Aims

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Main Objectives

1. To be world source on buffalo information
2. To provide literature search and photocopy services
3. To disseminate information in newsletter
4. To publish occasional publications such as an inventory of ongoing research projects

Buffalo Bulletin is published quarterly in March, June, September and December. Contributions on any aspect of research or development, progress reports of projects and news on buffalo will be considered for publication in the bulletin. Manuscripts must be written in English and follow the instruction for authors which describe at inside of the back cover.

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ABSTRACT

A case of bilateral hydrosalpinx with multiple affections in oviducts is reported in present communication.

Keywords: buffalo, oviduct, bilateral hydrosalpinx

INTRODUCTION

Genital organ disorders are an important cause of infertility and sterility in buffaloes causing high economic losses (Azawi, 2008). Generally, female animals are culled and sent to the slaughterhouse either because they are uneconomic to maintain or else because they have some disease problem. Hence, abattoirs are a good source of material for studying pathological lesions of buffalo reproductive organs that are severe enough to cause infertility and even sterility (Dobson and Kamonpatana, 1986). Earlier surveys on morbid genitalia revealed higher incidence of structural abnormalities in buffaloes as compared to cattle. The oviducts are important for fertilization and maintenance of the embryo until its arrival in the uterus. Salpingitis, hydrosalpinx and pyosalpinx are common diseases affecting the oviduct in domestic mammals, especially cattle (McEntee, 1990). All these the oviductal affections cause hindrance to proper gamete transport, and hence, fertilization fails to occur. Published reports on the incidence of oviductal abnormalities in buffaloes, though not very common in India (0.31 to 0.62%), are considerably higher in areas like Latin America (1.3 to 5.2%), Egypt (1.7 to 5.9%), and Pakistan (10.9%) (Kumaresan and Ansari, 2002; Vale et al., 1988). Hydrosalpinx, an oviductal disorder in buffalo, has been reported to occur in 1.8 to 2.2% cases of slaughtered animals in India (Dwivedi and Singh, 1971). In a survey of morbid genitalia, Azawi (2009) reported 71.4% of the hydrosalpinx were unilateral while the rest were of the bilateral type. Reports of multiple affections of the oviduct in a single case are rare.

A number of morbid genitalia were collected from the central buffalo slaughter house, Bareilly and transported to the laboratory for evaluation within two hours of slaughter. Each specimen was grossly examined in the laboratory in order to determine the nature of the reproductive abnormality and its location in the tract. Of these, one genitalia had bilateral hydrosalpinx; hence, the specimen was subjected to thorough observation (Figures 1 and 2).

Left oviductal portion: From the pictorial view, it was clear that the left ovary was...
completely ingrained by the ovarian bursa and adnexa. The oviductal parts seemed to be tightly coiled with extensive enlargement of ampullae and infundibulum with accumulation of clear viscous fluid. However, the isthmic portion appeared to be normal.

Right oviductal portion: The ovary was free from any adhesion and was functional with a corpus haemorrhagicum. A tough membranous attachment from the dorsal part of uterus to the oviduct was present. Ampulary and infundibular conditions were similar to those of the left oviductal part. The ostium abdominal, opening of

Figure 1. Bilateral hydrosalpinx in buffalo genitalia.

Figure 2. Close view of enlarged infundibulum and ampullae of buffalo oviduct.
the infundibulum was tightly closed and placed apart from the ovary hence, failed to pick up the ovum at ovulation.

On macroscopical observation no pathological lesions were observed in the uterine horns. Endometrial cytology, carried out using Giemsa staining technique, indicated that the animal was negative for endometritis. The rest of the organ was normal without any affection.

The exact mechanism by which hydrosalpinx develops is still not established. The propositions put forth so far by various researchers are mainly concerned with the blockage or inflammatory conditions responsible for development of hydrosalpinx. According to Azawi et al. (2007) severe inflammation of the uterine tissue could be extended to utero-tubal junction or the end part of isthmus resulting in fibrosis and tubal obstruction, and leading to accumulation of fluid. Miller and Campbell (1978) claimed that hydrosalpinx is a sequel to a localized salpingitis resulting in oviductal obstruction. If the condition is unilateral, the fertility of the affected animal is maintained to some extent. If it is bilateral, complete sterility occurs; this might be the reason for the slaughter of the buffalo reported in this case. The case here was no doubt a multiple affection including the oviduct and the ovary, but we failed to detect any apparent inflammatory condition. This atypical case by its origin could be explained by the suggestion of Ellington and Schlafer (1993), who supposed that hydrosalpinx might be a congenital condition affecting oviductal segments.

REFERENCES


ABSTRACT

A successful delivery of a dicephalic monster through fetotomy was recorded.

Keywords: dicephalic monster, buffalo, congenital defect, fetotomy

INTRODUCTION

Monstrosities are malformed fetuses, which are rare in buffaloes (Chauhan and Verma, 1995 and Bugalia et al., 2001). Incidence among all calves seems to range from 0.2 to 3.0 percent with 40 to 50 percent born dead and only a small fraction of reported defects not being externally visible. The most frequently encountered congenital defect involves the skeletal system (Morrow, 1986). Monstrosities are associated with either congenital defects or infectious disease (Arthur et al., 2001) and may or may not interfere with birth. Abnormal duplication of the germinal area during embryogenesis of a monozygotic fetus will give rise partial duplication of body structures (Sharma et al., 2010). Duplication of the cranial portion of the fetus is more common than the caudal portion (Roberts, 2004). Dystocia is common sequelae of fetal monstrosities. Fetotomy offers a good alternative to the caesarean for relieving a fetal monster causing dystocia (Vermunt, 2009). In the present study, a dicephalic fetal monster was relieved by fetotomy.

CASE HISTORY AND CLINICAL EXAMINATION

A five-and-half-year-old she Murrah buffalo with full term gestation was presented to the Teaching Veterinary Clinical Complex, Mathura University, with a complaint of unsuccessful straining, lasting ten hours. Clinical examination revealed an increase in respiration and pulse rate with normal rectal temperature. Obstetrical examination revealed a dicephalic fetus, in anterior longitudinal presentation, dorso sacral position with bilaterally flexed knee joint.

TREATMENTS AND DISCUSSION

Obstetrical procedure-Percutaneous fetotomy was indicated, as attempts to extract the morbid monster by forced traction would pose great risk to the dam. The animal was restrained in lateral recumbency following low epidural anesthesia with 2% of lignocaine hydrochloride. A fetotome was partially threaded on one side,
and a sand snare introducer was applied at end of the wire. The wire was then carried in the birth canal with loose loops to pass over the deviated head and retrieved from ventral side; the fetotome was then completely threaded outside, and the loop was positioned near the base of the head attachment. A final check was made to ensure the wire rested behind the ears at the base of the attached head. Now, the head of fetotome was carried into the birth canal and positioned at the juncture of the two heads ventrally. Finally, sawing was done, initially with short strokes followed by a continuous full hand strokes till the head was amputated. The amputated head was extracted by applying Krey-Schottler double jointed eye hook. After thorough lubrication of the birth canal, the remaining fetus was extracted by simultaneous three point traction on both extended fore limbs and head with the help of obstetrical chains ensuring minimum damage to the birth canal. Thorough examination of fetus revealed two heads conjoined at the base of the medial ears approximately at 45º (Figure 1) and both heads attached to single neck. One of the heads had better alignment with the vertebra than the other. The neck, thorax, abdomen and limbs were grossly normal. These observations are in consonance with the earlier findings (Sharma et al., 2010 and Fisher et al., 1986).

Dicephalus monsters have been reported in goats (Pandit et al., 1994), buffaloes (Chauhan and Verma, 1995; Raju et al., 2000; Bugalia et al., 2001; Srivastava et al., 2008) and cows (Chandrahasan et al., 2003; Patil et al., 2004; Abraham et al., 2007). Jones and Hunt (1983) stated that the causes of many congenital anomalies are essentially unknown; however, the important known causes are prenatal infection with a virus, teratogens ingested by mother, vitamin deficiency (A and folic acid), genetic factors and/or combination of these factors. The zygote (< 14 days) is susceptible to genetic mutations and chromosomal aberrations. During the embryonic period (day 14 to 42 days), the embryo is highly susceptible to teratogens, and the effect decreases gradually as embryo matures to fetus (Morrow, 1986). Sharma et al., (2010) stated that monstrosities are malformed monozygotic individuals due to abnormal duplication of the germinal area giving rise to a fetus whose body structures are partially duplicated. The embryonic disk differentiates on the 13th day. If the split occurs after day 13, then the twins will share body parts in addition to sharing their chorion and amnion (Finberg, 1994) and it is thought that these factors are responsible for the failure of twins to separate after the 13th day after fertilization (Srivastava et al., 2008).

![Figure 1. Dicephalic monster.](image-url)
Thus the present report describes relieving dystocia due to dicephalic monsters in buffaloes by fetotomy and its preventive measures. The technique is safer and less time consuming, it can be used successfully as an alternative to the caesarean operation seems expensive to the farmer. Identification of specific cause will aid in ensuring the preventive measures. This can be achieved by genetic analysis of the dam and sire of a defective fetus and discouraging the breeding of positive reactors.

REFERENCES


INTRODUCTION

All over the world, mastitis, or swelling of the udder, is considered to be the most important production-limiting disease of the dairy industry. This dairy scourge not only reduces the milk yield of affected animals (nearly 15 to 20%) but also renders the milk unsuitable for human consumption. Mastitis results from the growth of germs in the udder. It occurs in two forms: (1) Clinical mastitis in which there is visible swelling in the udder/teat and noticeable changes in milk such as flakes, clots etc. (2) Sub-clinical, or hidden, mastitis in which swelling in the udder is so mild that there is no visible change in the milk or in the udder/teat.

The subclinical, or hidden, form of mastitis is 15-40 times more common than clinical mastitis. Clinical cases are preceded by the hidden form of mastitis. Research conducted in Pakistan over the past four decades has shown that about 25% of cows and 15% of buffaloes are afflicted with the subclinical form of mastitis. An early diagnosis of hidden mastitis is imperative to save the udder and prevent transmission of disease-producing organisms to other animals in the herd. It is also important from the public health viewpoint in so far as the milk of animals affected with sub-clinical mastitis contains disease producing bacteria, their toxins, abnormal milk constituents as well as pus cells.

Mastitis, like any other inflammation in the body, causes a tremendous increase in the number of white blood cells in the milk (pus cells). In the context of milk, these cells are called milk somatic cells. Researchers at the Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad, (Pakistan) have pioneered a study demonstrating that a 3% solution of a household detergent viz. Surf Excel (Unilever Pakistan Ltd.) can be used for an early farmer’s level detection of sub-clinical (hidden) mastitis. This test has been named the Surf Field Mastitis Test. A test kit utilizing locally available materials has also been fabricated and is now commercially available from Rainman Pvt. Ltd. 5 Shalimar Link Road, Lahore 54840 Pakistan; Mobile # 0321-9469359.

Unique attributes of Surf Field Mastitis Test

The desirable features of this innovative mastitis detection test include:

a. Compatibility with the technical capabilities of farmers who happen to be mostly illiterate in the developing countries. Owing to a facile nature of the test procedure, even an illiterate farmer can learn to conduct this test within a few minutes.

b. Desirable sensitivity (72.81 and 66.22 in cows and buffaloes respectively) of detection vis-à-vis other expensive similar tests like California Mastitis Test (75.73 and 70.27 cows and buffaloes
respectively; Muhammad et al., 2010, Tropical Animal Health Production 42: 457-464) and the gold standard of mastitis diagnosis i.e. microbiological examination of aseptically collected milk samples.

c. Availability of the required reagent, i.e. Surf Excel Powder (Unilever Pak Ltd.) in almost every village.

d. User friendly nature of the test.

**Procedure of Surf Field Mastitis Test and its interpretation:**

a) **Procedure**

1. Prepare a 3% solution of the household detergent viz. Surf Excel (Unilever Pak Ltd.). To this end, dissolve 5-6 teaspoonfuls of the Surf Excel powder in ½ liter of ordinary water. Pour this solution into a plastic bottle, apply a lid and place the bottle in a dark place. This reagent is good for about 3 months.

2. Collect 10-15ml of milk from each teat in separate container like tea cups. If the Surf Field Mastitis Test paddle is available, there is no need to collect milk samples into tea cups as the milk from individual quarters of cow and buffalo can be collected into individual receptacle of this paddle.

3. Mix the milk from an individual teat and the Surf solution (3%) in approximately equal proportions (i.e. add 10-15 ml 3% Surf solution).

4. Rotate the mixture of milk and the Surf solution for about 15-20 seconds.

5. Examine the mixture for thickening or any other change.

b) **Interpretation**

If the hidden form of mastitis (subclinical mastitis) is present in the quarter of udder, the mixture (milk + Surf solution) will thicken (i.e. gel formation) within 15 seconds. The udder is free of subclinical mastitis if the mixture remains liquid and there is no thickening of mixture of milk and Surf solution.

As mentioned earlier, a Surf test kit is now commercially available from Rainman Pvt. Ltd. 5 Shalimar Link Road, Lahore 54840 Pakistan; Mobile # 0321-9469359. The milk from Surf test positive quarters of udder is not wholesome for human consumption and should be discarded. As subclinical mastitis is antecedent to clinical mastitis, many Surf test positive quarter(s) will develop clinical form of the disease in the future.

**Uses of the Surf Field Mastitis Test in mastitis treatment, control and public health**

1. All farmers should conduct Surf Field Mastitis Test on all quarters of all milch animals at fortnightly intervals. In the event of a positive test reaction in one or more udder quarter(s), immediately contact the local veterinarian for treatment and advice on mastitis treatment and control.

2. Whenever there is reduction in quarter yield, the Surf Field Mastitis Test should be conducted to rule out the possibility of mastitis as the cause of reduced milk yield.

3. Lactating animals should be divided into Surf test positive and Surf test negative groups. The Surf test negative group (mastitis free animals) should be milked first because the germs, which cause mastitis are transmitted from mastitis-affected (Surf test positive) to healthy animals through milkers’ hands at the time of milking.

4. Always conduct the Surf Field Mastitis Test when purchasing new cows and buffaloes. Purchase only Surf test negative cows and buffaloes.

5. Milk from Surf test positive animals is unfit for human consumption because it contains a lot of germs, their toxins and pus cells (somatic cells) and abnormal milk constituents.
6. The processing of milk by the milk processing plants which gives a positive Surf test reaction results in sub-optimal and substandard finished products like yogurt, cheese, etc. The dairy industry (milk plant) management should, therefore, train their field staff and farmers about the procedure and uses of this innovative and nifty mastitis detection test.

Adoption of innovative the Surf Field Mastitis Test (SFMT) in Pakistan and other countries

Because of the ease of execution, the desirable sensitivity of detection, and the inexpensive and user-friendly nature of the SFMT, thus far it has found applications/ recognition with the organizations indicated hereunder:

i. Military dairy farms throughout Pakistan conduct this test on a routine basis under instructions from GHQ.

ii. The test has become a part of the training curricula of Remount Veterinary School, Sargodha, Livestock Production Research Institute, Bahadur Nagar, Okara, Livestock Extension Training Centre, Bahadur Nagar, Okara, In-Service Animal Husbandry Training Institute (IAHTI) Peshawar, Buffalo Research Institute Bhunikey etc.

iii. It is a routine test at veterinary hospitals in Punjab and other provinces.

iv. The Pakistan Dairy Development Company (State Cement Building Corp., near Lahore Race Club, Kot Lakhpat, Lahore, Pakistan; Tel: 042-9262065-68) has registered nearly 1000 dairy farmers from all over Pakistan. Through its network of Farm Production Advisors, Pakistan Dairy Development Company is promoting the use of the Surf Field Mastitis Test among the registered farmers for the purpose of mastitis treatment and control and improvement in milk quality.

v. The Directorate of Animal Disease Surveillance and Reporting, Livestock and Dairy Development Department (Punjab) through its field staff is training the dairy farmers in Punjab on the use of the Surf Field Mastitis Test in diagnosis, treatment and control of mastitis.

vi. The test is being used in other countries like UAE, China, Nepal etc.

vii. SAIC (SAARC Agricultural Information Centre, Dhaka, Bangladesh) has developed a video on SFMT for demonstration of the test to farmers and extension agencies operating in SAARC countries. This video can be had from SAIC (www.saic-dhaka.org). SAIC has also recognized the Surf Field Mastitis Test as a success story in the book entitled “SUCCESS STORIES ON TRANSFER OF FARM TECHNOLOGY IN SAARC COUNTRIES” compiled and edited by Muhammad Abdullah and published in 1998 by SAARC Agricultural Information Centre (SAIC) BARC Campus, Farmgate, Dhaka 1215, Bangladesh, pages: 71-75. The title of the success story described in these pages is Surf Field Mastitis Test (SFMT): an inexpensive tool for evaluation of wholesomeness of fresh milk.

viii. Several NGOs (e.g. SUNGI, Carritas International, Bunyad) have disseminated information on this innovative test to end-users (farmers).

ix. In order to improve the quality of raw milk procured for processing and to increase the profitability of dairying in Punjab, Nestlé Milkpak is currently training its field staff on the test procedure and its uses in the early spotting of hidden mastitis. Nestle Milk Pak is paying a milk quality bonus 20 paisa per litre of milk to those farmers who adopt the Surf Field Mastitis Test on regular basis. Other dairy companies (Chaudhry Dairies, Olpers, Nurpure milk etc.) are likely to follow suit of Nestlé Milkpak Ltd. Pakistan Dairy Development
Company, headed by Bill McD Stevenson (General Manager, Farm Production), is yet another addition to local dairy organizations who through their network of field workers are training of farmers in the diagnosis of sub-clinical mastitis by using the Surf Field Mastitis Test.

x. UM Enterprises Pakistan Ltd. has recently signed an MoU with Business Incubation Center (email: qamarbic@uaf.edu.pk), University of Agriculture, Faisalabad, Pakistan for the legalized production of the Surf Field Mastitis Test kit.

xi. A team of workers, as a part of rendition of farmer advisory services under the Endowment Fund project entitled ‘A Rural and Peri-Urban Outreach Mastitis Control Program Focusing on Transfer of Technologies Developed by University of Agriculture, Faisalabad’, is training dairy farmers in the diagnosis of sub-clinical mastitis with the help of the Surf Field Mastitis Test.

The innovative Surf Field Mastitis Test is the apotheosis of the onsite test for the determining the wholesomeness of milk available to the general public and to milk processing industry. The adoption of this technology epitomizes how investment in research pays rich dividends.

Figure 1. Surf Field Mastitis Test kit.

Figure 2. Milk from a buffalo suffering from clinical mastitis in 3 of the 4 quarters, there is no need to execute the Surf Field Mastitis Test.
Figure 3. Addition of 3% Surf solution to individual quarter milk samples approximately in equal proportions.

Figure 4. Positive the Surf test reaction (sub-clinical mastitis) in the two lower milk samples. Notice that the milk from the two lower quarters became thick (like the white of egg) on addition of the Surf test solution to milk.
Figure 5. Farmers being trained in the execution of the Surf Field Mastitis Test for diagnosis of sub-clinical (hidden) mastitis.

Figure 6. A youth being trained in the execution of the Surf Field Mastitis Test for diagnosis of sub-clinical (hidden) mastitis.
INTRODUCTION

Udder edema is generally a periparturient disorder characterized by excessive accumulation of fluids in the intercellular tissue spaces of the mammary gland. Incidence and severity are greater in pregnant heifers than in cows (Erb and Grohn, 1988). Udder edema can be a major discomfort to the buffalo and cow and causes management problems such as difficulty with milking, increased risk for teat and udder injuries, and mastitis, and may also reduce milk production. The present study deals with clinical manifestation and therapeutic management of periparturient udder edema in Jaffrabadi buffaloes and Gir cows.

Keywords: Jaffrabadi buffaloes, Gir cows, therapeutic management, udder edema

CASE HISTORY AND OBSERVATION

Post parturient udder edema

Two Jaffrabadi buffaloes and three Gir cows in between their first and third lactations were presented at the Cattle Breeding Farm, J.A.U., Junagadh with swelling of the udder and teats and with the history of calving in the previous week. The edema involved all the quarters, and the animals showed discomfort. Few animals showed painful condition. On palpation, edematous area and swelling showed pitting on pressure. Clinical examination revealed normal rectal temperature, respiration rates and feeding and watering. The potency of teats was normal in all the buffaloes and cows. In all the animals, milk from each quarter had no abnormal colour and had normal consistency and normal pH ranging from 6.4 to 6.7. The edematous swelling of udder in one buffalo was very severe and extended up to the navel causing severe discomfort to the buffalo and even difficulty in walking; the animal was partially off-feed.

Pre partum udder edema

The two Jaffrabadi buffaloes and four Gir cows in their 1st and 2nd lactation were treated at same farm having history of severe edematous swelling of udder and teat. The swelling of udder was so greatly enlarged in a few of these animals leading to severe discomfort and even to difficulty in walking. The animals were due for calving within week.

THERAPEUTIC MANAGEMENT

Post parturient udder edema

The animals showing signs of post partum edematous swelling were milked thrice a day and treated with diuretics (Furosemide) 1-2 mg/kg of body weight i/m, an antihistaminic (Chlorpheniramine maleate) 10 ml i/m and an
anti-inflammatory (Ketoprofen) 3 mg/kg of body weight i/m. One buffalo with extensive edematous swelling at the navel was additionally given Inj. Vit. B-Complex with liver extract 10 ml and hot fomentation of udder. All the animals successfully recovered within 3-5 days of treatment without affecting milk composition or milk production.

**Pre partum udder edema**

The animals with severe edematous udder swelling were milked by few striping twice a day to reduce the teat and udder tension. All the affected buffaloes and cows were treated the same as in post-partum udder edema. All the animals successfully recovered within 3-5 days of treatment and had normal parturition and milk production.

**RESULT AND DISCUSSION**

Udder edema begins shortly before calving when blood flow increases to the udder in preparation for lactation. It is normal for most cows to experience some degree of udder edema before calving. Under normal conditions, the edema will clear from the udder within a week or two post-calving. Physiologically, a developing calf can restrict the flow of blood and lymph away from the udder while at the same time metabolic changes, especially hormonal fluctuations, cause an increased blood supply to the area. This combination can lead to the excessive pooling of fluid (Merck’s Veterinary Manual).

The clinical manifestation recorded in the present study agree with those reported by Sharma et al. (2005). Dentine and McDaniel (1983) also observed that udder edema is more severe in heifers than cows.

The treatment with diuretics, antihistaminic and anti-inflammatory were help in draining out excessive accumulation of interstitial fluid, reducing histamine release and reducing swelling of udder and relieving pain respectively. Treatment includes massages and hot compresses on the

Figure 1. Primiparous cow heifer showing udder edema extentened up to nevel.
affected areas. This stimulates blood flow, which aids in the removal of the excess fluid.

Factors like prepartum heavy grain feeding to heifers (Dentine and McDaniel, 1983) and high sodium and potassium intake in housed cattle might be predisposing factors for the udder edema. Malven et al. (1983) concluded that prepartum edema was positive for plasma estrone and estradiole-17a and was negative for estradiole 17b and progesterone.

Udder edema is frequently seen in primiparous cows. This could be due to the immaturity in the vascular structure of the udder being more vulnerable to fluid retention. Heredity also may play a part increasing the susceptibility of the cow to udder edema (Merck’s Veterinary Manual).

Udder edema does not seem to be caused by just one factor but rather a combination of factors; genetic predisposition, management and nutrition, large foetus size, heavy concentrate feeding and incomplete developed mammary vein may be responsible for severe udder edema in the buffaloes and cows. Prevention through proper nutrition is the easiest route, but treatment is successful in controlling this condition.

REFERENCES


INTRODUCTION

Bovine mastitis is recognized as one of the most costly diseases in dairy cattle across the world. It has been estimated that bovine mastitis reduces milk yield by approximately 21% in infected cattle herd. Inflammation of the udder, or mastitis, is commonly observed in cattle and buffaloes due to their anatomical predisposition, especially during the peripartum period. Mycotic mastitis had been documented to be caused by various genera of moulds and yeasts. Besides bacterial mastitis, the most frequently encountered fungi are Candida spp., Aspergillus spp., Trichosporon spp., Cryptococcus spp., Saccharomyces spp., Penicillium spp., etc. (Costa et al., 1993). The involvement of Geotrichum candidum in mastitis is extremely rare (Krzyzanowski and Sielicka, 1996). Aspergillus flavus produces aflatoxin that is secreted in milk and has a hazardous effect on human and animal health (Reddy et al., 2005).

The present report documents clinical mastitis in Jaffrabadi buffaloes and Gir cows with histories of protracted mastitic conditions despite the administration of different parenteral and intramammary antibiotics and other supportive treatment. Clinically, the buffaloes and cows had not displayed any feature of systemic infection. The affected udders were moderately fibrous. The milk from all the quarters was watery with thick flakes and in a few of these animals slightly blood tinged.

Keywords: Mastitis, Peripartum period, PVP-iodine

CASE HISTORY

Three Jaffrabadi buffaloes and 14 Gir cows in early phases of different lactation were diagnosed positive for clinical mastitis. They were diagnosed based on clinical histories of no systemic reactions, having normal feed and water intake, and rectal temperatures within the normal physiological range (101-102°F).

Clinical signs were reduced milk yield, change in pH (alkaline) and the presence of obvious clinical symptoms like the appearance of visid mucoid gray white mammary secretion, secretions consist of a few yellow clots in watery supernatant fluid, and in a few of these animals, slightly blood tinged milk.

PRODUCT PROFILE

PVP-iodine is a stable complex of polyvinylpyrrolidone (PVP) and elemental iodine. The antimicrobial properties of PVP-iodine are...
related to non-complexed, freely mobile elemental iodine, I₂. The effective pH range of PVP-iodine is between 2.5 and 7 with a optimum range of 3 to 6 (Pharmaceutical and technical bulletin).

THERAPEUTIC MANAGEMENT

The affected quarters of all the 17 animals (three Jafferabadi buffaloes and fourteen Gir cows) were drained out completely and treated with antibiotics viz., penicillin, oxytetracyclin, gentamicin. enrofloxacin and ceftriaxone at standard therapeutic dose rates and intervals for 3 days, but none of the animals responded well to any of these antibiotics, and the mastitic condition became aggravated.

Treatment I

Affected quarters of six cows were drained out completely. The cows were administered 10 ml of 5% povidone-iodine solution (0.5% iodine, povindone solution) mixed with 500 ml 25% dextrose intravenously slowly 3 times on alternate days, and along with this, lavage of affected quarters was carried out by infusing 10% of providone-iodine (Betadine) dissolved in distilled water, slightly massaging the udder, and then draining the debris in the udder tissue twice a day. Out of these three cows, two successfully recovered within 4 days post treatment and two cows recovered after 6 days post treatment.

Treatment II

The three buffaloes and eight Gir cows which failed to respond to antibiotic treatment were treated with lavage of the affected quarters carried out by infusing 10% of povidone-iodine (5% povidone-iodine solution, 0.5% iodine) dissolved in distilled water. Slight massaging was done, and then the debris of udder tissues were drained out twice a day. Of these, nine animals successfully recovered within 6-10 days of post lavage treatment. However, two animals (one buffalo and one cow) remained unresponsive to this treatment.

RESULT AND DISCUSSION

According to Hamzaa et al. (2006) the treatment of mycotic infection is problematic as compared to the bacterial infection. Few antifungal drugs are available, and there are many side-effects and the possibility of resistance. For many years povidone-iodine has been used in humans and veterinary applications to quickly get rid of wide variety of bacteria, virus, fungi, protozoa and yeast on the skin. Its antimicrobial activity is a non-specific mode of action with no tendency to form resistance (International Specialty Products, 2011).

According to Loftsgard and Lindquist (1960), Candida spp. utilize nitrogen from penicillin and tetracycline antibiotics. The use of such antibiotics encourages establishment of the infection by damaging the mammary epithelium. Similarly, during this case study, it was observed that initially, the use of antibiotics worsened the condition of clinical mastitis. Chahota et al. (2001) documented that association of G. candidum with mastitis is greater in those patients which have been subjected to prolonged irrational antibiotic therapy, as is evident in this case.

During this study, six animals were completely recovered by using intravenous PVP-iodine along with 10% PVP-iodine intra mammary lavage, and eleven animals responded with only 10% PVP-iodine intra mammary lavage. Similarly
Blood et al. (1983) also stated that none of the animals responded well to antibiotic therapy, but treatment with iodine, either sodium iodide intravenously, organic iodides by mouth or iodine in oil as an intra mammary infusion might be of value.

Looking to the symptoms, non-response to antibiotics, aggravated condition after use of antibiotics and response to iodine treatment might be suggestive of fungal infection. Hence, animals suffering from clinical mastitis and not responding to antibiotic therapy may be considered for iodine treatment and simultaneous confirmation of fungal or any other organisms might be taken up.

REFERENCES


STUDIES ON THE EFFICACY OF POLYVINYL PYRROLIDONE (P.V.P.) IN PREVENTION OF INTRA-ABDOMINAL ADHESIONS IN BUFFALO CALVES (Bubalus bubalis)

K.K. Gupta¹, B.P. Shukla² and Rayees Ahmad¹

ABSTRACT

Efficacy of PVP as an antiadhesive in prevention of intra-abdominal adhesions in buffalo calves subjected to adhesions of transmural etiology was evaluated. Twelve clinically healthy male buffalo calves, aged 4 to 10 months and weighing 52 to 105 kg, were used to investigate haematobiochemical, gross and histomorphological aspects of peritoneal adhesions. Mean Hb% values showed non-significant (p>0.05) differences in both control and treatment groups at different time periods and between time periods. Mean TLC showed a significant increase (p<0.05) on day 1 in control group, which was non-significant in treatment group. Mean neutrophil (%) showed significant increase on day 1 in the control group, whereas it was non-significant in treatment group. Lymphocyte count showed a significant decrease on day 1 for the control and the treatment groups and a non-significant difference was observed between control and treated groups. Eosinophils, basophil and monocyte count showed non-significant changes in both the groups. Mean plasma fibrinogen values showed a significant (p<0.05) difference in both the groups at different periods and between control and treatment groups. Mean serum proteins did not differ significantly in both the groups. Mean serum creatinine values showed a significant increase in the control group on day 1. Mean serum CK and BUN values showed significant differences between control and treatment groups and also at different periods in both the groups.

Animals of the control and the treatment groups developed intra abdominal adhesions of varying degree and severity. In the control group, adhesion scores of +4 grade were observed in four out of six animals. However, in two animals, the adhesions were localized and dense (+2). Adhesions (+1 to +3) were also observed in three animals of the control group between the omentum and the sutured peritoneal incision. In the treatment group, there were almost no adhesions.

Histopathological assessment of sections of the ileum showed +3 and +2 serosal thickening with proliferation of fibrillary and invasion of inflammatory cells in the control group. In treatment (PVP) group, serosal thickening and the presence of inflammatory cells were not observed. These results indicated that there was less inflammation in the treatment group as compared to the control group.

Keywords: buffalo calves, prevention, polyvinylpyrrolidone, PVP, abdominal adhesions

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INTRODUCTION

Post-surgical adhesion formation is an everyday problem in clinical practice, a major cause of morbidity and expense, and an occasional cause of mortality; however, many surgeons have become inured to this problem and lack full awareness of its magnitude and consequences. There are several reasons for this. First, adhesive complications occur unpredictably up to decades after a procedure and often are treated by a physician or specialist other than the initial surgeon. Second, the etiology of adhesion formation remains incompletely understood and whatever knowledge has been gained, has not always been successfully defused from the laboratory to the operating theater. Lastly, traditional adhesion prevention modalities have accumulated a century-long track record of failure or limited applicability. Adhesion may be defined as abnormal attachment between tissue and organs (Backer, 1996) and may be classified as congenital or acquired (Ellis, 1983). Slatter (1985) and Wiseman (1994) defined adhesion as a fibrinous or fibrous band that forms an abnormal union between two or more surfaces that are normally lined with serosa.

Adhesions, which occur after 67% to 93% of abdominal operations, represent a major clinical problem, resulting in intestinal obstruction, infertility, pain and considerable economic loss. The magnitude and seriousness of the problem of adhesion have been underappreciated. Moreover, efforts to prevent or reduce adhesions have largely been unsuccessful, hindered by their empirical basis, the lack of good predictive models, and the biochemical complications of adhesiogenesis (Risberg, 1997). Polyvinylpyrrolidone (PVP) is a synthetic polymer. Its high molecular weight (MW=40,000) solution has been claimed to be effective with conflicting reports in preventing post operative adhesions and it has been recommended for intraperitoneal use in human beings at laparotomy (Ducans et al., 1988). On perusal of the above literature and the hypothesis advanced, it was thought worthwhile to study the efficacy of the drug PVP in minimizing adhesions in male buffalo calves with the objectives of evaluating and comparing the haematobiochemical alterations in control and treatment animals and evaluating the extent and grade of adhesions by gross and histopathological studies.

MATERIALS AND METHODS

The present study was conducted on 12 clinically healthy male buffalo calves aged 4-10 months and weighing between 52 to 105 kg. The animals were kept under standard managemental conditions for a week. All calves were dewormed with Ivermectin 1% 0.5 ml / 25 kg bodyweight subcutaneously. Calves were fed with green grass and dry fodder and wad access to fresh drinking water ad lib. The calves were randomly allotted to two groups of six animals each to evaluate efficacy of the agent PVP. In Group 1 (the control group), animals after creating model of small intestinal adhesion (For producing adhesions a piece of small intestine was selected and its mesenteric blood supply was ligated for 70 minutes and a loop of intestine was also tied at both ends. After 70 minutes, all the ligatures were removed. This period of ischaemia was already proved to be sufficient for producing adhesions of transmural etiology by Shukla (2001). No treatment was given. In Group 2 (the treatment group), 30 % solution of PVP was used during pre-, peri- and post-surgical stages. The animals were kept off feed for 24 h and water was
withheld for 12 h prior to surgery. Animals were restrained in lateral recumbancy and the site at the right paralumbar fossa was prepared for aseptic surgical intervention. Regional anaesthesia of the prepared right paralumbar fossa was achieved by infiltrating lignocaine HCL (2%) in inverted “L” block fashion. After taking all aseptic precautions, the abdominal cavity was entered through a 15 cm long posterior flank incision. An ileal loop, 60 cm in length was exteriorized and immediately one third solution of PVP was instilled over the bowel loop identified for inducing adhesions. All mesenteric blood vessels supplying to the ileal segment were ligated. The transmural occlusion at either end of this ileal segment was also done by applying file tags (Lundin et al., 1983 and Singh, 1999). This strangulated segment of bowel was returned to the peritoneal cavity and the laprotomy wound was closed temporarily by applying a layer of continous sutures to the skin using cotton thread.

After 70 minutes of ischaemic period, the strangulated segment was re–exteriorized. Again one-third of the prepared solution containing the PVP was instilled over the strangulated segment. All the sutures and file tags were removed after placing a single suture of chromic catgut No 2/0 as a marker in least vascular area of mesentery, the loop was finally returned to the abdominal cavity and the remaining portion (one third) of the PVP solution was again instilled over it. All animals were given parenterally amoxycillin and cloxacillin combination 6 mg/kg body weight by the intramuscular route twice daily for five consecutive post-operative days. The laparotomy wound in all the animals were dressed with betadine solution and skin sutures were removed on the 10th post-operative day.

Group 1: The animal of this group were not given any treatment to prevent adhesions and acted as control.

Group 2: The animals of this group were given 30% solution of PVP to prevent adhesions.

Parameters investigated

Haematobiochemical parameters: Venous blood samples from each animal were aseptically collected by jugalar venipuncture on day 0 prior to operation and on post operative days 1, 2, 3, 5, 10 and 12 using 18 gauge hypodermic needle. About 5 ml of blood was collected in the vials containing EDTA 2 mg/ml for TLC, DLC, Hb and fibrinogen estimation and 6 ml of blood was collected in a sterile centrifuge tube to separate the serum for estimation of biochemical parameters. All these haematological parameters were studied as per the procedure described by the Jain (1986). Among the biochemical parameters fibrinogen (refractometer method, Jain (1986)), total protein (Biuret method (Tietz, 1986), blood urea nitrogen (DAM method, Marsh et al., 1965), creatinine (alkaline picrate method, Bonses and Tausskey, 1945) and creatinine phosphokinase (CK Nac method (DGKC, 1977)).

Gross evaluation and categorization of adhesions

The animals were reopened on 12th postoperative day following the same anaesthetic procedure through a large incision parallel and 5 cm posterior to the previous incision. The peritoneal cavity, abdominal viscera, previous incision site and the ischaemic ileal loop were examined for degree of adhesions.

Grading of adhesions was done according to the criteria followed by Fredrick et al. (1986) as given below.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No adhesions</td>
</tr>
<tr>
<td>1 (+)</td>
<td>Localized flimsy adhesions</td>
</tr>
<tr>
<td>2 (+ +)</td>
<td>Localized dense adhesions</td>
</tr>
</tbody>
</table>
Figure 1. Control group, complete ischaemic strangulation of the ileal segment created by ligating mesenteric blood vessels with cotton thread.

Figure 2. Control group, gross appearance of strangulated ileal segment after 70 minutes of ischaemia. Note the distension of the ischaemic ileal loop.

Figure 3. Treatment group (PVP), gross appearance of strangulated ileal segment after 70 minutes of ischaemia with PVP solution instilled over it.
Histopathological evaluation

Samples of ileum for histopathological examination were collected by resecting the small portion of intestine, where the trauma was given for producing adhesions. Intestine was repaired by end to end anastomosis with the Schemedian technique and routine closure of laparotomy incision was performed. The lumen was washed with tap water and 10% formalin was poured. Finally the intestinal segment was ligated at either ends to be preserved in a wide-mouth bottle containing 10% neutral buffered formalin. For histomorphological studies, ileal tissue with or without adhesions from each fixed specimen was collected, processed, embedded in paraffin, cut in to 7 micron thick sections and stained with haematoxylline and eosine (H & E) and Van Gieson stain (Luna, 1986). The sections were examined for serosal reaction, collagen and fibrin deposition and also for infiltration of inflammatory cells and were graded +1, +2 and +3 depending upon mild, moderate and severe reactions respectively. The data of control and treatment (PVP) group was analysed statistically using CRD as described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Haematobiochemical parameters

No significant changes were noticed in haemoglobin concentration of either the control or the treatment groups. There was a slight increase (p>0.05) in Hb g% on day 1 post operatively, more in control animals than treatment animals. The increase in the Hb% might have been due to tissue alteration in acute inflammation (surgical trauma) and increased viscosity of the blood (Vegad, 1995), which was greater in the control group because no adhesive drug was used. There was no significant change in values of Hb% recorded from day 2 onward within each group and between the groups. Regarding TLC, there a was significant (p<0.05) rise in the value of TLC on first day post operatively in control group, which remained significant throughout the observation period. The increase in the value on the 1st day may have been due to an intense systemic response due to surgical trauma (Vegad, 1995; Benjamin, 2001). In the treatment group, there was no significant change in the values of TLC, showing a less intense inflammatory reaction. This might be due to the use of the anti adhesive drug in the animals of this group. Similar findings have also been recorded by Dabas (1991). As for DLC, increase in neutrophil count was significant (p<0.05) on the 1st and 2nd post operative days in the animals of the control group as compared to the base value; however, the values came down to normal and remained non-significant from day 3 to day 12. In treatment group, there was no significant changes in neutrophil count throughout the study period. The initial rise in the neutrophil count in the control group might have been due to a more intense systemic response to surgical trauma (Blood et al., 1983; Vegad, 1995). In the treatment group, the use of PVP might have reduced the intensity of inflammation causing non-significant changes in values. These findings are also supported by Dabas (1999). There was a significant (p<0.05) decrease in lymphocyte count on day 1 and day 2 post operatively, whereas in the treatment group, there was a decrease only on day 1. The rest of the values within each group and between the groups were non-significant. Initial lymphopenia with relative neutrophilia might be due to systemic response to
inflammation (Vegad, 1995). Similar findings were observed by Dabas (1991) in dogs. No significant changes were recorded in eosinophils, basophils and monocytes in either control or treatment group animals.

Regarding biochemical values, plasma fibrinogen increased significantly (p>0.05) on day 1 and day 2 in the control group and remained till day 5. The values came down (p<0.05) to the base values on day 10. In treatment group (PVP), the values also increased significantly (p<0.05) on day 1 and day 2 and came down to base values on the 5th post-operative day. The significant differences between control and treatment group from day 1 onwards post operatively and within group might have been due to acute inflammatory condition (Kaneko, 1989) and thereafter decrease in the intensity of inflammation resulted in decline of fibrinogen concentration as also observed by Singh (1999) in buffalo calves, Singh et al. (2001), in buffaloes and Shukla (2001) in cow calves, and Murphy et al. (2002) in dogs. In the PVP group change in values of fibrinogen concentration was much less as compared to the control group, which reflects the fact that inflammatory changes were minimal in this group, as also confirmed by gross and histopathological observations. Goldberg et al. (1980) revealed that significant serosal injuries are prevented by coating tissue with PVP solutions. Electron microscopy also showed a major reduction in tissue damage following the application of PVP solution. The serum total protein concentration in the control group was not affected significantly at any stage of observation. In the treatment group, the serum total protein concentration decreased significantly on day 1, whereas at subsequent intervals, there was general trend of decline, when compared with base value. The pre-operative mean concentration was highest in both the control and treatment groups. Singh (1999) in buffalo calves, Rathore (2000) and Shukla (2001) in cow calves observed similar findings. The decrease in serum total proteins has been attributed to protein catabolism in the body (Kaneko, 1989). The decrease in serum total protein in the present study might be due to increase in peritoneal fluid plasma proteins. The mean serum creatinine concentration values in the control group increased significantly (p<0.05) on day 1. From day 2 onwards, there was a gradual decrease towards the base value till day 12. In the treatment (PVP) group, mean values showed a declining trend from day 1 to day 12. However, there was no significant difference between periods. Kaneko (1989) stated that the quantity of creatinine which formed did not differ due to a single factor but was influenced by number of factor such as dietary intake, rate of synthesis of creatinine and muscle mass. In the present study, the increase in the serum creatinine value in the control group on day 1, was in agreement with the findings of Yadav et al. (1987) and Shukla (2001). Singh (1999) observed that value of serum creatinine decreased after 24 h of ischaemia in the control and hyaluronic acid treatment groups. Decrease in serum creatinine value in the treatment (PVP) group observed on day 1 was in agreement with the findings of Singh (1999) in buffalo calves and Shukla (2001) in cow calves. In the control animals, the values of creatinine phosphokinase (CPK) increased significantly on day 1 and remained higher till day 3 post-operatively. Further, the values became insignificantly different from day 5 onward. The higher values of CPK in the control group suggest its association with adhesions. In the control group, values reached peak level on day 1 and then gradually decreased; however, there was no significant difference in values in different periods of time. The increase in CPK activity 24 h
after surgery might have been the result of muscle damage due to tissue ischaemia (Smith and Healy, 1968: Vad den Hende et al., 1976 and Akhtas et al., 1994). Shukla (2001) also recorded dramatically increased CPK values on day 1 in all the groups of cow calves. The significant rise in CPK values in the control group might have been due to intense inflammatory response leading to muscle damage due to surgical trauma and ischaemia. In the treatment animals, where PVP was used, although the values increased but not to the significant level as compared to the base value, indicating the role of PVP in minimizing the inflammatory response to surgical trauma, ischaemia and tissue dehydration. As far as blood urea nitrogen (BUN) was concerned, its values in both the groups increased significantly ($p<0.05$) on day 1 post-operatively, and thereafter, showed a declining trend and reached the normal level on day 12; however the increase in the level of values was more significant in control group. The greater increase in the level of BUN in the control group than the treatment group might have been due to maximum inflammation and tissue protein catabolism caused by ischaemia and also because no treatment was given to this group, which was also supported by Shil (1963), Dossetor (1966), Singh (1999) and Shukla (2001).

**Gross evaluation**

For gross evaluation of adhesions, the animals were reopened on the 12th post-operative day following the same anaesthetic procedure through large incision parallel and 5 cm posterior to the previous laprotomy site. In the control group, all the animals developed adhesions. The widespread, dense adhesions (+4) were noticed within the ischaemic loop in four out of six animals, and localized and dense (+2) adhesions were observed in two animals of this group. Adhesions between affected and adjacent loops of the bowel were observed in the form of widespread dense bands of adhesions (+4) in two animals. Adhesions between the omentum and sutured peritoneal incision were observed in four animals (No. 1, 2, 4 and 5). The adhesions were graded as flimsy, localized to widespread flimsy (+1 to +3). Swanswick et al. (1973), after studying the healing of peritoneal defects and peritoneal midline incision in horses, concluded that the suturing of the peritoneum resulted in a higher incidence of adhesions after surgery than the non-suturing of it. Extensive fibrosis and adhesions formation of ischaemic segment was exhibited in adult horses after 1 month (Sullins et al., 1991).

Similar findings have been observed by Thakker (1994), Singh (1999), Shukla (2001) in cow calves and Singh (2003) in dogs in the affected ileal segment.

In the treatment group, the animals showed almost 0 adhesions as compared to the control group, and clumping of the ischaemic loop was absolutely absent. A thin transparent film of adhesions (+1) was present over the entire ischaemic loop in one animal (No. 5) only. From the above findings, it can be concluded that polyvinylpyrrolidone (PVP) is a very effective anti-adhesive drug in minimizing peritoneal adhesions, both in terms of degree and severity.

**Histopathological parameters**

The histopathological findings of the present study substantiated the reports of other workers. The serosa indicated degree of inflammation and tissue repair. This was in agreement with Ellis (1962) who opened the adhesions stimulated vascular grafts eliciting fibroblastic response, leading to the formation of scar tissue. In the treatment
Figure 4. Control group, wide spread, dense adhesions (+4) between affected and adjacent loop of bowel.

Figure 5. Control group, wide spread, flimsy adhesions (+3) between the omentum and sutured peritoneal incision.

Figure 6. Control group, localized and dense adhesions (+2).
Figure 7. PVP group, no adhesions within ischaemic ileal loop and also with adjacent bowel.

Figure 8. PVP group, 0 adhesions in an ischaemic ileal loop.

Figure 9. Section of ileum showing +3 thickening of serosa (arrow) [Control, H&E x240].
Figure 10. Section of ileum showing +2 thickening of serosa (arrow) [Control, H&E x240].

Figure 11. Section of ileum showing minimal thickening of serosa (arrow) [PVP group, H&E x240].

Figure 12. Section of ileum showing a normal presence of collagenous material between serosa and muscularis mucosa (arrow) [PVP group, VG x].
group (PVP), all animals showed minimal serosal thickening. No inflammatory cells and proliferating blood vessels were seen in the serous and subserous layer. This findings established the fact that PVP treatment group showed less inflammation. PVP tissue percoating has been proven to be effective in animal studies for prevention of peritoneal adhesions (Duncans et al., 1988 and Yaacobi et al., 1993).

REFERENCES


*Continue on page 135
ABSTRACT

The present study was primarily carried out to trace the behavioral, physiological and adrenal changes during the first stage of labour in cases of dystocia, either in parturient buffalo heifers or cows and their clinical significance. Out of 118 investigated buffalo herds, 40 cows (dystocia = 20 and eutocia = 20) and 40 heifers (dystocia = 20 and eutocia = 20) were used in this study. Cows approaching actual birth were transferred to a calving box with straw bedding. The behavior of the studied animals was recorded from the time that the animal was moved to the calving box until the emergence of the foetal limbs in the vulval lips. If no progress in parturition was observed through two hours after the rupture of fetal sacs, a vaginal examination was carried out, and these cases were considered as suffering dystocia and needed help. By the emergence of the foetal limbs in the birth canal, animals were examined clinically to determine their average pulse rate, respiratory rate and their body temperature. Blood samples were collected to determine its cortisol level. Results of the present study indicated that dystocia was accompanied by increase in pain stress, disturbed physiological status of cows and heifers and cortisol levels. These more drastic changes in dystocia than eutocia should be included in the practical monitoring system of parturient animals. Moreover, observation and measuring of behavioral, physiological and adrenal elements should highlight the possibility of a calving problem during the first stage of labour.

Keywords: buffaloes, dystocia, behavior, physiology, adrenal

INTRODUCTION

The birth of a healthy calf is one of the main basic requirements for economic efficiency in cattle and buffaloes production. Calves from difficult parturitions show a clearly increased mortality during the first 24 h after parturition (Patterson et al., 1987; Wittum et al., 1993; Nix et al., 1998). Therefore, it is of particular interest to detect difficulties during parturition as early as possible.

The parturition process is divided into three phases. The first stage of labour begins with the dilatation of the cervix and ends with the rupture of the choioallantois in the vagina (Jackson, 1995). In the second stage of labour, the calf is visible in the rime vulvae and is expelled, followed by the foetal membranes in the third stage.

The parturient behavior in most animals during the 1st stage of parturition begins with

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exploration of the surrounding environment of the parturient box accompanied by extensive olfactory check of the ground with pushing of the straw with their heads and front limbs to form a resting place. With the advancing of the first stage of parturition, most parturient animals lie down and get up several times, scraping the floor, looking back at the abdomen. Rupture of the foetal sac leads to an apparent relief and some of the parturient animals begin to lick up the escaped amniotic fluids (Wehrend et al., 2006).

In fact, there are some earlier studies carried out on the behavior of parturient cows and heifers during the antepartal period as well as the expulsion phase (Dufty, 1971; O’Mary and Hillers, 1976; Kharche et al., 1982; Berglund et al., 1987; Mukasa and Mattoni, 1990; Wehrend et al., 2005); however, there are hardly any studies, especially on buffaloes, about their behavior during the first stage of labour.

As the incidence of dystocia among buffalo herds is high, and so, the mortality rate of the delivered calves is also high, the aim of the present study is to observe the behavioral, physiological and adrenal changes of buffalo cows and heifers during the first stage of parturition and how to use these changes as a sign of a calving problem during parturition to solve it as early as possible.

MATERIALS AND METHODS

Animals used and management

Eighty parturient buffalo cows and heifers of total 118 members of investigated herds were used in this study during their first stage of parturition (20 heifers with eutocia; 20 heifers with dystocia; 20 multiparous cows with eutocia and 20 multiparous cows with dystocia). The average age of the experimented heifers and cows at the time of parturition was 3 and 7 years, respectively. Each animal was separated from the herd one week before expected parturition and was kept in a separate well-ventilated and well-lighted house with freely available water and food. All separated animals were checked continuously for externally visible signs of parturition. Cows and heifers being in the first stage of parturition (edematous vulva, relaxation of pelvic ligaments, leakage of colostrums from the teats and appearance of the amniotic sac in the cervix through vaginal examination), were transferred to a calving box with straw bedding.

Behavioral observations

Behavior of the experimental animals was recorded and analyzed according to Wehrend et al. (2006). The parturient animal was observed from a distance of 5 meters outside the calving box starting from the time that the animal was moved to the calving box until the emergence of the foetal limbs in the vulval lips. The recorded behavior was analyzed as follows:

Exploratory behavior, degree of restlessness (degree 1: calm, degree 2: restless with frequent change of activities, degree 3: very restless with permanent interruption in case of slight environmental stimuli), lying down and getting up, scraping on the floor with the forefeet, pawing, ingestion of the amniotic fluid, looking back at the abdomen, hunching of the back, vocalization, grooming and rubbing against the wall. If no progress in parturition was observed for two hours after the fetal sacs had ruptured, a vaginal examination was carried out, and these cases were considered as dystocia where the living calf could be pulled out after correction of its position and posture.
Physiological measurements

By the emergence of the foetal limbs in the rim vulvae, experimented animals were examined clinically according to Blood and Henderson (1974) and Blood and Radostits (1990) to determine their average pulse rate, respiratory rate and their body temperature.

Blood sampling and analysis

By the emergence of the foetal limbs, blood samples were collected from the jugular vein of the experimented animals. Samples were allowed to coagulate at room temperature, and the serum was separated by centrifugation. The sera were frozen at -20°C till further analysis to determine its cortisol level using TD x FLx system with fluorescence polarization and competitive binding technique according to Dandliker and Feigen (1970) and Dandliker and Saussure (1973).

Statistical analysis

Statistical analyses of the collected data were carried out according to procedures of completely random design, SAS (1995).

RESULTS AND DISCUSSION

Results are presented in Tables 1-5 and Figure 1.

Studying an animal’s behavior plays an important role in measuring its welfare. As the incidence of dystocia among buffalo herds is high, and so, the mortality rate of the delivered calves is also high, studying the parturient behavioral pattern of buffalo cows and heifers during the first stage of labour is very important in order to be able to use the changes of this pattern as a sign of impending dystocia. The findings of this study indicated that, as a result of the presence of excessive apprehension, most of the experimented heifers and cows that suffered form dystocia showed very prominent disturbances in their parturient behavioral pattern, especially restlessness, pawing the floor, looking back at the abdomen and hunching of the back. These findings agreed with those of Kharche et al. (1982); Mukasa and mattoni (1990); Dargartz et al. (2004); Wehrend et al. (2006). These parameters can be assessed as signs of pain and should be included in the practical monitoring system of parturient animals. However, the observation of these behavioral elements should highlight the possibility of a problem during the first stage of labour, and so bring into consideration the necessary precautions and solutions to help and save lives of both the mother and the living calf. The data collected during the present study showed the effect of the state of parturition on pulse rate, respiratory rate and body temperature of the investigated animals. These results revealed that both pulse and respiratory rates of heifers and cows with dystocia were significantly increased during the 1st stage of parturition compared with those with eutocia; however, their measured rectal temperatures were not significantly affected. This finding indicated that parturition was accompanied with some physiological disturbances that were more prominent during dystocia than eutocia as a result of some changes in the animal’s body to meet the more stressful situation of dystocia (Banerjee, 1982; Berglunmd et al.; 1987; Radostits et al., 1994). The data in the present study illustrated that cortisol level during the 1st stage of parturition was significantly higher in the serum of parturient animals suffering from dystocia. The significant increase in the blood cortisol level of parturient heifers and cows that suffered from dystocia
Table 1. Behavioral pattern of heifers with eutocia or dystocia during the 1<sup>st</sup> stage of labour.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Eutocia</th>
<th>Dystocia</th>
<th>“p” Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exploratory behavior</td>
<td>20 100</td>
<td>20 100</td>
<td>NS</td>
</tr>
<tr>
<td>Restlessness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degree 1: calm</td>
<td>6 30</td>
<td>0 0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Degree 2: restless</td>
<td>11 55</td>
<td>10 50</td>
<td>NS</td>
</tr>
<tr>
<td>Degree 3: very restless</td>
<td>3 15</td>
<td>10 50</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lying down and getting up</td>
<td>16 80</td>
<td>20 100</td>
<td>NS</td>
</tr>
<tr>
<td>Scraping on the floor</td>
<td>8 40</td>
<td>6 30</td>
<td>NS</td>
</tr>
<tr>
<td>Pawing</td>
<td>10 50</td>
<td>18 90</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ingestion of amniotic fluid</td>
<td>5 25</td>
<td>3 15</td>
<td>NS</td>
</tr>
<tr>
<td>Looking back at the abdomen</td>
<td>7 35</td>
<td>15 75</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hunching of the back</td>
<td>11 55</td>
<td>19 95</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vocalization</td>
<td>12 60</td>
<td>13 65</td>
<td>NS</td>
</tr>
<tr>
<td>Grooming</td>
<td>0 0</td>
<td>0 0</td>
<td>NS</td>
</tr>
<tr>
<td>Rubbing against the wall</td>
<td>0 0</td>
<td>0 0</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = Non-significant.

Table 2. Behavioral pattern of cows with eutocia or dystocia during the 1<sup>st</sup> stage of labour.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Eutocia</th>
<th>Dystocia</th>
<th>“p” Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exploratory behavior</td>
<td>20 100</td>
<td>20 100</td>
<td>NS</td>
</tr>
<tr>
<td>Restlessness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Degree 1: calm</td>
<td>8 40</td>
<td>0 0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Degree 2: restless</td>
<td>10 50</td>
<td>11 55</td>
<td>NS</td>
</tr>
<tr>
<td>Degree 3: very restless</td>
<td>2 10</td>
<td>9 45</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Lying down and getting up</td>
<td>17 85</td>
<td>20 100</td>
<td>NS</td>
</tr>
<tr>
<td>Scraping on the floor</td>
<td>9 45</td>
<td>8 40</td>
<td>NS</td>
</tr>
<tr>
<td>Pawing</td>
<td>8 40</td>
<td>16 80</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ingestion of amniotic fluid</td>
<td>3 15</td>
<td>1 5</td>
<td>NS</td>
</tr>
<tr>
<td>Looking back at the abdomen</td>
<td>5 25</td>
<td>13 65</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hunching of the back</td>
<td>9 45</td>
<td>19 95</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vocalization</td>
<td>7 35</td>
<td>9 45</td>
<td>NS</td>
</tr>
<tr>
<td>Grooming</td>
<td>0 0</td>
<td>0 0</td>
<td>NS</td>
</tr>
<tr>
<td>Rubbing against the wall</td>
<td>0 0</td>
<td>0 0</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = Non-significant.
Table 3. Health status measurements of heifers with eutocia or dystocia during the 1st stage of labour.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Eutocia</th>
<th>Dystocia</th>
<th>“P” Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse rate (No./min)</td>
<td>72±1</td>
<td>86±1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Respiratory rate (No./min)</td>
<td>28±1</td>
<td>40±1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Body temperature (ºC)</td>
<td>38.6±0.1</td>
<td>38.7±0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = Non-significant.

Table 4. Health status measurements of cows with eutocia or dystocia during the 1st stage of labour.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Eutocia</th>
<th>Dystocia</th>
<th>“P” Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse rate (No./min)</td>
<td>70±2</td>
<td>82±1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Respiratory rate (No./min)</td>
<td>28±1</td>
<td>38±1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Body temperature (ºC)</td>
<td>38.7±0.1</td>
<td>38.9±0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = Non-significant.

Table 5. Average serum cortisol level (µg/100 ml) of heifers and cows with eutocia or dystocia during the 1st stage of labour.

<table>
<thead>
<tr>
<th>Item</th>
<th>Eutocia</th>
<th>Dystocia</th>
<th>“P” Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heifers</td>
<td>0.74±0.01</td>
<td>0.98±0.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cows</td>
<td>0.69±0.01</td>
<td>0.91±0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Figure 1. Serum cortisol level of heifers and cows during 1st stage of labour with eutocia or dystocia.
indicated an incidence of a powerful acute stress due to this condition followed by a greater outpouring of ACTH which in turn caused the adrenal cortex to increase its secretion of glucocorticoids including cortisol (McDonald, 1969; Burchfi eld et al., 1980; Stephens, 1980; Kindahl et al., 2002).

CONCLUSION

In conclusion, the first stage of labour in parturient buffalo heifers and cows that suffered from dystocia was accompanied with more obvious changes in their behavioral, physiological and adrenal parameters. These changes can be assessed as signs of more pain and a more stressful condition and should be included in the practical monitoring system of parturient animals to highlight the possibility of a problem during labour and so bring into consideration the necessary precautions and solutions to help and save lives both of the mother and the living calf.

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*Continued from page 128


Yaacobi, Y., A.A. Israel and E.P. Goldberg. 1993. Prevention of postoperative abdominal adhesions by tissue pre-coating with
The aim of this study was to monitor and compare the ovarian follicular dynamics and serum progesterone profiles in heifers (HE) and buffalo cows (BC) during the Ovsynch protocol. A total of 17 apparently healthy female buffaloes received treatment regimen (8 HE and 9 BC). Twenty-two buffalo cows were used as reference for conception rate, control heifers and control buffalo-cow (CHE, n=10 and CBC, n=12). The heifers and buffalo-cows were cyclic. All treated animals were injected with GnRH on Day 0, PGF$_{2a}$ on day 7, and GnRH on day 9, and AI 16 h later. Ovarian structures were monitored daily by ultrasound and blood samples were collected for progesterone (P$_4$) analysis. All the heifers (8) and five of the cows had F>8 mm (LF) at the first GnRH injection. The first GnRH injection resulted in ovulation in seven HE (87.5%) and all five BC (100%) which had LF. Following the second GnRH, ovulation occurred in 100% of the HE and 88.8% of the BC. Ovulation began earlier in the BC (10.41 ± 7.6 h) following the second GnRH and extended for longer (22.6 ± 5.4 h) in the HE. The average P$_4$ concentrations of the HE were slightly greater than those of the BC on day 7 (P = 0.04). The conception rate in the HE was 62.5% (5/8) and was 60% (6/10) in the CHE, while only 11.11% (1/9) in the BC and 58.3% (7/12) in the CBC. It is suggested that the unsatisfactorily low conception rate in buffalo-cows compared to heifers may be attributable for the early ovulation and sub-functional CL.

**Keywords**: ovulation synchronization, follicular dynamics, P$_4$ level, Ultrasound, buffalo parity

**INTRODUCTION**

The water buffalo is used in many countries including Egypt as a source of milk and meat. The population of buffaloes in the Asian and Mediterranean areas is about 150 million, and in Egypt about 3.7 million (Borghese, 2004). Silent heat and long calving interval have been recognized as a major cause of infertility and low productivity in buffaloes. Seasonality in Egyptian buffaloes is not clear. The productivity of domesticated buffaloes is limited for reasons like inbreeding, feeding and health care, but the major problem seems to be infertility, which is much higher than that in cattle (Danell, 1987, Abol-Roos and Gaffar, 2000). Postpartum anestrus in buffaloes is responsible for long calving interval (Borghese et al., 1993; Campanile et al., 1993). Under typical management, upon reaching puberal weight and age, buffalo heifers are housed with female adult

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buffaloes for either natural mating or AI. AI has a significant contribution to the genetic improvement in cattle and has the potential to improve the genetic characters in buffaloes. Widespread use AI in buffalo is still limited due to relatively low expression of estrus behavior in buffalo (Seren et al., 1993; Ohashi, 1994). Variable duration of estrus (4-64 h) and difficulty in predicting time of ovulation make AI application in buffaloes difficult (Baruselli, 2001). This consideration indicated a need for estrus synchronization using fixed-time insemination for implementation of breeding programs in buffaloes (Presicce et al., 2004, Ali and Fahmy, 2007). Estrus synchronization protocols, largely derived from cattle, have yielded variable results in buffalo (Singh et al., 1984, Barile et al., 1997, Zicarelli et al., 1997, Neglia et al., 2003, Presicce et al., 2004, Campanile et al., 2007). Although failure of timed ovulation in synchronized buffaloes has been suggested as an important cause of poor fertility (Hattab et al., 2000, Baruselli, 2001), yet it has not been fully studied. There is limited use of artificial insemination (AI), due to a low conception rate following estrus synchronization (Zicarelli et al., 1997). There are some reports of ovarian follicular dynamics in buffalo (Manik et al., 1994; Taneja et al., 1995a, b, and 1996, Baruselli et al., 1997) but a critical comparison of the effects of age and parity on ovarian follicular dynamics and hormonal profiles has not been conducted on a large scale (Presicce et al., 2004). The Ovsynch program, developed in cattle (Pursley et al., 1995), has been applied in nulliparous and multiparous (Presicce et al., 2004), cyclic and non-cyclic buffaloes (De Rensis et al., 2005, Ali and Fahmy, 2007). However, application of ovulation synchronisation program in Egyptian buffalo is not wide spread. Characterization of follicular turnover using ultrasonography and hormonal profile in heifers and buffaloes during the ovsynch program under local conditions in Egypt has not been critically studied. The aim of the present study was to monitor and compare the ovarian follicular dynamics and serum progesterone profile in heifers and post-partum Egyptian buffalo-cows (Bubalus bubalis) during the ovulation synchronization protocol.

**MATERIALS AND METHODS**

**Animals and management**

Seventeen Egyptian river buffalos (Bubalus bubalis) were included in this study. Out of them, eight were heifers (HE), 24 ± 0.8 months old (range: 19-27 months) weighing 350-420 kg. The other nine were buffalo-cows (BC) having one to five births before, weighing 415-530 kg, and at 45-65 days post-partum. Ten heifers (CHE) and twelve buffalo-cows (CBC) inseminated on natural estrus using the same semen within the same season were used for conception rate reference. The treated group gave normal parturition and were cyclic. The animals were housed in an open yard on the animal farm of Al-Azhar University, Assiut-Campus. The buffalo-cows were milked twice daily and fed on 40% forage dry matter (Egyptian clover) and 60% concentrate mixture. Wheat straw was also available ad libitum. This ration provided 14% CP and 67% TDN. The experiment was conducted during Dec-Feb (57.29 ± 2.3% relative humidity and 13.45 ± 0.8ºC maximum atmospheric temperatures). A body condition scoring (BCS) system from 1 = very thin to 5 = very fat was evaluated for each cow (Edmonson et al., 1989). Only cows between 2.5 and 3.5 BCS were included.
Experimental design

Before starting the Ovsynch program, the reproductive tract of all the HE and BC was examined rectally and ultrasonographically to record the ovarian and uterine findings for at least one cycle for each animal. The examination started after day 25 from parturition in BC. In the control group, the routine rectal palpations were done before insemination. No significant differences were detected between groups in BCS or the days from parturition to beginning of treatment.

Ovsynch program

The HE and BC buffalo cows were treated on day 0 (1st day of the program) with 100 mg GnRH im, (Buserelin, Receptal®, Intervet International B.V., Boxmeer, Holland). Seven days later (Day 7), 25 mg PGF2α (Dinoprost, Lutalyse, Pfizer, Pharmacia and Upjohn Company, NY, USA) was administered im. Forty-eight hours later (Day 9), the animals received a second dose of 100 mg GnRH im. Timed AI was performed for all cows at 16-21 h following the second GnRH treatment, with frozen-thawed semen from a buffalo bull of known high fertility.

Ultrasound examination

Ovarian structures of all animals in the treated group were monitored ultrasonographically using a real-time, B-mode, diagnostic scanner equipped with a transrectal 5/7.5 MHz linear array transducer (Hitachi, EUB-405B, Japan). Ultrasound examinations were performed once daily from days 0 to 9, and each 12 h thereafter until ovulation or for a maximum of 48 h. All follicles >3 mm and CL were measured, and mapped individually for each cow. Ovulation was considered to have occurred when a large growing antral follicle that had been identified and followed for several days was no longer observed. Emergence of a follicular wave was defined as occurring on the day on which the retrospectively identified dominant follicle was 4 mm (Ginther et al., 1997). Follicle luteinization was considered when a follicle did not ovulate, instead lutein tissue gradually developed and was detected as echogenic ring that increased in thickness later and filled the whole follicular antrum. The CL was examined and an image of the largest cross-sectional area was estimated. Luteal regression following PGF2α treatment was considered when P4 concentration was <1 ng/ml. The following ovarian characteristics were determined and compared between groups: (1) ovulation rates after the first and second GnRH treatments; (2) diameter of the ovulatory follicles; (3) interval from treatment to emergence of a new follicular wave after the first GnRH treatment; (4) number and diameter of the CL; (5) luteal regression rate after PGF2α treatment. Pregnancy diagnosis was performed by ultrasonography 30 days after AI. The CR was determined and compared between groups.

Serum hormonal analysis

Blood samples were collected from the jugular vein of the treated group into non-heparinized tubes at days 0, 2, 4, 7, 9 and 10 of the program. The samples were transported in an ice box to the laboratory within 20-30 minutes, centrifuged at 3000 rpm for 20 minutes, and the serum was stored at -20°C until analyzed for P4. The P4 concentration was determined using direct ELISA technique. Kits were provided by Diagnostic System Laboratory Co. (DSL, Catalogue No. 3900, USA). The coefficient of variance of intra- and interassay were 4.8 and 9.2%, respectively. The sensitivity of the assay was 0.12 ng P4.
Statistical analysis

The data were presented in mean ±S.E.M.; statistical analysis was carried out using SPSS program, version 10.0. Differences in ovulation rates after GnRH treatment, luteal regression rates after PGF$_{2a}$ treatment, and CR between HE and CHE heifers; BC and CBC cows were evaluated by $\chi^2$-test. A t-test was used to compare groups for follicle and CL diameters within the examination dates, and the interval from treatment to ovulation and interval to wave emergence. Differences among the HE, CHE, BC and CBC groups in the serum P$_4$ level were evaluated by ANOVA. Level of significance was set at P < 0.05.

RESULTS

Follicle turnover

At the first GnRH injection, the mean number of the small follicles (2-5 mm) in the HE was slightly lower than that in the BC (65 versus 73). The mean number of small follicles in the HE increased after GnRH injection more than that in the BC (P < 0.05). The mean number of medium-sized follicles (5-8 mm) was nearly equal in both groups at the time of the first GnRH injection (11 in HE Vs 10 in BC), and then increased in both groups with larger number in the HE than the BC, but the differences were not significant (P= 0.3) in the course of treatment. The number of the large follicles (>8mm) at the first GnRH injection was higher in the HE (7LF) than in the BC (3LF) and stayed higher in the HE than in the BC (Figure 1) especially at 10th day of the program (8 vs. 4 LF).

Ovarian response to First GnRH

The collective ovarian response of the HE and BC buffaloes to the Ovsynch program is summarized in Table 1. The proportion of the heifers that ovulated to the first GnRH treatment was 87.5 and that for the BC group was 100% (P = 0.88). Follicle luteinization was observed only in one follicle (11.1%) in the HE group. A new follicular wave was recruited in all heifers after nearly two days (51.0 ± 3.4 h); and in eight out of nine cows a new dominant follicle developed within nearly the same period as in heifers (52.6 ± 2.4 h) (Table 1).

Ovarian Findings on day 7

At day of PGF$_{2a}$ injection (Day 7), three HE had single CL and five had double CL; two of the five were already present at the time of the first GnRH injection and the others resulted from the ovulation of LF. One buffalo cow only had double CL, and four others had single CL. The mean diameter of the CL was significantly greater in the BC (P = 0.003). A follicle larger than 8 mm in diameter was detected in all the heifers (8/8) and in five of the nine buffalo cows (55.5%). The mean diameter of the LF2 was significantly larger in the BC than in the HE (10.11 ± 0.59 vs. 12.90 ± 0.18 mm). The mean growth rate of the LF between days 0 and 7 was higher (P = 0.03), and the maximum diameter was larger (P = 0.01) in the BC.

Ovarian response to the PGF$_{2a}$

Luteolytic responses to PGF$_{2a}$ treatment were 87.5% and 20.0% for the HE and BC which had corpora lutea at PGF$_{2a}$ injection, respectively. In the HE, the CL regressed from an average diameter of 14.85 ± 0.72 mm on day 7 to 10.33 ± 0.21 mm on day 8, and 9.14 ± 0.7 mm on day 9. In the BC cows, the CL decreased from 16.20 ± 1.20 mm on day 7 to 12.46 ± 0.5 mm on day 8, and 9.06 ± 0.6 mm on day 9. The diameters of the dominant follicles were 10.68 ± 0.69 mm and 11.66 ± 0.91
Table 1. Ovarian response of the heifers (HE) and buffalo-cows (BC) to the Ovsynch program.

<table>
<thead>
<tr>
<th>Ovarian Findings</th>
<th>Heifers (HE, n=8)</th>
<th>Buffalo-Cows (BC, n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Findings at first GnRH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animals had LF (n)</td>
<td>8/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5/9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LF number (n)</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LF diameter (mm)</td>
<td>9.5 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.8 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MF number (n)</td>
<td>11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF number (n)</td>
<td>65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CL (n)</td>
<td>4/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4/9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CL size (mm)</td>
<td>15.48 ± 1.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.90 ± 1.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Response to first GnRH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovulation (n)</td>
<td>7/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5/5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Time to ovulation (h)</td>
<td>48.7 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.2 ± 5.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>OvF size (mm)</td>
<td>10.5 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.4 ± 4.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F atresia</td>
<td>11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F lutenization</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>FW (n)</td>
<td>8/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8/9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Time to FW (h)</td>
<td>51.0 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.6 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FGR (days 0-7, mm day&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.57 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.86 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Findings at PGF&lt;sub&gt;2α&lt;/sub&gt; (Day 7)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL (n)</td>
<td>3/8 had 1 CL</td>
<td>4/9 1 CL</td>
</tr>
<tr>
<td>CL size</td>
<td>14.85 ± 0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.20 ± 0.95&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Animals had LF</td>
<td>8/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5/9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LF number (n)</td>
<td>10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>LF diameter (mm)</td>
<td>10.11 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.90 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FGR (days 0-7, mm day&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.57 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.86 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Response to PGF&lt;sub&gt;2α&lt;/sub&gt; (Day 9)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luteolysis (n)</td>
<td>8/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1/5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Animals had LF (n)</td>
<td>8/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7/9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>LF (n)</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F size (mm)</td>
<td>10.68 ± 0.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.66 ± 0.91&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FGR (days 7-9, mm day&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.59 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.58 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Respose to second GnRH (Day 10)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovulation (n)</td>
<td>8/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8/9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Interval to ovulation (h)</td>
<td>22.6 ± 5.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.41 ± 7.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>OvF size (mm)</td>
<td>10.94 ± 0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.64 ± 0.89&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Second CL size</td>
<td>15.45 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.7 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(mm, developed at D7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR (n)</td>
<td>5/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1/9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CR %</td>
<td>62.5±%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.1±%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values in means ± S.E.M. F: follicle; CL: corpus luteum; Lut F: luteinized follicle; FW: follicular wave; FGR: follicle growth rate; OvF: ovulatory follicle; CR: conception rate; day 0: day of first GnRH; day 7: day of PGF2α. Values with different letters (a and b) differ significantly.
Figure 1. Follicular population in heifers (n=8) and in buffalo cows (n=9) on both ovaries (Mean ± S.E.M., small follicle 2-5 mm, medium follicle 5-8 mm, large follicle >8 mm.)
mm in the HE and the BC, respectively.

**Ovarian response to the second GnRH**

In response to the second GnRH injection, the ovulation rate was 100% in the HE and it was 88.8% in BC. Ovulation time averaged 22.6 h (range 16-36 h) and 10.4 h (range 6-24 h) in the heifers and the buffalo cows, respectively (P = 0.01). The mean diameter of the CL developed at day 7 of the program was 15.45 ± 0.8 mm and 19.7 ± 1.3 in the HE and the BC, respectively (P = 0.03).

**P₄ concentrations**

Changes in the P₄ levels during the program are illustrated in Figure 2. P₄ level slightly increased up to 4th day and then sharply increased at 7th day. The average P₄ concentrations of the HE were greater than those of the BC on day 7 (P = 0.04). Two days after PGF₂α treatment (day 9), mean P₄ levels dropped in the treated groups In general, there was a positive correlation at day 0 of the program (r = 0.6, P = 0.005) between the diameter of the CL and the level of P₄ in the serum.

**Conception rate**

In the treated group, five of the eight HE (62.5%) and only one of the nine BC (11.11%, 1/9) had conceived after one insemination (P = 0.01). In the control heifer group, six out of 10 CHE (60.0%) and seven out of 12 CBC (58.3%) had conceived after the first insemination with straws from the same semen.

**DISCUSSION**

This study aimed to describe the differences between the heifers and buffalo cows in response to different steps of the Ovsynch program. In this program, each animal received three injections. The first GnRH was designed to cause the ovulation of the large functional follicle and to induce a new

---

Figure 2. Progesterone levels in serum of the heifers (n = 8) and the buffalo cows (n = 9) treated with the Ovsynch program, in comparison to the untreated control group (control heifers n = 10, control cows, n=12). Values with different letters (a, b) differ significantly (P < 0.05).
follicular wave. The second injection of PGF$_{2\alpha}$ was to increase the percentage of animals synchronized by lysis of both the cyclic CL and that resulting from ovulation of LF1 (Pursley et al., 1995).

The study showed that 87.5% of the HE and 55.5% of the BC responded by ovulation of the DF1 after the first GnRH injection. In previous studies, ovulation rate of approximately 86% was recorded in cyclic buffaloes (Rao and Venkatramiah, 1991, De Araujo Berber et al., 2002) and about 90% in cyclic and 50% in non-cyclic buffaloes (Negila et al., 2003, Ali and Fahmy, 2007) and 82-90% in cyclic cattle (Pursley et al., 1995, Wiltbank, 1998, Frike et al., 1998) following the first GnRH administration. The results in this work in coincide with those reported in buffaloes by Negila et al. (2003) and disagree with those recorded in cattle (Pursley et al., 1995, Hussein, 2003). The difference between the results recorded in this work and other works may be due to the fact that the BC had a small number of DF1 at the time of first GnRH injection. The time of ovulation after GnRH injection depends mainly on the DF1 diameter at the time of injection (Wiltbank, 1998, Hussein et al., 2002, Hussein, 2003). That the first GnRH was successful in synchronizing a new follicular wave 1-3 days after treatment agrees with Negila et al., 2003, Ali and Fahmy, 2007. In cattle, the new wave started 1-2 days after GnRH treatment regardless of whether the ovulation had occurred or not (Frike et al., 1998; Hussein, 2003; Hussein et al., 2004). This wave resulted in the development of a new DF (DF2) in most treated animals. Our results are in agreement with the previous studies where all HE showed new follicular wave and eight from nine of the BC showed a new follicular wave. The DF2 developed faster from days 0 to 7 and reached a larger diameter in the BC; this may be attributed to the subnormal P$_4$ level. The sub-

luteal circulating P$_4$ has been reported to increase the frequency of LH pulses and prolong the growth phase of the dominant follicle (Bridge and Fortune, 2003). When the dominant follicle after the PGF$_{2\alpha}$ injection has not ovulated, the new wave of small follicles needs some time to grow and become able to produce estradiol-17β that will lead to induction of LH-surge for ovulation (Bridge and Fortune, 2003).

PGF$_{2\alpha}$ was injected on day 7 to regress all CL. If a CL resulted from the initial injection of GnRH, the 7 day interval should have provided sufficient time for the CL to mature in order to respond to PGF$_{2\alpha}$ (Wiltbank, 1998, Ali and Fahmy, 2007). In the present study, all the treated HE showed on the day of PGF$_{2\alpha}$ at least one CL or double CL, while in the BC only five cows had CL. Most of the HE (7/8) had a follicle larger than 8 mm. This high synchrony between animals, the presence of functional CL, and a large active follicle at the same time is the result of the first GnRH treatment. A synchrony rate of 84% was previously recorded in cattle (Frike et al., 1998). Regression of corpora lutea was recorded in this study for all HE and BC, but the difference was only in the number of animals (eight in the HE vs. five in the BC).

In order to increase synchrony of ovulation, a second GnRH was injected to ovulate the preovulatory follicle at a precise time (Wiltbank1998). In the present study, the DF2 ovulated in 100% of the HE and in 88.8% of the BC animals. Our results were in agreement with previous reports. Ovulation rates of 90-93% were recorded in cyclic buffaloes (Paul and Prakash, 2005, Rao and Venkatramiah, 1991, De Araujo Berber et al., 2002) and of 86-100% in cyclic cattle (Wiltbank1998, Frike et al., 1998, Hussein, 2003). In the current study, the BC started to ovulate earlier
(6 h after second GnRH) than the inseminating time (16 h after second GnRH). Furthermore, those animals ovulated over a relatively long time (40 h). Early and asynchronous ovulation as well as early application of this program in the post partum BC seemed to be problems of the BC, and this might explain the very low CR of this group (11.11% in the BC vs. 62.5%). Negila et al. (2001) observed a pregnancy rate of 45% in buffalo cows synchronized with with PGF$_2$α alone and 48.8% when PGF$_2$α was combined with GnRH injection at the time of AI. In other study, the pregnancy rate in cyclic buffaloes synchronized with Ovsynch (36%) compared with PRID® (28.2%) (Negila et al., 2003).

In the current study, the P$_4$ level indicated precisely the presence or absence of a CL and reflected its size and activity. The level of P$_4$ in the present study is in agreement with the levels recorded by others on skim milk in buffaloes (Qureshi et al., 2000) It was found that the serum progesterone levels in the HE and BC treated with the Ovsynch program, in comparison to the control groups were not significantly different till the fifth day of treatment. At the seventh days of the program, serum progesterone values differed significantly in the HE, BC and control groups (P < 0.05). The results showed that the CL of the BC group seemed to be less active than that of the HE group, and this may also explain the low CR recorded in the BC group (only 11.11% in BC). Accordingly, it could be suggested that a significant improvement in CR in the BC cows can be achieved with the supplementation of exogenous progesterone or progestagen from days 0 to 7. It has been previously suggested that high P$_4$ level at the time of PGF$_2$α application may be an important factor in improving conception on subsequent insemination (Hussein 2003). By contrast, Pursley et al. (1997) reported that P$_4$ on day of PGF$_2$α injection had no effect on the probability of pregnancy.

In conclusion, the application of Ovsynch program in heifers could be better than in buffalo cows. The conception rate in buffalo heifers was acceptable and satisfactory, while in buffalo cows, it was very low. The difference may be attributed to ovulation time occurring too early in buffalo cows or over a long period. This and the development of seemingly sub-functional CL as well as the early application of the program post partum may be the causes for low conception rate in buffalo-cows. Our suggestion is that further studies should be focused on this point to analyze the factors affecting the conception rate following application of the Ovsynch program.

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ABSTRACT

The present study was carried out to characterize the morphological variations of bubaline oviductal epithelial cells derived during various stages of the oestrous cycle and maintained as short term cultures. The oviducts were collected from slaughtered sexually mature female buffaloes and divided into three groups based on the stage of the oestrous cycle, viz., early luteal phase (EP), mid luteal phase (MP) and late luteal phase (LP). A total of 18 trials, with six from each group, were conducted. The oviductal epithelial cells (OECs) were extruded and cultured for seven days. Morphological characterization revealed that OECs appeared as sheets of cells on day 0, changed into aggregates of worm-like or globular structures by day 1-2, revealed cell vacuolation and initiation of attachment by day 2-3 and reached sub-confluence by day 6. It was found that on day 1, the percentage of motile cells was significantly greater in the EP and LP groups (65.0 ± 10.5 and 63.3 ± 10.3 respectively) of OEC culture when compared to the MP group (48.3 ± 7.5%). On day 6, still significantly greater numbers of actively rotating motile cells were found in the EP (28.3 ± 9.8%) and LP (20.0 ± 6.3%) groups, while only 6.7 ± 8.2 percent of motile cells were observed in the culture of the MP group. Early initiation of attachment (day 1.5 ± 0.6) of a greater number of cells was noticed in the MP group when compared to the other two groups (day 2.3 ± 0.8 and day 2.0 ± 0.6 respectively). In the MP, the attached cells reached 70-80 percent confluence by day 6 of the culture, while only 30-40 percent confluence was observed in the EP and LP groups. It was thus concluded that oviductal cells isolated from the early luteal stages with increased ciliary activity and motile cells might be more appropriate for embryo co-culture experiments.

Keywords: Bubalus bubalis, oviductal epithelial cell culture, morphology, stage of oestrous cycle

INTRODUCTION

The oviduct plays a pivotal role in mammalian reproduction, providing an optimal environment for oocyte maturation, sperm capacitation, fertilization, and transport of gametes and embryos (Hunter, 2003). The oviduct epithelium consists mainly of two different cell types, viz., ciliated and non-ciliated (secretory) cells, and their relative proportions and morphology change markedly during the oestrous cycle (Leese et al., 2001). Cultured oviduct epithelial cells have been employed to overcome the developmental block at the 8-16 cell stage embryos generated in-vitro in ruminants (Galli et al., 2003). Undoubtedly, there is a practical need to increase knowledge about the
first environment to which embryos are exposed to improve IVF success rates and to ensure the normality of the embryos created. So, the present study was carried out to place on record the characteristics of the bubaline oviductal epithelial cells derived during various stages of the oestrous cycle and developmental pattern as short term cultures.

MATERIALS AND METHODS

The genital tracts were collected from sexually mature female buffaloes (5-8 years old) slaughtered at Corporation Abattoir, Chennai and transferred to the laboratory in a cool container (4°C) within 30-45 minutes for analysis. The reproductive organs were examined for normality and divided into three groups based on the stage of the oestrous cycle, viz., early luteal phase (EP), mid luteal phase (MP) and late luteal phase (LP), which was determined by the corpus luteum (CL) and follicular characteristics as described by Ireland et al. (1979). A total of 18 trials, with six from each group, were conducted.

Preparation of buffalo oviductal epithelial cell culture

The oviducts, ipsilateral to the ovary with CL, were excised from the reproductive tract, trimmed of excess connective tissue, ligated at the infundibular end and at the utero-tubal junction and washed twice in warm (37-39°C) sterile 0.9% saline. Oviducts were dipped in ethanol and washed with phosphate buffered saline before removing the ligature in a laminar flow hood. The oviductal epithelial cells (OECs) were extruded from the lumen of the oviduct by the stripping motion of a clean microscope slide over the exterior surface of the oviduct (Way, 2006). The extruded OECs from the lamina propria, which appeared as a yellowish paste, were collected in culture medium TCM-199 (Invitrogen) supplemented with 10 percent foetal bovine serum and 0.25 mg/ml gentamicin (Sigma). The cell suspension was pipetted 10-15 times with a 1000 μl filter tip, and cells were dissociated. After three steps of washing, each followed by 25-min sedimentation in culture medium in the water bath, cellular suspensions were dispensed into 25 cm² flasks (Falcon, Oxnard, USA) and cultured at 38.5°C in a humidity-saturated atmosphere of 5% CO₂ in air (day 0). The primary cultures were maintained for a short term of seven days (day 0-6) till they finally settled as a monolayer.

RESULTS AND DISCUSSION

Morphological variations in OECs maintained as short term culture was represented
in Table 1. In general, freshly (day 0) isolated bubaline OECs appeared as sheets of cells with vibratory movements due to the presence of cilia on the periphery of epithelial cells. The mechanically obtained flat cell sheets changed into aggregates of worm-like or globular structures during the day 1-2 of culture and this morphology was maintained throughout culture. Cell aggregates with numerous cilia at the periphery remained in rapid and constant motion in the culture medium due to vigorous ciliary beating, but some cells without cilia were found to initiate their attachment process to the bottom of the flasks even as early as day 1-3 of the culture. Cell vacuolation was often detected by day 2-3, and ciliary beating was not found in such vacuolated cells (Hishinuma et al., 1989). On day 4 of the culture, the attached cells spread out extensively. The proportion of cells with ciliary activity decreased on day 5 of the culture. After attachment to the surface, the cilia could not be observed. The primary cultures reached sub-confluency by day 6. These observations were in concurrence with the findings of Rosselli et al. (1994) and Rottmayer et al. (2006).

The ciliated cells were spherical with cilia on their surface, and the cell mass showed the tendency to rotate in the medium. Proportions of ciliated and non-ciliated cell types were reported to change during the oestrous cycle (Yaniz et al., 2000). In the present study, as observed on day 1, it was found that the percentage of motile cells was significantly (P < 0.05) greater in the EP and LP groups (65.0 ± 10.5% and 63.3 ± 10.3% respectively) of OEC culture when compared to the MP group (48.3 ± 7.5%) as reported by Walter and Miller (1996) and Tienthai et al. (2009). On day 6, still significantly (P < 0.01) greater numbers of actively rotating motile cells were found in EP (28.3 ± 9.8%) and LP (20.0 ± 6.3%) groups while only 6.7 ± 8.2 percent of motile cells were observed in the culture of the MP group during the same period. A non-significantly increased proportion of motile cells was recorded in the EP group than the LP group. It was suggested that large numbers of ciliary cells in the oviduct were found during the follicular phase, but that their morphology altered by extensive atrophy and deciliation during the luteal phase due to cyclic changes affecting the hormonal environment (Suuroia et al., 2002).

The two cell populations of the oviduct epithelium showed different adhesion behaviour. Since the ciliated cells were in constant motion, the non-moving secretory cells seem to adhere easier and faster. This was best observed by the early initiation of attachment (day 1.5 ± 0.6) of a greater number of cells in the MP group, while in the EP and LP groups, the initiation of attachment of cells was noticed on day 2.3 ± 0.8 and day 2.0 ± 0.6 respectively. However, there were no significant differences between groups. This observation was in concurrence with Thibodeaux et al. (1992) who reported that a portion of oviductal cells often attached and formed monolayer (approximately 20%) during the 48-h incubation period. In the MP, the attached cells reached 70-80 percent confluency by day 6 of the culture, while only 30-40 percent confluency was observed in the EP and LP groups. The high percentage of confluency in the MP group could be attributed to the increased proportion of non-motile cells.

Thibodeaux et al. (1992) opined that oviductal cells with increased ciliary activity, viability, and efficiency of attachment and growth, were suitable for in vitro embryo production studies. It could be concluded, based on the morphological characterization, that buffalo OECs derived from the early luteal stages of the oestrous cycle, during which highly motile cells were dominant,
<table>
<thead>
<tr>
<th>Day of culture</th>
<th>Buffalo oviductal cells in culture</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td><img src="image1" alt="Image" /></td>
<td>Sheets of OECs with cilia in periphery</td>
</tr>
<tr>
<td>Day 1</td>
<td><img src="image2" alt="Image" /></td>
<td>Flat cell sheets changed into aggregates of worm-like or globular structures</td>
</tr>
<tr>
<td>Day 2</td>
<td><img src="image3" alt="Image" /></td>
<td>Vacuolation of cells</td>
</tr>
<tr>
<td>Day 3</td>
<td><img src="image4" alt="Image" /></td>
<td>Attachment and spreading out of OECs</td>
</tr>
<tr>
<td>Day 4</td>
<td><img src="image5" alt="Image" /></td>
<td>Confluent mono layer</td>
</tr>
<tr>
<td>Day 5</td>
<td><img src="image6" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>Day 6</td>
<td><img src="image7" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Figures of morphological changes in buffalo oviductal cells during short term culture.
might be more appropriate for embryo co-culture experiments.

REFERENCES


GENETIC POLYMORPHIC STUDY OF $\alpha_{S_2}$-CASEIN GENE IN DIFFERENT BREEDS OF BUFFALOES

G. Darshan Raj*, Swathi Shetty, M.G. Govindaiah, C.S. Nagaraja, S.M. Byre Gowda and M.R. Jayashankar

ABSTRACT

The present investigation was aimed at studying 1.26 kb $\alpha_{S_2}$-casein promoter gene in $\alpha_{S_2}$-casein gene locus between the three buffalo breeds. $\alpha_{S_2}$-casein promoter gene was amplified by PCR using oligonucleotide primers standardized for Bos taurus species. The sizes of the amplification products were similar. PCR amplicons are subjected to restriction digestion by the enzyme Eco RV, only an intact PCR fragments were observed in all the breeds studied. No variation was found when the Same PCR amplicons of $\alpha_{S_2}$-casein promoter gene subjected to nucleotide sequence analysis. The BLAST alignment of 1262 bp nucleotides of buffalo $\alpha_{S_2}$-casein gene showed homogeneity with the Bos taurus and Bos grunniens $\alpha_{S_2}$-casein gene. The milk components and milk yield parameters could not be associated with buffalo $\alpha_{S_2}$-casein genotypes due to their monomorphic haplotype.

Keywords: polymorphism, oligonucleotide primers, PCR amplicons, BLAST, monomorphic haplotype

INTRODUCTION

India produces more than 84 million tons of milk, with 80 percent of it being produced by both buffaloes and cows on small-scale farms with herd sizes of two to eight animals. These micro enterprises are operated by an estimated 11 million farmers located in remote villages, who are members of 96,000 odd village dairy co-operatives. The producers, mainly landless laborers with underprivileged families, enjoy a relatively steady income through the sale of surplus milk. This income is vital for their well being and economic security (Anon, 1998; 2003). Well-defined breeds of buffaloes with standard qualities are found mainly in India and Pakistan. There are 18 River buffalo breeds in South Asia, which are further classified into five major groups designated as the Murrah, Gujarat, Uttar Pradesh, Central Indian and South Indian breeds. The best known breeds are Murrah, Nili Ravi, Jafarabadi, Surti, Mehsana, Kundi and Nagpuri. Most of the remaining buffaloes of the Indian subcontinent belong to a nondescript group known as the Desi buffaloes.

Karnataka state possesses mainly nondescript buffalo populations. Animals of Surti and Murrah breeds are widely distributed in northern parts of Karnataka, along with the local animals reared by the Gowli tribe. Local breeds of buffalo like South Kanara are present in and around Dakshina Kannada districts.

Currently, direct sequencing is one of the high throughput methods for mutation detection and is the most accurate method for determining the
exact nature of a polymorphism. Sanger dideoxy-sequencing can detect any type of unknown polymorphism and its position when the majority of the DNA contains that polymorphism. Fluorescent sequencing can have variable sensitivity and specificity in detecting heterozygotes because of the inconsistency of base-calling of these sites (Chadwick et al., 1996; Yan et al., 2000). Thus, it has only limited utility when the polymorphism is present in a minor fraction of the total DNA (for example, in pooled samples of DNA or in solid tumors) due to low sensitivity. DNA sequencing is usually used as a second step to confirm and identify the exact base altered in the target region previously identified as polymorphic by using scanning methods.

Schild and Geldermann (1996) identified a total of 34 variable sites in approximately 1.2 kb 5'-flanking regions of genes encoding bovine calcium-sensitive caseins: 17, 10 and 8 for \( \alpha_{s1} \), \( \alpha_{s2} \) and \( \beta \)-casein, respectively. The sequenced 5'-flanking regions of the genes were computer-analyzed for homologies with consensus sequences for mammary gland-specific factors, hormone receptor sites and ubiquitous transcription factors. Seventeen of the 34 mutations (50%) were located within known potential regulatory sequences, suggesting an influence of these mutations on the binding of transcription factors. Thus, these variants may affect the expression of the Ca-sensitive casein-encoding genes. They applied the cell culture and transfection techniques to directly determine the effect of the mutations on gene expression. The results indicated that differences in expression of the casein genes could not be attributed to the influence of the variability at single sites, but rather to the additive effect of intragenic haplotypes within the 5'-flanking regions. Sequence variability in the 5'-flanking regions of milk protein genes of species other than cattle was studied only sporadically.

Pappalardo et al. (1997), while sequencing the goat \( \beta \)-casein gene, detected a polymorphic MseI restriction site at the 5'-flanking end of the gene in region containing the putative regulatory elements. Two alleles were observed, viz., allele A showing three fragments (197 bp, 124 bp and 63 bp) and the B allele showing only two fragments (321 bp and 63 bp). Nucleotide sequences showed that in position -161, the A allele is characterized by an adenine and allele B by a guanine.

Schild and Geldermann (1996), Koczan et al. (1991), and Bleck et al. (1996) identified mutations in the 5' flanking regions of bovine casein genes. In their experiments, nine of these mutations were screened to see whether they might change affinity to nuclear proteins - transcription factors affect expression of relevant genes and quantitatively influence composition of milk proteins. Eighty-one Polish Black and White (BW) and 195 Polish Red (PR) cows were screened for polymorphism using this approach. Both breeds were found to differ significantly in the distribution of various genotypes in the 5'-flanking regions of \( \alpha_{s1} \) and \( \alpha_{s2} \)-casein genes. No polymorphism was found in the 5'-flanking region of the \( \beta \)-casein gene. However, preferential associations were found between individual promoter genotypes and protein variants of \( \alpha_{s1} \)- and \( \beta \)-caseins. In all PR individuals with the CT promoter variant of the \( \alpha_{s2} \)-casein gene (position-186), only variant A of the \( \alpha_{s1} \)-casein was found (statistically significant at \( P \leq 0.001 \)). On the contrary, all BW cows with the CC genotype at position -1084 had the A1 allele of \( \beta \)-casein (\( P \leq 0.02 \)). This provides strong evidence for the existence of specific haplotype combinations, including both coding and regulatory sequences at the casein locus in the Polish cattle breeds. These results showed that nucleotide sequence variations
in the promoter regions of bovine casein genes might change the affinity of these regions to nuclear proteins, transcription factors, and thus affect the expression of relevant genes that quantitatively influence the composition of milk proteins (Martin et al., 2002).

MATERIALS AND METHODS

A total of 150 lactating buffaloes comprising of 50 South Kanara, 50 Surti, and 50 Murrah buffaloes from different villages and farms of Karnataka were chosen for the present study.

DNA isolation was carried out adopting the high salt method as described by Millers et al. (1988).

The purity and concentration of DNA samples were estimated by spectrophotometer (Shimdz, Japan). The ratio of 260/280 nm 0D was calculated. A ratio of 1.7 to 1.9 was considered as high purity of DNA.

PCR amplification

The oligonucleotide primers (Sigma-aldrich, Bangalore) for the 5’ flanking region of α_{s2}-casein gene were designed based on the bovine nucleotide information as described by Schild and Geldermann (1996). The nucleotide sequence information used for the PCR amplification of 5’flanking region of α_{s2}-casein gene in buffalo was as follows.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS1</td>
<td>5’TATGACATGTGCAGAAATGAG 3’</td>
</tr>
<tr>
<td>CS2</td>
<td>5’TTCGAACAAATGCTATTAGGT3’</td>
</tr>
</tbody>
</table>

All the reactions were carried out in 200 μl reaction tubes. Just before setting of the reaction, a master mix was prepared combining 10X PCR buffer (500 mM KCl, 100 mM Tris.HCl, pH 8.3) (MBI Fermentas), 2.0 mM MgCl₂ (MBI Fermentas), 100 mM dNTP's) (MBI Fermentas), 1.0 unit of Taq DNA polymerase (MBI Fermentas), 2.5 M.mol of each primer and filtered Milli Quartz (FMQ) water. Each reaction mix consisting of 19 μl of master mix and 1 μl (100 ng) of template DNA was then placed in the thermal cycler block.

An initial denaturation at 94°C for two minutes was done, and subsequently, denaturation and primer extension were carried out at 94°C for one minute and 72°C for one minute, respectively. However, the annealing temperature was determined empirically between 50°C and 65°C with increments of 2°C keeping the annealing time constant at one minute. The number of cycles was kept constant at 35. After the last cycle, a final primer extension was carried out at 72°C for ten minutes, and the samples were then cooled to 15°C.

Restriction enzyme digestion of the PCR product of casein genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Enzyme used</th>
<th>Site of action</th>
<th>Change at position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α_{s2}</td>
<td>ECORV</td>
<td>GAT</td>
<td>ATC</td>
<td>-186 C → T</td>
</tr>
</tbody>
</table>

Based on the nucleotide base changes, specific restriction enzymes were chosen. The restriction enzyme digestion of the PCR product was done by using the enzyme ECORV.
until retrieved.

A 20 μl digestion mixture, consisting of 15 μl of the PCR product, 1x of recommended buffer, 2 μl of FMQ and 6 units of (0.6 μl) restriction enzyme was placed in a 0.5 ml microcentrifuge tube. The digestion mixture was mixed thoroughly in a vortex mixer and incubated for three and half hours in a dry bath. The temperature of the dry bath was adjusted based on the optimum temperature of the individual enzyme. The digested product was run on two percent gel along with the standard DNA marker at 50 volts for three hours and observed under a UV transilluminator. Agarose gel photographs were taken by using a gel documentation instrument.

Nucleotide sequencing

The PCR products were concentrated to 50 ng/μl by pooling several tubes and precipitated by isopropanol procedure. In order to obtain clean fragments for sequencing, the PCR products were separated by electrophoresis in a TAE agarose gel containing ethidium bromide using standard protocols. The desired PCR product band was excised using a clean, sterile razor blade or scalpel (band was visualized with a medium or long wavelength (e.g., ≥300 nm) UV light, and excised quickly to minimize exposure of the DNA to UV light). The minimum agarose slice was transferred to a 1.5 ml microcentrifuge or screw cap tube and then purified by using commercially available gel extraction kits (Qiagen). Quantification was done by loading one μl of eluted sample in 1% agarose gel and comparing with a standard molecular marker (Phi X 174 DNA ladder or 100 bp DNA ladder). Only samples with good concentration (≥50 ng/μl) were selected. Samples were labeled and sent to sequencing. Sequencing was done by Avesthagen, Bangalore and Macrogen, South Korea.

Sequence data analysis

Sequences were edited and initially aligned using the Sequencher demo version and then optimally aligned visually. Multiple sequences were aligned by Clustal format for T-COFFEE Version 1.41 on line application. Polymorphic sites were analyzed. Polymorphic sites were again confirmed by electropherogram results. Coding sequences were translated to amino acids by online EBI (European Bioinformatics Institute tools, Translation tool, www.ebi.ac.uk).

Database search

The database search of sequences for a possible match to the DNA sequence of casein gene was conducted using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI, Bethesda, MD). Translated protein sequences of different casein genes were also subjected to the BLAST algorithm.

Milk sample analysis

Milk samples were collected from target animals and preserved by potassium dichromate powder (0.2%). Protein, fat, solid-not-fat, total solids and lactose of milk samples were analyzed by Milko Scan 1338. (Auto Analyzer, Denmark) at Quality Control Unit, Mother Dairy, Bangalore.

RESULTS AND DISCUSSION

\( \alpha_{s2} \)-casein gene

A 1267 bp fragment of 5’-noncoding regulatory sequence of \( \alpha_{s2} \)-casein gene was amplified by PCR using oligonucleotide primers reported by Koczkan et al. (1991) and Schild and Geldermann (1996). The sizes of the amplification products were similar for all the buffaloes, indicating
that this region was conserved in the buffalo breeds studied (Figure 1). Amplified products of similar size were also observed by Koczan et al. (1991) and Schild and Geldermann (1996) in several Bos taurus breeds (German Friesian, German Brown Swiss, German Simmental, Jersey, Galloway, Scottish Highland, Galloway and zebu), suggesting conservation of \( \alpha_{s_2} \)-casein gene between Bos taurus and Bubalus bubalis species.

**PCR-RFLP’s of \( \alpha_{s_2} \)-casein gene detected by Eco RV**

EcoRV enzyme did not produce any restriction fragments of PCR products of the \( \alpha_{s_2} \)-casein gene in any of the buffaloes studied, indicating the absence of restriction site for this enzyme in the PCR product. A restriction site existed for the enzyme EcoRV when base ‘T’ was present at position -186 bp, but the same was lost when ‘T’ was replaced by base ‘C’. Contrary to the present results in buffaloes, Schild and Geldermann (1996) obtained 965 and 302 bp fragments after digestion of the 1267 bp fragment with EcoRV, which was genotyped as TT. They did not find CC genotype in any of the thirteen cattle breeds. Further, the sequence data in the present study was completely different, which could be attributed to the absence of restriction digestion of the 1267 bp fragment with EcoRV. The -186 bp positions had ‘C’ base; however, the sequence data showed that it was ‘T’. This led to the conclusion that whole restriction site might have been mutated in Bubalus bubalis species. Though the base ‘T’ was present at the -186 bp position, the other bases at the -184 and -185 positions were mutated (AGTATC instead of GATATC) (Figure 2) leading to loss of restriction site for EcoRV in Bubalus bubalis. All the buffaloes were genotyped as TT, which is in accordance with the report of Schild and Geldermann (1996) in Bos taurus and Bos indicus species. From these results, it appears that EcoRV has limited application in identifying haplotypes in Bubalus bubalis.

**Sequencing and genotyping of \( \alpha_{s_2} \)-casein gene**

In the present study, 1.26 kb \( \alpha_{s_2} \)-casein promoter gene was sequenced in the South Kanara, Surti and Murrah breeds. In each breed, four animals were taken as representative samples and ten variable sites were analyzed. Out of these ten sites, six transitions (2 A/G; 4 T/C) and four transversions (3 A/C; 1 T/G) sites were recorded.

The nucleotide sequencing of the amplified Bubalus bubalis \( \alpha_{s_2} \) promoter region indicated that the product size is 1262 bp, in contrast to the 1267 bp amplified fragment size product of the cattle for the same set of primers.

The sequence pattern of all the three buffalo breeds (South Kanara, Surti and Murrah) indicated monomorphic nature of the \( \alpha_{s_2} \) promoter region, as evidenced by similar nucleotide sequence pattern and the same type of transitions and transversions in all the 10 variable sites studied (Figure 2). No polymorphism was found within or between the breeds studied for 10 variable sites at the \( \alpha_{s_2} \)-casein gene promoter region.

The first polymorphic site R 2.1 at nucleotide position +57 bp of the non coding exon I showed a transversion from G→T, whereas the second polymorphic site R 2.2 at position +7 bp of the first intron showed a transition of T→C. The third polymorphic site R 2.3 at the position -7 bp of the downstream 5’ non-coding region showed a transversion of base C→A, and the polymorphic site R 2.4 which was studied by the enzyme EcoRV for restriction digestion at the -186 bp position of the 5’ non-coding region showed a transition from C→T. The restriction site analyses indicated a change in the adjacent bases within the restriction site.
Figure 1. PCR amplification of the *Bubalus bubalis* $\alpha_s$-casein gene.
Lane 1 and 2 - South Kanara breed.
Lane 3 and 4 - Surti breed.
Lane 5 - Murrah.

Figure 2. Electropherogram showing monomorphic results for the buffalo breeds at nine polymorphic sites of the $\alpha_s$-casein gene (5’untranslated region).
Instead of GAT↓ATC the restriction site sequence was AGT↓ATC in all the twelve samples sequenced (Figure 2). This was one of the exclusive mutations observed in the case of *Bubalus bubalis* species. The fifth polymorphic site R 2.5 at position -317 bp showed a transition from G → A (Figure 2). The sixth polymorphic site at position -399 bp of the 5’ non-coding region revealed a transition from G → A. On the other hand, the seventh polymorphic site at position -433 bp showed a transition from T → C. Another polymorphic site at position -1084 upstream 5’ non-coding region showed a transition from C→ T. Two transversions were seen from C → A at positions -1100 bp and -1101 bp upstream of the non-coding promoter region. αs2-casein gene based on the sequence patterns recorded for the different variable sites, and the possible genotypes are presented in Tables 1 and 2. The sequence pattern of all the three breeds (South Kanara, Surti and Murrah) indicated monomorphic nature for the αs2-casein gene studied, as evidenced by similar nucleotide sequence patterns.

Schild and Geldermann (1996) recorded 1267 bp nucleotide fragment in several cattle breeds (German Friesian, German Brown Swiss, German Simmental, Jersey, Scottish Highland, Galloway and zebu) by sequencing with the same set of primers and identified 10 variable sites distributed at several locations within the 5’ flanking region of the αs2-casein gene. They also identified several haplotypes for each of these variable sites. The nucleotide sequence pattern of buffalo breeds in the present study showed all the 10 variable sites (Figure 2); however, different variants for these sites were not identified between or within the buffalo breeds studied.

In the present study, all the buffaloes showed the base ‘T’ at -1084 and ‘A’ at -317 bp positions, Hence, the polymorphic sites were genotyped as TT and AA, respectively, for the -1084 and -317 bp positions (Figure 2). However, Schild and Geldermann (1996) reported genotypes CC and CT for the -1084 bp and AA, GG and AG for the -317 bp polymorphic positions in several cattle breeds.

According to αs2-casein gene *EcoRV* digestion results, it was predicted that the -186 bp position had “C” allele. However, when the restriction site was analyzed, it had the sequence AGTATC in contrast to GATATC in cattle (*Bos taurus*). The sequence data indicated that the polymorphic site -186 bp had “T” instead of “C” and the adjacent bases at the -184 and -185 positions were altered leading to loss of the restriction site. The restriction site of *EcoRV* in *Bubalus bubalis* species was mutated. The base present at the -184 bp position was “A” instead of “G”, and was “G” instead of “A” at the -185 bp position. This was one of the exclusive mutations observed in the case of the *Bubalus bubalis* species (Figure 2).

Schild and Geldermann (1996) recommended *EcoRV* enzyme to detect polymorphism at the -186 bp position of the αs2-casein gene in cattle species. These workers observed the TT genotype in eleven of the thirteen cattle breeds studied. However, in the present study, no polymorphisms were observed using *EcoRV*, indicating that the same enzyme cannot be used in buffaloes to identify mutations at this restriction site.

Further, Schild and Geldermann (1996) identified polymorphism at seven variable sites including the above mentioned three variable sites. The seven variable sites were +57, +7, -7, -399, -433, -1100 and -1101 in 13 cattle breeds. However, no such polymorphisms were observed in the buffaloes in the present study. This may be attributed to species differences, but further work
Table 1. Variants along with base position and transitions/tranversions observed in buffalo breeds.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Position</th>
<th>Base transitions/Tranversions</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2.1</td>
<td>+57</td>
<td>G→T</td>
</tr>
<tr>
<td>R2.2</td>
<td>+7</td>
<td>T→C</td>
</tr>
<tr>
<td>R2.3</td>
<td>-7</td>
<td>C→A</td>
</tr>
<tr>
<td>R2.4</td>
<td>-186</td>
<td>C→T</td>
</tr>
<tr>
<td>R2.5</td>
<td>-317</td>
<td>G→A</td>
</tr>
<tr>
<td>R2.6</td>
<td>-399</td>
<td>G→A</td>
</tr>
<tr>
<td>R2.7</td>
<td>-433</td>
<td>T→C</td>
</tr>
<tr>
<td>R2.8</td>
<td>-1084</td>
<td>C→T</td>
</tr>
<tr>
<td>R2.9</td>
<td>-1100</td>
<td>C→A</td>
</tr>
<tr>
<td>R2.10</td>
<td>-1101</td>
<td>C→A</td>
</tr>
</tbody>
</table>

Table 2. Genotypes of polymorphic positions recorded in buffalo breeds.

<table>
<thead>
<tr>
<th>Breeds</th>
<th>Polymorphic positions (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R2.1</td>
</tr>
<tr>
<td>Position</td>
<td>+57</td>
</tr>
<tr>
<td>Base present</td>
<td>T</td>
</tr>
<tr>
<td>South Kanara</td>
<td>TT</td>
</tr>
<tr>
<td>Surti</td>
<td>TT</td>
</tr>
<tr>
<td>Murrah</td>
<td>TT</td>
</tr>
</tbody>
</table>

Table 3. Base deletions and insertions recorded at αsz-casein nucleotide sequence (5’ untranslated region) of *Bubalus bubalis* by comparing with *Bos taurus* species.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Position</th>
<th>Base deletion and insertion in <em>Bubalus bubalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>172</td>
<td>Del of A</td>
</tr>
<tr>
<td>2</td>
<td>276</td>
<td>Ins of A</td>
</tr>
<tr>
<td>3</td>
<td>379</td>
<td>Ins of C</td>
</tr>
<tr>
<td>4</td>
<td>487</td>
<td>Del of seq (catggagacaca)</td>
</tr>
<tr>
<td>5</td>
<td>515</td>
<td>Ins of seq (CTTAAGA)</td>
</tr>
<tr>
<td>6</td>
<td>809</td>
<td>Del of G</td>
</tr>
<tr>
<td>7</td>
<td>821</td>
<td>Del of G</td>
</tr>
</tbody>
</table>
is needed.

**Blast search for \( \alpha_{s_2} \)-casein gene**

The BLAST analysis of sequence data of \( \alpha_{s_2} \)-casein gene in the present study matched with the same sequence data reported earlier in cattle. However, the product size of the promoter region was 1262 bp in contrast to 1267 bp reported by Schild and Geldermann (1996). The difference in the number of bp in buffaloes and cattle may be attributed to the insertions and deletions at various positions in the gene sequences of the two species (Table 3).

The exact effect of this mutation has to be detected in the case of *Bubalus bubalis* species. The \( \alpha_{s_2} \) promoter gene sequence for *Bubalus bubalis* species was not available in NCBI gene bank. The present work is the first report on \( \alpha_{s_2} \) promoter gene sequence for *Bubalus bubalis* species. The highest match of this gene was with bovine casein type A protein (CASAS2) gene (Groenen *et al.*, 1994). The alignment of 1262 total nucleotides showed 96 percent homogeneity. The second highest match was with *Bos grunniens* (domestic yak) \( \alpha_{s_2} \)-casein gene promoter region (Fan *et al.*, 1999) with 97 percent homogeneity.

**Correlation with milk constituents**

All the 150 animals in the current investigation were of homozygous (monomorphic) genotype for the \( \alpha_{s_2} \)-casein gene, and hence, it was not possible to assess the relationship between genetic variant and milk constituents.

**REFERENCES**


ABSTRACT

Trypanosoma evansi, a blood protozoan parasite, causes a serious disease known as ‘surra’, which runs a chronic course in buffaloes. It is an arthropod-borne disease, and Tabanus spp. has been implicated as the main vector. The long-term debilitating effects of parasitic infestations in buffaloes assume greater importance in terms of production losses. Study was conducted to screen the sera and blood samples of buffaloes by monoclonal antibody based latex agglutination test (MAb-LAT) and TE-PCR respectively. Forty-six buffalo serum samples collected from different parts of Haryana were screened by MAb-LAT to detect the circulating antigens of T. evansi and their corresponding blood samples by wet blood film (WBF) and TE-PCR using synthetic oligonucleotide primers (21 mer sense and 22 mer antisense) targeted to a repetitive nuclear DNA sequence of T. evansi. Out of 46 blood and sera samples from buffaloes, 2% were positive by WBF, 78.26% positive by MAb-LAT and 76.09% by TE-PCR. Diagnostic sensitivity of MAb-LAT was found to be more than WBF and PCR. MAb-LAT, being simple to perform, rapid, convenient, and cost-effective, could be quite suitable for field-level diagnosis. Thus MAb-LAT provides a better tool for the early diagnosis and thereby effective control of trypanosomosis, which leads to improved productivity in buffaloes.

Keywords: Trypanosoma evansi, diagnosis, buffalo, latex agglutination, polymerase chain reaction

INTRODUCTION

The animal husbandry sector plays a pivotal role in the national economy and socio-economic development of the country. India has the largest livestock population in the world, but recovery of produce from this sector is lower than its potential. The long term debilitating effects of parasitic infestations in buffaloes assume greater importance in terms of production losses. Trypanosomosis, an arthropod borne disease, runs a chronic course in buffaloes, affecting the overall performance of the animals. Trypanosoma evansi is the most widely geographically distributed pathogenic trypanosome in Africa, South and Central America and Asia (Luckins, 1998; Pathak and Khanna, 1998; Wernery and Kaaden, 1995). In India, T. evansi infection is widely prevalent in different parts of the country and has its impact in livestock production. The clinical course of disease is variable depending on virulence of the parasite and susceptibility of the host and hence clinical signs do not indicate a
pathognomonic feature for diagnosis. Chronically infected animals may survive up to 3-4 years, causing heavy production losses due to lowered milk and meat yield, abortions, premature births and also inability to feed the young ones apart from acting as carriers of the infection (Ngaira et al., 2003). This infection is distributed in hot or warm countries and in temperate climates particularly where arid desert and semi-desert steppes exist, but may occur in other types of climate as well (Hoare, 1956). Recently a case of surra infection in a man has been reported in India (Joshi et al., 2005; Powar et al., 2006; Shah et al., 2011). This report assumes significance, for it indicates a possible zoonotic threat in future (Laha and Sasmal, 2007).

Though trypanosomes have been studied over the past 130 years, their definite diagnosis still suffers from low sensitivity and specificity, and as a result, the epidemiology of the disease is far from completely understood. The true account of epidemiological picture of surra in India is lacking because the infections often do not manifest any real pathognomonic clinical signs. Clinical signs such as emaciation and anaemia are used as a provisional diagnosis but are unsatisfactory when considering successful measures of control. The parasitological examinations frequently fail to detect patent infections because parasitaemia is scanty in peripheral blood in the chronic forms (Killick-Kendrick, 1968). Biochemical methods viz., formol-gel, mercuric chloride, flocculation and turbidity tests, and concentration methods like haematocrit centrifugation and DEAE-cellulose adsorption are less sensitive than animal inoculation methods (Pegram and Scott, 1976). Serological diagnosis using antibody detection is hampered by its inability to distinguish between current and past infections because of persistent titres. Though animal inoculation methods are more sensitive for diagnosis of surra, yet they are laborious, time consuming and unsuitable for large-scale use in the field. Further, the SPCA (Society for Prevention of Cruelty to Animals) does not permit the use of experimental animals if alternatives are available.

Accurate diagnosis of ‘surra’ is extremely important to identify animals for treatment, to track the prevalence of the disease and to avoid misuse of the trypanocidal drugs. So development of cost-effective and field-oriented diagnostic test(s)/test kit(s) is required for large-scale screening of animals for effective control of the disease. Despite improvement in parasitological techniques for detection of trypanosomes, a high proportion of infections are never detected (Luckins, 1992). One major reason for this is the constant antigen variation (Jones and Mckinnell, 1984). The identification of circulating variable antigen types (VAT) would be of great value in developing more sensitive diagnostic tests. The present study was conducted with an objective of detecting circulating antigen of Trypanosoma evansi, indicating the active infection in buffaloes, by monoclonal antibody based latex agglutination test and testing its efficacy by comparing with highly sensitive PCR.

**MATERIALS AND METHODS**

**Collection of blood and serum samples:** Forty-six blood samples from buffaloes were collected from different places of Haryana in separate vials with and without anticoagulant. The blood samples with anticoagulant (heparin, 10 units/ml of blood) were used for detecting *T. evansi* in WBF and for extraction of parasite DNA for PCR. The blood samples without anticoagulant were collected to separate serum for use in the latex agglutination test. The serum was stored at -70°C.
Wet blood film: A drop of blood from each sample was used for preparation of WBF in triplicate on a clean glass slide and examined for *T. evansi* at magnifications of 100x and 400x according to the method of Killick-Kendrick (1968). A blood sample positive for *T. evansi* parasites by WBF was inoculated in 0.2 ml volume into disease-free adult Wistar rats by the intra-peritoneal (i/p) route for bulk harvests of parasites for extraction of DNA.

Monoclonal antibody based latex agglutination test: The sera samples were tested with monoclonal antibody based latex agglutination test (MAb-LAT) according to the method of Rayulu *et al.* (2007). Latex reagent test was prepared by mixing one part of polystyrene latex beads (Sigma, USA) with mean particle size of 0.8 μm to nine parts of anti-*T. evansi* murine monoclonal antibody (IgA isotype) (MAb) produced against cell membrane antigens of *T. evansi*. The suspension was stirred for two hours at RT. The suspension was centrifuged at 2400 x g for 10 minutes at 4°C and the supernatant discarded. The MAb coated latex beads were resuspended in PBS, pH 8.0 and the centrifugation was repeated thrice. Finally the pellet containing MAb coated beads was resuspended in PBS, pH 8.0 with 0.1% BSA. This reagent was used to screen the sera samples collected for detection of *T. evansi* antigens.

Twenty microlitres of the latex reagent were taken in the cavity of the slide and an equal volume of field serum sample was added to it. The reagent and the serum sample were mixed by gentle swirling motion of the slide for five to ten minutes. When clumps or granular aggregates formed within five minutes, the sample was scored as strong positive, and when within ten minutes, the sample was scored as weak positive. Controls including known positive and negative rat serum samples were also used in parallel.

Extraction of *T. evansi* DNA: The genomic DNA was extracted from *T. evansi* positive rat blood samples according to the method described by Sambrook and Russell (2001) with some modification. Briefly, 5 ml of *T. evansi*- positive rat blood was centrifuged at 1500 rpm for 10 minutes to separate plasma from the blood cells. Buffy coat was then separated by using a wide bored pipette tip without disturbing the pelleted erythrocytes. *T. evansi* DNA was then extracted separately from erythrocytes, buffy coat and plasma. About 3.0 ml of pelleted erythrocytes were transferred to a 50 ml centrifuge tube and 30 ml of lysis buffer was added, mixed well and incubated for 1 h at 37°C. Proteinase K was then added to a final conc. of 100 μg/ml and mixed by inverting the tube. The tube was incubated at 50°C for 3 h with intermittent shaking. An equal volume of phenol: chloroform: isoamyl alcohol (PCI) (25:24:1) (Appendix II) was added and mixed by gentle rocking for 3 minutes. The tube was centrifuged at 3000x g for 10 minutes at room temperature (RT) and the upper aqueous phase collected in a clean centrifuge tube. The aqueous phase was once more treated with an equal volume of PCI and then once with an equal volume of chloroform: isoamyl alcohol (24:1). The upper aqueous phase was finally collected after centrifugation at 3000x g for 10 minutes at RT.

To the aqueous phase was added one-tenth volume of 3M sodium acetate, pH 5.2 and DNA was precipitated by adding an equal volume of ice-cold isopropanol and incubating the mixture at 4°C overnight. The DNA pellet was obtained by centrifugation at 4000x g for 15 minutes at RT, and the pellet was washed once in 70% ethanol and air-dried. The dry pellet was suspended in 250 μl 10 mM tris-HCl pH 8.5, kept at RT for 30 minutes for dissolving DNA and then stored at -70°C until
The purity of the DNA isolated was recorded using a biophotometer. Similarly, DNA was isolated from the field blood samples for detection of *T. evansi*.

**Polymerase chain reaction:** PCR reactions were standardized using different magnesium chloride concentrations, Taq DNA polymerase concentrations, primer concentrations, annealing temperatures and numbers of cycles in the thermocycler (temperature gradient XP Cycler, Bioer Technology, China). A set of primers specific to *T. evansi* repetitive DNA sequence probe pMUTec 6.258 as described by Wuyts et al. (1994) were used for amplification by TE-PCR.

Forward primer (21 mer)-
5’-TGCAGACGACCTGACGCTACT-3’

Reverse primer (22 mer)-
5’-CTCCTAGAAGCTTCGGTGTCCT-3’

The PCR reaction was performed in a thermocycler with a reaction volume of 50 µl. DNA isolated from *T. evansi* infected rat blood was taken as the positive control, nuclease free water was taken as the negative control, and the PCR mixture without template was taken as the PCR control to check for the possibility of contamination.

**Analysis of PCR products:** After amplification, ten microlitres of amplified products were subjected to electrophoresis in 2% agarose gel prepared using 0.5X Tris Borate EDTA Amresco) containing ethidium bromide at a concentration of 0.5 µg/ml. A 100 bp gene ruler (Fermentas) was used as a marker. Electrophoresis was carried out at 6.5 V/cm of gel in 0.5X TBE running buffer in a submarine electrophoresis apparatus, using a power supply (Amersham Pharmacia Biotech) for one hour. The gel was visualized using a UV transilluminator (Gel documentation system, Spectroline®, USA).

**RESULTS AND DISCUSSION**

Wet blood film WBF of forty-six blood samples revealed the presence of trypanosomes in only one sample, i.e., 2.17% of the total samples. One obvious reason for the low number of positive samples by WBF is the inherent low sensitivity of the test. Similar observations have been made by numerous workers during the past two decades in India (Pathak et al., 1993; Swarnkar et al., 1993; Singh et al., 1995a; Baghel et al., 1996; Singh et al., 2004; Rayulu et al., 2007) and in other countries (Masake and Nantulya, 1991; Trial et al., 1991; Olaho-Mukani et al., 1993; Davison et al., 2000; Ngaira et al., 2003). Jeyabal et al. (2003) found 3.6% and 4.5% cattle positive by WBF and MHCT, respectively. Another probable reason why WBF figures were low was the treatment of animals for further use.

<table>
<thead>
<tr>
<th>Diagnostic tests</th>
<th>Buffaloes (Total- 46)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive</td>
</tr>
<tr>
<td>WBF</td>
<td>1</td>
</tr>
<tr>
<td>MAb-LAT</td>
<td>36</td>
</tr>
<tr>
<td>TE-PCR</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 1. Comparison of different diagnostic tests for detection of *T. evansi* in buffaloes.
trypanosomosis on symptomatological basis--quite a common practice in the field in India, including the state of Haryana. In the field, even today, this least sensitive diagnostic test, which misses about 50-80% positive cases, is still being used.

MAb-LAT detected thirty-six samples positive for trypanosomosis in the field serum samples, which is about 78.26%. Since MAb-LAT detects circulating antigen, it indicates the presence of active infection. The number of positive samples will be certainly higher than shown by WBF, since the majority of the samples were collected from areas where there is higher vector density, and moreover, samples were collected in the monsoon season, when there is abundance of tabanid flies in these areas. Besides, latent infections with low parasitaemia are common in buffaloes (Woo, 1977; Losos, 1980; Pathak and Singh, 2005), which could be a reason that many samples were missed by WBF. The monoclonal antibody-based latex agglutination test (Suratex®) developed by Nantulya (1994) detected the antigens in 53 (88.3%) of 60 blood samples collected from experimentally infected rabbits in comparison to 22 (36.7%) and 2 (2.3%) by buffy coat and WBF, respectively. Olaho-Mukani et al. (1996) screened 549 camels by Suratex® and found T. evansi antigens in 254 (46.3%) camels. Rayulu et al. (2007) using a latex agglutination test (LAT) declared overall 42.59% positive out of 1538 samples. Overall, both MAB-LAT reagents (Nantulya’s and Rayulu’s) could detect far more samples positive than those detected by WBF, indicating thereby higher sensitivity of the LAT than of the WBF. The information on specificity of LAT then could be better obtained by comparing it with another highly sensitive test, such as PCR or mouse inoculation. In the present study, PCR was therefore employed on the blood samples of the same animals on which LAT and WBF had been performed at the same time. All the samples positive by PCR gave a positive reaction in MAb-LAT, indicating the higher sensitivity of MAb-LAT.

History of treatment of all forty six buffalo samples was recorded, and no animals were treated with antiprotozoals at the time of collection or before one month. This was done to eliminate any false positive cases due to the presence of antigens released from the killed parasites due to treatment. An inherent limitation of Ag-detecting LAT is a high probability of declaring the recently-treated animals as positive. This is because of the fact

![Figure 1. Monoclonal antibody based latex agglutination test for detection of T. evansi antigens.](image)

[Spot A: positive rat serum sample showing granular aggregates; Spot B: negative rat serum sample showing milkiness; Spot C: diluent negative control].
Figure 2. *T. evansi* - specific PCR using DNA extracted directly from *T. evansi* infected rat blood. [Lane M: 100 bp DNA ladder; Lane 1: 5 μl *T. evansi* DNA from rat blood, Lane 2: 10 μl *T. evansi* DNA from rat blood, Lane 3: Negative control with Nuclease free water].

Figure 3. *T. evansi* - specific PCR using DNA extracted from field blood samples. [Lane M: 100 bp DNA ladder; Lane 1: positive control, Lane 2 - Lane 9 field samples].
that the antigens released from the killed parasites remain in blood circulation up to nearly four weeks after treatment (Wernery et al., 2001). These animals showed intermittent fever, emaciation, and poor body condition, which significantly correlated with positive serological status by MAb-LAT as well as trypanosome DNA detection by PCR.

TE-PCR was carried out in 50 μl reaction mixtures containing 10x PCR buffer with KCl (100 mM tris-HCl pH 8.8 at 25°C, 500 mM KCl, 0.8% Nonidet P₄₀), 1.5 mM MgCl₂, 200 μM each dNTP, primers each at 20 pM and 2U of thermostable Taq DNA polymerase. The cycles included an initial step at 95°C for 4 minutes followed by 29 cycles of denaturing at 95°C for 1 minute, primer annealing at 60°C for 1 minute and primer extension at 72°C for 1 minute. This was followed by last cycle of denaturing at 95°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 10 minutes and hold at 4°C for an indefinite time.

TE-PCR was first standardized using parasite template DNA from the infected rat blood and further extended to blood samples collected from buffaloes. A distinct PCR product of approximately 227 bp size was obtained. Among 46 blood samples examined, 35, i.e., 76.09 %, were found positive by TE-PCR. However, most of these samples showed a weak band of 227 bp by TE-PCR. This indicated that most samples either contained a low number of parasites or some inhibitors being in the DNA during its extraction from blood samples. PCR detected much larger number of positive samples than WBF could in the present study. Several investigators have reached similar conclusions about the relative sensitivities of the two tests. Mugittu et al. (2001) using PCR and DNA probes detected trypanosome DNA in 27 (43.55%) of the 62 parasitologically-negative samples. In another study, Omanwar et al. (1999b) using the Wuyts’ primers in PCR detected 3 (15%) out of 20 parasitologically-negative blood samples from camels in Rajasthan. Shahardar et al. (2007) in which PCR assay using ribosomal DNA amplifiers (20-mer sense and 16-mer antisense primer) based on structural 18S and 5.8S ribosomal sequence specific for kinetoplastida taxon detected six (60%) out of 10 Indian dromedary camels positive for T. evansi. There are speculations on the duration that T. evansi DNA remains in circulation following the trypanocidal treatment. It would be interesting to investigate the persistence of released parasite DNA in circulation so as to comment on the PCR positivity in animals treated for trypanosomosis in the past.

In the process of validation of MAb-LAT, the cross-reactivity of the monoclonal antibody in the LAT reagent used in the present study also needs to be examined with antigens from T. theileri, the only other trypanosome reported from some parts of India. Babesia bigemina and Theileria annulata infected cattle serum samples collected from the field were used to determine the specificity of the MAb-LAT. These samples did not show any agglutination reaction in MAb-LAT, which excludes the cross reactivity of monoclonal antibodies with other haemoprotozoan parasites like Babesia and Theileria to a certain extent. Further validation should be carried out with a considerable number of experimentally infected buffaloes inoculated with T. evansi, which could be possible in a larger study.

The present study represents the first report in which an Ag-detecting monoclonal Ab-based latex agglutination test has been compared with a DNA-detecting PCR for the diagnosis of T. evansi in buffaloes. In brief, the results reported in this study have reinforced the findings of earlier workers and emphasized the adoption of this
simple and sensitive field-level diagnostic test for trypanosomosis in order to improve management of this economically important disease in buffaloes. Being a rapid and more sensitive method than WBF, LAT can be used as a useful screening test for detection of latent and cryptic infections. Moreover, the test is simple to perform neither requiring multiple and complex procedural steps, nor the use of sophisticated equipment for reading the results. It declared far more number of samples positive, when compared with routinely used wet film and was nearly as sensitive as PCR. In this manner, the test could be quite suitable for field-level diagnosis and screening of trypanosomosis in buffaloes.

REFERENCES


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