., 1996). The infection has serious economic implications for India, which is emerging as the world’s biggest milk producer and has the world’s largest cattle and buffalo population.

Transmission of the virus is thought to occur primarily via the respiratory route. However, the virus can also be transmitted venereally and by BHV-1 contaminated semen from virus-shedding bulls (Kupferschmied et al., 1986 and Afshar and Eaglesome, 1990). Bulls may shed virus in semen during both clinical and subclinical infections (Oirscholt et al., 1993). After genital infection and seroconversion, BHV-1 localizes and persists, latently, in sacral ganglia (Ackermann and Wyler, 1984). Shedding of virus reoccurs during periodic reactivation of viral replication. Viral reactivation from the latent state is generally thought to be stress-induced but can also be induced by the injection of

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The use of semen from BHV-1 infected bulls necessitates the identification of BHV-1 contaminated semen samples to prevent transmission of virus to the recipient cow. To prevent the transmission of BHV-1 by artificial insemination, only semen that is free of BHV-1 should be used. The virus is excreted through secretions (nasal and ocular), and is present in the placenta of aborted animals and semen. Bovine semen is stored and handled in conditions that are ideal for preserving the viral pathogen, so contaminated semen presents a potential threat to the cattle industry. BHV-1 can spread through artificial insemination (AI), causing a variety of genital tract disorders such as endometritis, infertility and abortion.

The present methods of BHV-1 detection in diagnostic laboratories are virus isolation (VI), the fluorescent antibody test (FAT) of tissues, and screening for specific antibodies either in paired serum samples or single serum samples. Due to the latent nature of most of BHV-1 infections and intermittent shedding of the virus in semen, the situation is complex since the semen collected at the AI centres is distributed for the female dairy animals, which further results into dissemination of the viral agent to the virus-free herds. Such a situation causes huge economic losses to the dairy industry. Keeping above points in view, the present study was designed to detect the presence of BHV-1 in the semen of breeding bulls by a direct fluorescence test using a direct immunofluorescence kit made available by VMRD, Inc. Pullman, USA, since the dairy industry plays pivotal role in the economy of the Gujarat state.

MATERIALS AND METHODS

Reference reagents

Direct FA conjugate (Catalog no.: 210-69-IBR, VMRD) was made available by VMRD, Inc. Pullman, USA.

Reference virus

IBR seed virus (7th passage) was procured from PD-ADMAS, Bangalore. The virus was processed for the 8th and 9th passages at the Disease Investigation and Monitoring Laboratory, NDBD, Anand. This 9th passage IBR seed virus was used as reference virus for standardization of protocol for direct fluorescence.

Semen samples

A total of 101 semen samples were collected from cattle (49 samples) and buffalo breeding bulls (52 samples) of five different AI centres of Gujarat. Samples were collected in a screw capped plastic vials and transported on ice to the laboratory and were stored at -80°C temperature for future use.

Standardization of direct FAT

In a 25 ml tissue culture flask containing MDBK cell monolayer, 100 µl of reference virus was inoculated. The flask was incubated at 37°C for 1 h and then 20 ml of RPMI medium was added. The flask was incubated at 37°C for 24-36 h till 50% CPE appeared. Then RPMI medium was discarded and MDBK cell monolayer was fixed in 80% acetone.

In another method, 50 µl of reference virus was added to 1 ml of neat semen and vortexed. A smear was prepared from 50 µl of this mixture and fixed in an acetone-methanol (3:1) solution. The fixed cell layer and smear were further processed for direct FAT as per the test protocol (from step 3 onwards).

Test protocol

Direct FAT was performed as per the protocol outlined in the user manual supplied with the VMRD kit, as follows.
1. Smears were prepared from 50 µl of semen samples on clean slides.
2. Smears were air dried overnight at room temperature and fixed for 20 minutes in acetone-methanol (3:1) at room temperature.
3. Slides were stained with 50-75 µl direct FA conjugate for 30 minutes at 37°C in a humid chamber.
4. Slides were gently rinsed in 1X FA rinse buffer (4X FA rinse buffer composition Na₂CO₃:11.4 gm; NaHCO₃:33.6 gm; NaCl:8.5 gm; DI/dH₂O:Q.S. to 1 liter, pH:9.0) and then soaked for 10 minutes in 1X FA rinse buffer.
5. Slides were drained and their backs and edges were dried with a paper towel without allowing the stained surface to dry.
6. Slides were mounted with FA mounting fluid (glycerol/ 1X FA rinse buffer, pH 9.0, 1:1) and
viewed with fluorescent microscope at 100X-250X. Confirmation was done at 400X.

**Observation of Result**

Slides in which green fluorescence was observed were considered as positive. Slides in which no fluorescence was observed were taken as negative.

**RESULTS**

**Overall incidence**

Out of 101 samples tested for BHV-1 antigen by direct FAT, green fluorescence was observed in 33. The remaining 68 samples did not show any green fluorescence and thus were negative. Thus, overall incidence of BHV-1 was recorded as 32.67%. Table 1 represents location wise, species wise, breed wise incidence of BHV-1.

**Locationwise incidence**

Semen samples were collected from five AI centres of Gujarat, located in different regions of the state. Out of the five AI centres, Centres I and IV were in the North Gujarat region, Centre II was in the South Gujarat region, Centre III in the Saurashtra region, and Centre V in the central Gujarat region. Maximum incidence of BHV-1 by direct FAT was observed in AI centre V (50.00%), followed by IV (39.12%), III (38.10%) and II (21.43%). All the samples from one centre (AI Centre I) were found negative.

**Species wise incidence**

Semen samples from different centres included both cattle as well as buffalo bull semen. Out of 101 samples, 49 and 52 samples, respectively, were from cattle and buffalo bulls. Out of 49 samples from cattle, 16 (32.65%) were found positive, whereas 17 (32.69%) of 52 samples were found positive from buffalo bulls. Thus there was an equal distribution of incidence of BHV-1 in cattle and buffalo bulls.

**Breed wise incidence**

Cattle: Out of 49 samples, 10 and 39 samples, respectively, were from Gir cattle bulls and crossbred bulls. Greater incidence of BHV-1 by direct FAT was observed in Gir cattle bulls (50.0%) than in crossbred bulls (28.2%).

Buffalo: Out of 51 samples, 38, 7 and 7 samples, respectively, were from Mahesani, Surti and Jafrabadi buffalo bulls. Maximum incidence of BHV-1 by direct FAT was observed in Mahesani buffalo bulls (36.84%) followed by Jafrabadi buffalo bulls (28.57%) and Surti buffalo bulls (14.28%).

**DISCUSSION**

During present investigation, overall incidence of BHV-1 was found to be 32.67%. Due to the lack of published literature on detection of BHV-1 infection in semen samples using direct FAT, it is difficult to compare this study and to reach meaningful conclusions. However, Elazhary et al. (1980) demonstrated BHV-1 in sperm heads from the bull by direct FAT. Mishra et al. (1982) in Orissa detected viral antigen in semen and found a lower incidence of 18% by immunofluorescence technique. Misra and Mishra (1987) cultured 15 samples (tissues of aborted foetus, uterine mucus and semen) in primary bovine kidney cells and isolated BHV-1 from five samples and then confirmed by direct FAT in cell culture.

Like wise other workers, viz., Reed et al. (1971); Popisil et al. (1972); Martell et al. (1974); Terpstra (1979); Edwards et al. (1986) and Silim and Elazhary (1983), detected BHV-1 antigen by direct FAT from nasal, tracheal, vaginal, preputial and conjunctival swabs and aborted foetal tissues. Thus from the present study, it is deduced that the direct FAT can be a valuable test to find the incidence of BHV-1 within a short period of time.

Considering the fact than BHV-1 can be transmitted through artificial insemination, the findings of this study are alarming for the Gujarat State. Therefore, the results of this study should be taken as an indicator of evidence of infection foci and warrant large scale state wide surveys using appropriate sampling techniques for a meaningful assessment of the disease situation in the bovine population so as to help in planning state level control programmes.
Table 1. BHV-1 antigen in semen of bulls by direct FAT.

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Numbers tested</th>
<th>Number positive</th>
<th>Percent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>[A] Centre/Location:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Himmatnagar</td>
<td>11</td>
<td>00</td>
<td>00.00</td>
</tr>
<tr>
<td>II. Surat</td>
<td>14</td>
<td>03</td>
<td>21.43</td>
</tr>
<tr>
<td>III. Rajkot</td>
<td>21</td>
<td>08</td>
<td>38.10</td>
</tr>
<tr>
<td>IV. Mahesana</td>
<td>51</td>
<td>20</td>
<td>39.12</td>
</tr>
<tr>
<td>V. Anand</td>
<td>04</td>
<td>02</td>
<td>50.00</td>
</tr>
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<td><strong>Total</strong></td>
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<td>32.67</td>
</tr>
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<td><strong>[B] Specieswise:</strong></td>
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<td></td>
</tr>
<tr>
<td>Cattle</td>
<td>49</td>
<td>16</td>
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</tr>
<tr>
<td>Buffalo</td>
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<td>17</td>
<td>32.69</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>101</td>
<td>33</td>
<td>32.67</td>
</tr>
<tr>
<td><strong>[C] Breedwise (Cattle):</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross bred</td>
<td>39</td>
<td>11</td>
<td>28.20</td>
</tr>
<tr>
<td>Gir</td>
<td>10</td>
<td>5</td>
<td>50.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
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<td>16</td>
<td>32.65</td>
</tr>
<tr>
<td><strong>[D] Breedwise (Buffalo):</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mahesani</td>
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<td>Jafrabadi</td>
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<tr>
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</tr>
<tr>
<td><strong>Total</strong></td>
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<td>17</td>
<td>32.69</td>
</tr>
</tbody>
</table>

**ACKNOWLEDGEMENT**

The authors are thankful to Dr. J.V. Solanki, The Dean, Anand Veterinary College, for providing all essential facilities to conduct this study. Authors are also thankful to Dr. S. K. Rana, Disease Investigation and Monitoring Laboratory, NDDB, Anand for providing cell culture facilities. We are also thankful to the Incharges of different semen collection stations of Gujarat State for providing semen samples for this study.

**REFERENCES**


EVALUATION OF PCR AND INDIRECT ENZYME LINKED IMMUNOSORBENT ASSAY ON MILK SAMPLES FOR DIAGNOSIS OF BRUCELLOSIS IN BOVINES

T.J. Patel, A.N. Kanani, Lata Jain, C.G. Joshi and J.H. Purohit

ABSTRACT

Polymerase chain reaction (PCR) was used for detection of Brucella DNA in bovine milk. A genus specific primer pair which amplified a 223 bp fragment of gene encoding a 31-kDa Brucella abortus antigen was used in assay. The efficacy of the PCR was evaluated by comparing with the result of milk-ELISA for the diagnosis of Brucella infection in bovines. The milk-ELISA resulted in more positive samples as compared to PCR, since it detected Brucella antibodies in 15 milk samples out of 53 bovines while Brucella DNA detected the antibodies in nine out of 53 milk samples. The results suggest that ELISA is a better screening test than PCR, and their simultaneous application could be more useful than one test alone for a rapid screening of brucellosis in bovines.

INTRODUCTION

Bovine brucellosis is found worldwide, and although it has been eradicated from many countries, it is one of the most serious diseases in developing countries. The rates of infection vary greatly from one country to another and between regions within a country. The highest prevalence is seen in dairy cattle. In India, brucellosis was first recognized in 1942 and is now endemic throughout the country. The disease has been reported in cattle, buffaloes, sheep, goats, pigs, dogs and humans.

Despite the advances made in diagnosis and therapy, brucellosis is still widespread, and its prevalence in many developing countries is increasing. The disease has a considerable impact on human and animal health, as well as socioeconomic impacts, especially, where rural income relies largely on livestock breeding and dairy products.

Probably the main route of entry is ingestion. The practice of sharing equipment between various farms is also a potential danger. It has also been observed that calves fed on infected milk harbour infection and excrete Brucella organisms in their faeces for up to 4 weeks after the cessation of feeding. The high rate of isolation of the Brucella from the udder and the supramammary lymph nodes is reflected in the numbers excreted in milk and can vary from a few hundreds up to 2,000,000 organisms/ml of milk (Corbel, 1988). Thus, the milk is an important material to be processed for assessing the prevalence of brucellosis in a particular area.

The most certain test for an accurate diagnosis of brucellosis is the bacteriological isolation of Brucella spp. It has the advantage of detecting the organisms directly, but it is time consuming since it takes about 10 days or longer for proper identification of the causative agents and it has reduced sensitivity in chronic infection. Besides, the culture materials must be handled carefully, as the organisms are class III pathogens. Also, when testing large numbers of animals, this direct diagnostic test is often impractical, and so indirect tests detecting antibodies in milk are used routinely to screen for animals suspected for brucellosis. The milk ring test (MRT) has been used for many years for detection of dairy cows infected with B. abortus, since milk constitutes a highly desirable source of antibody for routine screening purposes and for the identification of infected individuals as sample collection is simple and non-invasive (Roepke et al., 1950 and 1974; Alton et al., 1975). It is particularly useful on bulk milk samples and is effective for screening and
monitoring small dairy herds for brucellosis. In large herds (>100 lactating animals) and in early infection the MRT does not show sufficient sensitivity. Furthermore, the MRT when performed using undiluted whole milk from an individual may give false positive results shortly after parturition, near the end of lactation, and when mastitis is present (Alton et al., 1988). Considering the limitations of MRT several indirect enzyme-linked immunosorbent assays (ELISA) have been developed and successfully tested on milk samples for detection of antibodies to *B. abortus* (Thoen et al., 1983). However, antibody detection is not wholly satisfactory because not all infected animals produce significant levels of antibodies and several bacteria can produce cross reacting antibodies (Alton et al., 1988). Because of these difficulties, the development of new diagnostic tests for the direct detection of *Brucella* spp. in milk, or other samples is increasingly interesting. PCR assay has been shown to be a valuable method to detect *Brucella* DNA in varieties of clinical samples including tissues (mainly aborted foetuses and associated maternal tissues), blood, milk and semen (Fekete et al., 1992; Leal-Klevezas et al., 1995; Amin et al., 2001; Kanani, 2007) but, no comparisons with serological tests have been made. The aim of this study was to compare a PCR assay with indirect ELISA on milk samples for the diagnosis of bovine brucellosis.

**MATERIALS AND METHODS**

**Samples:**

Milk samples were collected from 13 cattle and 40 buffaloes. Milk samples were stored at -20°C until the extraction of bacterial DNA or serological studies were performed.

**Reference Bacterial Strains**

The *Brucella abortus* biovar 1 strain 544 procured from the Biotechnology Laboratory, National Dairy Development Board, Anand, and *Brucella abortus* live vaccine strain 19 (Bruvex, Indian Immunologicals Limited, Hyderabad) were used as reference bacterial strains for standardization of PCR.

**Samples Processing for PCR**

**DNA extraction**

DNA was extracted from milk samples using the procedure described by Romero and Lopez-Goni (1999). For standardization of the DNA extraction protocol, milk was spiked with known reference bacterial strains and was processed in a way similar to the field milk samples. Frozen milk samples was thawed at room temperature, and 500 µl of the sample was mixed with 100 µl of NET buffer {50 mM NaCl-125 mM EDTA-50 mM Tris-HCl (NET)} (pH 7.6) and 85 µl of 24% SDS, thoroughly mixed by vortexing. After incubation at 80°C for 10 minutes. the mixture was cooled on ice for 10 minutes. and digested with 12 µl proteinase K (20 mg/ml, w/v) and the mixture was incubated at 50°C for 2-3 h. DNA was extracted by the standard protocol with phenol-chloroform-isoamyl alcohol, precipitated with isopropanol, washed with 70% ethanol, and air dried. The DNA pellet was resuspended in 100 µl of sterile distilled water or 0.3 X TE and kept in a water bath at 65°C for one hour and stored at -20°C until further use. DNA extracted from a milk sample spiked with *Brucella abortus* strain 544 was used as a positive PCR control.

**Amplification and Detection of Brucella DNA by PCR**

The oligonucleotide primers used were B4 (TGG CTC GGT TGC CAA TAT CAA) and B5 (CGC GCT TGC CTT TCA GGT CTG) designed from the nucleotide sequence of the 31-kDa *Brucella abortus* antigen (Baily et al., 1992). PCR was performed in 25 µl volumes containing 12.5 µl PCR master mix (MBI Fermentas), 1 µl of each primer (10 pmol/µl), 3 µl of extracted DNA and 7.5 µl of DNase free water. The amplification was performed in a thermal cycler (MyCycler, Bio-Rad, USA) with a cycling conditions consisted of an initial denaturation step at 93°C for 5 minutes, followed by 35 cycles of 90°C for 1 minutes, 64°C for 30 s, 72°C for 1 minutes and a final extension at 72°C for 10 minutes. The negative control consisted of sterile water instead of DNA template. After amplification, 5 µl of the reaction mixture was electrophoresed in a 2.0% agarose gel, stained with ethidium bromide, and photographed on an
UV transilluminator. A clear, compact band of 223 bp was regarded as a positive result.

**Milk-ELISA**

Standard protocol of the ELISA kit provided by the manufacturer (VMRD, Inc., USA) was strictly followed.

**Test Procedure**

a) **Loading of samples and controls**: Using a pipettor set at 100 µl, samples and controls were transferred to the antigen-coated plate. The loaded assay plate was gently mixed by tapping the side of the plate several times, taking care not to spill samples from well to well. The plate was incubated 30 minutes at room temperature (21-25°C).

b) **Washing of wells**: After incubation, the plate was washed four times by manual washing. For manual washing, the contents of the wells were dumped into a sink and the remaining sera and controls were removed by striking sharply the inverted plate four times on a clean paper towel. Each well was filled immediately with 1X wash/diluent solution using a multichannel pipettor, then the solution was dumped and the inverted plate struck sharply on a clean paper towel as above. This washing procedure was repeated thrice (total four washes).

c) **Adding conjugate**: 100 µl of diluted (1X) antibody-peroxidase conjugate was added to each well. The plate contents were gently mixed by tapping the side of the plate several times. The plate was incubated uncovered for 30 minutes at room temperature (21-25°C).

d) **Washing of wells**: After incubation, the washing procedure was repeated as per Step b (total four washes).

e) **Adding of substrate solution**: 100 µl of substrate solution was added to each well. The contents were gently mixed by tapping the side of the plate several times. The plate was incubated 10 minutes at room temperature (21-25°C). Exposure of plate to the direct sunlight was avoided.

f) **Adding of stop solution**: 100 µl of stop solution was added to each well. The contents were gently mixed by tapping the side of the plate several times.

g) **Reading and recording of the test result**: Immediately after adding the stop solution, the plate was read on a plate reader. The optical density (OD) reading wavelength was set to 620 nm.

**Validation of Test**

The test was considered valid when mean OD of the negative controls was <0.250 and the mean OD of the positive controls was > 0.500 and ≤ 1.800.

**Observation of Result**

The SP ratio was calculated as follows:

\[ SP = \frac{\text{sample OD} - \text{mean NC}}{\text{mean PC} - \text{mean NC}} \times 100 \]

where:

- \(SP\) = sample/positive control ratio
- \(\text{sample OD}\) = OD value of sample
- \(\text{mean NC}\) = mean OD value of negative control
- \(\text{mean PC}\) = mean OD value of positive control

Samples producing an SP ratio <25 were considered negative, whereas those with SP ratios ≥ 25 were considered positive.

**RESULTS AND DISCUSSION**

In this study, milk samples collected from 13 cattle and 40 buffaloes were subjected to the PCR assay for detection of *Brucella* DNA and milk-ELISA for detection of *Brucella* antibodies.

<table>
<thead>
<tr>
<th></th>
<th>ELISA positive</th>
<th>ELISA negative</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PCR positive</td>
<td>6</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>PCR negative</td>
<td>9</td>
<td>35</td>
<td>44</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>15</td>
<td>38</td>
<td>53</td>
</tr>
</tbody>
</table>

Table 1. Comparison of PCR and milk-ELISA results in 53 bovines.
Of the 53 bovines whose milk was tested, 15 bovines revealed the presence of *Brucella* antibodies while nine bovines showed the presence of *Brucella* DNA. In the present study six out of 15 animals positive for *Brucella* antibodies in milk revealed the presence of *Brucella* DNA in PCR assay; thus, *Brucella* DNA could not be detected in nine bovines which were positive for *Brucella* antibodies. However, *Brucella* DNA could be detected by PCR from three of the 38 bovines negative for milk antibodies. Agreement between these two methods was found to be 77.35%. Similarly, Romero *et al.* (1995) found a higher sensitivity of milk-ELISA (98.2%) as compared to PCR (87.5%) for detection of *Brucella* infection when they tested 56 *Brucella* milk culture positive cattle. They found one PCR positive sample to be negative by milk-ELISA and seven milk-ELISA positive samples turned out to be PCR negative, yielding an observed proportion of agreement of 0.91 for the two tests. They also found 100% specificities of both the tests when testing the milk samples from *Brucella*-free cattle. Evangelista *et al.* (2005) found that PCR was less sensitive than the serological methods. Guarino *et al.* (2000) found five blood samples positive by PCR from CFT and ELISA negative buffaloes. Leal-Klevezas *et al.* (2000) found 86% of the blood samples positive by PCR, while 60% were found positive by serological test in goat. Gupta *et al.* (2006) found higher sensitivity and specificity of the PCR than serological methods in a study conducted for diagnosis of brucellosis in goat. They found 12 samples exclusively positive in PCR, which were not detected in serology.

The study revealed the presence of *Brucella* antibodies as well as *Brucella* organisms in the bovine milk. Simultaneously the bovine milk negative for *Brucella* antibodies also revealed the presence of *Brucella* organisms and vice versa. This might be due to the previous exposure and possibility of periodic shedding or no shedding of the *Brucella* in milk (Corbel, 1988). Thus under Health Control Programme to eradicate brucellosis from animals as well as from the public health point of view, proper measures must be taken at the State level for controlling brucellosis. Therefore all animals must be tested periodically for detection of both *Brucella* antibody and presence of organisms.

**REFERENCES**


ABSTRACT

The maintenance of reduced glutathione (GSH) content in erythrocytes is essential for their viability as this tripeptide protects the cell components from oxidative damage. The reduced glutathione level has been estimated in six different breeds of buffaloes. The overall GSH level in buffaloes was found as 19.76±0.12 mg/100 ml blood, with the highest in the Murrah breed (22.79±0.35) followed by Badhawari (21.64±0.48), Nili Ravi (21.49±0.72), Surti (19.76±0.53), Jaffarabadi (19.35±0.46) and lowest in non-descript breeds as 18.86±0.14. The effect of breed was found highly significant (p<0.01) on glutathione level. Higher levels of GSH were found in young animals as compared to calves and adults. Again, healthy animals showed a higher level of GSH as compared to emaciated animals, which indicates the GSH level is highly related to the health status of an animal. Good correlations between glutathione level and age at first calving (0.03), total milk yield (0.46), lactation length (-0.23) and average milk yield (0.48) were found.

Keywords: reduced glutathione (GSH), Murrah, Jaffarabadi, Surti, Nili Ravi, Badhawari

INTRODUCTION

Glutathione is a tripeptide consisting of glutamic acid, cysteine and glycine. It is widely distributed in living tissues and is maintained there in relatively large amounts as compared to most other soluble low molecular weight components of the tissues. The major part of this tripeptide (99%) is present in the reduced form (GSH) and a very minor part is present in the oxidized form (GSSG) (Srivastava and Beutler, 1969). It functions in oxidative reduction reactions and in amino acid transport across the cellular membrane, protects and activates thiol dependent enzymes, and is also used as a co-factor for some enzymes. It is considered to be of utmost importance in mitosis, in the functioning of hormones, and as a reactant to detoxification mechanism. The demonstration of glutathione in relation to some drug induced haemolysis (Beutler, 1957) provided a great impetus to workers in the fields of genetics and biochemistry. Glutathione has been found to be associated with physical health and longevity and can be used as an predictor of morbidity and mortality in man. It is also being used as a putative indicator of health in man (Lang et al., 1992). Although a number of investigations have been carried out in the haematology and blood protein polymorphism systems of buffaloes (Archer and Jeffcot, 1977; Bachmann et al., 1978; Elamin and Saha, 1980), we are unaware of any comprehensive study of blood reduced glutathione (GSH) level in this species of ruminant. In view of the very little information on the above topic in buffaloes, it is deemed important to find out the status of GSH in Indian breeds of buffaloes. Accordingly we have measured the activity of GSH in six different breeds of buffaloes. This study also throws genetic interactions with diseases and production traits in buffaloes.
MATERIALS AND METHODS

Blood samples were collected from 1527 buffaloes of six different breeds i.e. Murrah, Jaffarabadi, Bhadawari, Surti, Nili Ravi and nondescript are categorized by for age group as adult, young and calves, by health status as healthy and emaciated and by sex. Reduced glutathione (GSH) in whole blood was estimated by the DTNB methods of Prins and Loss (1969). The advantage of this method was that the colour formed by DTNB was related to the amount of glutathione in such a fashion that determination of standard glutathione solution was not necessary after initial spectrophotometric standardization. Calculation of glutathione concentration in whole blood for buffaloes was done by using the formula = 45.098x O.D. at 412 nm wave length. Haemoglobin concentration (gm/100 ml blood) was estimated calorimetrically according to the methods described by Richterich (1969) at 578 nm wave length. All the calculations and statistical analyses were done following the procedures described by Snydecor and Cochran (1967).

RESULTS AND DISCUSSION

The reduced glutathione (GSH) levels were estimated calorimetrically in different age groups, health status, and sex in six different breeds of buffaloes and are presented in Table 1. Overall glutathione level in buffalo was 19.76±0.12 mg/100 ml blood. Whereas glutathione levels in Murrah, Jaffarabadi, Bhadawari, Surti, Nili Ravi and nondescript breeds were found as 22.79±0.35, 19.35±0.46, 21.64±0.48, 19.76±0.53, 21.49±0.72 and 18.86±0.14 mg/100 ml blood respectively. From the analysis of variance, it was found that the effect of breed was highly significant (p<0.01) on glutathione level. This significant effect may be due to significantly higher level of glutathione in Murrah, Bhadawari and Nili Ravi breeds which did not differ among themselves but differed significantly from Jaffarabadi, Surti and non-

Table 1. Reduced glutathione (GSH) levels in different age groups, health status and sex in different breeds of buffaloes.

<table>
<thead>
<tr>
<th>Category</th>
<th>Breeds</th>
<th>Murrah (209)</th>
<th>Jaffarabadi (121)</th>
<th>Bhadawari (123)</th>
<th>Surti (93)</th>
<th>NiliRavi (24)</th>
<th>Nondescript (957)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age Group</strong></td>
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<tr>
<td>Adult</td>
<td></td>
<td>22.56±0.55</td>
<td>18.81±0.57</td>
<td>20.78±0.69</td>
<td>19.25±0.83</td>
<td>22.30±0.88</td>
<td>18.28±0.17</td>
</tr>
<tr>
<td>Young</td>
<td></td>
<td>23.91±0.50</td>
<td>20.11±0.70</td>
<td>22.40±0.67</td>
<td>20.15±0.70</td>
<td>21.08±0.99</td>
<td>19.56±0.22</td>
</tr>
<tr>
<td>Calf</td>
<td></td>
<td>19.84±0.96</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>18.90±0.75</td>
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<tr>
<td><strong>Health Status</strong></td>
<td></td>
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<tr>
<td>Healthy</td>
<td></td>
<td>23.19±0.36</td>
<td>20.86±0.56</td>
<td>20.97±0.50</td>
<td>21.72±0.54</td>
<td>21.94±0.72</td>
<td>20.64±0.16</td>
</tr>
<tr>
<td>Emaciated</td>
<td></td>
<td>19.62±1.23</td>
<td>17.27±0.67</td>
<td>17.84±0.19</td>
<td>16.01±0.83</td>
<td>19.22±2.34</td>
<td>16.36±0.18</td>
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<tr>
<td><strong>Sex</strong></td>
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</tr>
<tr>
<td>Male</td>
<td></td>
<td>24.35±0.61</td>
<td>19.54±0.87</td>
<td>22.22±0.74</td>
<td>20.16±0.98</td>
<td>22.27±0.79</td>
<td>18.97±0.21</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>22.06±0.43</td>
<td>19.25±0.54</td>
<td>21.35±0.62</td>
<td>19.62±0.63</td>
<td>20.90±1.05</td>
<td>18.78±0.18</td>
</tr>
<tr>
<td><strong>Overall (1527)</strong></td>
<td></td>
<td>22.79±0.35</td>
<td>19.35±0.46</td>
<td>21.64±0.48</td>
<td>19.76±0.53</td>
<td>21.49±0.72</td>
<td>18.86±0.14</td>
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</tbody>
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Values in parenthesis are the number of animals.
descript breeds, in which three breeds GSH levels were found lower and almost the same. This supports the native geographical distribution of Indian buffalo breeds. The overall glutathione level in adults, young and calves were found as 19.06±0.16, 20.54±0.19 and 19.39±0.16 mg/100 ml blood, respectively. Higher levels of glutathione were found in young animals as compared to adults, which agrees with the findings of Annunziata and Iorio (2004). The overall glutathione level was found significantly higher in healthy animals than in emaciated animals in all six breeds of buffaloes. These differences in GSH values in healthy and emaciated buffaloes indicated that GSH content in the blood appeared to be regulated by nutritional status of the animals also. A similar observation was made by Agar (1975) and Singari et al. (1989). The glutathione level was found as 19.96±0.02 in males and 19.63±0.15 mg/100 ml of blood in females, the two sexes thus not differing significantly. The analysis of variance showed that the effect of breed, age group and health status had a significant effect on GSH level, but the effect of sex was found nonsignificant. A good correlation between glutathione level and age at first calving (0.03), total milk yield (0.46), lactation length (-0.23) and average milk yield (0.48) were also found in this study.

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