

ISSN: 0125-6726

Volume 29 No. 2

BUFFALO *BULLETIN*

Editor: S. Sophon



INTERNATIONAL BUFFALO
INFORMATION CENTRE



International Buffalo Information Centre

(IBIC)

BUFFALO BULLETIN

ISSN : 0125-6726

Aims

IBIC is a specialized information center on water buffalo. Established in 1981 by Kasetsart University (Thailand) with an initial financial support from the International Development Research Center (IDRC) of Canada. IBIC aims at being the buffalo information center of buffalo research community through out the world.

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.

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

Editor

S. Sophon

Publisher

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

Online available:

<http://ibic.lib.ku.ac.th/e-Bulletin>

BUFFALO BULLEITN
IBIC, KASETSART UNIVERSITY, P.O. BOX 1084
BANGKOK 10903, THAILAND

URL : *http://ibic.lib.ku.ac.th*

E-mail : *libibic@ku.ac.th*

Tel : 66-2-9428616 ext. 344

Fax : 66-2-9406688

DYSTOCIA DUE TO UTERINE TORSION IN A BUFFALO

G.K. Das, Firdous A. Khan, S. Deori and Uma Shanker

ABSTRACT

The present communication reports a case of dystocia due to uterine torsion in a pluriparous buffalo and its successful treatment using modified Schaffer's method followed by mutation.

Keywords: dystocia, torsion, buffalo

INTRODUCTION

Uterine torsion is the most frequent cause of dystocia in buffalo, followed by incomplete dilatation of cervix and uterine inertia (Ahmad, 2001). It is observed commonly in pluriparous animals at the time of parturition or during the last month of gestation (Roberts, 1986). The condition accounts for about 29.5 to 30.6% of dystocia cases in this species (Amer *et al.*, 2008). In the present communication, a case of dystocia due to uterine torsion in a buffalo and its successful treatment is reported.

HISTORY AND OBSERVATIONS

A 6-year old buffalo in its third parity was presented to the Referral Veterinary Polyclinic of the Institute with a history of complete gestation and straining from the last 48 h but without any further progress. On general examination, the animal appeared dull and reluctant to move; the rectal

temperature was normal but the pulse and respiratory rates were elevated. Per rectal examination of the animal indicated more than 180° right-sided uterine torsion. Per vaginal examination revealed no fluid discharges, and the os-cervix could not be palpated. The case was diagnosed to be maternal dystocia due to right-sided uterine torsion.

RESULTS AND DISCUSSION

The animal was cast on its right side and uterine torsion was corrected using modified Schaffer's method by rolling the animal in the direction of torsion. After the first roll, per vaginal examination was done to assess the degree of detorsion. A second roll was needed to detort the uterus completely. This was followed by profuse voiding of fluid from the uterus. Per vaginal examination revealed adequate dilatation of the cervix and anterior longitudinal presentation and dorso-sacral position of the foetus but with lateral deviation of the head. After proper lubrication of the birth canal, the foetus was repelled into the abdominal cavity and the deviation corrected. This was followed by application of snares to both the forelimbs and lower jaw, and a dead male foetus (Figure 1) was removed by gentle caudal traction. Following delivery, the animal was treated with Enrofloxacin (1500 mg IM once daily for 5 days), Meloxicam (75 mg IM), Furazolidone (4 boli IU), DNS (1 litre IV) and a herbal ecboic Uterotone (150 ml orally for 3 days).



Figure 1. A dead male foetus delivered after correction of torsion.

Dystocia is less common in the buffalo than cattle, and among buffaloes, the stabled riverine type is more prone than the free-ranging swamp type (Ahmad, 2001). Uterine torsion accounts for most of the cases of maternal dystocia in this species, and the direction is to the right in more than 90% of the cases (Roberts, 1986). Predisposing factors include relatively long uterine ligaments, the low number of smooth muscle cells in the broad ligament, constant confinement, and hilly terrain (Ahmad, 2001). Timely diagnosis and correction of the condition is favorable for both the dam as well as the foetus since hypoxia can result from placental separation even in the absence of unruptured membranes (Sloss and Dufty, 1980). The death of the foetus in the present case may be attributed to the delay in presentation to the clinics leading to foetal hypoxia due to separation of foetal membranes.

REFERENCES

- Ahmad, N. 2001. Reproduction in the buffalo, p. 789-799. In Noakes, D.E., Timothy J. Parkinson, Gary C.W. England (eds.) *Arthur's Veterinary Reproduction and Obstetrics*. W.B. Saunders Company, Philadelphia.
- Amer, H.A., M.A. Hashem and A. Badr. 2008. Uterine twisting during pregnancy in buffaloes: relationship between clinical findings and biochemical indices. *J. Applied Biol. Sci.*, **2**: 31-39.
- Roberts, S.J. 1986. Diseases and accidents during the gestation period, p. 230-233. In Roberts, S.J. (ed.) *Veterinary Obstetrics and Genital Diseases (Theriogenology)*. Woodstock, VT.
- Sloss, V. and J.H. Dufty. 1980. Obstetrical pathology, p. 105-111. In Sloss, V. and J.H. Dufty (eds) *Handbook of Bovine Obstetrics*. Williams and Wilkins, Baltimore, U.S.A.

SURGICAL MANAGEMENT OF CERVICAL ESOPHAGEAL OBSTRUCTION IN A BUFFALO: A CASE REPORT

R.V. Suresh Kumar, N. Dhana Lakshmi, P. Veena, P. Sankar and P. Yasotha

ABSTRACT

Obstruction of the esophagus is infrequent in ruminants. Intra-luminal obstruction of esophagus is commonly referred to as choke occurs in buffaloes due to attempts to swallow of a whole fruit like turnips, large lemons, apples, phytobezoars (Tyagi, 1993), and pieces of leather and rubber. (Salunke *et al.*, 2003), ingestion of cloth or rexin material in buffaloes (Sivaprakash *et al.*, 1998 and Sivaprakash, 2003), mango seeds, potatoes, placenta, gunny bags and even stones (Ojha and Mohanty, 1970; Verma, 1974; Nagam *et al.*, 1978; Frank, 1981; Umakanthan, 1995 and Dilipkumar *et al.*, 1995), tarpaulin cloth (Ravikumar *et al.*, 2003), and coconut shell (Madhava Rao *et al.*, 2009). In the cervical part, the esophageal lumen appears trumpet shaped, and obstruction is common in the cervical part of the oesophagus (Venugopalan, 1997). An unusual case of cervical esophageal obstruction in a buffalo is reported here.

CASE HISTORY AND OBSERVATIONS

An 8-year-old she buffalo was presented to the Department of Veterinary Surgery and Radiology, College of Veterinary Science, Tirupati, with a history of chronic anorexia, (however taking water) absence of defecation, decreased urination, regurgitation of feed material through nostrils for the past three days. Clinical examination revealed a

hard swelling at left ventro-lateral aspect of proximal cervical region. Temperature, pulse rate, heart rate, were within normal range. Based on the owner history and clinical examination, this case was tentatively diagnosed as esophageal obstruction, and it was decided to perform surgery.

TREATMENT AND DISCUSSIONS

Pre-operatively, the animal was given dextrose normal, 1500 ml, and Ringers lactate 1500 ml intravenously. The animal was sedated with xylazine hydrochloride 0.05 mg/kg body weight (since there was not complete obstruction), and the surgical site was prepared aseptically.

The animal was positioned in right lateral recumbancy. To produce local analgesia, 2% lignocaine hydrochloride was infiltrated around the swelling. A longitudinal incision was made in the skin of the cervical part over the obstructing foreign bodies between the trachea and the sternocephalic muscle. The oesophagus was exposed and umbilical tape was applied proximal and distal to the obstruction to prevent contamination of surgical area and also to prevent the movement of the mass. On incision the hard mass was removed. The esophagus was thoroughly cleaned with normal saline. The esophagus was closed with a two-layer suture pattern. In the first layer, the mucosa was closed with the continuous suture pattern and the sub mucosa and muscularis were closed with the cushing

pattern using 2-0 chromic catgut. Skin apposed with horizontal pattern using silk. The suture line was sealed with tincture benzoine soaked cotton.

Post-operatively, the animal was given Enrofloxacin 15 ml, Melonex 15 ml intramuscularly for 7 days. Oral feeding was withheld and animal was maintained with Dextrose normal saline 3 l and Ringers lactate 3 l daily for 5 days. The animal was given rice gruel mixed bran from the 7th day onwards and chopped green grass and dry fodder, each 1 kg, daily. The sutures were removed on the 12th post-operative day and animal recovered unevenfully.

REFERENCES

- Dilipkumar, D.M., V.R. Kasaraliker and B.V. Muralikrishna. 1995. Oesophageal obstruction by a stone in a bullock. *Indian Vet. J.*, **72**: 385-386.
- Frank, E.R. 1981. *Veterinary Surgery*, CBS, 175-176.
- Madhava Rao, T., S. Bharathi and K.B.P. Raghavender. 2009. Oesophageal obstruction in a buffalo- a case report. *Intas Polivet*, **10**: 1-3.
- Nigam, J.M., G.R Singh and D. Krishnamurth Y. 1978. Thoracic oesophageal obstruction in a calf. *Indian Vet. J.*, **55**: 819-820.
- Ravikumar, S.B., P. Arunkumar and A. Madhusudan. 2003. Oesophageal obstruction in a buffalo - a case report. *Intas Polivet*, **4**(1): 48-49.
- Saluke, V.M., M.S. Ali, A.P. Bhokre and V.S. Panchbhi. 2003. Oesophagotomy in standing position - An easy approach to successful treatment of oesophageal obstruction in buffaloes - a report of 18 cases. *Intas Polivet*, **4**(2): 366-367.
- Shivaprakash, B.V., Mohd. Amanulla and S.M. Usturge. 1998. *Indian Vet. J.*, **75**: 159.
- Sivaprakash, B.V. 2003. Pregnancy and young age: prone factors of esophageal obstruction in buffaloes. *Intas Polivet*, **4**(2): 284-288.
- Tyagi, R.P.S. and Jit Singh. 1993. *Ruminant Surgery*, CBS, Delhi. 192-193.
- Umakanthan, T. 1995. Surgical management of choke in cows-clinical report. *Indian Vet. J.*, **72**: 275-276.
- Verma, R.J. 1974. *U.P. Vet. J.*, **2**:38.
- Venugopal, A. 1997. *Essentials of Veterinary Surgery*, 7th ed. Oxford and IBH Publisher, New Delhi. 316.

DYSTOCIA DUE TO FETAL ASCITIS WITH WRY NECK IN A GRADED MURRAH BUFFALO: A CASE REPORT

P. Vidya Sagar¹, Krishna Veni², K.S. Sai Krishna³ and K.S. Vadde³

ABSTRACT

This communication reports a case of dystocia due to multiple congenital abnormalities which include fetal ascitis, wry neck and arthogryposis in a graded Murrah buffalo.

Keywords: dystocia, fetal ascitis, wry neck

No fetal parts were observed in the birth canal. A detailed per vaginal examination revealed a dead calf with an abnormally distended abdomen with lateral deviation of head and neck. Attempts made to correct lateral deviation of head failed because of its rigidity. The animal was quite active and was taking feed and water. A slight elevation in temperature, pulse and respirations were noticed.

INTRODUCTION

Dropsical conditions such as fetal ascitis, fetal anasarca, and edema of the allantochorion and hydrops of the amnion or allantois or both are reported causes of dystocia (Roberts, 1971). Fetal ascitis is seen as an occasional cause of dystocia in many species but occurs more frequently in the cow and is associated with a dropsical condition of the uterus, mesotheliomas of the fetal abdomen and brucellosis. The present report describes a case of dystocia due to fetal ascitis and wry neck in a graded murrah buffalo.

CASE HISTORY AND OBSERVATIONS

A 6-year-old graded Murrah buffalo in third calving was presented to the Veterinary Poly Clinic, Gudiwada, with difficulty in parturition for the previous 12 h after the rupture of allantochorion.

TREATMENTS AND DISCUSSION

In the present case, caesarean section was considered to be appropriate to deliver the foetus. Caesarean section was performed through left paramedian approach as per the procedure under pre anesthetic medication with Triflupromazine (Siquil) and local infiltration with 2% lignocaine. The dead foetus was delivered and post operative care was followed. The animal had an uneventful recovery.

Gross examination of the fetus revealed multiple congenital abnormalities that include an abnormally distended abdomen (ascitis) with wry neck. The forelimbs were ankylosed and rigid at all the joints (arthogryposis). Muscular atrophy was noticed in all the limbs (Figure 1).

Exploration of the abdomen revealed straw colored fluid; no other abnormalities were found and confirmed the condition as ascitis. However, other treatment options recommended in such cases was

¹Veterinary Poly Clinic, Gudivada, Krishna District 521301 India

²Veterinary Poly Clinic, Visakhapatnam, India

³N.T.R. College of Veterinary Science, Gannavaram, India



Figure 1.

partial fetotomy to reduce the size of the abdomen. (Jackson, 1995 and Hoparkhe *et al.*, 2003). Wry neck is usually noticed in equines with transverse pregnancy but rarely observed in bovine fetuses. It is characterized by ankylosis, atrophy and contracture of the neck muscles resulting the head and neck being fixed in lateral direction along the side of the body. Fetus was quite small, but the distended abdomen with wry neck caused it to become wedged in the pelvic inlet, and this was the primary reason for dystocia as observed in the present case.

REFERENCES

- Hoparkhe, M., Ajeet Kumar and V.K. Gandotra. 2003. Dystocia due to accumulation of fluid in peritoneal cavity and intestines of fetus in a cross breed cow. *Indian J. Anim. Reprod.*, **24**(1): 83-84.
- Jackson, P.G. 1995. *Handbook of Veterinary Obstetrics*. W.B. Saunders Co. Philadelphia. 12, 41-43.
- Roberts, S.J. 1971. *Veterinary Obstetrics and Genital Diseases*, 2nd ed. CBS Publishers, New Delhi.

MANAGEMENT OF UTERINE TORSION IN A SHE BUFFALO

Qazi Mudasir, S.P. Shukla, S.P. Nema, R. Ali and S.S. Mahor

ABSTRACT

A pluriparous full-term pregnant Murrah buffalo was presented to Teaching Veterinary Clinical Service Complex, with history of restlessness and excessive straining for the previous 8 h. Per vaginal examination revealed right-sided uterine torsion more than 180 degrees. The detorsion was achieved by laying down the animal in right lateral recumbent position with rolling in the same direction as that of torsion. A live foetus was delivered on application of traction.

Keywords: pluriparous, buffalo, straining, torsion, foetus

INTRODUCTION

Uterine torsion is defined as the revolution or twisting of the uterus on its long axis (Roberts, 2004). It is the complication of late first stage or early second stage labour, and excessive foetal weight and movements at the time of parturition seem to be the predisposing factors for causing uterine torsion (Arthur *et al.*, 2001). Srinivas *et al.* (2007) stated that uterine torsion was the most important cause of maternal dystocia in graded Murrah buffaloes with an incidence of 83.33%.

CASE HISTORY AND OBSERVATIONS

A pluriparous full term pregnant Murrah buffalo was presented to Teaching Veterinary Clinical Service Complex, College of Veterinary Science and Animal Husbandry Mhow with history of restlessness and excessive straining from last 8 h. The animal had temperature of 101°F and was off fed with lack of rumination, rapid pulse and respiration rate and continuous switching of tail was observed. The vaginal mucosa was dry and vulval lips were drawn in. Per vaginal examination revealed right sided uterine torsion more than 180 degrees.

OBSTETRICAL MANAGEMENT

The animal was laid down in right lateral recumbent position with fore and hind limbs tied separately (Figure 1) and rolled in the same direction as that of torsion (Figure 2). After rolling through 180° body of the buffalo was pushed slowly over the legs and sternum (Figure 3) so as to continue rolling in the same direction. The vaginal passage of the animal was examined after each roll to find out whether the rolling was effective. After giving two complete rolls, the foetal fluids gushed out of the uterus (Figure 4) and the foetal head was now easily palpable.

On application of gentle traction to the foetus live buffalo calf was delivered (Figure 5). Intra uterine antibiotics 4 Furea bolus (Nitrofurazone



Figure 1. Buffalo laid down.

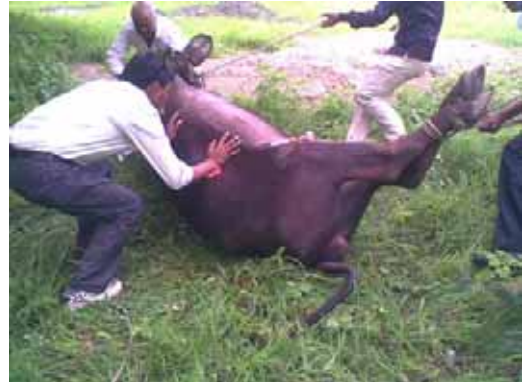


Figure 2. Rolling of buffalo.



Figure 3. Buffalo brought in sternal.



Figure 4. Appearance of foetal fluids recumbancy.



Figure 4. Delivery of live buffalo calf.

60 mg + urea 6 gm) were left in the uterus and parenteral antibiotic therapy using Strepto-Penicillin (5 gms) was given twice daily with other supportive treatments including anti-inflammatory and analgesics (Pheniramine maleate 15 ml I/M and Meloxicam 15 ml I/M) for the next 5 days. Inj. Calcium borogluconate (450 ml), 250 ml I/V and remaining 200 ml was given S/C to restore the normal body condition. The animal expelled placenta normally within 8 h of parturition.

RESULTS AND DISCUSSION

Rolling of the dam is the simplest method for relieving uterine torsion (Sane *et al.*, 1994). The objective of rolling is to suddenly and rapidly rotate the dam's body in the same direction while the uterus remains stationary during the procedure. Right-sided uterine torsion is more common than left-sided uterine torsion. (Srinivas *et al.*, 2007). The incidence of 180° to 270° uterine torsion is more common as compared to torsion of more than 270°. (Mathara and Prabhakar, 2001). In the present case, successful management of right-sided uterine torsion of more than 180° with delivery of a live foetus on application of traction was achieved.

REFERENCES

- Arthur, G.H., D.E. Noakes, T.J. Parkinson and G.C.W. England. 2001. *Veterinary Reproduction and Obstetrics*, 8th ed. WB Saunders Company Ltd., London. 233.
- Matharu, S.S. and S. Prabhakar. 2001. Clinical observations and success of treatment of uterine torsion in buffaloes. *Indian J. Anim. Reprod.*, **22**(1): 45-48.
- Roberts, S.J. 2004. *Veterinary Obstetrics and Genital Diseases (Therionology)*, 2nd ed. CBS Publishers and Distributors, India. 186.
- Sane, C.R., B.R. Deshpande, A.S. Kaikini, D.P. Velhankar, S.B. Kodagali, S.N. Luktuke, V.B. Hukeri and V.L. Deopurkar. 1994. *Reproduction in Farm Animals*. Varghese Publishing House, Bombay. 86.
- Srinivas, M., M. Sreenu, N.L. Rani, K.S. Naidu and V.D. Prasad. 2007. Studies on dystocia in graded Murrah buffaloes: a retrospective study. *Buffalo Bull.*, **26**(2): 40-45.

DYSTOCIA DUE TO LATERAL DEVIATION OF THE HEAD AND FOETAL EMPHYSEMA IN A SHE BUFFALO

Qazi Mudasir, S.P. Shukla, S.P. Nema, L. Patidar and R. Ali

ABSTRACT

A case of dystocia due to lateral deviation of head and foetal emphysema in a she buffalo was presented to Teaching Veterinary Clinical Service Complex. The foetal death had occurred 24 h earlier as per history and clinical examination. The postural defect of the foetus was corrected and several deep incisions were given over the foetal skin to relieve gases and subsequent dystocia. The animal made an uneventful recovery.

Keywords: dystocia, deviation, emphysema, buffalo, recovery

INTRODUCTION

Foetal emphysema is a frequent complication of parturition and primary cause of dystocia in farm animals (Arthur *et al.*, 2001). There is putrefaction characterized by formation of gases in the subcutis within 24-72 h, subsequent to death of foetus and the foetus becomes soft, decomposed and distended with gases (Sane *et al.*, 1994). Srinivas *et al.* (2007) reported that 40.84 percent of dystocia in graded Murrah buffalo are due to fetal cause, among which head deviations were 42.22 percent. The present communication describes a case of dystocia due to lateral deviation of head, further complicated by foetal emphysema in a she buffalo.

CASE HISTORY AND OBSERVATIONS

A pluriparous full term pregnant Murrah buffalo was presented to Teaching Veterinary Clinical Service Complex, College of Veterinary Science and Animal Husbandry Mhow with a history of dystocia. The animal was straining continuously and forelimbs appeared through the birth canal. Pervaginal examination revealed presence of an emphysematous foetus in normal presentation and position with lateral deviation of the head. The birth canal was dry, fully dilated and the vulva of the animal was swollen. The foetal skin was dry, cool with scanty foetal fluids and crepitant feeling of the subcutis was palpable. The animal had temperature of 103.2°F.

OBSTETRICAL MANAGEMENT AND DISCUSSION

The animal was put under caudal epidural analgesia with 8 ml of 2% xylocaine and the birth canal was copiously lubricated using liquid paraffin. The foetus was first repelled in the abdominal cavity and eye hook with snare was applied in the eye socket.

On giving traction to the eye hook, the postural defect of foetus was corrected. Snares were also applied to both fore limbs separately. On application of traction to snares and eye hook, the



Figure 1. Foetal emphysema in a she buffalo.

foetus was not expelled; either the foetal head or only the limbs were able to pass through the birth canal. Hence, it was decided to give traction to foetal head only. Upon expulsion of foetal head several deep incisions over skin of neck region were given with help of sharp knife to relieve gases. This subsequently reduced the foetal volume and now on application of traction to the snares, the foetus was expelled. The birth canal was lubricated at intervals and several incisions were again given over the foetal abdomen to cause further reduction in foetal mass. The putrid, emphysematous foetus was expelled in this manner (Figure 1).

The animal was given immediate fluid therapy (5 l of normal saline), intra uterine antibiotics 4 Furea bolus (Nitrofurazone 60 mg + urea 6 gm) and 20 ml oxytetracycline LA (200 mg/ml) once daily with other supportive treatment including analgesics and anti-inflammatory drugs for next 3 days. The animal expelled the placenta normally after 6 h and made an uneventful recovery.

The present communication describes the successful management of dystocia due to lateral deviation of head further complicated by foetal emphysema in a she buffalo.

REFERENCES

- Arthur, G.H., D.E. Noakes, T.J. Parkinson and G.C.W. England. 2001. *Veterinary Reproduction and Obstetrics*, 8th ed. WB Saunders Company Ltd., London. 864p.
- Sane, C.R., B.R. Deshpande, A.S. Kaikini, D.P. Velhankar, S.B. Kodagali, S.N. Luktuke, V.B. Hukeri and V.L. Deopurkar. 1994. *Reproduction in Farm Animals*, 2nd ed. Varghese Publishing House, Bombay, India. 97.
- Srinivas, M., M. Sreenu, N.L. Rani, K.S. Naidu and V.D. Prasad. 2007. Studies on dystocia in graded Murrah buffaloes: a retrospective study. *Buffalo Bull.*, 26(2): 40-45.

UTERINE LEIOMYOMA IN A BUFFALO: A CASE REPORT

O.I. Azawi¹ and H.I. Al-Sadi²**ABSTRACT**

This report describes a uterine leiomyoma in a buffalo cow. This is the first description of a leiomyoma in the uterus of a buffalo cow.

INTRODUCTION

Leiomyomas are benign smooth muscle neoplasia (Hulland, 1990). Although, these tumors are the most common uterine neoplasia in humans (Crum, 1999) and “middle aged” or older dogs (MaClachlan and Kennedy, 2002; Sontas *et al.*, 2008), but is rare in domestic ruminants (Kennedy *et al.*, 1998; Corpa and Martinez, 2008). Little is known about the etiology and pathogenesis of leiomyoma (Sendag *et al.*, 2008). In the bitch, these tumors are often multiple neoplasms, not only in the uterus, but also in the cervix and vagina associated with ovarian follicular cysts or estrogen secreting tumors (Kennedy and Miller, 1993). The present report describes a clinical case of uterine leiomyoma in a buffalo cow.

Case description

A 12-year old buffalo cow, weighing 400 kg, was presented to the Department of Surgery and Theriogenology, College of Veterinary Medicine, University of Mosul, with a history of repeat breeding of more than one year and calving normally before

16 months. Her estrous cycles were irregular. Accompanying complaints were straining, decreased milk production and weight loss. This animal was a part of private buffalo dairy herd consisting of 150 buffaloes. The animals were kept outdoors near a river where they wallowed and were milked twice daily. A balanced nutritional diet including green fodder and concentrated mixture were fed to these animals.

Clinical examination

Physical examination of the buffalo cow revealed a normal temperature, respiration and pulse rate. The vulva was wiped clean with damp clean towels, then disinfected with iodine-povidine, and then washed again with water. A sterile vaginal speculum was lubricated with sterile Vaseline and then inserted into the vagina up to the level of the external os of the cervix. Inspection of the cervix and vagina was performed with illumination from a penlight and revealed a purulent discharge. Rectal examination of uterus revealed a very thick uterine wall and enlarged cervix and multiple follicular cysts in both left and right ovaries. Sampling for bacteriological examination was performed immediately after vaginal examination. After parting the vulval lips, an outer protective sterile plastic soft drink straw sheath was advanced into the vagina and fixed in the external opening of the cervix. A guarded inseminating pipette was advanced through the outer tube into the uterus by cervical manipulation

¹Department of Surgery and Theriogenology, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

²Department of Pathology, College of Veterinary Medicine, University of Mosul, Mosul, Iraq

similar to the technique for artificial insemination. The sterile swab, which was fixed on the inseminating pipette was pushed out of its protective sterile plastic drink straw sheath (to protect from contamination with cervix), and moved about slightly in the body of the uterus. After retraction into its cover, the swab was removed from the vagina, with an assistant parting the vulval lips. Swabs were transferred into sterile tubes containing thioglycolate broth as a transport media, transported to the laboratory at 4°C, and immediately processed for bacteriological examination. Biopsies were taken following the culturing procedure. Separate tissue samples were obtained from each uterine horn and the uterine body with the biopsy instrument.

RESULTS AND DISCUSSION

Histopathological examination of the uterus of this buffalo showed a tissue of dense cellularity composed of regularly interlacing and undulating

bundles of elongate staplike smooth muscle cells and fibers (spindle cells) extensively infiltrating and expanding the uterine submucosa (Figure 1). The spindle cells had a moderate nuclear pleomorphism and were separated by collagenous matrix. The etiology of uterine leiomyoma is not known (Sendag *et al.*, 2008). Steroid hormones, especially estrogens, are thought to play a role in the pathogenesis of leiomyomas (Fiorito, 1992), as this case a multiple follicular cyst was found. Administration of exogenous hormones has been associated with the development of epithelial tumors of the endometrium in the bitch (Pena *et al.*, 2006), a true cause and effect association has not been proved. Leiomyoma in this buffalo could be due to long-term estrogen production by the multiple ovarian follicular cysts that might have played a part in the formation of the uterine leiomyoma.

Results of bacterial isolation from the uterus showed three bacterial isolates. These isolates were *Arcanobacterium pyogenes*, *Escherichia coli* and *Proteus mirabilis*. Buffalo cow bacterial

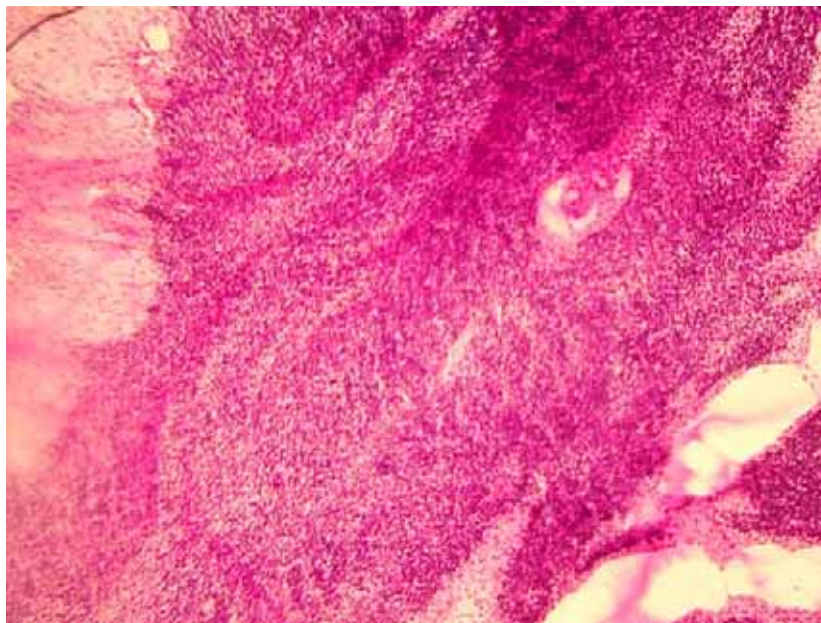


Figure 1. Leiomyoma in a buffalo cow with chronic metritis. Note that the neoplastic cells are intersecting at right angles. (H&E X 200).

contamination of the vagina and other external reproductive organs might occur during wallowing and coitus or insemination (Azawi, 2008; Azawi *et al.*, 2008). It could be suggested based on clinical outcome, bacteriological and histopathological examinations, that leiomyoma of this buffalo might predispose for bacterial infection causing metritis by lowering the uterine defense mechanism. A retrospective online search of necropsy and probably record for uterine leiomyoma in buffalo cows identified no such tumor was recorded.

REFERENCES

- Azawi, O.I. 2008. Review: Postpartum uterine infection in cattle. *Anim. Reprod. Sci.*, **105**: 187-208.
- Azawi, O.I., S.N. Omran and J.J. Hadad. 2008. A Study of endometritis causing repeat breeding of cycling Iraqi buffalo cows. *Reprod. Domest. Anim.*, **43**: 735-743.
- Corpa, J.M. and C.M. Martinez. 2008. Uterine leiomyoma in a sheep. *Reprod. Domest. Anim.*, In press.
- Crum, P.C. 1999. The female genital tract, p. 1053-1091. In Cortan, R.S., V. Jumar, T. Collins (eds.) *Pathologic Basis of Disease*. WB Saunders, Philadelphia.
- Fiorito, D.A. 1992. Hyperestrogenism in bitches. *Compend. Cont. Educ. Pract. Vet.*, **14**: 727-729.
- Hulland, T.J. 1990. Tumors of the muscle, p. 88-101. In Moulton, J.E. (ed.) *Tumors of Domestic Animals*. Los Angeles University, California Press.
- Kennedy P.C. and R.B. Miller. 1993. The female genital system, p. 349-470. In Jubb, K.F.V., P.C. Kennedy and N. Palmer (eds.) *Pathology of Domestic Animals*, 4th ed., Vol. 3. Academic Press, San Diego, CA.
- Kennedy, P.C., J.M. Cullen, J.F. Edwards, M.H. Goldschmidt, S. Larsen and S. Nielsen. 1998. Tumors of the uterus, p. 32-33. In Schulman, F.Y. (ed.) *Histological Classification of Tumors of the Genital System of Domestic Animals*. Armed Forces Institute of Pathology, Washington, DC.
- MaClachlan, N.J. and P.C. Kennedy. 2002. Tumors of the genital system, p. 547-573. In Meuten, D.J. (ed.) *Tumors in Domestic Animals*, 4th ed. Iowa State Press, Iowa.
- Pena, F.J., J.A. Gines, J. Duque, V. Vieitez, R. Martinez-Perez, L. Madejon, I. Nunez Martinez, J.M. Moran and S. Fernandez-Garcia. 2006. Endometrial adenocarcinoma and mucometra in a 6-year-old Alaska Malamute Dog. *Reprod. Domest. Anim.*, **41**: 189-190
- Sendag, S., Y. Cetina, M. Alana, F. Ilhana, F. Eskia and A. Wehrendb. 2008. Cervical leiomyoma in a dairy cattle. *Anim. Reprod. Sci.*, **103**: 355-350.
- Sontas, B.H., H. Ozyogurtcu, O. Turna, S. Arun and H. Ekici. 2008. Uterine leiomyoma in a spayed poodle bitch: A case report. *Reprod. Domest. Anim.*, In press.

DETECTION AND CHARACTERIZATION OF *LISTERIA* SPECIES FROM BUFFALO MEAT

J.B. Nayak, M.N. Brahmhatt, C.V. Savalia, C.D. Bhong, A. Roy, I.H. Kalyani and B.C. Parmar

ABSTRACT

The isolation of *Listeria* spp. from buffalo meat samples sold in retail meat market was evaluated. Isolation of the *Listeria* was attempted from the samples by selective enrichment in University of Vermont Medium (UVM) and plating onto PALCAM, Oxford and Dominguez-Rodriguez isolation agar (DRIA). The pathogenicity of the isolates was tested by Christie, Atkins, Munch Petersen (CAMP) test and sugar fermentation patterns were used for identification of the isolates. Out of 150 buffalo meat samples examined, 10 (6.7%) samples were found positive for *Listeria* species, of which 4 (2.7%) were positive for *L. monocytogenes*, 2 (1.3%) for *L. innocua*, 3 (2.0%) for *L. seeligeri* and 1 (0.7%) for *L. welshimeri*. PALCAM yielded a cent percent isolates where as recovery rate on DRIA and Oxford agar was 90.0 and 60.0 percent, respectively. The PCR assay targeting *iap* gene was used for species specific detection *L. monocytogenes* isolates up to 2×10^1 CFU/ml.

Keywords: *Listeria*, buffalo meat, selective media, PCR

INTRODUCTION

India has made rapid strides in meat exports to about 50 countries of the world. Quality meat is

produced adopting OIE guidelines and international quality standards. Out of total meat production of more than 6 million tones, the buffalo alone contributes about 1.47 million tones and India is 5th largest exporter of buffalo meat in the world. (Agnihotri, 2008). Meat, whether wholesome or unwholesome, fresh or spoiled, has been held responsible for a number of food borne infections in human beings. Listeriosis, caused by *Listeria* spp. is one of the important food-borne bacterial zoonotic infections worldwide. Among the different species, *Listeria monocytogenes* is known to cause listeriosis in humans and in more than 40 species of animal and 22 species of birds (Gray and Killinger, 1966). Listeriosis is a relatively rare disease, but fatality rate ranges from 15.0 to 30.0 percent with the highest hospitalization rates (90.5%) amongst known food-borne pathogens (CDC, 2000). Keeping in view these facts, many countries have adopted “zero tolerance limit” for *L. monocytogenes* in ready-to-eat food products. A number of reports have indicated the occurrence of the organism in various meat and meat products with overall incidence rate varying from 0 to 92.0 percent (Farber and Peterkin, 1991). There is paucity of comprehensive information regarding occurrence of *Listeria* spp. in buffalo meat in India. Therefore, keeping in view the above facts, the magnitude of the problems and the gap in knowledge regarding these aspects, the present study was carried out with a view to isolate and identify *Listeria* spp. from different samples of buffalo meat.

Department of Veterinary Public Health, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand - 388 001, India

MATERIALS AND METHODS

Altogether 150 samples of buffalo meat collected from retail meat shops were studied.

Isolation and identification

The method used for isolation of *Listeria* spp. from meat samples was divided in two phases 1) enrichment of samples and 2) selective plating on the three different media.

Samples were processed by two-step enrichment in UVM broth (Donnelly and Baigent, 1986) and loopful of inoculum was streaked directly onto DR1A, PALCAM and Oxford agar separately. The isolates were confirmed on the basis of colony characters, biochemical tests (Cheesbrough, 1991; Cowan and Steel, 1993) and also tested for xylose,

rhamnose and α-methyl D-mannopyranoside fermentation patterns as per the method of Cowan and Steel (1993) for characterization up to species level. The CAMP test was performed as per the method of Bureau of Indian Standards (1994).

Confirmation of *L. monocytogenes* by PCR

Listeria monocytogenes isolates were confirmed by PCR assay targeting *iap* gene (Bubert *et al.*, 1999) using primer sequence narrated in Table 1. The tenfold serial dilution was followed (Barros *et al.*, 2007). The standard strain of *L. monocytogenes* (MTCC1143) was obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India, was used as positive control during the assay.

Table 1. Primer sequence for detection of *L. monocytogenes*.

Species	Primer sequence (3' to 5')		Size	Reference
<i>L. monocytogenes</i>	Forward	TTA TAC GCG ACC GAA GCC AAC	660 bp	Bubert <i>et al.</i> , 1999
	Reverse	CAA ACT GCT AAC ACA GCT ACT A		

Table 2. Thermal cycling protocols for detection of *L. monocytogenes*.

Species	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
<i>L. monocytogenes</i>	95°C, 5 minutes	95°C, 45 seconds	50°C, 1 minute	72°C, 1 minute	72°C, 10 minutes
		Repeated for 31 cycles			

RESULTS

Isolation and identification

Out of 150 buffalo meat samples examined, 10 (6.7%) samples were found positive for *Listeria* species, of which four (2.7%) were positive for *L. monocytogenes*, two (1.3%) for *L. innocua*, three (2.0%) for *L. seeligeri* and one (0.7%) for *L. welshimeri*.

Recovery of the *Listeria* spp. on different selective plating media

From 10 positive buffalo meat samples, PALCAM yielded a recovery rate of 100 percent of isolates whereas the recovery rates on DRI and Oxford agar were 90.0 and 60.0 percent, respectively (Table 3).

PCR assay for detection of *L. monocytogenes* in raw buffalo meat

All 4 (2.7%) *L. monocytogenes* isolates identified by biochemical tests were subjected to PCR, and all these isolates were successfully amplified the desired amplicon of 660 bp. The PCR was performed from each diluted culture and showed the amplification up to as low as 2×10^1 CFU/ml using primer pair of *iap* gene.

DISCUSSION

Out of 150 buffalo meat samples examined, 10 (6.7%) samples were positive for *Listeria* spp. The results in the present study are in close proximity to the findings of Barbudhe (1996) and Barbudhe *et al.* (2002) who isolated *Listeria* spp. from 5.4 percent and 10.7 percent buffalo meat samples, respectively. However, Yucel *et al.* (2005) observed a higher prevalence of 10.7 percent in buffalo meat, which might have been due to differences in climatic conditions or sample size.

The recorded prevalence rate of *L. monocytogenes* in present study was 1.3 percent which is lower than earlier reports of Barbudhe *et al.* (2002), Chaudhari (1997) as well as Brahmabhatt and Anjaria (1993) who recorded 2.4, 3.0 and 5.5 percent prevalence, respectively. In contrast, Biswas *et al.* (2008) recorded a lower prevalence (0.9%) of the pathogen in the samples screened from buffalo meat packing plants where all the sanitary measures were observed to minimize the microbial contamination.

Lower prevalences of 1.3 percent, 2.0 percent and 0.7 percent of *L. innocua*, *L. seeligeri* and *L. welshimeri*, respectively, were observed in present study than in previous reports. Yucel *et al.*

Table 3. Recovery of *Listeria* spp. on different selective plating media.

Sr. no	<i>Listeria</i> spp.	No. of positive sample	PALCAM agar	DRI Agar	Oxford agar
1	<i>L. monocytogenes</i>	4	4	4	2
2	<i>L. innocua</i>	2	2	2	1
3	<i>L. seeligeri</i>	3	3	2	2
4	<i>L. welshimeri</i>	1	1	1	1
Total		10	10 (100%)	9 (90.0%)	6 (60.0%)

(2005) observed higher prevalence rates of 63.1 percent and 5.2 percent for *L. innocua* and *L. welshimeri*, respectively. Jalali and Abedi (2008) also reported 2.6 percent and 7.8 percent prevalences of *L. innocua* and *L. seeligeri* from fresh beef, respectively. This could be due to the lower overall prevalence of *Listeria* spp. in the present work.

Comparison of efficacy of different selective media

From 10 positive buffalo meat samples, PALCAM yielded a recovery rate of 100 percent of isolates whereas DR1A and Oxford agar yielded nine (90.0%) and six (60.0%), respectively. Scotter *et al.* (2001) observed more sensitivity of PALCAM (92.2%) than Oxford agar (91.1%) for the detection of *L. monocytogenes* from beef. Similarly, Gunasinghe *et al.* (1994) found recovery rates of 40.0 percent for *Listeria* spp. and 13.0 percent for *L. monocytogenes* in various meat products on PALCAM; the recovery rate was lower (23.0 percent and 7.7 percent, respectively) when Oxford medium was used. Capita *et al.* (2001) reported significantly higher results of isolation of *Listeria* spp. with PALCAM than modified Oxford medium (95.0% and 87.0%, respectively) and also a higher rate of *L. monocytogenes* with PALCAM (31.0%) when compared with the modified Oxford agar (27.0%). As many workers have tried these media for the isolation of *Listeria* spp., an attempt was not made to compare the efficacy of PALCAM with DR1A for the isolation of *Listeria* spp. Nevertheless, PALCAM agar was found to be superior for the recovery of the *Listeria* spp. including *L. monocytogenes* from buffalo meat samples.

PCR assay for confirmation of *L. monocytogenes*

A major 60-kDa extra-cellular protein *i.e.* p60, encoded by *iap* gene plays a vital role in

intestinal invasion and *in vivo* survival and all the isolates of *L. monocytogenes* secrete a protein of 60 kDa as a major extracellular product (Kuhn and Goebel, 1989) encoded by the *iap* gene. Primers targeting *iap* gene were used to amplify 660 bp amplicon for confirmation of *L. monocytogenes*. In the present study, standard culture and all four *L. monocytogenes* isolates identified by biochemical tests were successfully amplified desired amplicon of 660 bp which is in accordance with Zeng *et al.* (2006) who used *iap* gene as PCR - target for the species specific detection of *L. monocytogenes* from various samples including meat. It has been found that PCR amplification of *iap* gene is useful for the identification of *L. monocytogenes* to detect very low number of bacteria up to 2×10^1 CFU/ml in the samples.

Thus it can be concluded from the study that 1.3 percent prevalence of *L. monocytogenes* was observed in raw buffalo meat samples. PALCAM agar was found to be superior to DR1A and Oxford agar for the recovery of the *Listeria* spp. Moreover, PCR assay targeting *iap* gene proved useful for species specific detection of *L. monocytogenes* up to level of 2×10^1 CFU/ml in the meat samples.

REFERENCES

- Agnihotri, M.K. 2008. Quality meat production and product processing. p. 105-111. *In Proceedings of short course on 'Development of various value added products from goat milk and meat and their quality evaluation'. Central Institute for Research on Goats.* Makhdoom, India.
- Barbuddhe, S.B., S.P. Chaudhari and S.V.S. Malik. 2002. The occurrence of pathogenic *Listeria monocytogenes* and antibodies against

- Listeriolysin-O in buffaloes. *Journal of Veterinary Medicine Series.*, **49**(4): 181-184.
- Barbudhhe, S.B. 1996. *Studies on the kinetics and efficacy of listeriolysin O in the serodiagnosis of Listeria monocytogenes infection*. Ph. D. Thesis, Indian Veterinary Research Institute, Izatnagar, India.
- Barros, M.A.F., L. Nero, A. Silva, L.C. d'Ovidio, L. Monteiro, F.A. Tamanini, R. Fagnani, E. Hofer and V. Beloti. 2007. *Listeria monocytogenes*: Occurrence in beef and identification of the main contamination points in processing plants. *Meat Sci.*, **76**(4): 591-596.
- Biswas, A.K., N. Kondaiah, K.N. Bheilegaonkar, A.S. Anjaneyulu, S.K. Mendiratta, C.H. Jana and R.R. Singh. 2008. Microbial profiles of frozen trimmings and silver sides prepared at Indian buffalo meat packing plants. *Meat Sci.*, **80**(2): 151-154.
- Brahmbhatt, M.N. and J.M. Anjaria. 1993. Analysis of market meat for possible contamination with *Listeria*. *Indian J. Anim. Sci.*, **63**(6): 687.
- Bubert, A., Z. Sokolovic, S.K. Chun, L. Papatheodorou, A. Simm and W. Goebel. 1999. Differential expression of *Listeria monocytogenes* virulence genes in mammalian host cells. *Mol. Gen. Genet.*, **261**: 323-336.
- Bureau of Indian Standards. 1994. Committee draft, General guidelines for the detection of *Listeria monocytogenes*. *Microbiol.*, CD11290: 11-12.
- Capita, R., C. Alonso-Calleja, M. Prieto, M.C.G. Fernandez and B. Moreno. 2001. Comparison of PALCAM and modified Oxford plating media for isolation of *Listeria* species in poultry meat following UVMII or Fraser secondary enrichment broths. *Food Microbiol.*, **18**: 555-563.
- Centers for Disease Control and Prevention. 2000. Annual Report. CDC/USDA/FDA Foodborne Disease Active Surveillance Network. CDC's Emerging Infections Program. Revised 27 June 2003. Available at: <http://www.cdc.gov/foodnet/annual/1998/html/1998p>
- Chaudhari, S.P. 1997. *Studies on natural and experimental infection of buffaloes with pathogenic Listeria monocytogenes*. M.V. Sc. Thesis submitted to the Indian Veterinary Research Institute, Izatnagar, India.
- Cheesebrough, M. 1991. *Medical laboratory Manual for tropical countries. Volume II: Microbiology*. Tropical health technology, Doddington, Cambridshire-PE15OTT.
- Cowan, S.T. and K.J. Steel. 1993. *Manual for the Identification of Medical Bacteria*. The Syndics of the Cambridge University Press, London, U.K.
- Donnelly, C.W. and G.J. Baigent. 1986. Method for flow cytometric detection of *Listeria monocytogenes* in milk. *Appl. Environ. Microbiol.*, **52**: 689- 695.
- Farber, J.M. and P.I. Peterkin. 1991. *Listeria monocytogenes*, a foodborne pathogen. *Microbiol. Rev.*, **55**: 476-511.
- Gray, M.L. and A.H. Killinger. 1966. *Listeria monocytogenes* and listeric infections *Bacteriological Reviews*, **30**(2): 309-382.
- Gunasinghe, C.P.G.L., C. Henderson and M.A. Rutter. 1994. Comparative study of two plating media (PALCAM and Oxford) for detection of *Listeria* species in a range of meat products following a variety of enrichment procedures. *Lett. Appl. Microbiol.*, **18**: 156-158.
- Jalali, M. and D. Abedi. 2008. Prevalence of *Listeria* species in food products in Isfahan, Iran. *Int. J. Food Microbiol.*, **122**(3): 336-340.
- Kuhn, M. and W.Goebel. 1989. Identification of an extracellular protein of *Listeria*

Continued on page 94

GENOTYPING THE PROLACTIN GENE IN PANDHARPURI BUFFALOES BY PCR-RFLP

V.S. Madnalwar, M.P. Sawane, V.D. Pawar, P.A. Patil, A.P. Fernandis and A.S. Bannalekar

ABSTRACT

The present study was undertaken to detect polymorphism at exon I of the prolactin locus using polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) in the Pandharpuri buffalo breed. DNA from 50 Pandharpuri buffaloes was extracted by the phenol - chloroform method. A pair of bovine specific primers (forward 5'-ATTATCTCTCAT TTCCTTTTACA-3' and reverse 5'-ACTCTGCTGTCACTGTCTGTATT-3') were used for amplification of PRL gene exon I. PCR was carried out in a final reaction volume of 25 µl and the reaction mixture was subjected to standard PCR protocol. The PCR product digested with *Hae* III (RE). Digestion with *Hae* III revealed an intact product of 857 bp. The results of PCR-RFLP using *Hae* III show a different band pattern from cattle and previously observed studies in Jaffarabadi buffaloes. This indicated there might be absence of *Hae* III restriction sites in this buffalo breed. However, this would require confirmation by sequencing of amplified region to find the exact sequence variation.

INTRODUCTION

Lactation is under the physiological influence of the endocrine system. The milk protein and hormone genes are excellent candidate genes for linkage analysis with quantitative trait loci (QTL) because of their biological significance on the

quantitative traits of interest. Among several hormones that regulate lactation and reproduction in bovines, prolactin is an important anterior pituitary hormone (Ladani *et al.*, 2003).

The polypeptidic hormone prolactin is responsible not only for triggering lactation but also for mammary gland growth and lactogenesis (Tucker, 1981; Collier *et al.*, 1984). It also plays an important regulatory function in expression of milk protein genes. Therefore, the bovine prolactin gene (*bPRL*) seems to be an excellent candidate for linkage analysis with quantitative trait loci (QTL) affecting milk production traits (Brym and Kaminski, 2005). Considering the importance of the prolactin hormone gene, the present study was undertaken. The objective, was to investigate polymorphism within prolactin gene using PCR RFLP technique in Pandharpuri buffalo.

MATERIALS AND METHODS

Experimental material for the present study comprised of blood samples from 50 Pandharpuri buffaloes. The animals were unrelated and selected at random.

Genomic DNA was extracted by the phenol-chloroform method as described by John *et al.* (1991) with minor modification. The quality and quantity of DNA were checked and quantitation done by UV spectrophotometry and agarose gel electrophoresis. The DNA samples were diluted 1:50 by dissolving 2 µl of DNA in 98 µl of autoclaved

distilled water and used for spectrophotometry. DNA samples with an OD 260/280 ratio of 1.8 to 2.0 were further subjected to electrophoresis as a quality check; this was done on the 0.8 percent agarose in 0.5 X TBE buffer.

A pair of bovine specific primers (forward 5'-ATTATCTCTCAT TTCCTTTTTACA-3' and reverse 5'-ACTCTGCTGCTACTGTCTGTTT-3') (Zhang *et al.*, 1994) were used in the present study to amplify PRL gene exon 1. PCR was carried out in a final reaction volume of 25 µl. Each reaction volume contained 200 µM of each dNTP, 10 pmole of each primer and 0.65 unit of Taq. polymerase and 90 ng of template DNA in 1X PCR buffer. The reaction volume was subjected to 36 cycles comprising of denaturation at 94°C, annealing at 56°C for 1 minute and extension at 72°C for 1 minute followed by a final extension step at 72°C for 10 minutes.

The PCR products were digested with 10 units of *Hae* III at 37°C for 6 h in a final reaction

volume 25 µl. The RE digests were electrophoresed on agarose along with 100 bp DNA ladder.

RESULTS AND DISCUSSION

The primers amplified an 857 bp fragment from prolactin gene in all the Pandharpuri buffalo DNA samples. The PCR amplification was confirmed by running 5 µl of PCR product along with 100 bp DNA ladder in 0.8% agarose gel. (Plate1) The amplified PCR product of 857 bp was visualized as a single compact fluorescent band of the expected size under the U.V. transilluminator and was confirmed by comparing its distance from the well with that of the ladder and documented by the gel documentation system.

The amplified PCR product was digested with *Hae* III enzyme and after restriction digestion the PCR products were electrophoresed on 2.5 percent agarose gel containing ethidium bromide

181	aatgactgct	ataattttat	agttcctcta	actcaaaacta	gtctccagat	etcaccatc{a
241	ttatctctct	catttccttt	cagtctaatt	aatcaaaaatc	cttctagat	gttcatttct
301	ggtcagtatg
481	aggacgagag	cttctggtg	aagtgtgtg	cttgaaatca	teaccacat	ggacagcaaa
541	ggttcgctgc	agaaaggtat	gtacagcage	ttgtggagt	gttgggttt	atccatgttc
601	caatgggggc
781	ggttgtctta	atatggggca	ggggtgcaac	tggtgccagg	gggtagagaa	cagagatatt
841	gctaagcata	ctacaatgca	caagttagcc	cccagaacaa	gtacttatcc	agctcagggt
901	gg↓ccaatcat	gtcaaagttg	agaaaactta	gactggaata	agacaaaaa	tgtctctgag
961	tccaattcat	cacaactcca	gaaggtagaa	acaacattt	tctagtacc	aagattctta
1021	tgtggtgtgg	ctaagatgag	tcagtctgat	gaaacttta	actctgaagc	tata aatacag
1081	acagtgacag	cagagt agtc	teetacaata	ctttgttgac		

↓ Restriction sites for *Hae* III in cattle

Figure 1. Nucleotide sequence bovine prolactin gene 5' flanking region and exon 1 (Camper *et al.*, 1984).

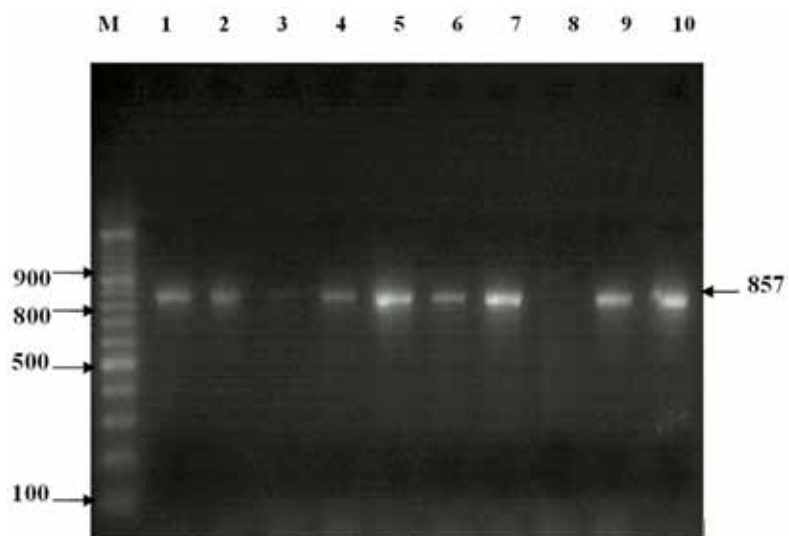


Plate 1. PCR product of Pandharpuri buffaloes.

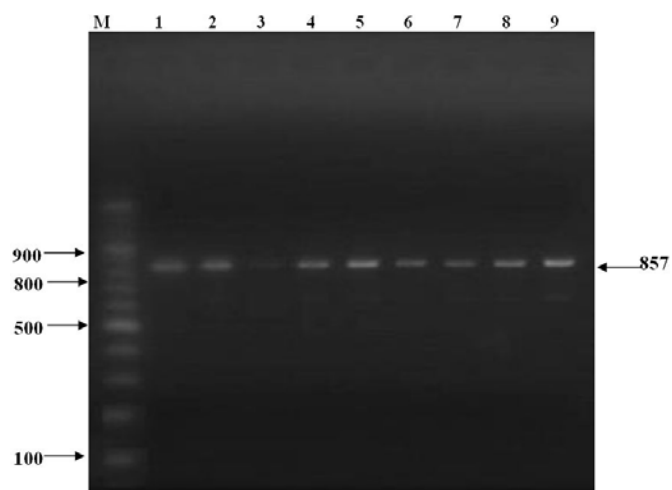


Plate 2. RE digestion of the PCR product of Pandharpuri buffaloes by *Hae III*.

Well No.	Sample No.
M	100 bp ladder
1	undigested PCR product
2	A
3	B
4	C
5	D
6	E
7	F
8	G
9	H

1 percent 5 µl/100 ml with a submarine gel electrophoresis apparatus at a constant voltage of 80 V for 90-120 minutes. The length of each fragment compared with the markers lane and fragment size was estimated. However, in all samples, an intact product of 857 bp was revealed (Plate 2). This suggests that a restriction site might be absent/abolished and reveals variation in nucleotide sequence of the PRL gene in Pandharpuri buffalo.

The present findings are in agreement with Ladani *et al.* (2003) who reported that *Hae* III has no restriction site for PRL gene (exon 1) and generates only one fragment of 857 bp in Mehsani and Surti buffaloes. However, they observed different restriction pattern in Jaffarabadi buffalo suggesting the presence of *Hae* III restriction sites.

The results obtained supports the existence of PRL sequence variation in Pandharpuri buffaloes that is different from other buffalo breeds. Comparison of above finding with published sequence of bovine 5' flanking region (Figure 1.) and exon I, it seems possible that point mutation (transition or transversion) could have abolished *Hae* III sites in this buffalo breed. However, this would require confirmation by sequencing of amplified region to find the exact sequence variation.

REFERENCES

- Brym, P. and S. Kaminski. 2005. Nucleotide sequence polymorphism within exon of the bovine prolactin gene and its associations with milk performance traits. *J. Appl. Genetics*, **45**(2): 179-185.
- Camper, S.A., D.N. Luck, Y. Yao, R.P. Woychik, R.G. Goodwin, R.H. Lyons and F.M. Jr, Rottman. 1984. Characterization of the bovine prolactin gene. *DNA*, **3**(3): 237-249.
- Collier, R.J., J.P. McNamara, C.R. Wallace and M.H. Dehoff. 1984. A review on endocrine regulation of metabolism during lactation. *J. Anim. Sci.*, **59**: 495-510.
- John, S.W.M., G. Weitzner, R. Rozen and C.R. Scriver. 1991. A rapid procedure for extracting genomic DNA from leukocytes. *Nucleic Acid Research*, **19**: 408.
- Ladani, D.D., D.L. Pipalia, B.P. Brahmkshtri, D.N. Rank, C.G. Joshi, P.H. Vataliya and J.V. Solanki. 2003b. PCR-RFLP polymorphism at prolactin locus in buffaloes. *Buffalo Journal*, **2**: 237-242.
- Tucker, H.A. 1974. Physiological control of mammary growth, lactogenesis and lactation. *J. Dairy Sci.*, **4**: 1403- 1421.
- Zhang, H.M., S.K. Denise and R.L. Ax. 1994. Rapid communication: diallalic single stranded confirmation polymorphism detected in bovine prolactin gene. *J. Anim. Sci.*, **72**: 256.

CLINICAL MANAGEMENT AND HAEMATO-BIOCHEMICAL CHANGES IN BABESIOSIS IN BUFFALOES

N. Lakshmi Rani*, C. Sreedevi, P. Annapurna and K. Aswani Kumar

ABSTRACT

Babesiosis in four graded Murrah she buffaloes aged between 6-8 years with symptoms of haemoglobinuria, anorexia, suspended rumination, reduced milk yield, depression and reluctance to move was studied. The clinical examination revealed elevated temperature ranging from 102.5°F to 104.2°F, accelerated heart rate and respiration and icteric mucus membranes with mild to moderate tick infestation. Haematological studies revealed reduced Hb, PCV and TEC. Serum chemistry revealed hyperglycemia, hyperbilirubinemia, BUN, AST and hypoproteinemia. Urine was coffee coloured and positive for haemoglobin, glucose and bile pigments. All the affected animals were treated with Diminazine aceturate along with supportive therapy, except for one animal which succumbed to illness, all the animals became afebrile by 24 h. The colour of urine became normal only after 3 days, while milk production was restored to its normal level by 3 weeks.

Keywords: babesiosis, haemoglobinuria, Diminazine aceturate, coffee coloured urine, jaundice

INTRODUCTION

Babesiosis is an important tick borne haemoprotozoan disease of cattle in many tropical countries including India. It causes great economic

loss due to reduced production and occasional mortality (Banerjee *et al.*, 2005). It is characterized by high rise of temperature, jaundice, weakness and haemoglobinuria. The disease may be so acute as to cause death within a few days, during which the PCV falls below 20% with parasitaemia, which is usually detectable once the clinical signs appear (Urquhart *et al.*, 1996). The present communication reports clinico-biochemical changes and the response to therapy in four buffaloes affected with babesiosis.

CASE HISTORY AND CLINICAL OBSERVATIONS

Four graded Murrah she buffaloes aged between 6-8 years with symptoms of haemoglobinuria, anorexia, suspended rumination, reduced milk yield, depression and reluctance to move that were brought to the Teaching Veterinary Clinical Service Complex over a period of six months from August 2005 to January 2006 were included in the present study. The clinical examination revealed elevated temperature ranging from 102.5°F to 104.2°F, accelerated heart rate and respirations. The mucous membranes of the ailing animals were icteric. In one animal, nervous signs like trembling and mild convulsions were observed. Mild to moderate tick infestation was also found in all the cases. Blood was collected for parasitological as well as routine hematological examinations, and serum was

collected for biochemical estimations. The Giemsa stained blood smears revealed the presence of babesia organisms. Haematological studies revealed 6.78 ± 0.75 g/dl Hb, $20.33 \pm 2.32\%$ PCV, 4.58 ± 0.41 millions/cmm total RBC, 9.2 ± 0.63 thousands/cmm total WBC. The differential leucocytes count exhibited $45.75 \pm 7.81\%$ neutrophils, $50.25 \pm 7.55\%$ Lymphocytes, $2.00 \pm 0.41\%$ monocytes and $2.00 \pm 0.82\%$ eosinophils. Serum chemistry revealed 5.70 ± 0.41 g/dl total protein, 98.5 ± 7.85 mg/dl glucose, 4.34 ± 0.97 mg/dl bilirubin, 35.91 ± 2.73 mg/dl blood urea nitrogen and 62.3 ± 5.81 units/ml AST. Urine samples of all the affected animals were coffee coloured and were positive for haemoglobin, glucose and bile pigments, which were in agreement with the findings of Bhikane *et al.* (2001).

TREATMENT AND DISCUSSION

All the affected animals were treated with Diminazine aceturate 10 mg/kg body weight intramuscularly along with supportive therapy (Inj. Imferon 5-10 ml IM, Inj. B complex 10 ml IM, and Inj. Chlorpheniramine maleate 15 ml IM on the first day). The animals became afebrile by 24 h after therapy. Out of the four animals, one succumbed to illness in spite of treatment; this might have been due to infection in an advanced stage prior to initiation of therapy. The colour of urine became normal only after 3 days, while the milk production was restored to its normal level by 3 weeks. Prolonged convalescent period results in considerable loss of production for a long period in babesiosis (Urquhart *et al.*, 1996). Supportive therapy with B complex vitamins was continued for 4 days while oral haematinic mixture was given till complete recovery.

The clinical signs can be attributed to multiplication of organisms in peripheral vessels and resultant intravascular hemolysis. The hemolysis

results in profound anemia, jaundice and hemoglobinuria. The neurological signs could be attributed to cerebral thrombosis and hypotension by activation of plasma kallikrein and disseminated intravascular coagulation leading to high mortality rates in cerebral babesiosis (Radostits *et al.*, 2000).

In the present case, significantly lower values of Hb, PCV and TEC were observed. In acute infections there is large scale destruction of erythrocytes with intravascular hemolysis, indiscriminate phagocytosis of infected / non-infected erythrocytes by activated macrophage system and suppression of erythropoietic activity of bone marrow, and these contribute to anemia. Anemic hypoxia results in elevated pulse and respiratory rates. This could be due to compensatory mechanism of the body for proper oxygenation of the tissues (Tufani *et al.*, 2009). These findings are in agreement with those of Ali *et al.* (1995) and Vivek *et al.* (1996). Leucocytosis may be due to stress associated with acute babesiosis (Bhikane *et al.*, 2001).

Hyperglycemia might be due to intravascular hemolysis resulting in anemic anoxia, and the glucose values observed in the present report are in agreement with those of Ashok Kumar *et al.* (1995). Intravascular hemolysis results in marked hyperbilirubinemia and icterus. Diminazine aceturate has been reported to be highly effective drug against babesiosis which acts by blocking the replication of DNA of the parasite (Bhatt *et al.*, 2005 and Bipin Kumar *et al.*, 2008).

REFERENCES

- Ali, Md.K., A.K. Pramanik and C. Guha. 1995. Babesiosis in calves and its treatment. *Indian Vet. J.*, **72**(12): 1289-1290.
- Ashok, Kumar and B.P. Joshi. 1995. Studies on blood glucose levels as a metabolic profile in clinical

- appraisal in buffaloes. *Indian J. Vet. Med.*, **15**(2): 71-73.
- Banerjee, P.S., Suresh Dabas, Stuti Vatsya, P. Bhatt and C.L. Yadav. 2005. Babesiosis in a cross bred-cow with clumping of parasitized red blood cells. *J. Vet. Parasitology*, **19**(2): 153-154.
- Bhatt, P., Suresh Kumar and S. Kumar. 2005. Babesiosis in a cross bred calf a case study. *Indian J. Vet. Med.*, **25**(1): 60.
- Bhikane, A.U., B.W. Narladkar, L.G. Anantwar and A.P. Bhokre. 2001. Epidemiology, clinico-pathology and treatment of babesiosis in cattle. *Indian Vet. J.*, **78**(8): 726-729.
- Bipin, Kumar, S.P. Verma, Bidasankhar Sinha and Sudhanshu Shekar. 2008. Therapeutic efficacy of alum-boric acid in bovine babesiosis. *Indian Vet. J.*, **85**(10): 1107.
- Radostitis, O.M., C.C. Gay, D.C. Blood and K.W. Hinchcliff. 2000. *Veterinary Medicine A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses*, 9th ed. W.B. Saunders. 1289p.
- Tufani, N.A., A. Hafiz, H.U. Malik, F.U. Peer and D.M. Makhdoomi. 2009. Clinico-therapeutic management of acute babesiosis in bovine. *Intas Polivet*, **10**(1): 49-50.
- Urquhart, G.M., J. Armour, J.L. Duncan, A.M. Dunn and F.W. Jennings. 1996. *Veterinary Parasitology*, 2nd ed. Book Power, Blackwell Science. Scotland. 243-244.
- Vivek, R., Kasarlikar, C. Ansar Kumar, S. Prasanna Kumar, K. Ganesh Udupa and P.M. Thimma Reddy. 1996. Babesia bigemina infection in a neonatal calf. *Indian Vet. J.*, **73**(11): 1185-1186.
-

Continued from page 87

- monocytogenes* possibly involved in intracellular uptake by mammalian cells. *Infect. Immunity*, **57**(1): 55-61.
- Scotter, S.L., S. Langtonb, B. Lombardc, S. Schultend, N. Nagelkerked, P.H. Int Velde, P. Rollier and C. Lahellec. 2001. Validation of ISO method 11290 Part 1- Detection of *Listeria monocytogenes* in foods. *Int. J. Food Microbiol.*, **64**: 295-306.
- Yucel, N., S.C. Cýtak and M. Onder. 2005. Prevalence and antibiotic resistance of *Listeria* species in meat products in Ankara, Turkey Short communication. *Food Microbiol.*, **22**: 241-245.
- Zeng, H., X. Zhang, Z. Sun and W. Fang. 2006. Multiplex PCR identification of *Listeria monocytogenes* isolates from milk and milk-processing environments. *J. Sci. Food Agr.*, **86**(3): 367-371.

SEROPREVALENCE OF LEPTOSPIROSIS IN SHE-BUFFALOES
(*Bos bubalis*) AT SLAUGHTER IN CHENNAI, INDIA

J. Selvaraj¹, B. Murali Manohar¹, R. Govindarajan², Vajiravelu Jayakumar²,
T.V. Meenambigai³ and C. Balachandran⁴

ABSTRACT

Seroprevalence of leptospirosis in she-buffaloes in Chennai, India were detected by microscopic agglutination test. Leptospirosis was detected in 88 percent of 125 sera samples tested. The most prevalent serogroup observed was Pomona (54.4 percent). Highest titre of 6400 was detected with the serogroup Pomona (0.4 percent).

Keywords: buffalo, leptospirosis, microscopic agglutination test, Pomona, seroprevalence

INTRODUCTION

Buffaloes are reared for milk, meat and ploughing/draft purposes in India. Out of a population of 1.65 million buffalo Tamilnadu, 2.3 lakhs are reared in Chennai, Kancheepuram and Tiruvallur districts (Anon, 2004). Animals are sold for slaughter because they are uneconomical to maintain for milk production or unsuitable for breeding due to infertility or sterility and at times because their owners are in need of money. Leptospirosis, a major zoonotic disease of animals and man, is of significant public health importance. It is one of the incriminating agents for jaundice (John *et al.*, 1980) and abortion (Sharma *et al.*, 1982) in buffaloes causing heavy economic losses to livestock farmers. Epidemiological studies carried out in different

countries have shown that leptospirosis occurs in buffaloes (*Bos bubalis*) and that both clinical and subclinical infection can occur (Kujumgiev, 1963; Andreani *et al.*, 1974; Arora and Baxi, 1978; Farina, 1989; Ciceroni *et al.*, 1995). In India, Adinarayanan *et al.* (1960) were the first to report leptospirosis among buffaloes in Uttar Pradesh. Subsequently, seroprevalence among buffaloes has been reported from many parts of India (Pande *et al.*, 1961; Bhatnagar *et al.*, 1967; Arora and Baxi, 1978; Srivastava and Kumar, 2003).

In southern peninsular India, the seroprevalence of leptospiral antibodies in buffaloes has been reported by many authors (Hussain, 1973; John *et al.*, 1980; Basha *et al.*, 1982; Ratnam *et al.*, 1983; Ramakrishna, 1986; Seenivasan, 1995; Ramani Pushpa and Punya Kumari, 2005; Selvaraj *et al.*, 2005). Information on the current status of seroprevalence among buffaloes in Tamilnadu state is inadequate. As a continuous study on the seroprevalence of leptospirosis in buffaloes is needed, the present work was undertaken.

MATERIALS AND METHODS

Sera samples were collected from 125 she-buffaloes, which were brought to slaughter at Corporation Slaughterhouse, Chennai. These buffaloes were apparently healthy adults brought by private farmers of Chennai, Kancheepuram and

¹Central University Laboratory, ²Leptospirosis Research Laboratory, Centre for Animal Health Studies, Madhavaram Milk Colony, Chennai-600 051, India

³Animal Biotechnology Unit, Madras Veterinary College, Madhavaram Milk Colony, Chennai-600 051, India

⁴Department of Veterinary Pathology, Madras Veterinary College, Chennai 600 007, India

Tiruvallur districts. All the sera samples were inactivated at 56°C for 30 minutes and stored at -20°C until tested.

The microscopic agglutination test (MAT) was performed as described by Cole *et al.* (1973). A panel of 12 strains representing 12 serovars of *Leptospira interrogans* known to circulate in Chennai were used. These strains were grown in Ellinghausen - McCullough / Johnson - Harris (EMJH) basal medium containing 10 percent EMJH enrichment medium (Difco) and 4 to 7 day old cultures were used as antigen. Antigen panel included live culture representing serogroups Australis, Autumnalis, Ballum, Canicola, Grippytyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes, Sejroe and Tarassovi.

Initially each serum was diluted 1 in 50 in phosphate buffered saline and 25 µl of it was added to an equal volume of each of the antigens' mixed and incubated for two hours at 37°C and agglutination tested by dark field microscopy at X100 magnification. The reacting sera were then titrated against the respective antigen. The endpoint was defined as the highest serum dilution that showed an agglutination of 50 percent or more of leptospire.

A microscopic agglutination (MA) titre of 50 or greater was considered positive in this study. When the serum was reacting to more than one antigen, the one giving highest MA titre was considered as the reacting serogroup and the remaining as cross reacting serogroups.

RESULTS AND DISCUSSION

Out of the 125 sera samples of she-buffaloes tested, 111 (88.8 percent) reacted with one or an other of the leptospiral antigens used (Table 1). The anti leptospiral antibodies that occurred in the sera in the frequency of descending order were: Pomona 68 (54.4 percent), Australis 52 (41.6

percent), Sejroe and Hebdomadis each 31 (24.8 percent), Pyrogenes 29 (23.2 percent), Tarassovi 23 (18.4 percent each), Autumnalis 17 (13.6 percent), Canicola 6 (4.8 percent) Icterohaemorrhagiae 3 (2.4 percent) Ballum and Grippytyphosa each 2 (1.6 percent) and Javanica 1 (0.8 percent). Serogroups like Ballum, Grippytyphosa and icterohaemorrhagiae were not the primary reacting serogroup but they were observed to be only cross reacting serogroups in this study.

The MA titres against various serogroups ranged between 50 and 6400 (Table 2). Out of 265 positive reactions 51 (19.3 percent), 82 (30.9 percent), 68 (25.7 percent), 30 (11.3 percent), 21 (7.9 percent), 10 (3.8 percent), 2 (0.8 percent) and 1 (0.4 percent) had MA titres of 50, 100, 200, 400, 800, 1600, 3200 and 6400, respectively. High seroprevalence observed in the present study is in accordance with Seenivasan (1995), who reported 85.7 percent seropositivity in buffaloes, whereas Srivastava and Kumar (2003) reported only 2.7 percent seropositivity in buffaloes in India. The prevalence of antileptospiral antibodies observed in the present study to different serogroups was also previously reported (Ratnam *et al.*, 1983; Venugopal *et al.*, 1986; Gupta, 1997; Selvaraj *et al.*, 2005).

Ratnam *et al.* (1994) observed that 39.3 percent of 56 buffalo sera samples were positive for leptospiral antibodies and the titres were 1:50 and above with the predominance of Autumnalis followed by Pomona in Tamilnadu; whereas Ramakrishna (1986) detected Pomona in 31.65 percent and Autumnalis 10.12 percent out of 79 MAT positive buffalo sera samples. Selvaraj *et al.* (2005) detected Pomona in 45.33 percent out of 75 sera samples screened. In the present study also Pomona was the most common serogroup detected in buffaloes (54.4 percent) which indicated the continuous high prevalence of the serogroup in buffaloes in Tamilnadu. The high percentage of positivity among the buffaloes could be due to their

habitation. Most of the private owners rear buffaloes in small herds and allow their buffaloes to wallow in sewage drainage (Coovam river) and dried ponds in and around Chennai as water sources are scarce in the city. As water buffaloes are considered as shedders of leptospira (Carlos *et al.*, 1970) they could perpetuate the cycle of transmission and could endanger the life of unprotected human and animal hosts. Hence, a continuous systematic seroprevalence study for a considerable period and isolation of local isolates may provide useful information to decide on the serogroup to be included in the vaccine production to control the disease in domestic livestock.

REFERENCES

- Adinarayanan, N., N.C. Jain, N.K. Chandiramani and S.K. Hajela. 1960. Studies on leptospirosis among bovines in India. *Indian Vet. J.*, **37**: 251-254.
- Andreani, E., A. Vaccaeo and C. Robert. 1974. *Atti della Societa Italiana delle Scienze Veterinarie*, **28**: 762.
- Anon. 2004. Department of Economics and Statistics, Statistical Handbook, Tamilnadu Government. http://www.tn.gov.in/deptst/tab07_01.htm. Accessed on 6-1-06.
- Arora, B.M. and K.K. Baxi. 1978. A note on the sero-evidence of leptospiral antibodies in domestic animals in Punjab State. *Indian J. Anim. Sci.*, **48**: 836-838.
- Basha, M., T.G. Abdul Khadar, J.R. Rao and N. Rhagavan. 1982. Survey of leptospirosis in man and animals by microagglutination lysis test. *Cheiron*, **11**: 114-116.
- Bhatnagar, R.K., M.V. Sant and H.I. Jhala. 1967. Prevalence of leptospirosis in Bombay - Studies in man and animals. *Indian J. Pathol. Bacteriol.*, **10**: 324-331.
- Carlos, E.R., W.D. Kundin, C.C. Tsai, G.S. Irving, R.H. Watten and C. Batungbakal. 1970. *Acta Medica Philippina*, **6**: 154.
- Ciceroni, L., P. D'Aniello, N. Russo, D. Picarella, D. Nese, F. Lauria, A. Pinto and B. Cacciapuoti. 1995. Prevalence of leptospire infections in buffalo herds in Italy. *Vet. Rec.*, **137**: 192-193.
- Cole, J.R., C.R. Sulzer and A.R. Pursell. 1973. Improved microtechnique for the leptospiral microscopic agglutination test. *Appl. Microbiol.*, **25**(6): 976-980.
- Farina, R. 1989. *Microbiologia Medica*, **4**: 87.
- Gupta, S.K. 1997. Serological evidence of *Brucella* and leptospiral infection in cases of abortion in bovines. *Indian J. Anim. Reprod.*, **18**: 75-76.
- Hussain, J.M. 1973. *A survey on the incidence of leptospirosis among domestic animals in and around Madras City*. M.V. Sc. Thesis, Madras University, Chennai, India.
- John, M.C., R. Simon and T.G. Abdul Khadar. 1980. Leptospiral jaundice among buffaloes in Madras City. *Indian Vet. J.*, **57**: 681-683.
- Kujumgiev, I. 1963. *Veterinaria Italiana*, **10**: 790.
- Seenivasan, N.K. 1995. *A study on the prevalence of leptospiral infections in some defined groups of animals and man*. Ph. D. Thesis, Tamilnadu Dr. MGR Medical University, Chennai, India.
- Pande, P.G., P.C. Sekhariah, P.K.R. Iyer and R.R. Shukla. 1961. Cerebrospinal leptospirosis in buffalo-calves due to *Leptospira hebdomadis*. *Curr. Sci.*, **30**: 147-148.
- Ramakrishna, J. 1986. *Studies on immune response to experimental leptospirosis in buffalo calves*. Ph. D. Thesis, Tamil Nadu Agricultural University, Coimbatore, India.

*Continued on page 108

IDENTIFICATION OF *Brucella* spp. FROM ANIMALS WITH REPRODUCTIVE DISORDERS BY POLYMERASE CHAIN REACTION ASSAYSanjay Ghodasara¹, Ashish Roy¹, D.N. Rank² and Bharat B. Bhandari¹

ABSTRACT

Brucella could recovered in 10 samples of vaginal swabs, abortion and placenta (two from cattle, one from buffaloes, four from goats, and one from a bitch) of the 248 samples processed by cultural, morphological, biochemical characteristics. Among isolates, eight from abortion cases (two from cow, one from buffalo, four from goat and one from a bitch) and two isolates from reproductive disorder (one from a cow and one from a buffalo) were recovered. Three different sets of primers were compared for the detection of *Brucella* species. The three pairs of primers amplified three different fragments viz., (i) B4/B5 primer pair amplified a 223 bp (ii) F4/R2 primer pairs amplified a 905 bp (iii) JPF/JPR primer pair amplified a 193 bp. Of these primer pairs, B4/B5 was found to be more sensitive as it detected 10 *Brucella* isolates. Whereas the other two primer pairs, F4/R2 and JPF/JPR, detected eight samples of *Brucella* organisms. The isolates identified as *Brucella* organisms were subjected to species differentiation using combinatorial PCR to identify the species of genus *Brucella* simultaneously. Four pairs of primers targeting the gene encoding cell surface protein (*BCSP31*) and outer membrane protein (*omp2b*, *omp2a* and *omp31*) were used. PCR using these primers gives rise to a specific pattern of amplification for each *Brucella* species. Out of 10 isolates of *Brucella*,

the five isolates from cattle and buffaloes could be identified as *B. abortus* when fragments of *BCSP31* and *omp2b/2a* were amplified by *B. abortus*-specific primers, whereas isolates from goats could be identified as *B. melitensis* by the amplification of fragments of *BCSP31*, *omp2b/2a* and *omp31* using primer B4/B5, JPF/JPR-ab and *omp31*. Identification of *B. canis* from the bitch isolates could be made by amplification of *BCSP31* and *omp31*.

Keywords: *Brucella*, PCR, reproductive disorders

INTRODUCTION

Brucellosis is a zoonotic disease caused by *Brucella* species and is an economically important infectious disease of livestock with worldwide distribution. The disease is enzootic in many states of India (Mehra *et al.*, 2000; Renukaradhya *et al.*, 2002 and Sarumathi *et al.*, 2003). Nine species in the genus of *Brucella* are currently recognized on the basis of their phenotypic characteristics, antigenic properties and host distribution (Scholz *et al.*, 2008, Foster *et al.*, 2007). *B. abortus*, *B. melitensis* and *B. canis* is the main etiological agent of brucellosis in large ruminants, small ruminants and canines, respectively, although cross infectious among hosts have been reported.

¹Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat, India

²Department of Animal Genetics and Breeding, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat, India

Brucellosis causes economic losses due to abortion, infertility and loss of calves in the livestock animals. Conventionally diagnosis is based on serological tests, which although rapid, give a non specific reaction that often leads to false positive reaction. Cultural isolation is time consuming and not routinely practiced (Mayfield *et al.*, 1989).

In the recent years, polymerase chain reaction (PCR) based detection of organisms have been found to be convenient as compared with cultural isolation. Different target genes, primers, PCR techniques and extraction procedure have been previously published for detection of genus *Brucella* (Baily *et al.*, 1992 and Romero *et al.*, 1995) and few of the studies reported use of these primers for animal isolates (Romero *et al.*, 1995 and Leal-Klevezas *et al.*, 1995) and human isolates (Zerva *et al.*, 2001). However, limited attempts have been made to compare the sensitivity three different primer pairs B4/B5 (Baily *et al.*, 1992), JPF/JPR and F4/F2 (Romero *et al.*, 1995) for the detection of the *Brucella* spp. using colony PCR and limited reports appear for species identification by PCR for *B. abortus* and *B. melitensis* (Bricker and Halling, 1994), *B. abortus* and *B. suis* (Fayazi *et al.*, 2002), *B. canis* (Kim *et al.*, 2006).

The aim of the present study was to compare specificity of the three different primer pairs B4/B5, JPF/JPR and F4/F2 for the detection of the *Brucella* genus as well as identification of *Brucella* species (*B. abortus*, *B. melitensis*, *B. suis*, and *B. canis*) by combinatorial PCR method using culturally confirmed *Brucella* isolates obtained from the specimens of animals with reproductive disorders in animals from different villages of Anand town Gujarat state, India.

MATERIALS AND METHODS

Sample collection

In the present investigation, a total of 248 cases of recently aborted and reproductive disorders comprising of deep vaginal swabs, placenta, fetal abomasal content and spleen were collected aseptically for cultural isolation from cows (107), buffaloes (73), goats (51) and bitches (17) from villages of Anand, Gujarat, India.

Bacteriological isolation and identification of *Brucella* organism

Samples were inoculated on *Brucella* agar medium (BAM) (Hi media, Bombay) plates in duplicate and one plate was incubated aerobically in an incubator at 37°C (without CO₂), and the other incubated at 37°C aerobically in an atmosphere of 5% CO₂ in a CO₂ incubator (Binder, Germany) and observed for growth at every 24 h for 15 days. The suspected colonies were identified as *Brucella* spp. by morphologic, cultural and biochemical properties such as oxidase, H₂S production, urease, CO₂ requirement and dye inhibition test.

Identification of culturally confirmed *Brucella* isolates by PCR assays

DNA Extraction

After identification of *Brucella* by morphologic, cultural and biochemical characters methods, suspected loopful cultures were suspended in 100 µl of phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM KH₂PO₄, pH 7.2). The samples were kept at 95°C for 15 minutes in a thermal cycler (MyCycler, Bio-Rad, USA), cell debris were removed by centrifugation at 3000 rpm for 15 minutes and 3 µl of the supernatant was used as a template.

Identification *Brucella* by genus specific PCR assays and Identification *Brucella* at species level by PCR assays

The following primer pairs were used for the identification of genus *Brucella*: (i) B4/B5 for the expected amplified product of 223 bp (for the region of the sequence encoding a 31 kDa immunogenic *bcs*31). (ii) F4/R2 for the expected amplified product of 905 bp (region of the sequence 16S rRNA of *B. Abortus*). (iii) JPF/JPR for the expected amplified product of 193 bp (the region of the sequence encoding an outer membrane protein *omp*2) (Table 1). A combination of four pairs of primers targeting genes coding the cell surface immunogenic protein (*BCSP31*) and outer membrane protein (*omp*2*a*, *omp*2*b* and *omp*31) were used. The identification of isolates up to species level was carried out by specific pattern of amplification obtained by this combination of four pairs of primers (Table 1). PCR based species identification of *Brucella* isolates was done as per Koichi *et al.* (2007). Primers (Bangalore Genei, India) details (Table 1), steps and conditions of thermal cycling for different primer pairs are given in Table 2.

The PCR was standardized for the detection of the above genes by following the methodologies described Romero *et al.*, 1995; Navarro *et al.*, 2002 and Koichi *et al.*, 2007 with suitable modifications.

The specificity of the PCR was tested by using the standard strain of pathogenic *Brucella abortus* 544 (reference strain procured from National Dairy Development Board, Anand, Gujarat, India) for positive control and standard strains of MTCC 1144-*Staphylococcus aureus* and MTCC 1143-*Listeria monocytogenes* 4b were procured from IMTECH, Chandigarh, India, as a negative control. The DNA template preparation from the test organisms and other PCR conditions were similar to those described earlier.

The PCR reaction was carried out in 25 µl reaction mixture of 12.5 µl 2x PCR-Master-Mix (0.05 units/µl *Taq* DNA Polymerase in reaction buffer, 4 mM MgCl₂, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, (Sigma Aldrich, USA). To make a final concentration of 1X, 1 µl of forward and reverse primers (10 pmol/µl), 3 µl of DNA template, and nuclease free water was added to make 25 µl final volume. The DNA amplification reaction was performed in a Master Cycler Gradient Thermocycler (Eppendorf, Germany) with a preheated lid. The resultant PCR products were further analyzed by agarose gel electrophoresis (1.5%; low melting temperature agarose L), stained with ethidium bromide (0.5 µg/ml) and visualized by a gel documentation system (SynGene, Gene Genius Bio Imaging System, UK).

RESULTS

Results of cultural and biochemical identification

According to the results of morphological, cultural and biochemical properties of the isolates, 10 *Brucella* isolates were obtained: three from cows (C1, C2, C3), two from buffaloes (B1, B2), four from goats (G1,G2,G3,G4) and one from a bitch (D1) and subjected to PCR based identification.

Confirmation by genus specific primer pairs

Three genus *Brucella* specific sets of primers B4/B5, F4/R2 and JPF/JPR were used. The results of amplification using various sets of primers are depicted in Table 3. In the present study, the desired product of 223 bp (Figure 1) using B4/B5 primer pair was amplified in all the 10 isolates and the reference strain, whereas the isolate C2 and B1 did not yield a desired product of 193 bp (Figure 2) using primer pair JPF/JPR even after repeated trials. Similarly isolates G2 and D1 did not produce a desired

amplicon of 905 bp (Figure 3) using primer pair F4/R2 on repeated trials.

Species level identification of *Brucella* isolates using combinatorial PCR

After confirming all the 10 *Brucella* isolates using the three sets of genus specific primers, species level identification was carried out by specific patterns of amplification obtained by the combinatorial PCR for four primer pairs B4/B5, JPF/JPR-ab, JPF/JPR-ca, and 1S/1AS were compared

for the detection of *Brucella* species. Primer pair B4/B5 produced the desired amplicons of 223 bp in all 10 isolates and reference strain. However, primer pair JPF/JPR-ab produced the desired amplicons of 186 bp in those isolates which were isolated from the cows, buffaloes and goats, whereas primer pair JPF/JPR-ca produced the desired amplifications of 187 bp for the isolates from bitch isolates only. The primer pair 1S/1AS produced the desired amplified product of 249 bp from the all the isolates of goats and bitches (Table 4 and Figure 4).

Table 1. List of genus and species specific primers.

Target Gene	Primer name	Sequence 5'-3'	Target Length bp	References
BCSP31	B4(F)	TGGCTCGGTTGCCAATATCAA	223	Bailey <i>et al.</i> (1992), Koichi <i>et al.</i> (2007)
	B5(R)	CGCGCTTGCCCTTTCAGGTCTG		
16S rRNA	F4 (F)	TCG AGC GCC CGC AAG GGG	905	Romero <i>et al.</i> (1995)
	R2 (R)	AAC CAT AGT GTC TCC ACT AA		
<i>Omp2</i>	JPF(F)	GCGCTCAGGCTGCCGACGCAA	193	Leal-Klevezas <i>et al.</i> (1995)
	JPR (R)	ACC AGC CAT TGC GGT CGG TA		
	JPF(F)	GCGCTCAGGCTGCCGACGCAA	186	Koichi <i>et al.</i> (2007)
	JPR-ab(R)	CATTGCGGTCCGGTACCGGAG		
	JPF(F)	GCGCTCAGGCTGCCGACGCAA	187	
	JPR-ca(R)	CCTTTACGATCCGAGCCGGTA		
<i>Omp31</i>	1S(F)	GTTCGCTCGACGTAACAGCTG	249	
	1AS(R)	GACCGCCGGTACCATAAACCA		

DISCUSSION

In the present study, the ability of the three primer pairs to detect *Brucella* DNA from an isolated colony on *Brucella* agar medium were compared. Among the three different genus specific primers, the B4/B5 primer pair was found to be more sensitive for identification of *Brucella* organisms. The isolates C2 and B1 did not yield the desired product of 193 bp using primer pair JPF/JPR even after repeated trials. The non-amplification might have been due to a probable mutation in primer attachment sites, particularly at 3' end. Further, in the absence of sequence information on the annealing site of field isolates, no conclusive inference could

be drawn about the behavior of this primer pair (Kanani, 2007). Navarro *et al.* (2002) observed a slightly different sensitivity of these three primer pairs and concluded that difference in sensitivity might be due to the sample pretreatment methods and extraction methods of the DNA. Thus the B4/B5 primers were the most effective for detection among the three. Kanani (2007) and Patel (2007) also observed differences in the sensitivity among the same three primer pairs with high sensitivity by B4/B5 primer. Analyses of 16S rRNA gene have been extensively used for molecular detection or taxonomic analyses of many different bacterial species (Moreno *et al.*, 2002; Unver *et al.*, 2003 and Gee *et al.*, 2004). 16S rRNA gene sequences

Table 2. Steps and conditions of thermal cycling for different primer pairs in PCR Primers used for genus and species level identification.

Primers (Forward and Reverse)	Cycling conditions				
	Initial denaturation	Denaturation	Annealing	Extension	Final extension
B4 (F) B5 (R)	93°C 5 minutes	90°C 1 minute	64°C 30 seconds	72°C 1 minute	72°C 10 minutes
Repeated for 35 cycles					
JPF (F) JPR (R)	94°C 4 minutes	94°C 1 minute	60°C 1 minute	72°C 1 minute	72°C 3 minutes
Repeated for 35 cycles					
F4 (F) R2(R)	95°C 5 minutes	95°C 30 seconds	54°C 90 seconds	72°C 90 seconds	72°C 6 minutes
Repeated for 30 cycles					
JPF (F) JPR-ab (R)	95°C 5 minutes	95°C 1 minute	60°C 1 minute	72°C 1 minute	72°C 7 minutes
Repeated for 35 cycles					
JPF (F) JPR-ca (R)	95°C 5 minutes	95°C 1 minute	60°C 1 minute	72°C 1 minute	72°C 7 minutes
Repeated for 35 cycles					
1S (F) 1AS (R)	95°C 5 minutes	95°C 1 minute	57°C 1 minute	72°C 1 minute	72°C 7 minutes
Repeated for 35 cycles					

Table 3. Confirmation of *Brucella* isolates by PCR.

Sr. No.	Isolates	Primer pairs		
		B4/B5	JPF/JPR	F4/R2
1.	C1	+	+	+
2.	C2	+	-	+
3.	C3	+	+	+
4.	B1	+	-	+
5.	B2	+	+	+
6.	G1	+	+	+
7.	G2	+	+	-
8.	G3	+	+	+
9.	G4	+	+	+
10.	D1	+	+	-
11.	Reference strain <i>B. abortus</i> 544	+	+	+

+ = Amplified desired product - = Did not amplify desired product

For positive samples amplified product sizes for different primers were: B4/B5 - 223bp, JPF/JPR - 193 bp and F4/R2 - 905 bp

Table 4. Result of PCR using various primers for detection of *Brucella* species.

Sr. No.	Gene, primers Name → Isolates No.	BCSP31	<i>omp2b/2a</i>		<i>Omp31</i>	Species identified
		B4/B5	JPF/JPR-ab	JPF/JPR-ca	1S/1AS	
1	C1	+	+	-	-	<i>B. abortus</i>
2	C2	+	+	-	-	<i>B. abortus</i>
3	C3	+	+	-	-	<i>B. abortus</i>
4	B1	+	+	-	-	<i>B. abortus</i>
5	B2	+	+	-	-	<i>B. abortus</i>
6	G1	+	+	-	+	<i>B. melitensis</i>
7	G2	+	+	-	+	<i>B. melitensis</i>
8	G3	+	+	-	+	<i>B. melitensis</i>
9	G4	+	+	-	+	<i>B. melitensis</i>
10	D1	+	-	+	+	<i>B. canis</i>
11	Reference strain <i>B. abortus</i> 544	+	+	-	-	<i>B. abortus</i> strain 544

+ = Amplified desired product, -- = Did not amplify desired product

For positive samples amplicon sizes for different primers are: B4/B5 - 223 bp, JPF/JPR-ab - 186 bp, JPF/JPR-ca - 187 bp and 1S/1AS - 249 bp

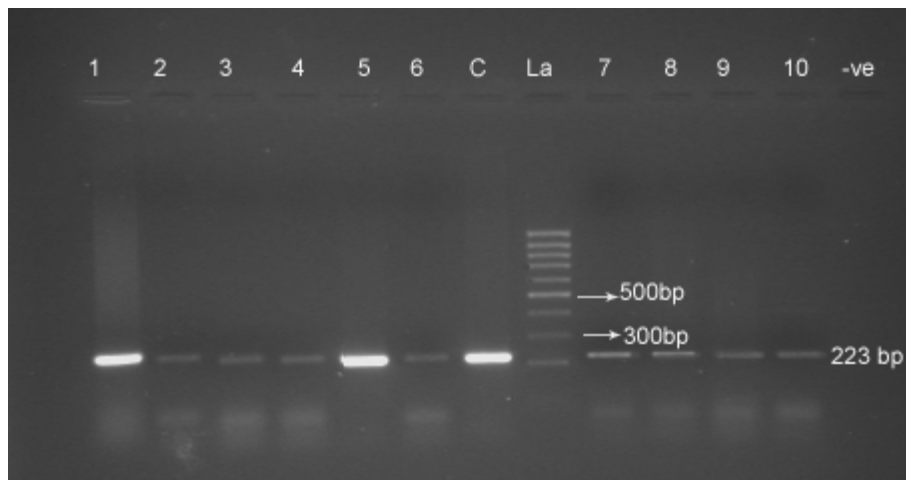


Figure 1. Agarose gel showing PCR amplification product (223 bp) for BCSP31 gene using primer pair B4/B5 of *Brucella*.

223 bp of product amplified with primer pair B4/B5

La : DNA molecular weight ladder 100 bp

C : Control (reference strain *B. abortus* 544)

-ve : Negative control 1 to 10 Field samples (Isolates)

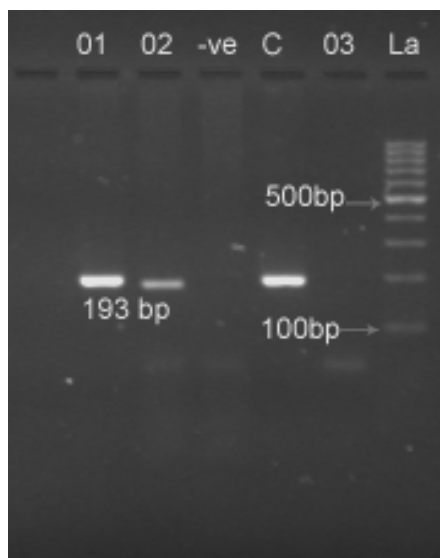


Figure 2. Agarose gel showing PCR amplification product (193 bp) for omp2 gene using primer pair JPF/JPR of *Brucella*.

La : DNA molecular weight ladder 100 bp

C : Control (*B. abortus* 544)

-ve : Negative control 01 to 03 : Field samples (Isolates)



Figure 3. Agarose gel showing PCR amplification product (905 bp) for 16S rRNA gene using primer pair F4/R2 of *Brucella*.

La : DNA molecular weight ladder 100 bp

C : Control (*B. abortus* 544)

-ve : Negative control

1 : Field samples (Isolates)

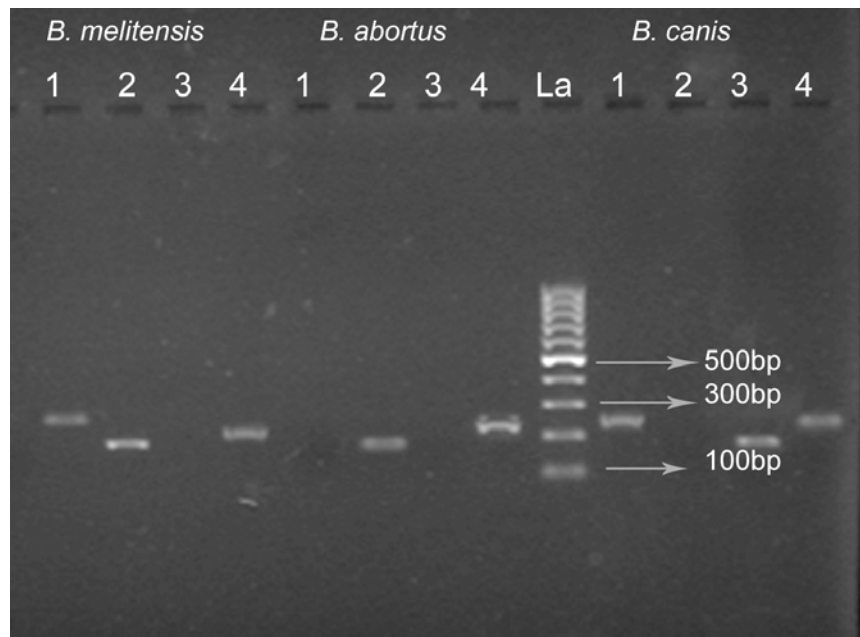


Figure 4. PCR amplified products obtained using various primer sets for detection of various genes in *B. abortus*, *B. melitensis* and *B. canis*.

Lane 1 - 1S/1AS of 249 bp size

2 - JPF/JPR-ab of 186 bp size

3 - JPF/JPR-ca of 187 bp size

4 - B4/B5 of 224 bp size

La - Ladder of 100 bp size

among *Brucella* species and strains are identical or significantly conserved and it was recently reported that 16S rRNA gene sequencing is a reliable tool for rapid genus level identification of *Brucella* spp. (Gee *et al.*, 2004). PCR utilizing different gene targets has recently become the most common way of diagnosis for human and animal brucellosis (Herman and Ridder, 1992; Bricker and Halling, 1994). Even though it is more sensitive, more rapid and less biohazardous than cultural techniques, the isolation of the organism is still accepted as the gold standard. The culture isolation followed by the confirmation by PCR in this study is an another approach of diagnosis, since PCR confirmation can rapidly identify at species level.

Based on the amplified products obtained, the isolates were identified as *B. abortus* for the isolates obtained from cows and buffaloes, based on an amplified product size of 223 bp and 186 bp with primer set B4/B5 and JPF/JPR-ab, respectively. The isolates obtained from goats were identified as *B. melitensis* based on an amplified product size obtained of 223 bp, 186 bp and 249 bp with primer set B4/B5, JPF/JPR-ab and 1S/1AS, respectively. Similarly the lone isolate of bitch was identified as *B. canis* based on an amplified product obtained of 223 bp, 187 bp and 249 bp with primer set B4/B5, JPF/JPR-ca and 1S/1AS. The findings of PCR based *Brucella* spp. identification of the isolates is in accordance with report of Koichi *et al.* (2007), who identified each *Brucella* species using the specific pattern of amplification obtained. Various other workers used species specific primer sets for the identification of species and biotypes of *Brucella*. It is important not only to detect but also to identify the species of *Brucella* implicated in natural infections, thus the present study using a combination of primer pairs makes species differentiation of various *Brucella* species possible and could be useful to study *Brucella* organism at species level.

Since brucellosis is a zoonotic disease and the fight against this disease in humans and animals relies mainly on veterinary sanitation measures focused on the reduction or eradication of this disease in farm animals, a critical tool for the success of these measures is, without a doubt, an accurate and early diagnosis of the disease. Thus the present finding could be useful for the specific and confirmatory detection of *Brucella* organisms from cases of abortion and other reproductive disorders from various species of animals using isolation and direct detection of *Brucella* DNA by colony PCR using various sets of primer pairs to the genus and species levels. It provides a strong basis for the confirmatory diagnosis of *Brucella* infection in animals.

ACKNOWLEDGEMENTS

The authors are thankful to the Professor, Department of Animal Biotechnology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, for providing facilities to carry out the work.

REFERENCES

- Baily, G.C., J.B. Kraahn, B.S. Drasar and N.G. Stokeer. 1992. Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. *Amer. J. Trop. Med. Hyg.*, **95**: 271-275.
- Bricker, B.J. and S.M. Halling. 1994. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. *J. Clin. Microbiol.*, **32**: 2660-2666.
- Fayazi, Z., A. Ghadarsohi and R.G. Hirst. 2002. Development of *Brucella suis* specific

- hybridization prob and PCR which distinguish *B. suis* from *B. abortus*. *Vet. Microbiol.*, **84**: 253-261.
- Foster, G., B.S. Osterman, J. Godfroid, J. Isabelle and A. Cloeckaert. 2007. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *Int. J. Syst. Evol. Microbiol.*, **57**: 2688-2693.
- Gee, J.E., B.K. De, P.N. Levett, A.M. Whitney, R.T. Novak and T. Popovic. 2004. Use of 16S rRNA gene sequencing for rapid confirmatory identification of *Brucella* isolates. *J. Clin. Microbiol.*, **42**: 3649-3654.
- Herman, L. and H. De Ridder. 1992. Identification of *Brucella* spp. by using the polymerase chain reaction. *Appl. Environ. Microbiol.*, **58**: 2099-2101.
- Kanani, A.N. 2007. Serological, cultural and molecular detection of *Brucella* infection in breeding bulls. A thesis submitted to Anand Agricultural University, Anand.
- Kim, S., D.S. Lee, H. Suzuki and M. Watarai. 2006. Detection *Brucella canis* and *Leptospira interrogans* in canine semen by multiplex PCR. *J. Vet. Med. Sci.*, **68**: 615-618.
- Koichi, I., K. Masanobu, S. Michio, K. Tsuneo and Y. Akio. 2007. Simultaneous detection of the genus *Brucella* by combinatorial PCR. *Jon. J. Infect. Dis.*, **60**: 137-139.
- Leal-Klevezas, D.S., V.I.O. Martinez, M.A. Lopez and S.J.P. Martinez. 1995. Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. *J. Clin. Microbiol.*, **3**: 3087-3090.
- Mayfield, J.E., J.A. Bantle and D.R. Ewalt *et al.* 1989. Detection of *Brucella* cell components, p. 98-115. In Nielsen, K. and J.R. Duncan (eds.) *Animal Brucellosis*, CRC Press, Boca Raton, FL.
- Mehra, K.N., N.S. Dhanesar and V.K. Chaturvedi. 2000. Sero-prevalence of brucellosis in bovines of Madhya Pradesh. *Indian Vet. J.*, **77**: 571-573.
- Moreno, E., A. Cloeckaert and I. Moriyón. 2002. *Brucella* evolution and taxonomy. *Vet. Microbiol.*, **90**: 209-227.
- Navarro, E., J. Escribano, J.A. Fernandez and J. Solera. 2002. Comparison of three different PCR methods for detection of *Brucella* spp. from human blood samples. *Immu. Medical Microbiol.*, **34**: 147-151.
- Patel, T.J. 2007. Serological, cultural and molecular detection of *Brucella* infection in bovines including quantification in milk by real-time PCR. Master Thesis, Anand Agricultural University, Anand.
- Renukaradhya, G.J., S. Isloor and M. Rajasekhar. 2002. Epidemiology, zoonotic aspects, vaccination and control/eradication of brucellosis in India. *Vet. Microbiol.*, **90**: 183-195.
- Romero, C., M. Pardo, M.J. Grillo, R. Diaz, J.M. Blasco and I. Lopez-Goni. 1995. Evaluation of PCR and indirect enzyme-linked immunosorbent assay on milk samples for diagnosis of brucellosis in dairy cattle. *J. Clin. Microbiol.*, **33**: 3198-3200.
- Sarumathi, C., T.V. Reddy, B. Sreedevi and U.V.N.M. Rao. 2003. Comparison of avidin-biotin ELISA with RBPT and STAT for screening of antibodies to bovine brucellosis. *Indian Vet. J.*, **80**: 1106-1108.
- Scholz, H.C., Z. Hubalek, I. Sedláček, G. Vergnaud, H. Tomaso, S.A. Dahouk, F. Melzer, P. Kämpfer, H. Neubauer, A. Cloeckaert, M. Maquart, M.S. Zygmunt, A.M. Whatmore, E. Falsen, P. Bahn, C. Göllner, M. Pfeffer, B. Huber, H. Busse, and K. Nöckler. 2008. *Brucella microti* sp. nov., isolated from the

common vole *Microtus arvalis*. *Int. J. Syst. Evol. Microbiol.*, **58**: 375-382.

Unver, A., Y. Rikihisa, M. Kawahara and S. Yamamoto. 2003. Analysis of 16S rRNA gene sequences of *Ehrlichia canis*, *Anaplasma platys*, and *Wolbachia* species from canine blood in Japan. *Ann. N. Y. Acad. Sci.*, **990**: 692-698.

Zerva, L., K. Bourantas, S. Mitka, A. Kansouzidou and N.J. Legakis. 2001. Serum is the preferred clinical specimen for the diagnosis of human brucellosis by PCR. *J. Clin. Microbiol.*, **39**: 1661-1664.

*Continued from page 97

Ramani Pushpa, R.N. and B. Punya Kumari. 2005. Seroprevalence of leptospirosis in domestic animals. *Indian Vet. J.*, **82**: 670-671.

Ratnam, S., C.O.R. Everard, J.C. Alex, L. Suresh Babu, B. Suresh, V. Jayakumar and K. Manickavel. 1994. Leptospiral antibodies among cattle and buffaloes in Tamil Nadu. *Indian J. Anim. Sci.*, **64**: 594-596.

Ratnam, S., S. Subramanian and T. Sundararaj. 1983. Evidence of leptospiral antibodies among domestic animals in Madras City. *Cheiron*, **12**: 176-179.

Sharma, M.C., N.N. Hung and N.V. Vuc. 1982. Buffalo Breeding Res. Cen. Song Be Annual. **5**: 33.

Selvaraj, J., B. Murali Manohar, R. Govindarajan, V. Jayakumar, T.V. Meenambigai, C. Balachandran and A. Koteeswaran. 2005. Prevalence of leptospiral antibodies in buffaloes (*Bos bubalis*) at slaughter. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.*, **26**: 125-127.

Srivastava, S.K. and A.A. Kumar. 2003. Seroprevalence of leptospirosis in animals and human beings in various regions of the country. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.*, **24**: 155-159.

Venugopal, K., S. Ratnam, N. Raghavan and K. Nachimuthu. 1986. Prevalence of leptospiral antibodies in aborting and repeat breeding cattle. *Cheiron*, **15**: 80-83.

EVALUATION OF DIFFERENT METHODS OF DNA EXTRACTION FROM SEMEN OF BUFFALO (*Bubalus bubalis*) BULLS

Anju Manuja¹, Sonia Manchanda², Balvinder Kumar¹, S. Khanna² and R.K. Sethi²

ABSTRACT

Microbes excreted in the semen of infected or carrier bulls can be disseminated to susceptible animals through artificial insemination. The polymerase chain reaction (PCR) has been employed successfully to detect infectious agents in tissues and body fluids. PCR inhibitors present in the semen pose serious problems in detection of microorganisms by inhibiting the amplification of the target DNA template. These inhibitors need to be removed completely during DNA extraction to amplify the target sequences in semen by PCR. DNA was extracted using seven different protocols from semen of buffalo bulls, and the quantity and quality was evaluated spectrophotometrically. Chelex-100 and Qiagen modified methods for extraction of DNA from semen were found to be superior qualitatively as compared to the other methods. In the Qiagen modified protocol, the semen was treated with two extra buffers containing EDTA to chelate the metals. Additional treatment of semen with proteinase K was included to completely degrade cellular proteins. DNA extracted by the phenol-chloroform and CTAB methods yielded high value of residual RNA and other contaminants. The chelex-100 method has the potential advantage of requiring a smaller volume of semen to extract good quality of DNA.

Keywords: DNA extraction, PCR inhibitors, semen, *Bubalus bubalis*, buffalo bull

INTRODUCTION

Several pathogenic microbes are excreted in the semen of infected/carrier bulls and due to lack of effective quality control practices, the infections are disseminated to susceptible animals through artificial insemination. It is, therefore, important that measures be undertaken to screen the bulls against various semen borne infections prior to their entry into breeding programmes. The polymerase chain reaction (PCR) technique has become an important tool to detect infectious agents. During DNA extraction procedures, when the cells are lysed to release nucleic acids, endogenous nucleases, enzymes that degrade nucleic acids (DNA and RNA), are also released. Thus, when minimally degraded (i.e., high integrity) nucleic acid is desired, nuclease activity should be minimized as much as possible. The success of PCR depends on the quality of the DNA; it must be free of contaminants and nucleases of the DNA that impair the amplification process. Inhibitory components of PCR may be present naturally in the sample or added during processing of clinical samples while preparing for PCR.

Spermatozoa and non-sperm cells also inhibit PCR. Semen is composed of cellular (spermatozoa, urogenital epithelial cells, round cells etc.) and non-cellular components. There is also a portion of the cells loaded with a special protein that anchors the sperm cell to the egg. Biochemically, there is a lot of nucleic acid (DNA), some glycogen

¹National Research Centre on Equines, Sirsa road, Hisar-125001, Haryana, India

²Central Institute for Research on Buffaloes, Sirsa road, Hisar-125001, Haryana, India

as an energy source, lots of protein, but few fats and lipids. Besides this, the prostate produces zinc which protects the sperm chromatin condensation from becoming superstabilized as a result of excessive disulfide bond crosslinking of the protamines. So, it contains metal ions too. Thus, there is a need for methods and compositions for isolating high integrity, i.e., high molecular weight, nucleic acid molecules in a rapid and efficient manner. It is also very critical to keep an account of the inhibitory factors while interpreting the results to avoid false negative results. Although modified DNA extraction procedures from semen have been reported in cattle, the information on buffalo bull semen is scanty. Previously we reported a rapid, reliable and convenient method for DNA extraction from buffalo bull semen using Chelex 100 resins, and DNA was used successfully for the molecular detection of BHV-1 in *Bubalus bubalis* (Manuja *et al.*, 2006). But no previous work seems to have been done on systematic comparison of these extraction procedures with conventional methods. In this study, as a preliminary for further work, we have evaluated different methods for DNA extraction from semen of *Bubalus bubalis* quantitatively and qualitatively.

MATERIALS AND METHODS

Semen samples

Fresh semen samples of four healthy Murrah buffalo bulls from organized farms in India were collected in Eppendorf tubes and stored at -20°C till further use. Bulls passed a complete physical examination and a breeding soundness evaluation. Strict hygienic conditions were maintained during semen collection.

DNA extraction from Semen

DNA was extracted from semen of four bulls by the following extraction methods.

Phenol: chloroform method

DNA was extracted as per method described by Birren *et al.* (1997) with slight modifications. Briefly, 200 µl of semen was treated with 0.1 volume of 10% sodium dodecyl sulphate (SDS) and 0.1 volume of 2M sodium acetate and incubated for 1 h at 56°C in a water bath. After the addition of an equal volume of saturated phenol:chloroform:isoamylalcohol (PCI mixture in the ratio of 25:24:1) and vortexing and centrifuging at 10,000 x g for 10 minutes in refrigerated centrifuge, the upper aqueous layer taken in another tube was extracted with an equal volume of 24:1 chloroform: isoamylalcohol (CI) mixture. The PCI extraction was repeated until the interface was clear. To the final aqueous solution, a 0.1 volume of 3M sodium acetate pH 5.2 and an equal volume of isopropanol was added and mixed gently. The DNA was precipitated by keeping at -20°C overnight. The DNA was pelleted by centrifuging at 10,000 x g for 10 minutes and washed with pre-chilled 70% ethanol to remove the excess salts. The resultant pellet was air dried and dissolved in tris-EDTA buffer (TE).

Cetyl Trimethyl Ammonium Bromide (CTAB) method

DNA was extracted from semen as described by Ausubel *et al.* (1990) with some modifications. A mixture of 500 µl semen and 10 ml extraction buffer was incubated at 60°C for 1 h. After addition of 20 µl CI and centrifugation at 3000 x g for 20 minutes, the upper phase was taken in fresh tube and was precipitated with 1 volume of isopropanol at -20°C. The pellet obtained after centrifugation at 3000 x g for 20 minutes was extracted again with CI. The upper phase was mixed with 0.4 ml of 10% CTAB (Amresco, USA), 1 volume of CI and centrifuged at 3000 x g for 20 minutes. Then the upper phase mixed with equal volume of 1% CTAB was incubated at room temperature for 30 minutes. The pellet obtained after

centrifugation at 3000 x g for 20 minutes was washed with 70% ethanol, 10 mM ammonium acetate and finally redissolved in 1 ml of TE.

Chelex-100 method

DNA was extracted by Chelex-100 method as previously described by Walsh *et al.* (1991) and modified by Santrude *et al.* (1996) and Manuja *et al.* (2006). Briefly, a 25 µl of sample was added to 200 ml of 5% Chelex-100 resin (Sodium form, Sigma, USA). Proteinase K (MBI fermentas, EU) at 0.1mg/ml concentration and 31 mM dithiothreitol (DTT) were added to 225 ml of semen and mixed thoroughly. After incubation at 56°C for 45 minutes, the mixture was incubated in a boiling water bath. After vigorous vortexing for 10 seconds, the material was centrifuged at 10,000 x g for 3 minutes. Then the supernatant was collected and stored at -20°C to be used for PCR assay as template.

Qiagen Protocol

DNA was extracted from 200 µl semen using mini QIAamp DNA mini kit (Qiagen, Germany) as per manufacturer's instructions.

Qiagen Modified Protocol

Before proceeding to the Qiagen protocol, semen was treated with two additional buffers and proteinase K. Briefly, 200 µl of semen and 10 ml of lysis buffer (150 mM NaCl and 10 mM EDTA, pH 8.) were mixed and centrifuged at 2500 x g for 10 minutes. The pellet was resuspended in 300 µl buffer containing 100 mM Tris-Cl, pH 8.0, 10 mM EDTA, 500 mM NaCl, 1% SDS and 2% 2-mercaptoethanol and then 100 µl of proteinase K was added. After incubation at 56°C for 2 h, another 20 µl proteinase K was added and incubated again as specified above. After addition of lysis buffer supplied with the kit and ethanol in 400 µl quantity each, the mixture was applied to the mini spin column (Qiagen, Germany) and centrifuged at 6000 x g for 1 minute

and the tube containing filtrate was discarded. The bound DNA was washed and eluted as per the manufacturer's instructions.

Trizol method

TRI reagent method was used for total DNA isolation as per the manufacturer's instructions (Sigma, USA). To 200 µl of semen an equal volume of TRI reagent was added and allowed to stand for 5 minutes at room temperature. Following addition of 40 µl chloroform, it was centrifuged after 15 minutes at 12,000 x g for 15 minutes at 4°C. After transferring the interface to a fresh tube, 100 µl of ethanol was added and centrifuged after 10 minutes at 12,000 x g for 10 minutes at 4°C. The pellet was washed with pre-chilled 75% ethanol, air-dried and dissolved in TE.

One step-RNA reagent method

DNA was extracted using one-step RNA reagent (Bio Basic Inc.) as per the manufacturer's protocol. To 500 µl of semen, after centrifugation at 3000 rpm for 1 minute an equal quantity of reagent was added and incubated for 5 minutes at room temperature. After addition of 100 µl of chloroform, tubes were shaken vigorously, incubated at room temperature for 3 minutes and centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase containing RNA was discarded. DNA was precipitated from the interface and organic phase with 150 µl of absolute ethanol and incubated at room temperature for 3 minutes and then centrifuged at 2000 x g for 5 minutes at 4°C. The DNA pellet was washed twice with 0.1 M sodium citrate in 10% ethanol. At each wash, DNA pellet was kept in washing solution for 30 minutes at room temperature, centrifuged at 2000 x g for 5 minutes at 4°C. Finally the DNA pellet was washed with pre-chilled 75% ethanol, air-dried and dissolved in TE.

Quantification and Purity of DNA

The quantity and quality of extracted DNA was determined by spectrophotometer (Bechman, USA). The concentration of DNA was calculated based on the approximation that an absorbance reading of 1 of the purified DNA at 260 nm was taken to correspond to 50 µg/ml (50 ng/µl). DNA purity was estimated by determining the A_{260}/A_{280} ratio and the reference value considered for purity was 1.8.

Statistical Analysis

Quantitative and qualitative analysis was done by Duncan's multiple range test (Steel and Torrie, 1984).

RESULTS AND DISCUSSION

Current methods for isolating nucleic acids from cells generally include lysing the cells and inactivating nucleases using chaotropic salts and a nonionic surfactant. The released nucleic acids are then selectively precipitated from the solution. The combination of chaotropic salts and nonionic surfactants, however, are ineffective when they have to go through tough contents to release nucleic acid. Semen contains a lot of nucleic acid (DNA), glycogen, proteins, fats and lipids and some metal ions, some of which may inhibit PCR. Different scientists have developed different methods of DNA extraction for the removal of these unknown inhibitors from the semen so as to obtain better results in PCR (Santrude *et al.*, 1996; Masri *et al.*, 1997; Manterola *et al.*, 2003; Gupta *et al.*, 2006; Manuja *et al.*, 2006). But a comparative study of these procedures with the conventional methods is lacking. Therefore, in this study, we have evaluated seven different protocols for extraction of DNA from the semen of buffalo bulls.

Qualitative analysis of all the four replicates of DNA samples extracted from semen of buffalo bulls by different extraction methods is shown in Table 1. Relative qualitative analysis of different DNA extraction methods employing all the four replicates was calculated by subtracting the A_{260}/A_{280} of DNA samples from the value of 1.8, which was the purity value of DNA taken as the reference (Figure 1). Values above or below 1.8 imply impurities in the DNA (Birren *et al.*, 1997). All the four samples extracted by the phenol- chloroform method yielded values higher than 1.8 whereas two samples from CTAB exhibited values less than 1.8 (Table 1). Phenol-chloroform and CTAB methods have mean values much greater than 1.8 indicating high residual RNA so these methods should include treatment with RNase A, so as to obtain pure DNA preparations. These two methods are similar as per Duncan's multiple range tests for variables. These are followed by Qiagen modified, Chelex-100, Qiagen, one-step RNA reagent, Trizol, and all these methods are similar (Table 1, Figure 1). The Qiagen and one-step RNA reagent methods have less than 1.8 A_{260}/A_{280} ratios for extracted DNA which indicates protein contamination, maybe due to decreased protease activity. The Chelex-100 method has a mean value of 1.8, so the DNA obtained is pure enough. The Qiagen modified method is also a method of choice as the DNA obtained was purer than other methods in this case. A potential advantage of the Chelex-100 method is the volume of semen, i.e. 25 µl; only one straw can be used for extraction of DNA from semen (Manuja *et al.*, 2006). This method also removes the polyvalent metal ions, which have potential lesive effects on DNA extraction at high temperatures (Santrude *et al.*, 1996; Manuja *et al.*, 2006) so this method should be the method of choice. The Chelex-100 method has also been used for molecular detection of bovine herpes virus-1 (BHV-1) in *Bubalus bubalis* and was found to be rapid, convenient and accurate

Table 1. Qualitative analysis of DNA extracted from semen by different methods.

Semen samples	Quality of DNA extracted from semen in ng/ μ l						
	Phenol-Chloroform	CTAB	Chelex-100	Qiagen	Qiagen modified	Trizol	One Step RNA Reagent
1	2.87	1.70	1.79	1.70	1.70	1.70	1.62
2	2.86	1.70	1.99	1.70	2.04	1.66	1.78
3	2.63	3.19	1.84	1.72	1.7	1.7	1.70
4	2.64	3.02	1.67	1.73	2.04	2.04	1.78
Mean	2.75 ^A	2.40 ^A	1.82 ^B	1.71 ^B	1.87 ^B	1.68 ^B	1.70 ^B
Std. Dev	0.08	0.49	0.02	0.01	0.12	0.01	0.06

Means bearing different superscripts (A&B) are significantly different with respect to Duncan's multiple range test ($P < 0.01$).

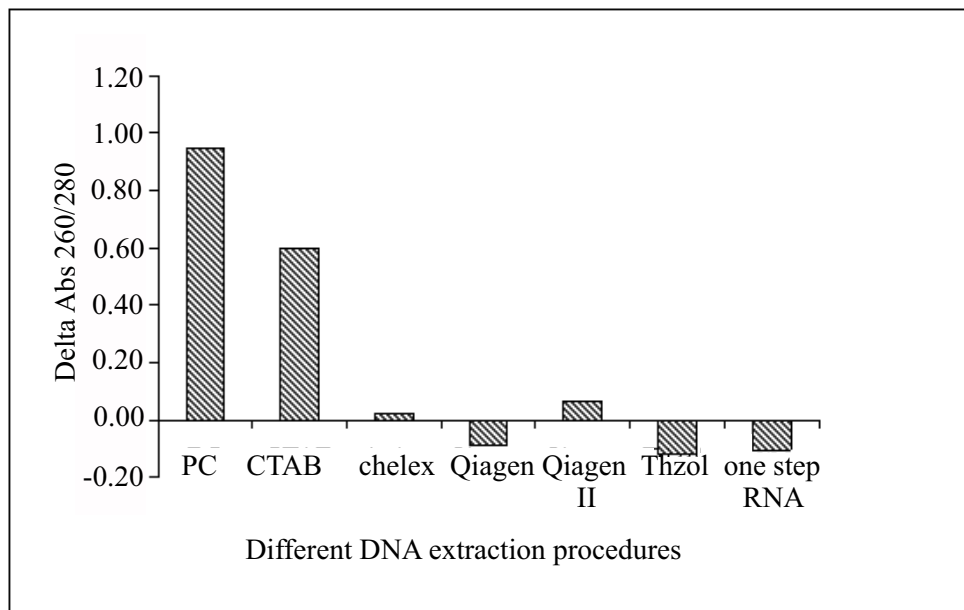


Figure 1. Extent of DNA contamination using different extraction procedures.

(Manuja *et al.*, 2006). In the Qiagen modified protocol, the semen is treated with two extra buffers containing 10 mM EDTA which is used to chelate the metals. Sequestration of Mg ions by EDTA serves to inhibit nuclease activity. Additional treatment of semen with proteinase K is included to completely degrade cellular proteins. Therefore, this method provides good quality of DNA.

Quantitative analysis of different DNA extraction methods showed the highest mean value for all the four replicates of DNA extracted by Trizol followed by Qiagen, phenol-chloroform, one-step RNA reagent, Chelex-100, Qiagen modified, CTAB, which were all quantitatively different as per Duncan's multiple range test for variable. The quantity of DNA obtained was greater with the Trizol and Qiagen methods in comparison to the other methods studied (data not shown). Since the DNA was extracted from variable amounts of semen according to the given protocols, the comparison was determined on the basis of 100 µl of semen.

A further attempt to use of DNA extracted by various methods for the detection of infectious agent using PCR and subsequent comparative analysis is required. However, we have reported earlier a rapid, simple method of DNA extraction using Chelex-100 from semen of buffalo bulls for its use in PCR for detection of BHV-1. Inhibition in a PCR assay due to excess host genomic DNA is suggested to be a simple consequence of decrease in the rate of diffusion of macromolecular components in the reaction mixture (Cogswell *et al.*, 1996). It has been reported that higher amounts of host genomic DNA could interfere with the PCR detection of the infectious agent (Cogswell *et al.*, 1996). Higher dilution of DNA from clinical samples have also been reported to increase the sensitivity of the PCR assay (von Stedingk *et al.*, 1995).

It is concluded that the Chelex-100 and Qiagen modified methods for extraction of DNA from semen were superior qualitatively as compared

to the other methods. If cost is to be considered then phenol-chloroform method is the cheapest method to follow, but the expensive method used in preparing high quality DNA is well worth since this will be stable for years, eliminating the need to repeat extractions. Moreover, the samples derived from the unique conditions are frequently not replaceable.

REFERENCES

- Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl. 1990. *Current Protocols in Molecular Biology*. Greene Publishing Association and John Wiley & Sons. 2.4.2-2.4.3.
- Birren, B., E.D. Green, S. Klapholz, R.M. Myers and J. Roskams. 1997. Standard methods used for isolating DNA. In *Genome Analysis: A Laboratory Manual*. Cold Spring Harbor Laboratory Press **1**: 617-629.
- Cogswell, F.B., C.E. Bantar, T.G. Hughes, Y. Gu and M.T. Philipp. 1996. Host DNA can interfere with detection of *Borrelia burgdorferi* in skin biopsy specimens by PCR. *J. Clin. Microbiol.*, **34**: 980-982.
- Gupta, P.K., M. Saini and A. Rai. 2006. Rapid and sensitive PCR based test for detection of Bovine herpesvirus-1 in semen. *Indian J. Virol.*, **17**: 23-27.
- Manterola, L., A. Tejero Garces, A. Ficapal, G. Shopayeva, J.M. Blasco, C.M. Marin and G.I. Lopez. 2003. Evaluation of a PCR test for the diagnosis of *Brucella ovis* infection in semen samples from rams. *Vet. Microbiol.*, **92**: 65-72.
- Manuja, A., M. Prasad, B. Kumar, G. Prasad and R.K. Sethi. 2006. Molecular detection of BHV-1 genome in semen of buffalo (*Bubalus bubalis*) bulls. *Indian Buffalo J.*, **4**(1): 8-11.

*Continued on page 128

STUDIES ON THE THYROID PROFILE (T_3 , T_4 AND TSH) IN ESTROUS, CYCLIC AND ANOESTROUS BUFFALOES

Raja Kumar P.¹, Lakshmana Swamy P.¹, Aswani Kumar K.¹ and Somasekhara Rao K.²

ABSTRACT

In the present study, serum concentrations of the thyroid profile (T_3 , T_4 and TSH) were estimated in normal cyclic, estrous and anoestrous buffaloes. The heifers, both in estrous and anoestrous, were in the age range from 16 months to 24 months, and the cyclic buffaloes were aged 3-6 years. The method adopted for the estimation was the EIA method. The T_3 values observed were 3.179 ± 1.660 , 2.936 ± 1.260 and 2.238 ± 0.912 , The T_4 values observed were 5.920 ± 1.963 , 5.093 ± 1.614 and 5.500 ± 1.208 , and the TSH values were 0.035 ± 0.059 , 0.038 ± 0.030 , and 0.014 ± 0.025 in estrous, cyclic and anoestrous buffaloes, respectively. The overall thyroid diagnosis in estrous was 47%, in cyclic, 34%, and in anoestrous, 19%. The occurrence of the thyroid profile in controls and anoestrous varied significantly. The thyroid profile was low in anoestrous buffaloes when compared with the controls. The variation ranged about 15-18% between controls and anoestrous buffaloes.

Keywords: anoestrous, estrous, cyclic, heifer, thyroid profile, reproductive activity

INTRODUCTION

Endocrinology has the important position of being able to correlate anatomy, physiology, genetics

and biochemistry (LE Mc Donald *et al.*, 1985). The thyroid gland is very important in governing various body functions. The hormones T_4 and T_3 and calcitonin (CT) are related with maintenance of BMR and growth. Low levels of T_4 and T_3 are related with delayed puberty and reproductive disorders, so the levels of thyroid hormones are important in the reproductive activity of the animals (Chandra Sekhar *et al.*, 1985). The reproductive performance is very poor primarily because there is a high incidence of anoestrus (63-83%) especially during summer which has been attributed to heat stress together with an overriding effect of nutritional deficiencies owing to scarcity of green fodder. Negative energy balance (NEB) decreases circulatory concentrations of glucose, insulin and innulin-like growth factor (IGF-I), leading to increased losses of body condition score (BCS) and higher incidence of anoestrous in cows and buffaloes. Abnormalities in reproduction are common when breeding animal develops hypothyroidism. Lack of libido and reduction in sperm count occurs in males, whereas abnormal or absent estrus cycles with reduced conception rates may result in females. Obesity and changes in behavior resulting from hypothyroidism often have detrimental effects on reproduction. Iodine deficiency is a health problem of considerable magnitude in India. A minimum of 60 μg of elemental iodine per day is required for thyroid hormone synthesis. Thyroid disorders are more common in female than in male animals

¹Department of Veterinary Biochemistry, NTR College of Veterinary Science Gannavaram, Krishna (Dt)-521102, Andhra Pradesh, India

²Acharya Nagarjuna University PG Centre, Nuzvid-521201, Andhra Pradesh, India

(Larsen *et al.*, 2003). Measurement of TSH is more reliable in the diagnosis of thyroid hormone abnormalities than the measurement of thyroid hormone levels. A sensitive TSH assay is now an accepted initial screening test of thyroid function. T_4 and T_3 circulate in the blood as equilibrium mixtures of free and protein bound hormones. Carrier proteins bind 99.97% of T_4 and 99.7% of T_3 . Only a small fraction of the total is free and physiologically active (Carl *et al.*, 2000). Enzyme immunoassays (EIA) are rapid and provide a high degree of specificity and sensitivity over the traditional radioimmunoassays (RIA). Immunometric assays offer not only improved sensitivity for TSH measurement but also rapid turnaround time and a wide linear measurement range as compared with RIA methods (Odell *et al.*, 1965). There is no previous study on the thyroid profile of cattle or buffaloes. The values obtained from this study of cyclic and estrous buffaloes are considered as control values with which to compare anoestrous values.

MATERIALS AND METHODS

The present study was conducted from September 2006 to August 2007. During this period, buffaloes brought to veterinary hospitals of rural areas were selected. The present study comprised a total of 126 buffaloes, out of which 50 heifers ranging in age from 12-18 months were in anoestrous and 50 heifers (aged from 12-18 months) were in estrous, and 26 buffaloes were cyclic (aged from 3-6 years) and active in their reproductive performance were used as controls. The buffaloes are selected after thorough examination by per rectal palpation of internal genitalia and ovaries. The buffaloes with thyroid disorders were excluded. A sample of 5 ml of blood was withdrawn from the each buffalo and the serum samples were stored at 2-8°C. The serum samples were analyzed for T_3 ,

T_4 and TSH by using an EIA kit (VEDA LABS Product number T 012)

A monoclonal antibody to thyroid hormones (T_3 , T_4 and TSH) was immobilized on the micro wells. A purified (T_3 , T_4 and TSH) polyclonal antiserum conjugate to the enzyme horseradish peroxidase was used to detect T_3 , T_4 and TSH in the given serum samples. For the measurement of T_3 , T_4 and TSH, the EIA method was employed (Odell *et al.*, 1965).

For TSH concentration, a standard curve was prepared by plotting the average absorbance values against the concentration of the standards in μ IU/ml on a linear-log graph paper using the mean absorbance value for each sample. The concentration of TSH in μ IU/ml was determined from the standard curve (Odell *et al.*, 1965).

For T_3 concentration, % A/A_0 was calculated for each standard and test sample by dividing the average absorbance (A) of each standard and test sample by the average absorbance (A_0) for the 0 ng/dl standard and multiplying by 100. A standard curve was prepared by plotting the % A/A_0 for standards against the concentration of the standards on linear-log graph paper. Using the % A/A_0 value for each sample, the concentration of T_3 in ng/dl is determined from the standard curve (Cavaliere *et al.*, 1977).

For T_4 concentration, the average absorbance (A) of each standard and test sample was divided by the average absorbance (A_0) for the 0 μ g /dl standard and multiplied by 100 to give % A/A_0 for each sample and standards. A standard curve was prepared by plotting the % A/A_0 for standards against the concentration of the standards on linear-log graph paper. Using the % A/A_0 value for each sample the concentration of T_4 in mean absorbance value for each sample μ g/dl was determined from the standard curve (Evered *et al.*, 1976).

RESULTS AND DISCUSSION

The values of T_3 in the present study were as follows: 3.179 ± 1.660 ng/ml, 2.238 ± 0.912 ng/ml, and 2.936 ± 1.26 ng/ml estrous, anoestrous and cyclic buffaloes. The T_3 values were lower in the anoestrous heifers than in the controls, so it was concluded that the thyroid-stimulating hormone can regulate the secretion of the triiodo thyronine, T_3 in low, levels leads to hypothyroidism in the anoestrous buffaloes. For the expression of the estrous condition, normal levels of T_3 are required. So, low levels of T_3 can depress the reproductive performance in the buffaloes. The values of T_4 in the present study in estrous, anoestrous and cyclic buffaloes were 5.920 ± 1.963 μ g/dl, 5.500 ± 1.208 μ g/dl, and 5.930 ± 1.614 μ g/dl, respectively. The T_4 values were also lower in the anoestrous than in cyclic buffaloes and estrous buffaloes, but these values were very close in these three cases when compared with the T_3 and TSH values.

The TSH values observed were 0.035 ± 0.059 , 0.038 ± 0.030 and 0.014 ± 0.025 , in estrous, cyclic and anoestrous buffaloes, respectively.

The anoestrous condition is generally seen as 65.9% in winter, 59.75% in spring, 55.99% in summer, 57.38% in the rainy season and 66.73% in autumn, and as 48% the entire year. In heifers generally the reproductive problems are 23.53% in all the seasons, and they may gradually decrease by calving status of the buffaloes. (1st calving 18.86%, 2nd calving 18.19% and > 3rd calving 13.02%.)

The table represents the thyroid profile of the estrous, cyclic and anoestrous buffaloes. These values were obtained by the use of biostatistical formulae i.e. mean \pm SD. The overall thyroid profile diagnosis in anoestrous was 19%, in estrous 47%, and in the controls, are 34%. The thyroid profile was low in anoestrous buffaloes when compared with the controls. The variation between controls and anoestrous buffaloes ranged 15 to 28%. The maximum anoestrous was due to low protein and other nutrients in the diet. The range of thyroid profile, according to the RIA method the values obtained for normal and reproductively inactive animals were similar in level, but it was concluded that there was little variation between the thyroid profiles of normal and anoestrous buffaloes. Finally, the buffaloes provided a good environment, highly nutritious feed, and thyroid hormonal ingestion give good results in reproduction.

Table. The mean concentrations of thyroid profile of estrous, cyclic and anoestrous buffaloes with standard deviation.

Thyroid Profile	Estrous (50)	Cyclic (26)	Anoestrous (50)
TSH in μ IU / ml)	0.035 ± 0.059	0.038 ± 0.030	0.014 ± 0.025
T_3 in ng/ml	$3.179 \pm 1.660^*$	$2.936 \pm 1.260^*$	2.238 ± 0.912
T_4 in μ g / dl	$5.920 \pm 1.963^*$	$5.918 \pm 1.392^*$	$5.500 \pm 1.208^*$

Mean \pm SD at * $P < 0.05$ -statistical significance compared.

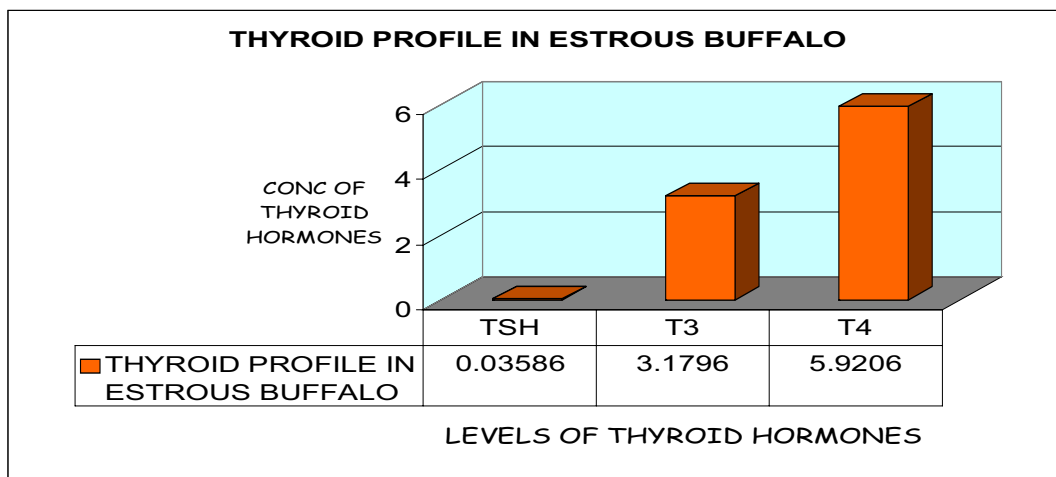


Figure 1. Thyroid profile in estrous buffaloes.

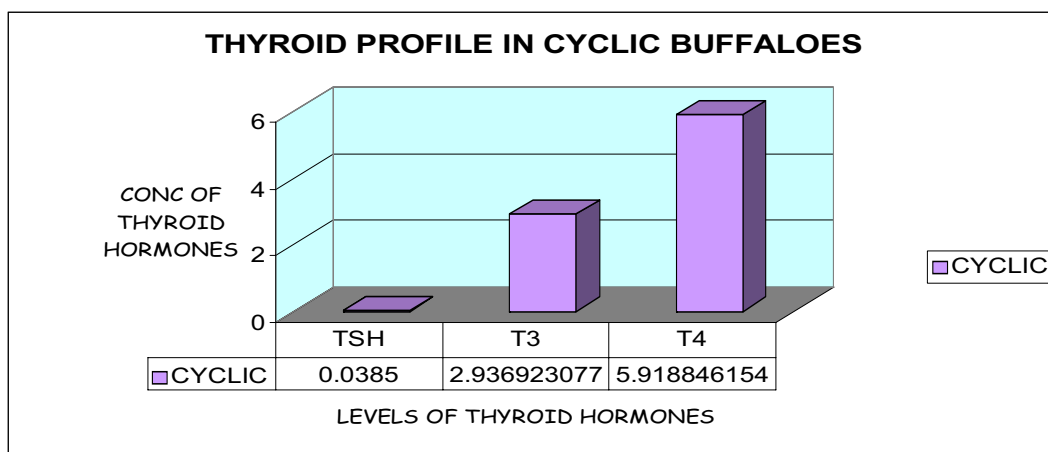


Figure 2. Thyroid profile in cyclic buffaloes.

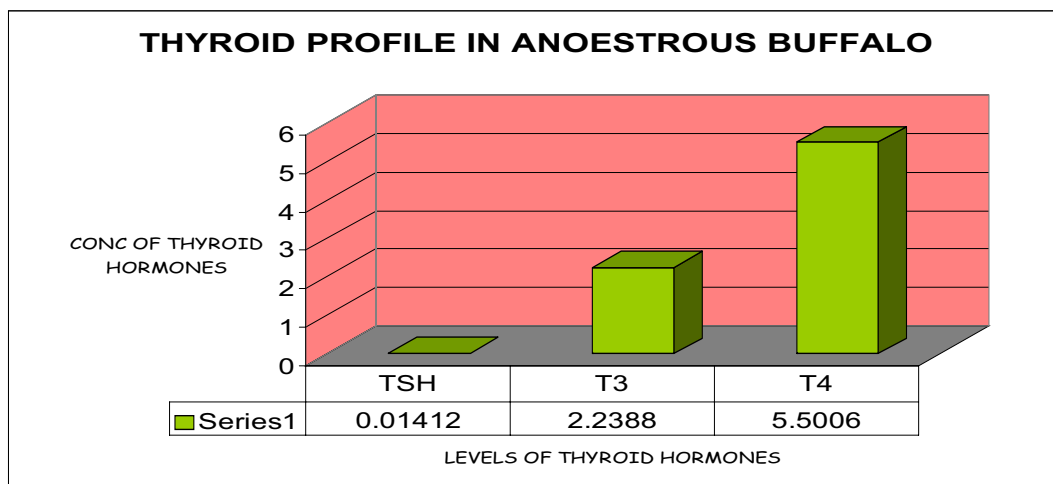


Figure 3. Thyroid profile in anoestrous buffaloes.

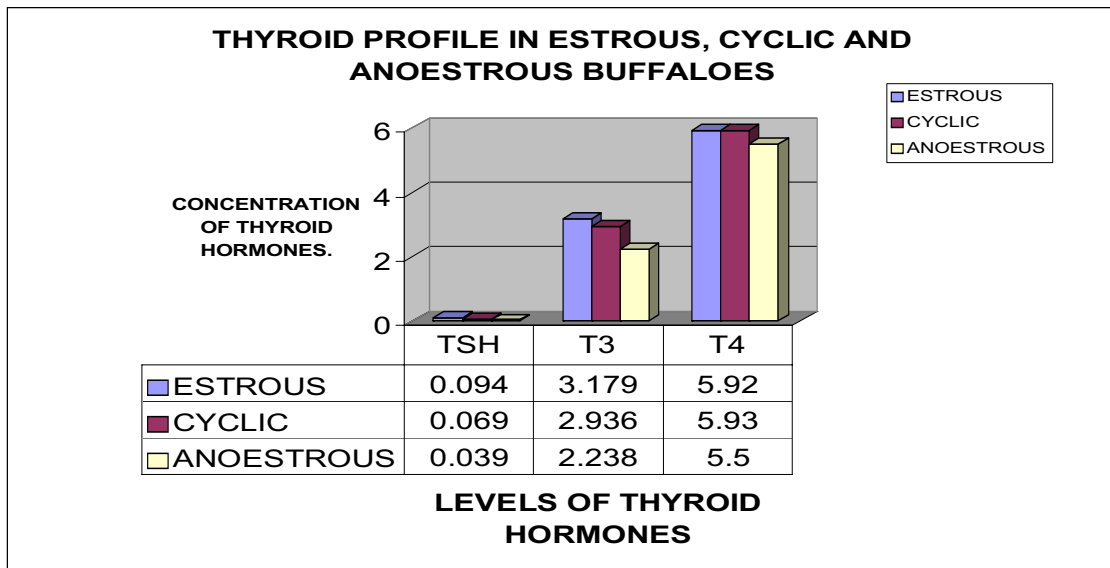


Figure 4. Thyroid profile in estrous, cyclic, and anoestrous buffaloes.

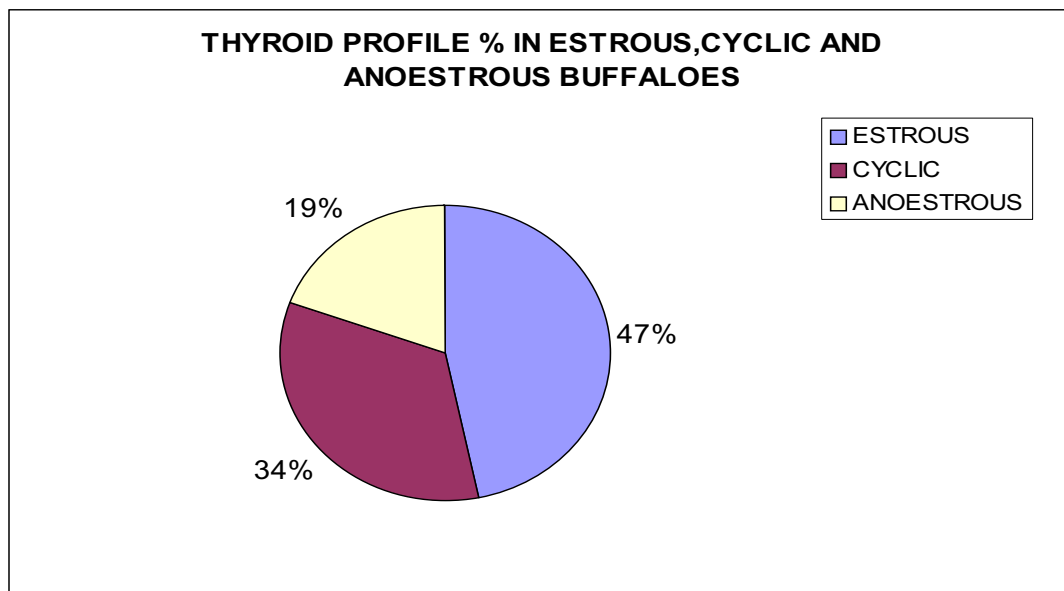


Figure 5. %Thyroid profile in estrous, cyclic, and anoestrous buffaloes.

REFERENCES

- Acebedo, G., A. Hayek, M. Klegerman, L. Crolla, E. Bermes and M. Brooks. 1975. A rapid ultramicro- radioimmunoassay for human thyrotropin. *Biochem. Biophys. Res. Commun.*, **65**: 449-455.
- Burke, C.W. and C.J. Eastman. 1974. Thyroid hormones. *Brit. Med. Bull.*, **30**: 93-99.
- Burr, W.A., E.G. Black, R.S. Griffiths, R. Hoffenberg, H. Meinhold and K.W. Wenzel. 1975. Serum triiodothyronine and reverse triiodothyronine concentrations after surgical operation. *Lancet*, **306**(Issue 7948): 1277-1279.
- Carl A. Burtis and Edward R. Ashwood. 1998. *Tietz Textbook of Clinical Chemistry*, 3rd ed. Philadelphia, PA, WB Saunders. 1917p.
- Cavalieri, R.R. and B. Rapoport. 1977. Impaired peripheral conversion of thyroxine to triiodothyronine. *Annu. Rev. Med.*, **28**: 57-65.
- Chandra Sekhar *et al.* 1985 and Brooks *et al.* 1964. (research article).
- Evered, D. 1976. *Diseases of the Thyroid*. John Wiley & Sons, New York. 29.
- Hamburger, J.I. 1973. Perspectives on diagnostic techniques. In *Thyroid Today: an Endocrine Update*, Inger, S.H. (ed.) MEDCOM Learning Systems, Deerfield, IL.
- Hershmann, J.M. and J.A. Pittman. 1971. Utility of the radioimmunoassay of serum thyrotrophin in man. *Ann. Intern. Med.*, **74**: 481-490.
- Hsu, T.H. and S.S.L. Yang. 1978. Thyroid function tests, p. 34-54. In Rothfeld, B. (ed.) *Nuclear Med: Endocrinal*. JB Lippincott Co., Philadelphia.
- Larsen, P.R., H.M. Kronenberg, S. Melmed, K.S. Polonsky. 2003. *Williams Textbook of Endocrinology*, 10th ed. Philadelphia, Pa, Saunders. 1927p.
- Mc Donald, L.E. and D.V.M. 1985. *Textbook Veterinary Endocrinology and Reproduction*. 61-63, 72.
- Mc Catty D.G. *et al.* 1975. Thyroid hormone levels and prognosis in patients with serious non-thyroid illness. *Lancet II*: 275-276.
- Odell, W.D., J.F. Wilber and W.E. Paul. 1965. Radioimmunoassay of thyrotropin in human serum. *J. Clin. Endocrinol. Metab.*, **25**(9): 1179-1188.
- Oppenheimer, J.H. 1968. Role of plasma proteins in the binding, distribution and metabolism of the thyroid hormones. *N. Engl. J. Med.*, **278**: 1153-1162.
- Patel, Y.C., H.G. Burger and B. Hudson. 1971. Radioimmunoassay of serum thyrotropin: sensitivity and specificity. *J. Clin. Endocrinol. Metab.*, **33**(5): 768-774.
- Sluiter, W.J., F. Van Kersen, A.K. Van Zanten, H. Beckhuis and H. Doorenbos. 1972. A radioimmunoassay of human TSH, employing a solid phase second antibody, and a purified globulin preparation for standardization of non specific protein interactions. *Clin. Chim. Acta.*, **42**: 255-262.
- Spector, D.A., P.J. Davis, J.H. Helderman, B. Bell and R.D. Utiger. 1976. Thyroid function and metabolic state in chronic renal failure. *Ann. Intern. Med.*, **85**(6): 724-730.
- Sterling, K., S. Refetoff and H.A. Selenkow. 1970. T3 Thyrotoxicosis: Thyrotoxicosis due to elevated serum triiodothyronine levels. *J.A.M.A.*, **213**(4): 571-575.
- Tietz, N.W. 1983. *Clinical Guide to Laboratory Tests*. W.B. Saunders Co., Philadelphia, 1296p.
- Utiger, R.D. 1965. Radioimmunoassay of human plasma thyrotropin. *J. Clin. Invest.*, **44**(8): 1277-1286.

*Continued on page 128

VALUE ADDED BUFFALO MEAT SAUSAGE WITH POTATO FLOUR AS BINDER

R. Ponsingh¹, R. Narendra Babu², S. Wilfred Ruban³ and V. Appa Rao⁴**ABSTRACT**

A study to determine the optimum level of inclusion of three different levels of potato flour (3, 5 and 7 percent) in buffalo meat sausage incorporated with 30 percent low-value meat was carried out. The optimum level of potato flour was determined by assessing the physico-chemical (emulsion pH, product pH, emulsion stability, cooking yield and shear force value) and sensory characteristics. A highly significant ($P < 0.01$) increase in emulsion and product pH was observed with increase in level of inclusion. Similarly, 7 percent flour recorded better emulsion stability and cooking yield compared to other levels. Sensory evaluation revealed that sausages prepared with 5 percent potato flour had superior scores ($P < 0.01$) compared to 3 and 7 percent. Sausages with 5 percent level had higher scores for appearance, texture, flavour and overall palatability except for juiciness. Sausages prepared with a 5% level of PF were packed and stored under refrigeration ($4 \pm 1^\circ\text{C}$) for 30 days. The product was subjected to storage stability studies based on evaluation of pH, shear force, TBARS and tyrosine value. The results revealed that during storage there was a highly significant ($P < 0.01$) decrease in pH and shear force value, and significant increases in TBARS and TV value. Sausages

prepared with 5 percent PF were acceptable up to 21 days of refrigerated storage

Keywords: buffalo sausage, potato flour, emulsion, sensory evaluation

INTRODUCTION

India ranks first in the world buffalo population (96.9 million) and possess about 58 percent of world population (FAO, 2003). In India, the production of buffalo meat is 142 million tonnes (FAO, 2003), and this accounts for 35.7 percent of the total meat production in the country contributing significantly for human nutrition and gross domestic product. Although buffalo meat is rated superior to beef (Keshava Rao and Kowale, 1986), the meat from aged buffalo is not preferred because of its toughness.

Utilization of tough meat like head meat and other offal meat in the production of value added comminuted meat products will promote the meat industry. Buffalo meat has been used for processing of products like sausages (Sachindra *et al.*, 2005), loaves (Suresh *et al.*, 2004) burgers (Modi *et al.*, 2003), patties (Suman and Sharma, 2003) and nuggets (Thomas *et al.*, 2006). These offal meat and low value cuts are low in emulsifying and water

¹Hind Agro Foods Limited, Aligarh, India

²Department of Meat Science and Technology, Madras Veterinary College, Chennai- 605 007 India

³Department of Livestock Products Technology, Veterinary College, Hebbal, Bangalore- 560 024 India, E-mail: rubanlpt@gmail.com

⁴Department of Meat Science and Technology, Madras Veterinary College, Chennai India

binding capacities (Whiting, 1989 and Hendrick *et al.*, 1994). Binders, especially those of plant origin, can be used in the product formulations to compensate low functionality of such meat and offals (Bawa *et al.*, 1998). Potato has long been used by meat processors and can be processed into starches and flour which can be used in sausages as a binder or extender to increase water binding and improve cooking yield (Berry, 1997 and Hughes *et al.*, 1998). Hence this study was designed to evaluate the optimum level of inclusion of potato flour as a binder in improving the stability of buffalo meat sausage.

MATERIALS AND METHODS

Fresh buffalo lean meat, head meat, heart, tongue and fat were obtained from the buffaloes slaughtered at the Chennai Corporation slaughter house, Perambur. Meat from the head and cheek

was isolated and separable fat was removed. The heart was cut open along its longitudinal axis and clotted blood was removed. The epithelial layer of tongue was scraped off, and then the tongue was cut into small pieces. The lean meat, head meat, tongue, fat, and heart were packed in polyethylene bags separately and frozen at -20°C. Potato flour was prepared by scalding fresh potatoes at 80°C for 10 minutes and then peeling off the skin. Scalded potatoes were cut into small pieces and dried in an oven at 60°C overnight. The dried potatoes were ground into a fine powder using a Cyclotec of 1 mm sieve.

Preparation of Sausage: Sausages were prepared by using 50 percent lean meat, 30 percent low-value meat (LVM- head, heart and tongue), 20 percent fat and with 3, 5 and 7 percent levels of potato flour individually (Table 1). Frozen meat, LVM and fat were tempered at 4°C and were cut into small pieces

Table 1. Formulation for preparation of buffalo meat sausage.

Ingredients	Percentage
Lean Meat	50
Low value meat (<i>head, heart and tongue meat in the ratio of 70:15:15</i>)	30
Buffalo fat	10
Vegetable oil (sunflower)	<u>10</u> <u>100</u>
Sodium Tripolyphosphate	0.3
Salt	2.0
Added water (Ice)	10.0
Spice mix	1.5
Green condiments	4.0
Sodium nitrite	0.012
Binder (Potato Flour)	3 or 5 or 7 percent

and minced using 4.5 mm plate in Electrolux mincer (Omas, Model-16789). LVM was minced twice. Additives (sodium tripolyphosphate -0.3 percent, salt-2 percent and sodium nitrite-120 ppm), spice mix-1.5 percent, green condiments, chilled water(Ice)-10 percent were added and chopped along with meat and fat in a meat chopper. At the final step, potato flour was added and chopped for 1.5 minutes. During chopping, care was taken to maintain the emulsion temperature between 10-13°C. From this emulsion, samples were taken for pH and emulsion stability. Sausage emulsion was stuffed in sheep casing of 19 mm diameter, using a manual sausage stuffer and linked manually. Stuffed sausages were kept in a refrigerator ($4\pm 1^\circ\text{C}$) for 1 h to ensure proper setting. Sausages were then cooked in a water bath at 80°C for 15 minutes until a core temperature of $72\pm 3^\circ\text{C}$ was reached. Sausages prepared were subjected to physicochemical and sensory evaluation.

Analysis: The pH was measured using a digital pH meter (Cyberscan pH 510, Merck). Emulsion stability was estimated as per the method outlined by Baliga and Madaiah (1971). The cooking yield was calculated as the difference in the weight of the sausage before and after cooking. The shear force values of cooked sausages were assessed using Warner Bratzler Shear Press (Model No. 04347, The G.R. MFG. Co., Manhattan, U.S.A.) and the results were recorded as per method of Rao *et al.* (1999). Thio-barbituric acid reactive substance (TBARS) number and tyrosine value (TV) were determined using method of Tarladgis *et al.* (1960) and Strange *et al.* (1977), respectively. Organoleptic quality was evaluated by a semi-trained panel using a nine-point hedonic scale (where nine was extremely desirable and one was extremely undesirable).

Statistical Analysis: Data were analyzed by the statistical method of one way ANOVA using a SPSS@ software package developed as per the procedure of Snedecor and Cochran (1994) and means were compared by using Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSION

The mean values on physicochemical and sensory characteristics of buffalo meat sausages with different levels of inclusion of potato flour are presented in Tables 2 and 3.

The pH of both the emulsion and the product showed a highly significant ($P<0.01$) increase with increase in the levels of potato flour. The pH of the emulsion increased irrespective of the level of inclusion upon cooking; this might be attributed to the fact that in the cooking range of $55\text{-}80^\circ\text{C}$, new cross linkages are formed along with loss of free acidic groups from meat protein (Reddy and Vijayalakshmi, 1998). A highly significant ($P<0.01$) enhancement in emulsion stability was observed in sausages incorporated with 5 and 7 percent levels of potato flour. This might be attributed to high starch content of PF and binding of greater amounts of water favoring stable emulsion formation (Berry and Wergin, 1993). Incorporation of increasing levels of potato flour had a positive effect ($P<0.01$) on cooking yield, which is a reflection of increased emulsion stability (Hachmeister and Herald, 1998). A highly significant ($P<0.01$) increase was observed in shear force with increase in levels of potato flour. Bushway *et al.* (1982) observed an increase in shear value in potato flour + potato starch incorporated frankfurters than frankfurters with potato starch alone. Similarly, Carballo *et al.* (1996) established a direct relationship between starch content and hardness of frankfurters. Addition of starch generally caused an increase in penetration force (Hughes *et*

Table 2. Mean \pm S.E. values of physico-chemical characteristics of buffalo meat sausage with different levels of potato flour.

PARAMETERS	LEVELS OF POTATO FLOUR		
	3%	5%	7%
Emulsion pH	5.75 \pm 0.01 ^a	5.83 \pm 0.01 ^b	5.88 \pm 0.01 ^c
Product pH	5.80 \pm 0.012 ^a	5.88 \pm 0.010 ^b	5.93 \pm 0.010 ^c
Emulsion stability (%)	92.79 \pm 0.23 ^a	94.71 \pm 0.17 ^b	95.98 \pm 0.24 ^b
Cooking Yield (%)	91.64 \pm 0.17 ^a	94.31 \pm 0.26 ^b	95.58 \pm 0.22 ^c
Shear Force (Kg/ 19 mm dia)	0.54 \pm 0.01 ^a	0.63 \pm 0.019 ^b	0.68 \pm 0.01 ^c

Means bearing different superscripts (a, b, c) between rows differ significantly (P<0.01 or P<0.05).

Table 3. Mean \pm S.E. values of Sensory characteristics of buffalo meat sausage with different levels Potato flour.

PARAMETERS	n	LEVELS OF POTATO FLOUR		
		3%	5%	7%
Appearance	6	7.40 \pm 0.17 ^a	7.32 \pm 0.18 ^a	6.84 \pm 0.11 ^b
Flavour	6	6.69 \pm 0.05 ^a	7.48 \pm 0.07 ^b	6.65 \pm 0.012 ^c
Texture	6	6.8 \pm 0.04 ^a	7.43 \pm 0.05 ^b	6.09 \pm 0.18 ^c
Juiciness	6	6.79 \pm 0.07 ^a	7.43 \pm 0.05 ^b	6.24 \pm 0.27 ^c
Overall acceptability	6	6.69 \pm 0.04 ^a	7.6 \pm 0.07 ^b	6.24 \pm 0.15 ^c

Means bearing different superscripts (a, b, c) between rows differ significantly (P<0.01 or P<0.05).

Table 4. Mean \pm S.E for physico-chemical characteristics of buffalo meat sausage with 5 percent potato flour at refrigerated storage ($4\pm 1^\circ\text{C}$).

Parameter	Storage Days					Mean \pm S.E
	0	7	14	21	30	
pH	5.88 \pm 0.01 ^a	5.91 \pm 0.01 ^a	6.07 \pm 0.01 ^b	6.01 \pm 0.01 ^b	5.94 \pm 0.008 ^a	5.96 \pm 0.01 **
Shear Force Value	0.57 \pm 0.01 ^a	0.67 \pm 0.01 ^b	0.77 \pm 0.006 ^c	0.85 \pm 0.01 ^d	0.99 \pm 0.007 ^e	0.77 \pm 0.01 **
TBARS (mg of malanoidhyde/Kg)	0.35 \pm 0.004 ^a	0.65 \pm 0.008 ^b	0.75 \pm 0.007 ^c	0.85 \pm 0.008 ^d	0.97 \pm 0.005 ^e	0.71 \pm 0.01 **
Tyrosine Value (mg/100g of sample)	1.89 \pm 0.05 ^a	4.35 \pm 0.2 ^b	5.04 \pm 0.08 ^c	7.2 \pm 0.21 ^d	9.44 \pm 0.06 ^e	5.58 \pm 0.13 **

Means bearing same superscripts (a,b,c,d,e) between rows do not differ significantly ($P < 0.01$ or $P < 0.05$).

** Highly Significant ($P < 0.01$).

al., 1998). This may be due to the fact that starch in flour favours formation of strong heat induced structure through swelling of starch granules embedded in a protein matrix (Berry and Wergin, 1993).

A highly significant difference was observed with respect to appearance of sausage, with lower scores for sausage incorporated with the 7 percent level followed by the 5 and 3 percent levels. The 5 percent level recorded better flavor scores compared to the other levels. An enhancement of flavour release during mastication may be due to the slow release of bound water during physical breakdown allowing more effective flavour release (Trout *et al.*, 1992).

A highly significant ($p < 0.01$) difference in texture scores was evident between the different treatments of potato flour. Upon sensory evaluation, sausages showing the highest texture score were those with the 5 percent level for potato flour. This result was in accordance with Bushway *et al.* (1982) who found an improvement in texture of frankfurters with inclusion of potato starch. Sausages containing 3 percent level had higher scores for juiciness than other levels; this can be attributed to higher water binding properties of potato starch making moisture unavailable for early juice release during mastication (Berry and Wergin, 1993). The results revealed that, sausages with the 7 percent inclusion level recorded better emulsion stability and cooking yield followed by the 5 and 3 percent levels. The sensory scores revealed sausages with 5 percent level had higher scores than 7 and 3 percent except for juiciness. Hence potato flour at 5 percent level was considered optimum in preparation of buffalo meat sausage with 70 percent lean meat and 30 percent low-value meat.

The mean values on physicochemical characteristics of sausages with optimum level (5 percent) of PF under refrigerated storage ($4 \pm 1^\circ\text{C}$) are presented in Tables 4.

A highly significant ($P < 0.01$) increase in pH values was observed from 0 day to 20 days of storage and a slight decrease at 30 days of storage. This is in congruence with Reddy and Rao (1997) who observed increase in pH with increase in storage period in patties. They thought that this increase might be due to the liberation of metabolites resulting from bacterial activity. On the contrary, a significant decrease in pH during refrigerated storage was observed by Choi and Chin (2003) and Devatkal *et al.* (2004). Shear force values showed a highly significant ($P < 0.01$) increase during the storage period. Similar findings were observed by Thompson (1984) and Sahoo and Anjaneyulu (1997) who suggested that increase in firmness could have been caused by dehydration of cooked sausages. There was a progressive increase ($P < 0.01$) in TBARS number during storage, which is in concurrence with Drerup *et al.* (1981) and Bentley *et al.* (1987) and is a reflection of the advance in oxidative changes in buffalo meat sausage during storage. The tyrosine value showed a linear and highly significant ($P < 0.01$) increase with the increase in storage days from an initial value and was in accordance with findings of Bentley *et al.* (1987).

Sausages prepared with 5 percent inclusion of potato flour were acceptable till 21 days of storage at refrigerated temperature and their stability were based on the rancidity and protein degradation.

REFERENCES

- Baliga, B.R. and N. Madaiah. 1971. Preparation of mutton sausages. *J. Food Sci.*, **36**: 607-610.
- Bawa, A.S., W.R. Osborne and H.L. Orr. 1998. Interaction among meat, fillers, extenders in meat emulsion system. *J. Food Sci. Technol.*, **25**(2): 78-83.
- Bentley, D.S., J.O. Regan, N.A. Cox and J.S. Bailley. 1987. Effect of meat type, storage time and

- temperature on various physical, chemical and microbial characteristics of ground pork. *J. Food Protect.*, **50**(11): 948-951.
- Berry, B.W. 1997. Sodium Alginate plus modified potato flour improves properties of low fat beef patties. *J. Food Sci.*, **62**(6): 1245-1249.
- Berry, B.W. and W.P. Wergin. 1993. Modified pregelatinized potato starch in low fat ground beef patties. *J. Muscle Foods*, **4**: 305-320.
- Bushway, A.A., P.R. Belyea, R.H. True, T.M. Work, D.O. Russell and D.F. McGann. 1982. Potato starch and flour in frankfurters: Effect on chemical, sensory properties and Total viable count. *J. Food Sci.*, **47**: 402-404, 408.
- Carballo, J., P. Fernandez., G. Barreto, M.T. Solas and F.J. Colmenero. 1996. Morphology and texture of bologna sausage as related to content of fat, starch and egg white. *J. Food Sci.*, **61**: 652-655.
- Choi, S.H. and K.B. Chin. 2003. Evaluation of sodium lactate as a replacement for conventional chemical preservatives in comminuted sausages inoculated with *Listeria monocytogenes*. *Meat Sci.*, **5**: 531-537.
- Devatkal, S., S.K. Mendiratta and N. Kondaiah. 2004. Quality characteristics of loaves from buffalo meat, liver and vegetables. *Meat Sci.*, **67**: 377-383.
- Drerup, D.L., M.D. Judge and E.D. Aberle. 1981. Sensory properties and lipid oxidation in prerigor processed fresh pork sausage. *J. Food Sci.*, **46**: 1659-1661.
- Duncan, D.B. 1955. Multiple range and multiple F tests. *Biometrics*, **1**: 1-8.
- FAO, Stat. 2003. Food and Agricultural Organization of the United Nations. FAOSTAT database.
- Hachmeister, K.A. and T.J. Herald. 1998. Thermal and rheological properties and textural attributes of reduced fat turkey batters. *Poultry Sci.*, **77**: 632-638.
- Hendrick, H.B., E.D. Aberle, M.D. Judge and J.C. Forrest. 1994. *Principles of Meat Science*. W.H. Freeman and Co., San Francisco, 198.
- Hughes, E., A.M. Mullen and D.J. Tray. 1998. Effect of fat level, tapioca starch and whey protein on frankfurters formulated with 5 and 12 percent fat. *Meat Sci.*, **48**: 169-180.
- Modi, V.K., N.S. Mahendrakar, D. Narasimha Rao and N.M. Sachindra. 2003. Quality of buffalo meat burger containing legume flours as binders. *Meat Sci.*, **66**: 143-149.
- Reddy, K.P. and B.J. Rao. 2000. Effect of binders and precooking on quality of chicken loaves. *J. Food Sci.*, **37**: 551-553.
- Reddy, K.P. and K. Vijayalakshmi. 1998. Effect of incorporation of skin, gizzard, heart and yolk on the quality of frozen chicken meat sausage. *J. Food Sci. Technol.*, **35**(3): 276-278.
- Sachindra, N.M., P.Z. Sakhare, K.P. Yashoda and D.R. Narasimha. 2005. Microbial profile of buffalo sausage during processing and storage. *Food Control*, **16**(1): 31-35.
- Sahoo, J. and A.S.R. Anjaneyulu. 1997. Effect of natural antioxidants and vacuum packaging on the quality of buffalo meat nuggets during refrigerated storage. *Meat Sci.*, **47**(3/4): 223-230.
- Snedecor, G.W. and Cochran. 1994. *Statistical Methods*. First East West Press Edition, New Delhi.
- Strange, E.D., R.C. Benedict, J.L. Smith and G.E. Swift. 1977. Evaluation of rapid tests for monitoring alteration in meat quality during storage. I Intact Meat. *J. Food Prot.*, **40**: 843-847.
- Suman, S. P. and B.D. Sharma. 2003. Effect of grind size and fat levels on the physico-chemical and sensory characteristics of low-fat ground buffalo meat patties. *Meat Sci.*, **65**(3): 973-976.

- Tarladgis, B.G., B.M. Walts, M.T. Mounatham and L.R. Dugan. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. *J. Amer. Oil Chem. Soc.*, **37**: 44.
- Thomas, R., A.S.R. Anjaneyulu and N. Kondaiah. 2006. Quality and shelf life evaluation of emulsion and restructured buffalo meat nuggets at cold storage(4±1°C). *Meat Sci.*, **72**: 373-379.
- Trout, E.S., N.C. Hunt, D.E. Johnson, J.R. Claus, C.L. Kashner and O.H. Krolpf. 1992. Chemical, physical and sensory Characteristics of ground beef containing 5-30 percent fat. *J. Food Sci.*, **57**(1): 25-29.
- Whiting, R.C. 1989. Contribution of collagen to properties of comminuted and restructured meat product. *Reciprocal Meat Conference Proceedings*, **42**: 149-155.
-

*Continued from page 114

- Masri, S.A., W. Olson, P.T. Nguyen, S. Prins and D. Deregt. 1996. Rapid detection of *bovine herpesvirus-1* in the semen of infected bulls by a nested polymerase chain reaction assay. *Can. J. Vet. Res.*, **60**(2): 100-107.
- Santrude, G., N.D.V. Siva, R. Tabares, E. Solana, A. Bautista and J.M. Castro. 1996. Rapid and high sensitivity test for direct detection of bovine herpes virus-1 genome in clinical samples. *Vet. Microbial.*, **49**: 81-92.
- Steel, R.G.D. and J.H. Torrie. 1984. Multiple Comparisons. In *Principles and Procedures of Statistics*. McGraw-Hill International Book Company Singapore. **2**: 172-194.
- Von Stedingk, L.V., I. Olsson, H.S. Hanson, E. Asbrink and A. Hovmark. 1995. Polymerase chain reaction for detection of *Borrelia burgdorferi* DNA in skin lesions of early and late Lyme borreliosis. *Eur. J. Clin. Microbiol. Infect. Dis.*, **14**: 1-5.
- Walsh, P.S., D.A. Metzger and R. Higuchi. 1991. Chelex®100 as medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques*, **10**: 506-513.
-

*Continued from page 120

- Utiger, R.D. 1974. Serum triiodothyronine in man. *Ann. Rev. Med.*, **25**: 289-302.
- Wilson and Walker. 2000. *Practical Biochemistry*. Wiley Eastern, New Delhi.
- Yao, Y. 1975. Thyrotropin releasing hormone, clinical uses, *NY State J. Med.*, **75**(9): 1413-1416.
- Young, D.S., L. Pestaner and U. Gibberman. 1975. Effects of drugs on clinical laboratory tests. *Clin. Chem.*, **21**(5): 3660.

EFFECT OF HOUSING SYSTEM ON MILK YIELD, CLEANLINESS AND LAMENESS IN MURRAH BUFFALOES

R.M.V. Prasad, K. Sudhakar, E. Raghava Rao, B. Ramesh Gupta and M. Mahender

ABSTRACT

Buffaloes maintained at the Buffalo Research Station, Venkataramanna Gudem in Andhra Pradesh state of India during the period from August, 2007 to January, 2008 were utilized for the study. Significant differences were observed in the milk yield, cleanliness score and the lameness score between the housing systems. The milk yield was higher in loose housing (8.12 ± 0.02 kg) when compared to conventional housing (7.77 ± 0.02 kg). The cleanliness score was higher in the loose housing system (2.80 ± 0.05) compared to that in conventional housing (2.41 ± 0.05). The mean lameness score in the case of the conventional barn (0.10 ± 0.02) was greater compared to that in loose housing (0.01 ± 0.01). A greater number of animals showed lameness in the conventional system of housing.

Keywords: Murrah buffaloes, housing system, welfare, lameness, cleanliness, milk yield

INTRODUCTION

Buffaloes are well adapted to the hot and humid climate of India and play a distinct role in improving the rural economy which is primarily based on agricultural production systems. Buffaloes are considered a financial asset since they serve as an insurance against the risk of crop failure due to natural calamities (Dhanda, 2004).

Over time buffalo rearing has shifted from the backyard to commercial farms and large business enterprises. The housing system has changed from the loose housing system to the conventional tie stall system with intensive systems of management. But, very little attention is paid to the welfare of animal and very little is known about the effect of stress on productive performance and the well-being of buffaloes (De Rosa *et al.*, 2005). Better animal welfare will be reflected in normal behavioural activities and milk production.

Livestock housing conditions and all animal husbandry practices exert a considerable influence on animal behaviour, health and production. Integrating various aspects such as improved housing, nutrition, breeding and milking together are known to produce remarkable improvements in productive performance of buffaloes.

However, not many studies are conducted on buffaloes, and so cattle rearing techniques are often used for buffaloes, and these techniques may not always be appropriate for buffaloes. In view of the lack of literature on such aspects of buffalo production, an attempt was made to study the effect of housing on the milk yield, cleanliness and lameness in Murrah buffaloes.

MATERIALS AND METHODS

The material for the study comprised 24 lactating Murrah buffaloes in different lactations present on the farm. They were divided into two

groups of twelve animals each and kept in two housing systems viz., loose housing and conventional tie stall housing. An adaptation period of 14 days was given for the animals which were kept in the conventional tie stall, as they were accustomed to the loose housing prior to the experiment.

Loose housing

The loose housing had a covered area with simple asbestos sheet roofing and a sand filled open area surrounded by metal railings up to a height of 1.5 meters on the three sides and had the provision of water throughout the day. Sufficient free area was also available for free movement of the animals.

Conventional tie stall (Conventional housing)

The conventional tie stall was a completely enclosed structure having asbestos cement roofing with concrete flooring. All the animals were kept in individual partitions in tethered positions. The buffaloes were untied only at the time of milking, when they were sent to a milking parlor for milking with a milking machine. Water was made available to these animals three times during the day.

Management of the animals

The management of the buffaloes, including feeding, was uniform in both the groups except for the housing. Water was provided three times in a day to the animals in the tie -stall whereas the animals in the loose housing had water available throughout the day. All the buffaloes were washed with an automatic udder washing sprinkler before the start of milking.

Period and site of study

The experiment was conducted at the Buffalo Research Station, Venkataramannagudem in Andhra Pradesh state in India. The data was collected during the period from August, 2007 to January, 2008.

Milk yield: The milk yield was recorded every day during the morning and evening milking and the two yields were added to get the daily milk yield.

Cleanliness: Cleanliness of the animals was also observed in the two different housing types. A scoring system devised by Faye and Barnouin (1985) for cattle, dividing the pelvis into five parts viz., ano-genital, udder rear view, legs and thighs, under belly and udder lateral view was utilized. These parts were taken for scoring as these were important for the clean milk production. The score given was from 1 to 5, basing on the number of parts that were clean. The scoring was done before the evening milking, just before washing prior to milking.

Condition of lameness: Condition of lameness was also observed and lameness was taken as limping or dragging the feet. The lameness score was recorded after the afternoon milking while the buffaloes were passing out of the milking parlor. A score of '0 to 1' was used, where 0 was assigned when the animal was not lame, and 1 was given when the animal was lame.

Statistical analysis

The data obtained were analyzed using SPSS statistical package utilizing suitable statistical procedures to arrive at the conclusions.

RESULTS

The mean and standard error of milk yield, cleanliness score and the lameness score in the two housing systems are presented in Table 1. There was a significant differences in the milk yield, cleanliness score and the lameness score between the housing systems. The milk yield in the present study was higher in loose housing (8.12 ± 0.02 kg) when compared to conventional housing (7.77 ± 0.02

kg). The cleanliness score was higher in loose housing system (2.80 ± 0.05) compared to that of conventional housing (2.41 ± 0.05). The mean lameness score in the case of conventional tie stall (0.10 ± 0.02) was higher compared to that in the loose housing (0.01 ± 0.01). A greater number of animals showed lameness in the conventional system of housing.

DISCUSSION

The milk yield in the present study was higher in loose housing (8.12 ± 0.02) when compared to the conventional housing (7.77 ± 0.02) system. This finding was similar to that of Thomas *et al.* (1978), Yadav and Gupta (1985) and Singh *et al.* (1993) who reported that the loose housing system was more effective for getting higher milk yield. On the contrary, Egil Simensen (2007) reported that loose housed animals produced less milk compared to the tie stalled animals.

The present results suggest that the lack of space might have induced stress in the animals in the conventional tie stalls, which could be the reason for lower milk yields in the case of the conventional housing system. The animals in the loose housing system were provided adequate area to move to satisfy the expression of species specific behavioural urges.

The cleanliness score obtained in the present study was higher in loose housing system (2.80 ± 0.05) compared to that in conventional type of housing (2.41 ± 0.05). This finding was similar to that of Thomas *et al.* (1978). The results revealed that in loose housing, the animals had the freedom of choosing an area of their convenience for resting or lying down, whereas in the case of conventional system, there was no such choice. In the conventional system, the animal had to lie down in the area where it defecates or urinates. The dung

and the urine can cause dirt patches on the body parts. Fregonesi and Leaver (2002) also observed that the animals in the loose housing where more space was available were cleaner than those in conventional stalls where less space was available.

The evaluation of body cleanliness may give some information on animal comfort as well as farm hygiene. It can also give some indication about farm people's attitudes and care for animals. It may also be important for the purpose of clean milk production, but if the udder and other parts which come into contact with the milker or milking machine can be kept clean, this cleanliness score would not cause much problem. However, the cleanliness score indicates the attitude of the farm keepers and the quality of the farm management.

The results in the present study showed that the overall lameness score (Table 1) in the case of loose housing was lower compared to that of the conventional barn system. A greater number of animals exhibited lameness in the conventional system of housing. Lameness is a major welfare problem for dairy animals and indicates pain and discomfort. This may be caused by several factors, such as unbalanced nutrition, flooring and the time spent standing. The floor is an important part of the shed, and has a direct relation with lameness. Hard flooring in the conventional housing system may be a predisposing factor for this condition.

Livestock housing conditions may be important for animal welfare and may influence an animal's production. A higher number of buffaloes were found in the standing position in conventional housing compared to that in loose housing, and this could be because there is less comfortable space for lying in conventional housing. This might have made animals spend much time in a standing position predisposing them to foot lesions as is indicated in the literature (Galindo and Broom, 1993; Singh *et al.*, 1993; Leonard *et al.*, 1994). This was also substantiated by the significantly higher average

Table 1. Mean and standard error of milk yield, cleanliness score and lameness score in Murrah buffaloes in conventional tie stall and loose housing systems.

System of housing	N	Milk yield (Kg)		Cleanliness score		Lameness score	
		Mean	S.E.	Mean	S.E.	Mean	S.E.
Conventional housing	300	7.77 ^a	0.02	2.41	0.05	0.10 ^a	0.02
Loose housing	300	8.12 ^b	0.02	2.80 ^b	0.05	0.01 ^b	0.01
Total	600	3.697	0.01	2.61	0.03	0.06	0.01

lameness score observed in conventional housing in the present study.

The present study thus revealed that buffaloes appear to be more comfortable in loose housing system as indicated by the higher average daily milk yield, better cleanliness and less lameness when compared to the conventional tie stall system.

ACKNOWLEDGEMENT

The authors are highly thankful to the authorities of Sri Venkateswara Veterinary University for providing the necessary facilities for the study.

REFERENCES

Dhanda, O.P. 2004. Developments in water buffalo in Asia and Oceania, p. 17-28. *In Proceedings of the Seventh World Buffalo Congress*, Manila, Philippines.

De Rosa, G., F. Napolitano, F. Grasso, C. Pacelli and A. Bordi. 2005. On the development of a monitoring scheme of buffalo welfare at farm level. *Ital. J. Anim. Sci.*, **4**: 115-125.

Egil, Simensen, K. Camilla, E.B. Knut, E.R. Lars and N. Geir. 2007. Milk production, reproductive performance, milk somatic cell count and disease incidences in alternative housing systems for dairy cows. *Sixth International Dairy Housing Conference Proceeding*, 16-18 June 2007, Minneapolis, Minnesota 701P0507e.

Faye, B. and J. Barnouin. 1985. Objective assessment of the cleanliness of dairy cows and housing systems - the cleanliness index. *Bull. Tech.*, **59**: 61-67.

Fregonesi, J.A. and J.D. Leaver. 2002. Influence of space allowance and milk yield level on behaviour, performance and health of dairy cows housed in straw yard and cubicle systems. *Livest. Prod. Sci.*, **78**: 245-257.

Galindo, F. and D.M. Broom. 1993. The occurrence of lameness in relation to individual behavioural responses in dairy cows. *In Proceedings of the International Congress on Applied Ethology Berlin 1993: 3rd joint meeting* 318-320.

*Continued on page 140

DETECTION OF BPV-2 IN CUTANEOUS WARTS OF INDIAN WATER BUFFALOES (*BUBALUS BUBALIS*)

Vidya Singh*, R. Somvanshi, T. Yasotha, S.K. Subodh, S.K. Singh and Shanker Dayal

ABSTRACT

A study was undertaken to investigate the incidence and establish etiopathology of cutaneous warts (CWs) in Indian water buffaloes of the Murrah breed in parts of Uttar Pradesh district of India. Twenty cases of CWs were recorded. Grossly and microscopically they were cauliflower-like or dome-shaped and were diagnosed as fibropapilloma/papilloma. They were characterized by presence of koilocytes, keratohyaline granules and inclusion bodies. Negative staining and transmission electron microscopy (TEM) revealed bovine papilloma virus (BPV) like particles. BPV-2 was detected from CWs by PCR and was confirmed by nucleotide sequencing and phylogenetic analysis. Further, CWs were successfully transmitted to hamsters, cattle and buffaloes. Lesions produced in hamsters were early fibromatosis to fibromas and those in cattle and buffaloes were identical to those in natural cases in buffaloes. This is the first confirmed report about the incidence of CWs in Indian water buffaloes, its association with BPV-2, and its successful transmission in the laboratory as well as natural hosts.

Keywords: BPV-2, buffalo papillomatosis, cutaneous warts, fibropapilloma, koilocytes

INTRODUCTION

Cutaneous papillomatosis (warts) in bovine is a contagious hyperplasia or benign neoplasm

caused by BPV. BPV have specific tropism for squamous epithelial cells and full viral replication, including synthesis of DNA, capsid proteins and assembly of virions, occur only in the more terminally differentiated squamous epithelial cells (Campo, 2006). There are ten well characterized types: BPV-1 to -10, inducing papilloma and fibropapilloma (Shinichi *et al.*, 2008). Recently, BPV-8 has been detected in European bison and BPV-7, 9 and 10 have been isolated from cattle in Japan (Ogawa *et al.*, 2004; Ogawa *et al.*, 2007; Hatama *et al.*, 2008).

Cutaneous papillomatosis have been reported in cattle from around the world while in buffaloes there are only few sporadic reports and it is not a recognized disease entity. The aim of the present study was to establish prevalence of CWs in Indian water buffaloes as a separate ailment and characterize its etiopathology in natural and experimental hosts.

MATERIALS AND METHODS

Spontaneous Case Studies

Field Survey and Sample Collection

A survey was conducted in six organized dairy farms, local private dairies, large animal slaughter house, animal shows, etc. in U.P. and Uttarakhand, India, to record the incidence of CWs cases in buffaloes. The size, number, location of warts as well as age of animals and duration of affection were recorded. A total of 12 CW tissue biopsies and five blood samples were collected along

Table 1. Field survey for cases of bovine papillomatosis.

Sl. No.	Organized Dairies	Breed and herd strength	No of Cases	Incidence (%)
1	Dairy Farm IVRI, Izatnagar	MB (460)	6	1.30
2	Private Local Dairies, Bareilly	MB (250)	8	3.20
3	Large Animal Slaughter House	MB (550)	4	0.73
4	Farmers Fair, IVRI, Izatnagar	MB (60)	2	3.33
Total		1470	20	1.36

Note: MB- Murrah breed.

with skin and blood samples from apparently normal buffaloes.

DNA analysis

DNA was extracted from skin (one), lymphocyte (five) and CW (ten) samples using QIAamp DNA Mini Kit (Qiagen). Primers for conserved region of LI gene were used for BPV-1 (Fw: 5'-gga ggc cct gct aac tat agg a-3'; Rev: 5'-atc tgt tgt ttg ggt ggt gac-3') and BPV-2 (Fw: 5'-gtt ata cca ccc aaa gaa gac cct-3'; Rev: 5'-ctg gtt gca aca gct ctc ttt ctc-3' as described earlier (Leishangthem 2008a). PCR products were electrophoresed on 1.5% agarose gel and visualized by transillumination under UV light.

Phylogenetic analysis

The PCR products were directly sequenced commercially on ABIPRISM dye terminator DNA Sequencing Facility at South Campus, Jawahar Lal Nehru University, New Delhi, India, using primers, and the sequences were compared with published sequences (EMBL and NCBI) of BPVs by phylogenetic analysis using EDITSEQ and MEG ALIGN modules of LASERGENE software (DNASTAR Inc.).

Negative Staining and TEM

BPV was purified from CWs as described by Lancaster and Olson (1978). One mm² pieces of CWs were preserved in ice chilled 2.5%

gluteraldehyde in 0.2 M phosphate buffer (pH 7.4) and stored at 4°C. Samples were processed and examined under an electron microscope. (Morgagni-268 and Philips CM-10, Holland) at the Electron Microscope Facility, Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India.

Pathological Studies

CWs were biopsied and collected in 10% buffered formalin for routine histopathology and histochemistry (AgNOR). One sample of malignant urinary bladder tumor (transitional cell adenocarcinoma) was examined in order to compare the degree of malignancy (Crocker, 1992).

Apoptotic Studies

Single cell suspensions of CWs prepared as described earlier (Nicolleti *et al.*, 1991) were used for flow cytometric analysis using a Fluorescence Activated Cell Sorter (FACS) calibur (Becton Dickinson, San Jose, CA) at the Division of Biochemistry of the Institute. They were also examined for fluorescent pattern using the fluorescent dyes acridine orange-ethidium bromide (AO/EB, Sigma) and Hoechst (HO, Sigma) under a fluorescent microscope and the percentages of apoptotic cells were calculated.

Apoptotic index = (Total number of apoptotic cells / Total number of cells counted) X 100.

Experimental Transmission Studies

There were done in both natural as well as experimental hosts.

Hamsters

Seven adult male Syrian golden hamsters (*Mesocricetus auratus*), about 110-130 g in weight were used as infection and control groups containing four and three animals, respectively. They were infected by multiple scarification and inoculation (3 times) on the abdominal skin with homogenized suspension of CWs. All animals were observed regularly and sacrificed at 120 days post inoculation (DPI) and tissues were collected for routine histopathology and PCR analysis.

Cattle and Buffaloes

A group of six adult animals consisting of three cattle in Group I and three buffaloes in Group II were experimentally infected by multiple scarifications and subcutaneous inoculation on the neck, twice a week, with 1.0 ml of the homogenized suspension of CWs, tested positive for BPV-2. They were observed regularly for growth patterns on the site of inoculation and biopsies were collected in 10% buffered formalin on 70 DPI and processed for histopathology.

Both small and large animal experimentation were cleared by Institute Animal Ethics Committee. All the hamsters were sacrificed humanely under chloroform anaesthesia and skin biopsies from large animals were collected under local anaesthesia by skilled veterinarians.

RESULTS AND DISCUSSION

Clinical Observations

In the present study, 19 cases of CWs (Figure 1) and one case of teat papilloma were recorded in buffaloes. Grossly, warts were cauliflower-like with horny papillae or dome shaped with a smooth outer surface (0.5-2.0 cm in diameter) varying in number from one to six. Commonly affected sites were head, face, neck, legs and back. Most (14/20) of the cases were recorded in adult milking Murrah breed of buffaloes and only a few (6/20) cases were noticed in young animals. Bovine papillomatosis in cattle is a known disease while in buffaloes, only a few sporadic cases have been reported, and on the Indian subcontinent, it is an unrecognized entity (Joshi *et al.*, 1994; Sood *et al.*, 2006; Leishangthem *et al.*, 2008a).

Etiological Studies

PCR

BPV-2 was detected in CWs (Figure 2) and lymphocytes of CWs affected animals. On phylogenetic analysis of the sequence of PCR products (Accession Nos: GQ369511.1 and GQ369510.1), 98% and 100% homology was seen with the genome sequences (Ac. No: M 20219 and XO 1768) and (Ac. No: EF 151531 and EF 151532) of BPV-2, respectively. Presence of BPV DNA in the lymphocytes supports its latency in the lymphocytes. Earlier, BPV was detected from CWs, blood and various tissue fluids of BP affected animals (Freitas *et al.*, 2003; Leishangthem *et al.*, 2008b).

Negative Staining and TEM

Negative staining of purified virus suspensions of CWs showed BPVs-like particles of approximately 50-55 nm in size against dark background (Figure 3). Earlier Leishangthem *et al.* (2008a) also observed scanty virus particles by

negative staining of CWs from cattle. TEM revealed marked changes in the epidermocytes with high nucleus to cytoplasmic (N/C) ratio, pleomorphic and bizarre shaped nuclei, prominent heterochromatin, electron dense cyokeratin filaments, indistinct cell membrane and marked intercellular spaces. The cytoplasm had multiple, small vacuoles with pyknotic nucleus and small aggregates of electron dense PVs-like particles of about 50-55 nm in size (Figure 4-5). These findings are in accordance with those described for papillomatosis in cattle by earlier workers (Joshi *et al.*, 1994; Dorte *et al.*, 2004).

Pathological Studies

Gross and Histopathology

A total of 12 cases of CWs were studied in detail and two types of growth patterns were seen. Grossly, eight cases were of cauliflower-like with papillary outgrowths or dome shaped with smooth or undulating outer layer. Histologically, moderate to extensive cornification (hyperkeratosis) was seen with varying degree of parakeratosis (nuclear remnants), finger-like outgrowths (papillae) and dermal fibrous connective tissue core. The granular cell layer had prominent basophilic keratohyaline granules. Moderate to severe degrees of acanthosis (thickening of stratum spinosum) were observed with irregular rete pegs projecting deep into the dermis with lateral interconnections forming islands of dermal connective tissue surrounded by hyperplastic epidermal cells. The stratum spinosum revealed koilocytes, degenerating cells and intracytoplasmic eosinophilic inclusion bodies. The basal cell layer was hyperplastic with few mitoses. In the hypodermis, there were engorged capillaries with occasional infiltration of mononuclear cells and neutrophils. Extensive fibrocellular proliferative changes were seen in the hypodermis. These cases were diagnosed as fibropapilloma (exophytic, cauliflower-like or dome shaped).

Four cases were cauliflower-like with papillary outgrowths. Histopathologically, extensive hyperkeratosis and moderate to severe parakeratosis with basket weave appearance was seen in the stratum corneum. There was extensive acanthosis and uniformly downward growing columns of epidermal cells with thin cores of dermal tissue (Figure 6). The whole epidermal layer was hyperplastic with hyperchromatic nuclei and few mitoses in the basal cell layer. The stratum granulosum had intensively stained basophilic keratohyaline granules with few koilocytes, and intracytoplasmic eosinophilic inclusion bodies were noticed in the upper layers. There were no fibrocellular proliferative changes in the hypodermis. These cases were diagnosed as papilloma (endophytic, cauliflower-like).

Earlier, this type of histological classification was not attempted for BP. However, fibropapilloma in cattle with acanthosis has been described by Lancaster and Olson (1982) with koilocytes, keratohyaline granules and inclusion bodies (Jelinek and Tachezy, 2005; Tomita *et al.*, 2007) but it is for the first time an endophytic type of papilloma has been described and further study is needed as such lesions were observed only in buffaloes and not in cattle.

Histochemistry (AgNORs count)

The AgNOR dots were in general large and round in CWs, and the average count was 1.9 ± 0.43 whereas in malignant transitional cell adenocarcinoma, it was 3.9 ± 0.34 indicating the relatively benign nature of CWs.

Apoptosis Studies

FACS revealed high values of apoptotic cells (5 to 35%) indicating large aggregates of dead keratinocytes and confirm earlier findings (Leishangthem *et al.*, 2008a). With fluorescent dye, an apoptotic index of 12-20 (AO/EB) and 8-15 (HO)



Figure 1. Cauliflower (arrow) like growth on fore leg.

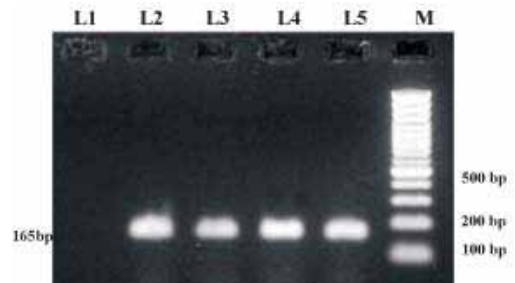


Figure 2. Dairy, IVRI. Buffalo: L1-negative control, L2-L5 CWs, M-100bp marker.

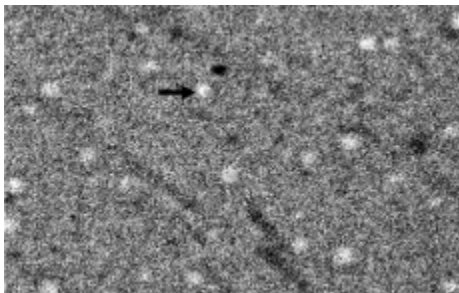


Figure 3. Purified virus: virus-like particles (arrow) in buffalo Cutaneous Warts negative stain, EM x1,50,000.

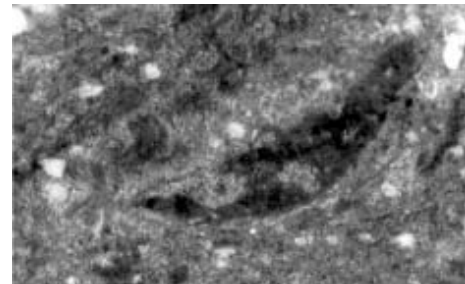


Figure 4. Epidermocyte with pyknotic nucleus (pN) and crystalline array of virus particles in nucleus (N), EM x 4400.

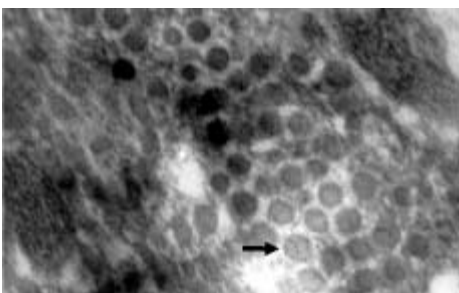


Figure 5. Papilloma virus-like particles (arrow) of 50-55 nm (scale not shown) in size. EM x 3,00,000.



Figure 6. Acanthosis, uniformly downward growing columns of rete pegs with no dermal fibrous hyperplasia. HE x100.



Figure 7. Buffalo CWs infection: Multiple (10-12), small lentil sized tumours (arrow) on abdominal skin of hamster.

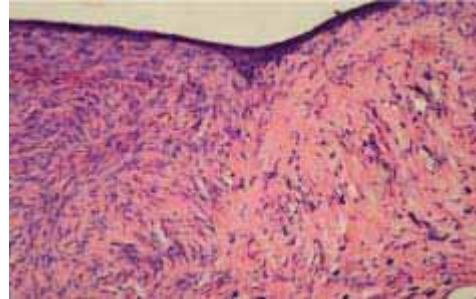


Figure 8. Hamster dermal tumour: Areas with extensive connective tissue proliferation and well formed fibromas. HE x 100.

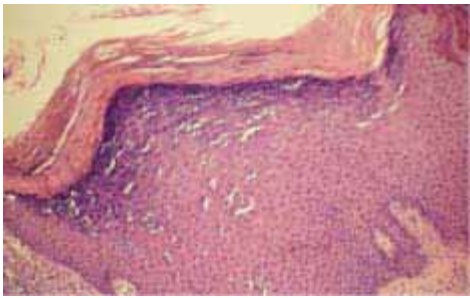


Figure 9. Buffalo infected with BCWs: Hyperkeratosis, acanthosis and proliferative changes in the dermal fibrous connective tissue, Fibropapilloma. HE x100.

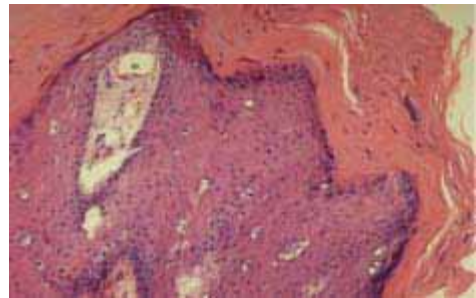


Figure 10. Cattle infected with BCWs: Finger-like papillae, hyperkeratosis, acanthosis, fibroproliferative changes in the dermis. Fibropapilloma. HE x 100.

was recorded in CWs while that for normal skin was 3-5. With fluorescent staining, apoptotic live and dead cells could be differentiated and this is in accordance with earlier report that PVs sensitizes cells to apoptosis through DNA fragmentation (Dorte *et al.*, 2004).

Experimental Transmission Studies

Hamsters

Grossly, few to multiple (3-12) small, lentil-to pea-sized, nodular growths were noticed on the abdomen region in all the four animals in the infected

group (Figure 7). The epithelium was intact with moderate to extensive proliferative changes in the dermal fibrous connective tissue. Fibroblasts and fibrocytes were seen with homogenous a glossy mass of eosinophilic collagen fibers in a radiating or whirling pattern (Figure 8). There was no encapsulation, and the mass was merged with the surrounding connective tissue and muscle. No mitoses were observed in the fibroblasts. Hyperplasia of sebaceous glands was seen with some epithelial proliferation around hair follicles. These cases were diagnosed as early fibromatosis

to fibromas. The findings are similar to the earlier reports of progressing fibromas, fibromas and fibroblastic tumors induced by inoculation of cattle CWs (Lancaster and Olson, 1982; Somvanshi *et al.*, 1988).

Cattle and Buffaloes

Grossly, small millet- to peanut-sized wart-like growths were seen along the line of scarifications on 60-70 DPI in both groups, in two cows and two buffaloes. In buffaloes, the lesions were similar to those in natural cases, both grossly and microscopically and were diagnosed as cauliflower-like exophytic fibropapilloma (Figure 9) and endophytic papilloma in buffaloes while fibropapilloma (exophytic, dome shaped) in cattle (Figure 10). This indicated that buffalo CWs are transmissible to both cattle as well as buffaloes. Experimental transmission of cattle CWs have been reported in calves (Meischke, 1979). Recently, CWs in a cattle and European bison in Japan were not found identical to any of the BPVs and were established as new types.

To sum up, this is the first systematic study of spontaneous cases of CWs in Indian water buffaloes with an aim to establish as new disease entity. BPV-like particles were demonstrated by negative staining and TEM of CWs in buffaloes. CWs of buffaloes are associated with BPV-2, are transmissible to hamsters as well as cattle and buffaloes and induce hyperproliferation of keratinocytes and fibroblasts. The lesions produced in hamsters were fibromatosis to fibroma where as in cattle and buffaloes fibropapilloma along with endophytic papilloma, which have not been described earlier. This is only the second example of interspecies transmission of papilloma viruses in any species, first being equine sarcoids due to BPV-1 and -2. However, complete genome sequencing of viral DNA from buffalo CWs and its comparison with that of BPV-2 needs to be done to know per

cent of homology between the two. Also, cases of endophytic papilloma, observed in both spontaneous and experimental cases of CWs in buffalo need to be investigated further, as it is not common in cattle.

ACKNOWLEDGEMENTS

Thanks are due to the Director, IVRI, Izatnagar, and U. P. for extending all facilities and also to ICAR for providing a Junior Research Fellowship to the first author.

REFERENCES

- Campo, M.S. 2006. Bovine papillomavirus: old system, new lessons? Chap 23. In *Papillomavirus research: from natural history to vaccine and beyond*, Pub. Caister Academic Press, England.
- Crocker, J. 1992. Nucleolar organizer regions and fibrillar centres, pp. 221- 238. In Herrington, C.S. and J.O.D. McGee (eds.) *Diagnostic Molecular Pathology: A Practical Approach, Vol. I*. Oxford University Press, Oxford, UK.
- Dorte, W.K., B. Silas, M. Astrid, G. Simon, B. Jesper, R.O. Carsten and N.O. Bodil. 2004. Simultaneous human papilloma virus type-16 E7 and CDk inhibitor p21 expression induces apoptosis and cathepsin B activation. *Virology*, **320**: 301-312.
- Freitas, A.C., C. Carvalho, O. Brunner, H. Eduardo, A. Dellalibera, J. Fernando, L. Gregory, W. Becak and R. Santos. 2003. Viral sequences in peripheral blood and vertical transmission of the virus: a discussion about BPV-1. *Braz. J. Microbiol.*, **34**(1): 76-78.
- Hatama, S., K. Nobumoto and T. Kanno. 2008. Genomic and phylogenetic analysis of two

- novel bovine papillomaviruses, BPV-9 and BPV-10. *J. Gen. Virol.*, **89**: 158-163.
- Jelinek, F. and R. Tachezy. 2005. Cutaneous papillomatosis in cattle. *J. Comp. Pathol.*, **132**(1): 70-81.
- Joshi, D.V., B.J. Patel and S.N. Sharma. 1994. Fibropapilloma of mammary gland in a buffalo calf. *Indian J. Vet. Pathol.*, **18**(1): 61-62.
- Lancaster, W.D. and C. Olson. 1978. Demonstration of two distinct classes of bovine papillomavirus. *Virology*, **89**: 371-379.
- Lancaster, W.D. and C. Olson. 1982. Animal papillomaviruses. *Microbiol. Rev.*, **46**: 191-207.
- Leishangthem, G.D., R. Somvanshi and D.R. Lauren. 2008a. Pathological studies on Bovine Papilloma virus-fern interaction in hamsters. *Indian J. Exp. Biol.*, **46**: 100-107.
- Leishangthem, G.D., R. Somvanshi and A.K. Tiwari. 2008b. Detection of bovine papilloma-viruses in cutaneous warts/papillomas in cattle. *Indian J. Vet. Pathol.*, **32**(1): 15-20.
- Meischke, H.R. 1979. In vitro transformation by bovine papilloma virus. *J. Gen. Virol.*, **43**: 471-487.
- Nicolleti, I., G. Miglorati, M.C. Pagliacci, G. Grignami and C. Riccardi. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Method.*, **139**: 271-279.
- Ogawa, T., Y. Tomita, M. Okada and H. Shirasawa. 2007. Complete genome and phylogenetic position of bovine papillomavirus type 7. *J. Gen. Virol.*, **88**(7): 1934-1938.
- Ogawa, T., Y. Tomita, M. Okada, K. Shinozaki, H. Kubonoya, I. Kaiho and H. Shirasawa. 2004. Broad-spectrum detection of papillomaviruses in bovine teat papillomas and healthy teat skin. *J. Gen. Virol.*, **85**: 2191-2197.
- Shinichi, H., N. Kiyoko and K. Toru. 2008. Genomic and phylogenetic analysis of two novel bovine papillomaviruses, BPV-9 and BPV-10. *J. Gen. Virol.*, **89**: 158-163.
- Somvanshi, R., P.K.R. Iyer, B. Sharma, G.L. Koul and J.C. Biswas. 1988. Transmission studies on bovine cutaneous papillomas in hamsters. *Indian J. Anim. Sci.*, **58**: 752-758.
- Sood, S., S. Azmi, T.R. Mohanti, M.S. Bhadwal and A.K. Gupta. 2006. Fibropapilloma in a buffalo. Compendium, p. 56. *In XXIII Annual Conference of IAVP*, Madras Vety. College, TANUVAS, Chennai, Tamil Nadu, India.
- Tomita, Y., I. Literak, T. Ogawa, Z. Jin and H. Shirasawa. 2007. Complete genomes and phylogenetic positions of bovine papillomavirus type 8 and a variant type from a European bison. *Virus Genes*, **35**: 243-249.

*Continued from page 132

- Leonard, F.C., J. O'Connell and K. O'Farrell. 1994. Effect of different housing conditions on behaviour and foot lesions in Friesian heifers. *Vet. Rec.*, **134**: 490-494.
- Singh, J., R.S. Yadav and O.P. Mehla. 1993 Effect of housing and feeding systems on physiological reactions in lactating buffaloes during hot-humid season. *Indian Journal of Animal Production and Management*, **9**: 48-50.
- Thomas, C.K., V.N. Tripathi, N.S.R. Sastry, R.S. Yadav, L.R. Gupta, Singh Kripal and R.N. Pal. 1978. Loose housing versus conventional tie barns for lactating crossbred and buffalo cows during winter under two levels of feeding. *Indian Journal of Dairy Science*, **31**: 128-134.
- Yadav, J.L. and L.R. Gupta. 1985. Effect of different housing systems on the production of buffaloes during summer. *Indian Journal of Dairy Science*, **38**: 245-249.

SERUM BIOCHEMICAL REFERENCE VALUES FOR FEMALE BUFFALOES IN EGYPT

Mahmoud Rushdi Abd Ellah

ABSTRACT

The goal of the present study was to establish a reference values for serum biochemical constituents in female buffaloes in Egypt. A total number of 50 non-pregnant lactating buffaloes (above 5 years old) were included in the study. Selection criteria were established for the reference sample population, which was selected according to the recommendations of the International Federation of Clinical Chemistry on the Theory of Reference Values. Serum samples were used for measuring serum total protein, albumin, globulins, albumin/globulin ratio, glucose, triglyceride, cholesterol, blood urea nitrogen, creatinine, chloride, potassium, phosphorus and magnesium levels, and for measuring serum activities of creatine phosphokinase, aspartate aminotransferase, alanine aminotransferase, gamma glutamyltransferase, lactate dehydrogenase and alkaline phosphatase. The reference value, reference limits, 90% confidence intervals for the lower reference limit and 90% confidence intervals for the upper reference limit were calculated for each serum biochemical constituents. In conclusion, the current study established a reference values for 19 serum biochemical constituents in female buffaloes in Egypt.

Keywords: buffaloes, reference values, serum, Egypt

INTRODUCTION

The buffalo (*Bubalus bubalis*) was originally an Asian animals, and the majority of buffaloes are distributed in tropical and sub-tropical Asia. The buffaloes have been classified into swamp and river buffaloes based on their appearance, wallowing habits and uses. The swamp buffaloes are raised in countries extending from Assam to China and used for drought power, the latter are found in countries ranging from the Indian sub-continent to the Mediterranean countries and Egypt and used primarily as a source of milk and meat (Cockril 1974, 1977).

Reference values from an individual or a group of individuals are only meaningful when the individual(s) and methods of production of values are adequately described. The international Federation of Clinical Chemistry *et al.* (1979) specified some factors that must be established when using reference values, such as the characterization of the reference population in respect to age and sex, the criteria used for including or excluding individuals from the reference sample group, the physiological and environmental conditions under which the reference population was studied and the specimens collected from the reference sample group. The current study aimed to establish a reference values for some serum biochemical constituents in female buffaloes in Egypt. These values will be used by the veterinary teaching hospital, Assiut University, Egypt.

Clinical Laboratory Diagnosis, Department of Animal Medicine, Faculty of Veterinary Medicine, Assiut University 71526, Assiut, Egypt, E-mail: mrushdi@aun.edu.eg

MATERIALS AND METHODS

Animals

A total number of 50 non-pregnant lactating buffaloes (above 5 years old) were included in the study. The buffaloes were selected from one slaughterhouse (Mosha, Assiut governorate, Assiut, Egypt) and during the period from March to June 2008. During the study environmental temperature ranged between 30-35°C.

Animals were inspected before slaughtering for presence of any abnormal clinical

signs and rectal temperature was recorded. After slaughtering, animals that had abnormal clinical findings or gross pathological lesions are excluded from the study. The decision of inclusion or exclusion of the animal was done by one observer. A number of selection criteria were established for the reference sample population (Table 1); these were selected according to the recommendations of the International Federation of Clinical Chemistry (IFCC) on the Theory of Reference Values (International Federation of Clinical Chemistry *et al.*, 1979, 1987a, b, 1988 and International Federation of Clinical Chemistry, 1991).

Table 1. Selection criteria for female buffaloes.

Observed criteria
Age: above 5 years
None pregnant
Lactating buffaloes
Samples collected half an hour after transportation to slaughterhouse
Rectal temperature: 38.5-39°C
Good body condition score
General attitude: alert
No loss of skin elasticity
Normal mucous membrane: pink
No colic or diarrhea in previous 7 days
No urogenital abnormalities in previous 7 days
No muscular abnormalities in previous 7 days
No medication in previous 7 days
Absence of skin lesions or alopecia
Absence of abnormal clinical science
Absence of postmortem pathological lesions

Sample

Blood samples were collected from the jugular before slaughtering in plain vacutainer tubes and were processed for separation of serum (Coles, 1986). All measurements were done by one person. Serum measurements were performed on the second day after collection. The following serum constituents were measured: total protein, albumin, glucose, triglyceride, total cholesterol, blood urea nitrogen (BUN), creatinine, chloride, potassium, phosphorus and magnesium, creatine phosphokinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) using commercial test kits supplied by Spectrum Diagnostics (Egyptian Company for Biotechnology, Cairo, Egypt) and by means of Digital VIS/ Ultraviolet Spectrophotometer (Cecil instruments, Cambridge, England, Series No. 52.232).

Statistical Analysis

Data analysis was carried out according to IFCC Approved Recommendations on the Theory of Reference Values [13]. The 95% reference intervals were calculated by removing the upper and lower 2.5% of the range for each serum biochemical constituent to give the 2.5 and 97.5 percentiles. Confidence intervals (CI) were calculated for each reference limit, to determine whether their precision was sufficient for clinical use (International Federation of Clinical Chemistry *et al.*, 1987a).

Data analysis was performed using Medical Calculator Version 11 (© 1883-2009, Frank Schoonjans) and according to the Clinical and Laboratory Standards Institute (CLSI) guidelines C28-A2 and C28-A3 for estimating percentiles and their 90% confidence intervals. Normal distribution of data was tested using Kolmogorov-Smirnov test. For the 90% confidence intervals of the reference limits, the CLSI guidelines were followed, and

conservative confidence intervals were calculated using integer ranks (and therefore the confidence intervals are at least 90% wide).

RESULTS

From the total number of 73 animals examined, only 50 buffaloes met the clinical criteria. The remained animals did not meet the selection criteria and were excluded from the study.

Table 2 summarize the reference value, reference limits, 90% CI for the lower reference limit and 90% CI for the upper reference limit for each serum biochemical constituents.

Normal serum biochemical constituents from clinically healthy buffaloes are summarized in Table 3.

DISCUSSION

Serum biochemical reference values are essential for diagnosing diseases and are also used as a guide for the normal serum levels. The current study aimed to establish a reference value for female buffaloes in Assiut Governorate, Egypt. Currently there is no reference value for buffaloes in Egypt and the current study is the first to estimate these values. Samples were collected from animals in the slaughterhouse to ensure that the animals under study were healthy both clinically and at postmortem. The IFCC sets out guidelines for the reference value and limits, and the current study carefully selected the animals according to the criteria in Table 1.

Comparison of the reference limit of serum biochemical constituents in the present study with normal serum constituents in clinically healthy buffaloes of previous studies (Table 3) revealed that the reference values for the current study are slightly higher or lower than mean values for previously published data. The latter data were in accordance with those of the current study when compared with

Table 2. Serum biochemical reference intervals in buffaloes.

	Reference value	Reference limit	90% CI for lower reference limit	90% CI for upper reference limit
Total protein (g/l)	68	53-79	52-58	77-83
Albumin (g/l)	35	22-44	21-26	42-47
Globulin (g/l)	33	21-54	20-25	41-46
A/G ratio	1.07	0.44-1.69	0.45-0.69	1.44-1.69
Glucose (mmol/l)	3.49	1.38-5.88	1.44-2.27	4.77-5.60
Cholesterol (mmol/l)	2.03	0.67-3.58	0.88-1.32	2.73-3.19
Triglyceride (mg/dl)	19	7-38	2-9	29-35
BUN (mmol/l)	5.35	2.49-8.92	1.78-3.21	7.14-8.56
Creatinine (μ mol/l)	60	32-114	21-37	83-99
Chloride (mmol/l)	102	88-119	87-93	111-117
Potassium (mmol/l)	4.8	3.3-6.9	2.78-3.55	5.89-6.66
Phosphorus (mmol/l)	2.04	1.23-2.83	1.18-1.54	2.64-3.00
Magnesium (mmol/l)	1.37	0.65-1.80	0.70-0.96	1.78-2.04
CK (U/l)	63	29-137	10-31	96-117
GGT (U/l)	19	8-52	6-11	27-32
AST (U/l)	81	44-125	34-52	110-129
ALT (U/l)	16	7-38	3-8	25-30
LDH (U/l)	309	193-492	126-198	420-492
ALP (U/l)	81	23-137	22-46	117-141

Reference value = mean value; reference limits = 2.5 and 97.5 percentiles; reference interval = interval between, and including, two reference limits.

Table 3. Normal serum constituents in clinically healthy buffaloes.

	Mean \pm SD	Authors
Total protein (g/l)	75.9 \pm 5.6	Hedaoo <i>et al.</i> (2008)
	72.60 \pm 6.40	Abd-El-All <i>et al.</i> (1991)
	63.30 \pm 1.50	Kumar <i>et al.</i> (1982)
Albumin (g/l)	37.60 \pm 6.80	Abd-El-All <i>et al.</i> (1991)
	34.80 \pm 8.50	Kumar <i>et al.</i> (1982)
Globulin (g/l)	26.50 \pm 6.80	Abd-El-All <i>et al.</i> (1991)
	29.50 \pm 1.00	Kumar <i>et al.</i> (1982)
A/G ratio	1.54 \pm 0.7	Abd-El-All <i>et al.</i> (1991)
	1.17 \pm 0.15	Kumar <i>et al.</i> (1982)
Glucose (mmol/l)	3.58 \pm 0.61	Abd-El-Salam <i>et al.</i> (1998)
Total cholesterol (mmol/l)	2.06 \pm 0.39	Singh and Nigham (1982)
BUN (mmol/l)	3.81 \pm 0.49	Hedaoo <i>et al.</i> (2008)
Creatinine (μ mol/l)	114 \pm 26	Akhtar <i>et al.</i> (2007)
Potassium (mmol/l)	4.7 \pm 0.3	Hasanpour <i>et al.</i> (2008)
Phosphorus (mmol/l)	1.9 \pm 0.03	Akhtar <i>et al.</i> (2008)
	1.77 \pm 0.52	Ahmed <i>et al.</i> (2005)
	1.60 \pm 0.03	Pandey <i>et al.</i> (2007)
Magnesium (mmol/l)	0.95 \pm 0.01	Akhtar <i>et al.</i> (2008)
	0.98 \pm 0.21	Ahmed <i>et al.</i> (2005)
GGT (U/l)	14.00 \pm 3.99	Abd-El-Salam <i>et al.</i> (1998)
AST (U/l)	87.5 \pm 6.5	Hasanpour <i>et al.</i> (2008)
	74.50 \pm 14.96	Singh and Nigham (1982)
ALT (U/l)	8.32 \pm 0.64	Singh and Nigham (1982)
	7.20 \pm 1.68	Sethuraman and Verma (1979)
ALP (U/l)	104.72 \pm 6.66	Kumar <i>et al.</i> (1982)

the reference limit. For example, reference value for total protein is 68 g/l, which is less than previously reported mean values of 75.9 g/l (Hedao *et al.*, 2008) and 72.6 g/l (Abd-El-All *et al.*, 1991) and higher than mean value of 63.30 g/l (Kumar *et al.*, 1982); at the same time, all of them are included within the reference limit of the present study 53 - 79 g/l (Table 2). In conclusion, the current study established a reference values for 19 serum biochemical constituents in female buffaloes in Egypt.

REFERENCES

- Abd-El-All, Th.S., M.N. Ismail and H.A. Daghsh. 1991. Variations in some blood constituents in Egyptian buffaloes. *In Proceedings of 1st Scientific Congress, Egyptian Society for Cattle Diseases*, Egypt.
- Abd-El-Salam, M.N., A.S. Sayed, M. Moubark and M.R. Abd Ellah. 1998. Clinical, biochemical and pathological studies of some liver affections in buffaloes. *In Proceedings of 8th Scientific Congress*, Egypt.
- Ahmed, S., I. Ahmad, L.A. Lodhi, N. Ahmad and H.A Samad. 2005. Clinical, haematological and serum macro mineral contents in buffaloes with genital prolapse. *Pak. Vet. J.*, **25**: 167-170.
- Akhtar, M.S., L.A. Lodhi, I. Ahmad, Z.I. Qureshi and G. Muhammad. 2008. Serum concentrations of calcium, phosphorus and magnesium in pregnant Nili-Ravi buffaloes with or without vaginal prolapsed in irrigated and rain fed areas of Punjab, Pakistan. *Pak. Vet. J.*, **28**: 107-110.
- Akhtar, M.Z., A. Khan, M.Z. Khan and G. Muhammad. 2007. Haemato-biochemical aspects of parturient haemoglobinuria in buffalo. *Turk. J. Vet. Anim. Sci.*, **31**: 119-123.
- Cockril, W.R. 1977. *The Water Buffalo*. FAO, Rome.
- Cockril, W.R. 1980. The ascendant water buffalo key domestic animal. *World Anim. Rev.*, **33**: 2-13.
- Coles, E.H. 1986. *Veterinary Clinical Pathology*, 4th ed. Saunders Co. Philadelphia, London, Toronto.
- Hasanpour, A., G.A. Moghaddam and A. Nematollahi. 2008. Biochemical, hematological, and electrocardiographic changes in buffaloes naturally infected with *Theileria Annulata*. *Korean J. Parasitol.*, **46**: 223-227.
- Hedao, M.K., K.P. Khllare, M.D. Meshram, S.K. Sahatpure and M.G. Patil. 2008. Comparative studies of certain bio-chemical constituents of normal cyclic and anoestrus surti buffaloes. *Vet. World*, **1**: 105-106.
- International Federation of Clinical Chemistry. 1991. IFCC recommendation - theory of reference values. Part 4: control of analytical variation in the production, transfer and application of reference values. *Clin. Chem. Acta.*, **202**: S5-S12.
- International Federation of Clinical Chemistry, Committee on Standards, Expert Panel on the Theory of Reference Values. 1979. Provisional recommendation on the theory of reference values. Part 1: the concept of reference values. *Clin. Chem.*, **25**: 1506-1508.
- International Federation of Clinical Chemistry, Scientific Committee, Clinical Section Expert Panel on the Theory of Reference Values. 1987a. Provisional recommendation on the theory of reference values. Part 5: statistical treatment of selected reference values. Determination of reference limits. *Clin. Chem. Acta.*, **170**: S13-S32.

International Federation of Clinical Chemistry, Scientific Committee, Clinical Section Expert Panel on the Theory of Reference Values. 1987b. Provisional recommendation on the theory of reference values. Part 2: selection of individuals for the production of reference values. *Clin. Chem. Acta.*, **170**: S3-S12.

International Federation of Clinical Chemistry, Scientific Committee, Clinical Section Expert Panel on the Theory of Reference Values. 1988. Provisional recommendation on the theory of reference values. Part 3: preparation of individuals and collection of specimens for the production of reference values. *Clin. Chem. Acta.*, **177**: S1-S12.

Kumar, M., K.M.L. Pathak and S.P. Pachauri. 1982. Clinical pathological studies on naturally occurring bovine fascioliasis in India. *Brit. Vet. J.*, **138**: 241-246.

Pandey, A.K., S.P. Shukla and S.P. Nema. 2007. Certain haemato-biochemical alterations during post-partum uterine prolapse in buffaloes (*Bubalus bubalis*). *Buffalo Bull.*, **26**(1): 20-22.

Sethuraman, V. and B.B. Verma. 1979. The pathogenesis of fascioliasis. *Vet. Rec.*, **78**: 98-105.

Singh, A.P. and J.M. Nigham, 1982. Clinico-Biochemical study on Experimental common bile duct obstruction in bovine. *Jap. J. Vet. Sci.*, **44**: 349-353.

OCT-4 GENE EXPRESSION PATTERN IN DIFFERENT GRADES
OF BUFFALO (*Bubalus bubalis*) OOCYTES

Ch. Srinivasa Prasad¹, V.S. Gomathy¹, A. Palanisamy², G. Dhinakar Raj²,
A. Thangavel¹ and S. Satheshkumar²

ABSTRACT

The developmental competence of oocytes may be a function of the presence/abundance of specific transcripts in their mRNA pools, since the earlier stages of embryogenesis in mammals and other animals are regulated by maternally-inherited RNAs and proteins stored within the oocyte. This study was undertaken to test the hypothesis that naked oocytes which are proved to be of low developmental potential are devoid of *Oct-4* gene expression. It was observed that culture grade ('A' and 'B' grade) oocytes showed strong *Oct-4* expression whereas naked oocytes showed faint or no detectable signal. A fragment of 196 bp product was detected in *Oct-4* positive oocytes. It was observed that culture grade oocytes showed strong *Oct-4* expression with a dense band whereas naked oocytes showed a faint band. Out of twenty trials, a thick fragment of 196 bp product (EU661360) was detected in all trials of culture grade oocytes whereas in naked oocytes only a faint band was observed in twelve trials (60%) and no band at all in the remaining eight (40%) trials. Based on the results, it was concluded that *Oct-4* may be one of those specific genes whose expression is dependent on oocyte quality, suggesting that the lower developmental potential of naked oocytes might be due to the absence of *Oct-4* transcripts.

Keywords: *Oct-4*, gene expression, oocyte, buffalo, *Bubalus bubalis*

INTRODUCTION

Cumulus cells (CC) form a multilayered cell mass that surround the oocyte during maturation process within the follicle as well as after ovulation. These cells not only protect the oocytes but also provide nutrients to the oocyte through gap junctions (Mori *et al.*, 2000). Due to this direct association between the oocyte and CC, these cells may influence the development of the oocyte (Perez and Tilly, 1997). Germinal vesicle (GV) oocytes constitute a potential resource for in vitro production of embryos, but their developmental competence is questionable especially when surrounding cumulus cells are removed. The intercellular factors/mechanisms underlying such poor embryonic competence may originate at a nuclear and/or ooplasmic level.

The developmental competence of oocytes may be a function of the presence/ abundance of specific transcripts in their mRNA pools since the earlier stages of embryogenesis in mammals and other animals are regulated by maternally-inherited RNAs and proteins stored within the oocyte. Therefore, oocyte quality could be correlated with the prevalence of transcripts in the oocyte itself as

¹Centralized Embryo Biotechnology Unit, Department of Animal Biotechnology, Madras Veterinary College, Tamilnadu Veterinary and Animal Sciences University, Chennai - 600 007, India

²Department of Animal Biotechnology, Madras Veterinary College, Tamilnadu Veterinary and Animal Sciences University, Chennai - 600 007, India

well as the prevalence of these maternal messages in early embryos. Moreover, since activation of the embryonic genome must be mediated by maternal proteins, oocyte quality could have an effect on this event and, therefore, on the prevalence of transcripts synthesized after activation of the embryonic genome.

Oct-4 is a transcriptional regulator of genes involved in maintaining the undifferentiated pluripotent state and may also prevent expression of genes activated during differentiation. It is normally found in the pluripotent stem cells of pregastrulation embryos, including oocytes, early cleavage-stage embryos, and the inner cell mass of the blastocyst (Rosner *et al.*, 1990; Palmieri *et al.*, 1994). The *Oct-4* gene encodes a nuclear protein that belongs to a family of transcription factors containing the POU DNA binding domain (Rosner *et al.*, 1990). It activates transcription via Octamer motifs located proximal or distal to transcriptional start sites. The POU domain of *Oct-4* is a conserved DNA binding domain that binds as a monomer to the Octamer sequence motif 5'-ATGCAAAT-3'. This cis-acting element is important in controlling the activity of many promoters and enhancers of house-keeping and cell type-specific genes. *Oct-4* -binding sites have been found in various genes, including *fgf-4* (fibroblast growth factor-4), *pdgfr* (platelet-derived growth factor- receptor), osteopontin, Nanog etc (Lanza *et al.*, 2006).

Very few studies (Pfeffer *et al.*, 2007; Wrenzycki *et al.*, 2007) have examined the effect of oocyte quality on the expression of specific genes in oocytes of domestic animals. This study of *Oct-4* expression in different grades of oocytes to correlate the *Oct-4* expression with developmental competence was undertaken to test the hypothesis that naked oocytes which are proved to be of low developmental potential are devoid of *Oct-4* gene expression.

MATERIALS AND METHODS

Buffalo ovaries were collected from Chennai Corporation abattoir and transported in saline supplemented with 50 mg/ml gentamycin (37°C) to the Centralized Embryo Biotechnology Unit, Department of Animal Biotechnology, within 30 minutes for further processing. The ovaries were trimmed of extra-ovarian tissues and washed. Oocytes were collected from washed ovaries by aspiration. The oocytes were screened under a stereo zoom microscope and transferred to 35 mm petri dish containing oocyte collection medium and then graded as described by Nandi *et al.* (1998). Culture grade ('A' and 'B' grade) oocytes, as well as naked oocytes were segregated and subjected to gene expression studies in two groups.

Oct-4 was detected by reverse transcription-polymerase chain reaction (RT-PCR). Primers were designed based on the sequence (NCBI Ac. No: DQ487023) of Chinese swamp buffalo available in Pub med GenBank by using Lasergene software DNASTAR and were compared with the same gene of the other species by BLAST.

RT-PCR for *Oct-4* was carried out in an Eppendorf Mastercycler by using the forward primer (5' TGCTGCAGAAGTGGGTGGAGGAAG 3') and the reverse primer (5' CCGAGCTGCTGGGCGATGTG 3') with the following cycling profile: cDNA synthesis for 15 minutes at 50°C, initial denaturation at 95°C for 2 minutes followed by 36 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 45 seconds at 72°C, and a final extension for 10 minutes at 72°C. These primers amplified a fragment of 196 bp. *Oct-4* RT-PCR product was analyzed by gel electrophoresis along with a standard 100 bp ladder as marker. Then the gel was visualized under UV light gel documentation unit. RNA equivalent of ten to fifteen oocytes (approx. 200 ng) was utilized

per reaction and twenty trials were conducted for each group of oocytes.

The sequences of the RT-PCR products were obtained using specific primers from DNA sequencing facility available at the Department of Animal Biotechnology, Madras Veterinary College, Chennai. The sequences were analyzed for phylogenic conservation and the sequence homology across the species was established. Homologies of *Oct-4* gene (mRNAs) were compared with reported sequences of other species retrieved from web pages of the National Centre of the Biotechnology information (www.ncbi.nih.gov) in the BLAST search mode. The cDNA sequences of *Oct-4* gene were phylogenetically analysed using Lasergene version 4.1 (DNASTAR Package, USA).

RESULTS

It was observed that culture grade ('A' and 'B' grade) oocytes showed strong *Oct-4* expression

whereas naked oocytes showed only a faint or no detectable signal (Figure 1). Out of twenty trials, a thick fragment of 196 bp product was detected in all trials of culture grade oocytes whereas in naked oocytes only a faint band was observed in 12 trials (60%) and no band at all in the remaining eight trials. The DNA sequence of the *Oct-4* RT-PCR product submitted to NCBI Pub med GenBank was given Accession number: EU661360.

The nucleotide sequence of Indian water buffalo (*Bubalus bubalis*) *Oct-4* gene had 88 - 97% homology across the phylogeny; specifically, it had 97 percent homology with Chinese swamp buffalo, *Bos taurus*, *Bos grunniens* and pig *Oct-4* gene, 92 percent homology with rat *Oct-4* gene, 91 per cent homology with human and dog *Oct-4* gene and 88 percent with mouse *Oct-4* gene. The phylogenetic analysis of *Oct-4* gene by clustralW method revealed two major clusters of which the *Oct-4* gene lineage of cattle (USA), Chinese swamp buffalo, horse, domestic yak, pig were grouped to first cluster and the second cluster revealed a lineage covering

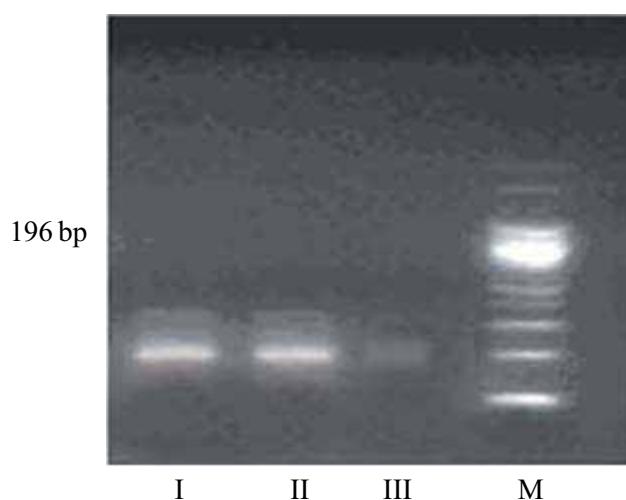


Figure 1. Gel photo of *Oct-4* gene expression in culture grade and naked buffalo oocytes.

- Lane I & II : Culture grade oocytes
- Lane III : Naked oocytes
- Lane M : 100 bp ladder

the cattle (Netherlands), Norway rat and Indian water buffalo.

Amino acid analysis of *Oct-4* gene revealed six hypervariable regions at the positions namely 247-251, 260-264, 275-280, 283-286, 299-303 and 367-370. The variations were in relation with species specificity.

DISCUSSION

Communication between the oocyte and its surrounding cumulus cells is critical for the development of a competent oocyte at ovulation. As an oocyte grows and matures, it acquires the ability to resume and complete meiosis, successfully undergo the fertilization process, and initiate and sustain embryonic development (First *et al.*, 1988). In the course of acquiring these competencies, cytoplasmic changes occur that may include such cellular processes as mRNA transcription (Hunter and Moor, 1987; Farin and Yang, 1992), protein translation (Sirard *et al.*, 1989), post-translational modification of proteins (Levesque and Sirard, 1995) etc. During growth of the oocyte within the follicle, many genes are expressed (Schultz, 1986). Some of these proteins, such as maturation promoting factor are involved in meiotic progression and cell cycle control. Other gene transcripts and their respective protein products may be involved in cellular processes critical for developmental success before and after activation of the zygotic genome (Barron *et al.*, 1989; Watson *et al.*, 1999).

Bilodeau-Goeseels (2003) compared the relative abundance of transcripts for α -actin, ribosomal protein L30 (two abundant housekeeping proteins), PDGF receptor α (PDGFR α), bFGF, and the bFGF receptor (bFGFR) in oocytes of high versus low potential for development. Oocyte quality (as assessed by visual appearance) did not have any significant effects on the relative abundance of two

mRNA transcripts encoding housekeeping proteins (L30 and α -actin) and three mRNA transcripts of lower abundance (PDGFR α , bFGF, and bFGFR), suggesting that the lower developmental potential of oocytes was not due to the absence of these transcripts, or dramatically lower level of expression of these genes, or increased degradation of the transcripts. Studies of Bilodeau-Goeseels (2002 and 2003) indicate that oocyte quality would dramatically affect the level of expression of a small number of specific genes only. *Oct-4* may be one of those specific genes whose expression is dependent on oocyte quality suggesting that the lower developmental potential of naked oocytes might be due to the absence or lower levels of *Oct-4* transcripts, as *Oct-4* is essential for antiapoptosis of cells in response to stress through the STAT3/Survivin pathway mediated effects (Guo *et al.*, 2008).

Donnison and Pfeffer (2004) compared and reported 1.2 fold increase in *Oct-4* transcript in more competent oocytes derived from large follicles (≥ 5 mm) relative to those less competent oocytes derived from small follicles (≤ 2 mm).

Manuela *et al.* (2006) suggested a potential twofold role for *Oct-4* gene in the recruitment of oocytes for initiating growth and in the selection of oocytes for ovulation. Kehler *et al.* (2004) reported that *Oct-4* plays an essential role not only in maintaining pluripotency in early embryonic cells but also for viability of primordial germ cells.

Observed lack/ dearth of *Oct-4* expression in naked oocytes compared to culture grade ('A' and 'B') oocytes in this study strengthen the version of Chang *et al.* (2005) who opined that developmental defect(s) appear to develop primarily in the ooplasm of oocytes that are void of their cumulus cells prior to in vitro maturation. Such oocytes result in embryos with poor developmental competence. These defects result in anomalies in

cell number and *Oct-4* expression during the morula-blastocyst developmental transition.

ACKNOWLEDGEMENT

The work was carried out in a scheme on “Establishment of Buffalo ES-Cell Line” funded by the Department of Biotechnology, Government of India. The authors are thankful to the funding agency.

REFERENCES

- Barron, D.J., G. Valdimarsson, D.L. Paul, and G.M. Kidder. 1989. Connexin 43, a gap junction protein, is a persistent oocyte product through preimplantation development. *Develop. Genetics*, **10**: 318-323.
- Bilodeau-Goeseels, S. 2003. Effect of oocyte quality on the relative abundance of specific gene transcripts in bovine mature oocytes and 16-cell embryos. *Canadian J. Vet. Res.*, **67**(2): 151-156.
- Bilodeau-Goeseels, S. and P. Panich. 2002. Effects of oocyte quality on development and transcriptional activity in early bovine embryos. *Anim. Reprod. Sci.*, **71**: 143-155.
- Chang, H.C., H. Liu, J. Zhang, J. Grifo and L.C. Krey. 2005. Developmental incompetency of denuded mouse oocytes undergoing maturation in vitro is ooplasmic in nature and is associated with aberrant *Oct-4* expression. *Hum. Reprod.*, **20**(7): 1958-1968.
- Donnison M. and P.L. Pfeffer. 2004. Isolation of genes associated with developmentally competent bovine oocytes and quantification of their levels during development. *Biol. Reprod.*, **71**: 1813-1821.
- Farin, C.E. and L. Yang. 1992. Inhibition of germinal vesicle breakdown by 5,6-dichloro-benzimidazole riboside in bovine oocytes matured in vitro. *Theriogenology*, **37**: 208.
- First, N.L., M.L. Leibfried-Rutledge and M.A. Sirard. 1988. Cytoplasmic control of oocyte maturation and species differences in the development of maturational competence. *Prog. Clin. Biol. Res.*, **267**: 1-46.
- Guo, Y., C. Mantel, R.A. Hromas, H.E. Broxmeyer. 2008. *Oct-4* Is Critical for Survival/Antiapoptosis of Murine Embryonic Stem Cells Subjected to Stress: Effects Associated with Stat3/Survivin. *Stem Cells*, **26**(1): 30-34.
- Hunter, A.G. and R.M. Moor. 1987. Stage dependent effects of inhibiting ribonucleic acids and protein synthesis on meiotic maturation of bovine oocytes in vitro. *J. Dairy Sci.*, **70**: 1646-1651.
- Kehler, J., E. Tolkunova, B. Koschorz, M. Pesce, L. Gentile, M. Boiani, H. Lomely, A. Nagy, K.J. McLaughlin, H.R. Schöler and A. Tomilin. 2004. *Oct-4* is required for primordial germ cell survival. *EMBO Rep.*, **5**: 1078-1083.
- Lanza, R., J. Gearhart, B. Hogan, D. Melton, R. Pederson, J. Thomson, E.D. Thomas and M. West. 2006. *Essentials of Stem Cell Biology*. Elsevier Academic Press.
- Levesque, J.T. and M.A. Sirard. 1995. Effects of different kinases and phosphatases on nuclear and cytoplasmic maturation of bovine oocytes. *Mol. Reprod. Dev.*, **42**: 114-121.
- Manuela, M., G. Silvia, R. Carloalberto and Z. Maurizio. 2006. Gonadotropins affect *Oct-4* gene expression during mouse oocyte growth. *Mol. Reprod. Dev.*, **73**(6): 685-691.
- Mori, T., T. Amano and H. Shimizu. 2000. Roles of gap junctional communication of cumulus cells in cytoplasmic maturation of porcine oocytes cultured in vitro. *Biol. Reprod.*, **62**: 913-919.
- Nandi, S., M.S. Chauhan and P. Palta. 1998. Influence of cumulus cells and sperm concentration on cleavage rate and subsequent

- embryonic development of buffalo (*Bubalus bubalis*) oocytes matured and fertilized in vitro. *Theriogenology*, **50**: 1251-1262.
- Palmieri, S.L., W. Peter, H. Hess and H.R. Schöler. 1994. *Oct-4* transcription factor is differentially expressed in the mouse embryoduring establishment of the first two extraembryonic cell lineages involved in implantation. *Develop. Biol.*, **166**: 259-267.
- Perez, G.I. and J.L. Tilly. 1997. Cumulus cells are required for the increased apoptotic potential in oocytes of aged mice. *Hum. Reprod.*, **12**: 2781-2783.
- Pfeffer, P.L., B. Sisco, M. Donnison, J. Somers and C. Smith. 2007. Isolation of genes associated with developmental competency of bovine oocytes. *Theriogenology*, **68**(Sup. 1): S84-S90.
- Rosner, M.H., M.A. Vigano, K. Ozato, P.M. Timmons, F. Poirier, P. Rigby and L.M. Staudt. 1990. A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature*, **345**: 686-692.
- Schultz, R.M. 1986. Molecular aspects of mammalian oocyte growth and maturation. p. 195-237 In Rossant, J. and R.A. Pedersen (eds.) *Experimental Approaches to Mammalian Embryonic Development*. Cambridge Univ. Press, Cambridge, U.K.
- Sirard, M.A., H.M. Florman, M.L. Leibfried-Rutledge, F.L. Barnes, M.L. Sims and N.L. First. 1989. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. *Biol. Reprod.*, **40**: 1257-1263.
- Watson, A.J., M.E. Westhusin, P.A. De Sousa, D.H. Betts and L.C. Barcroft. 1999. Gene expression regulating blastocyst formation. *Theriogenology*, **51**: 117-133.
- Wrenzycki, C., D. Herrmann and H. Niemann. 2007. Messenger RNA in oocytes and embryos in relation to embryo viability. *Theriogenology*, **68**(Sup. 1): S77-S83.

Instructions for Authors

Buffalo Bulletin is published by International Buffalo Information Centre under the authorization of Office of University Library, Kasetsart University, Thailand. Contributions on any aspect of research or development, progress report of projects and news on buffalo will be considered for publication in the bulletin.

General editorial policies

Authorship criteria

Authorship is restricted to those who (1) have contributed substantially to one or more of the following aspects of the work and (2) are willing to assume public responsibility for the validity of the work.

Copyright

Copyright to published manuscripts becomes the sole property of International Buffalo Information Center.

Criteria for manuscript acceptance

Manuscript acceptability is based on clarity of objectives; originality; appropriateness of the experimental design, methods and statistical analysis; substance of the results; thoroughness with which the results are discussed; and appropriateness of the conclusions.

Following acceptance of a paper and prior to publication, the author will be received the acceptance letter.

Manuscript requirements

Manuscripts preparation

Manuscripts on original research in English language should include at least the following elements.

Title

- Full title (be concise)
- Name(s) of author(s) and the first author affiliation with complete address.

Abstract

- An abstract not exceeding 250 words; all acronyms and abbreviations defined; no references cited. State what, where and how it was done, major results.
- Five key words.

Introduction. Review pertinent work, cite key references, explain importance of the research, and state objectives of your work.

Materials and Methods. Provide sufficient detail so work can be repeated. Describe new methods in detail; accepted methods briefly with references.

Use of trade names. Trade names are to be avoided in defining products whenever possible.

Use of abbreviations and acronyms. At first text use, define in parentheses. Do not use abbreviations and acronyms in titles.

Results and discussion. Present results concisely using figures and tables as needed. Do not present the same information in figures and tables. Discuss principles and relationship, point out exception. Show agreement with published research work. The significances of work or conduction should be presented in the end of discussion.

Tables. Number each table with Arabic numerals. Place a descriptive caption at the top of each table.

Figures. (graphs, charts, line drawings, photographs) Number each figure with Arabic numerals under the illustration. Lettering, data lines and symbols must be sufficiently large so as to be clearly visible when the figure is reduced to a size commonly used in the journal.

References. List only those references cited in the text. Required format of described below.

Reference cited format

Manuscripts should follow the name-year reference format. Cite only necessary publications. Primary rather than secondary references should be cited, when possible. It is acceptable to cite work that is "in press" (i.e., accepted but not yet published) with the pertinent year and volume number of the reference.

In text. Cite publications in text with author name and year. Three or more authors use "et al.". In parenthetical citations, separate author and year with a comma. Use suffixes a, b and c to separate publications in same year by the same author. Semi-colon separate citations of different authors. Cite two or more publications of different authors in chronological sequence, from earliest to latest. For example:

....used liquid nitrogen vapour freezing technique from Verma *et al.* (1975)

....liquid nitrogen vapour freezing technique (Verma *et al.*, 1975)

...and buffaloes (Singh *et al.*, 1983; Shah *et al.*, 1987; Misra, 1996; Pant *et al.*, 2002)

In reference cited. List only those literature cited in the text. References should be listed alphabetically by the first author's last name. Single author precedes same author with co-authors. Type references flush left as separate paragraphs. Do not indent manually. Write the name of book or journal in italic letters. Use the following format.

- *Journal articles:* Author(s). Year. Article title. *Journal title*, volume number: inclusive pages.

Example: Citation in text: Chaudhary *et al.* (1981)

Choudhary, P.C., B. Prasad and S.K. Misra. 1981. Note on the use of rumen liquor in the treatment of chronic alkaline indigestion in cows. *Indian J. Anim. Sci.*, **51**: 356-360.

- *Books:* Author(s) or editor(s). Year. *Title*. Publishername, Place of publication. Number of pages.

Example: Citation in text: Snedecor and Cochram. (1980)

Snedecor, G.W. and W.G. Cochram. 1980. *Statistical Methods*, 7th ed. The Iowa State University Press, Ames, Iowa, USA. 593p.

Sattar, A. 1995. *Studies on the effect of immunopotential of vaccinated pregnant buffaloes and cows on neonatal antibody titre and hematological profile*. Ph. D. Thesis, University of Agriculture, Faisalabad, Pakistan. 208p.

- *Chapter:* Author(s) of the chapter. Year. Title of the chapter, pages of the chapter. In author(s) or editor(s). *Title of the book*. Publisher name, Place of publication.

Example: Citation in text: Sloss and Dufty. (1980)

Sloss, V. and J.H. Dufty. 1980. Disorders during pregnancy, p. 88-97. In Sloss, V. and J.H. Dufty (eds.) *Handbook of Bovine Obstetrics*. Williams and Wilkins, Baltimore, U.S.A.

Sabrani, M., K. Diwyanto and M. Winugroho 1994. A critical review of buffalo research and development activities in Indonesia. Past performance and future strategies, p. 78-89. In *Proceedings of 1st Asian Buffalo Association Congress*, Thailand.

Submission manuscript

Submit the following items.

Cover letter. Identify the corresponding author and provide his/her full name, address, numbers for telephone and fax, and e-mail address.

Manuscript. In 12 point Times or Times New Roman. Type on one side of A4 paper. Use one inch margins. Number all pages. Send an original manuscript and 1 photocopy.

Disk. Include an IBM-formatted, 3-1/2" disk or 4-3/4" CD-ROM, containing the manuscript in Microsoft Word.

Mail manuscript to:

By post: International Buffalo Information Centre
Office of University Library
Kasetsart University,
50 Pahonyothin Road, Chatuchak,
Bangkok 10900, Thailand
Tel. 66-2-942-8616

By e-mail: libibic@ku.ac.th

CONTENTS

	Page
Case Report	
Dystocia due to uterine torsion in a buffalo <i>G.K. Das, Firdous A. Khan, S. Deori and Uma Shanker</i>	69
Surgical management of cervical esophageal obstruction in a buffalo: A case report <i>R.V. Suresh Kumar, N. Dhana Lakshmi, P. Veena, P. Sankar and P. Yasotha</i>	71
Dystocia due to fetal ascitis with wry neck in a graded Murrah buffalo: A case report <i>P. Vidya Sagar, Krishna Veni, K.S. Sai Krishna and K.S. Vadde</i>	73
Management of uterine torsion in a she buffalo <i>Qazi Mudasir, S.P. Shukla, S.P. Nema, R. Ali and S.S. Mahor</i>	75
Dystocia due to lateral deviation of head and foetal emphysema in a she buffalo <i>Qazi Mudasir, S.P. Shukla, S.P. Nema, L. Patidar and R. Ali</i>	78
Uterine leiomyoma in a buffalo: A case report <i>O.I. Azawi and H.I. Al-Sadi</i>	80
Short Communication	
Detection and characterization of <i>Listeria</i> species from buffalo meat <i>J.B. Nayak, M.N. Brahmabhatt, C.V. Savalia, C.D. Bhong, A. Roy, I.H. Kalyani and B.C. Parmar</i>	83
Genotyping of Prolactin gene in Pandharpuri buffalo by PCR-RFLP <i>V.S. Madnalwar, M.P. Sawane, V.D. Pawar, P.A. Patil, A.P. Fernandis and A.S. Bannalikal</i>	88
Clinical management and haemato-biochemical changes in babesiosis in buffaloes <i>N. Lakshmi Rani*, C. Sreedevi, P. Annapurna and K. Aswani Kumar</i>	92
Seroprevalence of leptospirosis in she-buffaloes (<i>Bos bubalis</i>) at slaughter in Chennai, India <i>J. Selvaraj, B. Murali Manohar, R. Govindarajan, Vajiravelu Jayakumar, T.V. Meenambigai and C. Balachandran</i>	95

CONTENTS

	Page
Original Article	
Identification of <i>Brucella</i> spp. from the animals with reproductive disorders by polymerase chain reaction assay <i>Sanjay Ghodasara, Ashish Roy, D.N. Rank and Bharat B. Bhanderi</i>	98
Evaluation of different methods of DNA extraction from semen of buffalo (<i>Bubalus bubalis</i>) bulls <i>Anju Manuja, Sonia Manchanda, Balvinder Kumar, S. Khanna and R.K. Sethi</i>	109
Studies on thyroid profile (T_3 , T_4 and TSH) in estrous, cyclic and anoestrous buffaloes <i>Raja Kumar P., Lakshmana Swamy P., Aswani Kumar K. and Somasekhara Rao K.</i>	115
Value added buffalo meat sausage with potato flour as binder <i>R. Ponsingh, R. Narendra Babu, S. Wilfred Ruban and V. Appa Rao</i>	121
Effect of housing system on milk yield, cleanliness and lameness in Murrah buffaloes <i>R.M.V. Prasad, K. Sudhakar, E. Raghava Rao, B. Ramesh Gupta and M. Mahender</i>	129
Detection of BPV-2 in cutaneous warts of Indian water buffaloes (<i>Bubalus bubalis</i>) <i>Vidya Singh, R. Somvanshi, T. Yasotha, S. K. Subodh, S.K. Singh and Shanker Dayal</i>	133
Serum biochemical reference values for female buffaloes in Egypt <i>Mahmoud Rushdi Abd Ellah</i>	141
<i>OCT-4</i> gene expression pattern in different grades of buffalo (<i>Bubalus bubalis</i>) oocytes <i>Ch. Srinivasa Prasad, V.S. Gomathy, A. Palanisamy, G. Dhinakar Raj, A. Thangavel and S. Satheshkumar</i>	148

BUFFALO BULLETIN
IBIC, KASETSART UNIVERSITY, P.O. BOX 1084
BANGKOK 10903, THAILAND
URL : <http://ibic.lib.ku.ac.th>
E-mail : libibic@ku.ac.th
Tel : 66-2-9428616 ext. 344
Fax : 66-2-9406688