# DYSTOCIA DUE TO CEBOCEPHALUS MONSTER IN A GRADED MURRAH BUFFALO

**Vidya Sagar Pentyala, M. Sreenu, V. Karuna Sri and K. Rajesh**

## ABSTRACT

In this report, an atypical form of Cebocephalus monster is described in a graded Murrah buffalo male calf. The changes were confined to the head and abdomen. The most significant malformation was the presence of a centrally located orbit with two eye balls which were not fused. Other defects included fetal ascitis and congenital hairlessness (hypotrichiosis). The possible cause of this congenital defect could not be ascertained.

**Keywords**: Dystocia, Cebocephalus, buffalo

## INTRODUCTION

Cebocephaly is a developmental defect in which the orbits are abnormally close together and incorrectly oriented rostrally during embryonic development giving monkey face appearance (Noden, 1985)

## CASE HISTORY AND CLINICAL OBSERVATIONS

A 5-year-old graded Murrah buffalo in its second parity was brought to the Veterinary Poly Clinic with the history of active labour for the preceding 6 h. The amniotic bag had already been ruptured and the limbs were visible in the birth canal. On detailed vaginal examination, an abnormally dome shaped head was noticed. On deeper per vaginal examination, an abnormally distended fluid filled abdomen was noticed and the condition was diagnosed as a foetal monster. It was decided to deliver the fetus by Caesarean section.

## TREATMENTS AND DISCUSSION

Caesarean section was performed through left para medial approach as per the routine procedure under pre-medication with Triflupromazine followed by local infiltration with 2% lignocaine hydro chloride. A male dead foetal monster was delivered.

Gross examination revealed severe reduction of facial features; the calf had a centrally located orbit (Figure 1) with two eye balls. The

[1]Veterinary Poly Clinic, Gudiwada, Krishna District, 521301, A.P, India
[2]Department of Surgery and Radiology, N.T.R. College of Veterinary Science, Gannavaram, India
[3]N.T.R. College of Veterinary Science, Gannavaram, India
[4]Department of Medicine, N.T.R. College of Veterinary Science, Gannavaram, India

eye lids and all the skeletal structures of the nose were absent. The calf had a dome-shaped head, centrally located orbit with protruded tongue giving it a monkey-face-like appearance. It had an abnormally distended abdomen due to the accumulation the fluid (ascitis) and was hairless. On exploration, straw-colured fluid was observed. No other abnormalities were found.

Cebocephaly and cyclopia have been classified under teratological defects of embryonic development (Roberts, 1971). Compared with cyclopia, cebocephaly is less a severe form of holoprosencephaly. It was a developmental defect commonly seen in pigs and sheep but observed in all species. In the present case the exact cause is unknown, but this condition is known to be due to ingestion of *Veratum californicum* in sheep (Binns *et al*., 1960; 1963).

In the available literature, there were very few reports available on cebocephaly in buffalo calves.

## REFERENCES

Binns, W.M, L.F. James, J.L. Shupe and G. Evert. 1963. A congenital cyclopian type malformation in lambs induced by maternal ingestion of a range plant Veratum californicum. *Am. J. Vet. Res.*, **24**(103): 1164.

Noden, D.M. and A. de Lahunta. 1985. Digestive system, p. 292-311. *In The Embryology of Domestic Animals*. Williams & Wilkins, Baltimore,

Roberts, S.J. 1971. *Veterinary Obstetrics and Genital Diseases*, 2nd ed. CBS Publishers, New Delhi.

Figure 1. Legends: Photomicrograph showing cebocephalus calf.

# CONGENITAL UMBILICAL DEFECT WITH VISCERAL EVENTRATION IN A BUFFALO CALF - A CASE REPORT

**P. Veena, P. Sankar, S. Kokila, R.V. Suresh Kumar and N. Dhana Lakshmi**

## ABSTRACT

This communication reports a case of congenital umbilical defect with visceral eventration in a buffalo calf which was operated successfully without any complication.

**Keywords:** umbilical defect, eventration, buffalo calf

## INTRODUCTION

Congenital ventral abdominal defects are very common in calves. Defects in the development of somatopleura lead to various defects in the body wall especially in the ventral median parts. The umbilical opening is present to provide passage of the urachus, the umbilical vein carrying placental blood and the two large umbilical artries carrying blood to the placenta. Exposure of the abdominal viscera is very common in schistosomus reflexes which include spinal inversion in bovine fetal monsters (Denis and Meyer, 1965; Denis, 1972) and in found to be one of the most important fatal congenital disorders (Cavalieri and Farin, 1999), a defect resulting from faulty closure of abdominal wall along its ventral mid line along with protrusion of abdominal viscera (Willis, 1962). The present paper records a rare case of congenital prolapse of abdominal viscera through the defect in the umbilicus in a buffalo calf and its surgical correction.

## CASE HISTORY AND OBSERVATIONS

A newly born male buffalo calf was brought to the college clinic with the history of prolapse of abdominal viscera contained through the umbilical opening since birth (Figure 1) On clinical examination, the abdominal viscera contained congested abomasum and intestinal loops (Figure 2) The abdominal viscera were covered with parietal peritoneum and there was a rise in temperature, i.e. 39°C, respiratory rate and heart rate.

## TREATMENTS AND DISCUSSION

The animal was given fluid therapy using normal saline (0.89%). The protruded visceral mass was washed with normal saline. After aseptic preparation of the site, lignocaine 2% was infiltrated around the hernial ring, which was about one inch in diameter. Reduction of the contents was impossible through the umbilical opening; hence it was enlarged cranio - caudally. The abdominal viscera were replaced in to the abdominal cavity after replacing the viscera; the ballooning of the peritoneum was

Department of Veterinary Surgery and Radiology, College of Veterinary Science, Sri Venkateswara Veterinary University, Tirupati, Andhrapradesh, India

Figure 1. Eventration of abdominal viscera in a buffalo calf.

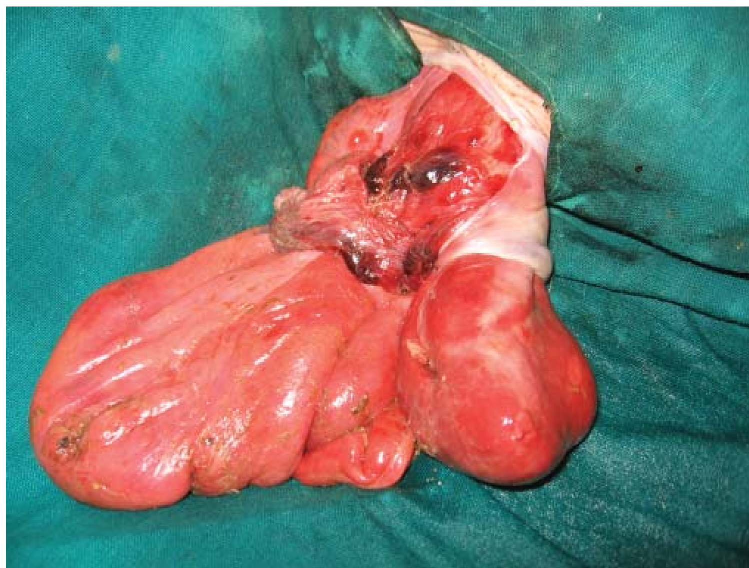

Figure 2. Congested abomasum covered with parietal peritoneum.

trimmed from the base. The peritoneum, abdominal muscles and skin were sutured in routine manner. Post - operative care included injection Megapen (Ampicillin 125 mg and Cloxacilline 125 mg BID, Aristo Pharmaceutical Pvt Ltd) and Melonex 0.5 ml s/c SID for 3 days. Antiseptic dressing of the wound was done with betadine till suture removal. The animal recovered completely and started natural suckling after 2 days treatment. Sutures were removed on the 9th post operative day.

Faulty closure of the abdominal wall in the prenatal development results in the eventration of parts of visceral organs with its serous sac. The condition can be corrected successfully and it should be done immediately to avoid contamination and injury to organs. When contamination of the sac is noticed it is advised to remove the sac at the level of fissure. Congenital intestinal prolapse through the persistent umbilical opening in the new born calf has been reported by Sharma (2003); Jana and Ghosh (2005).

## REFERENCES

Cavalieri, J. and Farin. 1999. Birth of a holstein freemartin calf co - twinned to a schistosomus reflexes fetus. *Therigenology*, **52**: 815-826.

Dennis, S.M. and E.P. Meyer. 1965. Schistosomus reflexes in a sheep. *Vet. Rec.*, **77**: 1386-1388.

Dennis, S.M. 1972. Schistosomus reflexes in conjoined twim lambs. *Vet. Rec.*, **19**: 509-510.

Jana, D. and Ghosh. 2005. *Indian Vet. Med. J.*, **29**: 329.

Sharma, A. 2003. Passage of abdominal viscera through persistant umbilical opening ina newly born female buffalo calf and its surgical correction. *Intas Poly Vet.*, **4**: 335.

Willis, R.A. 1962. *The Borderland of Embryology and Pathology*, 2nd ed. Butterworths, London, 288-289.

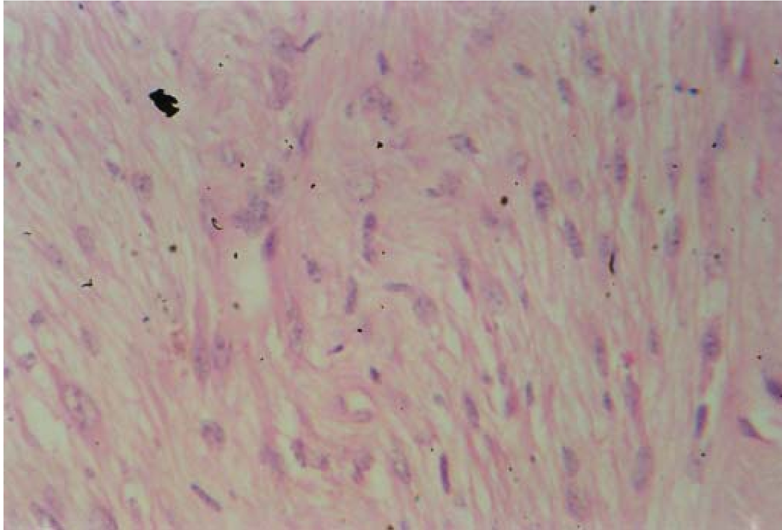

Figure 1. Section showing densely packed cells admixed with collagen. Vangieson stain. x280.

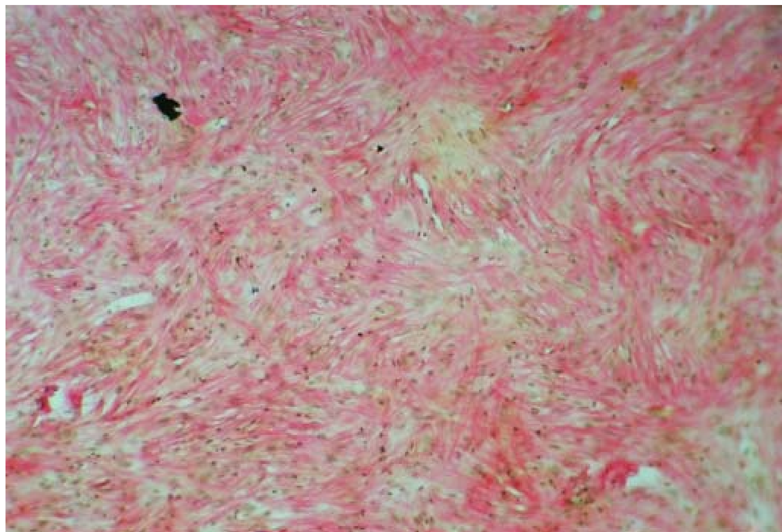

Figure 2. Note several mitotic figures in the cells. Hand E. x280.

# IDENTIFICATION OF BUFFALO (*Bubalus bubalis*) MEAT USING PCR TARGETING MITOCHONDRIAL D-LOOP GENE

**Deepak Kumar*, S.P. Singh, V. Umapathi, K. Nagappa and Abhishek Gaurav**

## ABSTRACT

A novel polymerase chain reaction (PCR) was developed for the identification of buffalo (*Bubalus bubalis*) meat using newly designed primers targeting the mitochondrial D-loop region. Buffalo-specific primers were designed against a conserved region of mitochondrial d-loop that amplified buffalo specific region of 358 bp in size. The specificity of primers was confirmed by PCR analysis of DNA from related domestic animal meats i.e. cattle, goat, sheep, pig and chicken. The PCR assay was checked for repeatability using DNA isolated from different buffalo meat samples and was validated. Buffalo species-specific PCR developed in this study presents a means of identification of buffalo meat as a reliable tool to avoid the fraudulent substitution and adulteration of buffalo meat.

**Keywords:** meat, adulteration, buffalo, d-loop, PCR

## INTRODUCTION

In India, there are two major factors associated with the consumption of buffalo meat (carabeef). Firstly, the Hindus have reservation towards the consumption of buffalo meat, while the Muslim community prefers to consume carabeef in view of ban on the slaughter of the cows (beef) in this country. Secondly, there is a malpractice among meat vendors to mix the low priced carabeef (even sometimes the banned cow meat) meat with other costlier meats like goat (chevon) and sheep (mutton) meats to gain monetary benefits. Under such circumstances, the consumers would have questions pertaining to the surety and authenticity of the origin of meat. Also, every year a huge number of veterolegal cases are registered involving buffalo killing in India including the wild ones. Keeping in view these peculiarities in India, carabeef identification has become an essential element for food quality control and forensic analysis. A number of analytical procedures have been evolved to correctly differentiate various food animal species. Most of analytical methods employed are based on the protein analysis by either electrophoretic (Vallejo *et al*., 2005), chromatographic (Toorop *et al*., 1997), or immunochemical assays (Chen and Hsieh, 2000). However, most proteins get denatured at high temperatures, resulting in changed antigenicity and electrophoretic mobility of molecules (Giovannacci *et al*., 2004). Recently, DNA-based methods particularly polymerase chain reaction (PCR) has proved to be a reliable tool for rapid detection and identification of organisms at the species level. Using an appropriate primer pair, mitochondrial sequences have been amplified in

*Research Associate, Division of Veterinary Public Health, Indian Veterinary Research Institute (IVRI), Bareilly, Uttar Pradesh, India

many species, and the resulting differences used for species identification (Di Pinto, *et al*., 2005).

Several mitochondrial genes including cytochrome-b gene (Forrest and Carnegie 1994; Verma and Singh, 2003), the 12S and 16S ribosomal RNA genes (Rodrıguez *et al*., 2003; Fajardo *et al*., 2006), and the displacement loop gene (d-loop) (Lopez *et al*., 1996; Gao *et al*., 2004; Kierstein *et al*., 2004) have been targeted for species identification. The D-loop is the most rapidly evolving region of the mt DNA molecule and is one of the most commonly used markers (Kocher *et al*., 1989; Foran *et al*., 1997) when determining evolutionary relationships among closely related species and subspecies. Keeping in mind the need for a reliable technique for identification of buffalo meat, the present study was aimed to develop a buffalo specific PCR assay for the authentic identification of buffalo meat (carabeef).

## MATERIALS AND METHODS

### 1. Meat samples

Meat samples from buffalo (Bubalus bubalis), cattle (Bos indicus), goat (Capra hircus), sheep (Ovis aries), pig (Sus domesticus) and chicken (*Galus gallus*) were used in the present study. Cattle, goat, sheep, pig and chicken meats were used to check the specificity of the designed primers. Approximately, 50 gm of meat samples were collected from local markets, slaughter houses and veterinary clinics under sterile conditions and were transported to laboratory in an icebox containing gel cool packs. Meat samples were kept in deep freezer maintained at 20 ºC till further use.

### 2. DNA Extraction

The DNA was isolated from the samples using a Wizard® Genomic DNA purification kit (Promega, Madison, USA) following the manufacturer's instructions. Purity, quality and concentration were determined as per standard protocols.

### 3. Designing of primers

Buffalo specific primers were designed targeting mitochondrial d-loop (DNAStar, Inc., 1996). The buffalo mitochondrial d-loop sequences were downloaded from the NCBI and aligned using "*Megalign*" software (DNAStar, Inc., 1996). A conserved region was identified and oligonucleotide primers were designed using "*Primer-Select*" software (DNAStar Inc., 1996) so as to yield a PCR product of 358 bp specific for buffalo. Later, the selected primers were confirmed for specificity by using the PRIMER-BLAST of NCBI. Finally, selected primers were custom synthesized from IDT, USA and used for PCR amplification. The primer sequences were forward (DAF-01, 5`-TTCTTCAGGGCCATCTCATC-3`) and reverse (DBR-03, 5`-TCGAATAAGCATC TAGGGAGAA-3`).

### 4. Standardization of PCR

The PCR conditions were standardized so as to obtain the desired amplicon of 358 bp for buffalo. A 25 µl reaction mixture was prepared containing 2.5 µl of 10X assay buffer [25 mM MgCl2, Bioron, GmbH], 0.5 µl (200 µM each) of dNTP mix [sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water i.e., 40 mM total, pH 7.5, Promega, USA], 0.8 µl (20 Pico moles) each of forward and reverse primers (Integrated DNA Technologies - IDT, Madison, USA), 1U Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron, Germany), 50 ng of purified DNA and nuclease free water (Merck, Germany) to make the volume. The

tubes were flash spun and the PCR was performed in a Thermal cycler (Gene AMP® PCR System 9700, Applied Biosystems).

The cycling conditions were as follows: an initial denaturation (95°C for 5 minutes) followed by 30 cycles of denaturation (95°C, 30 seconds), primer annealing (52°C, 30 seconds) and extension (72°C, 30 seconds) and final extension (95°C, 5 minutes). The PCR products were held at 4°C until electrophoresis. Agarose gel (2%) was prepared in 0.5X TBE buffer and the PCR products (8 µl) stained with 6X gel loading dye (2 µl) were electrophoresed at 50V for 1.5 h along with 100 bp DNA ladder (M/s. Bangalore Genei, India). The amplified products were visualized using a gel documentation system (AlphaImager® HP, Alpha Innotech Corp.).

## RESULTS AND DISCUSSION

Fraudulent substitution of buffalo meat with other costlier meats demands the development of simple and authentic method for detecting buffalo meat. The present investigation was undertaken with the objective to develop a simple and specific PCR based molecular diagnostic techniques for the identification of buffalo meat.

### 1. Standardization of buffalo specific PCR assay

A fragment of 358 bp from the targeted buffalo mitochondrial D-loop region (Accession no. AF197216, location 490-847) was amplified (Figure 1). Primer concentration of 20 pico moles per reaction and an annealing temperature of 52°C were found ideal for amplification. Different primer concentrations ranging from 18-22 pico moles were attempted to obtain the desired PCR product of 358 bp and finally a primer concentration of 20 pico moles was selected for amplification. Similarly, different annealing temperatures ranging from 48-58°C were used for the standardization and lastly 52°C was selected as an optimum annealing temperature. In a similar study, Malisa *et al*. (2006) reported PCR amplification of mitochondrial D-loop for identification of buffalo meat species but with different primer sequences. This study is in accordance with the work done by Nagappa (2008), who differentiated six food animal species including buffalo, targeting mitochondrial D-loop with species-specific primers; and who also employed species-specific PCR assays for the detection of origin of meat species in raw, heat treated as well as adulterated meat samples. Similarly, Guoli *et al*. (1999) reported PCR amplification of a 218 bp product specific for buffalo DNA by targeting 1.709 satellite DNA.

### 2. Specificity and repeatability of standardized PCR assay

Possibility of cross amplification of buffalo specific primers was eliminated by testing buffalo specific primers with DNA of cattle, goat, sheep, pig and chicken. The buffalo specific primer pair was able to produce amplicon of 358 bp in buffalo DNA only (Figure 2). No amplification was observed in the DNA of other species tested including negative control and thus the specificity of designed buffalo specific primers was confirmed. Repeatability of buffalo specific primers was confirmed by testing primers with DNA isolated from different buffalo meat samples (5 each) collected from different places. Invariably, the amplicon of 358 bp specific for buffalo DNA was obtained (Figure 3).

```
1   AATATTATTAATATACCTCCACAAATGCAAAGAGCCTTCTCAGTATTAAATTCACTAAAACTTGCAACAACTTAACACTGACTTTACACTCTAACCTAAC AF197216.SEQ
1   ---------------------------------------------------------------------------------------------------- common forward.SEQ
1   ---------------------------------------------------------------------------------------------------- Buffalo reverse.SEQ

101 ATTAGAAATAACTGCAACCATCAACACACCTAACCTCGCATGTACGGCATACAACATATGGTCTTACCACTCCGAATGGGGAATGTACATAACATTAAT AF197216.SEQ
1   ---------------------------------------------------------------------------------------------------- common forward.SEQ
1   ---------------------------------------------------------------------------------------------------- Buffalo reverse.SEQ

201 GTAACAAGGACATAATATGTATATAGTACATTACATTATATGCCCCATGCGTATAAGCAAGTACATAAACATGCATGATAGTACATAGTACATATAATTA AF197216.SEQ
1   ---------------------------------------------------------------------------------------------------- common forward.SEQ
1   ---------------------------------------------------------------------------------------------------- Buffalo reverse.SEQ

301 TTGATCGTACATAGCACATTTAAGTCAAATCCATTCTTATCAACATGCGTATCCCTTCCACTAGATCACGAGCTTGATCACCATGCCGCGTGAAACCAGC AF197216.SEQ
1   ---------------------------------------------------------------------------------------------------- common forward.SEQ
1   ---------------------------------------------------------------------------------------------------- Buffalo reverse.SEQ

401 AACCCTTCAGGCAAGGATCCCTCTTCTCGCTCCGGGCCCATGTTATGTGGGGGTAGCTATTTAATGAATTTTAACAGGCATCTGGTTCTTTCTTCAGGGC AF197216.SEQ
1   -----------------------------------------------------------------------------------------TTCTTCAGGGC common forward.SEQ
1   ---------------------------------------------------------------------------------------------------- Buffalo reverse.SEQ

501 CATCTCATCTAAAATCGCCCATTCTTTCCTCTTAAATAAGACATCTCGATGGACTAATGTCTAATCAGCCCATGCTCACACATAACTGTGCTGTCATACA AF197216.SEQ
12  CATCTCATC                                                                                            common forward.SEQ
1   ---------------------------------------------------------------------------------------------------- Buffalo reverse.SEQ

601 TTTGGTATTTTTTTATTTTGGGGATGCTTGGACTCAGCTATGGCCGTCAAAGGCCCCGACCCGGAGCATAAATTGTAGCTGGACTTAACTGCATCTTGA AF197216.SEQ
20                                                                                                       common forward.SEQ
1   ---------------------------------------------------------------------------------------------------- Buffalo reverse.SEQ

701 GCACCAGCATAATGGTAGGCATGGGACATTACAGTTAATGGTCACAGGACATAAATATATTATTCATTCTTCCCCCCCTCCTATCAAACCCCCCCCTTAA AF197216.SEQ
20                                                                                                       common forward.SEQ
1   ---------------------------------------------------------------------------------------------------- Buffalo reverse.SEQ

801 ATATTTACCACCATTTTTAACATGCTTCTCCCTAGATGCTTATTCGAATTTATCACACTTCCAATACTCAAATTAGCACTCCAAACAAAGTAACTATATA AF197216.SEQ
20                                                                                                       common forward.SEQ
1   -------------------------TTCTCCCTAGATGCTTATTCGA                                                     Buffalo reverse.SEQ

901 AGCACCTAAACCATCACCTCAC                                                                               AF197216.SEQ
20                                                                                                       common forward.SEQ
22                                                                                                       Buffalo reverse.SEQ
```

Forward primer

Reverse primer

Figure 1. Alignment of buffalo specific primers (DAF-01 and DBR-03) to mitochondrial D-loop region of buffalo (AF197216).

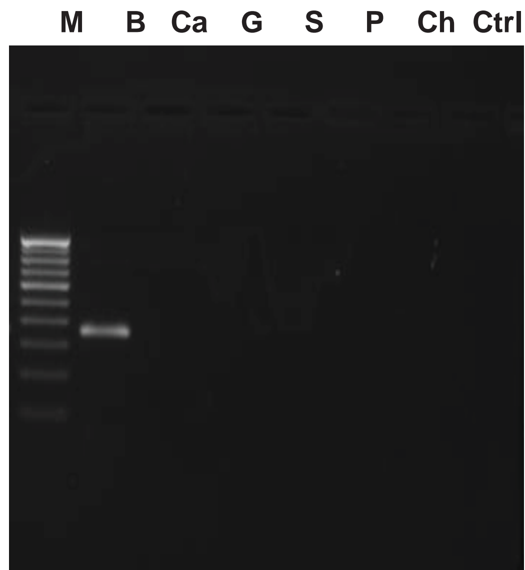


Figure 2. PCR amplification of buffalo DNA (358 bp). M-100bp DNA marker, B-Buffalo, Ca-Cattle, G-Goat, S-Sheep, P-Pig, Ch-Chicken and Ctrl-Negative control.

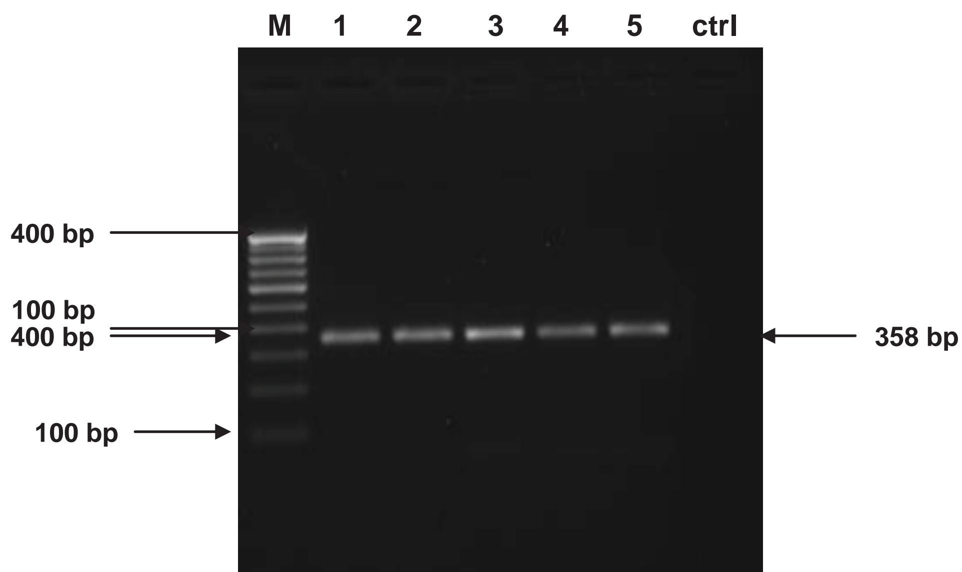


Figure 3. PCR amplification of buffalo DNA (358 bp). M-100bp DNA marker, 1-5 (buffalo meat samples) and Ctrl-Negative control.

## CONCLUSION

The conventional methods available for meat species identification lack specificity and repeatability. To overcome these problems DNA based techniques are employed for species identification. Specific PCR assay was developed for identification of buffalo meat by amplifying a conserved region of mitochondrial D - loop gene. The assay was found to be highly specific. The single step PCR assay developed for identification of buffalo meat presents a reliable tool to solve adulteration, falsification and veterolegal problems related to buffalo meat.

## REFERENCES

Chen, F.C. and Y.H. Hsieh. 2000. Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. *J. AOAC Int.*, **83**: 79-85.

Di Pinto, A., V.T. Forte, M.C. Conversano and G.M. Tantillo. 2005. Duplex polymerase chain reaction for detection of pork meat in horse meat fresh sausages from italian retail sources. *Food Control*, **16**: 391-394.

Fajardo, V., I. Gonzalez, I. Lopez-Calleja, I. Martın, P.E. Hernandex, T. Garcıa and R. Martın. 2006. PCR-RFLP authentication of meats from Red Deer (Cervus elaphus), Fallow Deer (Dama dama), Roe Deer (Capreolus capreolus), cattle (Bos taurus), sheep (Ovis aries), and goat (Capra hircus). *Journal of Agriculture and Food Chemistry*, **54**: 1144-1150.

Foran, D.R., K.R. Crooks and S.C. Minta. 1997. Species identification from scat: An unambiguous genetic method. *Wildlife Soc. B.*, **25**(4): 835-839.

Forrest, A.R.R. and P.R. Carnegie. 1994. Identification of gourmet using FINS (Forensically informative nucleotide sequencing). *Biotechniques,* **17**(1): 24-26.

Gao, H.W., C.Z. Liang, Y.B. Zhang and L.H. Zhu. 2004. Polymerase chain reaction method to detect canis materials by amplification of species-specific DNA fragment. *J. AOAC Int.*, **87**: 1195-1199.

Giovannacci, I., C. Guizard, M. Carlier, V. Duval, J.L. Martin and C. Demeulemester. 2004. Species identification of meat products by ELISA. *Int. J. Food Sci. Tech.*, **39**: 863-867.

Guoli, Z., Z. Mingguang, Z. Zhijiang, O. Hongsheng and L. Qiang. 1999. Establishment and application of a polymerase chain reaction for the identification of beef. *Meat Sci.,* **51**: 233-236.

Karabasanavar, Nagappa. 2008. *Molecular identification of animal species using Polymerase Chain Reaction based techniques.* Ph. D. Thesis. G.B. Pant University of Agricurtural and Technology, Pantnagar, Uttarakhand, India.

Kierstein, G., M. Vallinoto, A. Silva, M.P. Schneider, L. Iannuzzi and B. Brenig. 2004. Analysis of mitochondrial D-loop region casts new light on domestic water buffalo (*Bubalus bubalis*) phylogeny. *Mol. Phylogenet. Evol.,* **30**(2): 308-24

Kocher, T.D., W.K. Thomas, A. Meyer, S.V. Edwards, S. Paabo and F.X. Villablanca. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing of conserved primers, p. 86. *In Proceedings of the National Academy Sciences of the United States of America*.

Lopez, J.V., S. Cervarlo and S. Brien. 1996.

Complete nucleotide sequence of domestic cat (*Felis catus*) mitochondrial genome and a transposed mtDNA tandem repeat (NUMT) in the nuclear genome. *Genomics,* **33**: 229-246.

Malisa, A.L., P. Gwakisa, S. Balthazary, S.K. Wasser and B.M. Mutayoba. 2006. The potential of mitochondrial DNA markers and polymerase chain reaction-restriction fragment length polymorphism for domestic and wild species identification. *Afr. J. Biotechno.,* **5**(18): 1588-1593.

Rodriguez, M.A., T. Garcia, I. Gonzalez, L. Asensio, B. Mayoral, I. Lopez-Calleja, P.E. Hernandez and R. Martin. 2003. Development of polymerase chain assay for species identification of goose and mule duck in *foie gras* products. *Meat Sci.,* **65**: 1257-1263

Toorop, R.M., S.J. Murch and R.O. Ball. 1997. Methodology and development of prediction equations for the determination of pork substitution in veal. *Food Res. Int*., **30**: 629-636.

Vallejo, B., A.F. Gonzalez, M.A. Mazorra and R. Rodrıguez. 2005. Capillary electrophoresis for the analysis of meat authenticity. *J. Sep. Sci.*, **28**: 826-836.

Verma, S.K. and L. Singh. 2003. Novel universal primers establish identity of enormous number of animal species for forensic application. *Mol. Ecol. Notes*., **3**: 28-31.

# STUDIES ON REPEAT BREEDING OF BUFFALOES

**Rajesh Kumar[1], Dharmendra Kumar[2] and Biswajit Roy[3]**

## ABSTRACT

The present study was carried out in outdoor clinics of the Veterinary College and private farms in and around Patna, Bihar, India. After gynecological examination 18 buffaloes from clinics as well as private farms were selected as true repeat breeders. The buffaloes were examined for ectoparasites, and animals having parasitic infestation were treated accordingly with albendazole 10 mg/Kg body wt. Mineral mixture was given to all the animals at the dose rate of 30 gm/animal/day to rule out any marginal nutritional deficiencies. Animals having short or irregular estrus cycle, purulent or mucopurulent discharge, or having ovulatry disturbance were excluded from the present study. Cervical mucous samples were collected by taking all possible sterile precautions. The colour and consistency of cervical mucous was studied in respect of its cleanliness and transparency. The animals harboring turbid, translucent, opaque cervical mucous or cervical mucous with flakes or pus were excluded from the present study. The consistency of cervical mucous was studied in respect of thin and thick. The pH of cervical mucous was studied immediately after collection of sample with the help of narrow range pH paper (range 6.5 to 9.00) having the difference of 0.5 only. The incidence of repeat breeding in buffalo was found 8.82%. Highest incidence of repeat breeding was observed in second parity (27.77%) and lowest incidence was observed in 4$^{th}$ and onward partum (11.11%). The mean pH $\pm$ S.E. of cervical mucous of repeat breeder buffaloes was found to be 8.027 $\pm$ 0.110 with the coefficient of variation of 5.84%. The consistency of cervical mucous of repeat breeder buffaloes was found to be thin in 55.55% and thick in 44.44%. The conception rate found was 62.50% and 50.00% respectively for thin and thick consistency of cervical mucous.

## INTRODUCTION

The buffalo plays an important role in maintaining a sustainable food production system in the developing countries (Nanda and Nakao, 2003). The success of the dairy farm lies in ensuring proper and optimal reproductive rhythm of each individual female in the herd within the normal physiological limits. Any deviation in breeding rhythm results in progressive economic losses due to widening of the dry period, the calving interval as well as lactation during the life time of the animals. Infertile buffaloes mean a loss in milk production whereas fewer calves reduce the efficacy of selection in dairy herd improvement. Efficient dairying and breeding demand that an

[1]Veterinary college, Patna, Bihar, India
[2]Department of surgery and Radiology, College of Veterinary Science and A.H., Rewa, M.P. India
[3]College of Veterinary Science and A.H., Jabalpur, M.P. India

animal shall give birth to a healthy calf every twelve months and be in milk for at least 300 days in lactation. Effort should therefore be made to enhance fertility in dairy animals by narrowing down their dry period to the barest minimum range of 60 to 90 days. Thus, fertility of milch animals appears to play a major role in dairy economics. The productivity of buffaloes, however, remains low largely due to poor management of health, nutrition (Bal Krishnan and Bakagopal, 1994) and breeding (Rane *et al*., 2003).

One of the most important and commonly encountered sub fertile conditions in buffalo which plays a vital role in dairy economics is repeat breeding. The repeat breeding syndrome is defined as a condition in which dairy animal have a regular estrus cycle and appear normal on superficial clinical examination but fail to become pregnant following three or more breeding (Bartlett *et al*.,1986). The condition may occur due to defects in gametes, failure of gametic encounters, endocrine dysfunction, infection, nutritional defects etc., which ultimately leads to either fertilization failure or early embryonic death. Earlier works indicated 39.7% conception failure due to non fertilization and 39.2% due to early embryonic mortality (Tanabe and Casida.1949). The incidence of the repeat breeding condition in buffaloes varies depends upon the mangemental condition of the farm.

The productivity of buffaloes remains low largely due to poor management of health, nutrition (Bal Krishnan and Bakagopal, 1994) and breeding (Rane *et al*., 2003). Anestrus due to ovarian dysfunction and silent ovulation and repeat breeding are two major reproductive disorders in buffaloes (Goley and Kadu, 1995). A high incidence of infertility and repeat breeding in buffaloes mainly of infectious nature has been reported by several workers (Malik *et al*., 1987). Exploration of possible causes and measures for restoring fertility in repeat breeding animals has been the objective of reproductive biologists since the beginning. In spite of good progress made, the causes of conception failure are largely not well understood and repeat breeding remains the biggest problems of the dairy industry.

Therefore, the present investigation was carried out to see the rate of repeat breeding in buffaloes, physical characteristics of cervical mucous and conception rate after treatment of repeat breeder buffaloes.

## MATERIALS AND METHODS

The present study was carried out in outdoor clinics of the Veterinary College and private farms in and around Patna, Bihar, India. A total of 68 buffaloes were brought to the clinics and out of which six were found to be repeat breeders. After gynecological examination, 18 buffaloes from clinics as well as private farms were selected as true repeat breeders, i.e. animals that had regular estrus cycle and periods but had failed to become pregnant following three or more breedings with fertile semen. Gynecological examination of such animal did not reveal any gross abnormalities of the genital organs. The buffaloes were examined for ectoparasites and animals having parasitic infestation were treated accordingly with albendazole 10 mg/Kg body wt. Mineral mixture were given to all the animals at the dose rate of 30 gm/animal/day to rule out any marginal nutritional deficiencies. Animals having short or irregular estrus cycles, purulent or mucopurulent discharges or having ovulatry disturbances were excluded from the present study.

**Collection of cervical mucous**

Cervical mucous sample were collected taking all possible sterile precautions. The vulvar and perineum region were cleaned and dried. The vulvar lips were spread by an assistant and a sterilized insemination gun along with an assembled factory sterilized sheath were passed through the vagina. Rectally, the cervix and insemination gun was manipulated until the tip of the sheath was introduced into os - cervix. Then the insemination gun was withdrawn leaving the sheath in the cervix and cervical mucous was aspirated (Dabas and Maurya 1988). Aspirated mucous was then transferred to a sterilized test tube to study the physical characteristics, *viz.* colour, consistency and hydrogen ion concentrations.

**Examination of physical characteristics of cervical mucous:**

The colour and consistency of cervical mucous was studied in respect of its cleanliness and transparency since only those animals which had clean and transparent cervical mucous were selected. The animals harboring turbid, translucent, opaque cervical mucous or cervical mucous with flakes or pus were excluded from the present study. The consistency of cervical mucous was studied in terms of thin and thick (Sukhdeo and Rao 1971). Thin cervical mucous flowed easily on a glass slide kept inclined at a 45 degree angle.

**Examination of pH of cervical mucous:**

Hydrogen ion concentration (pH) of cervical mucous was studied immediately after collection of the sample with the help of narrow range pH paper (range 6.5 to 9.00) having the difference of 0.5 only.

**Treatment of repeat breeding animals and insemination**

All the 18 animals which were marked as repeat breeder were investigated for cervical mucous consistency and pH. Treatment of the animals was done with various antibiotics, and 12 animals were investigated and inseminated after treatment.

**Conception rate**

Between 45 and 60 days after insemination, the animals were checked for pregnancy by per rectal examination to know the efficacy of each treatment.

**Statistical analysis:**

Standard statistical procedure was applied to test the various parameters (Snedecor and Cochran, 1968).

## RESULTS

**Incidence of repeat breeding**

The incidence of repeat breeding is presented in Table 1. A total of 68 buffaloes were examined in the clinics of The Veterinary College, Patna, out of which six were found to be repeat breeder. Therefore, the percentage of repeat breeding was 8.82 Incidence of repeat breeding of 18 repeat breeding buffaloes, selected from private farms as well as college clinics were analyzed parity - wise (Table 1).

**Physical characteristic of cervical mucous:**

Different scores of physical characteristics of cervical mucous, *viz.* colour, consistency and hydrogen ion concentration (pH), were examined. The results of physical characteristics (colour and

consistency) of cervical mucous of repeat breeder buffaloes in relation to conception rate has been presented in Table 2.

It is evident from Table 2 that the colour of cervical mucous was clean and transparent in all 18 buffaloes selected for present study. Out of 18 animals, 12 animals were investigated and inseminated till 2nd heat after treatment. A total conception rate of 58.33% was obtained as revealed in Table 2. The consistency of cervical mucous of repeat breeder buffaloes was found to be thin in 55.55% and thick in 44.44% of the animals before treatment. Whereas thin consistency was found in 66.66% and thick in 33.33% animals after treatment. The conception rate found was 62.50% and 50.00%, respectively, for thin and thick consistency of cervical mucous. The percentage of conception was higher among buffaloes having thin consistency of cervical mucous than those having thick consistency (Table 2).

The pH of cervical mucous of all the selected repeat breeder buffaloes was taken prior to treatment. Similarly, the same was recorded after treatment and analysis of variance of pH before and after treatment were calculated. The result obtained has been depicted in Table 3.

Analysis of variance of pH of cervical mucous was done which indicated that pH before and pH after treatment were statistically significant ($p < 0.01$). The mean pH $\pm$ S.E. and CV percent of the cervical mucous were also calculated before and after treatment. The result obtained has been presented in table 3.

The mean pH $\pm$ S.E. of cervical mucous of repeat breeder buffaloes before treatment was found to be $8.027 \pm 0.110$ with the coefficient of variation of 5.84% whereas after treatment the mean pH $\pm$ S.E. and coefficient of variation obtained was $7.458 \pm 0.114$ and 5.32%, respectively as depicted in Table 3.

## DISCUSSION

In the present investigation, out of 68 buffaloes brought to the clinics of Veterinary College, Patna six buffaloes were found positive for repeat breeding. Therefore, the incidence of repeat breeding was 8.82%. The present findings were in agreement with the findings of Hussain (1987) who reported an incidence of 8.06% in buffaloes. However, the results differs with the findings of Pandit *et al*. (1982); Rahumathulla *et al*. (1986); Samad *et al*. (1984) who reported comparatively higher incidences of repeat breeding between 12 to 56.44%. While Tomar and Tripathy (1986) reported slightly lower incidence (5%). The variation in the result of different workers might be due to differences in breed, climate, nutrition and management.

The present study revealed that maximum incidence of repeat breeding was observed during 2nd parity (27.77%) and minimum during 4th and onward parity (11.11%), which was in accordance with Hafez, (1987). Maximum incidence of repeat breeding in 2nd parity might be due to maximum milk production during this period, which causes lactational stress and hormonal imbalance. In contrast to these findings, Sah and Nakao (2006) reported maximum incidence (60%) of repeat breeding in heifers.

The colour of cervical mucous was clean and transparent in all the animals selected for the present study. After treatment, 12 buffaloes were inseminated, and overall 88.33% pregnancy was achieved. These findings were in agreement with Sukhdev and Roy (1971) who found that normally the estrus secretions of repeat breeder were clean, but differed from the findings of Mehta (1986),

Table 1. Incidence of repeat breeding in buffaloes in relation to parity.

| Parity - wise distribution of buffaloes | Parity - wise break up of figure | Parity - wise percentage |
|---|---|---|
| Heifer | 4 | 22.22 |
| Buffalo of 1st parity | 3 | 16.66 |
| Buffalo of 2nd parity | 5 | 27.77 |
| Buffalo of 3rd parity | 4 | 22.22 |
| Buffalo of 4th and above parity | 2 | 11.11 |

Table 1 indicated that highest incidence of repeat breeding was observed in second parity (27.77%) and lowest incidence was observed in 4th and onward parity (11.11%).

Table 2. Influence of thin or thick consistency of cervical mucous on conception.

| Observation | Number of clean and transparent sample taken | Consistency | |
|---|---|---|---|
| | | Thin | Thick |
| No of animal investigated before treatment | 18 | 10 (55.55) | 8 (44.44) |
| No of animal investigated after treatment | 12 | 8 (66.66) | 4 (33.33) |
| Conception occurred | 7 (58.33) | 5 (62.50) | 2 (50.00) |

Figure in the parentheses indicates corresponding percentage values.

Table 3. Calculation of mean pH $\pm$ S.E. and CV percent of repeat breeder buffaloes before and after treatment.

| Observation | No. of buffaloes | Mean pH $\pm$ S.E. | CV% |
|---|---|---|---|
| Before treatment | 18 | $8.027^{a} \pm 0.110$ | 5.84 |
| After treatment | 12 | $7.458^{b} \pm 0.114$ | 5.32 |

$^{ab}$ Mean with different superscript differ significantly ($p < 0.01$).

who reported that only 54.17% of repeat breeder animals had clean and transparent cervical mucous and of Vadodria and Prabhu (1990) who reported 46.67% conception in repeater cattle showing clear cervical mucous.

The consistency of cervical of mucous of repeat breeder buffaloes was found to be thin in 55.55% and thick in 44.44% before treatment, whereas it was thick in 66.66 and thin in 33.33% after treatment. The conception rate was found to be 62.50 and 50%, respectively for thin and thick consistency of cervical mucous. The result revealed that a higher conception rate was found in animals showing thin consistency of cervical mucous than a thick consistency of cervical mucous. This was found to be in agreement with Sukhdev and Roy (1971); Vadodria and Prabhu (1990), whose findings were more or less similar. One cause of Low conception rate in thick cervical mucous could be that muco - proteins are interwined and thus resist the penetration and progressive movement of spermatozoa (Odebald, 1968). Gebhard and Schumacher (1970) also reported that profuse watery and clear cervical mucous was favourable for sperm penetration and that thick scanty and opaque cervical mucous was unfavourable for sperm penetration. However, these findings differ from the finding of Dhaliwal and Sharma (1988) who reported that the animals showing thick cervical mucous had a significantly higher conception rate than those with thin cervical mucous.

The overall mean pH of cervical mucous prior to and after treatment was 8.027 $\pm$ 0.11 and 7.458 $\pm$ 0.11, respectively. Analysis of variance of mean pH of cervical mucous showed a significant difference before and after treatment. The result revealed that the mean pH of cervical mucous of repeat breeder animals prior to treatment was higher pH than after treatment. The present findings supported Salphale *et al*. (1993) who reported that the cervical mucous of repeater animals had higher mean pH than that of normal animals. In the present study, most of cervical mucous sample were found more alkaline in reaction in repeater animal prior to treatment than after treatment, and this might have been the cause of conception failure. One reason for this might be infectious organism present in genital tract of repeat breeder animals which cause inflammation and denudation of uterine mucosa. In addition, metabolites of bacteria and inflammatorty exudates might have altered the pH of uterine and cervical fluid to the alkaline side resulting in failure of conception due to death of spermatozoa (Raghaban *et al*., 1971). However, there was general consensus that pH above neutrality provides the most favourable condition for survival and oxidative metabolism of spermatozoa (Mann, 1964). Besides this breed, nutritional variation and electrolyte fluctuation especially Na and K contained in cervical mucous might also be the cause of pH variation (Bocic, 1962).

## REFERENCES

Bal Krishnan, V. and R. Bakagopal. 1994. Serum calcium, phosphorus magnesium, copper and zinc level in regular breeding buffaloes. *Indian Vet. J*., **71**: 23-25.

Bartlett, P.C. J.H. Krik and E.C. Mather. 1986. Repeated insemination in Michingan Holstein-Friesian cattle: incidence, descriptive epidemiology and estimated economic impact. *Theriogenology,* **26**: 309-322.

Bocic, M. 1962. The relation between quality of oestrus mucous and conception rate in cows. *Veterinaria*, **11**: 231-240.

Dabas, Y.P.S. and S.M. Maurya. 1988. A field methods for collections of bovine cervical mucous for microbiological studies. *Indian J. Anim. Reprod*., **9**: 138.

Dhaliwal, G.S. and R.D. Sharma. 1988. Physico-chemical properties of cervical mucous in PGF2 induced oestrous buffaloes in relation to fertility. *Indian J. Anim. Res*., **22**(1): 39-41.

Gebhard, F.B. and M.D. Schumacher. 1970. Biochemistry of cervical mucous. *Fertil. Steril.,* **21**(10): 967.

Goley, R.R. and M.S. Kadu. 1995. Efficacy of $PGF_{2\alpha}$ (Lutalyse), GnRH analogue (Receptal) a d HCG (Chorulon) in treatment of repeat breeding cows. *Indian Vet. J*., **72**: 472-475.

Hafez, E.S.E. 1987. *Reproduction in Farm Animals*. 5th Ed Lea and Febiger. Philadelphia.

Hussain, P.M. 1987. Infertility problems in bovines of northern Karnataka. *Indian. Vet. J.,* **64**: 529-530.

Malik, S.Z., M.A. Chaudhary, N. Ahmed and N. Rahman. 1987. Effect of different antibiotics in the treatment of uterine infection in repeat breeding cows in Faisalabad and Toba Tek sindh districts. *Pak. Vet. J.,* **7**(2): 60-61.

Mehta, G.B. 1986. A study on repeat breeding conditions in crossbred (K X J and K X HF) cattle with special reference to cervical mucous. *Indian J. Anim. Reprod*., **7**(2): 102-103.

Nanda A.S. and T. Nakao. 2003. Role of buffalo in the socio - economic development of rural Asia: current status and future prospects. *Anim. Sci. J.*, **74**; 443-455.

Odebald, E. 1968. The functional structure of human cervical mucous. *Acta Obestetrics and gynaecology*, Scandinavia.

Pandit, R.K., S.P. Shukla and S.R.P. Raman. 1982. Incidence of various reproductive disorders in cattle and buffaloes. *Livestock Advisor*, **7**: 51-53.

Raghaban, R., P.R. Nelkantam and P.K. Uppal. 1971. Studies of the bacteriology of bovine genital tract. *Indian Veterinary Medical Journal*, **48**(8): 779-783.

Rahumathulla, P.S., R.C. Raja Sundaram, and K. Gajendran. 1986. Incidence of various reproductive disorders among cattle and buffaloes. *Cheriosn*, **15**(3): 78-79.

Rane, R.S., R.H. Jadav, R.C. Mazkori and S.S. Swami. 2003. Efficacy of intra-uterine Enrogil in the treatment of repeat breeding in buffaloes. *Indian Vet. J*., **80**: 169-172.

Sah, S.K. and T. Nakao. 2006. Characteristics of repeat breeding buffaloes in Nepal. *Journal of Reproduction and Development,* **52**(3): 335-341.

Salphale, G.V., M.M. Kadu, M Fasihuddin and M.S. Kadu. 1993. Study of some physical properties of estral cervical mucous in synchronized normal and repeat breeder crossbred cows with reference to fertility. *Indian J. Anim. Reprod*., **14**(2): 77.

Samad, H.A., C.S. Ali, K.M. Ahmed and N.U. Rehman. 1984. Reproductive diseases of the water buffalo, p. 25-33. *In Proceedings of 10th International Congress on Animal Reproduction and Artificial Insemination*. Urbana-Champaign.

Snedecor, G.W. and W.G. Cochran. 1968. *Statistical Methods*, 6th ed (Ind.) Oxford and IBH publishing co. 66, New Delhi, India.

**Continued on page 187*

# STUDIES ON FERTILITY RESPONSE IN ANOESTRUS BUFFALOES USING A MODIFIED CIDR-BASED SYNCHRONIZATION PROTOCOL

**N.K. Caesar, S.N. Shukla*, O.P. Shrivastava, S. Agrawal and R.G. Agrawal**

## ABSTRACT

The present study was conducted on 42 true anoestrus buffaloes to observe the efficacy of CIDR implant alone and in combination with PMSG and estradiol valerate for synchronization of estrus and fertility response. The experimental animals were randomly divided into six groups each containing seven animals. All the animals were administered 20 ml Liquid Terramycin intra uterine except in Groups 1 and 3. Animals of Groups 1 and 2 served as control whereas Groups 3, 4, 5 and 6 were implanted with CIDR. Animals of Groups 5 and 6 were also administered, respectively estradiol and PMSG along with implant. On removal of implant, all the animals of G5 and G6 exhibited estrus within 30.42 $\pm$ 5.10 and 65.14 $\pm$ 11.39 h with 57.1 and 85.7% conception rate, respectively. However, in the animals of Groups 4 and 3, induction rates were 85.7 and 71.4% within 40.08 $\pm$ 2.09 and 72.00 $\pm$ 10.76 h of onset interval with 66.6% and 0.00% conception rates, respectively. In none of the animals of Groups 1 and 2 was estrus induced. Our results indicate that addition of PMSG to a progesterone-based estrus synchronization regimen substantially improve ovulation rate and fertility in non-cyclic buffaloes.

**Keywords:** buffalo, anoestrus, CIDR, PMSG, estradiol valerate

## INTRODUCTION

Buffaloes are difficult breeders because of their inherent susceptibility to environmental stress leading to anoestrus and sub estrus conditions. These two conditions are responsible for a prolonged inter calving period resulting in great economic losses to the dairy industry. A Clinical survey by Tanwar *et al*. (2003) revealed higher incidences of anoestrus and inactive ovaries in buffaloes (55.5 and 19.4%) than in cows (43 and 17.2%), respectively. Exogenous administration of progesterone exerts a negative feedback effect over the hypothalamus and pituitary and blocks the release of pituitary gonadotropin. Upon withdrawal of progesterone, the block is removed and larger quantities of gonadotrophins are released which in turn ensures growth and maturation of ovarian follicle and thus onset of estrus. Therefore, in view of the above, this study was conducted to observe the effect of CIDR alone and in combination with PMSG on fertility response in anoestrus buffaloes.

## MATERIALS AND METHODS

Study was conducted on 42 post partum (120 days onwards) true anoestrus buffaloes belonging to organized dairy farms. The true anoestrus was confirmed by two rectal palpations at an 11

*Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary Sciences and Animal Husbandry M.P.P.C.V.V. Jabalpur (M.P.) India

day interval and serum progesterone assay. The animals were randomly divided equally (n = 7) in to six groups. After confirmation of true anoestrus, 20 ml of Liquid Terramycin was administered intra uterine in all the animals except in Group 1 and 3 to rule out any subclinical infection of the genital tract. The CIDR was implanted intra vaginally to the animals of Group 3 to Group 6 for 9 days. The animals of Group 1 served as control for the animals of Group 2; whereas, animals of Group 2 served as control for the animals of Group 3 to Group 6. The treatment regimen followed was as below:

| Groups | Treatment |
|---|---|
| 1 | Untreated control. |
| 2 | 20 ml Liquid Terramycin (without CIDR). |
| 3 | CIDR alone. |
| 4 | CIDR + 20 ml liquid Terramycin. |
| 5 | CIDR + 20 ml liquid Terramycin + intramuscular injection of 1 mg estradiol valerate on the day of CIDR application and on the day of its withdrawal. |
| 6 | CIDR + 20 ml liquid Terramycin followed by intramuscular injection of 500 I.U PMSG on the day of CIDR withdrawal. |

1. EAZI BREED CIDR made in New Zealand, marketed by Pfizer Mumbai contains 1.38gm progesterone in one insert, 10 a insert.
2. Inj. Folligon (1000 IU serum gonadotrophins) Intervet International, Holland
3. Inj. Progynon depot (10 mg/ml oestradiol valerate) German remedies.
4. Liquid Terramycin I/U (each ml contains 50 mg oxytetracycline hydrochloride). Pfizer Ltd Mumbai.

Estrus was detected by parading a buffalo bull followed by observing behavioural symptoms and confirmed by rectal examination of genitalia. The animals showing estrus were bred with a fertile bull. The time taken for onset of estrus following withdrawal of treatment and fertility at induced and subsequent estrus was recorded and analyzed. Confirmation of pregnancy was done between 60 to 70 days after breeding.

## RESULTS AND DISSCUSSION

All the animals of G5 and G6 responded to the treatment for estrus induction (100%) followed by the animals of G Group 4 (85.7%) and Group 3 (71.4%). However, estrus was induced in none of the animals in either of the control groups (Group 1 and 2). The shortest duration of estrus induction was observed in animals of G5 (30.04 $\pm$ 5.11 h) followed by Group 4, Group 6 and Group 3 (40.8 $\pm$ 2.9, 65.1 $\pm$ 11.3 and 72.00 $\pm$ 10.76 h), respectively. The best conception rate was observed in Group 6 (85.7%) followed by Group 4 (66.6%) and Group 5 (57.1%). However, none of the animals of Group 3 conceived. Our finding for 100% estrus induction within the shortest duration in animals of Group 5 (CIDR + Estradiol) was in accordance with the findings of Nikam *et al*. (2002) who also reported 100% estrus induction within 35.66 $\pm$ 3.98 h using Crestar ear implant and administration of estradiol valerate on the day of implantation. Our findings were further supported by the reports of Nayak *et*

*al*. (2009) who also obtained 85.7% estrus induction with 71.42% conception in buffaloes using Crestar implant for 7 days and an intramuscular injection of 2 ml Crestar solution (3 mg norgestomet and 5 mg estradiol valerate) on the day of implantation.

The shortest duration for the onset of estrus with 100% induction in CIDR + Estradiol group might be due to the administration of estradiol valerate on the day of implantation and withdrawl of CIDR. The estradiol 17 is necessary for the pulsatile LH secretion that is prerequisite for maturation and ovulation the follicle and expression of estrus. It also induces premature regression of corpus luteum and enhances response to progestagens (Jainudeen *et al*., 2000).

Our results of fertility response in Group 6 (CIDR + PMSG) are in accordance with the findings of Dabas and Bardhan (2006), who treated anoestrus buffaloes with PMSG, progesterone and hCG. Estrus was induced in. Estrus was induced in all the buffaloes (100%) within 72 to 120 h with 100% conception. Our findings regarding induction of estrus, duration for onset, and conception rate are very close to the finding of Nayak *et al*. (2009) who also reported 100% estrus induction, within 2.75 $\pm$ 0.249 days with 75% conception using Crestar implant for 7 days with 2 ml of Crestar solution intramuscularly on the day of implantation and an intramuscular injection of 500 I.U. PMSG on the day of implant in postpartum anoestrus buffaloes.

The better conception rate (85.7%) in animals of Group 6 in comparison to Group 4 and 5 might be due to the combining effect of implant withdrawl with intramuscular injection of PMSG which stimulate follicular development and ovulation (Murugavel *et al*., 2000). Our results indicate that addition of PMSG (eCG) to a progesterone - based estrus synchronization regimen substantially improves ovulation rate and fertility in non - cyclic buffaloes.

## REFERENCES

Dabas, Y.P.S. and D. Bardhan. 2006. Oestrus and fertility after gonadotrophic treatment in anestrus buffaloes. *Intas Polivet*, **4**(2): 162-165.

Jainudeen, M.R., B. Hafez, and E.S.E. Hafez. 2000.

Table 1. Treatment response for estrus induction and conception.

| Groups | Animals (n) | Induction rate (%) | Onset of estrus interval (h) | Animals bred | Conception rate at induced estrus (%) |
|---|---|---|---|---|---|
| 1 | 7 | - | - | - | - |
| 2 | 7 | - | - | - | - |
| 3 | 7 | 5 (71.4) | 72.00 ± 10.76 | 5 | 0 (0.0) |
| 4 | 7 | 6 (85.7) | 40.08 ± 2.94 | 6 | 4 (66.6) |
| 5 | 7 | 7 (100) | 30.42 ± 5.10 | 7 | 4 (57.1) |
| 6 | 7 | 7 (100) | 65.14 ± 11.39 | 7 | 6 (85.7) |

*Cattle and Buffalo In: Reproduction in Farm Animals*, 7th ed., Lippincott Williams and Wilkins Philadelphia, U.S.A. 167p.

Murugavel, K., D. Antoine, M. Raju, F. López - Gatius. 2009. The effect of addition of equine chorionic gonadotropin to a progesterone-based estrous synchronization protocol in buffaloes (*Bubalus bubalis*) under tropical conditions. *Theriogenology*, **71**(7): 1120-1126.

Nayak, V., R.G. Agrawal, O.P. Shrivastava and M.S. Thakur. 2009. Induction of estrus in true anestrus buffaloes using Crestar implant alone and incombination with PMSG. *Buffalo Bull.*, **28**(2):51-54.

Nikam, P.T., S.K. Sahatpure, C.H. Pawshe, M. Fasihuddin, M.S. Patil and S.G. Deshmukh. 2002. Studies on oestrus synchronization by using Crestar ear implant in Nagpuri buffaloes during summer season, p. 81. *In Proceedings of XVIII Annual Convention of ISSAR and National Symposium; Nov 14-16*, IVRI, Izatnagar.

Tanwar, P.S., N.K. Rakha and J.B. Phogat. 2003. Challenges in buffalo infertility. *Intas Polivet*, **4**(2): 121-127.

---

**Continued from page 183*

Sukhdeo and D.J. Roy. 1971. Investigation on repeat breeding cows and buffaloes- studies on physical properties of cervical mucous. *Indian Vet. J.,* **38**: 479-484.

Tanabe, T.Y. and L.E. Casida. 1949. The nature of reproductive failures in cows of low fertility. *J. Dairy Sci.*, **32**: 237-246.

Tomar, S.S. and V.N. Tripathy. 1986. Incidence and inheritance of repeat breeding in Murrah buffaloes. *Indian J. Dairy Sci*., **39**(4): 363-367.

Vadodria, V.P. and G.A. Prabhu. 1990. Volume and pH of oestrual cervical mucous congenial for conception in Mehsani buffaloes and heifers. *Indian J. Anim. Sci*., **60**(4): 406-410.

Original Article

Buffalo Bulletin (September 2011) Vol.30 No.3

# *IN VITRO* ANTIBIOTIC SENSITIVITY PATTERN OF *Brucella* spp. ISOLATED FROM REPRODUCTIVE DISORDERS OF ANIMALS

**S.N. Ghodasara, A. Roy and B.B. Bhanderi***

## ABSTRACT

Out of 248 samples processed, a total 10 *Brucella* could recover, three from cows, two from a buffaloes, four from goats and one from a bitch by cultural, morphological, biochemical characteristics and PCR methods. Among the 20 antibiotics tested against the 10 *Brucella* isolates, variable sensitivity was observed. All the isolates were 100% sensitive to penicillin-G, streptomycin, gentamicin, choramphenicol, erythromycin, kanamycin, ciprofloxacin, tetracycline, oxytetracycline, doxycycline hydrocloride, amikacin and enrofloxacin. Whereas 80% of the isolates found sensitive to tobramycin, 70% to ampicillin/ salbactum, 60% to rifampicin, 50% to methicillin and 40% of the isolates found sensitive to cefriaxone. While cefuroxime and vancomycin were found only 20% sensitive and ampicillin / cloxacillin was found 100% resistant to *Brucella* isolates. Thus the present finding a could be useful to the clinician and veterinary practitioner to prevent the further progression of disease and further development of complications in infected human patients and animals by selecting appropriate antibiotics.

**Keywords:** *Brucella,* antibiotic sensitivity

## INTRODUCTION

Brucellosis is a worldwide re-emerging zoonoses causing high economic losses and severe human diseases. In the last decade, brucellosis has changed dramatically from being an occupational illness to a food-borne disease. Although ingestion is the major route of spread of infection in human and animals, even the aerosol route plays a role. Reproductive proficiency is one of the core economic considerations in any livestock production enterprise. Loss of a calf, lamb or kid due to abortion and its sequel frequently leads to infertility. It hardly needs to be emphasized that known causes of female infertility are many and involve a wide range of etiologic agents, both specific and nonspecific. Non-specific infectious agents are influenced by some perpetuating causes, whereas specific agents contribute directly to manifestation of infertility (Verma *et al.,* 2000). The appropriate antibiotic therapy for human brucellosis has been studied to some degree. Various drugs like doxycycline, rifampicin, streptomycin, and corticosteroids have been tried alone or in combinations in simple infection and chronic infection cases with high success rates; however, relapses have been reported in certain cases. But there is no prescribed treatment of brucellosis in animals. Information on antibiotic sensitivity of bacterial species is important for

Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry Anand-388 001, Gujarat, India, *E-mail: bbbhanderi@yahoo.co.in

the therapeutic outcome. Thus the present study was envisaged with a view to determine *in vitro* antibiotic sensitivity patterns of *Brucella* spp. from the reproductive disorders of animals in and around Anand city of Gujarat.

## MATERIALS AND METHODS

**Sample collection**

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

**Bacteriological isolation and identification of *Brucella* organism**

Samples were inoculated on *Brucella* agar medium (BAM) (Hi Media Ltd., Mumbai, India) plates in duplicate, one plate was kept at 37°C for incubation aerobically in incubator (without $CO_2$) and the other plate was incubated at 37°C aerobically in an atmosphere of 5% $CO_2$ in a $CO_2$ incubator (Binder, Germany) and observed for growth every 24 h for 15 days. Suspected colonies were identified as *Brucella* spp. by morphologic, cultural and biochemical properties such as oxidase, $H_2S$ production, urease, $CO_2$ requirement and dye inhibition test. Further, the isolates were also identified at the genus level and differentiated at the species level by the PCR method using different sets of primer as reported earlier by Baily *et al*. (1992), Romero *et al*. (1995), Leal-Klevezas *et al.* (1995) and Koichi *et al.* (2007). The *Brucella abortus* biovar 1 strain 544 procured from the Biotechnology Laboratory, National Dairy Development Board, Anand, Gujarat, India, was used as reference strain for cultural and PCR work.

The *Brucella* isolates which were recovered from suspected samples of *Brucella* infection, based on morphological cultural and biochemical character and PCR method were subjected to antibiotic sensitivity tests. Antimicrobial susceptibility testing was performed by the standard disk diffusion method using BAM. The *in vitro* antibiotic sensitivity test of the isolates was conducted as per the method of Bauer *et al*. (1966). Antibiotic discs (Hi Media Ltd., Mumbai, India) used in the present study were penicillin-G (10 units), streptomycin (10 mcg), gentamicin (10 mcg), choramphenicol (30 mcg), erythromycin (15 mcg), kanamycin (30 mcg), ciprofloxacin (30 mcg), tetracycline (30 mcg), oxytetracycline (30 mcg), vancomycin (30 mcg), doxycycline hydrochloride (30 mcg), amikacin (10 mcg), enrofloxacin (10 mcg), tobramycin (30 mcg), ampicillin/salbactum (10 mcg), rifampicin (5 mcg), methicillin (5 mcg), cefriaxone (30 mcg), cefurixime (30 mcg) and ampicillin/cloxacillin (30 mcg).

## RESULTS

**Results of cultural, biochemical and PCR methods for identification**

According to the results of morphological, cultural, biochemical characters and PCR testing 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). Further, the isolates from cows and buffaloes and *Brucella abortus* biovar 1 strain 544 were confirmed as *B. abortus,* isolates

from goats were confirmed as *B. melitensis,* and the isolate from the bitch was confirmed as *B. canis* using the PCR method at the species level.

All the 10 *Brucella* isolates were tested for *in vitro* antibiotic sensitivity to 20 antibacterial drugs, and the results of individual isolate to various drugs were interpreted according to the manufacturer's instructions (Hi Media Ltd., Mumbai, India). The results are presented in Tables 1 and 2.

**_In vitro_ antibiogram pattern of _Brucella_ isolates**

In the present study, *Brucella* isolates were found variably sensitive to the antibiotics tested. Overall, 100% of the isolates were sensitive to penicillin-G, streptomycin, gentamicin, choramphenicol, erythromycin, kanamycin, ciprofloxacin, tetracycline, oxytetracycline, doxycycline hydrocloride, amikacin and enrofloxacin. Whereas 80% of the isolates were found sensitive to tobramycin,

Table 1. *In-vitro* antibiotic sensitivity results of the *Brucella* isolates.

| **Isolate number, percentage/ Antibiotic** | ***B. abortus*** | | | **%** | ***B. melitensis*** | | | | **%** | ***B. canis*** | **%** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **C1, C2, and C3** | **B1** | **B2** | | **G1** | **G2** | **G3** | **G4** | | **D1** | |
| Penicillin-G | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Vancomycin | R | S | S | 40 | R | R | R | R | 0 | R | 0 |
| Gentamicin | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Kanamycin | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Methicillin | S | S | S | 100 | R | R | R | R | 0 | R | 0 |
| Choramphenicol | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Erythromycin | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Streptomycin | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Tetracycline | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Oxytetracycline | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Ampicilline/ Cloxacillin | R | R | R | 0 | R | R | R | R | 0 | R | 0 |
| Ciprofloxacin | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Enerofloxacin | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Amikacin | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Tobramycin | S | S | S | 100 | S | S | S | R | 75 | R | 0 |
| Doxycycline hydrocloride | S | S | S | 100 | S | S | S | S | 100 | S | 100 |
| Rifampicin | S | R | R | 60 | S | S | R | R | 50 | S | 100 |
| Ceftriaxone | S | S | R | 80 | R | R | R | R | 0 | R | 0 |
| Cefuroxime | R | R | R | 0 | R | R | R | S | 25 | S | 100 |
| Ampicillin/ salbactum | S | S | S | 100 | S | S | R | R | 50 | R | 0 |

S = Sensitive, R = Resistant

70% to ampicillin/salbactum, 60% to rifampicin, 50% to methicillin and 40% isolates were found sensitive to cefriaxone. While cefuroxime and vancomycin were found only 20% sensitive, and ampicillin/cloxacillin was found 100% resistant to *Brucella* isolates (Table 2).

**Species-wise antibiotic sensitivity of *Brucella* isolates to various antibiotics**

**a) Antibiotic sensitivity pattern *Brucella abortus* isolates**

All the isolates of *Brucella abortus* from cows (C1, C2, C3) and buffaloes (B1, B2) were found to 100% sensitive to penicillin-G, streptomycin, gentamicin, choramphenicol, erythromycin, kanamycin, ciprofloxacin, tetracycline, oxytetracycline, doxycycline hydrocloride, amikacin, enrofloxacin, methicillin, ampicillin/salbactum, tobramycin. While, 80% of the isolates were found sensitive to cefriaxone, 60% to rifampicin and 40% to vancomycin. Whereas all the *B. abortus* isolates were found resistant to ampicilline/cloxacillin and cefurixime (Table 1).

**b) Antibiotic sensitivity pattern of *B. melitensis* isolates**

All the isolates of *B. melitensis* from goats (G1, G2, G3, G4) were found to be 100% sensitive to penicillin-G, streptomycin, gentamicin, choramphenicol, erythromycin, kanamycin, cipro-floxacin, tetracycline, oxytetracycline, doxycycline

Table 2. Percent antibiotic sensitivity of *Brucella* isolates to antimicrobial agents.

| Sr. No. | Antimicrobial agent | Isolates (n = 10) | | | |
|---|---|---|---|---|---|
| | | Sensitive % | | Resistant % | |
| | | No. | % | No. | % |
| 1 | Penicillin-G | 10 | 100 | 0 | 0 |
| 2 | Vancomycin | 2 | 20 | 8 | 80 |
| 3 | Gentamicin | 10 | 100 | 0 | 0 |
| 4 | Kanamycin | 10 | 100 | 0 | 0 |
| 5 | Methicillin | 5 | 50 | 5 | 50 |
| 6 | Choramphenicol | 10 | 100 | 0 | 0 |
| 7 | Erythromycin | 10 | 100 | 0 | 0 |
| 8 | Streptomycin | 10 | 100 | 0 | 0 |
| 9 | Tetracycline | 10 | 100 | 0 | 0 |
| 10 | Oxytetracycline | 10 | 100 | 0 | 0 |
| 11 | Ampicilline/Cloxacillin | 0 | 0 | 10 | 100 |
| 12 | Ciprofloxacin | 10 | 100 | 0 | 0 |
| 13 | Enerofloxacin | 10 | 100 | 0 | 0 |
| 14 | Amikacin | 10 | 100 | 0 | 0 |
| 15 | Tobramycin | 8 | 80 | 2 | 20 |
| 16 | Doxycycline hydrocloride | 10 | 100 | 0 | 0 |
| 17 | Rifampicin | 6 | 60 | 4 | 40 |
| 18 | Ceftriaxone | 4 | 40 | 6 | 60 |
| 19 | Cefuroxime | 2 | 20 | 8 | 80 |
| 20 | Ampicillin/salbactum | 7 | 70 | 3 | 30 |

hydrocloride, amikacin and enrofloxacin. While 75% of the isolates were found sensitive to tobramycin, 50% to ampicillin/salbactum and rifampicin and 25% to cefurixime. Whereas 100% resistance was recorded for ampicillin/cloxacillin, vancomycin, methicillin and cefriaxone (Table 1).

**c) Antibiotic sensitivity pattern of the *B. canis* isolate**

The single *B. canis* isolate from a bitch (D1) was 100% sensitive to penicillin-G, streptomycin, gentamicin, choramphenicol, erythromycin, kanamycin, ciprofloxacin, tetracycline, oxytetracycline, doxycycline hydrocloride, amikacin, enrofloxacin, rifampicin and cefurixime. Whereas 100% resistance was observed in vancomycin, methicillin, cefriaxone, ampicillin/cloxacillin, tobramycin and ampicillin/salbactum (Table 1).

## DISCUSSIONS

With the great expansion of livestock industry, *Brucella* spp. has emerged as a problem of economic concern to all phases of the industry from production to marketing to consumer health significant, to clinicians, veterinarians and to the in contact persons due to emergence of multiple drug resistance and due to the fact that intracellular survival of the organism limits the effect of antibiotics.

In the present study, *Brucella* isolates were found variably sensitivity to the tested antibiotics. Higher percentages of sensitivity was observed to penicillin-G, streptomycin, gentamicin, choramphenicol, erythromycin, kanamycin, ciprofloxacin, tetracycline, oxytetracycline, doxycycline hydrocloride, amikacin and enrofloxacin. Similar results were obtained by Hall *et al.* (1970), who reported tetracycline was the most effective amongst the tested antibiotics. Jensen *et al.* (1996) showed susceptibility to tetracycline, amikacin, doxycycline hydrochloride, gentamicin, kanamycin, penicillin, streptomycin and tobramycin. Chahota *et al.* (2003) revealed one hundred percent sensitivity to streptomycin, chlortetracycline, ciprofloxacine, tetracycline and gentamicin. Nagal *et al.* (1994) reported that *B. melitensis* biotype III was sensitive to tetracycline and gentamicin but obtained contradictory result to the present study, revealing resistance to penicillin G and streptomycin. Turkmani *et al.* (2006) reported that all the isolates were susceptible to tetracycline, streptomycin, gentamicin, ciprofloxacin. Marianelli *et al.* (2007) reported higher sensitivity to doxycycline, ciprofloxacin. Bodur *et al.* (2003) reported the most sensitive drug against *Brucella* was doxycycline.

Whereas, in contrast to the present study, Khan *et al.* (1989) found lower sensitivity to streptomycin, tetracycline and rifampicin. Verma *et al.* (2000) recorded 85.71% sensitivity to gentamicin, tetracycline and streptomycin, while 71.43% isolates were sensitive to chloramphenicol and amikacin.

Similar to present study, for *B. canis* isolates, some similar results in the case of treated dogs were obtained by Wanke *et al.* (2006), who studied the effect of treatment enrofloxacin orally on *Brucella* positive dogs. They successfully eliminated infection with a 30-day treatment. Nicolatti *et al.* (1987) eliminated *B. canis* infection in foxhounds, with 500 mg tetracycline orally for 3 times daily for 30 days plus 34 mg/kg streptomycin intramuscularly on day 1-7 and 24-31 of the treatment period. Fountain *et al.* (1985) successfully cured infection with aminoglycosides like streptomycin, gentamicin and kanamycin for

the treatment of *Brucella* spp. (*B. canis* and *B. abortus*) infection in mice and guinea pig.

In the present study, methicillin, cefriaxone, ampicillin/salbactum, tobramycin and rifampicin were observed to be moderatly effective. Similar results were obtained by Bodur *et al.* (2003), who reported cefriaxone and rifampicin is moderately effective. Baykam *et al.* (2004) reported that rifampicin is more effective against *B. abortus* than *B. melitensis.* In contrast to present study, Jensen *et al.* (1996) reported rifampicin resistance in *Brucella abortus* isolates.

According to the present findings, penicillin-G, streptomycin, gentamicin, choramphenicol, erythromycin, kanamycin, ciprofloxacin, tetracycline, oxytetracycline, doxycycline hydrocloride, amikacin and enrofloxacin are the most effective antibiotics. Therefore, they could be useful to the clinician and veterinary practicener to prevent the further progress of disease and further development of complications in infected human patients and animals by selecting appropriate antibiotics. But, it is also essential to remember that from the public health point of view, prolonged treatment of infected domestic animals with a high dosage of antibiotics can not be undertaken due to the appearance of antibiotics in the human food chain, which interferes with the production of milk products. Moreover, as *Brucella* is facultative intracellular bacteria, relapses after treatment usually occur. Therefore, efforts should be directed at prevention or eradication of brucellosis.

## ACKNOWLEDGEMENT

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

## 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. *J. Trop. Med. Hyg.*, **95**: 271-275.

Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Turk. 1996. Antibiotic susceptibility testing by a standard disc method. *Am. J. Clin. Pathol.,* **45**: 493-496.

Baykam, N., H. Esener, O. Ergonul, E. Eren, A.K. Celikbas and B. Dokuzoguz. 2004. *In vitro* antimicrobial susceptibility of *Brucella* species. *Int. J. Antimicrob. Ag.,* **4**: 405-407.

Bodur, H., N. Balaban, S. Aksaray, V. Yetener, E. Akinci, A. Colpan and A. Erbay. 2003. Biotypes and antimicrobial susceptibilities of *Brucella* isolates. *Scand. J. Infect. Dis.,* **35**: 337-338.

Chahota, R., M. Sharmal, R.C. Katochl, S. Verma, M.M. Singh, V. Kapoor and R.K. Asrani, 2003. Brucellosis outbreak in an organized dairy farm involving cows and in contact human beings in Himachal Pradesh. *Vet. Arhiv*, **73**: 95-102.

Fountain, M.W., S.J. Weiss, A.G. Fountain, A. Shen and R.P. Lenk. 1985. Treatment of *Brucella canis* and *Brucella abortus in vitro* and *in vivo* by stable plurilamellar vesicle-encapsulated aminoglycosides. *J. Infect. Dis.,* **152**: 529-535.

Hall, W.H. and R.E. Manion. 1970. *In vitro* susceptibility of *Brucella* to various antibiotics. *Appl. Microbiol.,* **20**: 600-604.

Jensen, A.E., D.R. Ewalt, N.F. Cheville, C.O. Thoen and J.B. Payeur. 1996. Determination of stability of *Brucella abortus* RB51 by use of genomic fingerprint, oxidative metabolism, and colonial morphology and differentiation of strain RB51 from *B. abortus* isolates from Bison and Elk. *J. Clin. Microbiol.,* **34**: 628-633.

Khan, M.Y., M. Dizon and F.W. Kiel. 1989. Comparative *in vitro* activities of ofloxacin, difloxacin, ciprofloxacin, and other selected antimicrobial agents against *Brucella melitensis*. *Antimicrob. Agents Ch.,* **33**: 1409-1410.

Koichi, I., K. Masanobu, S. Michio, K. Tsuneo and Y. Akio. 2007. Simultaneous detection of the genus *Brucella* by combinatorial PCR. *Jpn. J. Infect. Dis.,* **60**(2-3): 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.

Marianelli, C., C. Graziani, C. Santangelo, M.T. Xibilia, A. Imbriani, R. Amato, D. Neri, M. Cuccia, S. Rinnone, V. Marco and F.S. Ciuchini. 2007. Molecular epidemiological and antibiotic susceptibility characterization of *Brucella* isolates from humans in Sicily, Italy. *J. Clin. Microbiol.,* **45**(9): 2923-2928.

Nagal, K.B., R.C. Katoch, M. Sharma, D.S. Sambyal and N. Kumar, 1994. *Brucella melitensis* abortions in a dairy herd. *Indian J. Anim. Sci.,* **64**: 132-134.

Nicoletti, P. and A. Chase. 1987. The use of antibiotics to control canine brucellosis. *Compadium on continuing education for the practicing veterinarian*, **9**(11): 1063-1066.

Romero, C., C. Gamazo, M. Pardo and I. Lopez-Goni. 1995. Specific detection of *Brucella* DNA by PCR. *J. Clin. Microbiol.,* **33**: 615-617.

Turkmani, A., A. Ioannidis, A.Christidou, A. Psaroulaki, F. Loukaides and Y. Tselentis, 2006. *In vitro* susceptibilities of *Brucella melitensis* isolates to eleven antibiotics. *Ann. Clin. Microbiol. Antimicrob.,* **5**: 24-28.

Verma, S., R.C. Katoch, M. Sharma and P. Nigam. 2000. Abortions and infertility in domestic livestock due to brucellosis in Himachal Pradesh, India. *Vet. Arhiv,* **70**: 75-82.

Wanke, M.M., M.V. Delpino and P.C. Baldi, 2006. Use of enrofloxacin in the treatment of canine brucellosis in a dog kennel (clinical trial). *Theriogenology,* **66**: 1573-1578.

# COMPARATIVE STUDY AND STORAGE STABILITY OF SERUM HEPATOBILIARY ENZYME ACTIVITIES IN MURRAH BUFFALOES

**P.D. Divya[1] and K.K. Jayavardhanan[2]**

## ABSTRACT

The present study was designed and conducted to find the appropriate physical baseline values for hepatobiliary enzymes such as, alanine aminotransferases (ALT), aspartate aminotransferases (AST), alkaline phosphatase (ALP) and gamma glutamyltransferases (GGT) in adult healthy Murrah buffaloes in hot humid climatic conditions and also to assess the ideal storage condition for buffalo sera samples meant for the assay of hepatobiliary enzymes by storing at room temperature (22 to 27ºC), 4ºC and -20ºC up to 14 days. The normal mean serum activities of ALT, AST, ALP and GGT were 50.0 ± 3.53, 130.0 ± 7.29, 323.6 ± 32.09 and 10.11 ± 1.28 IU/L, respectively. ALT and GGT were found to be sufficiently stable up to the study period of 14 days at both 4ºC and -20ºC but unstable at room temperature. AST was found to be stable for 11 days at 4ºC and 8 days at room temperature and was stable only 2 days at -20ºC. Alkaline phosphatase showed great variation upon storage as compared to the other hepatobiliary enzymes and it is suggested that its estimation should be performed in fresh serum samples to get a more accurate result. Thus, the present study reveals specific reference values for each serum hepatobiliary enzyme in Murrah buffaloes of the hot humid tropics. From these results it is also advisable to consider stability of each serum hepatobiliary enzymes for different animals separately before preserving sera samples to get more valid and reliable results.

**Keywords:** Murrah buffaloes, hepatobiliary enzymes, physical baseline values, days of storage, storage temperature

## INTRODUCTION

The measurement of serum enzymes is an important tool for disease diagnosis in veterinary and human clinical. The routinely used enzymes to evaluate hepatic damage in animals includes ALT, AST, ALP, GGT, sorbitol dehydrogenase (SDH), lactate dehydrogenase (LDH), ornithine carbamoyl transferase (OCT) and 5' nucleotidase (NTP) (Kaneko *et al*., 2008). The enzymes routinely used in human beings for disease diagnosis may not give true indications of hepatic injury in veterinary practice. There is also a lack of standard reference values for some species. Each animal species have its own specific hepatobiliary enzyme levels which vary from one species to another (Kaneko *et al*., 2008). The available data on hepatobiliary enzyme

[1]Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Pookot, Wayanad, Kerala, India, 673576, E-mail: divsjyothy@gmail.com

[2]Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India, 680651

levels from the literature shows widely divergent values among different species, and these data are mainly procured from the animals reared in a temperate climate. Even though considerable information is available on normal serum hepatobiliary enzyme levels of domestic animals of exotic breeds kept under different environment and management conditions, use of these serum enzyme levels for monitoring health status of indigenous breeds may mislead the diagnosis. So for more accurate clinical interpretation of hepatic diseases, it is a prerequisite to establish the reference values of these enzymes.

When large numbers of blood samples are collected or when many different analysis are required it is inevitable that the samples be stored. Different treatment of the blood before analysis like conditions of preservation, centrifugation, haemolysis and bacterial growth could account for the variations in the results, as could varying specificities of individual methods of analysis. At present, as there are conflicting data regarding the effect of different temperatures and durations of storage on the stability of the activities of hepatobiliary enzymes which are routinely analysed for clinical diagnostic use, it is of primary importance to reexamine the storage stability of these enzymes. Besides, data of this kind gained under hot humid tropical conditions are very meagre. Therefore, the present study aims to find out the effects of storage time and temperature on the measured activities of the hepatobiliary enzymes like ALT, AST, ALP and GGT in the sera samples of buffaloes under various storage conditions *viz*, at room temperature (22 to 27ºC), 4ºC and -20ºC for a period of two weeks.

## MATERIALS AND METHODS

Ten female Murrah buffaloes between 2 to 3 years of age maintained at the University Buffalo Farm, College of Veterinary and Animal Sciences, Kerala Agricultural University, Mannuthy, Thrissur were selected randomly for the study. Blood samples were collected by jugular venipuncture using sterile needles (18 gauge) directly into clean dry sterile glass tubes without anticoagulants. Serum was harvested after 30 to 45 minutes following clot formation and by centrifugation for 10 minutes at 2000 g.

The clear serum was immediately assayed for the following hepatobiliary enzymes ALT, AST, ALP and GGT within an hour of serum separation to serve as basal fresh values (day 0). The remaining sera were dispensed into 18 sample tubes, closed tightly and divided into three groups. One of each group was stored upright at room temperature (approximately 25ºC), 4ºC and -20ºC. The stored serum aliquots from all temperatures and time points were analysed together in one batch for hepatobiliary enzymes on 1, 2, 5, 8, 11 and 14 days post collection. The enzyme assay was performed using Ecoline - Merck diagnostic kits (Merck Specialities Pvt. Ltd, Mumbai) on an automated blood analyzer (Microlab 200). The stability of an enzyme activity under each temperature condition and time was determined by calculating the percentage change in concentrations from the mean fresh value (day 0) at each time-point for each animal.

The experimental results obtained were analysed statistically by using analysis of variance (ANOVA) technique followed by the Duncan Multiple Range Test and paired t-test as described by Snedecor and Cochran (1994) using computer software programme, SPSS .

## RESULTS AND DISCUSSION

The results showed that the mean serum activities of ALT, AST, ALP and GGT were 50.0 ± 3.53, 130.0 ± 7.29, 323.6 ± 32.09 and 10.11 ± 1.28 IU/L, respectively (Table 1). The mean value of ALT activity observed for female Murrah buffaloes in the present study was found to be 50.00 ± 3.53 IU/L with a reference range of 42.02 to 57.98 IU/L. The results support the findings of Terzano *et al.* (2005) and Grasso *et al.* (2004) who reported mean ALT values of 60 IU/L in buffalo heifers and with a range of 55.35 to 58.49 IU/L in adult female buffaloes kept at intensive and traditional system of management. However, a higher ALT activity of 176 to 219 IU/L and 83 to 116 IU/L was observed for buffaloes at different pre-post partum time intervals and early lactation, respectively (Terzano *et al.,* 2005). A significantly lower ALT level was reported by Mudgal *et al.* (2008) who found a mean ALT level of 37.15 IU/L for 8-to-9-month-old buffalo calves. Marked differences were also observed in mean serum ALT activity for adult buffaloes (Pal and Dasgupta, 2006) who reported 28.50 ± 1.32 IU/L, which was significantly lower than the present findings.

The 113.51 to 146.49 IU/L AST reference range observed is in close agreement with the reports of Randhawa *et al.* (1997) and Grasso *et al.* (2004), who reported a mean AST value of 134.6 ± 4.36 IU/L for adult healthy buffaloes and 146.84 IU/L for buffalo cows maintained under a intensive system of management, respectively. In contrast, a slightly increased AST value (164.68 IU/L) was observed for those under a traditional system of management (Grasso *et al.,* 2004). The present findings were also comparable with the observations of Terzano *et al.* (2005) who reported 101.2 IU/L of mean AST activity for adult buffaloes even though it was towards the lower margins of the present reference range. Contrary to the results of the present study, significantly lower AST values were reported by Pal and Dasgupta (2006) and Mudgal *et al.* (2008) who reported 54.00 ± 1.22 IU/L for adult healthy buffaloes and 62.47 IU/L for male buffalo calves, respectively.

The reference range of 251.00 to 396.19 IU/L ALP activity obtained in the present study is in close agreement with the studies of Grasso *et al.* (2004) who reported 370.11 IU/L of ALP activity in buffaloes maintained under an intensive system of management, whereas a higher ALP values was observed for those maintained under a traditional system (443.12 IU/L). A similar study was conducted by Terzano *et al.* (2005) on adult healthy buffaloes and the present findings were within reference range of 200 to 650 IU/L established by them. But Randhawa *et al.* (1997) presented comparatively lower ALP values (113.9 ± 4.25 IU/L) for buffaloes. ALP activity of 76.34 IU/L reported by Bharti *et al.* (2008) for male Murrah buffalo calves of 6 to 8 months of age was significantly lower than the present findings.

The mean GGT concentration of 10.11 ± 1.28 IU/L obtained for adult healthy buffaloes is within the range of 4.9 to 25.7 IU/L reported by Hilali *et al.* (2008). The findings of the present study are also comparable to the reports of Randhawa *et al.* (1997) who presented a GGT activity of 16.8 ± 0.82 IU/L for adult healthy buffaloes. However, the results of the present study were significantly lower than the reports of Terzano *et al*. (2005) and Grasso *et al.* (2004) who reported GGT levels of 21.2 IU/L and 26.95 to 27.43 IU/L, respectively.

The stability of ALT activity at room temperature was much less as compared to 4$^{o}$C and -20$^{o}$C (Table 2). The enzyme was highly unstable at room temperature and showed a significant

Table 1. Serum ALT, AST, ALP and GGT activities (IU/L) in buffalo (n=10).

| **Enzyme** | **Min.** | **Max.** | **Mean± SE** | **95 % confidence interval** |
|---|---|---|---|---|
| ALT | 30 | 64 | 50.00± 3.53 | 42.02 - 57.98 |
| AST | 105 | 172 | 130.00± 7.29 | 113.51 -146.49 |
| ALP | 175 | 479 | 323.60± 32.09 | 251.00 - 396.19 |
| GGT | 4 | 15 | 10.11± 1.28 | 7.15 - 13.07 |

Table 2. Activity of ALT and AST in buffalo sera samples preserved at 25ºC (room temperature), 4ºC and -20ºC for 14 days.

| **Days of storage** | **ALT** | | | **AST** | | |
|---|---|---|---|---|---|---|
| | **25ºC** | **4ºC** | **-20ºC** | **25ºC** | **4ºC** | **-20ºC** |
| 0 (Base line value) | 51.80±3.43<br>- | 51.80±3.43<br>- | 51.8±3.43<br>- | 129.80±8.70<br>- | 129.80±8.70<br>- | 129.80±8.70<br>- |
| 1 | 44.50±2.19*<br>(-14.09) | 47.30±2.39<br>(-8.69) | 51.30±3.12<br>(-0.97) | 132.80±7.50<br>(+2.31) | 127.00±7.60<br>(-2.16) | 128.30±7.49<br>(-1.16) |
| 2 | 44.30±2.46*<br>(-14.47) | 47.40±3.10<br>(-8.49) | 50.90±2.29<br>(-1.74) | 130.80±7.90<br>(+0.77) | 125.80±7.05<br>(-3.08) | 121.9±10.09<br>(-6.09) |
| 5 | 37.90±2.16*<br>(-26.83) | 47.40±2.61<br>(-8.49) | 46.30±1.74<br>(-10.62) | 125.60±8.30<br>(-3.23) | 125.30±7.62<br>(-3.47) | 118.40±10.69*<br>(-8.78) |
| 8 | 27.10±1.52*<br>(-47.68) | 47.60±1.91<br>(-8.11) | 46.60±2.03<br>(-10.04) | 114.30±16.40<br>(-11.94) | 127.60±9.40<br>(-1.69) | 116.30±7.9*<br>(-10.40) |
| 11 | 20.50±2.09*<br>(-60.42) | 46.20±3.22<br>(-10.80) | 45.80±3.80<br>(-11.58 | 77.10±12.90*<br>(-40.60) | 123.60±7.80<br>(-4.78) | 112.80±6.9*<br>(-13.09) |
| 14 | 14.8±1.85*<br>(-71.42) | 45.10±3.79<br>(-12.93) | 51.10±3.72<br>(-1.35) | 68.0±10.9*<br>(-47.61 | 121.4±7.8*<br>(-6.47) | 109.1±6.9*<br>(-15.95) |

Percentage change from initial activity in parenthesis, * P 0.05

decrease in activity from the very next day of blood collection. At the end of the experimental period, less than 30% of initial activity was retained in the serum samples whereas the storage of serum at 4ºC for two weeks did not result in any significant change in enzyme activity. The storage of serum at -20ºC was also considered to be suitable for ALT assay in buffalo. The activity remained unaffected up to the study period of two weeks. The observations for serum ALT stability were consistent with the study of Boyanton and Blick (2000) in human plasma. They observed a 20% decrease in ALT activity at 48 h and 56 h at room temperature and the reason given increased lactate concentration as a result of bacterial contamination. This study recommends either 4ºC or -20ºC for preservation of buffalo sera samples for ALT assay.

Sera samples stored at room temperature maintained the initial AST activity up to 8 days without any significant loss, but thereafter the

Table 3. Activity of ALP and GGT in buffalo sera samples preserved at 25ºC (room temperature), 4ºC and -20ºC for 14 days.

| **Days of storage** | **ALP** | | | **GGT** | | |
|---|---|---|---|---|---|---|
| | **25ºC** | **4ºC** | **-20ºC** | **25ºC** | **4ºC** | **-20ºC** |
| 0 (Base line value) | 310.20±36.6 - | 310.20±36.6 - | 310.2±36.6 - | 11.30±1.02 - | 11.30±1.02 - | 11.30±1.02 - |
| 1 | 254.5±44.10 (-17.96) | 261.3±45.17* (-15.76) | 274.6±44.7* (-11.48) | 13.60±0.95* (+20.35) | 12.80±0.58 (+11.27) | 12.70±0.57 +12.39 |
| 2 | 229.8±38.31* (-25.91) | 279.1±42.38* (-10.03) | 275.0±44.62* (-11.35) | 15.80±0.87* (+39.82) | 13.80±0.43 (+22.12) | 12.20±0.039 +7.96 |
| 5 | 165.4±38.59* (-46.68) | 274.5±43.75* (-11.51) | 311.5±34.19 (+0.42) | 17.60±0.80* (+55.75) | 12.60±0.56 (+11.50) | 12.50±0.41 +10.62 |
| 8 | 110.5±24.59* (-64.38) | 272.3±43.32* (-12.21) | 319.6±32.3 (+3.03) | 20.10±2.48* (+77.88) | 13.50±0.38 (+19.47) | 13.20±0.40 +16.81 |
| 11 | 77.6±14.99* (-74.98) | 270.9±41.95* (-12.67) | 284.1±41.22 (-8.41) | 18.00±0.52* (+59.29) | 13.80±0.58 (+22.12) | 13.80±0.58 +22.12 |
| 14 | 71.25±11.8* (-77.03) | 271.8±43.3* (-12.38) | 279.8±42.93 (-9.80) | 17.70±1.02* (+56.64) | 13.00±0.56 (+15.04) | 11.00±0.63 -2.65 |

Percentage change from initial activity in parenthesis, * P   0.05

values decreased to a point of statistical significance on the $11^{th}$ and $14^{th}$ day of storage, more than 40% decrease in activity was noticed during this period (Table 2). Only negligible changes were found in AST activity when the serum was stored at $4^{o}$C up to the $11^{th}$ day and these changes were not statistically significant. After 11 days, a clinically acceptable significant decrease in AST values (P 0.05) was seen. Results obtained for AST stability at -$20^{o}$C revealed a negligible variation on enzyme activity up to 2 days. Beyond this, a statistically significant (P 0.05) decline in activity was observed up to $14^{th}$ day. The AST activity under various storage conditions suggested for human serum was 3 days at room temperature, one week at 4ºC and one month -25ºC (Kaplan and Pesce, 1989). Due to significant decrease in AST activity at -20ºC, the present study suggests 4ºC as the better storage condition for buffalo sera samples.

The ALP activity in the sera samples stored at room temperature did not show any statistically significant change up to the first day, followed thereafter by a significant decline to below baseline values and only less than 23% of initial activity retained at the end of the experimental period. Results are presented in Table 3. At $4^{o}$C, ALP activities declined markedly beginning within 24 h of venipuncture, and the changes were statistically significant; the enzyme was totally unstable at this temperature. However, the percentage change in activity was comparatively less than that at room temperature. The specimens kept in the frozen state showed great fluctuations in ALP activity over the entire period. Even after 24 h of storage, a significant decline (P 0.05) in ALP activity was observed. These results were contradictory to the reports of Kaplan and Pesce (1989) in human sera samples where ALP activity increased with increase in temperature. The present results suggest the instability of buffalo ALP enzyme during preservation of sera samples 4ºC and -20ºC and the assay should be performed on the day of blood collection itself.

Time of storage had significant effect on GGT activity in the sera samples kept at room temperature (Table 3). The activities increased significantly over the time of storage with more pronounced degree of change on the $8^{th}$ day; an increase in activity of about 78% was observed. In the refrigerated and frozen states, the enzyme showed no appreciable change over a period of two weeks and the percentage change in mean activity was less than 23% in both the conditions. Between these two conditions, the storage of serum at -$20^{o}$C was considered to be more suitable for GGT assay of buffalo serum. The results were in accordance with the study of Donnley *et al.* (1995) on human serum; they stated that GGT was highly stable at 4ºC (14 days) and -20ºC (4 months) and reported a stability of 48 h at room temperature, while that in the present study was 24 h. The increase in serum GGT activity at room temperature may be due to bacterial contamination. A similar finding was reported by Lazaroni *et al.* (1958) who stated that, bacterial contamination can cause either an increase or decrease in the enzyme activity in human serum maintained at room temperature. The present study suggests -20ºC as the most suitable storage condition for GGT assay in buffalo sera samples.

## REFERENCES

Bharti, V.K., M. Gupta and D. Lall. 2008. Ameliorative effects of boron on serum profile in buffalo (*Bubalus bubalis*) fed high fluoride ration. *Trop. Anim. Health. Pro.,* **40**: 111-116.

Boyanton, B.L. and K.E. Blick. 2002. Stability studies of twenty-four analytes in human plasma and serum. *Clin. Chem.,* **48**: 2242-2247.

Donnelly, J.G., S.J. Soldin, D.A. Nealon and J.M. Hicks 1995. Stability of twenty five analytes in human serum at 22ºC, 4ºC and -20ºC. *Pediatr. Pathol. Lab. M.,* **15**: 869-874.

Grasso, F., G.M. Terzano, G.D. Rosa, C. Tripaldi and F. Napolitano. 2004. Influence of housing conditions and calving distance on blood metabolites in water buffalo cows. *Ital. J. Anim. Sci.*, **3**: 275-282.

Hilali, M., A. Abdel-Gawad, A. Nassar and A. Abdel-Wahab. 2006. Hematological and biochemical changes in water buffalo calves (*Bubalus bubalis*) infected with *Trypanosoma evansi*. *Vet. Parasitol.,* **139**: 237-243.

Kaneko, J.J., J.W. Harvey and M.L. Bruss. 2008. *Clinical Biochemistry of Domestic Animals,* 5th ed. Academic Press, California, USA. 932p.

Kaplan, L.A. and A.J. Pesce. 1989. *Clinical Chemistry, Theory, Analysis and Correlation,* 2nd ed. The C.V. Mosby Company, Baltimore, USA. 1149p.

Lazaroni, J.A., E.C. Maier and L.R. Gorczyca. 1958. The stability of lactic dehydrogenase in serum. *Clin. Chem.*, **4**: 379-381.

Mudgal, V., A.K. Garg, R.M. Dass and V.P. Varshney. 2008. Biological effect of selenium and copper supplementation on blood metabolic profile in male buffalo (*Bubalus bubalis*) calves. *Trace Elem. Res.*, **121**: 31-38.

Pal, S. and C.K. Dasgupta. 2006. Haemato-biochemical profiles of buffalo in anthelmintics treatment against *Fasciola gigantica* infection. *Buffalo Bull.,* **25**: 25-29.

Randhawa, C.S., R.S. Brar, D.R. Sharma and S.S. Randhawa. 1997. Biochemical responses in mixed chronic psoroptic and sarcoptic mange of buffaloes *(Bubalus bubalis). Trop. Anim. Health Pro.,* **4**: 253-254.

Snedecor, G.W. and W.G. Cochran. 1994. *Statistical Medhods,* 8th ed. Oxford and IBH Publishing Company, Calcutta. 564p.

Terzano, G.P., S. Allegrini, A. Borghese, C. Roncoroni and L. Alfieri. 2005. Metabolic and hormonal parameters in buffaloes, p. 219-248. *In* Borghese, A. (ed). *Buffalo Production and Research Paper No: 67.* Food and Agriculture Organization of the United Nations, Rome.

# EFFECTS OF NON-GENETIC FACTORS IN MILK PRODUCTION AND COMPOSITION IN EAST AZARBAIJAN NATIVE BUFFALOES OF IRAN

**Zhila Toopchi Khosroshahi[1], Seyed Abbas Rafat[2] and Djalil Shoja[2]**

## ABSTRACT

The buffalo is one of the most important genetic resources for milk production in Iran. The advantages of buffalo breeding are: ability to subsist on a low quality and high roughage diet; converting low quality roughages to high quality protein; high adaptability; and use of buffalo skin in leather industry. The aim of this study is to describe effects of non-genetic factors on buffalo milk of 672 animals in East Azerbaijan province. East Azerbaijan can be divided to three regions of cold, hot and temperate based on the mean temperature. From the cold, hot and temperate regions, 26, 17, 30 villages were randomly selected, respectively. From every village, one to three farmers were selected. Data of milk record, parity, season and year of recording were registered. Fat, protein, lactose, total solids, and solid non fat percentages were measured in the laboratory. Data were analysed by a nested design. Results showed that effects of city, village and farmer were not significant. The mean of daily milk production was 5.48± 2.31 kg. Effect of year on milk production was significant ($P<0.05$). Least square means of milk production at 1997, 1998, 1999, 2000 and 2001 were 5.71, 5.22, 5.02, 4.87 and 4.28 kg, respectively.

**Keywords**: *Bubalus baublis,* buffalo, milk production, milk composition, Iran

## INTRODUCTION

The buffalo has an essential role in rural household economy in developing countries, especially in Asia. Dairy products of buffalo have economic importance in India, Nepal and Pakistan, so farmers keep this animal and subsist on buffalo milk. A high capacity to face adverse environmental conditions and a remarkable longevity of the buffalo has also been appreciated in Italy. The buffalo is the major dairy animal in Pakistan, contributing approximately 67% of the total milk produced in the country (1). It has also important role in the agricultural economy of Azerbaijan province in Iran. Buffalo milk and dairy products have high quality so they have been sold in higher prices in comparison with other dairy products in this region. However, the buffalo has been noticed less than other domestic animals in this country. Food and Agriculture Organization data shows that the buffalo milk production increased between 1982 and 2001 by 58.2% in the world and by 57.9% in Asia. The increase in buffalo milk production in India,

[1]East Azerbaijan Research Center for Agriculture and Natural Resources, Tabriz, Iran, E-mail: Zhila_toopchi@yahoo.co.uk
[2]Animal Science Department, University of Tabriz

Pakistan, China and Italy for the same period was 59.0, 37.0, 63.5, 154.8%, respectively. According to FAO experts, Iranian buffalo are among the best buffalos in Asia (8). In order to enhance productivity of a dairy animal, it is necessary to understand the factors affecting milk production. The non-genetic factors such as herd, parity and season influence milk yield so there is need to assess these factors in applied production systems. In East Azarbaijan, the majority of the buffalo population is kept in small herds of four or fewer animals per farm. The buffalo has some advantages in comparison with cattle on small farms in regard to higher persistency of milk yield, higher fat yield, and easely separation of milk fat in the household (Afzal and Anvar, 2007). Genetic improvement of buffalo for milk traits is a necessity, but before application of any genetic improvement program, it is necessary to identify fixed effects on milk production.

The aim of this paper is to study of non-genetic factors on buffalo milk production and its composition in buffalo of Azerbaijan. Yet there is not a complete study on milk traits of this breed.

## MATERIALS AND METHODS

The data were 3,966 records of milk production traits recorded on 672 animals during the years 1997 to 2001 in 73 village of East Azerbaijan. Villages were divided into three regions and from each region 26, 17 and 30 villages were selected, which had cold, warm and temperate climates, respectively. From each village, one to three farmers that had recently calving buffalo were randomly selected for recording. Scaled bucket, were delivered to farmers to measure milk production of animals after calving. Milk production (morning and evening) during the lactation period was recorded monthly.

Sampling of milk and analysis of milk composition carried out by milkoscan. For keeping of the samples $K_2Cro_4$ was used in warm seasons. For sampling of milk after milking, container contents were mixed and sampled and kept in a refrigerator. Milk characteristics including fat, protein, lactose, total solids (TS), solids non fat (SNF) percent were measured after sample temperature reached 38-39°C,

### Statistical Analysis

The mixed model was used to estimate the effects of year, parity and birth season. Data were analyed by proc Mixed of SAS software. Effects of year, season and calving were considered as fixed effects. Effects of animal within herd, herd within village, village within city and city within region was considered as random effects.

## RESULTS AND DISCUSSION

This study was carried out using milk production records of buffalo herds recorded by the agricultural research center in East Azerbaijan during the period from 1997 to 2001, which were used to estimate non-genetic effects on total milk yield of recorded Azerbaijan buffaloes. In comparison with other reported results, in this paper we reported least square means which are more appropriate than raw means.

The least square mean of milk production was 5.48±2.31 whereas in India means of this trait were reported te be 7 to 10 kg (Young and Park, 2002). Effects of year on milk production was significant ($P<0.05$). The lowest production was 4.28 kg in the year of 2001. Decreasing trend in milk production could be attributed to annual

variations in feeding and management practices followed. Similarly highly significant effects of calving year in Indian and Pakistani buffaloes have been reported (Cady *et al*., 1983; Khosla and Gill, 1984; Reddy and Taneja, 1984 and Khola *et al*., 1987). The decrease in milk production during the 5 years was due to scarcity in 1998 to 2001 in this region. Year effect on fat was significant. During these years, milk production decreased and fat percentages increased relationship, it is normal. In Nilli-Ravi buffaloes of Pakistan also, year effects on fat was significant, but parity, season and age were not significant (Shah and Schermerhorn, 1983). Year differences were closely related to nutrition, and the change in fat percent of buffaloes corresponded to differences among cattle on high and low feeding. Also, year effects on protein percentage were significant (P<0.05). No significant difference among years was observed in lactose percentage (P>0.05). The effect of parity on milk production was significant (P<0.05). The milk production in the fourth and the sixth calvings were 5.19, 5.34 kg, respectively. The lowest milk production was in the first calving with 4.55 kg.

Effect of calving season was significant on all traits. Summer calving milk production is significantly less than other season. Hassan Raza *et al*. (1999) showed that in Nili Ravi buffaloes, the highest milk production was in autumn (25,528 L) and the lowest in summer (14,507 L). The increase in milk production in autumn was due to alleviation of summer heat stress. In summer, high ambient temperature adversely affects animal production. This results in reduction in feed consumption and a huge drop in production (Farhomand, 2001). Buffaloes in India and Pakistan are characterized by seasonal pattern of calving where the peak calving season is from August to October. The marked seasonality of buffalo milk production may be attributed to the scarcity of green fodder during summer (April to June) (Young and Park, 2002). Daily milk production over the whole period (1997-2000) were higher (P<0.01) in the animals that calved throughout summer and autumn than in those that calved in winter and spring. Milk fat content was higher (P<0.05) in animals that calved in winter and spring than that those calved in the summer and autumn (8.88 vs. 8.41%). Effects of parity, lactation length, calving season, sex and service period on milk yield in Nili Ravi buffaloes during 1988-2004 were evaluated. Their results showed milk production was lower in the first lactation than that in the 2nd, 3rd and 4th lactations (P<0.05). Milk yield per lactation increased with increasing lactation length (P<0.05). The season of calving had a significant effect on milk yield. Buffaloes calving in the spring showed the highest and those calving in the summer showed the lowest milk yield. It was concluded that parity and lactation length significantly affected milk production in Nili Ravi buffaloes (Afzal, and Anvar, 2007). No differences between groups were observed in milk protein content (4.7%) in water buffaloes from a single farm in southern Italy over a 3-year period (Bufano and Carnicella, 2006). Fat percentage of a buffaloes was influenced by environmental factors such as season of calving. Fat percentage was higher in animals that calved in the autumn and was lower in animals that calved in the summer. Macciotta *et al*. (2006) surveyed factors affecting the occurrence of atypical curves by a logistic regression model. Biological and environmental factors (age at calving and calving season, herd) and, mainly, the structure of data analysed (distance of the first recorded test from parturition) were significantly related to the probability of having an atypical shape. Effect of parity on milk production was significant, and the first parity was significantly

different from the others (P<0.05). Effects of parity, lactation length and calving season was surveyed on milk yield for 426 records in 134 Nili Ravi buffaloes maintained at the National Agricultural Research Center, Islamabad. The season of calving had the significant effect on milk yield. Buffaloes calving in the spring showed the highest and those calving in the summer showed the lowest milk yield (Farhomand, 2001). The correlation between milk production and fat percent, and protein, and TS, and SNF was negative in this study. The correlation between milk production and lactose was positive. In Murrah and Bulgarian buffalo cow crosses, the correlation between milk yield and fat content, and protein and dry matter was negative and low (Farhomand, 2001).

Nutritional comparison of cow and buffalo milk cheddar cheese was carried out in Pakistan. It was concluded that the nutritional value and acceptability of cheddar cheese manufactured from buffalo milk is much superior to that of cow milk. So, the buffalo milk because of its chemical composition, offers excellent opportunities for the development of different dairy products (Mian Anjum and Salim. 2008). Means (± standard deviation) of fat, protein, lactose, TS, SNF percentages in this province were 7.38±1.58, 4.23±0.64, 5.04±0.32, 17.73±1.69 and 10.12±0.61 respectively (Table 1). Pandya and Khan. (2006) reported that average composition (%) of buffalo milk, consisting of fat, protein, lactose, TS, SNF was 7.0, 4.0, 5.1, 9.8, 16.7 respectively. Tomas *et al*. (2004) reported that average composition (%) of Indian river-buffalo milk, consisting of fat, protein, lactose was 7.5, 4.2 and 5% respectively. The effect of various genetic and non-genetic factors on Murrah buffalo milk yield and milk constituent traits analysed and lactation average fat, average solids-not-fat, average total solids percentages, lactation at yield, solids-non-fat yield, total solids yield and 6% fat corrected milk yield were 2505.53±33.31 kg, 2342.47±27.20 kg, 8.06±0.50%, 9.58±0.03%, 17.65±0.51%, 201.22±10.10 kg, 240.13±3.28 kg, 441.35±11.35 kg and 3093.72±119.37 kg, respectively.

## ACKNOWLEDGEMENT

We thank to Jahad Keshavarizi of East Azrbaijan for providing data sets.

## REFERENCES

Afzal, M. and M. Anvar. 2007. Some factors affecting milk yield and lactation length in

Table 1. Analysis and variance of different traits.

| | **Milk production** | **Fat** | **Protein** | **Lactose** | **TS** | **SNF** |
|---|---|---|---|---|---|---|
| **Year** | *** | *** | *** | Ns | *** | *** |
| **Parity** | *** | Ns | Ns | Ns | Ns | Ns |
| **Calving** | *** | * | Ns | Ns | *** | Ns |
| **City** | ns | ns | ns | ns | ns | ns |

***: significant P<0.01 ns: non significant
TS = total solid, SNF = solid non fat * : P<0.01

Table 2. Effect of year on milk characteristics.

| Solid non fat | Total solid | Lactose | Protein | Fat | Production | Year |
|---|---|---|---|---|---|---|
| 9.90[bc] | 17.53[b] | 5.08 | 4.18[b] | 6.94[b] | 5.71[a] | 1997 |
| 10.02[b] | 17.89[ab] | 5.07 | 4.28[b] | 7.20[b] | 5.22[b] | 1998 |
| 10.51[a] | 18.08[ab] | 5.14 | 4. 56[a] | 7.31[b] | 5.02[bc] | 1999 |
| 10.62[a] | 18.37[a] | 5.09 | 4.60[a] | 7.91[a] | 4.87[c] | 2000 |
| 9.48[c] | 17.11[b] | 4.93 | 3.61[c] | 7.64[ab] | 4.28[d] | 2001 |

Different letters within column shows significant difference (P<0.05).

Table 3. Effect of season on milk characteristics.

| Calving season | Production | Fat | Protein | Lactose | Total solid |
|---|---|---|---|---|---|
| Spring | 4.92[bc] | 7.51[a] | 4.26 | 5.04 | 18.05[a] |
| Summer | 4.85[c] | 7.32[c] | 4.17 | 5.05 | 17.40[b] |
| Autumn | 5.19[a] | 7.44[b] | 4.21 | 4.99 | 17.75[ab] |
| Winter | 5.12[ab] | 7.32[c] | 4.34 | 5.18 | 18.00[ab] |

Table 4. Effect of parity on milk characteristics.

| Parity | Production | Fat | Protein | Lactose |
|---|---|---|---|---|
| 1 | 4.55[c] | 7.38 | 4.30 | 5.03 |
| 2 | 5.03[b] | 7.43 | 4.20 | 5.13 |
| 3 | 5.01[b] | 7.30 | 4.25 | 5.09 |
| 4 | 5.19[ab] | 7.45 | 4.25 | 5.07 |
| 5 | 4.99[b] | 7.43 | 4.23 | 5.06 |
| 6 | 5.34[a] | 7.43 | 4.37 | 5.09 |

Table 5. Means and standard deviation (SD) of milk yield, fat, protein, lactose, total solids and solid non fat percentages.

| Variable | N | LS Mean | STD Dev | Sum | Minimum | Maximum |
|---|---|---|---|---|---|---|
| Production | 3966 | 5.47973 | 2.31046 | 21733 | 0.50000 | 15.00000 |
| Fat | 3284 | 7.38762 | 1.58055 | 24261 | 3.20000 | 11.54000 |
| Protein | 725 | 4.23859 | 0.64556 | 3073 | 2.95000 | 6.25000 |
| Lactose | 543 | 5.04958 | 0.32687 | 2742 | 4.27000 | 5.57000 |
| TS | 632 | 17.73834 | 1.69407 | 11211 | 15.00000 | 22.00000 |
| SNF | 649 | 10.12932 | 0.61484 | 6574 | 9.01000 | 12.54000 |

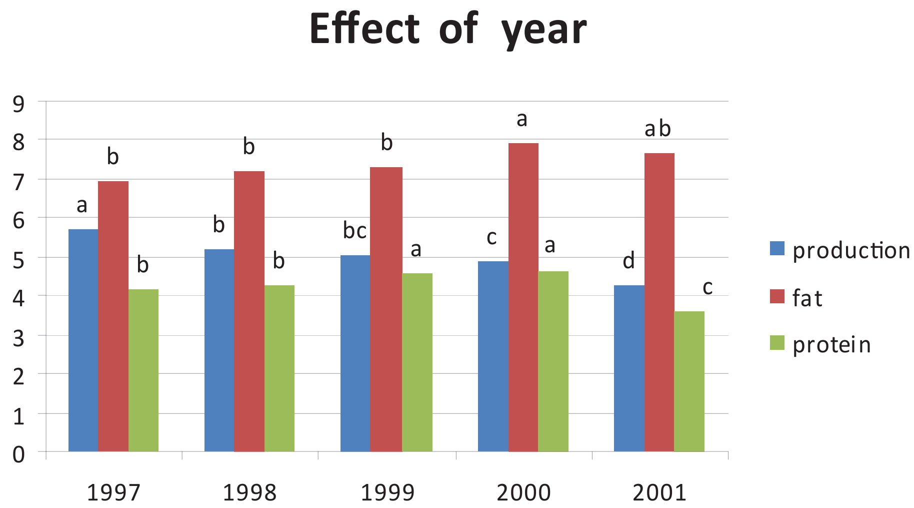


Figure 1. Effect of year on buffalo milk production and composition in East Azerbaijan.

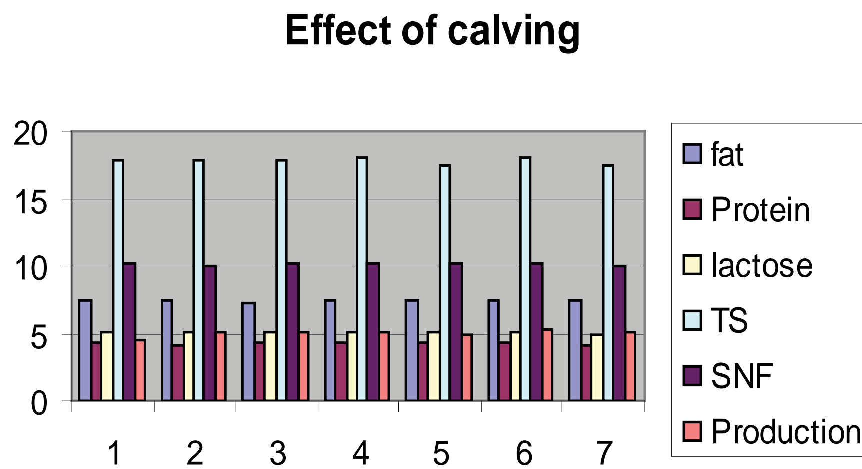


Figure 2. Effect of different parity on buffalo milk production and composition in East Azerbaijan.

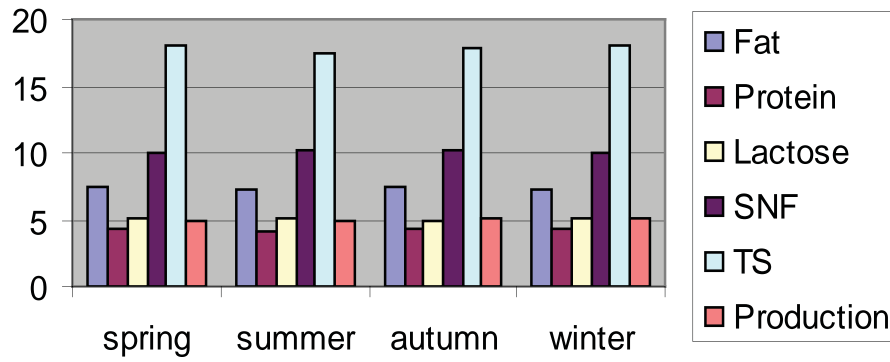


Figure 3. Effect of calving season on buffalo milk production and composition in East Azerbaijan.

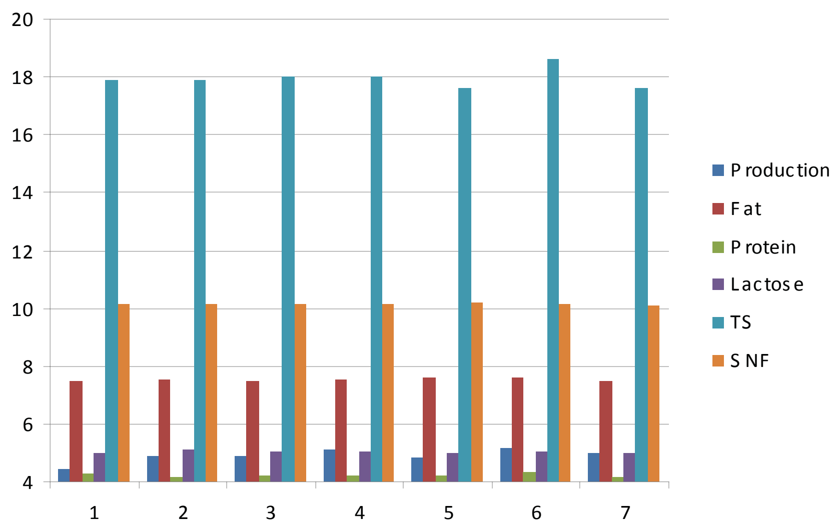


Figure 4. Effect of parity on buffalo milk production and composition in East Azerbaijan.

Niliravi buffaloes. *Pak. Vet. J*., **27**(3): 113-117.

Bufano1, G. and D. Carnicella. 2006. The effect of calving season on milk production in water buffalo *(Bubalus bubalis)*. *Arch. Latinoam. Prod. Anim*., **14**(2): 60-61.

Catillo, G. and N. Macciotta. 2002. Effects of age and calving season on lactation curves of milk production traits in Italian water buffaloes. *J. Dairy Sci*., **85**: 1298-1306.

Fahimuddin, M. and A. Rafat. 2000. *Domestic Water Buffalo*. West Azarbaijan Jahade Daneshgahi Publication. 347p.

Farhomand, P. 2001. *Buffalo Production*. Urmieh University Publication. 199p.

Hassan Raza, S., K.Z. Gondal Arshad Eqbal and A. Shafiq. 1999. A study on effect of seasons on milk production and calving pattern in Nili-Ravi buffaloes. *Buffalo Bull*., **18**(1): 16-19.

Peeva, T. 1997. Composition of milk, Correlation between components and effect of some factors on them, p. 15-16. *In Proceedings of 5th World Buffalo Congress*, Italy.

Mian Anjum, M. and R. Salim. 2008. Nutritional comparison of cow and buffalo milk cheddar cheese. *Pakistan. J. Nutr*., **7**(3): 509-512.

Macciota, N.P.P. 2000. *Lactation curves of milk production traits of Italian Water buffalo estimated by a mixed linear model*. Department Science, Sassari University, Italy.

Macciota, N.P.P., C. Dimauro, G. Catillo, A. Coletta and A. Cappio-Borlino. 2006. Factors affecting individual lactation curve shape in Italian river buffaloes. *Livest. Sci*., **104**(1-2): 33-37.

Sarkar, U and A.K. Gupta. 2006. Genetic and on-genetic factors affecting milk yield and milk constituents in Murrah buffaloes. *J. Dairy. Food. Home Sci.,* **25**(2): 44-56.

Shah, S.K and E.C. Schermerhorn. 1983. Factors affecting milk fat percent of Nili-Ravi buffaloes in Pakistan. *J. Dairy Sci.,* **66**: 573-577.

Tailor, S.P. and S.T. Broikar. 1992. Economics of milk production in Surti buffaloes. *Indian J. Dairy Sci*., **45**(4): 178-180.

Toupchi, Zh. 2001. *Primary study on buffalo breeding in East Azarbaijan and determination of lactation curve and milk composition*. Agricultural and Natural Resources Research Center of East Azarbaijan. Iran. 63p.

Tzankova, M. 2001. Influence of the factor number of lactation and lineage on buffalo milk composition. *Bulg. J. Agric. Sci*., 7(3): 5-7.

Young, W. Park. 2002. *Overview and Prospect of Buffalo Milk Production in the World*. Georgia Small Ruminant Research and Extension Center. Agricultural Research Station Fort Valley State University.

# CRYOPRESERVATION OF SEMEN AS A VENTURE FOR CONSERVATION OF WILD AND ENDANGERED TODA BUFFALO GERMPLASM

**R. Anil Kumar[1,*], M.N. Sundararaman[2], D.V. Patel[3], M. Iyue[1] and R. Kasiraj[3]**


## ABSTRACT

Conservation of endangered Toda buffaloes of the Nilagiri hills of South India in the form of cryopreservation of semen has been attempted. Toda bulls were reared from calfhood at this Research Station. Semen was collected from the bulls using an artificial vagina, evaluated and cryopreserved. The mean ejaculate volume of semen was 2.20 ± 0.25 ml and concentration was 1267.10 ± 107.78 million per ml. The pre-freeze motility and post thaw motility were 74.16 ± 3.60 and 43.14 ± 2.96 percent, respectively.

The motility characteristics of frozen semen were assessed by computer assisted semen analyzer (CASA). Average sperm motility of frozen thawed semen was 54.50± 9.72 percent with 28.00± 7.20 percent of sperm progressively motile. The means (± SE) for path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH), beat cross frequency (BCF) were 88.79± 8.23 µm/s, 74.19± 6.21 µm/s, 137.99± 14.90 µm/s, 6.78± 0.49 µm and 16.38± 2.09 Hz respectively. The percentage of straightness (STR) and linearity (LIN) were 83.88± 1.57 and 57.50± 2.50, respectively.

Semen samples with high post-thaw motility had significantly higher percentage of sperm motility (SM) and progressive motility (PSM). The samples with high PSM had higher path velocity, progressive velocity and track speed. Positive correlation was observed between VAP, VSL, VCL and ALH. Similarly, there was a high positive correlation between VSL and VCL. The mean linearity in Toda buffalo bull semen was above the acceptable threshold level of 50 percent and was of acceptable quality comparable to other buffaloes. The Toda buffalo bulls can be reared in a farm environment if they are trained from calfhood. By using a female Toda buffalo in estrum as a teaser, semen can be collected from Toda bulls in an artificial vagina. Cryopreservation can be successfully employed for conservation of Toda buffalo germplasm.


**Keywords:** conservation, Toda buffalo bulls, cryopreservation, sperm motility, computer assisted sperm analysis, CASA

## INTRODUCTION

Successful gamete storage can provide insurance for preserving the genetic materials of endangered species. Endangered species survive in fragmented habitats and are susceptible for

[1]Sheep Breeding Research Station, Tamilnadu Veterinary and Animal Sciences University, Sandynallah, Udhagamandalam-643 237, The Nilgiris, India, *E-mail: ootyanil@yahoo.com

[2]Farmers Training Centre, Kancheepuram, Tamilnadu, India

[3]Sabarmati Ashram Gaushala, Bidaj Farm, PO- Lali, Dist, Kheda, Gujarat - 387 120, India

environmental catastrophes, epidemics and to drastic shifts in social and political structures (Wildt *et al*., 2001 and Pukazhenthi *et al*., 2006). Toda buffaloes are one such endangered species. The population has come down drastically during the past three decades. The Toda tribes are socially and religiously more attached to their buffaloes, and hence, conservation of these buffaloes is highly essential.

Natural habitats of Toda buffaloes are fast disappearing; the grasslands have come down by a factor of six from 29,875 ha in 1849 to about 4700 ha, and in few areas of the district, there is a 100% reduction in grass land (Kumar, 1997). Hence, intense species management becomes essential for the Toda buffaloes, particularly for Toda buffalo bulls, which are wild in nature and come out of the forest cover and will be with the female herd only during the breeding season. Several Toda hamlets have reported no calvings during the last 5-6 years due to the non-availability of bulls. The objective of the present project was to collect and cryopreserve semen from Toda buffalo bulls as a conservation measure.

Germ plasm conservation of Toda buffalos was carried out as a collaborative project of Sabarmati Ashram Gaushala (SAG) managed by the National Dairy Development Board (NDDB) and Sheep Breeding Research Station, Tamil Nadu Veterinary and Animal Sciences University - (TANUVAS) Chennai, India. Computer assisted semen analysis (CASA) provides a more detailed and objective quantification of sperm motion characteristics than subjective (visual) assessment. Studies of motion characteristics in CASA have been carried out in bull, boar, goat (Tuli *et al*., 1992; Tardif *et al*., 1997 and Sundararaman and Edwin, 2005), human (Geyter *et al*., 1998) and stallion (Jasko *et al*., 1990). Studies are fewer in buffaloes (Rasul *et al*., 2001; Taraphder *et al*., 2002 and Koonjaenak *et al*., 2007). In the present study the motility characteristics of frozen thawed Toda semen was evaluated using computer assisted semen analysis (CASA) technique, as a measure of quality assessment of cryopreserved Toda bull semen.

## MATERIALS AND METHODS

### Toda buffalo

Toda buffaloes are medium-sized animals. The body is fairly long with a broad and deep chest. They have short, strong and sturdy legs. The have two characteristic white to light brown chevron markings, one around the jowl and other anterior to the brisket. The horns are typically long, set wide apart curved outward, slightly downward and upward with the points recurved inward forming a crescent shape or semi circle. Usually a herd consists of a few females with rarely one or two males. Toda bulls are known to stay in dense forests and will come out only during the breeding season.

### Experimental animals

The experimental animals consisted of three Toda bulls of 4-5 years of age, raised under organized farm conditions from calfhood age of 10-12 months. The study was undertaken during the period from February 2005 to June 2007 as a part of combined semen collection, embryo collection and conservation project. Female Toda buffaloes were also reared alongside the bulls.

### Housing, feeding and calfhood management

The Research Station is located at $11^{o}25'$

latitude N and 76°46' longitude E, about 13 km away from Udhagamandalam in the Nilagiri hills at an altitude ranging from 2090 to 2235 metres above mean sea level. The annual rainfall ranges from 848 to 3000 mm. The farm experiences a temperate climate with a maximum temperature of 24°C during the hottest days. During the winter, the night temperature falls to subzero levels.

The Toda bull calves were ferocious as they had been brought up under isolation in their natural condition. Initially it was very difficult to handle them as they were not used to being tethered or handled. Halters and nose ropes were applied for better control and were trained for handling and casting in trevis. Towards the end of the project period (June 2006 to December 2006), all the buffalo bulls could easily be handled.

The bull calves were purchased from Toda tribes people and had been maintained under zero concentrate feeding. Hence, they were very reluctant to take concentrate feed. They were constantly persuaded and trained to take concentrate feed. Gradually they accustomed to concentrate feeding and were fed with 2 kg concentrate/day/animal. All the animals were allowed to graze on natural pastures of farm land for 8 h and were housed in pucca sheds during night hours. During winter months due to frost there was reduction in availability of sufficient green fodder and hence the animals were fed with 3-4 kg of paddy straw /day / animal.

**Semen collection and cryopreservation**

Semen collections using an artificial vagina (AV) were tried in Toda bulls from the start of the project using other bulls as teaser. However, the bulls dismounted immediately once the handler with AV approached them. Hence during embryo collection (EC), the bulls were allowed for natural service. This was followed for four embryo collection programmes. During the fifth EC programme, intervention was made during natural mating and semen was collected successfully using an AV. Subsequently using the cows in estrum as teasers, semen was collected. Since semen collection was successful only when buffalo cows in estrum were used as teasers or mounts, a flexible collection schedule was adopted, even up to four collections in a day. Immediately after collection the semen samples were evaluated for macroscopic characters viz., colour, volume, consistency, mass activity and presence of foreign bodies. Kept in water bath at 37°C, the samples were transported to the Nucleus Jersey and Stud Farm, Udhagamandalam, for evaluation of initial motility and sperm concentration. Based on the motility and concentration the semen samples were extended in tris based diluent for a final concentration of 20-25 million spermatozoa per dose, filled in 0.25 ml French straws and were cryopreserved. The pre-freeze motility was analyzed after five hours of equilibration. Post-thaw motility was analyzed 24 h after cryopreservation. The pre-freeze and post-thaw motility were determined by phase contrast microscopy.

**Assessment of motility characteristics of sperm by CASA**

The frozen semen samples were transferred to the Semen Bank of the Department of Animal Genetics and Breeding, Madras Veterinary College, Chennai-7 for analysis with computer assisted semen analysis.

For analysis of semen by CASA the cryopreserved semen samples were thawed in a water bath at 37°C for 30 seconds. The thawed semen was further diluted for CASA analysis to reduce the sperm concentration. The CASA analysis was done

using Hamilton Thorne integrated visual optical system (HT-IVOS) version 10.9. The chamber temperature was set at $37^{o}$C. Using a micropipette, 1 µl of the prepared semen sample was loaded on the Makler counting chamber (Self-Medical Inst. Ltd) and cover glass was placed on the droplet. Ten microscopic fields were analyzed for each sample.

Sperm motility (SM) (%), progressive motility (PSM) (%), path velocity (VAS) (µm/s), progressive velocity (VCL) (µm/s), lateral amplitude of head displacement (ALH) (µm), beat cross frequency (BCF) (Hz), straightness (STR) (%) and linearity (LIN) (%) were the sperm motion characteristics studied.

**Statistical analysis**

The mean and standard error for all variables were calculated and the difference between the bulls and ejaculates were tested by least squares procedure (Harvey, 1990). All possible interactions with set of fixed effects were fitted initially and insignificant interaction effects were omitted. A linear statistical model was used for analysis of various traits. The differences between the least squares means for subclasses under a particular effect were tested by Duncan's multiple range test modified by Kramer (1957). Correlation between the motility characteristics were established with correlation coefficient. Differences at P 0.05 were considered to be statistically significant.

## RESULTS AND DISCUSSION

The present study describes for the first time the successful collection and cryopreservation of semen from Toda buffalo bulls, a wild and potentially endangered species of buffaloes in the Nilgiris district in the state of Tamil Nadu, South India. Semen collection in Toda bulls using an AV was successful only when she buffalo in estrum was presented as a teaser. The bulls in general had reaction time of 5-10 minutes, showed preference to particular Toda buffalo female in estrum. Bull no. TM2 was sluggish and had a longer reaction time (20-30 minutes).

The semen collected from these bulls was white in colour with a blue tinge, similar to the description given by Vale (1994). The consistency was thick in most of the collections except in the case of Bull no TM2 which frequently gave watery semen.

The ejaculate volume of buffalo semen as observed in several studies ranges from 1-8 ml based on breed and age of the bulls (Dhami and Kodagali, 1988; Vale, 1994; Mishra *et al*., 1994). The ejaculate volume obtained in this study was within the normal range. The average spermatozoal concentration in Toda buffalo was similar to the observation of Galli *et al*. (1993) and was higher than those reported by Aguiar *et al*. (1994), Kumar *et al*. (1993) in Murrah buffalo bulls and Javed *et al*. (2000) for Nili-Ravi buffaloes. Bull No.TM-8 showed significantly ($P<0.05$) higher concentration of sperm (1749.46 ± 181.41 million/ml) than TM-2 (1091.39 ± 144.27 million/ml) and TM-3 (960.46 ± 184.56 million/ml) (Table 1).

The mean (± SE) for pre-freeze motility and post-thaw motility were 74.16 ± 3.60 percent and 43.14 ± 2.96 percent respectively. Similar observations were made by Aguiar *et al*. (1994) and Galli *et al*. (1993). The motility observed in the present study was less than those observed by Dhami and Kodagali (1988) for Surti buffaloes and higher than those observed by Kumar *et al*. (1993) for Murrah buffaloes and Javed *et al*. (2000) for Nili-Ravi buffaloes.

Table 1. Mean (± SE) for physical characteristics of Toda buffalo semen.

| Particulars | Ejaculate volume (ml) | Sperm Concentration (million/ml) | Pre freeze motility (percent) | Post thaw motility (percent) |
|---|---|---|---|---|
| **Bull** | | | | |
| TM-2 | 2.09 ± 0.34 (10) | 1091.39 ± 144.27 [b] (10) | 74.39 ± 4.82 (10) | 48.43 ± 3.96 (10) |
| TM-3 | 2.20 ± 0.43 (5) | 960.46 ± 184.56 [b] (5) | 71.34 ± 6.17 (5) | 39.11 ± 5.07 (5) |
| TM-8 | 2.31 ± 0.44 (7) | 1749.46 ± 187.41 [a] (7) | 76.74 ± 6.27 (7) | 41.87 ± 5.15 (7) |
| **Ejaculate No.** | | | | |
| I | 1.85 ± 0.33 (10) | 992.07 ± 139.72 (10) | 57.90 ± 4.67[a] (10) | 33.92 ± 3.84 (10) |
| II | 2.19 ± 0.37 (7) | 1358.83 ± 160.24 (7) | 67.45 ± 5.36 [a] (5) | 47.59 ± 4.40 (5) |
| III | 2.20 ± 0.55 (3) | 1144.33 ± 234.95 (3) | 85.00 ± 7.86 [b] (7) | 46.67 ± 6.45 (7) |
| IV | 2.55 ± 0.69 (2) | 1573.18 ± 296.08 (2) | 86.29 ± 9.90 [b] (10) | 44.37 ± 8.13 (10) |
| **Overall** | **2.20 ± 0.25 (22)** | **1267.10 ± 107.78 (22)** | **74.16 ± 3.60 (22)** | **43.14 ± 2.96 (22)** |

Figures in parenthesis indicate number of observations.
Means in the same column within categories with different superscript differ significantly (P<0.05).

Table 1a. Least-squares analysis of variance for physical characteristics of Toda buffalo semen.

| Source of variation | Ejaculate volume | | Sperm concentration | | Pre freeze motility | | Post thaw motility | |
|---|---|---|---|---|---|---|---|---|
| | df | Mean squares | df | Mean squares | df | Mean squares | df | Mean squares |
| Bulls | 2 | 0.092 | 2 | 997638.897* | 2 | 35.347 | 2 | 169.902 |
| Ejaculate number | 3 | 0.305 | 3 | 244494.837 | 3 | 757.255* | 3 | 265.808 |
| Error | 16 | 0.904 | 16 | 165606.275 | 16 | 185.135 | 16 | 124.893 |

*(P<0.05)

**Motility characteristics of frozen semen by CASA**

The mean (±SE) sperm motility was 54.50±9.72 with 28.00±7.20 percent of the sperm were progressively motile. The means (± SE) for VAP (μm/s), VSL (μm/s), VCL (μm/s), ALH (μm), BCF (Hz) were 88.79±8.23, 74.19±6.21, 137.99±14.90, 6.78±0.49 and 16.38±2.09 respectively. The percentages of STR and LIN were 83.88±1.57 and 57.50±2.50 respectively (Table 2). The relative speeds of the spermatozoa (VAP, VSL and VCL) observed in this study were similar to Koonjanak *et al*. (2007) for Thai swamp buffaloes and were higher in Nili-Ravi buffaloes (Rasul *et al*., 2000) and Murrah buffaloes (Taraphder *et al*., 2002). The lateral head displacement observed in this study was higher than for Nili-Ravi buffaloes (Rasul *et al*., 2000) and Thai swamp buffaloes (Koonjanak *et al*., 2007) and was lower than the

Murrah buffaloes (Taraphder *et al*., 2002). The linearity was higher in the present study compared to other studies. Straightness was slightly higher than Nili Ravi buffaloes (Rasul *et al*., 2000).

**Correlation between PTM and motility characteristics**

Semen samples with high PTM had significantly higher sperm motility and progressive sperm motility (Table 3). The samples with high PSM had higher path velocity, progressive velocity and track speed. This was similar to the findings of Taraphder *et al*. (2002) for path velocity. The path velocity was significantly and positively correlated with progressive velocity, track speed and ALH.

The high positive correlation observed between VAP, VSL, VCL and ALH, between VSL and VCL and between ALH with VAP, VSL and VCL indicated that the velocity characteristics are interrelated among themselves and with head displacement. Linearity was significantly and negatively correlated with ALH. Taraphder *et al*.

Table 2. Motility characteristics of Toda buffalo sperm.

| Bull No. | SM (%) | PSM (%) | VAP (µm/s) | VSL (µm/s) | VCL (µm/s) | ALH (µm) | BCF (Hz) | STR (%) | LIN (%) |
|---|---|---|---|---|---|---|---|---|---|
| TM-3 | 74 | 47 | 112.00 | 90.40 | 192.40 | 8.40 | 26.70 | 80 | 49 |
| TM-2 | 21 | 3 | 54.40 | 47.80 | 83.10 | 4.90 | 14.40 | 88 | 59 |
| TM-2 | 46 | 22 | 83.80 | 75.50 | 120.40 | 5.30 | 12.10 | 89 | 64 |
| TM-2 | 85 | 45 | 94.70 | 82.90 | 135.90 | 6.60 | 18.10 | 88 | 68 |
| TM-2 | 80 | 39 | 88.80 | 76.40 | 127.40 | 6.50 | 18.60 | 86 | 63 |
| TM-8 | 36 | 6 | 55.90 | 46.40 | 86.50 | 6.30 | 11.10 | 81 | 55 |
| TM-8 | 18 | 9 | 110.40 | 84.10 | 178.30 | 8.90 | 8.80 | 77 | 50 |
| TM-8 | 76 | 53 | 110.30 | 90.00 | 179.90 | 7.30 | 21.20 | 82 | 52 |
| **OVER ALL** | **54.50± 9.72** | **28.00± 7.20** | **88.79± 8.23** | **74.19± 6.21** | **137.99± 14.90** | **6.78± 0.49** | **16.38± 2.09** | **83.88± 1.57** | **57.50± 2.50** |

Table 3. Correlation between the motility characteristics of Toda buffalo sperm and PTM.

| | SM | PSM | VAP | VSL | VCL | ALH | BCF | STR | LIN | PTM |
|---|---|---|---|---|---|---|---|---|---|---|
| **SM** | 1.000 | | | | | | | | | |
| **PSM** | **0.943*** | 1.000 | | | | | | | | |
| **VAP** | 0.471 | **0.688*** | 1.000 | | | | | | | |
| **VSL** | 0.592 | **0.779*** | **0.977*** | 1.000 | | | | | | |
| **VCL** | 0.382 | **0.628*** | **0.975*** | **0.918*** | 1.000 | | | | | |
| **ALH** | 0.139 | 0.323 | **0.803*** | **0.670*** | **0.862*** | 1.000 | | | | |
| **BCF** | **0.782*** | **0.836*** | 0.469 | 0.524 | 0.508 | 0.258 | 1.000 | | | |
| **STR** | 0.218 | 0.052 | -0.451 | -0.260 | -0.583 | **-0.859*** | 0.000 | 1.000 | | |
| **LIN** | 0.287 | 0.061 | -0.362 | -0.181 | -0.548 | **-0.688*** | -0.153 | **0.891*** | 1.000 | |
| **PTM** | **0.952*** | **0.927*** | 0.467 | 0.571 | 0.392 | 0.155 | **0.711*** | 0.148 | 0.220 | 1 |

* (P  0.05)

(2002) observed a negative correlation similar to the present study between LIN and ALH. They also found a highly significant negative correlation between VCL and LIN. A similar result was observed in this study.

The linear motility or percentage of linearity represents a sub population of spermatozoa with higher fertilization potential in comparison to the total motility percentage (Zhang *et al*., 1998; Amann, 1989 and Cremades *et al*., 2005). The proportions of such spermatozoa in a semen sample were correlated with pregnancy rates after A.I. (Zhang *et al*., 1998; Farrell *et al*., 1998 and Januskauskas *et al*., 2001). In bovine A.I. enterprises the acceptable level of percentage of linear motility is 50 percent (Januskauskas *et al*., 1999 and Hallap *et al*., 2004). The mean linearity in Toda buffalo bull semen is above the acceptable threshold, which shows that cryopreserved semen of Toda buffalo was of acceptable quality.

In conclusion, this experiment reveals that the wild Toda buffalo bulls can be tamed and reared in a farm environment if they are trained from calfhood. Semen can be collected from Toda buffalo bulls using an AV if female Toda buffaloes in estrum are used as teasers. The semen from Toda bulls is of good quality and is comparable to that of other buffaloes. Toda buffalo semen can be successfully frozen, and cryopreservation could be used as a method for conservation of the endangered germ plasm in haploid form.

## ACKNOWLEDGEMENTS

We are thankful TANUVAS and NDDP for allotting the necessary funds and permission to carry out the work successfully. We also thank the staff at Nucleus Jersey and Stud Farm, Ooty and Dr. R. Venkatramanan and Dr. C. Sreekumar, Assistant Professors, Sheep Breeding Research Station, Sandynallah, for their help during the project work.

## REFERENCES

Aguiar, P.H.P., V.J. Andrade, J.J. Abreu and N.B.N. Gomez. 1994. Physical and morphological semen characteristics of buffaloes aged from four to eight years old, Vol. 3, p. 486-488. *In Proceedings of 4th International Buffalo Congress*, Sao Paulo, Brazil.

Cremades, T., J. Roca, H. Rodriguez-Martinez, T. Abaigar, J.M. Vazquez and E.A. Martinez. 2005. Kinematic changes during the cryopreservation of boar spermatozoa. *J. Androl*., **26**(5): 610-618.

Dhami, A.J. and S.B. Kodagali. 1988. Seminal characteristics and their inter relationships in Surti buffalo. *Indian Vet. J*., **65**: 61-64.

Farrell, PB., GA. Presicce, CC. Brockett and R.H. Foote. 1998. Quantification of bull sperm characteristics measured by computer assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology*, **49**: 871-879.

Galli, A., V. Bornaghi, D. Balduzzi, L. Buttazzoni and R. Aleandri. 1993. Sexual behaviour and semen quality relating to Italian buffalo, Vol. 1, p. 562-570. *In Proceedings of 3rd World Buffalo Congress*, Varna, Bulgaria.

Geyter, Ch. De. , M. De. Geyter, B. Koppers and Nieschlag, 1998. Diagnostic accuracy of computer assisted sperm motion analysis. *Hum. Reprod*., **13**: 2512-2520.

Hallap, T., M. Haard, U. Jaakma, B. Larsson and H. Rodriguez-Martinez. 2004. Does cleansing of

frozen-thawed bull semen before assessment provide samples that relate better to potential fertility?. *Therigenology*, **62**: 702-713.

Harvey, W.R., 1990. *Mixed Model Least-squares and Maximum Likelihood Computer Programme. PC-2 version*. Ohio State University, Columbus.

Januskauskas, A., J. Gil, L. Soderquist, M.G. Haard, M.C. Haard, A. Johannisson and H. Rodriguez-Martinez. 1999. Effect of cooling rates on post-thaw sperm motility, membrane integrity, capacitation status and fertility of dairy bulls semen used for artificial insemination in Sweden. *Theriogenology*, **52**(4): 641-658.

Januskauskas, A., A. Johannisson and H. Rodriguez-Martinez. 2001. Assessment of sperm quality through fluorometry and sperm chromatin structure assay in relation to field fertility of frozen thawed semen from Sweden AI bulls. *Theriogenology*, **55**: 947-961.

Jasko, D.J., D.H. Lein and R.H., Foote. 1990. A comparison of two computer-automated semen analysis instruments for evaluation of sperm motion characteristics in the stallion. *J. Androl*., **11**: 453-459.

Javed, M.T., A. Khan and R. Kausar. 2000. Effect of age and season on some semen parameters of Nili-Ravi buffalo (*Bubalus bubalis*) bulls. *Vet. Arhiv*., **70**: 83-94.

Koonjaenak, S., P. Pongpeng, S. Wirojwuthikul, A. Johannisson, A. Kunavongkrit and H. Rodriguez-Martinez. 2007. Seasonality affects post-thaw membrane intactness and sperm velocities in spermatozoa from Thai AI swamp buffaloes (*Bubalus bubalis*). *Theriogenology*, **67**(9): 1424-1435.

Kramer, C.Y. 1957. Extension of multiple range tests to group correlated adjusted means. *Biometrics*, **13**:13-18.

Kumar, S. 1997. *Survey and mapping of shola forests and grass lands in the upper Nilgiri Plateau and assessment of human utilization of the vegetation*. Report submitted to World Wild Fund for Nature - India.

Kumar, S., K.L. Sahni and G.S. Bistha. 1993. Cytomorphological characteristics of motile and static semen of buffalo bulls. *Buffalo J*. **2**: 117-127.

Mishra, A.K., S.H. Patel, B.V. Joshi, R.S. Jaswal and K.R. Trivedi. 1994. Buffalo semen characteristics and its freezability under Indian conditions, Vol. 3, p. 495-497. *In Proceedings of 4th International Buffalo Congress*, Sao Paulo, Brazil.

Pukazhenthi, B., P. Comizzoli, A.J. Travis and D.E. Wildt. 2006. Application of emerging technologies to the study and conservation of threatened and endangered species. *Reprod. Fert. Develop*., **18**: 77-90.

Rasul, Z., N. Ahmad and M. Anzar. 2001. Changes in motion characteristics, plasma membrane integrity and acrosome morphology during cryopreservation of Buffalo spermatozoa. *J. Androl.*, **22**: 278-283.

Rasul, Z., M. Anzar, S. Jalali and N. Ahmad. 2000. Effect of buffering systems on post thaw motion characteristics, plasma membrane integrity and acrosome morphology of buffalo spermatozoa. *Anim. Reprod. Sci*., **59**: 31-41.

Sundararaman, M.N. and M.J. Edwin. 2005. Evaluation of pre-freeze and post-thaw sperm motion characteristics of goat spermatozoa by computer assisted semen analysis. *Indian J. Small Rumin*., **11**(1): 92-95.

Taraphder, S., A.K. Gupta and V.S. Raina. 2002. Assessment of post-thaw seminal

characteristics of Murrah buffalo bulls spermatozoa by conventional and computer assisted semen analyzer techniques. *Indian J. Dairy Sci*., **55**: 104-110.

Tardif, A.L., P.B. Farrell, V. Trouern-trend and R.H. Foote. 1997. Computer assisted sperm analysis for assessing initial semen quality and changes during storage at 5$^o$C. *J. Dairy Sci*., **80**: 1606-1612.

Tuli, R.K., R. Schmidt-Baulain and W. Holtz. 1992. Computer assisted motility assessment of spermatozoa from fresh and frozen thawed semen of the bull, boar and goat. *Theriogenology*, **38**: 487.

Vale, W.G., 1994. Collection, processing and deep freezing of buffalo semen. *Buffalo J*., **2**: 65-72.

Wildt, D.E., E. Ellis and J.G. Howard. 2001. Linkage of reproductive sciences: from 'quick fix' to 'integrated' conservation. *J. Reprod. Fertil. Suppl*., **57**: 295-307.

Zhang, B.R., B. Larsson, N. Lundeheim and H. Rodriguez-Martinez. 1998. Sperm characteristics and zona pellucida binding in relation to field fertility of frozen-thawed semen from dairy AI bulls. *Int. J. Androl*., **21**: 207-216.

## 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 conductions 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 immunopotentiation 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 performanceand 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<br>Office of University Library<br>Kasetsart University,<br>50 Pahonyothin Road, Chatuchak,<br>Bangkok 10900, Thailand<br>Tel. 66-2-942-8616

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

# CONTENTS

Page

**Case Report**

Dystocia due to Cebocephalus monster in a graded Murrah buffalo
*Vidya Sagar Pentyala, M. Sreenu, V. Karuna Sri and K. Rajesh*..................................................163

Congenital umbilical defect with visceral eventration in a buffalo calf - A case report
*P. Veena, P. Sankar, S. Kokila, R.V. Suresh Kumar and N. Dhana Lakshmi*.................................165

Intraoccular fibroleiomyosarcoma in a buffalo calf
*P. Amaravathi, Ch. Srilatha, K. Sujatha, R.V. Suresh Kumar*.......................................................168

**Original Article**

Identification of buffalo (*Bubalus bubalis*) meat using PCR targeting mitochondrial D-loop gene
*Deepak Kumar, S.P. Singh, V. Umapathi, K. Nagappa and Abhishek Gaurav*.............................170

Studies on repeat breeding of buffaloes
*Rajesh Kumar, Dharmendra Kumar and Biswajit Roy*.................................................................177

Studies on fertility response in anoestrus buffaloes using a modified CIDR-based synchronization protocol
*N.K. Caesar, S.N. Shukla, O.P. Shrivastava, S. Agrawal and R.G. Agrawal*................................184

*In vitro* antibiotic sensitivity pattern of *Brucella* spp. isolated from reproductive disorders of animals
*S.N. Ghodasara, A. Roy and B.B. Bhanderi*..................................................................................188

CONTENTS

Page

**Original Article**

Comparative study and storage stability of serum hepatobiliary enzyme activities
in Murrah buffaloes
*P.D. Divya and K.K. Jayavardhanan*.............................................................................195

Effects of non-genetic factors in milk production and composition
in East Azarbaijan native buffaloes of Iran
*Zhila Toopchi Khosroshahi, Seyed Abbas Rafat and Djalil Shoja*..............................................202

Cryopreservation of semen as a venture for conservation of wild
and endangered Toda buffalo germplasm
*R. Anil Kumar, M.N. Sundararaman, D.V. Patel, M. Iyue and R. Kasiraj*..................................210

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