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Editor
S. Sophon

Publisher
International Buffalo Information Centre,
Office of University Library,
Kasetsart University

Online available:
http://ibic.lib.ku.ac.th/e-Bulletin

BUFFALO BULLETIN
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BANGKOK 10903, THAILAND
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CONGENITAL ABOMASAL FISTULA IN A BUFFALO CALF 
AND ITS SUCCESSFUL TREATMENT

Ankur Sharma, S.V. Upadhye and Kawardeep Kour

ABSTRACT

This communication reports a case of a congenital abomasal fistula in a buffalo calf which was successfully treated surgically without any post operative complications.

Keywords: congenital, buffalo, calf

INTRODUCTION

Congenital anomalies in the bovine population have been associated with genetic factors (transgenes, chromosomes), environmental agents (infections, toxins, fertilization technique, management) or a combination of factors (Newman et al., 1999). Abomasal hernia and fistulation following trauma have been reported in an adult buffalo (Sobti et al., 1998) and a dairy cow (Balagopalan et al., 1993) but few reports of abnormalities in buffalo calves have documented a congenital abomasal fistula. The present case demonstrates the surgical management of congenital abomasal fistula in a buffalo calf.

HISTORY AND OBSERVATIONS

A 15-day-old non-descript buffalo calf produced by natural breeding was presented with a rare form of congenital abomasal fistula. History revealed that since birth, on suckling, milk was partially ingested and rest flowed out from the fistula (Figure 2). Physical examination revealed a fistula communicating to the exterior through the umbilical opening (Figure 1). The area surrounding the umbilicus was thickened and fibrosed. No other physical abnormality was manifested by the calf.

On clinical examination, the physiological parameters were found to be within the normal range. Haemato-biochemical parameters are depicted in Table 1. Haematological parameters and serum glucose levels were observed to be slightly lower than the normal physiological range.

TREATMENT

The animal was prepared for aseptic surgery, tranquilized with triflupromazine hydrochloride 0.1 mg/kg b.wt i/m and restrained in dorsal recumbency. Local infiltration with 2%
lignocaine hydrochloride was given encircling the lesion. An elliptical skin incision was then taken at the level of fistulous opening. It became clear at surgery that the fistula involved the abomasum. Haemorrhage was controlled and abomasum was freed of adhesions, freshened and sutured by double layer of inversion sutures using chromic catgut no.1. The muscle wall and skin was freshened and sutured routinely.

Post operatively dicysticine sulfate 1 gm i/m for 5 days and meloxicam 0.5 mg/kg b.wt for 3 days were administered. Antiseptic dressing of the surgical wound was carried out and skin sutures were removed on the 12th post operative day. Animal was kept on fluid therapy for three post operative days and thereafter a restricted soft diet was allowed. The calf recovered uneventfully and no recurrence of fistulation was noticed during a two-month observation period.

**DISCUSSION**

Fistulas of compound stomach have been frequently observed and usually associated with trauma. Due to the infrequency of abomasal

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<th>Haematological parameters:</th>
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<tr>
<td><strong>Hb (g%)</strong></td>
<td><strong>PCV (%)</strong></td>
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<tr>
<td>8.0</td>
<td>30</td>
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<table>
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<th>Biochemical parameters:</th>
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<tr>
<td><strong>Serum Glucose (mg%)</strong></td>
<td><strong>Serum Calcium (mg%)</strong></td>
</tr>
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<td>52.35</td>
<td>7.96</td>
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Table 1. Haemato-biochemical parameters observed on presentation.
disorders in calves, they need to be recognized and treated promptly to obtain a successful outcome (Nowrouzian, 1994). A case of reticular fistula in a buffalo (Singh, 2004) and omasal hernia with fistulation in a bullock (Bhardwaj et al., 2000) have been reported. The present case reports the successful surgical treatment of congenital abomasal fistula in a buffalo calf. Though abomasum has been usually involved in fistula in heifer calves (Rijkenhuizen and Sickmann, 1994), it is also reported in association with hernia in a cow (Balagopalan et al., 1993) and buffalo (Sobti et al., 1998), however associated with trauma. But the cause of the abnormality in the present case could not be ascertained.

REFERENCES


AN UNUSUAL CASE OF OESOPHAGEAL OBSTRUCTION
IN A FEMALE BUFFALO

N.V.V. Hari Krishna¹, Makkena Sreenu² and V.S.C. Bose³

ABSTRACT

This paper reports a rare case of oesophageal obstruction anterior to hiatus oesophagi along with ruminal tympany in a female graded Murrah buffalo caused by a palm kernel and its successful surgical treatment through rumenotomy.

Keywords: female buffaloes, Murrah, oesophageal

INTRODUCTION

Obstruction of the oesophagus is a rare occurrence in ruminants. Apart from cattle, oesophageal obstruction has been occasionally reported in buffaloes (Tyagi and Jit Singh, 1999). This paper reports a rare case of oesophageal obstruction at cardia, caused by a regurgitated palm kernel.

HISTORY AND CLINICAL SIGNS

A nine-year-old female graded Murrah buffalo weighing about 420 kg was presented to the clinic with a history of not taking feed and water and sudden development of bloat since the morning. It had calved three months before and had been sent out for grazing daily. The bloat was relieved using 16 G needle at a local hospital but the condition recurred. Clinical examination revealed distended left flank. Pulse, temperature and respiration were within normal physiological limits. On passage of a stomach tube, an obstruction was felt at the thoracic region anterior to the diaphragm. It was tentatively diagnosed as oesophageal obstruction, and it was decided to relieve the obstruction through rumenotomy, as thoracotomy in ruminants requires special equipment, and the procedure is a difficult one.

RESULTS AND DISCUSSION

Rumenotomy was performed following standard surgical procedure under inverted L - block using 2% Lignocaine hydrochloride. On opening the rumen, surprisingly, it was full of palm kernels. The obstructing palm kernel anterior to diaphragm was taken out by hand with force after clearing the entangled fibrous food material and all the remaining palm kernels found in rumen were also removed. Probiotics were placed in the rumen before closing it. The laparotomy wound was closed.

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³(PTT)
routinely. The animal was given streptopenicillin 5.0 g i/m for seven days, meloxicam 20 ml i/m for three days and DNS 2 L i/v for three days as postoperative care. The cutaneous sutures were removed on 10th postoperative day.

The intraluminal oesophageal obstruction, commonly known as choke, may occur in buffaloes due to vegetables, fruits and phytobezoars (Tyagi and Jit Singh, 1999) or to pieces of leather or rubber (Salunke et al., 2003). In ruminants, obstruction occurs mostly in the cervical region, and obstruction in the thoracic oesophagus is rare. In buffaloes, obstruction of the oesophagus has mostly been recorded in the distal cervical region as the lumen of the oesophagus narrows down at the junction of middle and distal third (Tyagi and Jit Singh, 1999). Madhava Rao et al. (2009) reported a case of cervical oesophageal obstruction due to coconut, and thoracic oesophageal obstruction has been reported by few workers (Ojha and Mohanthy, 1970; Yadav et al., 2008). Shivprakash (2003) found a higher incidence in pregnant buffaloes and young calves and attributed the same to nutritional deficiencies, pica and difficulty in adaptation during transition from milk to fodder, respectively. The prognosis of oesophageal obstruction is not always favourable as the oesophageal surgery is associated with various complications such as suture dehiscence, perforation or fistula, and stenosis due to scar.

In the present case, 92 palm kernels were recovered from the rumen (Figure 1). It may be due to the greediness of the animal during early rainy season, during which time ripened palm kernels are abundant in the fields and swallowed by animals sent out for grazing. The probable reason for the unusual site of the obstruction was that when the animal is ruminating one of the palm kernel has migrated and occluded at the hiatus oesophagi of oesophagus anterior to diaphragm, and this resulted in its present condition. Attempts to push the object with a probang were not successful, perhaps due to a change in position of kernel at the site, along with entangled food material which was removed.

Figure 1. Photographs showing the palm kernels recovered from the rumen.

*Continued to page 9
ABSTRACT

In this study, thiamine-responsive polioencephalomalacia (PEM) was detected in seven non-descript buffaloes of a dairy farm in Jammu and Kashmir State of India. Clinical cases showed variable signs including disorientation, aimless walking with a high stepping gait due to blindness, anorexia, opisthotonus or head retraction (star gazing), muscle tremor, convulsion and recumbency. The result of the study of clinical cases treated by parenteral injection of thiamine preparation showed a good response within 12 h after initial treatment. The immediate response to the specific treatment coupled with the reports of general hematology and other diagnostic test used routinely in field was diagnostic for PEM and differentiated it from other neurological conditions based on the animals’ response to injection of thiamine beside specific clinical findings.

Keywords: polioencephalomalacia, thiamine, buffaloes

INTRODUCTION

Polioencephalomalacia (PEM) is a non-infectious thiamine responsive neurological disease of buffaloes characterized by neurological manifestations (Debasis et al., 2009). The disease is seen sporadically or as a herd outbreak, and generally young animals on high concentrate diet are at high risk (Boyad et al., 1977). The probable mechanisms that may cause thiamine deficiency PEM in ruminants are disorders of absorption, synthesize and destruction of thiamine by thiaminase (Bakker et al., 1980; Debasis et al., 2009; Jeffrey et al., 1994; Radostit et al., 2007). The thiaminase produced by bacteria lead to decline in thiamine concentration in the digesta (Brent et al., 1984). Thiaminase-1 produced by the Bacillus thiaminalyticus and Clostridium sporogenes and the thiaminase-11 by Bacillus aneurinalyticus catalyze the cleavage of thiamine in the rumen and also chemicals in drinking water, toxins released by nitrate-utilizing bacteria, and some toxic plants in pastures are considered as the etiological agents (Dickie et al., 1979). Evidence linking thiamine with the ruminant PEM disorder includes clinical response to thiamine injection in some individuals (Mouli et al., 2004). Under the field conditions, presumptive diagnosis of PEM cases may depend on the pattern of clinical findings mentioned above which may be suggestive for the disease. However, other diseases such as lead poisoning, focal symmetrical encephalomalacia, hepatic encephalopathy, or head trauma may show similar clinical signs (Milad et al., 2009). Laboratory confirmation of thiamine deficiency can be based on histopathology and blood biochemistry.
including erythrocyte transketolase level, blood pyruvate and lactate levels (Kiupel et al., 2003). The purpose of the present investigation was to study and evaluate treatment with thiamine as a tool for rapid diagnosis of PEM and to differentiate it from other neurological diseases showing similar nervous signs under conditions of normal farm management in the absence of laboratory facilities.

MATERIALS AND METHODS

The clinical cases: In November 2009, a sporadic naturally occurring outbreak of PEM was reported from a farm house in Udhampur district of Jammu and Kashmir State (India). A total of seven animals were showing major complaint of inappetance, profuse salivation, sudden incoordination with occasional aimless movement, loss of vision and falling down. The clinical features revealing congested mucus membrane, swollen and bulging tendency of eye ball, profuse salivation with facial tremors and periodic convulsions. Although there is ruminal atony in some cases, temperature, pulse and respiration were all in normal ranges with slight variation along with normal micturation and defecation. Blood and urine samples were collected for further investigation. Blood samples were subjected to routine hematological tests like total leukocyte count, differential leukocyte count, blood glucose level, blood hemoglobin level and blood smear for blood protista examination, whereas urine samples were screened for presence of ketone bodies by Rothera’s test.

Treatment regimen: Initially the symptomatic treatment includes triflupromazine, a potent neuroleptic agent used 0.25 mg/kg BW intramuscularly (I/M), Injection of atropine sulphate 0.20 mg/kg BW I/M, antibiotic injection of dicrystine of 2.5 gm /animal I/M and intra venous (I/V) infusion of dextrose 10% 2000 ml /animal along with 20 ml of multivitamin injection mixed with dextrose for slow I/V infusion. However, once the hematological investigation revealed that all the vital blood parameters were in normal range with negative reports of blood smears and Rothera’s test, keeping in mind the blood picture and the other tests, the cases were presumed to be cases of thiamine associated PEM and accordingly high doses of neurotropic vitamin-B injection (Inj. Neuroxin-12-v) were given 10 ml I/M twice a day for five days.

RESULTS AND DISCUSSIONS

The thiamine associated PEM is a neurological disorder in buffaloes and is reported occasionally in India (Debasis et al., 2009) because of its difficult diagnosis under field conditions. The present outbreak was initially confused with poisoning, ketosis, other similar neurological disorders and blood protozoan infection. However, normal hemato-biochemical values coupled with negative blood smear and Rothera’s test led to the diagnosis of thiamine associated PEM, which was clinically confirmed by dramatic and instant response to vitamin-B therapy. All the animals showing signs of PEM responded to the treatment within 12 h of injection with normal vision, stoppage of salivation, and normal gait, and this was consistent with the findings of others in responsiveness toward thiamine treatment (Debasis et al., 2009; Gholami et al., 2003; Milad et al., 2009; Mouli et al., 2004). Laboratory tests used to confirm PEM in ruminants are based upon blood chemistry to determine the depressed level of blood thiamine and RBC’s transketolase.
activity and rising of pyruvate and lactate levels (Horino et al., 1994; Ramos et al., 2005; Ramos et al., 2006). Rumen and fecal samples may be tested for thiamine activity; however, these tests are not routinely available (Margo et al., 2002). However, signs of progressive CNS involvement coupled with the responsiveness to thiamine therapy as in the present study can be used as diagnostic for thiamine induced PEM (Strain et al., 1990; Milad et al., 2009; Mouli et al., 2004). Successful treatment of affected buffaloes in the present study was attributed to early and aggressive therapy with thiamine. The recovery in all animals was steady as all nervous signs and symptoms disappeared by the third day post therapy.

This study offers a method for early diagnosis of the PEM through rapid response to parenteral treatment with no need to wait for the results of laboratory tests, which takes time to confirm under field conditions. Early treatment is necessary for ruminants with PEM as the prognosis is considered favorable if the treatment is started early in the disease.

ACKNOWLEDGEMENT
The authors wish to thank the Dean, Faculty of Veterinary Science and Animal Husbandry, SKUAST-Jammu, for providing necessary facilities to carry out this work.

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*Continued from page 5*

first with fingers before retrieving the palm kernel from the oesophagus. The animal made uneventful recovery, and no complications were reported up to 6 months post surgery.

**REFERENCES**


AN OMPHALOCELE IN A BUFFALO CALF: A CASE REPORT

P. Vidya Sagar, K.S.Vadde, K.S. Sai Krishna and S. Venkateswarlu

ABSTRACT

An omphalocele in a buffalo calf immediately after birth with ruptured amnion and its prognosis was reported.

Keywords: buffalo calf, umbilicus, omphalocele

INTRODUCTION

An omphalocele is a congenital defect in the body wall in which eviscerated abdominal organs are covered by amnion rather than skin (Baird, 1993). It should be differentiated from umbilical hernias, in which skin covers over the herniated organs.

CASE REPORT AND CLINICAL HISTORY

A new born graded Murrah buffalo calf presented to the veterinary polyclinic within few hours of birth with a history of prolapsed bowels. On gross examination, it was noticed the herniated mass included the abomasum, small intestines and part of the colon through the abdominal defect at the umbilical region (Figure 1). The amnion covering the herniated mass was ruptured and congestion of the everted organs set in. An absence of abdomen musculature and skin to the extent of 10 cm caudal to the sternum was noticed. No other congenital abnormalities were found. It was diagnosed as an omphalocele, and it was decided to correct it by hernioraphy.

TREATMENT AND DISCUSSION

Prior to the surgery, the soiled herniated mass thoroughly washed with normal saline and the calf was sedated with triflupromazine hydrochloride 0.01 mg/kg bwt.

During laparotomy, it was found difficult to maintain the proper approximation of the edges because of the absence of the abdomen musculature and skin to a larger extent and with severe gross contamination of the abdominal cavity, it was decided to euthanize the calf.

An omphalocele is a hernia that occurs in the embryo in which the abdominal contents protrude through the umbilicus and remain in the umbilical stalk, therefore covered by the amnion (Noden and Lahunta, 1985). This probably results from the failure of normal withdrawal of developing intestinal loop. During early stages of fetal development the intestines rest partly within the extra embryonic celome of the umbilical card. Later, the body wall encloses this area and the intestines are internalized. Failure of the intestines to return or failure of the four body folds to migrate
normally results in an omphalocele (Noden and Lahunta, 1985). It is also called as congenital umbilical hernia, abdominal fissure, umbilical eventration and examphalocele.

The cause of isolated omphalocele is not known, and while it is a developmental defect, it is not necessarily a heritable anomaly (Baird, 1993) although it has been suggested that it may be a recessive genetic trait (Ko et al., 1990).

The prognosis with omphalocele is often poor if severe abdominal contamination and ischemic necrosis of the everted organs occurs and there is extensive loss of abdominal musculature and skin, as observed in the present case. Compared with the umbilical hernia, risk of mortality was high. However, successful surgical recovery was achieved in some cases in which the amnion covering of the herniated organs was present (Baird, 1993).

REFERENCES


DYSTOCIA DUE TO A CONJOINED TWIN MONSTER FOETUS
IN A FEMALE BUFFALO

S.P. Shukla, Qazi Mudasir and S.P. Nema

ABSTRACT

A case of dystocia due to a conjoined twin monster foetus with thoracopagus in a female buffalo is reported. An emergency cesarean section was decided upon to relieve the subsequent dystocia.

Keywords: female buffalo, twin monster, dystocia

INTRODUCTION

Foetal anomalies and monstrosities are common cause of dystocia in bovines (Shukla et al., 2007). Twin monsters are characterized by duplication of anterior, posterior or both parts of foetal body and are common in ruminants.

CASE HISTORY AND OBSERVATIONS

A pluriparous full-term pregnant Murrah buffalo was presented to the Teaching Veterinary Clinical Services Complex, College of Veterinary Science and Animal Husbandry, Mhow, with history of severe straining for the previous 12-14 h after the rupture of water bag. Two foetal legs were protruding from the vulva without any progress in parturition. Per vaginal examination after proper lubrication revealed that the foetus was in anterior longitudinal presentation, with two fore limbs protruding from the vulva and other two forelimbs in flexed positions. Repulsion and deeper exploration revealed a conjoined twin monster, with the presence of two foetal heads. The fetuses were dead, and it appeared to be a twin pregnancy as two foetal heads joined at the thorax were palpable. Hence, it was diagnosed as a dystocia due to a conjoined twin monster foetus and an emergency cesarean section was decided upon. Previous first calving of the animal was reported to be normal.

TREATMENT AND DISCUSSION

The buffalo was premedicated with xylazine (0.2 mg/kg b.wt. I/M) and caudal epidural analgesia and local infiltration anaesthesia was achieved using lignocaine Hcl. The left paramedian incision between linea alba and left subcutaneous abdominal vein was used for laparohysterotomy, and a dead conjoined twin monster foetus was delivered. The foetal membranes were also removed and eight Furea boli (Nitrofurazone 60 mg+urea 6 gm) were left in uterus. The uterus, peritoneum, muscles and skin were sutured in the routine manner. Post operatively the animal was given parenteral fluid therapy Inj. N.S (4 lts.) Inj. DNS (2 lts) Inj. Mifex (450 ml) and antibiotic

Department of Animal Reproduction Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry (Mhow) 453446, India
therapy using Strepto-Penicillin (2.5 gms) twice daily with other supportive treatment including anti-inflammatory and analgesics (Pheniramine maleate 15 ml I/M and Meloxicam 15 ml I/M) once daily for the next 5 days. Antiseptic dressing of the surgical wound was done on alternate days using povidone iodine solution, and sutures were opened on the 14th postoperative day. The buffalo made an uneventful recovery.

The twin monster had two normal heads, two necks, two pairs of fore and hind limbs with two separate abdominal areas but was joined at thorax (thoracopagus). Both the fetuses were of female sex. Postmortem examination of fetuses is presented in Table 1.

Conjoined or fused symmetrical twins are usually monozygotic in origin and represent incomplete division of one embryo into two components usually at the primitive streak of developmental stage and in the event they may develop into thoracopagus (Noden and Delahunta, 1985). Conjoined twins are always indentical twins and of the same sex (Arthur et al., 2001). Such twins are usually due to non-inherited defects and often lead to severe dystocia (Roberts, 2004). In such cases cesarean section undoubtedly is the method for choice for delivery (Sakthivel and Mathew, 2000).

**REFERENCES**


GENETIC POLYMORPHISM ANALYSIS OF MONOACYL GLYCEROL TRANSFERASE2 (MOGAT2) GENE IN MURRAH BUFFALO (Bubalus bubalis)

D.S. Kale and B.R. Yadav

ABSTRACT

The polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) was identified within the Murrah buffalo MOGAT2 gene. The result exhibited three SSCP patterns in the MOGAT2 gene with variable frequency in samples studied, indicating that Murrah buffaloes have genetic variability at that locus. This identified SSCP was confirmed by DNA sequencing which revealed one single nucleotide polymorphism (SNP) viz, c.193T>C within 245 bp fragment of the MOGAT2 gene spanning exon 5 of the Murrah buffalo. Identified SNP (c.193T>C) was used to genotype the 106 samples of Murrah buffalo in which frequencies for C and T variants were found to be 0.25 and 0.75, respectively. The frequency of genotypes within the breed group was in accordance with Hardy-Weinberg proportions. Murrah buffalo MOGAT2 allelic variant sequence was 95% pairwise similar with cattle sequence and comparison of the two revealed eleven computational SNPs. The statistical analysis using general linear model procedure (SYSTAT) for association study indicated that Murrah Buffalo MOGAT2 c.193T>C SNP genotypes did not differ significantly (P>0.01) from Murrah buffalo milk production traits.

Keywords: MOGAT2, SSCP, DNA sequencing, association study, Murrah buffalo

INTRODUCTION

India is rich in buffalo genetic resources having 97 million animals in 2003 (Annual Report, 2006) which accounted for 59.5% to total world buffalo population. Buffalo milk contributes 55.6% to the country’s total milk production. The Murrah buffalo is the most important dairy breed with superior genetic potential for milk production. However, their inherent potential for growth and production has not been exploited due to inadequate information about their genetic architecture. The river buffalo, along with domestic cattle, belongs to the subfamily Bovinae, and these species have been shown to be closely related, sharing homology in chromosome banding and gene mapping (Di Meo et al., 2005) and have been cytogenetically characterized in detail. The first generation whole genome RH map of the river buffalo was reported based on comparison to domestic cattle (Amaral et al., 2008). Therefore these preliminary maps, based on cattle-derived markers, demonstrated that the bovine genome is a useful source of markers for the buffalo genome mapping, allowing rapid
and efficient transfer of information from cattle to buffalo.

The publication of the entire genome sequences of several livestock species will allow easy identification of genetic markers in buffaloes, which will aid buffalo breeding and genetic improvement. The comparative genomics and genome analysis biotechniques have opened new possibilities for evaluation of the buffalo genome. The PCR-SSCP analysis (Orita et al., 1989) is a technique based on the principle that single-stranded DNA molecules form specific sequence-based secondary structures under non-denaturing conditions. The association of genetic polymorphisms with milk production traits and composition (Ganai et al., 2008) has stimulated interest in identifying the genetic markers influencing production traits which will be used in marker assisted selection (MAS) to improve productivity of farm animals.

In the marker assisted selection of dairy animals some genes are proposed as potential candidates associated with dairy performance traits. Fat is one of the major constituents of milk. Triglycerides are the major energy storage molecules in eukaryotes, and their final and presumably rate-limiting step of synthesis is catalyzed by a diacylglycerol acyltransferase (DGAT). A few years back, DGAT1 was the first identified gene encoding a protein with DGAT activity in which a mutation has been shown to be significantly associated with variation in milk fat percentage in cattle (Grisart et al., 2002; Gautier et al., 2007). DGAT-like activity has also been shown in other enzymes encoded by other genes and led to the detection of diacetylglcerol transferase2 (DGAT2), monoacyl glycerol transferases1 (MOGAT1) and monoacyl glycerol transferases2 (MOGAT2), which are members of the same family (Winter et al., 2003). The members of this gene family show similarity in their nucleotide sequences and arose from same ancestral gene by duplication. However, this family has not yet been fully characterized in any single mammalian species (Winter et al., 2003).

As the MOGAT2 gene is in the family related with QTL influencing milk production traits and its functional role similarity in triglyceride synthesis indicates that it might be a useful candidate to reveal genetic polymorphisms. As the Murrah buffalo is very important dairy breed of buffalo contributing the lion’s share in country’s milk production, it is necessary to screen candidate genes implicated for milk production, viz, the MOGAT2 in Murrah buffalo genome. Therefore the present study was undertaken to detect genetic variation in the MOGAT2 gene using PCR-SSCP followed by DNA sequencing and any find association with milk production traits.

**MATERIALS AND METHODS**

The study group included 106 Murrah buffaloes with milk production records from the Institute herd. Blood samples (10 ml) were collected by jugular vein puncture using vacuum tubes containing acid citrate dextrose solution (ACD) as an anticoagulant. Genomic DNA was isolated from blood using the phenol chloroform extraction protocol (Clamp et al., 1993) with some modifications. The integrity of the DNA was assessed following electrophoresis in a 0.8% agarose gel with ethidium bromide staining. In addition, the OD ratio 260/280 nm was measured to check for protein contamination and to calculate the DNA concentration. All stock DNA samples were kept at -80°C for longer storage, and the
working aliquots were maintained at -20°C.

The PCR primers were designed for exon V of the MOGAT2 gene on the basis of cattle gene sequence covering nucleotide substitution using PRIMER3 software (http://www-genome.wi.mit.edu). The polymerase chain reaction (PCR) was carried out on about 100 ng of genomic DNA in a 25 μL reaction volume. The reaction mixture consisted of 2.5 μL of 10x PCR assay buffer containing 1.5 mM MgCl2, 200 μM each of dNTPs, 0.75 unit Taq DNA polymerase and 10 pmole of each primer (Integrated DNA Technologies, Inc).

The primers (Forward primer: 5'-TTT GGT CTT ATG CCC TAC CG-3'; Reverse primer: 5'-GGA CAG GGT GAT CTT TTG GA-3') were used for amplification of exon V of the Buffalo MOGAT2 gene. Amplification was carried out in a Biometra thermal cycler using PCR cycling conditions as (95°C for 5 minutes) and 34 cycles of 45 seconds at 95°C, 65°C and 72°C consecutively, followed by a five minute final extension at 72°C. The PCR amplification was verified by electrophoresis of the PCR products with loading dye on 2% (w/v) agarose gel in 0.5 x TBE buffer using a 100 bp ladder as marker for confirmation of the length of the PCR products. The amplified products (5 μL) were detected on 2% agarose gel using 1 μL of loading dye as a stop dye, electrophoresed and visualized using UV light.

The MOGAT2 gene PCR products were resolved by SSCP analysis using PCR product (5 ul), acrylamide concentration (18%), presence glycerol (10%), voltage (200 volts), running time (24 hours) and temperature (15°C). PCR products were diluted in denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA) and heat denatured at 95°C for ten minutes. The PCR products were resolved on a non-denaturing 18% acrylamide: bis-acrylamide (49: 1) gel for SSCP analysis. Gels were silver-stained (Sambrook and Russell, 2001) and photographed using a digital camera for SSCP pattern analysis.

The PCR products representing different SSCP patterns were directly got sequenced. The nucleotide sequence analysis was carried out using Geneious software. The DNA sequence polymorphism observed was used to genotype Murrah buffalo population. The frequency of polymorphic allele variant, genotypes and their accordance with Hardy-Weinberg law was assessed by POPGENE 1.31 software (http://www.ualberta.ca/~fyeh). The association between polymorphic allelic variants of the MOGAT2 gene and milk production traits was analyzed using GLM procedure (SYSTAT). The following model was used,

\[ Y_{ijkl} = \mu + g_i + s_i + p_j + h_k + e_{ijkl} \]

- \( Y_{ij} \): bservation on jth animal ith genotype
- \( \mu \) : population mean
- \( g_i \) : effect of ith genotype (i=1, 2)
- \( s_i \) : effect of i season
- \( p_j \) : effect of j parity
- \( h_k \) : effect of k year,
- \( e_{ijkl} \) : random error

**RESULTS AND DISCUSSIONS**

The SSCP analysis of amplified gene fragments of exon 5 of the MOGAT2 gene resulted in three different patterns viz, A, B and C (Figure1) with the following frequencies in Murrah buffaloes (A = 0.49, B = 0.36, C = 0.15). This study has revealed the polymorphic nature of the 3’ UTR region of exon 5 of the buffalo MOGAT2 gene.

The direct DNA sequencing and nucleotide
sequence analysis of MOGAT2 amplified PCR products (Table 1) representing different SSCP patterns (A, B and C), revealed one SNP (T-C substitution) in exon 5 at the 193rd nucleotide position (denoted as c.193 T>C) within the MOGAT2 gene sequence of the Murrah buffalo (Figure 2). These MOGAT2 allelic variant nucleotide sequences were analyzed and submitted in NCBI GenBank with accession No. (EU239373, EU239374). The nature of mutation was T-C transversion between SSCP pattern A and B confirming them as variants (A and B). The SSCP patterns B and C had identical nucleotide sequences; therefore only two variants (A and B) were confirmed. The PCR products representing SSCP pattern B were homozygote: TT in position 193 while SSCP patterns A and B were heterozygote: TC in position 193.

The polymorphic Murrah buffalo MOGAT2 allelic variant sequence (EU239373) was compared with *Bos taurus* reference sequence (AJ534379) using alignment tool (Geneious Software) which revealed eleven computational mutations. The Murrah buffalo MOGAT2 variant sequence (EU239373) was 95% pairwise similar with, the cattle sequence (AJ534379). A neighbor-joining tree (Figure 3) was constructed based on comparison of the Murrah buffalo MOGAT2 consensus sequence (EU239373) and consensus GenBank sequences of cattle (AC149756, NM_0010011154 and AJ534379) and buffaloes (EF208205, EU239373) at the same MOGAT2 locus (Geneious Software). The phylogenetic tree based on partial consensus sequence agreed with taxonomic relationship of cattle and buffaloes.

The association analysis was carried out between MOGAT2 c.193T>C SNP and milk production traits to find any relationship between them. The ANOVA results indicated non-significant (P>0.01) effects of different Murrah buffalo MOGAT2 genotypes: c.193T>C TT and c.193T>C TC on 305 days milk yield, fat percentage as well as SNF percentage. The effect of non-genetic factors, viz. season, parity and year of calving on milk yield were found to be significant (p<0.01). The least squares mean values of the milk production traits studied in Murrah buffaloes differing in their MOGAT2 c.193T>C genotypes are given in Table 1.

Among different candidates, the DGAT/MOGAT2 gene family with DGAT-like activity seems to be a promising candidate due to its genetic and functional similarity to established cattle QTL (DGAT1) influencing milk fat percentage (Winter et al., 2002). It is believed that SNPs occurring within such genes may influence the milk production trait or at least be an effective DNA marker of a

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Milk Yield±SE</th>
<th>FAT*±SE</th>
<th>SNF*±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.193T&gt;C TT</td>
<td>52</td>
<td>1775.81NS±241.06</td>
<td>0.297NS±0.005</td>
<td>0.315NS±0.001</td>
</tr>
<tr>
<td>c.193T&gt;C TC</td>
<td>54</td>
<td>1678.21NS±264.85</td>
<td>0.297NS±0.005</td>
<td>0.314NS±0.001</td>
</tr>
</tbody>
</table>

Where,
* are scale-transformed values
superscript NS are means not differing significantly at p≤0.05.
Figure 1. MOGAT2 PCR-SSCP genotype patterns resolved on 18% PAGE and visualized by silver staining in Murrah buffalo.

Figure 2. Multiple sequence alignment using CLUSTAL W (1.83) indicating T-C substitution at 193rd position.

Figure 3. Neighbor-Joining Tree based on Murrah buffalo MOGAT2 variant A consensus sequence (EU239373) and related consensus sequences of cattle and buffaloes (Geneious Software).
sub-region of the dairy animal genome. In view of the above, the MOGAT2 gene was screened for polymorphism in Murrah buffalo using SSCP followed by sequencing and association study.

In the present study, one SNP has been identified within the population at the MOGAT2 gene locus of the Murrah buffalo. The identified SNP genotypes at the MOGAT2 gene locus did not differ significantly from Murrah buffalo milk production traits. However in their bovine MOGAT2 gene polymorphism study, Winter et al. (2003) found 15 SNPs outside exons and two silent exon SNPs (ID 358 and 363) and reported non-significant association of allele frequencies with breeding values for milk fat content in analyzed dairy breeds.

CONCLUSION

The present study revealed that PCR-SSCP followed by DNA sequencing is an effective molecular biological technique to detect DNA sequence variation at candidate gene loci in buffaloes. The identified SSCP within the MOGAT2 gene after DNA sequencing revealed one SNP (c.193T>C) in exon 5 of the Murrah buffalo. However, the statistical analysis revealed non-significant effect of the observed polymorphism genotypes on Murrah buffalo milk production traits. The possible reasons for non-significant effect might be the small size of the sample, absence of some genotypes, high standard error and uneven distribution of data.

The studies concerning associations between DGAT gene polymorphism and production traits of riverine buffaloes are, however, fairly scarce. Information regarding the actual physiological role of DGAT2 and the closely related MOGAT genes in vivo is only just becoming available. In view of this, it is necessary to screen all the regions of these genes in buffalo genome and to continue association studies as DGAT2/ MOGAT genes have genetic and functional similarity to DGAT1 influencing milk production traits in genetically related Bos taurus. The identified DNA polymorphism after validation study will open up possibilities for buffalo breeding and improvement in gene assisted selection.

ACKNOWLEDGMENTS

The financial grant as a National Fellowship to BR Yadav from the Indian Council of Agricultural Research and assistance in the laboratory by Mr. R.K. Tonk, Mr. Naresh Kumar and Mr. Nankoo Singh are acknowledged.

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This article reports studies on polymorphisms of the ATP-binding cassette superfamily G member 2 transporter (ABCG2) gene in 68 Chinese buffalo and 80 yak by DNA sequencing. We found four mutations: A deletion, G insertion, single nucleotide polymorphisms (SNPs) G/A and C/A. A deletion existed in the buffalo and yak breeds. G insertion, (G/A) and (C/A) SNPs existed only in the buffalo breed. Results showed the Chinese buffalo and yak breeds had four mutations. Especially, the buffalo breed had four mutations at least, and these probably affected the milk yields, milk fat, protein and dry matter percent. So the ABCG2 gene could be studied as a candidate gene effecting milk traits.

Keywords: Chinese buffalo, yak, polymorphisms, ABCG2, milk traits

INTRODUCTION

A candidate gene can be defined as a gene with biological effects on the physiology of a trait of function or as a gene closed linked to a functional gene. Polymorphisms within selected candidate genes can be tested for their association with quantitative traits and can be used in marker-assisted selection (MAS) programs (Wu et al., 2005). Many studies have found segregating QTL for milk production traits on Bos taurus chromosome (BTA) 6 in different cattle populations (Khatkar et al., 2004). There is strong evidence that a polymorphism of the ATP-binding cassette superfamily G member 2 transporter (ABCG2) gene located on BTA 6 is associated with effects on milk yield and composition in the Holstein cattle (Cohen-Zinder et al., 2005), dairy cattle (Olsen et al., 2007) and Indian cattle (Bos indicus) and buffalo (Bubalus bubalis) breeds (Tantia et al., 2006). Cohen-Zinder et al. (2005) found a single nucleotide polymorphism (A/C) in exon 14, encoding a substitution of tyrosine-581 to serine (Y581S), in the ABCG2 transporter gene. The ABCG2 gene was greatly induced during late pregnancy and especially during lactation (Jonker et al., 2005). The protein encoded by ABCG2, a member of the ATP binding cassette (ABC) superfamily, transports various xenobiotics and cytostatic drugs across the plasma membrane (Litman et al., 2000). Jonker et al. (2005) demonstrated that ABCG2 is responsible for the active secretion of clinically and toxicologically important substrates into mouse milk, and that mice homozygous for an ABCG2 knock-out mutation lack this function. Chinese buffalo and yak, distributed in the south and west of China, have a low milk yield, but high milk protein and fat percentages compared to cattle and have rarely been studied. The purpose of this article was to investigate the polymorphisms of the ABCG2 gene in the Chinese buffalo.
Bubalus bubalis) and yak (Bos grunniens) breeds.

MATERIALS AND METHODS

Sixty-eight Chinese buffalo (Anhui buffalo n=18, Fuling buffalo n=16, Jianghan buffalo n=17, Yunnan buffalo n=17) and 80 yak (Jiulong yak n=20, Maiwa yak n=20, Tianzhu yak n=20, Xizang yak n=20) blood samples were collected from the south and west of China. The individuals were unrelated based on the data of owners. Total genomic DNA was extracted by a standard phenol-chloroform extraction method. The primers for exon 14 of ABCG2 were: forward 5´-CAGGGCTGTTGGTAAATCTCA-3´ (nt 62491-511) and reverse 5´-GCACGGTGACAGATAAGGAGA-3´ (nt 62580-600) from NCBI database AJ871176. PCR amplifications were standardized using gradient PCR (iCycler, Hercules, CA, USA) from Tantia et al. (2006). Touch down PCR profile included 5 minutes at 95°C; 5 cycles 45s at 95°C, 45s at 57°C, 45s at 72°C; 15 cycles 45s at 95°C, 45s at 56°C, 45s at 72°C; 15 cycles 45s at 95°C, 45s at 55°C, 45s at 72°C; and a final 10 minutes at 72°C. PCR products were purified by using the Dinguo PCR columns (Dinguo Company, Beijing, China) and sequenced at a commercial facility using an ABI 377 automatic sequencer in both directions. The sequence of Bos taurus (AJ871176), Indian Bos indicus (DQ205445) and Bubalus bubalis (DQ205444) were downloaded and aligned using Clustal W program (Thompson et al., 1994).

RESULTS AND DISCUSSION

The sequence obtained for segment of ABCG2 in buffalo (GU183627) and yak (GU183628) have been submitted to the NCBI database. The length of the amplified sequence was 110-bp (this value excludes insertions/deletions). Comparison of the 148 sequences firstly revealed four variable sites located at 53, 73, 75 and 76bp. The variations were A deletion, G insertion, (G/A) and (C/A) SNPs, respectively. The number and frequency of the variations are given in Table 1.

Three of the variations cause changes of amino acid. A deletion, G insertion and C/A SNP cause Gln→His, Thr→Asp and Ala→Asp, respectively. However, our study did not find the ABCG2Y allele widely reported (Cohen-Zinder et al., 2005; Ron et al., 2006; Tantia et al., 2006; Olsen et al., 2007). The effects of the ABCG2 variations are economically favorable for most selection indexes used in dairy cattle breeding programs (Miglior et al., 2005). In this study, A deletion and single nucleotide polymorphism (C/A) are probably associated with increased dry matter and fat percent as well as decreased protein percentage and milk production compared to Holstein cattle. G insertion and single nucleotide polymorphism (G/A) are likely involved in increased dry matter and fat percent and increased milk yield. A deletion and G insertion

Table 1. Number and frequencies of mutations distributing between buffalo and yak breeds.

<table>
<thead>
<tr>
<th></th>
<th>A deletion</th>
<th>G insertion</th>
<th>GA</th>
<th>GG</th>
<th>AA</th>
<th>AC</th>
<th>CC</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffalo</td>
<td>68 1.0</td>
<td>64 0.9412</td>
<td>64 0.9412</td>
<td>4 0.0588</td>
<td>4 0.0588</td>
<td>64 0.9412</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>yak</td>
<td>80 1.0</td>
<td>--</td>
<td>--</td>
<td>80 1.0</td>
<td>--</td>
<td>80 1.0</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>
have potentially for use to study the separation of Chinese Bos Taurus, Bubalus bubalis and Bos grunniens. The ABCG2 gene could be studied as the candidate gene effecting milk traits. So the ABCG2 variation could be used in Chinese Bos Taurus, Bubalus bubalis and Bos grunniens cattle breeding.

CONCLUSION

This study provides evidence that the Chinese buffalo and yak breeds have unique mutations of the ABCG2 gene. The variations were likely involved in milk yields, milk fat, protein and dry matter percentage and can be used in cattle breeding programs.

ACKNOWLEDGMENT

This work was funded by the postdoctoral funds and “TWO-SUPPORT” Project of Sichuan Agricultural University. We would like to thank Shi-Yi Chen for sample collection and DNA extraction.

REFERENCES


*Continued on page 44
ABSTRACT

The bone morphogenetic proteins (BMPs) are multifunctional proteins that play critical roles in controlling development of follicles and ovulation. Genes encoding BMP and their receptors are involved in the function of reproductive organs in many animals and BMP15 is not an exception. The present study was aimed at molecular characterization of part of the exon 2 region of the BMP15 gene of Indian riverine buffalo (Bubalus bubalis) and finding out how it contrasts with other species of livestock. The study obtained the nucleotide sequences of two fragments of part of the partial exon-2 of the BMP15 gene of buffalo. The multiple alignment and homology analysis of these buffalo sequences with those of other species revealed different degrees of nucleotide variations present among them. The phylogenetic trees constructed on the basis of nucleotide and deduced amino acid sequences showed the relative closeness/distance among different species in the evolutionary time scale.

Keywords: alignment, BMP15, buffalo, characterization, exon 2, homology, phylogenetic analysis, sequence

INTRODUCTION

Many animals of different species and breeds give birth to more than one offspring per pregnancy. From genetic studies it has been observed that litter size and ovulation rate is under the genetic control of single genes with a major effect. These genes have been named fecundity (Fec) genes. Three of these genes identified in sheep and other species are the bone morphogenetic protein receptor type IB (BMPRIIB) known as Fec B located on chromosome 6 (Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001); growth differentiation factor 9 (GDF9), known as Fec G located on chromosome 5 (Hanrahan et al., 2004) and bone morphogenetic protein 15 (BMP 15) known as Fec X located on the X chromosome (Galloway et al., 2000; Hanrahan et al., 2004). All these three genes belong to the transforming growth factor-β (TGF-β) super-family.

The bone morphogenetic proteins (BMPs) are multifunctional proteins that regulate growth and differentiation in many cell types. They play critical roles in the fertility of mammals through modulation of essential growth factors controlling development of follicles and ovulation. Genes encoding BMP and their receptors are involved
in the function of reproductive organs including ovary and uterus, and in early fetal development of various species. The X-linked BMP15, also known as GDF9B, gene is expressed in oocytes in human, mouse and sheep (Davis et al., 1992; Dube et al., 1998). The mutations found in this gene have been found to be associated with different phenotypic effects. Several inactivating mutations have been reported in the BMP15 gene in some strains of sheep with large litter sizes (Galloway et al., 2000; Hanrahan et al., 2004; Chu et al., 2007). The ewe heterozygous carrier for any of these mutations has an increased fertility due to an increase in ovulation rate, whereas homozygous ewes are infertile as a result of blockage in folliculogenesis (Galloway et al., 2000; Hanrahan et al., 2004). When the BMP15 gene is knocked out in heterozygous mice, it does not show any phenotypic defects, but the homozygous mice have a decreased fertility because of defects in ovulation and early embryonic development (Yan et al., 2001). A 4-bp deletion identified in the exon 2 region of the gene in Chinese cattle resulted in a stop codon (Zhang et al., 2009), but there was no report of infertility among those cattle breeds. As in sheep, BMP15 is also reported to regulate the ovulation rate in cattle (Juengel et al., 2007). Thus, mutations in this gene can have species-specific differences in their mode of action. The present study was aimed at molecular characterization of part of the partial exon 2 region of the BMP15 gene of Indian riverine buffalo (Bubalus bubalis) and finding out how it contrasts with other species of livestock.

**MATERIALS AND METHODS**

**Experimental animals and samples**

Ten milliliters of blood was collected from the jugular veins of Bhadawari buffaloes from Bhadawari farm, Etawah, Uttar Pradesh state of north India, in a 15 ml sterile graduated polypropylene tube containing anticoagulant EDTA (0.5 M, pH = 8.0). The blood was mixed properly with the anticoagulant and kept in an ice box until transported to the laboratory. Blood samples were stored at -20°C.

**Isolation of genomic DNA**

Genomic DNA was isolated from the frozen blood samples using the standard phenol-chloroform-isoamyl alcohol extraction method of Sambrook and Russell (2001).

**Polymerase Chain Reaction**

Primers for two fragments of cert partial exon-2 region of the BMP15 gene of the buffalo were designed using FastPCR software (University of Helsinki, Finland). The details of primers are as follows:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer sequence (5’-3’)</th>
<th>Length</th>
<th>Amplified Region</th>
<th>Size of amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>GAGTGTTCAGAAGACCAAACCTC</td>
<td>23</td>
<td>Fragment I</td>
<td>222</td>
</tr>
<tr>
<td>R1</td>
<td>TGGGGAGCAATGATCCAGTGATCC</td>
<td>24</td>
<td>Fragment II</td>
<td>222</td>
</tr>
<tr>
<td>F2</td>
<td>CTACTGTAAGGGAGTGATGCTCC</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>CTGCATGTCAGGACTGGGCAA</td>
<td>22</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Primers for amplification of exon-2 fragments I and II of the BMP-15 gene of buffalo.
Different combinations of reaction components coupled with different thermal cycling conditions were tried to find out the optimum conditions for amplification of the fragments. The standardized combination of various reaction components for a 25 μl PCR reaction mixture for amplification of both partial exon-2 fragments I and II were 50 ng of DNA template, 200 μM dNTP mixture (Fermentas, Lithuania), 30 ng each of forward and reverse primers (Qiagen, Germany), 1.5 mM MgCl₂ (Fermentas, Lithuania), 1x PCR buffer (Fermentas, Lithuania) and 1U Taq DNA polymerase (Fermentas, Lithuania). The amplification conditions for these fragments were: initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes. The amplicons were run alongside a 100 bp molecular weight marker in 2% (w/v) agarose gel in 0.5 x TBE buffer and the size of the products were confirmed.

**Nucleotide Sequencing and sequence analysis:**

The amplified samples were subjected to sequencing by an automated DNA sequencer (ABI Prism, Applied Biosystems, USA). The obtained nucleotide sequences as well as their deduced amino acid sequences were used to generate multiple alignment reports, sequence similarity/distances and phylogenetic trees with the published sequences of different species using the ClustalW programme.

**RESULTS AND DISCUSSION**

The PCR amplification of the two fragments of exon-2 of the BMP15 gene of Bhadawari buffalo produced two amplicons each of the desired 222 bp length (Figure 1). The nucleotide sequences of the two fragments obtained through sequencing were analysed and compared with those of other species to produce the following results:

**A) Multiple alignment analysis**

**a) Alignment of exon-2 fragment I**

*Nucleotide sequence alignment:* The 222 bp sequence of fragment I of exon-2 of the BMP15 gene of Bhadawari buffalo was aligned with the published sequences of different species like cattle (GenBank Acc. No. AY304484), sheep (Acc. No AF236078S2), Boer goat (Acc. No. EU847289), Jining grey goat (Acc. No EU743938), Yunling goat (Acc. No. EU847284) and human (Acc. No. BC117264). Multiple alignment analysis revealed different nucleotide variations present in different positions of the Bhadawari sequence in comparison to the other species. The nucleotide alignment report is presented in Figure 2.

*Amino acid sequence alignment:* The deduced amino acid sequence of the buffalo nucleotide sequence, when aligned with the sequences of these species, also showed the variations in the amino acid sequences present among the different species. The amino acid alignment report is given as Figure 3.

**b) Alignment of exon-2 fragment II:**

*Nucleotide sequence alignment:* The multiple alignment report of fragment II of exon-2 of the BMP15 gene of Bhadawari buffalo was generated with that of cattle, sheep, Markhor goat (GenBank Acc. No. EU095935), Boer goat, Jining grey goat and human (Acc. No. BC117264) and is shown in Figure 4.

*Amino acid sequence alignment:* The deduced amino acid sequence of this fragment also identified the different variations present at
different positions, when subjected to multiple alignment analysis with the sequences of the above species. The alignment report is presented in Figure 5.

B) Sequence Homology

a) Homology of exon-2 fragment I:

The nucleotide sequence of this region of Bhadawari buffalo showed the highest similarity (98.6%) with the sequence of Boer goat (Figure 6). It had the second highest homology of 98.2% with the sequences of sheep, Jining grey goat and Yunling goat. With cattle and human, it had 97.7% and 72.1% similarity, respectively.

The amino acid sequence of Bhadawari buffalo showed the highest 98.6% similarity with the sequences of sheep, Boer goat and Yunling goat (Figure 7) whereas the similarities both with Jining grey goat and cattle were 97.3%. The sequence was 61.6% similar to that of human.

b) Homology of exon-2 fragment II:

The nucleotide sequence of exon-2 fragment II of BMP15 gene of Bhadawari buffalo had the highest (99.5%) homology with the cattle sequence (Figure 8). It was 98.6% similar to that of sheep, Boer goat and Jining grey goat. However, the homology with Markhor goat and human was 98.2% and 86%, respectively.

The deduced amino acid sequence of the region of buffalo revealed highest (98.6%) similarity with cattle (Figure 9). However, it was equally identical (95.9%) to the sequences of sheep, Boer goat, Markhor goat and Jining grey goat. It was least (84.9%) similar to the human sequence.

C) Phylogenetic analysis

The phylogenetic trees were constructed on the basis of nucleotide as well as their deduced
Figure 2. Nucleotide sequence alignment report of BMP15 gene exon 2 fragment 1 (222 bp) of Bhadawari buffalo with other species.
Figure 3. Amino acid sequence alignment report of BMP15 gene exon 2 fragment I (222 bp) of Bhadawari buffalo with other species.

<table>
<thead>
<tr>
<th></th>
<th>Majority</th>
<th>Bhadawari Buffalo</th>
<th>Cattle</th>
<th>Sheep</th>
<th>Boer Goat</th>
<th>Jining Grey Goat</th>
<th>Yunling Goat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Indicates the residues that match the consensus exactly and residues with yellow colour differ from the consensus*
Figure 4. Nucleotide sequence alignment report of BMP15 gene exon 2 fragment II (222 bp) of Bhadawari buffalo with other species.
Figure 5. Amino acid sequence alignment report of BMP15 gene exon 2 fragment II (222 bp) of Bhadawari buffalo with other species.

* * indicates the residues that match the consensus exactly and residues with yellow colour differ from the consensus.
Figure 6. Percent similarity/divergence among nucleotide sequences of BMP15 gene exon 2 fragment I (222 bp) of Bhadawari buffalo and other species.

<table>
<thead>
<tr>
<th>Percent Identity</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Divergence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>98.6</td>
<td>98.2</td>
<td>72.1</td>
<td>98.2</td>
<td>98.2</td>
<td>Bhadawari Buffalo</td>
</tr>
<tr>
<td>2</td>
<td>2.3</td>
<td>97.7</td>
<td>98.2</td>
<td>97.7</td>
<td>71.6</td>
<td>97.7</td>
<td>97.7</td>
<td>Cattle</td>
</tr>
<tr>
<td>3</td>
<td>1.8</td>
<td>2.3</td>
<td>99.5</td>
<td>99.1</td>
<td>72.1</td>
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<td>72.1</td>
<td>Sheep</td>
</tr>
<tr>
<td>4</td>
<td>1.4</td>
<td>1.8</td>
<td>0.5</td>
<td>99.5</td>
<td>72.5</td>
<td>99.5</td>
<td>72.5</td>
<td>Boer Goat</td>
</tr>
<tr>
<td>5</td>
<td>1.8</td>
<td>2.3</td>
<td>0.9</td>
<td>0.5</td>
<td>72.1</td>
<td>99.1</td>
<td>72.1</td>
<td>Jining Grey Goat</td>
</tr>
<tr>
<td>6</td>
<td>1.8</td>
<td>2.3</td>
<td>0.9</td>
<td>0.5</td>
<td>72.1</td>
<td>99.1</td>
<td>72.1</td>
<td>Yunling Goat</td>
</tr>
<tr>
<td>7</td>
<td>34.0</td>
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<td>34.0</td>
<td>33.3</td>
<td>34.2</td>
<td>34.0</td>
<td>34.0</td>
<td>Human</td>
</tr>
</tbody>
</table>

Figure 7. Percent similarity/divergence among amino acid sequences of BMP15 gene exon 2 fragment I (222 bp) of Bhadawari buffalo and other species.

<table>
<thead>
<tr>
<th>Percent Identity</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Divergence</th>
</tr>
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<td>98.6</td>
<td>97.3</td>
<td>61.6</td>
<td>98.6</td>
<td>61.6</td>
<td>Bhadawari Buffalo</td>
</tr>
<tr>
<td>2</td>
<td>2.8</td>
<td>98.6</td>
<td>98.6</td>
<td>97.3</td>
<td>61.6</td>
<td>98.6</td>
<td>61.6</td>
<td>Cattle</td>
</tr>
<tr>
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<td>1.4</td>
<td>100.0</td>
<td>98.6</td>
<td>63.0</td>
<td>100.0</td>
<td>63.0</td>
<td>Sheep</td>
</tr>
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<td>4</td>
<td>1.4</td>
<td>1.4</td>
<td>0.0</td>
<td>98.6</td>
<td>63.0</td>
<td>100.0</td>
<td>63.0</td>
<td>Boer Goat</td>
</tr>
<tr>
<td>5</td>
<td>2.8</td>
<td>2.8</td>
<td>1.4</td>
<td>1.4</td>
<td>61.6</td>
<td>98.6</td>
<td>61.6</td>
<td>Jining Grey Goat</td>
</tr>
<tr>
<td>6</td>
<td>1.4</td>
<td>1.4</td>
<td>0.0</td>
<td>0.0</td>
<td>63.0</td>
<td>1.4</td>
<td>63.0</td>
<td>Yunling Goat</td>
</tr>
<tr>
<td>7</td>
<td>49.9</td>
<td>49.9</td>
<td>47.3</td>
<td>47.3</td>
<td>49.9</td>
<td>47.3</td>
<td>47.3</td>
<td>Human</td>
</tr>
</tbody>
</table>

Figure 8. Percent similarity/divergence among nucleotide sequences of BMP15 gene exon 2 fragment II (222 bp) of Bhadawari buffalo and other species.

<table>
<thead>
<tr>
<th>Percent Identity</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Divergence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.5</td>
<td>98.6</td>
<td>98.6</td>
<td>98.6</td>
<td>86.0</td>
<td>98.6</td>
<td>86.0</td>
<td>Bhadawari Buffalo</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>99.1</td>
<td>98.6</td>
<td>99.1</td>
<td>86.5</td>
<td>99.1</td>
<td>86.5</td>
<td>Cattle</td>
</tr>
<tr>
<td>3</td>
<td>1.4</td>
<td>0.9</td>
<td>98.6</td>
<td>99.1</td>
<td>85.6</td>
<td>99.1</td>
<td>85.6</td>
<td>Sheep</td>
</tr>
<tr>
<td>4</td>
<td>1.8</td>
<td>1.4</td>
<td>1.4</td>
<td>99.5</td>
<td>85.1</td>
<td>99.5</td>
<td>85.1</td>
<td>Markhor Goat</td>
</tr>
<tr>
<td>5</td>
<td>1.4</td>
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<td>0.9</td>
<td>0.5</td>
<td>85.6</td>
<td>100.0</td>
<td>85.6</td>
<td>Boer Goat</td>
</tr>
<tr>
<td>6</td>
<td>1.4</td>
<td>0.9</td>
<td>0.9</td>
<td>0.5</td>
<td>85.6</td>
<td>100.0</td>
<td>85.6</td>
<td>Jining Grey Goat</td>
</tr>
<tr>
<td>7</td>
<td>15.8</td>
<td>15.3</td>
<td>16.5</td>
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<td>16.5</td>
<td>16.5</td>
<td>16.5</td>
<td>Human</td>
</tr>
</tbody>
</table>
Figure 9. Percent similarity/divergence among amino acid sequences of BMP15 gene exon 2 fragment II (222 bp) of Bhadawari buffalo and other species.

Figure 10. Phylogenetic analysis based on nucleotide sequences of BMP15 gene exon 2 fragment I (222 bp) of Bhadawari buffalo and other species.

Figure 11. Phylogenetic analysis based on amino acid sequences of BMP15 gene exon 2 fragment I (222 bp) of Bhadawari buffalo and other species.
amino acid sequences of Bhadawari buffalo for both the fragments.

a) Exon-2 fragment I:

The phylogenetic tree constructed on the basis of nucleotide sequences of different species revealed that Bhadawari buffalo form a cluster with cattle (Figure 10) indicating their closeness in the evolutionary timescale. All the goat breeds and sheep fall into a different group. Human was in a distinctly separate group.

The tree constructed from the deduced amino acid sequences of these species showed that Bhadawari buffalo, cattle and Jining grey goat were closer in terms of their origin (Figure 11). The distance of origin of this group gradually increased from Yunling goat, Boer goat and sheep. Human was distantly related to these species.

b) Exon-2 fragment II:

Phylogenetic analysis on the basis of nucleotide sequences produced a tree where cattle were the species most closely related to buffalo (Figure 12). Sheep was the next most closely related species and all the goat breeds came next in the hierarchy of closeness of origin. As usual human was most distant from these species.

From the phylogenetic tree based on amino acid sequences of these species, it was evident that Bhadawari buffalo and cattle were the species closest in origin (Figure 13). Among the others, sheep was the next most closely related species.
All the goat breeds had identical origin due to their same amino acid sequences. Human had a distinct point of origin located long ago compared to the other animals.

**CONCLUSIONS**

The present study obtained the nucleotide sequence of two fragments of part of the exon-2 of BMP15 gene in buffalo. The multiple alignment and homology analysis of these buffalo sequences with those of other species revealed different degrees of nucleotide variations present among them. The phylogenetic trees constructed on the basis of nucleotide and deduced amino acid sequences showed the relative closeness/distance among different species in the evolutionary time scale. The information generated can be useful for further DNA level studies of this gene in buffalo.

**REFERENCES**


*Continued on page 54*
ABSTRACT

Fifty-three faecal samples from diarrheic calves were collected from November 2008 to March 2009 and screened by LAT, and polyacrylamide gel electrophoresis (PAGE) to detect the presence of group A rotavirus antigen. Of the 53 samples screened by LAT, 17 (32.08%) tested positive for rotavirus antigen. When the results from the PAGE were compared to those from LAT, the “gold standard” for detection of bovine rotavirus in fecal samples, the sensitivity and specificity were found to be 52.94 and 100%, respectively. Latex agglutination is easy to perform in a short time and does not require expensive equipment or skilled personnel, and the reagents have long shelf lives. These factors make the LAT suitable and highly efficient for use in a clinical laboratory as a rapid screening test for bovine rotavirus.

Keywords: bovine rotavirus, latex agglutination, PAGE

INTRODUCTION

Livestock farming plays an important role in India. The future of any dairy operation depends upon a successful program of raising calves. Incidence of neonatal calf mortality varies from 8.7 to 64 percent throughout world. Among the infectious diseases of calves, neonatal diarrhoea is a matter of major concern, and multiple etiological agents have been involved (Steele et al., 2004; Gumusova et al., 2007). Rotavirus is a main cause of neonatal diarrhoea and has been documented worldwide. It has been reported that diarrhoea in calves from 5-10 days of age is commonly due to rotavirus and infected calves excrete rotavirus in their faeces up to the age of 6 to 8 weeks (Tzipori, 1985; Radostitis, 1986). Group A rotaviruses are morphologically identical but antigenically and electrophoretically distinct from other non-group A rotaviruses (B, C, D, E) (Saif et al., 1988). Group A rotaviruses, belonging to the family Reoviridae, are important viral diarrhoeal agents in children and young animals, including calves, worldwide. These viruses possess eleven segments of double-stranded ribonucleic acid (dsRNA) and two outer capsid proteins, VP4 and VP7, both of which are independently responsible for virus neutralisation (Estes, 2001). Antigenic specificity carried by the VP4 and VP7 proteins is termed P and G genotype/serotype, respectively (Estes and Cohen, 1989). At least, 15 G types and 26 P types have been recognized so far (Kapikian et al., 2001). In India, although the occurrence of BRV-related diarrhoea has been well documented, this paper describes the incidence and electropherotyping of bovine rotavirus in diarrhoeic buffalo and cattle calves by...
LA and RNA-PAGE.

MATERIALS AND METHODS

A total of 53 faecal samples were collected from nine buffalo calves and 44 cattle calves of 0-8 weeks of age from both organized and unorganized farms in and around the Anand area, including the Livestock Research Station, Anand, Gujarat.

Latex Agglutination Test

An approximately 10% (v/v) suspension of the faecal samples were made by using one ml extraction buffer to 0.1 ml of faecal sample in a centrifuge tube. The suspension was centrifuged at 1000Xg for 10 minutes and the supernatant was collected. Two separate drops of the supernatant from each sample were placed, one onto the left black circle, the other onto the right black circle of the test card from the Rotalex kit. The contents of the Rotalex latex reagent vial and the Rotalex control latex reagent vial were mixed by gently rolling the vials between the fingers. A drop of Rotalex latex reagent and Rotalex control reagent was added in left and right circles, respectively, already containing a drop of faecal supernatant. Using clean end of mixing sticks, the two droplets in each circle were mixed carefully trying to cover the full area of the black circle. The test card was tilted and rotated moving the reagents in a circular motion within the circles. It was observed for appearance of latex particles for evidence of agglutination occurring within two minutes.

Extraction of double-stranded ribonucleic acid

A 10% faecal suspension of each sample prepared in phosphate-buffered saline and clarified by centrifugation at 10,000 rpm for 30 minutes. at 4°C was used as the basis for extraction of rotavirus ribonucleic acid (RNA). Viral RNA extraction was done using the phenol chloroform method as described by Herring et al. (1982) with slight modification. In brief, 800 μl of faecal supernatant was treated with 0.1 ml of 10 % sodium dodecyl sulphate (SDS) and 0.1 ml of 2M sodium acetate pH 4.2 (Appendix), followed by incubation at 56°C for one hour in a water bath. An equal volume of tris-saturated phenol: chloroform: isoamylalcohol (25:24:1) mixture was added to the faecal suspension. It was then vortexed and centrifuged at 12,000 rpm for 10 minutes at 4°C. The upper aqueous layer was transferred carefully to another fresh tube without disturbing the interface. The phenol: chloroform: isoamylalcohol extraction was repeated till a clear interface was obtained. The resultant aqueous solution was mixed with an equal volume of chloroform: isoamylalcohol (24:1) and vortexed, and then the mixture was centrifuged again at 12000 rpm for 10 minutes, and the upper clear aqueous phase was transferred to a fresh microcentrifuge tube. To this aqueous solution, a 0.1 volume of 3 M sodium acetate (pH 5.2) was added and vortexed. After adding an equal volume of isopropanol, the eppendorf tube was inverted 4-5 times and left overnight for precipitation at -20°C. The precipitated RNA was pelleted by centrifuging at 12000 rpm for 30 minutes at 4°C. The pellet was then washed with one ml of prechilled 75% ethanol by centrifuging at 12000 rpm for 15 minutes at 4°C and air dried. The pellet was suspended in 20 μl DEPC treated MilliQ water and stored at -20°C till RNA PAGE analysis.

RNA-PAGE. The extracted viral dsRNA was analyzed by PAGE, which was performed according to the method of Laemmli (1970) with minor modifications. Briefly, PAGE was performed at 100 V for 5-6 h using 5% stacking and 8%
separating polyacrylamide gel. The extracted viral dsRNA was mixed with 0.25% w/v bromophenol blue solution and 40% w/v of sucrose and loaded in wells to perform PAGE.

Silver staining. Silver staining of the polyacrylamide gel was performed according to the method of Svensson et al. (1986). Briefly, the polyacrylamide gel was shaken for 30 minutes in a mixture of 10% (v/v) ethanol and 0.5% (v/v) acetic acid. The mixture was removed and the gel was shaken for 30 minutes in 0.1 M silver nitrate solution. The silver nitrate solution was removed, and the gel was washed three times in distilled water and then shaken for 15 minutes in a mixture of 0.75 M NaOH and formaldehyde. The gel was washed twice in distilled water, and shaken for 5 minutes in 5% (v/v) acetic acid solution, and the electropherotype was then identified.

RESULTS

Of a total of 53 diarrhoeic samples tested for rotavirus, 17 (32.08%) were found positive for rotavirus by the LA test as indicated by clear agglutination of latex particles in test samples. Out of nine buffalo calves tested, 7 (77.78%) were found positive for rotavirus. Similarly, 10 cattle calves out of 44 tested were positive for rotavirus with a 22.73% prevalence.

During the study, diarrhoeic samples collected from buffalo and cow calves were divided into two groups each to find the susceptibility of the animals according to age and sex. The results showed that female calves (38.46%) were more susceptible than the male calves (25.93%), as 10 out of 26 females calves and seven out of 27 male calves were positive (Table 1).

Agewise, seven, five, four and one samples were positive out of 20, 16, 12 and five samples collected from age groups of 0-2, 2-4, 4-6 and 6-8 weeks, respectively, yielding agewise incidences of 35.00, 31.25, 33.33 and 20.00 percent (Table 2).

Out of 53 faecal samples tested, nine (16.98%) samples were found positive for rotavirus by RNA-PAGE. PAGE of genomic RNA obtained from the nine positive faecal samples showed a well-defined and reproducible pattern of 11 segments. All the nine electropherotypes belonged to a long genome electropherotype pattern as per Tam et al. (1986). Further analysis of PAGE was done on the basis of migration and co-migration of 11 segments in the I, II, III and IV regions and were analysed as per Tam et al. (1986) and Rasool et al. (1989). The subgroup analysis revealed that all the nine positive samples belonged to the II subgroup as they revealed separate 4-2-3-2 patterns with a long migration pattern of the 10th and 11th segments. Of these, two samples (B4 and B29) from buffalo calves (belonging to the same farm) yielded the G pattern (distinct and separate 1, 2, 3 and 4 segments and co-migration of segments 7, 8 and 9 was designated as G), while the remaining seven showed the F pattern (Co migration of 2 and 3 and of 7, 8 and 9 as F). Of the seven the IIF subgroups, one was from a buffalo calf and all the six positive samples from cattle calves yielded IIF subgroup pattern (Table 3).

Relative sensitivity and specificity of LA and PAGE

Diagnosis of rotavirus infection is conventionally made by detection of the virus, viral RNA segments or viral antigen in the faeces. A variety of tests are available for this purpose. In the present study, relative sensitivity and specificity of LA and RNA-PAGE were compared using 53 faecal samples from the diarrhoeic bovine calves.
Table 1. Species-wise prevalence of rotavirus.

<table>
<thead>
<tr>
<th>Species</th>
<th>Samples tested</th>
<th>Rotavirus +ve</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo</td>
<td>09</td>
<td>07</td>
<td>77.78</td>
</tr>
<tr>
<td>Cattle</td>
<td>44</td>
<td>10</td>
<td>22.73</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>53</strong></td>
<td><strong>17</strong></td>
<td><strong>32.08</strong></td>
</tr>
</tbody>
</table>

Table 2. Age- and Sex-wise prevalence of rotavirus by LA.

<table>
<thead>
<tr>
<th>Age (Weeks)</th>
<th>Male calves screened</th>
<th>+ve by LA</th>
<th>Incidence (%)</th>
<th>Female calves screened</th>
<th>+ve by LA</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2</td>
<td>11</td>
<td>04</td>
<td>36.36</td>
<td>09</td>
<td>03</td>
<td>33.33</td>
</tr>
<tr>
<td>2-4</td>
<td>08</td>
<td>02</td>
<td>25.00</td>
<td>08</td>
<td>03</td>
<td>37.50</td>
</tr>
<tr>
<td>4-6</td>
<td>04</td>
<td>-</td>
<td>-</td>
<td>08</td>
<td>04</td>
<td>50.00</td>
</tr>
<tr>
<td>6-8</td>
<td>04</td>
<td>01</td>
<td>25.00</td>
<td>01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>27</strong></td>
<td><strong>07</strong></td>
<td><strong>25.93</strong></td>
<td><strong>26</strong></td>
<td><strong>10</strong></td>
<td><strong>38.46</strong></td>
</tr>
</tbody>
</table>

Table 3. Group, subgroup and electropherotypes of bovine rotaviruses.

<table>
<thead>
<tr>
<th>Source of Sample</th>
<th>Sample no.</th>
<th>Group as suggested by Parwani et al. (1995)</th>
<th>Sub group as suggested by Tam et al. (1986) and Rasool et al. (1989)</th>
<th>Long/short electropherotype as suggested by Tam et al. (1986)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle calf</td>
<td>C2</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>Cattle calf</td>
<td>C3</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>Buffalo calf</td>
<td>B4</td>
<td>Group A</td>
<td>IIG</td>
<td>Long</td>
</tr>
<tr>
<td>Cattle Calf</td>
<td>C10</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>,,</td>
<td>C16</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>,,</td>
<td>C20</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>,,</td>
<td>C21</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
<tr>
<td>Buffalo calf</td>
<td>B29</td>
<td>Group A</td>
<td>IIG</td>
<td>Long</td>
</tr>
<tr>
<td>,,</td>
<td>B31</td>
<td>Group A</td>
<td>IIF</td>
<td>Long</td>
</tr>
</tbody>
</table>
As LA detected a higher number of samples positive than PAGE, sensitivity and specificity of PAGE considering LA as reference test were calculated as per Samad et al. (1994). Out of 53 samples, LA detected 17 samples positive and 36 negative for BRV, while PAGE detected nine and 44 samples as positive and negative, respectively. Eight samples negative by PAGE were positive by LA, while none of the samples negative by LA was positive by PAGE. Thus, the relative sensitivity and specificity of PAGE to LA were 52.94 percent and 100 percent, respectively. Overall agreement between the two tests was 83.02 percent (Table 4).

### DISCUSSION

Out of nine buffalo calves tested, seven (77.78%) were found positive for rotavirus. Similarly, 10 cattle calves out of 44 tested were positive for rotavirus with a 22.73% prevalence. The results were in contrast with Singh et al., 1985, who showed overall prevalence of bovine rotavirus in cattle and buffalo calves as 46.29 and 25.65 percent, respectively. The higher percentage recorded in buffalo calves could be due to relatively smaller sample size in the present study. Overall incidence of rotavirus found in the present study was similar the studies by Herbst et al. (1986) and Erdogan et al. (2003), who observed 32.07% and 31.00% incidences, respectively. Other reports in India showed the lower prevalence of rotavirus (3% by Gandhi, 1992; and 6.12 % by Vaugh, 2009).

In this study, female calves (38.46%) were found more susceptible than the male calves (25.93%). This accords with the study by Kusumakar (2006), in which female buffalo calves were reported more susceptible (25.00%) than male calves (21.00%). However, the result was in contrast to Sharma (2004), who observed higher susceptibility of male bovine calves (42.85%) to BRV in comparison to females calves (28.20%).

Kapoor (1988) and Lyoo et al. (1989) reported higher prevalence of rotavirus infection in diarrhoeic cattle calves and buffalo calves under the age group of 4-14 days. Kaushick et al. (1983) and Shah (1989) reported higher prevalence of rotavirus in buffalo and cow calves of 4-8 weeks of age. In the present study, all the positive samples except one were from the calves below six weeks of age. None of the male calves in the age group of 4-6 weeks were positive, while four out of eight female calves in the same age group were positive. This discrepancy might be due to the different locations of these calves.

The prevalence rate observed in our study (16.98%) was in agreement with earlier reports from Haryana in which prevalence rates of 11-43% by RNA-PAGE were reported (Singh and Pandey, 1990; Chauhan and Singh, 1993; Grover et al., 1998). Three (33.33%) out of nine samples from buffalo calves and six (13.64%) out of 44 samples from cattle calves were positive. Earlier, Sharma (2004) reported 40 percent and 34 percent and

<table>
<thead>
<tr>
<th>Test</th>
<th>LA</th>
<th>Total</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Overall Agreement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAGE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>53</td>
<td>52.94</td>
<td>100</td>
<td>83.02</td>
</tr>
</tbody>
</table>

Table 4. Sensitivity and specificity of PAGE with LA for detection of BRV.
Kusumakar (2006) reported 23.16 percent and 21.43 percent incidences of bovine group A rotavirus by PAGE in cattle and buffaloes, respectively. The relatively higher incidence by PAGE in buffalo calves might be due to the smaller sample size in present study. However, incidence in cattle calves can be considered reflecting a true picture of the selected population due to the optimal sample size.

Out of nine, two samples (B4 and B29) from buffalo calves (belonging to same farm) yielded the G pattern (distinct and separate 1, 2, 3 and 4 segments and co migration of segments 7, 8 and 9 was designated as G), while the remaining seven showed the F pattern (Co migration of 2 and 3 and of 7, 8 and 9 as F). Of the seven IIF subgroups, one was from a buffalo calf, and all the six positive samples from cattle calves yielded the IIF subgroup pattern (Table 3). Rasool et al. (1989) reported that the IIC pattern of electropherotype was predominate, followed by the IIG electropherotype during a 10-year study, while Dash (2008) found that the IIG pattern of electropherotype was predominate, followed by the IIC and IIF patterns of electropherotype. The usefulness of RNA-PAGE for detection and/or characterization of bovine rotavirus has been documented by various workers (Hammami et al., 1990; Gulati et al., 1995; Jindal et al., 2000; Fodha et al., 2005; Sharma et al., 2008).

Plate 1. Electropherotype of bovine rotavirus.
Lane 1, 2 shows Co migration of 2, 3 and 7, 8 and 9 segments designet as F pattern.
Lane 4 shows Co migration of 7, 8 and 9 segments designet as G pattern.
Lane 3, 5 and 6 are negative.
In our study, the relative sensitivity and specificity of PAGE to LA were 52.94 percent and 100 percent, respectively. Overall agreement between the two tests was 83.02 percent. Hammami et al. (1990) found LA to be more sensitive than PAGE in detecting BRV. Garcia Sanchez et al. (1993) found 93.33 percent agreement between PAGE and LA for BRV diagnosis. Beer et al. (1997) compared electron microscopy, PAGE and LA for the detection of bovine rotavirus in faeces and found that LA test was slightly more sensitive than PAGE and EM. Nussbaum et al. (1999) found that QLAT had 92.5% sensitivity and 96.8% specificity with PAGE for detection of rotavirus in faeces of calves.

Thus, a unified interpretation of the experimental findings from the present study indicates prevalence of BRV infection in cattle and buffalo calves of this area, with 32.08% incidence as ascertained by the LA test. Female calves and calves under six weeks of age appeared to be susceptible with calves under two weeks of age showing comparatively higher incidences of BRV. Electropherotyping of the local BRV by RNA-PAGE revealed Group A rotaviruses with long electropherotype, the majority of them falling into subgroup IIF. VP7 based RT-PCR using Heminested approach by G-type specific primers resulted in identification of G10 and G6 genotypes with G10 type predominating in this area. RNA-PAGE was less (52.94%) sensitive than LA in detecting BRV from faecal samples.

**ACKNOWLEDGEMENT**

The authors are grateful to the Dean, College of Veterinary Science and Animal Husbandry, AAU, Anand, Gujarat, India, for providing necessary facilities.

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*Continued from page 23*
ABSTRACT

This study was undertaken to determine whether fully grown oocytes isolated from the early antral follicles (2-8 mm size) of frozen-thawed buffalo ovaries are viable and can be rescued to undergo maturation, fertilization, and embryo development in vitro. Ovaries were cryopreserved just after being collected during slaughter from the local abattoir using in situ oocyte (ISO) cryopreservation. ISO cryopreservation is a multistep procedure that involves aspiration of follicular fluid and then perfusion of antral follicles (10-12 mm size) and diffusion of whole buffalo ovaries with cryoprotectant agent (CPA), rapid cooling, storage, thawing and, finally, dilution and removal of the CPA with return to physiological environment.

Data analysis revealed the quality of follicular oocytes isolated from ISO cryo ovaries appeared similar to that from fresh ovaries, and the percentages of the morphologically normal immature oocytes from ISO cryo ovaries appeared also similar to those from fresh ovaries.

There were no significant differences in the maturation (80.1%), cleavage (46.9%) and buffalo embryo development (30.4%) produced by immature oocytes of ISO cryo ovaries in comparison to the three observations in fresh oocytes of fresh non-cryopreserved ovaries (88.4%, 56.5% and 38.1%, respectively, p<0.05). Therefore, fully grown oocytes in early antral follicles (2-8 mm size) survive the cryopreservation protocol, as demonstrated by maturation, fertilization and embryo development in vitro.

Keywords: buffalo, embryo, oocyte, ISO, in vitro

INTRODUCTION

Recent research investigating the in situ oocyte (ISO) cryopreservation of follicular oocytes has primarily been focused on the improvement of cryopreservation methods of immature oocytes to overcome cryoinjury and to regulate the cumulus-oocyte interface. ISO cryo is potentially a useful technology for the preservation of genetic resources of experimental, domestic and wild animals.

To achieve optimal cryoprotection, it is essential that freezing protocols allow uniform penetration of cryoprotectant agent (CPA) throughout the ovarian tissue. Thus, the rate of CPA permeation is an important determining factor in developing better cryopreservation protocols for ovarian tissues. To date, several factors have been determined that contribute to the development of optimal cryopreservation protocols for oocytes (Critser et al., 1997).

Whole ovaries from mice and rats survive freezing because of their smaller sizes. In these species effective cryoprotectant penetration can...
occur through simple diffusion. This is not the case for larger species, whose ovaries are larger and more fibrous (Candy et al., 1997; Yin et al., 2003). Therefore, a technique of ISO cryo needs to be adopted where by the cryoprotectant is perfused though the ovary via the antral follicles as well as diffusion of CPA in the entire ovary.

During the cryopreservation procedure, cells and tissues undergo volume changes due to different osmotic pressures between the intracellular and extracellular solutions (Oda et al., 1992). These changes in cell volume affect several parameters that play a role in the cryosurvival of oocytes, including integrity of the plasma membrane and subcellular organelles (Carroll et al., 1989; McWilliams et al., 1991).

The use of oocytes from early antral follicles for production of embryos could offer significant new ways for the propagation of valuable animal stocks. One approach to cryopreservation of female gametes is to freeze intact of ovaries. Significant success has been achieved in producing live offspring by natural matings after transfer of cryopreserved mouse ovaries to the ovarian bursa of ovariectomized recipient females (Gunasena et al., 1997 and Sztein et al., 1998). However, when cryopreserved ovaries were surgically implanted, only the small primordial and primary follicles survived and underwent further development (Sztein et al., 1998). Early antral follicles did not persist after transfer probably because of failure to vascularize appropriately before onset of follicular atresia. Moreover, because of the size and cellular complexity of antral follicles, it is also possible that follicular somatic cells or oocytes could become damaged by incomplete permeation of cryoprotectants. However, if oocytes of antral follicles could be rescued from cryopreserved ovaries before transplantation and matured and fertilized in vitro as was previously suggested (Harp et al., 1994), they could provide additional opportunities for producing offspring and would not be wasted.

In this study, fully-grown oocytes were isolated from early antral follicles of thawed cryopreserved ovaries and matured in vitro. They were assessed for competence to undergo development after fertilization. There were no reports available on the safety of using of ISO cryopreservation method for cryopreservation of buffalo ovaries. Therefore, the present study was conducted to study the influence of the ISO cryopreservation methods on the production of buffalo embryos from cryopreserved buffalo ovaries in vitro.

**MATERIALS AND METHODS**

All materials were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise indicated.

**EXPERIMENTAL DESIGN**

**Initial processing of ovaries**

Ovaries collected from mature non-pregnant buffaloes from a local slaughterhouse, Cairo were brought to the laboratory in warm (32 to 33°C) normal saline supplemented with gentamicin (50 μg/ml) within one hour of slaughter. Ovaries were washed 3 times in 0.9% normal saline in the laboratory, and extra ovarian tissues were removed followed by washing with Dulbecco’s phosphate buffer saline. Ovaries were used for the isolation of preantral, early antral follicles and collection of oocytes from visible antral follicles.
Isolation of large preantral follicles

Buffalo ovaries were brought to the laboratory in warm (32 to 33°C) normal saline supplemented with gentamicin (50 μg/ml) for the isolation of preantral follicles and in ice for the isolation of somatic cells within one hour of slaughter. Ovaries were washed thoroughly in 0.9 percent normal saline supplemented with gentamicin (50 μg/ml).

IN SITU OOCYTE (ISO) CRYOPRESERVATION TECHNIQUE

Immature buffalo oocytes were cryopreserved after collection of slaughters ovaries using the in situ oocyte cryopreservation technique. Oocytes from non-frozen ovaries subjected to the same in vitro fertilization technique were used as controls. Ovaries were obtained from buffalo that had just been slaughtered at a local abattoir. The buffaloes were between 5 and 15 years of age.

Abd-Allah (2009 a) described the in situ oocyte (ISO) cryopreservation procedure that was used in our study. Briefly, freshly collected ovaries with an antral follicle were dissociated from adipose tissue. The antral follicle (10-12 mm in diameter) was aspirated by a hypodermic needle 26 gauge (G) x 1/2˝ (0.45 x 12 mm) (Intermedica, Cairo) to remove part of the follicular fluid (2-5 mm³ or 2 ± 5 mm³ or 2.5 mm³) and then the follicle was infused by 10% glycerol (2-5 mm³ or 2 ± 5 mm³ or 2.5 mm³). Each ovary was transferred to a Petri dish containing 10% glycerol solution at 37°C. After a 30 minutes exposure to the 10% glycerol solution, each ovary was transferred to a 20 ml/cc Mediject syringe (Intermedica, Cairo) containing the 10% glycerol solution (15). The cryo syringes were plunged directly into liquid nitrogen (-196°C) for vitrification of the ovary and were stored for a month.

Thawing of ovaries

The cryo syringes were removed from the liquid nitrogen and held at room temperature for 2 minutes before being plunged into a 37°C water bath and gently agitated for 30-40 minutes. Each ovary was immersed, through the needle of the cryo syringe, into a thawing media consisting of Tissue Culture Medium-199 (TCM-199) supplemented with 10% fetal calf serum (FCS), 50 μg/ml gentamycine sulfates, 10% sucrose and, through the hypodermic needle 18 G × 1 1/2˝ (0.8 x 40 mm) attached to a 10 ml/cc mediject syringe (Intermedica, Cairo) containing warm (37°C) thawing media aspirate buffalo immature oocytes from antral follicles (2-8 mm in diameter). The ovaries were kept at 37°C in the final thawing media until analysis.

Retrieval of oocytes from the antral follicles

Oocytes were isolated by puncturing the antral follicles (2-8 mm) using an 18- gauge needle as described by Totey et al. (1992). The COCs were aspirated and washed three times with tissue culture medium (TCM-199), supplemented with 10% fetal calf serum (FCS) and 50 μg/ml gentamycin sulphate. The number of buffalo oocytes isolated from antral follicles (2-8 mm) of ISO cryo ovaries and fresh ovaries were recorded.

Grading of oocytes collected from antral follicle

The released immature buffalo oocytes were scored for granulose-oocyte cell adhesion as previously described (4): C+ for granulose-enclosed oocytes, C+- for partially granulose-enclosed oocytes, (whenever there were granulosa cell-free
regions on the oocyte surface), C- for granulose-free oocytes (Figure 1) (Combelles and Albertini, 2003).

**Assessment of survival of oocytes**

The recovered immature oocytes were observed under a stereomicroscope (M6C-10, N9116734, Russia). Oocytes were judged morphologically as survivors (Figure 1) when the spherical and symmetrical shape had no signs of lysis, membrane damage, swelling, degeneration or leakage of the cellular content; oocytes were considered abnormal (Figure 1) when a ruptured zona pellucida or a fragmented cytoplasm with signs of degeneration were present (Dhali et al., 2000). Follicles with normal appearance and without visible signs of degeneration were selected for the study.

**Rescue of oocytes of antral follicle**

*In vitro* maturation of oocytes

The oocytes were washed once with the aspiration medium and twice in the in vitro maturation medium (tissue culture medium (TCM-199), supplemented with 10% fetal calf serum (FCS) and 50 μg/ml gentamycin sulphate) in which they would be cultured. Oocytes in groups (8 to 12 oocytes) were transferred into 50 μl droplets of culture medium. The droplets containing oocytes were covered with warm (38.5°C) mineral oil and the Petri dishes were placed in a CO₂ incubator (38.5°C, 5% CO₂ in air, 90-95% relative humidity) for 24 h.

**Assessment of oocytes for in vitro maturation**

The evaluation of maturation rate of the oocytes was based on the visual assessment of the degree of expansion under zoom stereomicroscope (Kobayashi et al., 1994): degree 0: no expansion; degree 1 (moderate expansion): cumulus cells were non-homogeneously spread and clustered cells were still observed, and degree 2 (fully expanded): cumulus cells were homogeneously spread and clustered cells were no longer present. Only degree 2 and degree 1 were considered as matured.

**Sperm preparation and *in vitro* insemination**

Ejaculated frozen-thawed buffalo semen from two straws (0.25 ml, 20 million sperm cells per straw) were washed in Brackett and Oliphant (BO, Brackett and Oliphant, 1975) medium (without BSA) containing 10μg/mL heparin and centrifuged twice at 500g for 5 minutes. The sperm cells were suspended for swim-up in BO medium containing 10 mg/ml heparin and 2.5 mM caffeine. Progressively motile spermatozoa were placed in 100 ml droplets of BO medium containing 0.5 percent BSA, 10 mg/ml heparin and 2.5 mM caffeine in a Petri dish, covered with mineral oil, and placed in a CO₂ incubator for 1 h at 38.5°C before inseminating in vitro-matured oocytes. The sperm concentration was then adjusted to (8-10) x 10⁶ /mL before inseminating the oocytes.

**In vitro fertilization of oocytes**

The medium in the droplets containing the matured oocytes was removed and replaced by spermatozoa (8-10 million /ml) in BO medium with 0.5 % BSA. The dishes were then placed in 5% CO₂ incubator at 38.5°C for 16 h. After incubation period, the BO medium and unattached sperms were removed and replaced by TCM-199 supplemented by 10% FCS. The dishes were then placed again in CO₂ incubator at 38.5°C and incubated for a further period of 24 h.

**Assessment of in vitro fertilization**

After 40-42 h of insemination, presumptive
zygotes were evaluated under a stereo zoom microscope for evidence of cleavage. The cleaved embryos of the 2-4 cell stage or beyond were selected for *in vitro* culture study.

**In vitro development of oocytes**

Putative zygotes were cultured, under oil, in groups of 10 in 50 µl droplets of TCM-199 supplemented with 10% FCS and 50 µg gentamycin sulphate. Culture dishes were incubated for 5-7 days at 38.5°C in a humidified atmosphere of 5% CO₂, and the culture medium was changed every 48 h.

**Assessment of in vitro development of oocytes**

At 6-8 days post insemination, morula and/or blastocysts were evaluated under a stereo zoom microscope and the number of embryos which at least cleaved was calculated.

**Statistical analysis**

The experiment was replicated 10 times and the data were analyzed by chi square analysis (Snedecor and Cochran, 1980).

**RESULTS AND DISCUSSION**

Percentages for recovered oocyte classes and the morphology of buffalo oocytes isolated from cryopreserved and fresh ovaries are presented in Table 1 and in Figure 3. The data presented here demonstrate that the successful production of embryos *in vitro* in buffalo from cryopreserved ovaries using *in situ* oocyte cryopreservation method was successful. Percentages for oocyte classes and normal morphology of buffalo oocytes isolated from cryopreserved and fresh ovaries are presented in Table 1 and in Figure 3. The differences between the percentages of granulose-enclosed oocytes and granulose free oocytes yielded from the cryopreserved ovaries and fresh oocyte procedure were non-significant (p>0.05 or p>0.01), although the value of the fresh oocytes was higher (15.4% vs 19.4%, respectively).

The results of the present study (Table 1) revealed a percentage of post-thawing morphologically normal immature oocytes isolated from cryopreserved ovaries using *in situ* oocyte (ISO) technique. This may be attributed to ISO cryo that may prevent some of the apoptosis which can be induced by cryopreservation (Stroh *et al.*, 2002), as CPAs perfused directly to the follicular tissue would maximize cooling to facilitate vitrification and prevent ice crystal injury; it also resulted in less ultrastructural injury and hence improved tissue survival. ISO cryopreserving of buffalo oocytes is necessary from a cryobiology point of view because the rate of CPA/cellular water exchange is affected by the amount of tissue through which the CPA must diffuse. During the cooling stage of cryopreservation, the relative distance of cells within the ovary from the exterior affects the rate at which these cells undergo cooling.

Our statistical analysis (Table 2 and Figure 4) showed an insignificant (p>0.05 or p>0.01) difference in the maturation and cleavage rates in immature oocytes isolated from cryopreserved ovaries using *in situ* oocyte (ISO0 technique in comparison to those of fresh oocytes (80.1%, 46.9% vs. 88.4%,56.5 %, respectively).

The differences between the percentages of developed buffalo embryos resulting from the cryopreserved ovaries (Figure 2) and fresh oocyte procedure were non-significant (p>0.05 or p>0.01), although the value of the fresh oocytes was higher (30.4% vs. 38.1%, respectively). The high embryo rates of ISO cryo ovaries observed in the present
Table 1. The number and the percentages of recovered oocyte classes and morphologically normal oocytes isolated from early antral in the groups of ISO cry ovaries and fresh ovaries, scored for granulosa-oocyte adhesion.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>ISO cryo ovaries</th>
<th>Fresh ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovered immature oocytes (No.)</td>
<td>400</td>
<td>500</td>
</tr>
<tr>
<td>C+</td>
<td>60% (240/400)</td>
<td>70% (350/500)</td>
</tr>
<tr>
<td>Oocyte classes (No. and %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C+/-</td>
<td>30% (120/400)</td>
<td>22% (110/500)</td>
</tr>
<tr>
<td>C-</td>
<td>10% (40/400)</td>
<td>8% (40/500)</td>
</tr>
<tr>
<td>Morphological normal oocytes (No. and %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C+</td>
<td>83% (332/400)</td>
<td>88% (440/500)</td>
</tr>
<tr>
<td>C+/-</td>
<td>91.6% (220/332)</td>
<td>96.5% (338/350)</td>
</tr>
<tr>
<td>C-</td>
<td>55% (22/40)</td>
<td>50% (20/40)</td>
</tr>
<tr>
<td>Oocytes classes (No. and %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C+</td>
<td>75% (90/120)</td>
<td>70.7% (82/110)</td>
</tr>
<tr>
<td>C-</td>
<td>55% (22/40)</td>
<td>50% (20/40)</td>
</tr>
<tr>
<td>Number of damaged oocytes (%)</td>
<td>17% (68/400)</td>
<td>12% (60/500)</td>
</tr>
<tr>
<td>Types of cryoinjury</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ruptured zona pellucida (%)</td>
<td>17.64% (12/68)</td>
<td>16.6% (10/60)</td>
</tr>
<tr>
<td>Shrinkage of cytoplasm (%)</td>
<td>58.82% (40/68)</td>
<td>58.3% (35/60)</td>
</tr>
<tr>
<td>Leakage of cell content (%)</td>
<td>23.62% (20/68)</td>
<td>25% (15/60)</td>
</tr>
</tbody>
</table>

Within the same row, values with the same superscript are insignificantly different from each other (p>0.05). C+ for granulosa-enclosed oocytes, C+/- for partially granulosa-enclosed oocytes (whenever there were granulosa cell-free regions on the oocyte surface) and C- for granulosa-free oocytes.

Table 2. Preimplantation development of oocytes isolated from from ISO Cryo ovaries and fresh oocytes and matured in vitro.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>ISO cryo ovaries</th>
<th>Fresh ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cultured oocytes</td>
<td>332</td>
<td>440</td>
</tr>
<tr>
<td>Matured oocytes (%)</td>
<td>80.1% (266/332)</td>
<td>88.4% (389/440)</td>
</tr>
<tr>
<td>Cleaved oocytes (%)</td>
<td>46.9% (125/266)</td>
<td>56.5% (220/389)</td>
</tr>
<tr>
<td>Buffalo embryos (%)</td>
<td>30.4% (38/125)</td>
<td>38.1% (84/220)</td>
</tr>
</tbody>
</table>

Within the same row, values with the same superscript are insignificantly different from each other (p>0.05).
Figure 1. Representative sample of oocytes isolated from frozen-thawed ovaries. (a): Morphologically normal oocytes retrieved from antral in the groups of ISO cryo ovaries, scored for granulosa-oocyte adhesion. C⁺ for granulosa-enclosed oocytes, C⁻⁺ for partially granulosa-enclosed oocytes (whenever there were granulosa cell-free regions on the oocyte surface) and C⁻ for granulosa-free oocytes and (b) degenerated oocytes (a) Shrinkage of cytoplasm, (b) Leakage of cellular content, (c) Rupture of cell membrane (d) Non-shrinkage cytoplasm (morphological normal oocytes)

Figure 2. Representative sample of buffalo embryos produced from frozen-thawed ovaries.
Graph 3. Percentages of oocyte class and morphological normal oocytes retrieved from ISO cryo ovaries and fresh ovaries.

Graph 4. Percentages of maturation, cleavage and embryo production rate from immature oocytes isolated from ISO cryo ovaries and fresh ovaries.
study compare favorably with other reports in the cryopreservation of mouse oocytes (Candy et al., 1997; Sztein et al., 1998).

Here it is shown that oocytes in antral follicles survive the cryopreservation protocol, as demonstrated by maturation, fertilization and embryo development in vitro. It is possible that the oocytes in antral follicles do, in fact, sustain some damage inflicted by the freeze-thaw protocols. If so, either the damage is not serious, or serious damage is repaired during maturation in vitro (Schroeder et al., 1991). Since the percentage of oocytes that completed maturation and preimplantation development was always lower in the cryopreserved than the fresh group, it must be assumed that many oocytes that appeared morphologically normal must have suffered developmental lesions due to the cryopreservation protocol.

It is concluded that the in situ oocyte cryopreservation method used in this study is suitable for the freezing of ovarian tissue and immature buffalo oocytes on account of the high performance obtained.

REFERENCES


*Continued from page 35*


OVARIAN ACTIVITY AND HORMONAL RELATIONSHIPS IN PREGNANT BUFFALOES

M.M. Waheed

ABSTRACT

The morphological characters of ovaries were studied in 51 slaughtered pregnant Egyptian buffaloes including the weight and diameter of corpora lutea and the number and size of healthy follicles. Progesterone and estradiol-17β concentrations were estimated in both peripheral plasma and feces using enzymimmunoassay. The results revealed a decrease in number of all sized follicles as pregnancy advanced. Plasma and fecal progesterone concentrations increased and plasma estradiol-17β levels decreased with the progress of pregnancy. Positive correlation coefficients existed between the ovarian weight and weight of corpora lutea on one hand and between the number of follicles and estradiol-17β levels on the other. The correlation coefficients between the weight of corpora lutea and estradiol-17β levels were negative. Seasonal differences in ovarian activity occurred in pregnant buffaloes coinciding with the profound effect of season on the progesterone and estradiol-17β concentrations in the peripheral plasma.

Keywords: buffalo, pregnancy, ovary, progesterone, estradiol-17β

INTRODUCTION

Despite the buffaloes' importance in the animal production industry, there are relatively few studies regarding its physiology (Martin et al., 2008). Relatively few references exist concerning the morphological and physiological aspects of the buffalo ovary during pregnancy. In pregnant cows, corpora lutea on an ovary may modify the pattern of growth and atresia of follicles on the same ovary (Rexroad and Casida, 1975). Cows during pregnancy continue to manifest follicular waves at intervals of 8 to 10 days (Rexroad and Casida, 1975; Pierson and Ginther, 1987; Ginther et al., 1989; Taylor and Rajamahendran, 1991). The follicular development is characterized by waves of different patterns in pregnant buffaloes and new studies with hormone assays could be more elucidative (Martin et al., 2008).

The objective of the present study was to measure the ovarian activity of pregnant buffaloes in the form of morphological evaluation of corpora lutea and follicles as well as, the determination of progesterone and estradiol-17β concentrations in both peripheral plasma and feces of these buffaloes.
MATERIALS AND METHODS

Animals:
A total of 51 healthy pregnant, Egyptian buffalo-cows (*Bubalus bubalis*) aged from 5 to 10 years were used. These buffaloes were slaughtered at El-Warak slaughter-house, Giza, Egypt. Pregnancy was diagnosed per rectum before slaughter. Immediately after slaughter, the genital organs were so removed that the ovaries were maintained intact and transported to the laboratory. In the laboratory, the reproductive organs were dissected and stages of pregnancy were determined (Abdel Raouf and El Naggar, 1968; Luktuke, 1983).

Ovaries were weighed to the nearest mg. All the follicles on the surface of the ovaries were counted and their diameter was measured using a pair of vernier calipers. Follicular diameter was partitioned into three discrete size groups: [a] small (< 6 mm), [b] medium (6-10 mm) and [c] large (>10 mm; Schmidt *et al*., 1963; El-Wishy, 1965; Brantmeier *et al*., 1987). Corpora lutea were dissected free of extraneous tissue and their diameter and weight were recorded.

Blood and fecal samples:
At the time of rectal palpation, fecal samples were collected and then transported to the laboratory and stored at -20°C pending analysis. Blood samples (10 ml) were collected from the jugular vein at slaughter in labeled heparinized tubes (5 IU heparin/tube). In the laboratory, blood samples were centrifuged at 14,000 g for 10 minutes at 4°C. Blood plasma was stored at -20°C until analysis.

Progesterone and estradiol-17β enzymimmonoassay (EIA):
Progesterone and estradiol-17β were determined in the peripheral blood plasma and feces of pregnant buffaloes by enzymimmunoassay (Panchal *et al*., 1992; Palme and Mostle, 1993; Palme *et al*., 1993). In brief, progesterone and estradiol-17β were extracted from feces by adding 0.5 ml distilled water and 4.0 ml of absolute methanol to 0.5 g of feces. The mixture was shaken for 30 minutes to remove lipids then 3ml of petroleum ether was added. After thorough mixing in a vortex for 10 seconds, the mixture was centrifuged (1500 g) for 10 minutes. The methanol and ether layers were separated by cooling at 20°C for one hour. The methanol extract was drown and diluted 1:10 to 1:50 with assay buffer. A 0.01 ml of the extract was used for enzymimmonoassay (Panchal *et al*., 1992; Palme and Mostle, 1993; Palme *et al*., 1993).

Statistical analysis:
Pregnant buffaloes were categorized into two groups: Group A (1-<3 months pregnancy; n= 29) and Group B (3-6 months pregnancy; n= 22). The correlation coefficients and t test were calculated using a commercial software programme, Statistical for Windows, 1993.

RESULTS

Although there is no significant difference in the weight and diameter of corpora lutea between Groups A (1-<3 months pregnancy) and B (3-6 months pregnancy), the plasma and fecal progesterone concentrations were significantly (p<0.05) higher in Group B than Group A buffaloes (6.14 ± 0.61 Vs 4.09 ± 0.46 ng/ml and 2053.86 ± 232.28 vs 1100.26 ± 230.03 ng/g, respectively; Table 1).

Table 2 shows a decrease in the number of follicles of all diameters in the Group B buffaloes.
Table 1. Corpora lutea and progesterone concentrations (P₄) of pregnant buffaloes (mean±SEM).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>n</th>
<th>Group A (1-&lt;3 months pregnancy)</th>
<th>n</th>
<th>Group B (3-6 months pregnancy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian weight (g)</td>
<td>29</td>
<td>5.01 ± 0.38</td>
<td>22</td>
<td>5.01 ± 0.44</td>
</tr>
<tr>
<td>CL Ø (cm)</td>
<td>29</td>
<td>1.15 ± 0.07</td>
<td>22</td>
<td>1.20 ± 0.08</td>
</tr>
<tr>
<td>CL weight (g)</td>
<td>29</td>
<td>2.96 ± 0.13</td>
<td>22</td>
<td>3.26 ± 0.26</td>
</tr>
<tr>
<td>Plasma P₄ (ng/ml)</td>
<td>27</td>
<td>4.09a ± 0.46</td>
<td>22</td>
<td>6.14b ± 0.61</td>
</tr>
<tr>
<td>Fecal P₄ (ng/g)</td>
<td>19</td>
<td>1100.26a ± 230.03</td>
<td>21</td>
<td>2053.86b ± 232.28</td>
</tr>
</tbody>
</table>

Means with dissimilar superscripts in the same row are significantly different at p<0.05 Ø = diameter.

Table 2. Follicular activity (%) and estradiol-17β concentrations (mean ± SEM) of pregnant buffaloes.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>n</th>
<th>Group A (1 – &lt;3 months pregnancy)</th>
<th>n</th>
<th>Group B (3 – 6 months pregnancy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Ipsilateral with CL</td>
<td>Contralateral to CL</td>
<td>n</td>
</tr>
<tr>
<td>Follicles Ø &lt;6 mm</td>
<td>26</td>
<td>11 (42.3%)</td>
<td>15 (57.7%)</td>
<td>20</td>
</tr>
<tr>
<td>Follicles Ø 6-10 mm</td>
<td>25</td>
<td>9 (36%)</td>
<td>16 (64%)</td>
<td>14</td>
</tr>
<tr>
<td>Follicles Ø &gt;10 mm</td>
<td>15</td>
<td>4 (26.7%)</td>
<td>11 (73.3%)</td>
<td>3</td>
</tr>
<tr>
<td>Plasma estradiol (pg/ml)</td>
<td>27</td>
<td>1129.81a ± 137.47</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>Fecal estradiol (pg/g)</td>
<td>22</td>
<td>26509.38 ± 6373.90</td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>

Means with dissimilar superscripts in the same row are significantly different at p<0.05 Ø = diameter.

Table 3. Correlation Coefficients (r) between ovarian activity and hormonal concentrations of pregnant buffaloes (Group A, 1-<3 months pregnancy).

<table>
<thead>
<tr>
<th>Correlated parameters</th>
<th>n</th>
<th>Correlation Coefficients (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of the left ovary X weight of the CL</td>
<td>13</td>
<td>r = 0.66*</td>
</tr>
<tr>
<td>Weight of the right ovary X weight of the CL</td>
<td>16</td>
<td>r = 0.85*</td>
</tr>
<tr>
<td>Number of follicles &lt;6mm Ø X plasma estradiol level</td>
<td>18</td>
<td>r = 0.97*</td>
</tr>
<tr>
<td>Number of follicles &gt;10mm Ø X fecal estradiol level</td>
<td>12</td>
<td>r = 0.95*</td>
</tr>
<tr>
<td>Weight of the right CL X plasma estradiol level</td>
<td>12</td>
<td>r = - 0.79*</td>
</tr>
<tr>
<td>Weight of the right CL X fecal estradiol level</td>
<td>12</td>
<td>r = - 0.86*</td>
</tr>
</tbody>
</table>

p<0.05 Ø = diameter
This is coincided with the significant (p<0.05) decrease in plasma estradiol-17β concentrations in Group B (791.07 ± 75.59 pg/ml) in comparison to Group A (1129.81 ± 137.47 pg/ml). There was a clear reduction in the number of follicles of all sizes in Group A and medium-sized follicles in Group B with the presence of a corpus luteum (CL) on the same ovary (Table 2).

There was a positive correlation coefficient (p<0.05; r = 0.66 and 0.85) between the ovarian weight and the weight of CL of pregnant buffaloes (Table 3). Similarly, positive correlation coefficient (p<0.05) were present between the number of small follicles and plasma estradiol-17β concentrations (r = 0.97), and between the number of large follicles and fecal estradiol-17β concentrations (r = 0.95; Table 3). On the contrary, there are negative correlation coefficients (p<0.05) between the weight of corpora lutea and both plasma and fecal estradiol-17β concentrations of buffaloes (r = -0.79 and r = -0.86, respectively; Table 3).

The effect of season on corpora lutea and progesterone concentrations of pregnant buffaloes is shown in Table 4. In Group A, the ovarian weight and the diameter of CL decreased significantly (p<0.05) during summer compared to winter (4.68 ± 0.39 vs 5.14 ± 0.49 g and 0.94 ± 0.08 vs 1.27 ± 0.07 cm, respectively). However, in Group B, there was a significant (p<0.05) increase in plasma progesterone concentrations during summer in comparison to winter (7.26 ± 0.99 vs 5.01 ± 0.59 ng/ml, respectively; Table 4).

Table 5 shows that in Group A, despite the presence of high numbers of medium- and large-sized follicles during winter, the plasma estradiol-17β concentrations increased significantly (p<0.05) during summer in comparison to winter (1966.28 ± 234.98 vs 777.62 ± 80.58 pg/ml).

**DISCUSSION**

The mean weight and diameter of corpora lutea did not increase during the second trimester of pregnant buffaloes. Similar results were found by Hafez, 1955. Nevertheless, it was reported that the weight of CL increased slightly during the second period of gestation (75-132 days) in Egyptian buffaloes (El-Sheikh et al., 1969). Irrespective of persistent weight of corpora lutea during the second trimester of pregnancy, there was marked increase in progesterone concentrations in both peripheral plasma and feces during this period. The most plausible explanation for this increase in progesterone levels is the presence of additional sources of progesterone other than the CL such as the placenta, which secretes progesterone between days 150-250 of gestation in cattle (Thomas, 1997) and the adrenal glands, which may contribute 1-4 ng/ml of progesterone during gestation (Wendorf et al., 1983).

There were a decreased number of follicles with different diameters in the second trimester in pregnant buffaloes. This was accompanied by a significant decrease in plasma estradiol-17β concentrations in this period of gestation. The same relationship between the size and number of follicles and estradiol-17β concentrations was reported in cows (Brantmeier et al., 1987). However, Robertson and King (1979) stated that the concentration of estrogens in the peripheral blood increased as pregnancy progressed in cows. Ginther et al. (1996) observed a decrease in the dominant follicle diameter after day 90 of bovine pregnancy and attributed this to a decrease in luteinizing hormone (LH) pulse frequency and/or average LH concentrations, or to a low number of LH receptors in follicular granulosa cells. LH has a role in the growth and function of the largest
Table 4. Effect of season on corpora lutea and progesterone concentrations (P₄) of pregnant buffaloes (mean ± SEM).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>Winter (December - February)</th>
<th>Summer (May - July)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>mean ± SEM</td>
<td>n</td>
</tr>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1-&lt;3 months pregnancy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian weight (g)</td>
<td>19</td>
<td>5.14 ± 0.49</td>
<td>10</td>
</tr>
<tr>
<td>CL Ø (cm)</td>
<td>19</td>
<td>1.27 ± 0.07</td>
<td>10</td>
</tr>
<tr>
<td>CL weight (g)</td>
<td>19</td>
<td>2.95 ± 0.14</td>
<td>10</td>
</tr>
<tr>
<td>Plasma P₄ (ng/ml)</td>
<td>19</td>
<td>3.84 ± 0.54</td>
<td>8</td>
</tr>
<tr>
<td>Fecal P₄ (ng/g)</td>
<td>16</td>
<td>1126.56 ± 268.04</td>
<td>3</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3 - 6 months pregnancy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovarian weight (g)</td>
<td>11</td>
<td>4.96 ± 0.49</td>
<td>11</td>
</tr>
<tr>
<td>CL Ø (cm)</td>
<td>11</td>
<td>1.34 ± 0.11</td>
<td>11</td>
</tr>
<tr>
<td>CL weight (g)</td>
<td>11</td>
<td>3.19 ± 0.46</td>
<td>11</td>
</tr>
<tr>
<td>Plasma P₄ (ng/ml)</td>
<td>11</td>
<td>5.01 ± 0.59</td>
<td>11</td>
</tr>
<tr>
<td>Fecal P₄ (ng/g)</td>
<td>10</td>
<td>2611.60 ± 816.91</td>
<td>11</td>
</tr>
</tbody>
</table>

Means with dissimilar superscripts in the same row are significantly different at p<0.05
Ø = diameter.

Table 5. Effect of season on follicular activity (%) and estradiol-17B concentrations (mean ± SEM) of pregnant buffaloes.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>Winter (December - February)</th>
<th>Summer (May - July)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>(%)</td>
<td>n</td>
</tr>
<tr>
<td>Group A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1-&lt;3 months pregnancy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicles Ø &lt;6 mm</td>
<td>7</td>
<td>(50.00 %)</td>
<td>7</td>
</tr>
<tr>
<td>Follicles Ø 6-10 mm</td>
<td>13</td>
<td>(76.47 %)</td>
<td>4</td>
</tr>
<tr>
<td>Follicles Ø &gt;10 mm</td>
<td>11</td>
<td>(91.67 %)</td>
<td>1</td>
</tr>
<tr>
<td>Plasma estradiol (pg/ml)</td>
<td>19</td>
<td>777.62 ± 80.58</td>
<td>8</td>
</tr>
<tr>
<td>Fecal estradiol (pg/g)</td>
<td>16</td>
<td>25492.20 ± 7739.75</td>
<td>6</td>
</tr>
<tr>
<td>Group B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3 - 6 months pregnancy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicles Ø &lt;6 mm</td>
<td>4</td>
<td>(30.77 %)</td>
<td>9</td>
</tr>
<tr>
<td>Follicles Ø 6-10 mm</td>
<td>10</td>
<td>(76.92 %)</td>
<td>3</td>
</tr>
<tr>
<td>Follicles Ø &gt;10 mm</td>
<td>2</td>
<td>(66.67 %)</td>
<td>1</td>
</tr>
<tr>
<td>Plasma estradiol (pg/ml)</td>
<td>9</td>
<td>741.97 ± 124.07</td>
<td>11</td>
</tr>
<tr>
<td>Fecal estradiol (pg/g)</td>
<td>7</td>
<td>21821.71 ± 10715.46</td>
<td>11</td>
</tr>
</tbody>
</table>

Means with dissimilar superscripts in the same row are significantly different at p<0.05
Ø = diameter.
follicles (Martin et al., 2008). The dominant follicle is selected because it acquires LH receptors on its granulosa cells and this allows the cells to synthesize estradiol in response to LH (Xu et al., 1995; Fike et al., 1997). Moreover, the periods of follicular growth not exceeding 6.0 mm in diameter observed during buffalo pregnancy could be a consequence of inadequate follicle stimulating hormone (FSH) support (Martin et al., 2008).

There was a reduction in number of follicles with the presence of CL on the same ovary of pregnant buffaloes. In pregnant cows, corpora lutea on an ovary may modify the pattern of growth and atresia of follicles on the same ovary (Rexroad and Casida, 1975). The corpora lutea may act on follicles to alter their growth rates to result in atresia at a smaller size or to increase their rate of growth to a large size at which point they become atretic, thus increasing their turnover rate (Rexroad and Casida, 1975). One possibility of this action is locally high concentrations of progesterone (Rexroad and Casida, 1975). High luteal progesterone levels have been shown to reduce the diameter of the second dominant follicle in the bovine (Bergfelt et al., 1991; Fortune, 1993). Furthermore, progesterone has been shown to alter growth and atresia of follicles in rabbits (Wallach and Noreiga, 1970).

Positive correlation coefficients were calculated between the ovarian weight and the weight of CL of pregnant buffaloes. In the same direction, positive correlations between the weight of CL and ovary weight were reported in pregnant cows (Stormshak and Erb, 1961). Furthermore, in the current work, positive correlation coefficients were recorded between the number of follicles and plasma and fecal estradiol-17β levels. Mellin and Erb (1965) found an estrogenic biological activity in peripheral blood and feces from pregnant cows. About 70% of total estrogen is excreted in feces of cows (Monk et al., 1975). However, in the present study, there were negative correlation coefficients between the weight of corpora lutea and both plasma and fecal estradiol-17β concentrations. This may be attributed to the direct effect of corpora lutea on growth and atresia of follicles (Rexroad and Casida, 1975) through their high luteal progesterone levels (Bergfelt et al., 1991; Fortune, 1993).

In the current study, the ovary was heavier and the diameter of corpora lutea was larger in winter than in summer. The breeding efficiency of buffaloes is influenced by seasons (Yadava and Kushwaha, 1965; Rao and Pandey, 1982). Winter was proved to be the most favorable season for breeding of buffaloes (Yadava and Kushwaha, 1965; Roy et al., 1972). Similarly, in cows, McNatty et al. (1984) reported that the mean diameter of large follicles was greater and the corpora lutea were heavier in autumn and winter than in spring. The seasonal differences in ovarian activity are probably the consequence of seasonal differences in gonadotropin secretion (McNatty et al., 1984), climatic factors and vegetative growth of crops (Yadava and Kushwaha, 1965).

The plasma progesterone levels in the second trimester of pregnant buffaloes were higher in summer than in winter. On the contrary, Rao and Pandey (1982) stated that there was low progesterone level in hotter months in comparison to cooler ones. Apart from the seasonal effect, the explanation may again be the presence of additional sources of progesterone in the second trimester of bovine pregnancy (Wendorf et al., 1983; Thomas, 1997).

In the present study, the plasma estradiol-17β concentrations during the first trimester were higher in summer compared to winter. It is not clear whether this increase in plasma estradiol-17β level
is due to an imbalance in the pituitary gonadotropic complex as a result of neural stimuli from fetoplacental unit or to some other factors such as stress leading to certain endocrine disturbances (Batra et al., 1979; Rao and Pandey, 1982).

In conclusion, peripheral plasma and fecal progesterone concentrations increased and plasma estradiol-17ß levels decreased in the second trimester of pregnant buffaloes. Seasons exerted a significant effect on the ovarian activity during buffalo pregnancy.

REFERENCES


*Continued on page 99
ABSTRACT

In a 180 day feeding trial, 12 graded Murrah buffalo bull calves (127.8±4.1 kg and 11 to 12 months) were randomly divided into two equal groups of six animals each (control and treatment) taking into consideration their body weight. During the trial, calves in both the groups were offered a conventional concentrate mixture to meet the protein requirements for maintenance and growth 500 g/d (ICAR, 1998) and had access to chopped Guinea fodder ad libitum. The calves in the treatment group received yeast culture (Saccharomyces cerevisiae CNCM I-1077 strain) of 0.25 g/animal/day. The DMI (kg/d) increased (P>0.05) in bull calves fed diets supplemented with yeast culture as compared with the control group. The average daily gains (ADG) were significantly (P<0.05) higher in buffalo bull calves fed on the yeast culture supplemented diet compared to the control. The dry matter intake/kg gain for bull calves in the control and treatment groups were 9.94 and 9.52 kg, respectively. The cost of feed / kg gain for buffalo bull calves in the control and treatment groups was calculated as Rs 33.10 and 32.01, respectively. Thus, it can be concluded that inclusion of yeast culture (Saccharomyces cerevisiae CNCM I-1077 strain) of 0.25 g/animal/day increased ADG, improved feed efficiency and decreased the cost of feed per unit live weight in graded Murrah buffalo bull calves.

Keywords: yeast culture, DMI, ADG, feed efficiency, bull calves

INTRODUCTION

At present, increasing the productivity of the livestock through enhancement of nutrient utilization has become a great challenge. The addition of yeast culture is considered as a safe alternative approach in manipulating rumen fermentation to improve production performance in livestock. Reports on performance responses of ruminants fed on yeast culture have been variable. Growth rate and efficiency of gain were similar or reduced (Mutsvongwa et al., 1992; Kamra et al., 2002), while others suggested improved weight gain, feed consumption and feed efficiency of gain on yeast supplementation (Reddy and Bhima, 2003; Stella et al., 2007; Kishan Kumar and Ramana., 2008). Further, different yeast strains differ in their ability to produce such responses. Therefore, the present experiment was conducted to study the effect of a selected yeast culture Saccharomyces cerevisiae CNCM I-1077 strain on growth rate and feed efficiency in graded Murrah buffalo bull calves.
MATERIALS AND METHODS

A 180-day feeding trial was carried out at the Buffalo Research Station, Venkataramannagudem of Sri Venkateswara Veterinary University, Tirupati. Twelve graded Murrah buffalo bull calves (127.8±4.1 kg) of an average of 11 to 12 months of age were randomly divided into two equal groups of six animals each (control and treatment) taking into consideration their body weight. All the animals were housed in well-ventilated sheds provided with individual feeding and watering arrangements and were dewormed and vaccinated against HS and FMD before the start of the experiment. During the trial, calves in both the groups were offered a conventional concentrate mixture early in the morning at 8.00 AM to meet the protein requirements for maintenance and growth 500 g/d (ICAR, 1998) and had access to chopped Guinea fodder ad libitum. The calves in treatment group received yeast culture (Saccharomyces cerevisiae CNCM I-1077 strain) of 0.25 g / animal / day. The yeast culture was administered by top dressing over concentrate mixture every day. The animals were weighed before the start of the feeding trial and at intervals of every fortnight early in the morning before offering feed and water. Body weights were recorded for two consecutive days and the mean was taken as the actual body weight.

The data generated were subjected for the test of significance (Snedecor and Cochran, 1976).

RESULTS AND DISCUSSION

The chemical composition of feeds and fodders fed to bull calves during the present study are shown in Table 1. The average fortnightly body weights of the graded buffalo bull calves in both control and treatment groups recorded during the 180 day growth trial period are shown in Table 2. The DMI (kg/d) increased (P>0.05) in bull calves fed diets supplemented with yeast culture as compared with the control group. Similarly,

Table 1. Chemical composition (% DMB) of Guinea fodder and concentrate mixture fed to buffalo bull calves.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Guinea Fodder</th>
<th>Concentrate Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Matter</td>
<td>29.13</td>
<td>91.23</td>
</tr>
<tr>
<td>Organic Matter</td>
<td>89.48</td>
<td>92.34</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>8.96</td>
<td>19.63</td>
</tr>
<tr>
<td>Ether Extract</td>
<td>2.54</td>
<td>5.14</td>
</tr>
<tr>
<td>Crude Fibre</td>
<td>37.00</td>
<td>12.05</td>
</tr>
<tr>
<td>Nitrogen Free Extract</td>
<td>40.98</td>
<td>55.52</td>
</tr>
<tr>
<td>Total ash</td>
<td>10.52</td>
<td>7.66</td>
</tr>
<tr>
<td>Neutral Detergent Fibre</td>
<td>76.60</td>
<td>33.52</td>
</tr>
<tr>
<td>Acid Detergent Fibre</td>
<td>49.09</td>
<td>17.80</td>
</tr>
<tr>
<td>Cellulose</td>
<td>38.84</td>
<td>12.99</td>
</tr>
<tr>
<td>Hemi-cellulose</td>
<td>27.51</td>
<td>15.72</td>
</tr>
<tr>
<td>Calcium</td>
<td>1.29</td>
<td>1.39</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>0.52</td>
<td>0.42</td>
</tr>
</tbody>
</table>
increased DMI was reported by Kamra et al. (2002) in crossbred calves and by Kishan Kumar and Ramana (2008) in Deoni calves. The average daily gain (g/d) for graded buffalo bull calves in the control and treatment groups were 462.13 ± 15.96 and 549.91 ± 31.35, respectively (Table 3). The average daily gains were significantly (P<0.05) higher in buffalo bull calves fed on the probiotic supplemented diet compared to the control. Similar results were reported by Panda et al. (1995), Umesh Kumar et al. (1998), Saha et al. (1999), Reddy and Bhima (2003), Kishan Kumar and Ramana (2008), who observed significantly increased daily gain when animals were fed diets supplemented with yeast culture. The higher growth rate in yeast supplemented rations might be due to increased flow of microbial protein leaving the rumen and an enhanced supply of amino acids entering the small intestine.

Table 2. Average fortnightly body weights of calves fed diets supplemented with and without yeast culture in the diet.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Control Group</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>127.73</td>
<td>127.85</td>
</tr>
<tr>
<td>2.</td>
<td>134.73</td>
<td>136.8</td>
</tr>
<tr>
<td>3.</td>
<td>142.07</td>
<td>145.68</td>
</tr>
<tr>
<td>4.</td>
<td>149.52</td>
<td>153.97</td>
</tr>
<tr>
<td>5.</td>
<td>157.23</td>
<td>162.48</td>
</tr>
<tr>
<td>6.</td>
<td>163.87</td>
<td>170.47</td>
</tr>
<tr>
<td>7.</td>
<td>170.05</td>
<td>178.32</td>
</tr>
<tr>
<td>8.</td>
<td>176.78</td>
<td>186.45</td>
</tr>
<tr>
<td>9.</td>
<td>183.68</td>
<td>194.38</td>
</tr>
<tr>
<td>10.</td>
<td>190.32</td>
<td>202.37</td>
</tr>
<tr>
<td>11.</td>
<td>197.63</td>
<td>210.8</td>
</tr>
<tr>
<td>12.</td>
<td>204.13</td>
<td>218.85</td>
</tr>
<tr>
<td>13.</td>
<td>210.92</td>
<td>226.83</td>
</tr>
</tbody>
</table>

Table 3. Effect of inclusion of yeast culture in the diet on average daily gain (g/d) in buffalo bull calves.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Group</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (kg)</td>
<td>127.7±7.73</td>
<td>127.9±3.77</td>
</tr>
<tr>
<td>Final body weight (kg)</td>
<td>210.92±8.37</td>
<td>226.83±8.42</td>
</tr>
<tr>
<td>Total weight gain (kg)</td>
<td>83.2±2.87</td>
<td>98.98±5.64</td>
</tr>
<tr>
<td>Average daily gain (g/d)*</td>
<td>462.13±15.96</td>
<td>549.91±31.35</td>
</tr>
<tr>
<td>DMI (kg/d)</td>
<td>4.60</td>
<td>5.24</td>
</tr>
<tr>
<td>Feed efficiency (kg feed/kg gain)</td>
<td>9.94</td>
<td>9.52</td>
</tr>
<tr>
<td>Cost of feed/ kg gain</td>
<td>33.10</td>
<td>32.01</td>
</tr>
</tbody>
</table>

(* P<0.05)
intestine (Nagamalleswara Rao et al., 2003).

The dry matter intake / kg gain for graded buffalo bull calves in control and treatment groups were 9.94 and 9.52 kg, respectively (Table 3). The feed efficiency improved with inclusion of yeast culture in the diet and was reflected in the significantly increased ADG in buffalo bull calves fed yeast culture supplemented diets. These results are in agreement with the findings of Saha et al. (1999), Reddy and Bhima (2003), Kishan Kumar and Ramana (2008), who reported improved feed efficiency with yeast culture in the diets. The cost of feed / kg gain for buffalo bull calves in the control and treatment groups was calculated as Rs 33.10 and 32.01, respectively (Table 3). In the present study, the marginal decrease in cost of feed in the yeast culture supplemented group was attributed to improved feed efficiency in bull calves.

Thus, it can be concluded that inclusion of yeast culture (Saccharomyces cerevisiae CNCM I-1077 strain) of 0.25 g / animal / day increased ADG, improved feed efficiency and decreased the cost of feed per unit live weight in graded Murrah buffalo bull calves without any adverse effects.

REFERENCES


A STUDY ON THE PREVALENCE OF SOME PATHOLOGICAL ABNORMALITIES OF THE UTERUS DIAGNOSED AT POST MORTEM OF BUFFALOES IN MOSUL

O.I. Azawi* and A.J. Ali

ABSTRACT

A study was conducted to assess the type and prevalence of abnormalities occurring in the female reproductive tracts of 405 buffalo cows slaughtered at Mosul abattoir. Out of the 405 buffalo genital tracts examined, various abnormalities with different degrees of severity were observed in 216 (53.3%) of cases. Twenty-two (5.4%) were pregnant and the remaining 41.2% (167/405) were macroscopically normal. The most common abnormalities encountered were endometritis 50 (12.3%). Histological examination revealed a chronic endometritis (76%; 38/50), subacute endometritis (18 %; 9/50) and acute endometritis (6%; 3/50), while metritis cases (2.7%; 11/405) were of the chronic type of inflammation. All perimetritis cases (1.5%; 6/405) were characterized by increased thickness of uterine serosa with yellowish colored pus accumulation. Adhesions between vaginal serosa and rectum were observed. The parametritis cases (0.7%; 3/405) observed with severe abscesses and yellowish colored content and hard consistency were found in the ligamentum lata and ligamentum intercurnuale. One case of uterus didyphis was recorded. In conclusion, uterine abnormalities seem to be an important problem with possible subsequent infertility in buffalo cows in Mosul. The high proportions of endometritis and chronic metritis are the major problems in buffalo herds in Mosul leading to slaughter and economic losses.

Keywords: buffaloes, uterus, post mortem, prevalence, pathological abnormalities, Mosul

INTRODUCTION

Metritis is one of the most important disorders in buffaloes (Rao, 1982; Rao and Sreemannarayana, 1983, Azawi et al., 2007; Azawi, 2008), causing high economic losses due to prolonged days open and prolonged intercalving intervals, resulting in involuntary culling (Esslemont and Peeler, 1993). The incidence rate of uterine infection in buffalo cows was much higher than in cows (Jainudeen, 1986). In India, Raman and Bawa, (1977) found high prevalence of postpartum infections (38.54%) in buffalo cows. Metritis was recorded at an incidence rate of 25% (Sar et al., 1996). In Pakistan, Usmani et al. (2001) recorded an incidence of 24% of uterine infection among buffalo cows. In Malaysia, the incidence rate was the same as in India, with high incidence of ovarianbursal adhesions (Jainudeen, 1986). In Egypt, Ghanem et al. (2002) recorded a 22.4% incidence of endometritis in Egyptian buffalo cows. In Iran, Moghami et al. (1996) recorded an incidence of 33.2% of endometritis.
in buffalo cows, and recently, Moghaddam and Mamoei (2004) recorded an incidence of 29.4% of infertility problems including endometritis and metritis in Iranian local breed buffalo cows. In Iraq, Al-Fahad (2000) and Al-Fahad et al. (2004) recorded a 43.3% incidence of chronic endometritis in Basra buffalo cows. While Alwan et al. (2001) recorded a prevalence of 47.9% of endometritis in Baghdad buffaloes. The higher incidence of uterine infections in buffaloes than in cows might be due to poor hygiene, vaginal stimulation for milk let down and possibly, wallowing (Azawi, 2006; Azawi et al., 2008a; Azawi, 2009). Buffalo cows are culled and sent to slaughterhouse either because they are uneconomic to maintain or else because they have some disease problem. Hence, abattoirs are a good source for studying pathological lesions of buffalo reproductive organs that are severe enough to cause infertility and even sterility (Azawi et al., 2008b). The present study was conducted to determine the extent of metritis present in buffalo cows slaughtered in Mosul.

**MATERIALS AND METHODS**

Buffalo cow reproductive tracts of animals slaughtered at Mosul abattoir were collected at random intervals, from January 2006 to August 2009. A total of 405 of mature primiparous and pluriparous genital tracts were examined. The specimens were transported to the College of Veterinary Medicine, University of Mosul. Each specimen was examined grossly in the laboratory in order to exclude any specimen containing reproductive abnormality. All cases were examined for presence of fetuses. Pregnant specimens were discarded. Then the vagina, uterus and uterine tubes were visually inspected for cross lesions. The vagina and uterus were opened up to utero-tubal junction and examined. Ovaries were inspected for cross lesions and the number of corpora albicantia (CA) and side of the ovary with corpus luteum (CL) recorded. A pair of ovaries with either a corpus hemorrhagicum (CH), a large CL and > 5 mm follicle (s) in diameter or a regressing CL with follicle (s) > 6 mm in diameter were classified as active and the animals as cycling. When there was no CL or CH or the presence of a regressed CL without > 5 mm in diameter follicle (s), such ovaries were classified as inactive and the animals as noncycling. A regressing CL coupled with an incomplete involuted uterus was classified as post-parturient anestrous. Corpora albicantia replacing the corpora lutea of pregnancy are large and tend to persist indefinitely (Roberts, 1986). They are more prominent in buffaloes, and can therefore be used to estimate the parity of an animal (Jainudeen et al., 1983). An animal with more than 7-10 CA, with no CL or CH, and without > 5 mm diameter follicle (s) was regarded as being in anestrous due to old age or senility.

**RESULTS**

Reproductive organs from 405 animals were examined; 5.4% (22/405) of the animals were pregnant, and 41.2% (167/405) were cycling. Various abnormalities in the uterus with different degrees of severity were observed in 88 (21.7%) of the cases. The prevalence of the various uterine abnormalities of buffalo cows are presented in Table 1. One case (0.2%) of hydrometra was recorded. The uterus was thin-walled due to accumulation of clear and watery fluid (about 300-350 ml) in the lumen of corpus uteri and both uterine horns with stenosis of the cervical lumen. This lesion
was accompanied by atrophy of caruncular and follicular cyst in the right ovary. Mucometra were found in three (0.7%) cases; accumulations of 200-300 ml of clear mucinous fluid were detected in the lumen in corpus and cornu uteri. Mucometra was accompanied with follicular cyst and cystic corpus luteum in two cases. Two cases of pyometra were recorded. In these cases, accumulations of thick dense whitish-yellowish pus discharge of 300-500 ml were detected in uterine lumen. Inflammatory changes of endometritis were found in 50 (12.3%, 50/405) and classified according to histological examination as chronic endometritis (76%; 38/50), subacute endometritis (18%; 9/50) and acute endometritis (6%; 3/50), while metritis cases (2.7%; 11/405) were of the chronic type of inflammation. All perimetritis cases (1.5%; 6/405) were characterized by increased thickness of uterine serosa with yellowish colored pus accumulation. Adhesions between vaginal serosa and rectum were observed. The parametritis cases (0.7%; 3/405) observed with severe abscess and yellowish colored content and hard consistency were found in the ligamentum lata and ligamentum intercornuale. One case of uterus didylphis was recorded. This defect is characterized by presence of completely separated cervices, each one leading to a separate uterine horn.

DISCUSSION

Macroscopic and microscopic findings of endometritis and metritis were identical to those previously reported (Azawi, 2006) and in agreement with those found by Abalti et al., 2006, who reported 10.8%. On the other hand, higher incidence rates of endometritis of 22.4%, 24.7%, and 25% were obtained by Moghaddam and Mamoei, 2004; Sar et al., 1996; Ghanem et al., 2002) in Iranian, Indian and Egyptian buffaloes, respectively. Endometritis and metritis may

<table>
<thead>
<tr>
<th>Uterine abnormalities</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrometra</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Mucometra</td>
<td>3</td>
<td>0.7</td>
</tr>
<tr>
<td>Pyometra</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>Endometritis</td>
<td>50</td>
<td>12.3</td>
</tr>
<tr>
<td>Metritis</td>
<td>11</td>
<td>2.7</td>
</tr>
<tr>
<td>Perimetritis</td>
<td>6</td>
<td>1.5</td>
</tr>
<tr>
<td>Parametritis</td>
<td>3</td>
<td>0.7</td>
</tr>
<tr>
<td>Uterine edema</td>
<td>5</td>
<td>1.7</td>
</tr>
<tr>
<td>Perimetrial adhesions</td>
<td>3</td>
<td>0.7</td>
</tr>
<tr>
<td>Parametrial adhesions</td>
<td>2</td>
<td>0.5</td>
</tr>
<tr>
<td>Parametrial abscess</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Uterus didylphis</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Total uterine abnormalities</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Total reproductive tracts examined</td>
<td>405</td>
<td>100</td>
</tr>
</tbody>
</table>
result from inadequate hygienic conditions in the postpartum period or during parturition, retained placenta and traumatic lacerations due to dystocia. While studies concerning incidence of uterine diseases in southern Iraqi buffaloes (El-Dossokey and Juma, 1973; Alwan et al., 2001; Al-Fahad et al., 2004), showed much higher incidences than the present study. However, this disagreement can be accounted for largely by the differences in the definition of uterine infections between the present and previous studies. In addition, differences in breed, nutrition and management might play a role in the differences between the studies. Results of histopathological studies revealed a high incidence of chronic metritis. These observations were in agreement with Jajo-Azar (2000) and Al-Sharum (2000). Uterus didylphis, or true double cervix, recorded in this study is a congenital anatomical defect of the female genital tract of monotocous species, including cattle and humans (McEntee, 1990). This defect is characterized by presence of completely separated cervices, each one leading to a separate uterine horn. The condition has been attributed to failure of fusion of the caudal portions of the paramesonephric ducts during embryonic development, resulting in a double cervix / or a divided uterine fundus (Roberts, 1986). Noakes et al. (2002) claimed that these cases could conceive normally but may show dystocia due to a fetal limb entering each cervical canal. Case reports of uterus didylphis in cows have been sporadic (Fathalla, 2000). It is believed that this is the first report of uterus didylphis in buffaloes.

In conclusion, uterine abnormalities seem to be an important problem with possible subsequent infertility in buffalo cows in Mosul. The high proportions of endometritis and chronic metritis are the major problems in buffalo herds in Mosul leading to slaughter and economic losses.

REFERENCES


ABSTRACT

To see the effect of different physiological stages and managemental practices on the amount of milk somatic cells secreted from the udders of Murrah buffaloes, milk was collected from 64 Murrah buffaloes, which were divided into various groups according to their stage of lactation, parity, colostrums collected, body weight, body condition score (BCS), season, milking practices and fractionated. There were non-significant changes in milk somatic cell counts (SCC) in early, mid and late lactation. Milk SCC increased non-significantly from the 1st to 4th parity. Milk SCC were significantly higher (P<0.01) in day-1 colostrum samples and then decreased when colostrums transformed into milk. No relationship was found between milk SCC and body weight and body condition score. Milk SCC was significantly higher (P<0.01) in the summer season vis-a-vis winter season. SCC was higher in machine milking than hand milking. Our results indicate that milk SCC is greater in buffaloes of higher parity and during the months of summer. Therefore, proper care of these animals should be undertaken to maintain their milk quality.

Keywords: buffaloes, milk, SCC, physiological stages, managemental practices

INTRODUCTION

The buffalo population in India accounts for 57 percent of the world buffalo population. Improvement in milk production has been due to proper breeding, feeding and management of dairy animals. However, in spite of large volume of milk produced, the quality aspects of milk production have not received adequate attention, and this has been the major obstacle in realizing the large export potential of milk and milk products. Also, the vital aspect of clean milk production and herd health including udder health still remains a major concern. All the developed countries are using milk somatic cell counts (SCC) as a marker to determine the mammary health and quality of milk (Dang et al., 2007). Seeing the importance of milk SCC internationally, the present study was undertaken to estimate milk SCC in Murrah buffaloes in different physiological stages and reared under different managemental practices.

MATERIALS AND METHODS

Milk was collected from 64 Murrah buffaloes which were divided into various groups according to their stage of lactation (early, mid and late), parity (1, 2, 3 and 4), colostrums collected
from days 1 to 5, body weight, body condition score, season (summer, autumn and winter), and milking practices (hand and machine), milk collected from different fractions of the udder (stripping, fore milk and normal) as indicated in Table 1. The Number of animals in each group has also been represented in Table 1. Individual milk samples pooled for all four quarters from the entire animal were collected separately. About 100 ml of milk was collected aseptically in clean milk bottles. The samples were brought to the laboratory immediately after collection and placed in a refrigerator till use. For SCC, slides were prepared within one hour of collection of milk samples. Milk SCC was estimated microscopically (Dang et al., 2008). The SCC were measured under the microscope with a magnification of 100 X 10 in 200 fields, and the average number of cells per field was multiplied by the microscopic factor (8.81134633). The microscopic factor was determined by using ocular and stage micrometer.

Somatic cell counts/ml of milk (100,000) = Average cells count in one field x 8.81134633. The data obtained were subjected to statistical analysis using least square analysis of variance.

**RESULTS AND DISCUSSION**

Mean ± SE values of SCC (10^5 cells /ml) in milk during different physiological stages and under different managemental practices have been presented in Table 1. The values of milk SCC were within a range (Dang et al., 2007) as reported in Murrah buffaloes. There were non-significant changes in milk SCC in early, mid and late lactation. Little change in milk SCC has also been reported with stage of lactation in cows (Eberhart et al., 1982). On comparing the effect of parity on milk SCC, it was observed by milk SCC increased

Table 1. The effects of different physiological stages and management practices on milk SCC.

<table>
<thead>
<tr>
<th>Physiological effects</th>
<th>Managemental effects</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stages of lactation</strong></td>
<td>Body weight</td>
</tr>
<tr>
<td>Early (n = 17)</td>
<td>&lt;500 kg (n = 13) 0.99 ± 0.70</td>
</tr>
<tr>
<td>Mid (n=17)</td>
<td>500kg-600 kg (n = 24) 0.93 ± 0.70</td>
</tr>
<tr>
<td>Late (n = 17)</td>
<td>&gt;600 kg (n = 14) 1.03 ± 0.06</td>
</tr>
<tr>
<td><strong>Parity</strong></td>
<td>Body condition score</td>
</tr>
<tr>
<td>1 (n =14)</td>
<td>&lt;3.5 (n = 14) 0.98 ± 0.07</td>
</tr>
<tr>
<td>2 (n=16)</td>
<td>3.5-4.5 (n = 17) 0.94 ± 0.08</td>
</tr>
<tr>
<td>3 (n=10)</td>
<td>&gt;4.5 (n = 20) 1.03 ± 0.14</td>
</tr>
<tr>
<td>4 (n =11)</td>
<td>Summer (n = 51) 1.12 ± 0.17</td>
</tr>
<tr>
<td><strong>Days of colostrum</strong></td>
<td>Autumn (n = 51) 1.14 ± 0.05</td>
</tr>
<tr>
<td>1 (n = 13)</td>
<td>Winter (n = 51) 1.16 ± 0.05</td>
</tr>
<tr>
<td>2 (n = 13)</td>
<td>Machine (n = 22) 0.94 ± 0.05</td>
</tr>
<tr>
<td>3 (n = 13)</td>
<td>Hand (n = 29) 1.12 ± 0.11</td>
</tr>
<tr>
<td>4 (n = 13)</td>
<td>Fore milk (n = 12) 1.93 ± 0.07</td>
</tr>
<tr>
<td>5 (n = 13)</td>
<td>Normal milk (n = 12) 1.92 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>Stripping (n = 12) 1.82 ± 0.19</td>
</tr>
</tbody>
</table>

Figures with different superscripts within a column differ significantly from each other (P<0.01)
non-significantly from 1st to 4th parity. The reason may be that SCC is positively correlated with milk production and milk production increases with an increase in parity. Milk SCC were significantly higher (P<0.01) in day-1 colostrum samples and then decreased to 193,000 in day-5 samples when colostrums transformed into milk. The engorgement of the udder tissue during advanced pregnancy often results in very high level stress on the udder tissue which may be responsible for elevated SCC and neutrophil counts in day 1 colostrum. This returns gradually to normal values as the stress on the udder tissue is reduced by frequent emptying of the udder (Dang et al., 2007).

On seeing the effect of different managemental practices on milk SCC, there was no definite relationship between milk SCC and body weight in Murrah buffaloes, whereas, body weight was found to be positively correlated with the level of SCC in cows (Berry et al., 2007). Also there was no significant difference in different BCS classes with respect to milk SCC. Milk SCC was significantly higher (P<0.01) in the summer and autumn seasons as compared to the winter seasons as reported in milk of exotic cattle (Kelly et al., 2000).

In the present study, milk SCC was higher in machine milking than hand milking. One of the reasons behind relatively higher SCC (non significantly) in machine milking of Murrah buffaloes may be that they have not been selected for the udder traits and therefore have teats of variable shapes and sizes of teats. These are often not perfectly compatible with the milking machine which has one particular dimension and which are mostly suited to cows having uniform udder and teat characteristics (Dang et al., 2007).

There was a decrease in milk SCC in normal milk from that of fore milk, which again increased in stripping. A higher concentration of SCC in the stripping may be due to the sloughing off of more cells into milk, and an increase in fore milk SCC may be due to the presence of pathogens towards the teat end, which may promote movement of somatic cells towards it.

Our results indicate that as in cows, milk SCC also varies in the milk of Murrah buffaloes under different physiological stages and managemental practices. Buffaloes possess a powerful defence mechanism against mastitis due to their tight teat sphincter (Hogberg et al., 2007) and long narrow teat canal, which can be expected to effectively prevent micro-organisms from invading the udder (Uppal et al., 1994). However, with change in management systems (increased feeding and introduction of machine milkings), there is an increase in milk SCC which may increase the chance of mammary infection. Therefore, buffaloes of high parity, producing more milk and those exposed to summer stress require proper care and management to maintain their milk production.

REFERENCES


*Continued on page 99
ABSTRACT

In the present study, an attempt was made to compare the relative efficacy of different tests used for diagnosis of brucellosis in buffaloes at the field level. A total of 51 specimens, each of milk and serum from clinically suspected subjects in the field were studied. The true prevalence of brucellosis was 13.72% with apparent prevalence of 33.34% and 17.64% by the milk ring test (MRT) and the Rose Bengal plate agglutination test (RBPT), respectively. The overall agreement between RBPT and serum tube agglutination test (STAT) was higher (96.07%) as compared to MRT and STAT (80.89%). Both MRT and RBPT showed 100% sensitivity. Specificity of RBPT was higher (95.45%) than MRT (77.27%), when compared with STAT. The positive predictive value of MRT and RBPT was 0.412 and 0.778 respectively, with both the tests having negative predictive value of 1 when compared with STAT. The kappa value also revealed that RBPT and STAT had almost perfect agreement (kappa value = 0.852), whereas MRT and STAT (kappa value = 0.493) had substantial agreement.

Keywords: brucellosis, buffalo, diagnosis

INTRODUCTION

Brucellosis is considered as one of the most widespread zoonoses in the world by the Food and Agriculture Organisation (FAO), the World Health Organisation (WHO) and the Office International des Epizooties (OIE) (Schelling et al., 2003). The disease in buffalo is caused by Brucella abortus and is characterised by late term abortion, infertility and reduced milk production as a result of retained placenta and secondary endometritis, and excretion of the organisms in uterine discharges and milk. The diagnosis of brucellosis is usually carried out by serological testing, and it is assumed that the serological tests used for the diagnosis of brucellosis in cattle are also adequate for the diagnosis of brucellosis in buffalo (Alton, 1990). The absence of a perfect reference diagnostic test (gold standard) makes evaluation of serological tests difficult. On the other hand, isolation of the pathogen is considered as confirmatory test in bacterial infection, but in the case of brucellosis, bacterial cultures are often negative for infected animals because of the intra-cellular and fastidious nature of the pathogen, (Romero et al., 1995), and the test is relatively difficult to use in the field in rural areas. Hence, the use of bacterial culture as
gold standard will result in incorrect specificity estimation, thereby misdiagnosing infected animal as non-infectious. So the purpose of the paper was to determine relative efficacy (sensitivity, specificity and overall agreement) of different tests which are routinely used in diagnosis of brucellosis in the field in rural areas.

**MATERIALS AND METHODS**

Buffaloes having histories of frequent abortion, retention of placenta and repeat breeding were selected randomly from organized and unorganized dairy farms situated in and around the Jammu region of Jammu and Kashmir States (India). A total of 102 samples, fifty one each, of blood and milk were collected. The serum was separated from the whole blood sample by standard method and stored at -20°C until analysis. The samples were subjected to the milk ring test (MRT), the rose bengal plate agglutination test (RBPT) and the standard tube agglutination test (STAT).

MRT was done as per the standard protocol supplied with the Abortus bang ring test antigen, which was procured from the Indian Veterinary Research Institute (IVRI), Izatnagar (India).

RBPT was performed as per Morgan *et al.* (1969) using RBPT antigen procured from IVRI, Izatnagar. STAT was done according to the procedure described by Alton *et al.* (1975), employing two fold dilutions of the serum and the plain brucella agglutination antigen (IVRI). A titre of 80 I.U per ml and above was considered as positive, 40 I.U as doubtful and below 40 I.U as negative for brucellosis in case of STAT.

**Statistical analysis**

To test the significance, extent of agreement and the strength of association between the tests, Mc Nemars Chi-square value for STAT and MRT and between STAT and RBPT were calculated at 95 % confidence interval and the P-values were examined at critical probability of P<0.05 by using an online calculator (http://www.Graphpad.com / quickcals/contingency 2.cfm). In addition, the agreement between the tests was analyzed by using Kappa statistics (κ) as per the method suggested by Thrusfield, (1995). The sensitivity, specificity and overall agreement between the tests were analyzed by the statistical formula given by Samad *et al.* (1994).

**RESULTS AND DISCUSSION**

In the present study, brucellosis had a true prevalence of 13.72% with an apparent prevalence of 33.34% by MRT and 17.64% by RBPT. Our findings are in agreement with Sharma *et al.* (1995) and Aulakh *et al.* (2008), who reported 14.61% and 16.41% prevalence of brucellosis in buffaloes in Punjab. The results are in contrast to lower prevalences (1.8% and 4.6%) reported by Isloor *et al.* (1998) and Agrawal *et al.* (2007) for different states of India; 5.05% reposted by Ahmed *et al.*, 1995 for Pakistan and 4.2% reposted by Silva *et al.* (2002), for Sri Lanka. The higher prevalence of brucellosis obtained in the present study may be due to the fact that buffaloes having history of frequent abortion, repeat breeding and retention of placenta were used for study.

McNemars Chi-square value revealed higher significant value of Chi-square for MRT
and STAT, (4.413, P< 0.05) as compared to RBPT and STAT (0.074, P<0.05). Both MRT and RBPT showed 100% sensitivity. Specificity of RBPT was higher (95.45%) than MRT (77.27%), when compared with STAT (Table 2). Our results are in alignment with Singh et al. (2002), who suggested that the specificity of STAT is more than that of RBPT. Similarly, Thakur et al. (2004) reported that RBPT was more sensitive but less specific than STAT in different species of animals including humans. The differences in sensitivity and specificity determined by comparison of different tests vary because of variation in the number of false positives and false negatives detected by the various tests.

The overall agreement between RBPT and STAT (96.07%) was higher as compared to MRT and STAT (80.89%). Kappa statistics also revealed similar agreement as Kappa values suggested that RBPT and STAT had almost perfect agreement while MRT and STAT had substantial agreement (Table 2). Our results are in agreement with Thakur et al. (2004), who found 98% and 95% agreement between RBPT and STAT in cattle and sheep respectively. However, Sharma (2000) found 100% agreement between STAT and RBPT in humans.

In a nutshell, the findings suggest that brucellosis is widely prevalent in buffaloes of

Table 1. Prevalence of brucellosis in buffalo with abortion, retention of placenta (ROP) and repeat breeding condition.

<table>
<thead>
<tr>
<th>History</th>
<th>Total cases</th>
<th>Positive percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MRT</td>
<td>RBPT</td>
</tr>
<tr>
<td>Abortion</td>
<td>22</td>
<td>8 (36.37%)</td>
</tr>
<tr>
<td>ROP</td>
<td>17</td>
<td>6 (35.29%)</td>
</tr>
<tr>
<td>Repeat breeding</td>
<td>12</td>
<td>3 (25%)</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>17 (33.33%)</td>
</tr>
</tbody>
</table>

Table 2. Correlation between different diagnostic tests of brucellosis in buffalo.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>MRT vs. STAT</th>
<th>RBPT vs. STAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive predictive value</td>
<td>0.412</td>
<td>0.778</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Apparent Prevalence</td>
<td>33.34%</td>
<td>17.64%</td>
</tr>
<tr>
<td>True prevalence</td>
<td>13.72%</td>
<td>13.72%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Specificity</td>
<td>77.27%</td>
<td>95.45%</td>
</tr>
<tr>
<td>Overall agreement</td>
<td>80.89%</td>
<td>96.07%</td>
</tr>
<tr>
<td>Kappa value</td>
<td>0.493</td>
<td>0.852</td>
</tr>
</tbody>
</table>

Kappa value > 0.81 Almost perfect agreement, 0.61 - 0.80 Substantial agreement, 0.41 - 0.60 Moderate agreement, 0.21 - 0.40 Fair agreement, 0.01 - 0.20 Slight agreement, 0.00 Poor agreement.
the Jammu region and that a combination of tests should be used for its diagnosis at the field level, as a single test may lead to misdiagnosis.

ACKNOWLEDGEMENT

The authors are thankful to the Dean, Faculty of Veterinary Science and Animal Husbandry, SKUAST-Jammu, for providing necessary facilities and to the farmers of the study area for their co-operation.

REFERENCES


A new body condition score (BCS) system was developed for Murrah buffaloes. The skeletal check points were identified based on the anatomical features and carcass fat reserves. A new BCS chart with a 1-5 scale having 0.5 increments examining eight skeletal check points was developed. The ultrasonographic assessment of the precision of BCS system in 10 buffaloes for each point of the 1-5 scale indicated that BCS adequately reflected the actual fat reserves. The influence of body condition score at calving (BCS) on the reproductive and productive performance studied in 24 (4 x 6 completely randomised design) and 40 (4 x 10 completely randomized design) buffaloes, respectively, revealed that buffaloes of BCSc group 3.5-3.99 showed the best performance among the four BCSc groups with earlier (P < 0.05) resumption of ovarian activity (29.33 days), a shorter (P < 0.01) postpartum an estrus period (46.66 days), a shorter (P < 0.05) service period (58.83 days), fewer services per conception (1.50), a higher rate of first service conception (66.66%) with higher (P < 0.01) breeding efficiency (90.64 percent). The milk production traits like total milk yield up to 18 weeks of lactation (1658.67 kg), 305 day predicted lactation yield (3187.3 kg), peak milk yield (16.5 kg), milk protein and solids not fat were also higher in BCSc of 3.5-3.99 followed by the BCSc groups of 4.0-4.49, 3.0-3.49 and 2.5-2.99.

Keywords: body condition score, ultrasonography, reproduction, production, buffaloes

INTRODUCTION

The body condition score (BCS) system is a subjective scoring method of evaluating the energy reserves of dairy animals which provides a better understanding of biological relationship between body fat, milk production and reproduction that helps in adopting the optimum managemental practices to derive maximum production and maintain better health status. It is based on evaluation of the outer appearance of the animal that interacts with its body fat reserves and therefore is directly influenced by energy balance. It gives an immediate appraisal of the body state of the animal and is readily incorporated in operational decision making (Gransworthy, 1988). BCS systems have been developed earlier by many scientists like Jefferies (1961) using 0 to 5 scale in ewes, Lowman et al. (1976) using a 0 to 5 scale in beef cattle and Earle (1976) using an eight grade system in dairy cows. Edmonson et al. (1989) developed a chart for body condition scoring of Holstein dairy cows on

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2Dairy Technology Programme, College of Veterinary Science, Tirupati - 517 502, A.P., India.
a 1 to 5 scale using 0.25 increments. Sarjan Rao et al. (2002) and Anitha et al. (2005) have utilized this chart for scoring the crossbred dairy cows in India. India has the highest buffalo population of the world and is showing an increasing trend in the population growth (FAO, 2004). It is the native tract for the best buffalo breeds of the world. In order to derive the maximum potential from native buffaloes and for their better management, there is a need to develop a body condition scoring system to evaluate their fitness. There was no scale developed specifically for buffaloes, and such studies were meager in buffaloes. Hence, the present study was taken up to develop a score system for buffaloes taking into consideration the anatomical features and amount of fat reserves at various skeletal checkpoints and to validate the precision of scores with ultrasonic measurement of subcutaneous fat so that the scale developed can be used to assess and improve the reproduction and production status of buffaloes.

MATERIALS AND METHODS

Development of the new body condition score system

The skeletal check points were identified taking into consideration the anatomical features and amount of fat reserves in 50 slaughtered buffaloes. The amount of fat reserves were measured at six skeletal check points which include points between 12th and 13th ribs, spinous and transverse processes of lumbar vertebrae, sacral crest and tuber sacrale, sacral crest and hooks, hooks and pins, tail head and pins. Based on the amount of fat reserves the scores were prioritized on a 1 to 5 scale of the new BCS proposed. The new chart for condition scoring on a 1 to 5 scale using 0.5 increments was prepared. Diagrams were added to the text to convey the gradation of body changes and reduce the dependence on written descriptions. A score of 1 indicates emaciated, 2 indicates thin, 3 indicates average, 4 indicates fat and 5 indicates obese condition (Chart 1). Eight skeletal checkpoints were examined and merits within each area were used to indicate the body condition. The eight locations observed were:

1. Tail head to pin bones.
2. Spinous processes of the lumbar vertebrae.
3. Depression between the spinous and transverse processes.
4. Transverse processes of lumbar vertebrae.
5. Point between 12th and 13th ribs.
7. Depression between sacral crest and hooks.
8. Depression between hooks and pins.

A Murrah buffalo showing the skeletal checkpoints for BCS as presented in Figure 1. After each checkpoint was observed by vision and palpation the scores were recorded and an average BCS was assigned to a herd of 200 buffaloes.

The same buffaloes were rescored again using the same procedure without referring to the previously assigned scores to determine the accuracy. The scores assigned for the same herd of buffaloes by the faculty members of the department who had expertise in body condition scoring were also compared. The scores assigned by faculty members coincided with the scores of the researchers and so the method of score assigning by the researchers using the new score system was standardized.
Ultrasonographic assessment of the precision of new BCS system

The BCS system developed was subjected to testing for its precision in 10 buffaloes for each point (1, 2, 3, 4, 5) of the scale by ultrasonographic measurements of body fat reserves.

The BCS and ultrasonographic measurements were obtained independently for the same buffaloes on the same day. An LOGIQ α 100 ultrasound machine (GE Medical Systems) with a 5.5 MHz convex transducer was used to determine the amount of subcutaneous fat at five body locations through a coupling gel on each buffalo (Bruckmaier and Blum, 1992). Body locations were selected based on the skeletal checkpoints used for body condition scoring and ease of obtaining and reading ultrasonographic measurements.

The first location was the area between the tail head and pin bones; the second location was the lumbar area. The transducer was oriented parallel to the midline, midway between the spinous and transverse processes. The third location was the area between 12th and 13th ribs. The fourth was the area between the sacral crest and tuber coxae. The fifth area was located midway between hooks and pins above the greater trochanter of the femur.

Measurements were obtained by freezing the image on the screen of the ultrasound machine and then measuring the layer of subcutaneous fat in the centre of the screen (Domecq et al., 1995).

BCS in relation to reproductive performance

Twenty-four Murrah and graded Murrah buffaloes from the Buffalo Research Station, Venkataramannagudem, Sri Venkateswara Veterinary University, Tirupati, were selected to study the influence of BCS at calving (BCSc) on the reproductive performance. The buffaloes selected were in first to third lactation. The buffaloes selected were divided into four groups, six buffaloes each in group, in a 4 x 6 completely randomized design to study the relationship between BCSc and reproductive traits, which indicate the performance.

Postpartum resumption of ovarian activity

To study the postpartum resumption of ovarian activity, the serum progesterone (P₄) concentration was estimated as per the procedures of Hubl et al. (1982).

Blood Collection

Blood samples were collected from day 5 postpartum once every 5 days until 60 days postpartum. Approximately 6 ml of blood was collected into sterile test tubes by jugular vein puncture and allowed to clot by placing the test tubes in a slanting position. After one hour, the serum was separated and centrifuged at 3000 rpm for 5 minutes to get clear serum. The serum was stored at -20°C until utilized for the estimation of P₄ concentration.

Progesterone assay

The serum P₄ concentration was estimated by using Enzyme Linked Immuno Sorbent Assay (ELISA) technique with the help of P₄ kits (Biotron Diagnostics Inc. Hemet California, USA) and was expressed as ng/ml. The increase in serum progesterone concentration beyond one ng/ml for at least 5 days was considered as an indication of ovulation and corpus luteum formation. The day of first ovulation was presumed to be four days before the first rise in progesterone concentration of above one ng/ml. The day of first ovulation was presumed to be the day of postpartum resumption of ovarian activity.
**Post-partum estrus, service period, number of services per conception, first service conception rate**

Postpartum estrus was observed by the acceptance of a male by the female, which is the most prominent and reliable symptom of estrus in buffalo (Gordon, 1996). A vasectomized male was used for estrus detection on the farm.

The service period was calculated from the date of calving to date of successful service (Thomas and Sastry, 1991).

The data regarding number of services per conception were obtained from the records of the farm.

The first service conception rate was calculated by the percentage of experimental buffaloes conceiving out of the total buffaloes at first insemination (Rajagopal, 2008). The total numbers of services for successful conception also were recorded.

**Breeding Efficiency (BE)**

The breeding efficiency (BE) of experimental buffalo herd in relation to BCSc was calculated by using the formula:

\[
BE \text{ of buffalo} = \frac{n(365) + 1040}{AC + C_i} \times 100
\]

Where  
- \(n\) is the number of calving intervals  
- \(AC\) is the age at first calving  
- \(C_i\) is the calving interval in days  

(Jagdish Prasad and Neeraj, 2007).

The data regarding the calving intervals and age at first calving were obtained from the records of the farm.

**BCS in relation to productive performance**

The influence of body condition on milk production traits was studied in a herd of 40 buffaloes from calving up to 18 weeks postpartum in a 4 x 10 CRD (four groups divided based on BCSc).

**Milk yield (kg)**

The production data including the daily milk yield (kg) up to 18 weeks of lactation was measured every day both morning and evening after separating the milk for pail feeding the calves.

The peak milk yield (kg) pertaining to the test herd was obtained from the computed data of the farm.

The 305-day predicted lactation yields were calculated by using the ratio estimates of partial lactations of Murrah buffaloes (Thomas and Sastry, 1991). The lactation yield up to 18 weeks was multiplied by the corresponding ratio estimate of 1.9216 to obtain estimate of lactation yield.

**Milk components**

The milk components, including fat, protein and solids not fat (SNF), were studied in relation to BCS. Representative milk samples from individual buffaloes in the test herd were collected twice in sterile sample bottles during the study period (6-8 weeks after calving and again at 16-18 weeks after calving). The milk samples were analysed for fat, protein and SNF. The fat percent of the milk samples was determined in duplicate (IS: 1224, Part-I, 1977). The milk protein was estimated in duplicate as detailed in procedure (IS: 1479, Part II, 1961). The milk SNF was determined in duplicate (IS: 1224-1958).

**Statistical Analysis**

Analysis of variance was used to study the variation in carcass fat thickness at various skeletal check points, the variation in ultrasonographic fat
reserves within BCS and among different BCS groups, the relationship of BCSc with the parity and postpartum estrus, parity and service period, parity and number of services per conception, first service conception rate, resumption of ovarian activity, breeding efficiency, total milk yield up to 18 weeks after calving, predicted lactation, yield, peak milk yield, persistency index, milk fat, protein and SNF and for comparison of scores assigned with carcass fat reserves. Correlation coefficients were used to study the relationship among BCS, carcass, fat and ultrasonographic fat reserves (Snedecor and Cochran, 1994).

**RESULTS**

**Development of the new BCS system**

The anatomical features studied in slaughtered buffaloes showed that the narrow and pointed ends of the spinous and transverse processes of lumbar vertebrae make it possible to assess the fat reserves easily by vision and palpation on live animal. The convexity of the dorsal sacral crest and the sharp bony prominences of the hooks and pin bones help in the examination of fat cover in the pelvic area. The carcass fat reserves measured at six skeletal check points showed that the fat thickness (mm) at tail head to pin bones (6.28 ± 0.37) was significantly (P < 0.01) higher than the fat at other skeletal check points, followed by the fat thickness at the lumbar area (4.43 ± 0.28), between the 12th and 13th ribs (4.19 ± 0.27), sacral crest to tuber sacrale and sacral crest to hooks (3.56 ± 0.23), hooks to pins (3.22 ± 0.19). Based on the amount of fat reserves the scores were assigned on a 1 to 5 scale. The mean values of carcass fat thickness for the scores 1 to 5 at individual skeletal check points and the mean of all the six check points are presented in Table 1. Significant (P < 0.01) differences were observed in the carcass fat thickness among the five scores at all the individual check points as well as the mean of the six check points indicating that the scale was internally consistent.

The BCS chart with a 1 to 5 scale using 0.5 increments, examining eight skeletal check points was developed (Chart 1), and BCSs were assigned to a herd of 200 buffaloes using the chart.

**Ultrasonic assessment of the precision of the BCS system**

The ultrasonographic fat measurements at five BCS skeletal check points showed that the fat thickness was highest at the tail (P < 0.01), followed by the lumbar area, ribs, sacral crest to hooks and hook to pins. Figure 2, 3, 4, 5 and 6 show the ultrasonographic fat measurements in Murrah buffaloes of BCSSs 1, 2, 3, 4 and 5, respectively. The ultrasonographic measurements of mean body fat thickness for buffaloes of different body condition scores are presented in Table 1. Significant (P < 0.01) differences were observed in the fat thickness for buffaloes of various BCS groups. As the BCS increased, the amount of fat reserves also increased, indicating that BCSs were adequately reflected in the amount of actual fat reserves. BCS was significantly (P < 0.01) correlated with the carcass fat reserves (0.86) as well as ultrasonographic fat reserves (0.85).

**BCS in relation to reproductive performance**

Parity and the interaction of parity with BCS did not shown any significant influence on the reproductive performance whereas BCS had a significant effect on the reproductive performance in the test herd. The reproductive performances of buffaloes of various BCSc groups in the test herd
are presented in Table 2. The buffaloes of BCS group 3.5-3.99 had earlier (P < 0.05) resumption of ovarian activity, a shorter postpartum anestrus period (P < 0.01), a shorter service period (P < 0.05), fewer services per conception, higher first service conception rate, and higher breeding efficiency (P < 0.01) of 29.33 days, 46.66 days, 58.83 days, 1.5, 66.66 percent, and 90.64 percent, respectively, followed by buffaloes of BCS group 4.0-4.49 with 39.33 days, 55.16 days, 77.16 days, 1.83, 50 percent, and 87.48 percent, respectively, followed by buffaloes of BCS group 3.0-3.49 with 42 days, 65.66 days, 85.66 days 2.0, 33.33 percent and 80.58, respectively, followed by buffaloes of BCS group 2.5-2.99 with 47.25 days, 77.16 days, 125.16 days, 2.66, 16.66 percent and 70.49 percent, respectively. It was observed that the reproductive performance improved as the BCS increased up to 3.99, but beyond that a decline was noticed.

**BCS in relation to productive performance**

The relationship between BCS and milk yield in the test herd is presented in Table 3. Buffaloes of BCS group 3.5-3.99 had higher (P < 0.01) milk yields up to 18 weeks of lactation (kg), 305 day predicted lactation yield (kg), and peak milk yield (kg) of 1658.67, 3187.31 and 16.5, respectively, followed by buffaloes of BCS group 4.0-4.49 with 1359.92, 2613.23 and 13.75 respectively, followed by buffaloes of BCS group 3.0-3.49 with 1197.12, 2300.39 and 11.60 respectively, followed by buffaloes of BCS group 2.5-2.99 with 1030.93, 1981.05 and 9.50, respectively. As the BCS increased beyond 3.99, the milk yield showed a decline. Table 4 shows the relationship between BCS and milk components. Buffaloes of BCS group 4.0-4.49 had higher (P < 0.01) milk fat percent followed by BCS groups of 3.5-3.99, 3.0-3.49 and 2.5-2.99 at 6-8 weeks after calving as well as the 16-18 weeks after calving whereas buffaloes of BCS group 3.5-3.99 had higher (P < 0.01) milk protein and SNF percent followed by BCS groups of 4.0-4.49, 3.0-3.49 and 2.5-2.99 at 6-8 weeks and at 16-18 weeks after calving.

**DISCUSSION**

The concept of body condition scoring of dairy animals has gained widespread acceptance as a managemental aid in dairy production. In the present research work, a new BCS system was developed for Murrah buffaloes. The skeletal check points were identified by selecting the anatomical features which made it possible to assess the fat reserves easily and by measuring the amount of fat reserves in slaughtered animals. The scores were assigned on a 1 to 5 scale based on the amount of carcass fat reserves. The mean±SE (mm) values of carcass fat thickness for the scores 1 to 5 ranged from 1.67 ± 0.07 to 7.82±0.21 at the point between 12th and 13th ribs whereas the values recorded by Apple et al. (1999) by assigning scores to beef cows on a 9 point scale ranged from 0.5±1.5 to 27.3±1.5 at the 12th / 13th rib interface. The difference in these fat thickness measurements with those of the present study might be attributed to the species difference.

The new BCS chart with a 1 to 5 scale having 0.5 increments examining eight skeletal check points was developed to score Murrah buffaloes. For beef cattle, a 9-point scale is commonly used (Wagner et al., 1988). Concerning dairy cows, 8 and 10 point scales are used in Australia and New Zealand (Roche et al., 2004). The prevailing scoring systems in the United States and Ireland use a 5-point scale. The BCS is determined by vision and palpation of the skeletal check points in
the present study which was in tune with Wildman et al. (1982) and Ferguson et al. (1994) whereas Edmoson et al. (1989) evaluated the body locations only visually. BCS was assigned using the chart developed and the new BCS system was found to be precise and consistent. Thus, the present study suggested that anatomical studies, amount of fat reserves and the assessment of scores helped in the development of a valid BCS system.

An ultrasonographic machine with a 5.5 MHz convex transducer was used to determine the amount of subcutaneous fat whereas Domecq et al. (1995) used a 5 MHz linear array transducer. The ultrasonographic fat thickness measurements were significantly (P<0.01) higher at the check point between tail head to pin bones, and this was in accordance to the findings of Gentry et al. (2004) who observed that tail head area accounted for the majority of the variation in BCS in mares. As the BCS increased, the amount of fat reserves increased significantly (P<0.01) indicating that BCS adequately reflected the amount of actual fat reserves. Significant (P<0.01) correlation was observed between BCS and ultrasonographic fat reserves (0.85) and this was in accordance to the findings of Lubis and Fletcher (1985), who reported significant correlation (0.87) between subjectively determined BCS and ultrasonically determined back fat thickness in swamp buffalo cows.

The results of the present study highlighted the importance of body condition at calving in achieving good reproductive performance. Studies on the interaction of BCS with parity showed no significant effect on reproductive performance. However, Roche et al. (2007) reported that interaction of calving BCS with parity was consistent and suggested that cows in first and second parity may have good reproductive performance from greater BCS. Parity had no significant effect on reproduction in the present study whereas Buckley et al. (2003) reported that parity was associated with likelihood of pregnancy at first service.

The resumption of ovarian activity was observed at mean values of 47.25±2.39, 42.0±2.91, 29.33±3.33 and 39.33±4.21 days for the BCS groups of 2.5-2.99, 3.0-3.49, 3.5-3.99 and 4.0-4.49, respectively, whereas Nosier and Hussein (1988) report that postpartum ovarian activity resumed in the fourth week after calving in Egyptian buffaloes was earlier than the values of the present study. However, there was a great diversity in the postpartum interval to first ovulation in dairy cows and was reported as 17 days (Schams et al., 1978), 18 days (Stevenson and Britt, 1979), 19 days (Ducker and Morant, 1984), 21 days (Carruthers and Hafs, 1980; Fonseca et al., 1983) and 36 days (Butler et al., 1981). The findings on the influence of BCS on the resumption of ovarian activity were in agreement with the report of Langley and Sherington (1983) that cows of higher BCS had a shorter interval from calving to first ovulation.

The results showed that body condition at calving was the critical factor related to reestablishment of ovarian function. Buffaloes of BCS range 3.5-3.99 showed early resumption of ovarian activity, which is an indicator of good reproduction performance, whereas buffaloes of BCS range 2.5-2.99 took a longer period for resumption of ovarian activity, showing poor reproductive performance, which was in tune with the findings of Beam and Butler (1997) and Reksen et al. (2002), who reported the delayed resumption of luteal function in thinner cows. Similarly, Markusfeld et al. (1997) reported that thinner cows at calving were more likely to have inactive ovaries. Ramirez-Iglesia et al. (1992) also reported that body condition, reflecting the nutritional status of the cows at calving, favoured the onset of sexual
activity.

For every one unit increase in BCS, a decrease of 20.5 days in postpartum estrus period, 37.41 days in service period, 0.66 in the number of services per conception and an increase of 33.33 percent in the conception rate at first service was observed. The reproductive performance improved as the BCS increased to 3.99 but beyond that a decline was noticed. These findings were in agreement with the reports of Langley and Sherington (1983) who observed that cows with higher BCS at calving had a shorter interval to first detected oestrus. Hajurka et al. (1999) also reported that as BCS increased from 1 to 3.5, the number of days to first signs of oestrus decreased. Yaylak (2003) reported that as BCS increased (< more or > 3.50), service period became shorter. Lopez Gatius (2003) also reported that cows with a BCS of higher than 3.5 at calving showed significant reduction in the number of days open compared with cows of BCS less than 3.5 at calving and that pregnancy at first A.I. showed a significant drop in cows delivering in poor condition, which findings were in accordance with the present study.

Studies on the influence of BCS on productive performance showed that for every one unit increase in BCS, an increase of 395.27 kg, 795.55 kg and 4.57 kg was observed in the 18 weeks lactation yield, 305 day predicted lactation yield, and peak milk yield, respectively. These findings were in accord with the reports of Mohammed et al. (1988) that cows with BCS of 2.5 produced less milk than those with 3 to 3.5. Flamenbaum et al. (1995) also reported that milk production for 150 days of lactation was greater for cows at higher BCS (3.80±0.08) than for cows at low BCS (2.65±0.07). Similarly, Pedron et al. (1993) observed that BCS was related (P<0.05) to peak production, and one unit of BCS was associated with 422 kg 305 day milk production in Holstein cows, the value being lower than the predicted values of the present study, whereas Ruegg and Milton (1995) reported that BCS had no effect on 305 day milk yields in Holstein cows. The majority of investigations undertaken in grazing systems has reported a positive association between calving BCS and milk production (Stockdale, 2005; Roche et al., 2005) which was in accordance with the present study.

For every one unit increase in BCS, an increase of milk fat percent of 1.8 and 2.0 and milk protein / SNF percent of 0.55 and 0.54 was observed at 6-8 weeks after calving and 16-18 weeks after calving, respectively. Holter et al. (1990) observed that cows considered as underconditioned at calving had reduced milk fat concentration, which was in accordance with the present findings. Roche et al. (2007) reported that fat content increased with increasing BCS (0.1 and 0.02 percent fat up to 60 and 270 days in milk, respectively, per BCS unit at calving) which was less than the values observed in the present study, and that BCS at calving did not significantly affect milk protein content, which was in contrast to the present findings.

The results of the study revealed that the reproductive performance and milk production increased with BCS up to a score of 3.99, but beyond this there was a decline. The present study suggested that a BCS of 3.5-3.99 was ideal for better reproductive and productive performance of Murrah buffaloes, and hence the feeding management should be monitored such that the buffaloes maintain a BCS of 3.5-3.99 at the time of calving.
Table 1. The mean carcass and ultrasonographic fat thickness (mm) for the five scores of body condition.

<table>
<thead>
<tr>
<th>BCS</th>
<th>Between 12th and 13th ribs Carcass</th>
<th>Between sacral crest and tuber sacrale Carcass</th>
<th>Between sacral crest and hooks Carcass</th>
<th>Between hooks and pins Carcass</th>
<th>Between Tail head and pins Carcass</th>
<th>Mean of all check points Carcass</th>
<th>Mean of all check points Ultrasoundograph</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1.67</td>
<td>1.17</td>
<td>1.61</td>
<td>1.34</td>
<td>1.73</td>
<td>1.73</td>
<td>1.3</td>
</tr>
<tr>
<td>2.</td>
<td>2.68</td>
<td>2.37</td>
<td>2.08</td>
<td>2.37</td>
<td>2.37</td>
<td>2.37</td>
<td>2.8</td>
</tr>
<tr>
<td>3.</td>
<td>4.16</td>
<td>4.27</td>
<td>4.52</td>
<td>3.48</td>
<td>3.48</td>
<td>3.48</td>
<td>3.8</td>
</tr>
<tr>
<td>4.</td>
<td>6.06</td>
<td>6.24</td>
<td>6.62</td>
<td>5.20</td>
<td>5.52</td>
<td>5.20</td>
<td>5.6</td>
</tr>
<tr>
<td>5.</td>
<td>7.82</td>
<td>8.22</td>
<td>8.02</td>
<td>6.68</td>
<td>6.82</td>
<td>6.68</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Table 2. Reproductive performance of buffaloes of various BCSc groups in the test herd.

<table>
<thead>
<tr>
<th>Reproduction Parameters</th>
<th>BCSc 2.5-2.99</th>
<th>BCSc 3.0-3.49</th>
<th>BCSc 3.50-3.99</th>
<th>BCSc 4.00-4.49</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-partum resumption of ovarian activity</td>
<td>47.25 ± 2.39</td>
<td>42.00 ± 2.91</td>
<td>29.33 ± 3.33</td>
<td>39.33 ± 4.21</td>
</tr>
<tr>
<td>Post-partum estrus (days)</td>
<td>77.16 ± 5.33</td>
<td>65.66 ± 5.46</td>
<td>46.66 ± 4.26</td>
<td>55.16 ± 4.19</td>
</tr>
<tr>
<td>Service period (days)</td>
<td>125.16 ± 17.42</td>
<td>85.66 ± 5.83</td>
<td>58.83 ± 9.01</td>
<td>77.16 ± 14.76</td>
</tr>
<tr>
<td>No. of services per conception</td>
<td>2.66 ± 0.61</td>
<td>2.00 ± 0.40</td>
<td>1.50 ± 0.37</td>
<td>1.83 ± 0.52</td>
</tr>
<tr>
<td>1st service conception rate (%)</td>
<td>16.66</td>
<td>33.33</td>
<td>66.66</td>
<td>50</td>
</tr>
<tr>
<td>Breeding efficiency</td>
<td>70.49 ± 2.35</td>
<td>80.58 ± 2.01</td>
<td>90.64 ± 1.98</td>
<td>87.48 ± 1.10</td>
</tr>
</tbody>
</table>
Table 3. Relationship between BCSc and milk yield in the test herd.

<table>
<thead>
<tr>
<th>BCSc</th>
<th>Milk yield upto 18 weeks of lactation (kg)</th>
<th>‘F’ Value</th>
<th>Predicted lactation yield (kg)</th>
<th>‘F’ Value</th>
<th>Peak milk yield (kg)</th>
<th>‘F’ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5-2.99</td>
<td>1030.93 d</td>
<td>150.33**</td>
<td>1981.05 d</td>
<td>9.50 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0-3.49</td>
<td>1197.12 c</td>
<td>2300.39 c</td>
<td>150.33**</td>
<td>11.60 c</td>
<td></td>
<td>78.73**</td>
</tr>
<tr>
<td>3.5-3.99</td>
<td>1658.67 a</td>
<td>3187.31 a</td>
<td>16.50 a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0-4.49</td>
<td>1359.92 b</td>
<td>2613.23 b</td>
<td>13.75 b</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a, b, c, d : values with different superscripts vary significantly (P < 0.01).

Table 4. Relationship between BCSc and milk components in the test herd.

<table>
<thead>
<tr>
<th>BCSc</th>
<th>At 6-8 weeks after calving</th>
<th>At 16-18 weeks after calving</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fat %</td>
<td>Protein %</td>
</tr>
<tr>
<td>-----------</td>
<td>-------</td>
<td>-----------</td>
</tr>
<tr>
<td>2.5-2.99</td>
<td>5.82  d</td>
<td>3.12  d</td>
</tr>
<tr>
<td>3.0-3.49</td>
<td>6.80  c</td>
<td>3.47  c</td>
</tr>
<tr>
<td>3.5-3.99</td>
<td>7.76  b</td>
<td>3.96  a</td>
</tr>
<tr>
<td>4.0-4.49</td>
<td>8.46  b</td>
<td>3.74  b</td>
</tr>
</tbody>
</table>

a, b, c, d : values with different superscripts vary significantly (P < 0.01).
Figure 1. Murrah buffalo showing the skeletal check points for BCS.
Figure 2. Ultrasonographic fat measurements in a Murrah buffalo of BCS 1. (A) Fat thickness at the area between tail head and pins was 3 mm, (B) Fat thickness at the lumbar area was 2 mm, (C) Fat thickness at the area between 12th and 13th ribs was 2 mm, (D) Fat thickness at the area between sacral crest and tuber coxae was 2 mm, (E) Fat thickness at the area between hooks and pins was 1 mm.
Figure 3. Ultrasonographic fat measurements in a Murrah buffalo of BCS 2. (A) Fat thickness at the area between tail head and pins was 5 mm, (B) Fat thickness at the lumbar area was 4 mm, (C) Fat thickness at the area between 12th and 13th ribs was 3 mm, (D) Fat thickness at the area between sacral crest and tuber coxae was 3 mm, (E) Fat thickness at the area between hooks and pins was 2 mm.
Figure 4. Ultrasonographic fat measurements in a Murrah buffalo of BCS 3. (A) Fat thickness at the area between tail head and pins was 6 mm, (B) Fat thickness at the lumbar area was 5 mm, (C) Fat thickness at the area between 12th and 13th ribs was 4 mm, (D) Fat thickness at the area between sacral crest and tuber coxae was 4 mm, (E) Fat thickness at the area between hooks and pins was 3 mm.
Figure 5. Ultrasonographic fat measurements in a Murrah buffalo of BCS 4. (A) Fat thickness at the area between tail head and pins was 8 mm, (B) Fat thickness at the lumbar area was 6 mm, (C) Fat thickness at the area between 12th and 13th ribs was 6 mm, (D) Fat thickness at the area between sacral crest and tuber coxae was 5 mm, (E) Fat thickness at the area between hooks and pins was 4 mm.
Figure 6. Ultrasonographic fat measurements in a Murrah buffalo of BCS 5. (A) Fat thickness at the area between tail head and pins was 11 mm, (B) Fat thickness at the lumbar area was 8 mm, (C) Fat thickness at the area between 12th and 13th ribs was 8 mm, (D) Fat thickness at the area between sacral crest and tuber coxae was 7 mm, (E) Fat thickness at the area between hooks and pins was 6 mm.
Chart 1. Body condition scoring chart for Murrah and graded Murrah buffaloes on a 1 to 5 scale having 0.5 increments.
ACKNOWLEDGEMENT

The authors thank the Director of Research, Sri Venkateswara Veterinary University, Tirupati, for providing the facilities to carry out the research work.

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*Continued from page 62*


*Continued from page 74*


EFFECT OF SUPPLEMENTATION OF YEAST CULTURE IN THE DIET ON MILK YIELD AND COMPOSITION IN GRADED MURRAH BUFFALOES

D. Srinivas Kumar, J. Rama Prasad and E. Raghava Rao

ABSTRACT

A 180-day feeding trial was carried out at the Buffalo Research Station, SVVU, Venkataramannagudem, to study the effect of yeast culture supplementation on milk yield and composition in buffaloes. Twelve graded Murrah buffaloes (465.4±20.92 kg) in early lactation were divided into two groups of six animals each (Yeast unsupplemented, YU, and Yeast supplemented, YS) taking into consideration daily average milk yield, butter fat content, 6% FCM yield, number of lactations and stage of lactation. Animals in both the groups received a basal diet comprising of roughages and concentrates separately to meet the maintenance and production requirements (ICAR, 1998). Concentrates were offered twice daily at the time of milking and chopped hybrid Napier (APBN-1) was available ad libitum. The animals in YS group received yeast culture (\textit{Saccharomyces cerevisiae} CNCM I-1077 strain) 0.5 g/animal/day. The average dry matter intake increased (P>0.05) with yeast culture supplementation (YS) in the diet compared to the unsupplemented (YU) group. The average milk yield, 6\% FCM yield, solids not fat (SNF) percent and total solids percent increased (P>0.05) in YS group of buffaloes compared to the YU group. However, the butter fat percent decreased (P>0.05) marginally in YS group of buffaloes compared to the YU group. Further, it was observed that feeding of yeast culture resulted in increase in daily income of Rs 12.96/- per buffalo over the control. Thus, it is concluded that supplementation of yeast culture 0.5 g/animal/day showed a positive tendency in improving milk yield and is observed to be cost effective.

Keywords: yeast culture, DMI, milk yield, milk composition, buffaloes

INTRODUCTION

The buffalo has evoked world wide interest as an animal with potential for meeting the emerging demand for meat, milk and work in the developing countries. Further, in countries like India, the buffalo is the major milch animal, accounting for more than 50\% of the milk produced. However, it is often agreed that the huge amount of milk produced is not because of higher productivity but because of the higher population. The low productivity of buffaloes is primarily due to poor genetic potential, inadequate supply of nutrients an unscientific approach in feeding. Hence, in order to improve the productivity of buffaloes, there is a need to adopt scientific feeding strategies. The use of probiotic feed supplements containing \textit{Saccharomyces cerevisiae}, has been found to improve the performance of lactating animals (Dobicki \textit{et al.}, 2006; Phondba \textit{et al.}, 2009). Addition of yeast culture in the diet has increased
feed intake and milk production (Erasmus, 1992), functions as an oxygen scavenger in rumen (Dolezal et al., 2005) and results in overall improvement in the digestibility of high fibre feed, and this leads to improved nutrient availability in ruminants with positive effect on production performance. However, few trials have been conducted in buffaloes to illustrate the potential of this probiotic under Indian conditions. Thus, an attempt was made to ascertain the effect of feeding yeast culture on milk yield and composition in graded Murrah buffaloes.

**MATERIALS AND METHODS**

A 180-day feeding trial was carried out at the Buffalo Research Station, Sri Venkateswara Veterinary University, Venkataramannagudem, using 12 graded Murrah buffaloes (465.4±20.92 kg) in early lactation (1-2 months and 1-3 lactations). The animals were divided into two groups of six animals each (Yeast unsupplemented, YU and Yeast supplemented, YS) taking into consideration daily average milk yield (7.85 vs. 7.75 kg), butter fat content (5.38 vs. 5.40 %), 6% FCM yield (7.27 vs. 7.28 kg) number of lactations (1.67 vs. 1.33) and stage of lactation (31.17 vs. 29.17 days). Animals in both the groups received a basal diet comprising of roughages and concentrates separately to meet the maintenance and production requirements (ICAR, 1998). Concentrates were offered twice daily at the time of milking and chopped hybrid Napier (APBN-1) was available ad libitum. The animals in the YS group received yeast culture (*Saccharomyces cerevisiae* CNCM I-1077 strain) 0.5 g/animal/day. The yeast culture was administered by top dressing over the concentrate mixture every day. The chemical composition of feeds and fodders used in the present study is shown in Table 1.

The animals were completely milked twice daily at 5.00 A.M and 5.00 P.M throughout the experimental period, and the daily milk yield was recorded. Milk samples from individual buffaloes were collected at weekly intervals and were analyzed for fat, SNF, total solids and 6% FCM yield. Milk fat was determined by Gerber’s method (ISI 1977 IS: 1224 part-1) using special butyrometer and pipette with ISI marking. Based on milk yield and butter fat yield, the 6% FCM was calculated (Rice et al., 1970). The SNF was calculated using gravimetric method (ISI 1982 IS: 10083) based on estimation of specific gravity using corrected lactometer reading (CLR). The data generated were subjected for the test of significance (Snedecor and Cochran, 1976).

**RESULTS AND DISCUSSION**

The average DMI of buffaloes during the digestion trial was 11.24 and 11.95 kg/day, respectively, in the YU and YS groups (Table 2). Although differences in dry matter intake between the two groups were non-significant, but an improvement in intake was observed on yeast supplementation. Milk cows in early lactation normally cannot consume enough concentrate to meet their energy requirements for high milk production and are forced to use body reserves. This negative energy balance increases stress (Arambel and Kent, 1990). In the present study, mean daily DM intake was marginally increased by the addition of yeast culture to the diet. This might be attributed to the positive effect of yeast culture in relieving stress thereby maintaining the DM intake, which might have led to increased milk production. Similar results were observed by Garg
Table 1. Chemical composition (% DMB) of feedstuffs.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Hybrid Napier (APBN-1)</th>
<th>Concentrate Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry Matter</td>
<td>24.24</td>
<td>91.23</td>
</tr>
<tr>
<td>Organic Matter</td>
<td>91.20</td>
<td>92.34</td>
</tr>
<tr>
<td>Crude Protein</td>
<td>8.50</td>
<td>19.63</td>
</tr>
<tr>
<td>Ether Extract</td>
<td>2.19</td>
<td>5.14</td>
</tr>
<tr>
<td>Crude Fibre</td>
<td>36.84</td>
<td>12.05</td>
</tr>
<tr>
<td>Nitrogen Free Extract</td>
<td>43.49</td>
<td>55.52</td>
</tr>
<tr>
<td>Total Ash</td>
<td>8.98</td>
<td>7.66</td>
</tr>
<tr>
<td>Neutral Detergent Fibre</td>
<td>75.13</td>
<td>33.52</td>
</tr>
<tr>
<td>Acid Detergent Fibre</td>
<td>47.80</td>
<td>17.80</td>
</tr>
<tr>
<td>Acid Detergent Lignin</td>
<td>7.40</td>
<td>4.81</td>
</tr>
<tr>
<td>Cellulose</td>
<td>37.93</td>
<td>12.99</td>
</tr>
<tr>
<td>Hemi-cellulose</td>
<td>27.33</td>
<td>15.72</td>
</tr>
</tbody>
</table>

Table 2. Effect of feeding yeast culture on average milk yield and composition in graded Murrah buffaloes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dietary treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YU Group</td>
</tr>
<tr>
<td>DMI (kg / d)</td>
<td>11.24 ± 0.27</td>
</tr>
<tr>
<td>Milk yield (kg / d)</td>
<td>6.86 ± 0.35</td>
</tr>
<tr>
<td>6 % FCM yield (kg / d)</td>
<td>7.23 ± 0.36</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>6.52 ± 0.18</td>
</tr>
<tr>
<td>SNF (%)</td>
<td>8.65 ± 0.24</td>
</tr>
<tr>
<td>Total Solids (%)</td>
<td>15.17 ± 0.38</td>
</tr>
</tbody>
</table>

YU = Yeast unsupplemented group; YS = Yeast supplemented group.

Table 3. Economics of feeding yeast culture in the diet of graded Murrah buffaloes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dietary treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YU Group</td>
</tr>
<tr>
<td>Total feed cost (Rs/animal/day)</td>
<td>54.10</td>
</tr>
<tr>
<td>Cost of Probiotic (Rs/animal/day)</td>
<td>0.00</td>
</tr>
<tr>
<td>Total expenses (Rs/animal/day)</td>
<td>54.10</td>
</tr>
<tr>
<td>Average daily milk yield (kg/animal)</td>
<td>6.86</td>
</tr>
<tr>
<td>Cost of milk production (Rs/kg)</td>
<td>7.89</td>
</tr>
<tr>
<td>Daily income on milk sale (Rs/animal)</td>
<td>164.64</td>
</tr>
</tbody>
</table>

YU = Yeast unsupplemented group; YS = Yeast supplemented group.
et al. (2000) who reported increased DMI in HF cows by feeding yeast culture in the diet.

The average daily milk yield and its composition for different groups are presented in Table 2. It was observed that the average daily milk yield of buffaloes from the YS group was higher than the YU group, but the difference was not significant (P>0.05). Stimulatory factors for rumen bacteria, such as B vitamins or branched chain VFA (Higginbotham et al., 1994) and growth factors, such as malate (Nisbet and Martin, 1991) are present in yeast culture, and their absence may have contributed to the lack of significant milk production response in the present study. Lehloenya et al., 2008; Mahender et al., 2005 and Schingoethe et al., 2004 reported that yeast culture had no significant effect on milk yield while Bruno et al. (2009) and Phondba et al. (2009) reported significant improvement in milk yield with yeast culture supplementation in the diet. The 6% FCM yield of buffaloes increased (P>0.05) more in the YS group than in the YU group which might be due to higher average milk yield in the YS group compared to the YU group. Schingoethe et al. (2004) and Lehloenya et al. (2008) also reported non-significant increases in FCM yield with yeast supplementation in the diet.

The average milk fat % decreased marginally (P>0.05) in the YS group compared to the YU group of buffaloes. The marginal milk fat depression observed in the YS group of buffaloes may be attributed in part to dilution caused by increased milk yield compared to the YU group (Lehloenya et al., 2008). The non-significant decrease in milk fat observed in the present study was in agreement with the earlier findings (Li Shengli et al., 2004). The average SNF percent of buffaloes was higher (P>0.05) in the YS group as compared to the YU group (8.78 vs. 8.65). Increase in milk SNF percent is likely due to increase in milk components such as milk lactose or milk protein (Lehloenya et al., 2008). The non-significant increase in SNF percent observed in the present study was in agreement with the earlier findings (Erdman and Sharma, 1989; Arambel and Kent, 1990; Mahender et al., 2005 and Lehloenya et al., 2008). Similarly, the average total solids percent in milk of buffaloes from the YS group (15.17±0.38) increased non-significantly (P>0.05) as compared to the YU group (15.29±0.43). Similarly, Mahender et al. (2005) reported a non-significant increase in total solids in milk of buffaloes fed yeast culture in the diet. When the economics of milk production with supplementation of yeast culture was calculated (Table 3), it was observed that feeding of yeast culture resulted in a decrease in the cost of milk production by Rs 0.21/- per kg of milk and an increase in daily income of Rs 12.96/- per buffalo over the control.

Thus, it is concluded that supplementation of yeast culture 0.5 g/animal/day showed a positive tendency in improving milk yield and is observed to be cost effective.

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…liquid nitrogen vapour freezing technique (Verma et al., 1975)
…and buffaloes (Singh et al., 1983; Shah et al., 1987; Misra, 1996; Pant et al., 2002)

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CONTENTS

Case Report

Congenital abomasal fistula in a buffalo calf and its successful treatment
_Ankur Sharma, S.V. Upadhye and Kawardeep Kour_.

An unusual case of oesophageal obstruction in a female buffalo
_N.V.V. Hari Krishna, Makkena Sreenu and V.S.C. Bose_.

Diagnosis and management of polioencephalomalacia in Indian buffaloes under farm conditions
_Sumit Mahajan, Rajesh Agrawal and S.M. Rashid_.

An omphalocele in a buffalo calf: A case report
_P. Vidy Sagar, K.S.Vadde, K.S. Sai Krishna and S. Venkateswarlu_.

Dystocia due to a conjoined twin monster foetus in a female buffalo
_S.P. Shukla, Qazi Mudasir and S.P. Nema_.

Original Article

Genetic polymorphism analysis of monoacyl glycerol transferease2 (MOGAT2) gene in Murrah buffalo (_Bubalus bubalis_
_D.S. Kale and B.R. Yadav_.

ABCG2 polymorphisms of the Chinese buffalo (_Bubalus bubalis_) and yak (_Bos grunniens_) breeds
_Songjia LAI, Ming ZHANG, Changjun ZENG and Jie WANG_.

Molecular characterization of partial exon-2 of the bone morphogenetic protein 15 (BMP15) gene in Indian buffalo (_Bubalus bubalis_): Its contrast with other species
_S.S. Misra, T.A.S. Ganai, S.A. Mir and M.A. Kirmani_.

Page
CONTENTS

Original Article

Comparing relative sensitivity and specificity of LA and RNA-PAGE in detecting bovine rotaviruses
T.C. Singh and M.K. Jhala.................................................................36

Rescue of oocytes from early antral follicles isolated from cryopreserved buffalo ovaries using an in situ oocyte cryopreservation method: competence to undergo maturation, fertilization and development in vitro
Saber Mohammed Abd-Allah..........................................................................45

Ovarian activity and hormonal relationships in pregnant buffaloes
M.M. Waheed..........................................................................................55

Effect of dietary inclusion of yeast culture (Saccharomyces cerevisiae) on growth performance of graded Murrah buffalo bull calves
D. Srinivas Kumar, J. Rama Prasad and E. Raghava Rao......................................................63

A study on the prevalence of some pathological abnormalities of the uterus diagnosed at post mortem of buffaloes in Mosul

Effect of different physiological stages and managemental practices on milk somatic cell counts of Murrah buffaloes
Kalyan De, Joydip Mukherjee, Shiv Prasad and A.K. Dang.................................................72
CONTENTS

Original Article

A comparative evaluation of different diagnostic tests for brucellosis in buffaloes
Sumit Mahajan, Rajesh Agrawal and Nishi Pande.................................................................75

A body condition score (BCS) system in Murrah buffaloes
A. Anitha, K. Sarjan Rao, J. Suresh, P.R. Srinivasa Moorthy and Y. Kotilinga Reddy...............79

Effect of supplementation of yeast culture in the diet on milk yield and composition
in graded Murrah buffaloes
D. Srinivas Kumar, J. Rama Prasad and E. Raghava Rao.....................................................100