ISSN: 0125-6726 Volume 29 No. 1

BUFFALO BULLETIN

Editor: S. Sophon

International Buffalo Information Centre

International Buffalo Information Centre

(IBIC)

Aims

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

Main Objectives

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

BUFFALO BULLETIN
ISSN : 0125-6726

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

Editor
S. Sophon

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

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

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

HORMONAL CHANGES IN HEAT-STRESSED MURRAH BUFFALOES UNDER TWO DIFFERENT COOLING SYSTEMS

Anjali Aggarwal[1] and Mahendra Singh[2]

ABSTRACT

An experiment was carried out on twelve lactating Murrah buffaloes during early lactation of 50-70 days in the Cattle Yard of the National Dairy Research Institute, Karnal. Six buffaloes were kept under water showers (Group 1) while another group of buffaloes were allowed to wallow in a water pond (Group 2) from 11.00 A.M. to 4.00 P.M. daily for a period of 30 days. Blood samples were collected from buffaloes of both the groups at 3 day interval on day 1, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 of the experiment and analysed for plasma thyroxine (T_4), triiodothyronine (T_3), cortisol and insulin hormones. The thermal humidity index (THI)s was 80.3 and 83.6 during the hot-dry and hot-humid seasons, respectively. During the hot-dry season, average plasma T_4 and insulin levels were significantly ($P<0.01$) higher in Group 2 as compared to Group 1. Plasma T_3 levels did not vary significantly in Groups 1 or 2. Plasma cortisol concentration in Group 1 was higher ($P<0.01$) in comparison to Group 2 buffaloes (4.80 vs. 2.60 ng/ml). During the hot-humid season, average T_4, T_3 and insulin concentrations were significantly higher ($P<0.01$) in Groups 2 buffaloes than in Group 1 buffaloes. The overall average value of cortisol was higher in Group 1 when compared to Group 2 buffaloes. Feed intake and milk yield was significantly higher in Group 2 than Group 1 buffaloes during the hot-dry and hot-humid seasons, indicating the beneficial effects of wallowing as opposed to water showers.

Keywords: hormones, heat stress, buffaloes, water showers, wallowing

INTRODUCTION

Environmental stress has measurable effects on the endocrine profile of animals and influences milk production due to alteration of the metabolism. Metabolic hormones, such as thyroxine, triiodothyronine and insulin, can be used to indicate metabolic changes in relation to altered feed intake during different seasons. Plasma cortisol concentrations have been used as physiological markers of stress. During heat acclimation, there is reduction of plasma cortisol that helps the animal in reducing heat production (Stott and Wiersma, 1971). Insulin secretion and action change with the decrease in milk production following exposure of dairy animals to environmental stress (Sartin *et al.*, 1985). High ambient temperature markedly suppress thyroid hormone due to lower feed intake. Heat production and body temperature regulation are effectively controlled through the thyroid. There exists seasonal variation in thyroid gland activity, which is related to ambient temperature and humidity conditions (Khurana, 1983). Such information on the beneficial effect of cooling by showers or by

[1]Dairy Cattle Physiology Division, National Dairy Research Institute, Karnal-132001, India, E-mail: anjali.aggarwal23@yahoo.co.in, anj8204@yahoo.co.in

[2]ERS of National Dairy Research Institute, Kalyani, WB, India

wallowing on hormonal levels of lactating buffaloes is not available. The present study was undertaken to compare the differential effect of cooling by water showers and by wallowing on hormones, *viz.,* thyroxine, triiodothyronine, insulin and cortisol during the hot-dry and hot-humid seasons in buffaloes.

MATERIALS AND METHODS

Twelve Murrah buffaloes of II or III parity in early lactation of 50-70 days were selected from the Institute's herd. All the buffaloes were kept under a loose housing system and had access *ad lib* to green maize fodder and water to drink. Concentrate was offered based on milk production 1.0 kg/2.5 kg milk yield during the morning (6 A.M.) and evening (7 P.M.) milkings. The experiment was conducted for 30 days during the months of May-June (Hot-dry season) and August-September (Hot-humid season; Table 1). The animals were divided into two groups of six each. Group I buffaloes were kept under water showers (Group 1) while Group 2 buffaloes were allowed to wallow from 11.00 A.M. to 4.00 P.M. in a water pond. The buffaloes were handmilked by an expert milker, and the milk yield of each individual buffalo was recorded at each milking. The amount of feed offered and residue left were recorded on a group basis at weekly intervals during both the seasons. Rectal temperature and respiration rate were measured in both the groups of buffaloes in the morning at 8.00 A.M. and in the evening at 4.00 P.M. Blood samples were collected on days 1, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 of the experiment. Plasma levels of thyroxine, triiodothyronine and insulin were estimated by a radioimmunoassay method using RIA kits as per the standard procedure specified along with kits supplied by Board of Radiation and Isotope Technology (BRIT), Mumbai.

Plasma concentration of cortisol was estimated by a magnetic immunosorbent method using kits purchased from M/S Stat Diagnostics Services, Mumbai. The procedure of the assay is briefly described.

Assay procedure:

In the assay procedure, 10 µl each of standards, control and plasma samples were pipetted in plastic RIA tubes (10 x 75 mm) and 100 µl each of tracer (15000 cpm) and antiserum was added to all the tubes followed by vortexing of tubes for 2 minutes. The content of tubes were incubated at room temperature (24^{o}C) for 2 h and 500 µl of magnetic immunosorbent was added after shaking until homogeneity. The tubes were vortexed, incubated for 15 minutes and the bound fraction was separated by centrifugation at 3000 rpm for 15 minutes. The radioactivity in the pellets was counted using a gamma-counter (Model IC4702A). The inter-assay and intra-assay coefficients of variation were 6.72 and 9.40 percent, respectively.

Statistical analysis of the data was carried out using least square analysis of variance with interactions. Mean and standard errors were calculated as per Snedecor and Cochran (1980). The significant values were also tested using Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Average maximum temperature during the hot-dry and hot-humid seasons was 40.8 and 33.4^{o}C, respectively. Relative humidity reached >90 percent during the hot-humid season. The temperature humidity index (THI) was higher during the hot-humid season (83.6) than during the hot-dry season (80.7), indicating a higher level of stress during the hot-humid season (Table 1).

Average plasma T_4 during the hot-dry season was higher (52.27±0.67 ng/ml) in wallowing buffaloes than in buffaloes kept under water showers (50.65±0.50 ng/ml; Table 2). T_4 concentration varied (P<0.01) between periods of the study. Plasma T_3 did not vary between the two groups and between periods of the study. During the experiment, T_3 concentration fluctuated in both the groups and average T_3 concentration was 1.97±0.03 and 1.88±0.03 ng/ml in Groups 1 and 2, respectively. The overall average plasma insulin was significantly different between the groups (P<0.01) and was 10.86 vs. 8.30 μU/ml in Groups 2 and 1, respectively. Plasma cortisol concentration in Group 1 was higher (P<0.01) as compared to buffaloes of Group 2 (4.80 vs. 2.60 ng/ml).

During the hot-humid season, plasma T_4 concentration was higher (P<0.01) in wallowing buffaloes than in buffaloes under water showers (50.97 vs. 48.25 ng/ml). Plasma T_3 varied significantly between the groups (P<0.01) and was higher (1.99±0.03 ng/ml) in Group 2 than Group 1 buffaloes (1.83±0.04 ng/ml). Plasma insulin varied significantly between groups (P<0.01). Cortisol concentration declined (P<0.01) in wallowing buffaloes in comparison to buffaloes under showers. The overall average value of cortisol was 4.33±0.16 and 2.64±0.32 ng/ml (Table 2). Feed intake and milk yield were significantly higher in Group 2 as compared to Group 1 during the hot-dry and hot-humid seasons (Table 3). Similar increases in milk production and feed intake by providing water cooling to lactating buffaloes has been reported by other workers also (Chauhan *et al.*, 1998; Chauhan, 2004).

Rectal temperature and respiration rate in the morning were not significantly different in showering and wallowing groups of buffaloes. However, in the evening, the decline in rectal temperature and respiration rate was greater in the wallowing group of buffaloes thereby indicating more relief from heat stress in wallowing buffaloes (Table 4). Chikamune (1986) also reported that wallowing was more efficient in lowering body temperature and respiration rate of buffaloes than water shower or shading.

Khurana (1983) reported decreased plasma T_4 concentration (39.10 ng/ml) in buffaloes during the hot-dry season compared to 41.44 ng/ml during hot-humid season. During hot conditions animals in early lactation have lower levels of T_4 and T_3. Contrary to this, in the present study, the plasma T_3 level increased in the shower-cooled group. Abelardo *et al.* (2004) when comparing the two different cooling systems (spray/fan and evaporative cooling) found that either cooling system may be used increase the comfort of Holstein and Brown Swiss cows during summer in hot, dry climates.

The higher magnitude of reduction in thyroid activity in summer heat in buffalo than cows is mainly due to a difference in their capacity to adapt to subtropical conditions (Hassan and El-Nouty, 1985). Mean T_4 plasma ranged between 30.01±10.01 and 61.43±10.42 ng/ml in postpartum anoestrus buffaloes (Malik *et al.*, 2003). The increased feed intake concomitant to an increase in milk production suggests a major role for thyroid hormone metabolism in regulating homeorhetic responses involved in maintenance of high priority functions. Further, this fact was also evident from higher insulin level in the wallowing buffaloes in comparison to the shower-cooled ones.

Thus, cooling by wallowing was more efficient in reducing heat stress and restores the physiological responses to normal values without affecting quality of milk and health of animal.

Table 1. Average values of climatic variables during hot-dry (HD) and hot-humid (HH) seasons.

Periods of Experiment	Max. (°C)		Min. (°C)		RH				THI	
					8:AM		2:PM			
	HD	HH	HD	HH	HD	HH	HD	HH	HD	HH
1	40.5	34.6	24.0	22.8	46.0	78.0	21.0	69.0	80.3	81.9
2	39.6	38.5	26.4	20.2	58.0	75.0	13.0	73.0	80.4	80.4
3	40.3	33.2	24.5	21.8	46.0	66.0	10.0	69.0	81.8	85.3
4	40.1	33.4	22.6	26.9	47.0	78.0	14.0	74.0	78.4	84.9
5	42.2	30.8	21.6	27.8	46.0	79.0	17.0	88.0	79.5	84.3
6	43.1	30.4	24.6	25.6	47.0	86.0	14.0	84.0	82.4	82.4
7	39.6	34.6	19.4	29.2	57.0	89.0	23.0	80.0	78.7	84.9
8	39.0	31.6	24.6	31.4	31.0	84.0	34.0	82.0	79.8	83.3
9	40.6	31.2	24.8	25.0	54.0	91.0	32.0	83.0	81.7	82.5
10	43.8	35.4	26.2	27.8	56.0	87.0	35.0	81.0	84.3	86.2
Av.	**40.8**	**33.4**	**23.8**	**25.8**	**48.8**	**81.3**	**21.3**	**78.3**	**80.7**	**83.6**

Max. = Maximum temperature, Min. = Minimum temperature, RH = Relative humidity,
THI = temperature humidity index

Table 2. Mean±S.E. of Thyroxine, triiodothyronine, insulin and cortisol levels in group 1 and group 2 buffaloes during hot-dry and hot-humid season.

Parameter	Hot-dry		Hot-humid	
	Group 1	Group 2	Group 1	Group 2
Thyroxine (ng/ml)	50.65±0.50	52.57±0.67	48.25±0.54	50.57±0.61
Triiodothyronine (ng/ml)	1.97±0.03	1.88±0.03	1.83±0.04	1.99±0.03
Insulin (μU/ml)	8.30±0.26	10.86±0.27	7.86±0.33	9.62±0.30
Cortisol (ng/ml)	4.80±0.14	2.60±0.08	4.33±0.16	2.64±0.32

Table 3. Mean±S.E. of milk yield and feed intake in group 1 and 2 during hot-dry and hot-humid season.

Parameter	Hot-dry		Hot-humid	
	Group 1	Group 2	Group 1	Group 2
Milk yield (kg)	7.6±0.1	8.6±0.1	6.9±0.2	7.8±0.2
Feed Intake (kg)	39.3±0.5	43.9±0.8	40.8±0.8	45.0±0.5

Table 4. Mean (±S.E.) of respiration rate and rectal temperature of lactating Murrah buffaloes in the morning and evening during hot-dry (HD) and hot-humid (HH) seasons.

	Morning respiration rate/minute		Morning rectal temperature (^{o}F)	
	HD	HH	HD	HH
Group 1	24.1±0.4a	20.9±0.2a	101.4±0.1c	101.3±0.1c
Group 2	23.3±0.2a	19.2±0.2b	101.3±0.1c	101.2±0.1c
	Evening respiration rate/minute		Evening rectal temperature (^{o}F)	
	HD	HH	HD	HH
Group 1	21.2±0.2a	17.8±0.2a	101.2±0.1c	102.3±0.1c
Group 2	16.3±0.2b	15.2±0.2b	100.5±0.1d	100.7±0.1d

REFERENCES

Abelardo Correa-Calderon, Dennis Armstrong, Donald Ray, Sue DeNise, Mark Enns and Christine Howison. 2004. Thermoregulatory responses of Holstein and Brown Swiss Heat-Stressed dairy cows to two different cooling systems. *Int. J. Biometeorol.,* **48:** 142-148.

Chauhan, T.R. 2004. Feeding strategies for sustainable buffalo production, p. 74-83. *In Proceedings of XI Animal Nutrtion Conference*, Jabalpur, India.

Chauhan, T.R., S.S. Dahiya, R. Gupta, D.K. Hooda, D. Lall and B.S. Punia. 1998. Effect of climatic stress on nutrient utilization and milk production in lactating buffaloes. *Buffalo Journal*, **16:** 45-52.

Chikamune, T. 1986. Effect of environmental temperature on thermoregulatory responses and oxygen consumption in swamp buffaloes and Holstein cattle. *Buffalo Journal*, **2:** 151-160.

Hassan, G.A. and F.D. El-Nouty. 1985. Thyroidal activity in relation to reproductive status, seasonal variations and milk production in buffalo and cow heifers during their first lactation. *Indian J. Dairy Sci.,* **38:** 129-135.

Khurana, M.L. 1983. *Studies on T_3 and T_4 of dairy animals as influenced by climate*. Ph. D. Thesis, Kurukshetra University, Kurukshetra, India.

Malik, V.S., M.L. Kakkar and R.K. Malik. 2003. Thyroid hormone levels of post partum anoestrus buffaloes induced to cyclicity with crestar - folligon treatment during winter and summer seasons. *SARAS J. Livestock and Poultry Prod.*, **19:** 60-64.

Sartin, J.L., K.A. Cummins, R.J. Kemppainen, D.N. Marple, C.H. Rahe and J.C. Williams. 1985. Glucagon, insulin and growth hormone responses to glucose infusion in lactating dairy cows. *Am. J. Physiol.*, **248:** E108-E114.

Snedecor, G.W. and W.G. Cochran. 1980. *Statistical Methods*, 8th ed. Calcutta and Oxford, IBH Publishing Co.

Stott, G.H. and F. Wiersma. 1971. Plasma corticosteroids as indicators of gonadotrophin secretion and infertility in stressed bovine. *J. Dairy Sci.,* **53:** 652 (Abstr.).

COMPARATIVE STUDIES ON METABOLIC PROFILE OF ANESTROUS AND NORMAL CYCLIC MURRAH BUFFALOES

Sharad Kumar[1], Atul Saxena[2] and Ramsagar[2]

ABSTRACT

Two groups consisting 14 anestrous buffaloes (4-10 years), body weight (230-375 kg) with smooth and inactive ovaries and 10 normal cyclic buffaloes were studied to determine the impact of metabolic profile on infertility. Blood samples from anestrous buffaloes were collected at an interval of 10 days apart three times (42 samples), whereas from cyclic buffaloes blood samples were taken only at the time of A. I (a total of 10 samples). The normal cyclic animals had significantly ($P<0.01$) higher concentrations of haemoglobin, serum protein and inorganic phosphorus than did the anestrous buffaloes. However, the concentration of serum glucose and calcium differ red non significantly between these two groups. The ratio of Ca:P in normal cyclic animals was lower (2.51:1) as compared to the anestrous animals (4.59:1). It can be concluded from the present study that the level of haemoglobin, serum protein and inorganic phosphorus play major role in animal reproduction. The ratio of Ca:P should also be near to 2:1 for better reproduction.

Keywords: anestrous, haemoglobin, serum protein, calcium, inorganic phosphorus

INTRODUCTION

Low breeding efficiency is one of most serious and frustrating problems confronting the dairy industry: serious because of economic losses frustrating because the problem is well concealed and difficult to correct (Pelissier, 1976). Anoestrous is the most prevalent form of infertility encountered in buffaloes and is the most frustrating and challenging problem. Sreemannarayana and Narashimha Rao (1977) reported the incidence of anoestrus in rural buffaloes to be as high as 61% of cases, whereas Luktuke *et al*. (1973) reported 14.67% of cases of true anoestrus in non-discript buffaloes of Utter Pradesh. Amongst the various factors that causes anestrus, under-nutrition is a major problem (Francos *et al.*, 1977 and Bhaskaran and Patil, 1982). To some extent these problems can be detected through metabolic profile tests. Therefore, this study was conducted to assess the impact of nutritional deficiency on anestrus in buffaloes.

MATERIALS AND METHODS

Fourteen post partum (>90 days of calving), parous and lactating Murrah buffaloes aged between 4 to 10 years and having body weights between 230 kg to 375 kg belonging to the District

[1]Division of Veterinary Clinics and Teaching Hospital, Faculty of Veterinary Science and Animal Husbandry, R.S. Pura, Jammu, India

[2]College of Veterinary Science and animal Husbandry, Mathura, U.P. India

Dairy Demonstration Farm, College of Veterinary Science and Animal Husbandry, Mathura, were employed for the study.

Anestrus in these 14 animals was confirmed on the basis of their history and per-rectal examination of the genital organs twice at an interval of 10 days. All the animals had smooth and inactive ovaries with apparently normal genitalia without any palpable abnormalities on per- rectal examination. These animals were maintained on wheat straw, grain and concentrate ration and they were also allowed grazing. Normal cyclic animals selected from the A.I. center of the College of Veterinary Science and Animal Husbandry, Mathura, were used for comparative study.

The blood samples from the anoestrus buffaloes were collected at an interval of ten days three times (total 42 samples), whereas from the cyclic buffaloes, blood samples were taken only at the time of A. I (a total of 10 samples).

Haemoglobin concentration (g/dl) was estimated by using Shahlie's haemoglobinometer, glucose (mg/dl), total protein (g/dl) and inorganic phosphorus (mg/dl) were estimated by GOD/POD methods, modified Burette, Duma's method and Gomorrie's methods respectively by using kits supplied with a Span Diagnostic Ltd. The estimation of calcium was done with a atomic absorption spectrophotometer. The mean values for all the three collections of samples from anestrous animals were calculated and then compared with the normal cyclic animals. Statistical analysis was done as per Snedecor and Cochran (1967) utilizing paired 't' test.

RESULTS AND DISCUSSION

The mean serum values of different parameters have been presented in Table 1.

Metabolic Profile	Anestrous Buffaloes	Cyclic Buffaloes
Hb (g/dl)	12.20±0.23a	13.60±0.51b
Glucose (mg/dl)	54.17±3.88c	63.33±11.04c
Total Protein (g/dl)	6.32±0.22d	8.76±0.48e
Calcium (mg/dl)	7.91±0.48c	8.39±1.36c
P (mg/dl)	2.44±0.16f	3.96±0.25g
Ca:P	3.46:1h	2.15:1i

a vs b; t=2.08 (P<0.05)
d vs e;t=4.16 (P<0.01)
f vs g;t=4.79 (P<0.01)
h vs i;t=2.24 (P<0.05)

The normal cyclic animals had significantly ($P<0.05$) higher concentrations of Hb than those in the anestrous buffaloes. Significant differences in Hb concentration between anestrous and normal cyclic animals have also been reported by various earlier investigators (Dhoble and Gupta, 1981; Srivastava and Kharche, 1986; Yessein *et al.*, 1964). Though the importance of the Hb level has not been directly implicated in reproductive disorders, yet a decrease in Hb value is indicative of certain systemic disorders which could indirectly affect the functional activity of the reproductive organs. A low level of Hb influences tissue oxygenation of the reproductive tract, which in turn could affect the cyclicity (Ramakrishna, 1997).

In the present study, the normal cyclic animals had non-significantly higher glucose concentrations as compare to the anestrous animals, which is in accordance with Murthy *et al*., (1981); Devasri *et al*., (1984); Srivastava and Kharche (1986); Umesh *et al.*, (1995). Several workers have supported the view that the concentration of glucose reflects the energy status and reproductive activity of the animals (Mc Clure, 1965; Morrow, 1969; Richards *et al.*, 1987). Relative hypoglycemia in cows might possibly affect the expression of stress symptoms. Mc Clure (1965) observed that variations in blood glucose were clearly linked to cyclicity and fertility. The loss of ovarian activity in hypoglycemic animals is due to the effect of hypoglycemic state on the release of gonodotrophins from hypothalamus (Howland *et al.*, 1966). Richards *et al.*, (1987) suggested that the reduced concentration of glucose and insulin in blood were associated with nutritional anestrus. Contrary to above reports, Morrow (1969) reported that energy deficiency delayed puberty but did not affect estrus activity after puberty unless severe energy restriction occurs.

The normal cyclic animals had a significantly ($P<0.01$) higher concentration of serum protein as compared to the anestrus buffaloes. Similar findings have been reported by other workers (Devanathan and Quayam, 1983; Umesh *et al.*, 1995; Amanullah *et al.*, 1997).

The serum calcium level was lower in the normal cyclic animals than in the anestrous animals, but the difference was non-significant ($P>0.01$). This finding is in agreement with Pathak and Janakiraman (1987) who reported a lower value in estrus and higher values afterwards. However, Dhoble and Gupta (1987) reported a significantly higher concentration of Ca during the follicular phase as compared to the metestrus and diestrus phases. Roberts (1971) stated that the Ca deficiency may not cause reproductive failure in cattle.

Significantly higher ($P<0.01$) concentration of Pi was recorded in normal cyclic buffaloes compared to anestrous buffaloes. Higher P values in normal cyclic animals have also been recorded by several earlier workers (Ventaswarlu *et al.*, 1994; Umesh *et al.*, 1995; Jani *et al.*, 1995; Newar *et al.*, 1999). Lottammer *et al.* (1974) observed the role of P in the fertility in cattle and reported that both deficiency and excess can cause impaired fertility.

The ratio of Ca:P in normal cyclic animals was 2.15:1 compared to 4.59:1 in the anestrous animals. It has been reported that absorption of Ca and P was better from a diet having a Ca:P ratio of 2:1 than one in which the ratio was 1:1. Even higher Ca:P ratios have been reported to be associated with infertility (Webster, 1932 and Hignett, 1959). Many others have also supported the similar views (Cornahan, 1974 and Luca *et al.*, 1976). Our findings were well supported by Umesh *et al.* (1995) and Jani *et al.* (1995). They observed a disturbed Ca:P ratio in cyclic and noncyclic animals.

There were no observable clinical signs of deficiency. Subclinical nutrient inadequacies are the most probable cause for clinical anestrous and an integrated approach for treatment of multiple deficiencies is needed.

ACKNOWLEDGEMENT

The authors wish to express gratitude to the Dean, College of Veterinary Science and Animal Husbandry, Mathura, U.P. for providing necessary facilities for conducting this work.

REFERENCES

Amanullah, M., M.K. Tandle, S.S. Honnappagol, S.D. Sonwane, S.M. Kartikesh, B.C. Das and Jagjiwaniram. 1997. Serum cholesterol, calcium, phosphorus and total protein in relation to estrus and anoestrus in non-descript buffaloes. *Indian J. dairy. Sci.,* **50:** 410-412.

Bhaskaran, R. and R.V. Patil. 1982. Effects of precalving feed level on birth weight, calving difficulty and subsequent fertility. *J. Anim. Sci.*, **46:** 1522.

Cornahan, D.L. 1974. Mineral metabolism relationship to reproduction in dry lot dairy operations. *In Proc. Soc. Theriogeology.*

Devasri, K.R., P.S. Kavani, G.A. Prabhu and S.A. Kodagil. 1984. Blood glucose levels in different reproductive status in Surti buffaloes. *Indian J. Anim. Reprod.*, **15:** 128.

Dhoble, R.L. and S.K. Gupta. 1981. Total plasma protein and haemoglobin status during oestrous cycle and anoestrous in post-partum buffaloes. *Indian Vet. J.*, **58:** 547.

Dhoble, R.L. and S.K. Gupta. 1987. Biochemical parameters and response to gonadoliberin administration in anoestrus buffaloes. *Indian J. Anim. Sci.*, **57:** 403-407.

Francos, G., N. Davidson and E. Mayer. 1977. Influence of some nutritional factors on incidence of repeat breeder syndrome in high producing dairy herds. *Theriogenology,* **7:** 105-111.

Hignett, S.L. 1959. Some nutritional and other interacting factors which may influence the fertility of cattle. *Vet. Rec.*, **71:** 247.

Howland, B.E., P.L. Klarkaptrick, A.O. Pope and L.H. Casida. 1966. *J. Anim. Sci.*, **25:** 716.

Jani, R.G., B.R. Prajapati and M.R. Dave. 1995. Haematological and biochemical changes in normal fertile and infertile Surti buffaloes. *Indian J. Anim. Sci.,* **65:** 536-539.

Lottammer, K.K., L. Ahleswede, H. Meyer, Schulz and N. Hoffman. 1974. Cited from Sikka, P. *Indian J. Dairy Sci.,* **45:** 159-167.

Luca, L.J. De., J.H. Silva, R.J. Grimoldi and E.G. Capaul. 1976. Fertility in cattle and the practical application of some blood values, p. 972-974. *In Proceedings of 20th World Veterinary Congress*, Greece.

Luktuke, S.N., A.R. Bhattacharya, S.K. Singh and B.U. Khan. 1973. Studies on aberration in functional activity of ovaries in buffaloes. *Indian Vet. J.,* **50:** 876-881.

Mc Clure, T.J. 1965. A nutritional cause of low non-return rates in dairy herds. *Aust. Vet. J.*, **41:** 199.

Morrow, D.A. 1969. Phosphorus deficiency and infertility in dairy heifers. *JAVMA-J. Am.Vet. Med. Assoc.,* **154:** 761.

Murthy, T.S., P. Sreeramalu and K.V. Pillai. 1981. Studies on blood glucose in different physiological status in buffaloes. *Livestock Advisor,* **6:** 37-39.

Newar, S., K.K. Baruah, A. Baruah and D. Bhuyan. 1999. Studies on certain macrmineral status in anoestrus and cyclic post-partum swamp buffaloes. *Indian J. Anim. Res.*, **33:** 134-136.

Pathak, M.M. and K. Janakiraman. 1987. Blood serum calcium, inorganic phosphorus and magnesium at different stages of pregnancy in Surti buffaloes. *Indian J. Anim. Sci.*, **57:** 398-402.

Pelissier, A.R. 1976. Identification of reproductive problem and economic consequences, pp. 9-18. *In Proc: Natt. Invitational Dairy Cattle Reprod.* Workshop, Louisville, Ky. Ext. Comm. Policy Sci., Edc. Amin., U.S.D.A. 1982.

Ramakrishna, K.V. 1997. Comparative studies on certain biochemical constituents of anoestrus crossbred Jersey rural cow. *Indian J. Anim. Reprod.*, **18:** 335.

Richards, M.W., R.P. Wetteman, H.M. Schoeneman and S.D. Welty. 1987. Association between anestrus and blood glucose and insulin in Hereford cows. Animal Science Research Report, Agricultural Experiment Station, Oklahoma State University. M.P.-199, 75-80. (*Anim. Breed. Abst.* **57:** 1598).

Roberts, S.J. 1971. *Veterinary Obstetrics*, 2nd ed. College Book Store, 1701, Nai Sarak, Delhi.

Snedecor, G.W. and W.G. Cochran. 1967. *Statistical Methods*, 6th ed. Oxford and IBH Publishing Co., Calcutta, India.

Sreemannarayana, O. and A.V. Narashimha Rao. 1977. A comparative study in crossbred cows and buffaloes under village management. *Indian J. Reprod.*, **18:** 46-47.

Srivastava, A.K. and K.G. Kharche. 1986. Studies on some blood constituents in normal and abnormal cycling buffaloes. *Indian J. Anim. Reprod.*, **7:** 62-65.

Umesh, K.R., V. Sudhir, R. Chandra, A.S.S. Rao, E.E. Reddy, G.V.N. Reddy and C.C. Reddy. 1995. Studies on certain blood biochemical constituents of rural buffaloes during cyclic and post-partum anestrus periods. *Indian Vet. J.*, **72:** 469-471.

Venkateswarlu, M., V.S.C. Reddy, G.P. Sharma, V.S. Reddy, B. Yadagiri and C.E. Reddy. 1994. Calcium and phosphorus levels in cyclic and true anestrus buffaloes in flurosis endemic areas. *Indian J. Anim. Reprod.,* **15:** 135-137.

Webster, W.M. 1932. *Austr. Vet. J,.* **8**:199. Cited in Sikka, P. *Indian J. Dairy Sci.,* **45:** 159-167.

Yessein Saffaa, H. Shawki, M.M. Bashandy, S. Essawy and Abdallah Ibtihal. 1964. Clinico-pathological studies in female fertile buffaloes. *Indian J. Anim. Reprod.,* **15:** 14-18.

Buffalo Bulletin (March 2010) Vol.29 No.1

EFFECTS OF STRATEGIC DIETARY SUPPLEMENTATION OF BUFFALOES ON ECONOMICS OF THEIR MILK PRODUCTION

Ankur Khare and R.P.S. Baghel

ABSTRACT

A study was carried out to elucidate the effect of strategic dietary supplementation on the economics of milk production in lactating buffaloes. The body weight of the animals was recorded before and after the experiment. Body weight recorded before the start of experiment in different groups was 554.5 ± 4.66, 540.16 ± 5.62, 552.9 ± 4.36 and 542.1 ± 7.26 kg while, at the end of experiment it was 557.20 ± 5.0, 545.24 ± 4.1, 547.99 ± 4.1 and 538.88 ± 5.0 kg, respectively. Milk yield of the animals recorded in different groups during the experimental period was 7.48 ± 0.65, 7.54 ± 0.54, 7.23 ± 0.54 and 7.18 ± 0.65 kg, respectively. The quantity of feed reduction was 1.28 and 1.65 kg/ animal/day in Groups 3 and 4 as compared to control group of animals. The economics of milk production calculated in different groups was 12.27, 12.09, 11.25 and 10.86 Rs of feed/ kg of milk production by the animals.

Keywords: lactating buffaloes, strategic dietary supplementation, nutrient requirements, economics of milk production

INTRODUCTION

India is predominantly an agrarian society where lactating animals are the backbone of national economy. It has the largest livestock population in the world. According to 17th Livestock Census 2003, the total livestock population in India was 485.002 million. Madhya Pradesh contributed around 16.704 million to this total. The livestock population at Jabalpur was 7.11 lacs [7,110,000].

The buffalo population India, i.e., 97.92 million (17th Livestock Census, 2003) was the world's largest and was around 57 percent of the world's total buffalo population. While in Madhya Pradesh, the total buffalo population was 7.57 million which ranked fourth in India, The buffalo population in Jabalpur division of M.P. was around 99,374 (17th Livestock Census, 2003).

In 2005-06, the estimated milk production and per capita availability of milk in India was 97.1 million tons and 241 gm/day, respectively. In Madhya Pradesh milk production was 6.28 million tons (Basic Animal Husbandry Statistic, 2006). The major source of milk is buffaloes. For better and more efficient milk production buffaloes, should be provided an adequate balanced ration. Minerals play a very important role as co-factor for various vitamins as well as being required as a constituent of milk. Hence, it is essential that a lactating buffalo diet should be supplemented well with a good quality mineral mixture along with common salt. In most commercial dairies as well as in rural areas, mineral mixture is not used in the diet of buffaloes.

In India, ruminants depend on straw for their maintenance. The production requirement most often is met from protein supplements like groundnut cake, mustard cake or cottonseed cake (Lailer and

Department of Animal Nutrition, College of Veterinary Science and Animal Husbandry, Jabalpur-482001 (M.P.) India

Singh, 1998) and very seldom from compounded concentrate mixture (Prasad *et al.*, 1993), and this affects the farm economics. Therefore, to reduce the cost of milk, Das and Singh (2004) replaced half of the GNC with berseem and got better performance in growth rate of crossbred calves.

Every animal requires a different level of nutrients according to their physiological needs (Sharma and Thakur, 1991) but this concept is not put into practice on commercial dairy farms because they offer the same level of nutrients to all animals. This was the major factor responsible for increasing the cost of milk and also causes serious disturbances in the health status of animals. Thus, nutrient supplementation beyond the need of the animals may yield only diminishing returns and hence, to elicit the maximum benefit out of the supplementation a specific strategy must be chalked out prior to the start of the nutrient supplementation. This study was therefore planned to see the effect of strategic dietary supplementation on the economics of milk production in buffaloes.

MATERIALS AND METHODS

On a private dairy farm at Pariyat, Jabalpur, M.P., 900 breedable buffaloes were surveyed for their feeding regimes, and forty lactating Murrah buffaloes were selected from among. They were assigned to four dietary treatments, considering their body weights, milk yield, parity and stage of lactation.

Body measurements of all the forty buffaloes were taken in the beginning and at the end of the experiment. For body weights of the animals, measurements were taken before feeding and watering in the morning, and body weight was calculated by the Schaeffer's formula:

$$\text{Live weight (in pounds)} = \frac{\text{Length} \times \text{Girth}^2}{300}$$

The concentrate mixture which was used on the dairy farm included maize, mustard oil seed cake, rice polish, wheat bran, chuni, moong seed, and common salt. To increase the bulk of the concentrate mixture, wheat straw was also added to it. The detailed composition of the concentrate mixture is given in Table 1.

Group 1 was the control. The animals were fed the diet regularly used on the farm. It consisted of wheat straw ad lib., green berseem provided daily in the evening, and concentrate mixture was provided at the time of milking daily in the morning and evening. Group 2 animals were fed a similar diet to Group 1 but it was supplemented with mineral mixture 2% of the diet. Group 3 animals were maintained on strategic supplementation, i.e. the ration given exactly equalled their nutrient requirements. Group 4 animals of this group were fed a diet similar to that of Group 3; however it was devoid of minerals supplementation.

The concentrate mixture was offered at 2.30 AM and 3.00 PM, whereas the chaffed mixed roughage was offered at 3.00 PM. Samples of feed were analyzed for proximate composition, using A.O.A.C. (1990). The data obtained during experiment was analyzed by using the CRD method as described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Body weight of the Animals:

The body weight of all the animals was recorded before and after the experiment to observe the effect of strategic supplementation on change in their body weights. The average body weight of buffaloes in Group 1 at the start of the experiment was 554.5 kg, while after the termination, it was recorded as 557.20 kg. The average body weight recorded in Group 2 was 542.4 kg before the experiment, while at the end of the experiment, it

was 545.24 kg. In Group 3, the average weight of the animals before the start of experiment was 552.9 kg, while that at the end of experiment was 547.99 kg. In the last group, i.e. Group 4, the average body weight of the animals before the start of experiment was 542.1 kg, while that at the end of the experiment was 538.88 kg. Studies revealed that there was no significant effect of strategic supplementation on the body weight of animals. This result was comparable with the findings of Saha *et al.* (1997a); Saha *et al.* (1997b); Akter *et al.* (2004); Renquist *et al.* (2005). The average body weight of the animals before the start of the experiment is presented in Table 2.

The average body weight of the animals after the termination of the experiment is presented in Table 3.

Milk production of the animals:

Milk yield was recorded on a fortnightly basis, and the average milk yield of the animals before the start of experiment is presented in Table 4. In Group 1 (control group) the average milk yield of the animals was 7.91 litres, which was highest among all the four groups. In Group 2, the average milk yield was 7.25 litre. In Group 3, the average milk yield of the animals was 7.70 litre. In Group 4, the average milk yield of the animals before the start of experiment was 7.18 litre. The average milk production recorded before the start of experiment is presented in Table 4.

The average milk production recorded after the start of experiment is in Table 5. In Group 1 (control group), the average milk production of the animals was 7.48 litre. In Group 2, the average milk production of the animals after the experiment was 7.54 litre. In Group 3, the average milk yield of the animals was 7.23 litre. In Group 4, the average milk yield of the animals was 7.18 litre. The present study revealed that milk yield of the animals did not differ significantly due to strategic supplementation. This was in agreement with the work done by Sampath *et al.* (2004); Singh and Singh (2006); Soder *et al.* (2006). The average milk yields of the the groups are presented in Table 5.

Nutrient requirements of the animals:

Nutrient requirements of different animals of the different groups were calculated using ICAR (1998) feeding standards. The maintenance requirements of buffaloes were calculated on the basis of their body weights while production requirements were calculated on the basis of their milk yield. The nutrient requirements for different groups of buffaloes are furnished in Table 6.

Strategic supplementation to the animals

Animals were strategically supplemented exactly as per their nutrient requirements according to their maintenance and their production.

Percent excess and deficit of nutrients supplied to the animals

After calculating the total nutrients offered as well as the total nutrients required by the animals according to their maintenance as well as their production status, the percent ages of excess and deficit of nutrients were calculated by subtracting the total nutrients offered and total nutrients required by the animals. In Group 1, 12.85% excess DCP and 15.67% excess TDN were supplied in the feed. In Group 2, after calculating their nutrient requirements and nutrients supplied, the percent ages of excess of nutrients in terms of DCP was 22.75% while that in term of TDN was 23.29%; this was the highest among all the four groups. In Group 3, the percent ages of excess DCP and TDN in the diet were 14.43% and 16.90%. In Group 4, the percent ages of excess of nutrients in terms of DCP and TDN were 18.59% and 20.26%, respectively. These excess nutrients can be minimized to maintain the economics of milk production. In the present study, we found that the farmers fed of excess DCP

Table 1. Ingredient composition of concentrate mixture used at farm.

Ingredients	Quantity/day (kg)	Percentage (%)
Maize (yellow)	1120.00	19.05
Mustard oilseed cake	560.00	9.52
Cotton seed cake	280.00	4.76
Rice polish	360.00	6.12
Wheat bran	1540.00	26.19
Chuni	1080.00	18.36
Moong	140.00	2.38
Wheat straw	700.00	11.91
Common salt	100.00	1.71
Total	5880.00	100
Calculated		
DCP%	10.54	
TDN%	62.19	

Table 2. Average body weight of animals before the start of experiment.

Group 1	Group 2	Group 3	Group 4
554.5 ± 4.66	540.16 ± 5.62	552.9 ± 4.36	542.1 ± 7.26

Table 3. Average body weight of animals after termination of experiment.

Group 1	Group 2	Group 3	Group 4
557.20 ± 5.0	545.24 ± 4.1	547.99 ± 4.1	538.88 ± 5.0

Table 4. Average milk production of animals on a fortnightly basis before supplementation.

Group 1	Group 2	Group 3	Group 4
7.91 ± 1.02	7.25 ± 0.49	7.70 ± 0.78	7.16 ± 1.05

Table 5. Average milk production of the animals on fortnightly basis after strategic supplementation.

Group 1	Group 2	Group 3	Group 4
7.48 ± 0.65	7.54 ± 0.54	7.23 ± 0.54	7.18 ± 0.65

and TDN to the animals and were not using mineral mixture in the diet of animals, as was also reported by Ramesh *et al.* (2006) and Nagalakshmi *et al.* (2006b). These results were in agreement with the Nagalakshmi *et al.* (2006a); Singh *et al.* (2006); Tewatia *et al.* (2006). The studies reported by Singh *et al.* (1997) also indicated that CP and TDN intake was 16.74% and 22.01% higher in buffaloes. Similarly, Shahi and Saraswat (1997) also observed 31.25% higher TDN intake in milch cows and buffaloes. The percentages of excess and deficit of the nutrients are presented in Table 7.

Strategic supplementation

By strategic supplementation, we have reduced the feed supplied to different groups of animals on the dairy farm. Group 1 was control, so their feeding was as per their normal feeding schedule. In Group 2, there was only supplementation of mineral mixture along with feed (2% of concentrate mixture). In Group 3, there was a reduction of 1.28 kg of concentrate mixture per animal. While, in Group 4, there was reduction of 1.65 kg of concentrate mixture per animal. The total concentrate mixture reduction of Group 3 was 16.64 kg per day. While, that of Group 4 was 14.85 kg per day. The details on the reductions of concentrate mixture are presented in Table 8.

Economics of milk production

Cost of concentrate mixture

In the present study firstly we observe the ingredients used on the dairy farm which is mentioned in Table 1. For the computation of concentrate mixture and the mineral mixture used for supplementing the animals taking into consideration the existing market rate of the different feed ingredients used, thus the overall cost of the concentrate mixture was 823.53 per [1 quintal = 100 kg] including the mineral mixture; this cost was calculated on the basis of percent composition of the different ingredients used in the concentrate mixture. The percent composition, approved market rate of the feed ingredients and the cost of feed in Rs. per quintal is presented in Table 9.

Economics of milk production

The economics of milk production was calculated before and after the start of experiment to observe the change in cost of feed per kg of milk which governs the overall dairy practice. Thus, when we calculated the economics of the dairy farm in the same animals selected for the experiment before the start of the experiment, the cost of feed per kg of milk in Group I was Rs. 11.38. In Group 2, the cost of feed per kg of milk was Rs. 12.41 which was more than Group 1. In Group 3, the cost was less than that of Group 2 but slightly higher then Group 1; it was Rs. 11.67. In the last Group i.e. Group 4, the cost of feed was highest among all the four Groups; it was Rs 12.60. The variation observed in the cost of feed per kg of milk between Group 1 and 3 was Rs. 0.29. The data collected on the economics of milk production before the start of experiment is presented in Table 10.

The economics of milk production between the different experimental groups was again calculated after the start of experiment. The cost of feed per kg of milk production was Rs. 12.27 in Group 1. While, it was Rs. 12.09 in Group 2 slightly less than that of Group 1. In Group 3, the cost of feed per kg of milk was Rs. 11.25, which was lower than that of Group 1 or 2. In the last group, i.e. Group 4, the cost of feed per kg of milk was lowest among all the four groups; it was Rs. 10.86. The Most economic milk production was in Group 4, i.e. Rs. 10.86, but without the supplementation of mineral mixture. While, in Group 3, the cost was Rs. 11.25, which can be said to be the most profitable as it was strategically supplemented along with mineral mixture. The difference between the cost of feed

Table 6. Nutrient requirement of animals.

	Anim. B.W. (kg)	DCP (gm)	TDN (kg)	Avg. milk yield (ltr)	DCP (gm)	TDN (Kg)	DCP (gm)	TDN (Kg)
		Maintenance requirement			Production requirement		Total requirement	
GROUP 1								
Mean	554.53	320	3.9	7.91	498.4	3.639	818.4	7.53
GROUP 2								
Mean	542.48	345	3.90	6.67	420.25	3.068	726.09	6.85
GROUP 3								
Mean	552.92	318	3.88	7.70	485.10	3.53	750.63	7.42
GROUP 4								
Mean	542.12	313	3.83	7.16	451.15	3.29	764.48	7.12

Table 7. Percentage of excess or deficit of energy and protein in the diet of buffaloes of different groups.

Group	Supplied		Required		Excess (+) or Deficit (-)	
	DCP (gm)	TDN (kg)	DCP (gm)	TDN (kg)	Excess/ deficit of DCP (%)	Excess/deficit of TDN (%)
I	939.15	8.93	818.40	7.53	+ 120.75 (12.85%)	+ 1.4 (15.67%)
II	939.15	8.93	726.09	6.85	+ 213.66 (22.75%)	+ 2.08 (23.29%)
III	939.15	8.93	803.56	7.42	+135.59 (14.43%)	+ 1.51 (16.90%)
IV	939.15	8.93	764.48	7.12	+ 174.67 (18.59%)	+ 1.81 (20.26%)
Mean	939.15	8.93	774.07	7.20	161.16	1.70

Table 8. Feed reductions by strategic supplementation.

Groups	Excess DCP (gm)	Quantity of conc. mix. reduced (kg)
1	120.75	-
2	213.66	-
3	135.59	1.28
4	174.67	1.65

Table 9. Cost of concentrate mixture.

Ingredients	Composition in diet (%)	Rate (Rs/Q = quintal = 100 kg)	Cost of feed (Rs/Q = quintal = 100 kg)
Yellow Maize	19.05	939	178.87
M.O. Cake	9.52	1206	114.81
CSC	4.76	977	46.50
Rice polish	6.12	821	50.24
Wheat bran	26.19	779	204.02
Chuni	16.60	911	151.22
Moong grind	2.38	880	20.94
Common salt	1.70	340	5.78
Mineral mix.	2.00	1438.24	28.76
Wheat straw	11.91	187.99	22.39
Total	100		823.53

Table 10. Economics of milk production in the dairy before the experiment.

Treat	Roughage fed (kg)	Cost of Roughage (Rs)	Conc. fed (kg)	Cost of conc (Rs)	Tot. feeding cost (Rs)	Misc. exp. (Rs)	Total cost	Milk yield (kg)	Cost of feed/kg milk (Rs)
I	19.17	12.93	9.00	74.11	87.04	3	90.04	7.91	11.38
II	18.07	12.19	9.00	74.11	86.30	3	89.30	7.25	12.41
III	18.94	12.78	9.00	74.11	86.89	3	89.89	7.70	11.67
IV	19.46	13.13	9.00	74.11	87.24	3	90.24	7.16	12.60

Table 11. Economics of milk production of various group of animal after strategic supplementation.

Treat	Roughage fed (kg)	Cost of Roughage (Rs.)	Conc. fed (kg)	Cost of Conc (Rs.)	Total feeding cost (Rs.)	Misc. exp. (Rs.)	Total cost	Milk yield (kg)	Cost of feed /kg milk (Rs)
I	19.34	13.05	9.00	75.75	88.80	3	91.80	7.48	12.27
II	18.42	12.43	9.00	75.75	88.18	3	91.18	7.54	12.09
III	19.82	13.37	7.72	64.97	78.34	3	81.34	7.23	11.25
IV	19.53	13.17	7.35	61.86	75.03	3	78.03	7.18	10.86

per kg of milk production of Group 1 and 3 was Rs 1.02 per animal per day. Thus the owner of a dairy of 800 animals can save Rs. 24,480 per month by strategic supplementation. The cost of feed/kg milk in buffaloes was also reported by Nayak and Baghel (2004) who surveyed the dairies of the Mahakoshal region of MP. They also observed similar pattern of cost of milk production in buffaloes. Sohane (2006) concluded that the cost of milk production was reduced by providing the concentrate mixture to the animals. Olfadehan and Adewumi (2008) also studied the effect of strategic supplementation in prepartum Bunaji cows and observed that trategic supplementation was beneficial in improving the production of animals and reducing the cost of milk production. The Economics of milk production was calculated after the strategic supplementation, which is presented in Table 11.

REFERENCES

A.O.A.C. 1990. *Official Methods of Analysis,* 15th ed. Association of Official Analytical Chemists. Arlington, USA.

Basic Animal Husbandry Statistic. 2006. Department of Animal Husbandry Dairying and Fisheries, Ministry of Agriculture, Govt of India, New Delhi.

Das, A. and G. Singh. 2004. Effects of partial replacement of groundnut cake with green berseem on growth of crossbred calves. *Indian J. Anim. Nutr.,* **21**(4): 224-228.

ICAR. 1998. *Nutrient Requirement of Livestock and Poultry,* 2nd revised ed. Indian Council of Agricultural Research, Krishi Anusandhan Bhawan, PUSA, New Delhi.

Lailer, P. and G. Singh. 1998. Effects of different dietry nitrogen sources on growth performance of crossbred cattle. *Indian J. Anim. Sci.,* **68:** 76-78.

Nagalakshmi, D., D. Narsimha Reddy, M. Rajendra Prasad and P. Pavani. 2006a. Feeding Practices and nutritional status of dairy animals in North Telangana zone of Andhra Pradesh, p.180. *In Proceedings of VIth Biennial Conference of Animal Nutrition Association,* India.

Nagalakshmi, D. D. Narsimha Reddy, M. Sudhakar Reddy and P. Pavani. 2006b. Assesment of feeding Practices and nutritional status of animals in North coastal zone of Andhra Pradesh, p. 180. *In Proceedings of VIth Biennial Conference of Animal Nutrition Association,* India.

Nayak, S. and R.P.S. Baghel. 2004. Feeding practices and economics of milk production in buffaloes of Mahakosal region of M.P., p. 149. *In Proceedings of XIth Animal Nutrition Conference, Jabalpur, M.P., 5-7 January, 2004. Animal Nutrition Society of India, Souvenir and Abstracts.*

Olafadehan, O.A. and M.K. Adewumi. 2008. Milk production and economic impact of strategic supplementation of prepartum Bunaji cows in the peri-urban areas of derived savanna of southwestern Nigeria. http://www.lrrd.org/lrrd20/3/olaf20041.htm

Prasad, C., K. Sampath, M. Shivaramaiah and T. Walli. 1993. Dry matter digestibility and supplementation of slender and coarse straw. *In Proceedings of International Workshop*, India.

Ramesh, S., D. Nagalakshmi, Y. Ramana Reddy and A. Rajashekher Reddy. 2006. Assesment of feeding Practices and nutritional status of dairy animals in Mahaboobnagar district of Andhra Pradesh, p. 177. *In Proceedings of VIth Biennial Conference of Animal Nutrition Association*, India.

Renquist, B.J., J.W. Oltjen, R.D. Sainz, J.M. Connor and C.C. Calvert. 2005. Effects of supplementation and stocking rate on body

condition and production parameters of multiparous beef cows. http://journals.cambridge.org/action/displayAbstract?fromPage=online&aid=777696

Saha, R.C., R.B. Singh, P.K. Roy and R.A. Day. 1997a. Milk production of cows fed a concentrate mixture made of local feeds available in Nadia, Maldah and Murshidabad, p. 98. *In Proceedings of VIIIth Animal Nutrition Research work Conference,* India.

Saha, R.C., R.B. Singh, P.K. Roy and R.A. Day. 1997b. Milk production of cows fed a concentrate mixture made of local feeds available in Birbhum and west Dinajpur district of West Bengal, p. 99. *In Proceedings of VIIIth Animal Nutrition Research work Conference,* India.

Sampath, K., U. Praveen, and M. Chandrasekharaiah. 2004. Effect of strategic supplementation of finger millet straw on milk yield in crossbred cows on farm trial. *Indian J. Dairy Sci.,* **57**(3)**:** 192-197.

Shahi, M.V. and B.L. Saraswat. 1997. Nutritional status of dairy animals in rural area of Siddhrath nagar district of eastern Uttar Pradesh, p. 96. *In Proceedings of VIIIth Animal Nutrition Research Work Conference*, India.

Sharma, D and K. Thakur. 1991. Nutritional requirements of cross bred cattle. *In Proceedings of Ist International Animal Nutritional Workers Conferences for Asia and Pacific*, India.

Singh D., D. Kumar and Harpal Singh. 1997. Feeding pattern of concentrate and fodder to different categories of milch animals of Nainital district in Uttar Pradesh, p. 95. *In Proceedings of VIIIth Animal Nutrition Research Work Conference*, India.

Singh, J., K.K. Yadav and A.B. Mandal. 1997. Feeding of milch buffalo in Hissar district of Haryana, p. 91. *In Proceedings of VIIIth Animal Nutrition Research Work Conference*, India.

Singh, M.K., V.K. Vidyarthi and V.B. Sharma. 2006. Nutritional status of milch buffaloes in the rural areas nearby Chitrakoot, p. 201. *In Proceedings of VIth Biennial Conference of Animal Nutrition Association*, India.

Singh, S.P. and D. Singh. 2006. Effect of feeding balanced ration on milk production of buffaloes in Kanpur Dehat, p. 223. *In Proceeding of VIth Biennial Conference of Animal Nutrition Association*, India.

Snedecor, G.W. and W.G. Cochran. 1994. *Statistical Methods*, 6th ed. Oxford and IBH, New Delhi.

Soder, K.J., M.A. Sanderson, J. L. Stack, and L.D. Muller. 2006. Intake and performance of lactating cows grazing diverse forage mixtures. *J. Dairy Sci.,* **89:** 2158-2167.

Sohane, R.K. 2006. Effect of total mixed ration in milk production and fat percentage in cross bred dairy cows, p. 42. *In Proceedings of VIth Biennial Conference of Animal Nutrition Association*, India.

Tewatia, B.S., K.R. Yadav and V.S. Panwar. 2006. Assesment of feeding system and plane of nutrition of high yielding buffaloes of Hisar District, p. 170. *In Proceedings of VIth Biennial Conference of Animal Nutrition Association*, India.

17th Livestock Census. 2003. Department of Animal Husbandry Dairying and Fisheries, Ministry of Agriculture, Govt. of India. http://www.dahd.nic.in/relcensus.htm

EFFECT OF Zn SUPPLEMENTATION ($ZnSO_4$) ON SPERM MORPHOMETRY OF MURRAH BUFFALO BULLS (*BUBALUS BUBALIS*)

Biswajit Roy*, P.K. Nagpaul, P.K. Pankaj, T.K. Mohanty, V.S. Raina and S. Ghosh

ABSTRACT

The objective of the present study was to study the effect of zinc supplementation ($ZnSO_4$) on sperm morphometry of the Murrah buffalo bulls. Eight apparently healthy, sexually mature and clinically normal Murrah buffalo bulls of similar body weight and age group (nearly 3.0 to 6.5 years) were randomly divided into two groups: control and ING groups. The Diet for both the groups was the same except that the ING group was supplemented with 40 ppm zinc (zinc sulfate; analytical grade) for 150 days, including 30-day adaptation period. Zn was weighted as per the requirement of individual bulls and mixed with a weighed amount of concentrate mixture for feeding. The amount of Zn supplementation was adjusted at fortnight 14 intervals depending upon the total dry matter intake of individual bulls. Spermatozoa with intact acrosomes were selected and assessed using an immersion lens (1,000X) and standard illumination. The software made it possible to take linear measurements of each spermatozoon: head length, head width, head base, tail length, acrosomal cap length and acrosomal cap width. The results did not show any significant ($P>0.10$) effect of Zn supplementation on sperm morphometry. It can be concluded that Zn supplementation had no effect on sperm morphometry.

Keywords: buffalo, morphometry, sperm, zinc

INTRODUCTION

On a global scale, zinc (Zn) deficiency is the most widespread mineral deficiency and can occur through at least five mechanisms- inadequate intake, increased requirements, mal-absorption, increased losses and impaired utilisation. Sillanpaa (1982) concluded from the global study that deficiencies of Zn could be suspected in almost every country. Zn is an essential trace element in animal nutrition with a wide range of biological roles. It plays catalytic, structural or regulatory roles in the more than 200 Zn metalloenzymes that have been identified in biological systems. It plays important roles in polymeric organization of macromolecules like DNA and RNA, protein synthesis, cell division and stability of biomembranes (Chvapil, 1973). Zn plays a structural role in the formation of the so-called Zn fingers. Zn fingers are exploited by transcription factors for interacting with DNA and regulating the activity of genes. Another structural role of Zn is in the maintenance of the integrity of biological membranes resulting in their protection against oxidative injury. During spermatogenesis, a functional locomotor apparatus is formed in spermatozoa (Mohri and Ishijina, 1989) and a considerable amount of Zn is incorporated into the spermatozoa (Parizek *et al.*, 1966). Flagellar Zn is located mainly within the outer dense fibers (Calvin *et al.*, 1973), where it is bound to the sulfhydryl groups of cysteine. In the course of epididymal

Department of Livestock Production and Management, College of Veterinary Science and Animal Husbandry, Rewa Madhya Pradesh-486001, India *E-mail: drbiswajitroy@gmail.com

sperm maturation Zn content is reduced by approximately 60% (Kaminska *et al.*, 1987) leading to increased stabilization of outer dense fiber proteins by oxidation of sulfhydryl groups to disulfide bridges (Calvin *et al.*, 1973). Zn is also believed to regulate maturation of spermatozoa (Baccetti *et al.*, 1976). Zn deficiency adversely affects sperm integrity (Merrells *et al.*, 2009). With this in view, the following study was designed to study the effect of Zn supplementation on sperm morphometry of Murrah buffalo bulls.

MATERIALS AND METHODS

The investigation was carried out on eight apparently healthy, sexually mature and clinically normal Murrah buffalo bulls of similar body weight and age group (nearly 3.0 to 6.5 years). Bulls were randomly divided into two groups: control and ING groups. The Diet for both the groups was same except that the ING group was supplemented with 40 ppm zinc (zinc sulfate analytical grade) for 150 days, including a 30-day adaptation period. Except for Zn supplementation, the diets were the same for both the groups. Zn was weighted as per the requirement of individual bulls and mixed with weighed amounts of concentrate mixture for feeding. The amount of Zn supplementation was adjusted at fortnightly intervals depending upon the total dry matter intake of individual bulls.

Semen samples were diluted to 200 X 10^6 sperm/mL. To avoid individual technician variation, one person measured all the parameters from the captured image. A Dual staining procedure initially developed by Sidhu *et al.* (1992) was used with some modification to identify the clear acrosome structure of buffalo spermatozoa. One hundred microliters of semen were mixed with 0.2 percent trypan blue (in TALP medium without BSA) and incubated for 10 minutes on a clean glass slide at 37°C. After the incubation period, smears of the semen were prepared gently on the glass slides and allowed to dry for 15 minutes at room temperature. A 0.72 percent (W/V) Giemsa stock solution was prepared by dissolving 1 g of Giemsa dye in glycerol-methanol mixture (54:84). One gram of Giemsa was diluted five times with distilled water (final concentration of Giemsa working solution is approximately 0.15%). The smears of spermatozoa previously stained with trypan blue were then stained with Giemsa for 1hr at room temperature to evaluate the acrosomal status of the spermatozoa. Smears were dried between the folds of filter paper and stored. The dried smears were studied at 1000X under a light microscope using oil immersion without cover-glass. The slides were used for measurement within a week of preparation. A total of 700 spermatozoa were measured for the experiment.

Image Analysis Measurements:

Images were randomly selected from each slide by using an Nikon Eclipse E600 (Tokyo, Japan) microscope attached to an Nikon camera, interfaced to a PC computer and ACT1 software for measurement. The images were obtained by using

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

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

$$SF = (1 - e) \times \frac{P^2}{4\pi A}$$ (3)

100X objectives (oil immersion) in standard light transmission mode (transillumination). Only fresh images were used for the measurements. One speciality of this programme is that stored images cannot be used for measurements. The software was standardizing against a decimal scale. One hundred normal sperm were obtained for each bull from different days of semen sample to avoid any day-to-day variation.

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

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

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

RESULTS AND DISCUSSION

Various types of sperm morphometric parameters are presented in Table 1. There was no significant (P>0.10) difference between control and ING group. Individual bull variation was not found for the head length, width, base, head area and shape (width:length), acrosomal cap length and width, tail length, perimeter, ellipticity and shape factor.

The results of the present study indicated that Zn supplementation had no effect on the sperm morphometry. This is the first study of this kind. Zn seems to play an important role in the physiology of spermatozoa; it has been reported to influence the process of spermatogenesis in ram (Underwood and Somers, 1969). Production of spermatozoa necessitates extensive cell division, which requires a large amount of Zn as it is involved extensive1y in nucleic acid and protein metabolism and is hence fundamental to cell replication and differentiation. Zn is also believed to regulate maturation of spermatozoa (Baccetti *et al.*, 1976). Sperm morphometry, in combination with other objective traits, can be useful for developing a fertility index. Associations of abnormal spermatozoa with bull fertility have yielded varying results. Abnormal bull sperm morphology has been correlated with reduced

fertility (Sekoni and Gustafsson, 1987; Correa *et al.*, 1997). In particular, the occurrence of abnormal sperm head morphology is associated with lower fertility in the bull (Saacke and White, 1972; Sekoni and Gustafsson, 1987). However, a number of other studies have shown no correlation between sperm morphology and fertility (Bratton *et al.*, 1956; Linford *et al.*, 1976) with clear associations between normal bull sperm morphology and fertility continuing to remain elusive (Johnson, 1997). The nucleus of the mammalian spermatozoa becomes highly condensed during the latter stages of spermatogenesis (Zambani, 1971). This condensation is accompanied by biochemical changes involving the replacement of histones by the more basic arginine and cystein rich protamines (Gledhill *et al.*, 1966). The condensed sperm nucleus appears to be chemically more resistant than nuclei of other cells. This resistance or stability is probably due to the extensive disulphide (S-S) bridges existing between adjacent protamine molecules within sperm chromatin (Calvin *et al.*, 1975). These disulphide bonds are formed during spermatozoan transit through epididymis. Spermatozoan nucleus remains in condensed form before fertilization. Zn has a high affinity for thiols (Valle, 1959) and that removal of Zn is facilitated by thiol reacting compounds in spermatozoa from rats (Calvin and Bleau, 1974) and men (Kvist and Eliasson, 1980).

Inadequate uptake of Zn may jeopardize the normal stability of the chromatin, and could result in, or at least signify, a reduced potential of the spermatozoon to contribute to a normal embryonic development (Kvist *et al.*, 1998).

It can be concluded that Zn supplementation had no effect on sperm morphometry.

REFERENCES

Bacetti, B., V. Pallini and A.G. Burrini. 1976. The accessory fibers of the sperm tail. II. Their role in binding zinc in mammals and cephalopods. *J. Ultrastructure Res.,* **54:** 261-275.

Beatty, R.A. and R.A.N. Napier. 1960. Genetics of gametes. 1. A quantitative analysis of five characteristics of rabbit spermatozoa. *Proceedings of Royal Society, Edinburg,* **68:** 1.

Bratton, R.W., R.H. Foote, C.R. Henderson, S.D. Musgrave, H.O. Dunbar and J.P. Beardsly. 1956. The relative usefulness of combinations of laboratory tests for predicting the fertility of bovine semen. *J. Dairy Sci.,* **39:** 1542-1549.

Calvin, H.I. and G. Bleau. 1974. Zinc-thiol complexes in keratin like structures of rat spermatozoa. *Exptl. Cell Res.,* **86:** 280-284.

Calvin, H.I., F.H.F. Hwang and H. Wohlrab. 1975. Localization of zinc in a dense fibre connecting piece fraction of sperm tails analogous chemically to hair keratin. *Biol. Reprod.,* **13:** 228-239.

Calvin, H.I., C.C. Yu and J.M. Bedford. 1973. Effects of epididymal maturation, zinc (II) and copper (II) on the reactive sulfhydryl content of structural elements in rat spermatozoa. *Exp. Cell Res.,* **81:** 333-341.

Chvapil, M. 1973. New aspects in biological role of zinc: A stabilizer of macromolecules and biological membranes. *Life Sci.,* **13:** 1041-1049.

Correa, J.R., M.M. Pace and P.M. Zavos. 1997. Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bull in an artificial inseminations program. *Theriogenology,* **48:** 721-731.

Gledhill, B.L., M.P. Gledhill, R. Rigler and N.R. Ringer. 1966. Changes in deoxyribonucleic during spermatogenesis in the bull. *Exp. Cell Res.,* **41:** 652-665.

Hunt, C.D., P.E. Johnson, J. Herbel and L.K. Mullen. 1992. Effects of dietary depletion on seminal volume and Zinc loss, serum testosterone concentrations, and sperm morphology in young men. *Amer. J. Clin. Nutr.,* **56:** 148-157.

Johnson, W.H. 1997. The significance of bull fertility of morphologically abnormal sperm, pp. 255-270. *In* Van Camp, S.D. (ed.) Bull fertility. *Vet. Clin. N. Amer.-Food Anim. Pr.*, **13.**

Kaminska, B., L. Rozewicka, B. Dominiak, M. Mielnicka and D. Mikulska. 1987. Zinc content in epididymal spermatozoa of metoclopramide-treated rats. *Andrologia,* **19:** 677-683.

Kvist, U. and R. Eliasson. 1980. Influence of seminal plasma on the chromatin stability of ejaculated human spermatozoa. *Int. J. Androl.,* **3:** 130-142.

Kvist, U., S. Kjellberg, L. Bjorndahl, M. Hammar and G.M. Roomans. 1998. Zinc in sperm chromatin and chromatin stability in fertile men and men in barren unions. *Scand. J. Urol. Nephrol.,* **22:** 1-6.

Linford, E., F.A. Glover, C. Bishopand and D.L. Stewart. 1976. The relationship between semen evaluation methods and fertility in bull. *J. Reprod. Fertil.* **47:** 283-291.

Merrells, K.J., H. Blewett, J.A. Jamieson, C.G. Taylor and M. Suh. 2009. Relationship between abnormal sperm morphology induced by dietary zinc deficiency and lipid composition in testes of growing rats. *Brit. J. Nutr*., **18:** 1-7.

Mohri, H. and S. Ishijina. 1989. Epididymal maturation and motility of mammalian spermatozoa, p. 291-298. *In* Serio, M. (ed.) *Perspectives in Andrology*. New York, Raven Press.

Ostermeier, G.C., G.A. Sargeant, B.S. Yandell, D.P. Evenson and J.J. Parrish. 2001. Relationship of bull fertility to sperm nuclear shape. *J. Andrology*, **22:** 595-603.

Parizek, J., J.C. Boursnell, H.F. Hay, A. Babicky and D.M. Taylor. 1966. Zinc in the maturing rat testis. *J. Reprod. Fertil.,* **12:** 501-507.

Saacke, R.G. and J.M. White. 1972. Semen quality tests and their relationship to fertility, p. 22-27. *In NAAB Proceedings of the 4th Technical Conference on Artificial Insemination and Reproduction.*

Sekoni, V.O. and B.K. Gustafsson. 1987. Seasonal variations in the incidence of sperm morphological abnormalities in dairy bulls regularly used for artificial insemination. *British Vet. J.,* **143:** 312-317.

Siddhu, K.S., J.S. Dhindsa and S.S. Guraya. 1992. A simple staining procedure for detecting the true acrosome reaction in buffalo (*Bubalus bubalis*). *Biotechnology Histochemistry,* **67**(1): 35-39.

Sillanpaa, M. 1982. Micronutrients and the nutrient status of soils: a global study. *FAO Soils Bull*., **48**.

Snedecor, G.W. and W.G. Cochran. 1989. *Statistical Method*, 6th ed. The Iowa State University press, Ames, Iowa, USA.

Underwood, E.J. and M. Somers. 1969. Studies of zinc nutrition in sheep. I The relation of zinc to growth, testicular development and spermatogenesis in young rams. *Aust. J. Agr. Res.,* **20:** 889-897.

Valle, B.L. 1959. Biochemistry, physiology and pathology of zinc. *Physiol. Rev.,* **39:** 443-490.

Van Duijn, C. (Jr.). 1960. Mensuration of the head of bull spermatozoa. *Mikrokopie,* **14:** 265-276.

Zambani, L. 1971. *Fine Morphology of Mammalian Fertilization*. New York, Harper and Row.

THE EFFECT OF DIFFERENT PROTEOLYTIC ENZYMES ON THE DISSOLUTION OF THE ZONA PELLUCIDA OF IN VITRO PRODUCED BUFFALO (*BUBALUS BUBALIS*) EMBRYOS

Ch. Srinivasa Prasad, V.S. Gomathy, A. Palanisamy, G. Dhinakar Raj, A.Thangavel and S. Sathesh kumar

ABSTRACT

Removal of the zona pellucida is an important step in the isolation of inner cells from embryos. The zona pellucida could be removed either by mechanical means or by exposing embryos to proteolytic enzymes as the zona is made up of glycoproteins. Even though there were various methods to isolate inner cells from embryos, enzyme digestive method was the more convenient and better method. The present study was undertaken to compare the effect of different proteolytic enzymes (0.5 percent pronase, 0.5 percent trypsin and 0.5 percent papain) on zonalysis and quality of subsequently isolated inner cells/ stem cells from *in vitro*-produced preimplantation buffalo embryos. Out of those enzymes, 0.5 percent pronase, was found to be effective and superior. With 0.5 percent pronase, there was no damage to inner cells, whereas 0.5 percent trypsin and 0.5 percent papain resulted in observable morphological degradation of inner cells.

Keywords: zonalysis, pronase, papain, trypsin, buffalo, *Bubalus bubalis*

INTRODUCTION

Numerous investigators have extensively studied the effect of various proteolytic enzymes on zonalysis in a majority of lab and domestic animals including human being. However, the information on buffalo is scanty. Hence the present study was undertaken to study the effect of different proteolytic enzymes (0.5 percent pronase, 0.5 percent trypsin and 0.5 percent papain) on zonalysis and quality of inner cells derived subsequently from *in vitro*-produced preimplantation buffalo embryos. Removal of the zona pellucida is an important step in isolation of stem cells from embryos. The zona pellucida could be removed either by mechanical means or by exposing embryos to calcium ionophore (Surani *et al.*, 1977) or to proteolytic enzymes (Quinn *et al.*, 1982) as the zona is made up of glycoproteins. Even though there were various methods to isolate inner cells/ stem cells from embryos (Karasiewicz *et al.*, 1993; Ming *et al.*, 2004) enzyme digestive method (Li-Ming *et al.*, 2003 and 2004) was the convenient and more effective, as inner cells could be isolated without any damage to cells of embryos.

MATERIALS AND METHODS

In vitro-produced buffalo embryos (n=49) were utilized to assess the effect of proteolytic enzymes pronase, papain and trypsin. Embryos were incubated in solution containing 0.5 percent pronase/ 0.5 percent trypsin/ 0.5 percent papain until the zona was removed. Embryos were observed constantly under zoom stereo microscope until the zona was digested, and the zona-free embryos were washed with phosphate buffered saline containing 10 percent FBS.

Centralized Embryo Biotechnology Unit, Tamila Nadu Veterinary and Animal Sciences University

RESULTS AND DISCUSSION

The effect of proteolytic enzymes (pronase, papain and trypsin) on zonalysis and subsequent isolation of ES-cells is presented in Table 1.

In *vitro*-produced embryos (n=49) were utilized to assess the effect of proteolytic enzymes pronase, papain and trypsin. The only proteolytic enzyme that had a significant lytic effect on the zona pellucida of buffalo embryos was pronase. 0.5 percent pronase resulted in zonalysis within 2 minutes (approx. 1 minute) in 92 percent of the embryos, whereas for 0.5 percent papain and 0.5 percent trypsin, 83.33 percent and 91.67 percent of the zonae could not be denuded even in up to 90 minutes. With 0.5 percent pronase, there was no damage to blastomeres, whereas 0.5 percent trypsin and 0.5 percent papain resulted in observable morphological degradation of inner cells, especially with trypsin and to some extent in papain.

Table 1. Comparison of the enzymatic digestion of zona pellucida of in *vitro*-produced buffalo embryos.

S. No.	Enzyme (n = No. of embryos)	No. of embryos denuded at different time intervals (Percent)			No. of embryos not denuded in 90 minutes (Percent)
		< 2 minutes	2-30 minutes	30 - 90 minutes	
1.	Pronase (0.5%) (n = 25)	23 (92)	2 (8)	0	0
2.	Papain (0.5%) (n = 12)	0	0	2 (16.67)	10 (83.33)
3.	Trypsin (0.5%) (n = 12)	0	0	1 (8.33)	11 (91.67)

Values in the parenthesis indicate percentage.

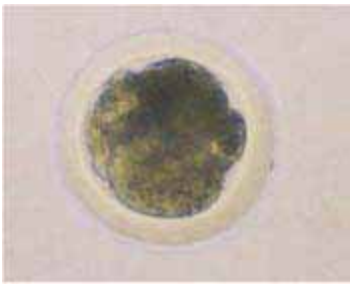

Figure 1. Pronase treated buffalo embryo X 200.

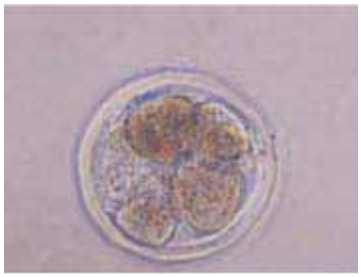

Figure 2. Trypsin treated buffalo embryo X 200.

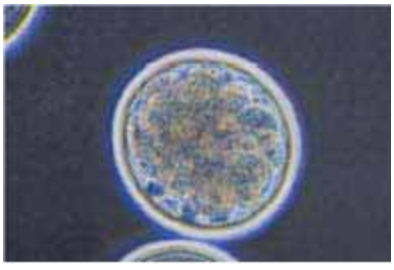

Figure 3. Papain treated buffalo embryo X 200.

The present results are in accordance with the findings of Fong *et al.* (1998), who reported that incubation of human blastocysts in the medium with pronase at a concentration of 10 IU/ml for approximately one minute resulted in initial stretching and softening of the zona. Moor and Cragle (1971) also reported that pronase was the only enzyme that had a significant lytic effect on the zonae of fertilized sheep eggs, denuding 34 percent of eggs in less than 5 minutes. On the contrary, Mangalagowri (2006) reported a higher duration of 15 - 90 minutes for zonalysis in a majority of mouse embryos (55%), which might be due to the use of in-vivo derived mouse embryos. It could be attributed to the fact that zonae of in vivo-derived embryos at various stages of development took much longer time for digestion than zonae of comparable in vitro-stages which might be due to structural changes in the zona pellucida or some sort of protective coating deposited while embryos reside in the oviduct supplying protection against the action of proteolytic enzymes (Kolbe and Holtz, 2005).

As pronase has the least specificity and greatest activity among proteolytic enzymes (Nomoto *et al.*, 1960) and because of its rapid action, which apparently does not harm the egg (Mintz, 1962) pronase can be used to dissolve the zona pellucida and to isolate inner cells/ stem cells from preimplantation buffalo embryos.

REFERENCES

Fong, C.Y., A. Bongso, S.C. Ng, J. Kumar, A. Trounson and S. Ratnam. 1998. Blastocyst transfer after enzymatic treatment of the zona pellucida: improving in-vitro fertilization and understanding implantation. *Hum. Reprod.,* **13**(10): 2926-2932.

Karasiewicz, J., M.A. Reed and J. Modlinski. 1993. Ovine embryonic stem cell-like line cultured over several passages. *Animal Science Papers and Reports Polish Academy of Sciences,* **11**(2): 131-134.

Kolbe, T and W. Holtz. 2005. Differences in proteinase digestibility of the zona pellucida of in vivo and in vitro derived porcine oocytes and embryos. *Theriogenology,* **63**(1): 1695-1705.

Li-Ming, Li-Yong Hai, Hou-Yi, Sun-Xiao Fang, Sun-Qing Yuan and Wang-Wei Hua. 2004. Isolation and culture of pluripotent cells from in vitro produced porcine embryos. *Zygote,* **12**(1): 43-48.

Li-Ming, Zhang-Dong, Hou-Yi, Jiao-LiHong, Zheng-Xing and Wang-Wei Hua 2003. Isolation and culture of embryonic stem cells from porcine blastocysts. *Mol. Reprod. Dev.,* **65**(4): 429-434.

Mangalagowri, A. 2006. *Isolation and characterisation of murine embryonic and bone marrow derived stem cells*. Ph. D. Thesis, Tamil Nadu Veterinary and Animal Sciences University, India.

Ming, X-X., Dou-Zhong Ying, Hua-Jin Lian and Ge-Xiu Guo. 2004. Isolation and culture of embryonic stem cells from BALB/c mice using immunosurgery. *Animal Biotechnology Bulletin,* **9**(1): 843.

Mintz. 1962. Experimental study of the developing mammalian egg: removal of the zona pellucida. *Sci.,* **138:** 594.

Moor, R.M. and R.G. Cragle. 1971. The sheep egg: enzymatic removal of the zona pellucida and culture of eggs in vitro. *J. Reprod. Feril.,* **27:** 401-409.

Nomoto, M., Y. Narahasi and M. Murakami. 1960. A proteolytic enzyme of *Streptomyces griseus*. VI. Hydrolysis by *Streptomyces griseus* protease. *J. Biochem.,* **48**: 593. VII. Substrate specificity of *Streptomyces griseus* protease. *J. Biochem.*, **48**: 906.

*Continued to page 38

PHENO-GENOTYPIC CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* FROM BOVINE CLINICAL MASTITIS

Mahendra Mohan Yadav, Ashish Roy, Bharat Bhanderi* and Chaitanya Joshi

ABSTRACT

The present study was carried out for pheno-genotypic characterization of *L. monocytogenes* (*L. monocytogenes*). A total of three isolates of *L. monocytogenes* were recovered from 85 mastitic milk samples (47 buffalos and 38 cows). Confirmation of the *L. monocytogenes* were based on biochemical tests followed by phenotypic characterization by hemolysis on sheep blood agar, the Christie Atkins Munch-Petersen (CAMP) test, phosphatidylinositol-specific phospholipase C (PIPLC) assay and phosphatidylcholine-specific phospholipase C (PIPLC) assay. The isolates were subjected to genotypic characterization with the help of PCR assay for five virulence associated genes namely, plcA, prfA, hlyA, actA and iap. The *L. monocytogenes* isolates were further subjected to multiplex-PCR based serotyping. All the three isolates of *L. monocytogenes* were hemolytic, CAMP positive, PI-PLC, PC-PLC positive, hlyA, pclA, actA, iap and prfA positive by PCR. All the three isolates of *L. monocytogenes* were serotyped as 4b.

Keywords: *Listeria monocytogenes*, clinical mastitis, PCR

INTRODUCTION

Mastitis is still a multi-etiological disease causing heavy economical losses to the dairy industry throughout the world. In India, the first report of mastitis by Dhanda and Sethi (1962) reported an annual economic loss of Rs. 529 million and thes increased to Rs. 60.5321 million annually in the year 2001 (Dua, 2001). Listeric mastitis, which is the most stubborn and difficult type to treat, results in culling of the infected animals from a herd (Stewart, 1998). It affects one or all the quarters, and the organism could be excreted for months, posing a potential threat to public health (Hird and Genigeorgis, 1990). Moreover, the naturally occurring cases of listeric mastitis cases may go unnoticed or undetected due to lack of suitable techniques employing specific media / antigen(s). The disease has been recognized as an emerging food-borne bacterial infection and public health peril. Outbreaks of food-borne listeriosis have been linked to dairy products, so attention has been focused in identifying animal reservoirs of listeriae in order to better understand the transmission of the disease (Farber and Peterkin, 1991). For both the sporadic and epidemic human listeriosis cases, ingestion of contaminated food is considered to be the primary source of infection (Schlech *et al.*, 1983). Since all major outbreaks of the invasive form of listeriosis are due to serovar 4b strains, an infrequent serovar in foods compared to 1/2a strains (Farber and Peterkin, 1991; Buchrieser *et al.*, 1993) and also major serovar responsible for ruminant listeriosis

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

(Rocourt and Seelinger, 1985; Radostitis *et al.*, 1994), the procedure adopted for outbreak investigations relies upon serovar characterization to provide valuable information for rapid screening of groups of strains. Although 13 serovars are described for the species *L. monocytogenes*, at least 95 per cent of the strains isolated from foods and patients are of serovars 1/2a, 1/2b and 4b (Seeliger and Hohne, 1979; Tappero *et al.*, 1995; Graves *et al.*, 1999). The pathogenic potential of *Listeria* isolates can be assessed by in vitro pathogenicity tests (phenotypic characters) such as hemolytic activity, phosphatidylinositol specific phospholipases C (PI-PLC) assay (Notermans *et al.*, 1991a), phosphatidylcholine specific phospholipase C (PC-PLC) assay and in vivo methods namely, chick embryo (Notermans *et al.*, 1991b) and mouse inoculation tests (Menudier *et al.*, 1991).

Polymerase chain reaction (PCR) has a tremendous potential for the detection of animal pathogens, and therefore, it has attracted much interest in clinical veterinary microbiology in recent years. However, the results obtained have rarely been analyzed in the light of the pathogenic potential of the isolate(s) by in vitro or in vivo pathogenicity test(s) or the natural cases of the disease or the phenotypic detection / expressions of virulence-associated genes. The rapid and reliable diagnosis of listeriosis has been suggested to be ideally based on the detection of virulence markers (genotypic characters) of *Listeria* spp. by molecular techniques, and preferably, on the expression of their activities by in vitro assays (Notermans *et al.*, 1991a). The present investigation was undertaken with a view to study the phenotypic and genotypic characters of *L. monocytogenes* from clinical mastitic cases of cows and buffaloes in and around Anand city of Gujarat state.

MATERIALS AND METHODS

Bacteria - The strains of *L. monocytogenes* 4b (MTCC 1143), Staphylococcus aureus (MTCC 1144), Rhodococcus equi (MTCC 1135), Escherichia coli (MTCC 443) used in the study were obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. The strains of Staphylococcus aureus (ATCC 25923), Str. agalactiae (NCIM 2401), Bacillus spp. (ATCC 6638), Ps. aeruginosa (ATCC 27853) were obtained from Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Anand, India.

Samples - A total of 85 mastitic milk samples were collected aseptically from buffaloes (47) and cows (38) of Gujarat state, India. All the samples were quickly transported to the laboratory under chilled conditions and stored at 4°C till processed.

Isolation of *Listeria* - Isolation of listeriae from the milk samples of the animals were attempted as per the US Department of Agriculture (USDA) method described by McClain and Lee (1988) after making necessary modifications.

Briefly, samples were enriched by two-step enrichment in University of Vermont (UVM) broth-I and II. In UVM-I, incubation was carried out at 30°C for 24 h, while in UVM -II medium incubation was carried out at 30°C up to 7 days, with intermittent streaking after 24 h, 48 h and after 7 days of incubation, simultaneously onto Dominguez-Rodriguez isolation agar (DRIA), PALCAM agar, Oxford agar.

Confirmation of the isolates - Morphologically typical colonies were verified by Gram's staining, catalase reaction, tumbling motility at 20-25°C, methyl red-Voges Proskauer (MR-VP) reactions, nitrate reduction, fermentation of sugars (rhamnose, xylose, mannitol and α-methyl-D-mannopyranoside).

Phenotypic characterization

Haemolysis on sheep blood agar (SBA) - All the *Listeria* isolates were tested for the type (α or β) and the degree (narrow or wider) of hemolysis on 7% sheep blood agar (SBA). Briefly, the isolates were streaked onto 7% SBA plates and incubated at 37°C in a humidified chamber for 24 h and examined for hemolytic zones around the colonies. Interpretation of the hemolytic reaction was based on the characteristic β-hemolysis in the form of wider and clear zone of hemolysis representing L. ivanovii while a narrow zone of α-hemolysis was the characteristic of *L. monocytogenes* or L. seeligeri.

Christie, Atkins, Munch and Petersen (CAMP) test - All the *Listeria* isolates were tested by CAMP test as per the method of Anonymous (1994) with some modifications. Briefly, the standard strains of Staphylococcus aureus and Rhodococcus equi were grown overnight on 7% SBA plates at 37°C and their colonies were again streaked onto freshly prepared 7% SBA plates in a manner such that the streaks were wide apart and parallel to each other. In between the parallel streaks of S. aureus and R. equi the *Listeria* isolates were streaked at 90°C angle and 3 mm apart before incubating them at 37°C for 24 h. The plates were examined for enhancement of the hemolytic zone from partial hemolysis to a wider zone of complete hemolysis, if any, between a *Listeria* strain and the S. aureus or R. equi strain owing to the synergistic effect of their hemolysins in case of a CAMP-positive reaction. The *Listeria* isolates with CAMP-positivity against S. aureus were characterized as *L. monocytogenes* and those with CAMP positivity against R. equi were characterized as L. ivanovii.

Phosphatidylinositol-specific phospholipase C (PI-PLC) assay - All the biochemically characterized *Listeria* isolates were assayed for PI-PLC activity as per the method of Leclercq (2004) with certain modifications. In brief, the *Listeria* isolates were grown overnight onto 7% SBA plates at 37°C. All *Listeria* isolates were streaked on L. mono differential agar (Hi Media Ltd, Mumbai, India) in order to assess PI-PLC activity. The inoculated plates were incubated at 37°C in a humidified chamber for 24 h. On L. mono differential agar, light blue colonies showing a halo formation around the inoculation site were considered positive for PI-PLC assay.

Phosphatidylcholine-specific phospholipase C (PC-PLC) Assay - The egg-yolk opacity test was done to examine the phosphatidylcholine-specific phospholipase C (PCPLC) activity of the isolates. Tryptic soy agar (Hi Media Ltd. Mumbai, India) plates were prepared with 2.5 per cent egg-yolk emulsion (Hi Media Ltd. Mumbai, India) and 2.5 per cent NaCl, pH 6.5-7. *Listeria* isolates were streaked onto the agar surfaces and incubated at 37°C for 36-72 h and observed for formation of opaque zones surrounding the growth (Coffey *et al.*, 1996).

Genotypic characterization

Polymerase chain reaction (PCR) based detection of multiple virulence-associated genes

The primers for the detection of hemolysin gene (hlyA), regulatory gene (prfA), phosphatidylinositol phospholipase C gene (plcA), actin gene (actA) and p60 gene (iap) of *L. monocytogenes* used in this study were synthesized by Sigma Aldrich, USA. The details of the primer sequences are shown in Table 1. The PCR was standardized for the detection of virulence-associated genes namely, plcA, prfA, hlyA, actA and iap of *L. monocytogenes* by following the methodologies described (Furrer *et al.*, 1991; Notermans *et al.*, 1991a; Paziak-Domanska *et al.*, 1999; Suarez and Vazquez-Boland, 2001) with suitable modifications. In brief, the standard strain of pathogenic *L. monocytogenes* (MTCC 1143) was grown overnight in brain heart infusion broth at

37°C. The culture (approximately 0.5 ml) was then centrifuged in a microcentrifuge (Sigma, USA) at 6000 xg for 10 minutes. The recovered pellet was resuspended in 100 µl of sterilized DNAse and RNAse-free milliQ water (Millipore, USA), heated in a boiling water bath for 10 minutes and then snap chilled in crushed ice. The obtained lysate (5 µl) was used as a DNA template in PCR reaction mixture. The PCR was standardized for the detection of virulence associated genes of *L. monocytogenes* by optimizing the different conditions that affect the sensitivity and specificity of the reaction such as the concentrations of biologicals namely, MgCl2 (1.5-2.2 mM), primers (0.1-0.5 µM), Taq DNA polymerase (0.5-2.0 U), annealing temperatures (50-60°C) and number of cycles for amplification of the target gene. Based on optimization trials, the standardized PCR protocol for a 50 µl reaction mixture included 5.0 µl of 10x PCR buffer (100 mM Tris-HCl buffer, pH 8.3 containing 500 mM KCl, 15 mM MgCl2 and 0.01% gelatin), 1 µl of 10 mM dNTP mix (a final concentration of 0.2 mM; Sigma, USA), 4 µl of 25 mM MgCl2 (a final concentration of 2 mM) and 10 µM of a primer set containing forward and reverse primers (a final concentration of 0.1 µM of each primer), 1 U of Taq DNA polymerase (Sigma, USA), 5 µl of cell lysate and sterilized milliQ water to make up the reaction volume.

The PCR tube (0.2 ml) containing the reaction mixture was tapped thoroughly with a finger and then flash spun in a micro centrifuge to settle the reactants at the bottom. The DNA amplification reaction was performed in a Master Cycler Gradient Thermocycler (Eppendorf, Germany) with a preheated lid. The cycling conditions for PCR included an initial denaturation of DNA at 95°C for 2 minutes followed by 35 cycles each of 15 seconds denaturation at 95°C, 30 seconds annealing at 60°C and 1 minute and 30 seconds extension at 72°C, followed by a final extension of 10 minutes at 72°C and held at 4°C. All the five sets of primers for virulence-associated genes were amplified under similar PCR conditions and amplification cycles. The resultant PCR products were further analyzed by agarose gel electrophoresis (1.5%; low melting temperature agarose L), stained with ethidium bromide (0.5 µg/ml) and visualized by a UV transilluminator (UVP Gel Seq Software, England).

Specificity of the PCR - The specificity of the standardized PCR was tested by screening the standard strains of *L. monocytogenes*, *Listeria* species as well as some other commonly prevalent and cross reacting bacterial species with the primers used in this study. The DNA template preparation from the test organisms and other PCR conditions were similar to those described earlier.

Multiplex PCR based serotype detection of *Listeria monocytogenes* isolates

The multiplex PCR assay was standardized for the detection of three major serovars of *L. monocytogenes* namely 1/2a, 1/2b and 4b, following the methodology as described by Doumith *et al.* (2004) with suitable modifications. The primers for detection of *L. monocytogenes* 0737 gene (lmo0737), transcriptional regulator gene (ORF2819), secreted protein gene (ORF2110) and phosphoribosyl pyrophosphate synthetase gene (prs) in this study were synthesized from Sigma Aldrich. The details of the primer sequence are shown in Table 1.

The PCR was set for 50 µl reaction volume. Initially for the detection of *L. monocytogenes* serotype by PCR, conditions were optimized by using varying concentrations of molecular biologicals (Sigma Aldrich), gradient annealing temperature and number of cycles for amplification of target genes. Based on these trials, the reaction mixture for PCR was optimized as follows: 5.0 µl of 10X PCR buffer (Ammonium sulphate) (consisting of 100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM MgCl2 and 0.01% gelatin), 1.5 µl dNTP mix (10 mM, with a

final concentration of 0.2 mM), 4 µl of 25 mM MgCl-2 (final concentration 2 mM) and 100 µM of forward and reverse primer of each set i.e. 1/2a, 1/2b and 4b (final concentration 0.1 µM each) and 10 µM of forward and reverse primer of each set of *Listeria* spp. (final concentration 0.1 µM each) 2 units of Taq DNA Polymerase, 5 µl of cell lysate and sterilized milliQ water to make up the reaction volume.

The PCR tube (0.2 ml) containing the reaction mixture was flash spun on a micro centrifuge (Remi, C 24) to get reactants at the bottom. The reaction was performed in Px2 Thermal cycler (Thermo electronic corporation, USA) with a pre-heated lid. The cycling conditions included an initial denaturation at 94°C for 5 minutes. followed by 35 cycles each of 30 seconds denaturation at 94°C, 1 minute. 15 seconds annealing at 54°C and 1 minute. 15 seconds extension at 72°C. This was followed by a final extension of 10 minutes. at 72°C and 30 minutes. held at 4°C. After the reaction, PCR products were kept at -20°C until further analysis by agarose gel electrophoresis.

Table 1. Primers for amplification of virulence associated genes and serotypes of *L. monocytogenes*.

Gene	**Primer Sequence (5'-3')**		**Product size (bp)**	**Reference**
plc A	Forward	CTGCTTGAGCGTTCATGTCTCATCCCCC	1484	Notermans *et al.* (1991a)
	Reverse	CATGGGTTTCACTCTCCTTCTAC		
prf A	Forward	CTGTTGGAGCTCTTCTTGGTGAAGCAATCG	1060	Notermans *et al.* (1991a)
	Reverse	AGCAACCTCGGTACCATATACTAACTC		
act A	Forward	CGCCGCGGAAATTAAAAAAAGA	839	Suarez and Vazquez Boland (2001)
	Reverse	ACGAAGGAACCGGGCTGCTAG		
hly A	Forward	GCAGTTGCAAGCGCTTGGAGTGAA	456	Paziak-Domanska *et al.* (1999)
	Reverse	GCAACGTATCCTCCAGAGTGATCG		
iap	Forward	ACAAGCTGCACCTGTTGCAG	131	Furrer *et al.* (1991)
	Reverse	TGACAGCGTGTGTAGTAGCA		
lmo0737 serovar1/2a	Forward	AGGGCTTCAAGGACTTACCC	691	Doumith *et al.* (2004)
	Reverse	ACGATTTCTGCTTGCCATTC		
ORF2819 serovar 1/2b and 4b	Forward	AGCAAAATGCCAAAACTCGT	471	
	Reverse	CATCACTAAAGCCTCCCATTG		
ORF2110 serovar 4b	Forward	AGTGGACAATTGATTGGTGAA	597	
	Reverse	CATCCATCCCTTACTTTGGAC		
prs serovar all *Listeria* pp.	Forward	GCTGAAGAGATTGCGAAAGAAG	370	
	Reverse	CAAAGAAACCTTGGATTTGCGG		

RESULTS

Isolation of *Listeria monocytogenes* - From 85 mastitis milk samples collected from cows and buffaloes, three were found positive for *Listeria* spp., all of which were *L. monocytogenes*. Among the isolates, two were from buffalo and one was from cow.

Phenotypic Characters - All the three isolates of *L. monocytogenes* showed the characteristic enhancement of hemolytic zone with S. aureus. All the three isolates of *L. monocytogenes* were found to be pathogenic by PI-PLC and PC-PLC.

Genotypic Characters - The standardized PCR allowed amplification of virulence associated genes of *L. monocytogenes* namely, plcA, prfA, actA, hlyA and iap to their respective base pairs, 1484 bp, 1060 bp, 839 bp, 456 bp and 131 bp PCR products, respectively, each represented by a single band in the corresponding region of the DNA marker ladder. Each of the primers was found to be specific to the target gene as it specifically amplified the PCR product of that gene, accordingly, all the five genes were detected in standard strains of *L. monocytogenes*, whereas, none of the genes was detected in the cultures of the other bacterial species cultures. The five virulence-associated genes were detected in all the three *L. monocytogenes* isolates

The multiplex PCR was standardized for detection of three major serotypes of *L. monocytogenes* viz., 1/2a, 1/2b and 4b by targeting various genes like lmo0737, ORF2819, ORF2110 and prs which were coding proteins like unknown protein, putative transcriptional regulator, putative secreted protein and putative phosphoribosyl pyrophosphate, respectively. All the three isolates showed amplification of three molecular size bands viz., 471 bp, 597 bp and 370 bp corresponding to their genes, ORF2819, ORF2110 and prs, respectively, while no amplification of lmo0737 gene. During the study, all the isolates were biochemically characterized as *Listeria*, all the three isolates amplified 370 bp products corresponding to gene prs, which served as internal amplification control. Employing the multiplex PCR assay serotyped, all the three *L. monocytogenes* revealed serotype 4b. In India, this the first report of isolation of *L. monocytogenes* serotype 4b from the bovine clinical mastitis.

DISCUSSION

Listeriosis is one of the important bacterial diseases of animals and a zoonosis with a broad distribution; it has considerable economic and public health significance. The milk industry in India is flourishing with cattle and buffalo playing the major role in milk production, but studies on occurrence of the important food borne pathogens like *L. monocytogenes* in animals and its environment have not yet been carried out in detail except for a few reports (Shakuntala *et al.*, 2006; Rawool *et al.*, 2007).

From 85 mastitis milk samples collected from cows and buffaloes, three were found positive for *Listeria* spp., all of which were *L. monocytogenes*. Pure cultures of *L. monocytogenes* were isolated from infected quarters. In India, isolation of *L. monocytogenes* from Holstein-Friesian cattle suffering from acute mastitis has been reported (Shome *et al.*, 2003) and in buffalo (Verma *et al.*, 2001), subclinical mastitis in cattle and buffalo (Rawool *et al.*, 2007). This is the first report on clinical mastitis caused by *L. monocytogenes* in Gujarat state, which is one of the leading dairy industry states in India. It is very well established that *L. monocytogenes* exists and multiplies as a saprophytic organism in the soil and on plants as well as in sewage and river water (Farber and Peterkin, 1991). Thus, it is obvious that a large source of *L. monocytogenes* exists in and

around milking cows and buffaloes. Cases of bovine clinical mastitis due to *L. monocytogenes* appear to be rare and the systematic literature on this subject was scanty.

With the exception of L. seeligeri being hemolytic but nonpathogenic, the pathogenic strains of *L. monocytogenes* are hemolytic. Hence, all the three *L. monocytogenes* isolates, which were hemolytic, could be considered as potentially pathogenic. All three isolates were found to be pathogenic in all the assays and possessed all the five virulence-associated genes. A number of factors are involved in manifestation of virulence of *L. monocytogenes* (Vazquez-Boland *et al.*, 2001). It has been demonstrated that the *L. monocytogenes* phospholipases are essential determinants of pathogenicity. Of these, the activity of virulence factor called PI-PLC and PCPLC is expressed only by pathogenic spp. of *Listeria* i.e., *L. monocytogenes* (Notermans *et al.*, 1991a) and L. ivanovii (Leimeister-Wachter *et al.*, 1991), and has been found to be a reliable marker for discrimination between pathogenic and nonpathogenic *Listeria* species (Notermans *et al.*, 1991a). The positivity of all the three isolates of *L. monocytogenes* in PI-PLC assay can be explained based on the common regulation of the hlyA gene and the plcA gene by the prfA encoded protein.

All the three isolates of *L. monocytogenes*, showed an opaque zone surrounding the growth. Similar Coffey *et al.* (1996) reported that *L. monocytogenes* produces an opaque zone surrounding the growth and Erdenlig *et al.* (2000) reported a zone of opacity on egg yolk agar around the growth of *L. monocytogenes* isolated from channel catfish. Thus our finding was supported by the earlier reports. One phenotype closely related with virulence of *L. monocytogenes* was egg yolk agar opacification, a reaction revealing lecithinase or phosphotidylcholine-phospholipase C (PC-PLC) activity. *L. monocytogenes* produces one or both of the two distinct types of reaction on egg yolk agar, either a faint halo or a dense zone of opacity surrounding the colony. The lipolytic activity of *L. monocytogenes* strains that produces a zone of opalescence around colonies on egg yolk or lecithovitellin agar is related to phopholipase C activity. PC-PLC was 29 kDa protein produced by all virulent strains of *L. monocytogenes*, whereas distinct lecithin degradation was not expressed by other *Listeria* spp. The presence of PC-PLC and another phospholipase enzyme (PIPLC) were required for virulence, although detection of one is sufficient for the identification of pathogenicity. As detection of virulence factors was useful to assist in the identification and differentiation of *Listeria* species, this report shows that lecithinase activity can conveniently be detected within 36 h on a relatively inexpensive medium.

The PCR employed in the present study turned out to be specific for the individual detection of five virulence-associated gene(s), namely plcA, prfA, actA, hlyA and iap, found in *L. monocytogenes* with respective sets of primers giving no cross-reactions with other bacteria. These findings are commensurate with the published work for detection of hlyA gene (Paziak-Domanska *et al.*, 1999), plcA and prfA genes (Notermans *et al.*, 1991a), iap gene (Furrer *et al.*, 1991) and actA gene (Suarez and Vazquez-Boland, 2001).

The multiplex PCR was standardized for detection of three major serotypes of *L. monocytogenes* viz., 1/2a, 1/2b and 4b by targetting various genes like Imo0737, ORF2819, ORF2110 and prs coding for proteins like unknown protein, putative transcriptional regulator, putative secreted protein and putative phosphoribosyl pyrophosphate, respectively. During the study, there was an absence of a 691 bp amplification product, corresponding to serotype 1/2a and serotype 1/2b was also not detected, since none of the isolates showed only amplification of 370 bp and 471 bp product. All the

three isolates showed amplification of three molecular size bands viz., 471 bp, 597 bp and 370 bp corresponding to their genes, ORF2819, ORF2110 and prs, respectively. This was in accordance with the results obtained for the aforementioned genes by Doumith *et al.* (2004) confirming that all the isolates to be from serotype 4b and this is the first report of isolation of *L. monocytogenes* serotype 4b from bovine clinical mastitis. The virulence of *Listeria* also depends upon serovar. Menudier *et al.* (1991) reported that serovar 4b was found to be more virulent as compared to other serovars (1/2a and 1/2c) and it helps to explain its association with listeriosis not only in animals but also in human beings. Furthermore, serotype 4b is the predominant serotype responsible for the animal listeriosis and *Listeria*-associated food-borne outbreaks, so it may be of immense importance to consider these three *L. monocytogenes* isolates for the further epidemiological investigation. A similar finding was also recorded by Yeh (2004), who observed 4b to be predominant serotype isolated from organic chicken carcasses.

In conclusion, recovery of potentially pathogenic *L. monocytogenes* from cow and buffalo mastitic milk samples signifies the zoonotic potential of listeriosis. Thus studies regarding epidemiological and zoonotic potential of this *L. monocytogenes* need special emphasis for improved diagnosis, control and surveillance measures in this part of the globe.

ACKNOWLEDGEMENT

We thank the Dean/Principal, College of Veterinary Science and Animal Husbandry, Anand, Gujarat, India for providing necessary facilities to carry out the research work.

REFERENCES

Annonymous, 1994. Bureau of Indian Standards: Microbiology - general guidance for the detection of *Listeria monocytogenes*. Committee Draft, CD, 11290.

Buchrieser, C., R. Brosch, B. Catimel and J. Rocourt. 1993. Pulsed-field gel electrophoresis applied for comparing *Listeria monocytogenes* strains involved in outbreaks. *Can. J. Microbiol.,* **39:** 395-401.

Coffey, A., F.M. Rombouts and T. Abee. 1996. Influence of environmental parameters on phosphatidylcholine phospholipase C production in *Listeria monocytogenes*: a convenient method to differentiate *L. monocytogenes* from other *Listeria* species. *Appl. Environ. Microbiol.,* **62:** 1252-1256.

Dhanda, M.R. and M.S. Sethi. 1962. Investigation of mastitis in India. *ICAR Res.* Series No. 35. New Delhi, India.

Doumith, M., C. Buchrieser, P. Glaser, C. Jacquet and P. Martin. 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *J. Clin. Microbiol.,* **42:** 3819-3822.

Dua, K. 2001. Incidence, etiology and estimated economic losses due to mastitis in Punjab and in India - An update. *Indian Dairyman,* **53:** 41-48.

Erdenlig, S., J.A. Ainsworth and F.W. Austin. 2000. Pathogenicity and production of virulence factors by *Listeria monocytogenes* isolates from channel catfish. *J. Food Protect.,* **63:** 613-619.

Farber, J.M. and P.I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev.,* **55:** 476-511.

Furrer, B., U. Candrian, C. Hoefelein and J. Luethy. 1991. Detection and identification of *Listeria monocytogenes* in cooked sausage products

and in milk by in-vitro amplification of haemolysin gene fragments. *J. Appl. Bacteriol.,* **70:** 372-379.

Graves, L.M., B. Swaminathan and S.B. Hunter. 1999. Subtyping *Listeria monocytogenes*, p. 251-297. *In* Ryser, E.T. and E.H. Marth (eds.) *Listeria, listeriosis and food safety.* Marcel. Dekker. Inc., New York, N.Y.

Hird, D.W. and C. Genigeorgis. 1990. Listeriosis in food animals: clinical signs and livestock as a potential source of direct (non-foodborne) infection for humans. *In* Miller, A.J., J.L. Smith, G.A. Somakuti (eds.) *Foodborne Listeriosis.* Elsevier Sci. Publ., Amsterdam, The Netherlands, 31p.

Leclercq, A. 2004. Atypical colonial morphology and low recoveries of *Listeria monocytogenes* strains on Oxford, PALCAM, Rapid'L.mono and ALOA solid media. *J. Microbiol. Meth.,* **57:** 251- 258.

Leimeister-Wachter, M., E. Domann and T. Chakraborty. 1991. Detection of a gene encoding a phosphatidylinositol-specific phospholipase C that is coordinately expressed with listeriolysin in *Listeria monocytogenes*. *Mol. Microbiol.,* **5:** 361-366.

McClain, D. and W.H. Lee. 1988. Devepolment of USDA-FSIS method for isolation of *Listeria monocytogenes* from raw meat and poultry. *J. Assoc. Offic. Anal. Chem.,* **71:** 660-663.

Menudier, A., C. Bosiraud and J.A. Nicolas. 1991 Virulence of *Listeria monocytogenes* serovars and *Listeria* spp. in experimental infection of mice. *J. Food Protect.,* **54:** 917-921.

Notermans, S.H., J. Dufrenne, M. Leimeister-Wachter, E. Domann and T. Chakraborty. 1991a. Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and non-pathogenic *Listeria* species. *Appl. Environ. Microbiol.,* **57:** 2666-2670.

Notermans, S.H, J. Dufrenne, T. Chakraborty, S. Steinmeyer and G. Terplant. 1991b. The chick embryo test agrees with the mouse bioassay for assessment of the pathogenicity of *Listeria* species. *Lett. Appl. Microbiol.,* **13:** 161-164.

Paziak-Domanska, B., E. Bogulawska, M. Wiekowska-Szakiel, R. Kotlowski, B. Rozalska, M. Chmiela, J. Kur, W. Dabrowski and W. Rudnicka. 1999. Evaluation of the API test, phosphatidylinositol-specific phospholipase C activity and PCR method in identification of *Listeria monocytogenes* in meat foods. *FEMS Microbiol. Lett.,* **171:** 209-214.

Radostits, O.M., D.C. Blood and C.C. Gay. 1994. *Veterinary Medicine,* 8th ed. ELBS Bailliere Tindall. London.

Rawool, D.B., S.V.S. Malik, I. Shakuntala, A.M. Sahare and S.B. Barbuddhe. 2007. Detection of multiple virulence-associated genes in *Listeria monocytogenes* isolated from bovine mastitis cases. *Int. J. Food Microbiol.,* **113:** 201-207.

Rocourt, J. and H.P.R. Seelinger. 1985. Distribution des especesdu genre *Listeria. Zbl. Bakt. Hyg.,* **259:** 317-330.

Schlech, W.F., P.M. Lavigne, R.A. Bortolussi, A.C. Allen, E.V. Haldane, A.J. Wort, A.W. Hightower, S.E. Johnson, S.H. King, E.S. Nicholls and C.V. Broome. 1983. Epidemic listeriosis-evidence for transmission by food. *N. Engl. J. Med.,* **308:** 203-206.

Seeliger, H.P.R. and K. Hohne. 1979. Serotyping of *Listeria monocytogenes* and related species. *Methods Microbiol.,* **13:** 31-49.

Shakuntala, I., S.V.S. Malik, S.B. Barbuddhe and D.B. Rawool. 2006. Isolation of *Listeria monocytogenes* from buffaloes with reproductive disorders and its confirmation by

polymerase chain reaction. *Vet. Microbiol.,* **117:** 229-234.

Shome, B.R., I. Shakuntala, R. Shome and A. Kumar. 2003. Isolation of *Listeria monocytogenes* from mastitis case in Holstein-Friesian cattle. *Indian Association of Veterinary Microbiologists, Immunologists and Specialists in Infectious Diseases. Annual Conference,* Umiam, Barapani, 7 Feb. Abs. No. PP:05, 52p.

Stewart, J. E. 1998. *Listeria monocytogenes.* http:www.bact.wisc.edu/scienceEd/Listeriamonocytogenes.html.

Suarez, M. and J.A. Vazquez-Boland. 2001. The bacterial actin nucleter protein ActA is involved in epithelial cell invasion by *Listeria monocytogenes.* PUBMED [Accession No. AF103807].

Tappero, J.W., A. Schuchat, K.A Deaver, L. Mascola and J.D. Wenger. 1995. Reduction in the incidence of human listeriosis in the United States. Effectiveness of prevention efforts. *JAMA-J. Am. Med. Assn.,* **273:** 1118-1122.

Vazquez-Boland, J.A., M. Kuhn, P. Berche, T. Chakraborty, G. Domi′Nguez-Bernal, W. Goebel, B. Gonza′Lez-Zorn, J. Wehland and J. Kreft. 2001. *Listeria* Pathogenesis and Molecular Virulence Determinants. *Cl. Microbiol. Rev.,* **14:** 584-640.

Verma, S., R.C. Katoch, R. Chahota and A.K Mahajan. 2001. *Listeria monocytogenes* - A cause of mastitis in an Indian buffalo (*Bubalus bubalis*). *Buffalo Journal,* **3:** 425-427.

Yeh, E.T. 2004. *Characterization of Listeria monocytogenes isolated from retail organic chicken*. M. S. Thesis, University of Maryland, U.S.

---

*Continued from page 28

Quinn, P., C. Barros and D.G. Whittingham. 1982. Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J. Reprod. Fertil.,* **66:** 161-168.

Surani, M.A.H., S.C. Barton and M.H. Kaufman. 1977. Development to team of chimeras between diploid parthenogenetic and fertilized embryos. *Nature,* **270:** 601-603.

PREVALENCE OF DIFFERENT PATHOLOGICAL AFFECTIONS OF OVARIES IN BUFFALOES (*BUBALUS BUBALIS*) IN MALWA REGION OF MADHYA PRADESH

Durgesh Mittal, U.K. Garg, G.P. Jatav, Supriya Shukla and Rakesh Sharda

ABSTRACT

A total number of 504 buffaloes were examined for different affections of ovaries collected from the Cantonment Board Slaughter House, Mhow (M.P). The present study indicated that out of the total buffaloes examined, 33 animals showed various pathological conditions of the ovary. Various ovarian affections observed during the present study revealed par ovarian cyst, ovarian hypoplasia, ovarian sclerosis, ovarian cysts i.e. follicular cyst and luteal cyst, embeded corpus luteum, intra follicular heamorrhage and oophoritis. Out of the total affected buffaloes, the incidence of par ovarian cyst was the highest (2.7%) followed by follicular cyst (1.78%), oophoritis (0.59%), ovarian Sclerosis (0.39%), embeded corpus luteum (0.39%), ovarian hypoplasia (0.19%), luteal cyst (0.19%) and intra follicular heamorrhage (0.19%).

Keywords: buffalo, embeded corpus luteum, follicular cyst, intra follicular heamorrhage, luteal cyst, oophoritis, ovarian hypoplasia, ovarian sclerosis and par-ovarian cyst

INTRODUCTION

The buffalo is the predominant domestic animal for milk and meat production in India. On average, buffaloes are about four times as the productive as average indigenous cows in India. India has the world's best dairy buffalo breeds and provides superior buffalo germplasm to several countries of the world (Kaikini, 1992). In our country, there are 93.8 million buffaloes (Anon, 2000), which contribute to more than half of the total buffalo population (164.9 million) in the world. Recently, India has emerged as the largest milk producer in the world. In spite of the huge buffalo population, animal husbandry and dairy sectors do not provide greater percentage of total agricultural income as low productivity of buffaloes is considerably affected by the inherent problems like late maturity, poor oestrus expressiveness in the female particularly during summer, long post partum interval, diseases of genital system and infertility. The present investigation was carried out to asses the health of the female genital organs with special reference to the ovary to observe the different pathological conditions in buffaloes.

MATERIALS AND METHODS

The materials for the present study were obtained from buffaloes brought from the different parts of the Malwa region and slaughtered as a source of meat at the Cantonment Board Slaughter House, Mhow, (M.P.). The ovaries of a total of 504 buffaloes ranging from 3 to 12 years of age were examined in-situ for gross abnormalities, if any. After this the ovaries were collected, brought to the laboratory for a careful examination of

Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Mhow, M.P., India

pathoanatomical abnormalities, wherever present. The ovaries were opened by frontal incision extending from free border to attached border; exposed parenchyma was examined for change in colour, nature of the fluid and alterations, if any. The observations were recorded and the affected organ was preserved in 10% formalin. After 48 to 72 h, formalin preserved tissues were washed overnight in running tap water, dehydrated in ascending grades of alcohol, cleared in benzene and embedded in paraffin wax of 60 - 62°C melting point. Sections of 4-6 micrometer thickness were cut through a Spencer's rotary microtome and stained with H & E as per the standard procedure recommended by Lillie (1954).

RESULTS AND DISCUSSION

Out of various affections, the ovarian affections were observed in 33 cases of buffaloes. Various affections observed included ovarian hypoplasia, ovarian sclerosis, ovarian cysts i.e.follicular cyst and luteal cyst, embeded corpus luteum, intra follicular heamorrhage and oophoritis (Table1).

The ovary and uterus involved directly with regular oestrus cycle and other reproductive processes like development of pregnancy, parturition, etc. Any alteration in hormonal balance or malnutrition makes this organ more prone to subsequent development of pathological lesions in the ovary and uterus (Cohrs, 1967; Jones and Hunt, 1983).

Ovarian hypoplasia was observed in one (0.19%) buffalo and was associated with underdeveloped genitalia and this finding was in agreement with the observation of Hansen (1970). Ovarian hypoplasia need be distinguished from functional anoestrus in which the ovaries are larger, rounded having smooth surface and the genital tract better developed. Gilmore (1949); Lagerlof and Boyd (1953) pointed that gonadal hypoplasia was hereditary and caused by two recessive genetic factors.

The occurrence of sclerosed ovaries was noticed in two (0.39%) animals as also reported by Rao and Rajya (1976). During the present study such lesions were mostly seen in the abattoir animals having poor conditions and, therefore, malnutrition might have significant role to play, as also mentioned by Elwishy (1965); Rao and Rajya (1976). However, El-Sawaf and Schmidt (1963) opined that the ovarian sub activity could be due to hypofunction of the thyroid as evidenced by low blood levels of thyrotrophic hormones in buffaloes having sclerosed ovaries (Abdo, 1962; Naser *et al.*, 1963).

The occurrence of follicular cyst was seen in nine (1.78%) cases, which corroborated with the reports of previous workers (Khan, 1970; Bhattacharya *et al*., 1970; Rao and Murthy, 1971 and Ohasi *et al.*, 1984). A higher incidence of 2.83 - 7.7% was observed by El-Sawaf and Schmidt (1963); Hansen (1970) and Potekar and Deshpande (1981). Garm (1949) considered the increased production of mineralocorticoids by the zona glomerulosa of the adrenals as the main factor responsible for the development of follicular cysts.

The incidence of luteal cyst (0.19%) observed in this study, appeared almost in consonance with the observations of Saxena (1966); Khan (1970); Luktuke *et al.* (1973) and Rao and Rajya (1976), but contradictory with the observation of Damodaran (1956), who reported a higher incidence of 1.15%.

The occurrence of par ovarian cysts was noticed in 2.7% of the cases. The par ovarian cysts develop from the vestigial remnant of Wolffian body and are not considered to have any bearing on reproductive potential of the animal (Dawson, 1963). However, if sufficiently large in size, it can create

Table 1. Pathological conditions observed in the ovaries of slaughtered female buffaloes.

S. No.	Pathological conditions	No. of cases	Overall percentage out of total animals examined
1	Par Ovarian cyst	14	2.70
2	Ovarian Hypoplasia	1	0.19
3	Ovarian Sclerosis	2	0.39
4	Follicular Cyst	9	1.78
5	Luteal Cyst	1	0.19
6	Embeded Corpus Luteum	2	0.39
7	Intra Follicular Haemorrhage	1	0.19
8	Oophoritis	3	0.59

problem in clinical diagnosis per rectum and, thus, is important from differential diagnostic point of view.

Embeded corpus luteum was encountered in 0.39% cases. Almost similar incidence was reported by Shalash and Salama (1960) the continuous production of progesterone from such ovaries might enhance the anestrous condition in the animals.

The intra follicular haemorrhage (0.19%) encountered in this investigation was similar to the observations made by Bhattacharya *et al.* (1954); Damodaran (1956); Sharma *et al.* (1967); Rao and Rajya (1976). Chronic oophoritis was encountered in three buffaloes (0.59). These conditions have also been recorded by Bhattacharya *et al.* (1954) and Rao and Rajya (1976).

REFERENCES

Abdo, M.S. 1962. Local cited from Naser *et al*., 1963. Level of Gonadotropic, Thyrotrophic and ACTH in the Blood of Buffaloes with Inactive Ovaries. IVth Arabian Vet. Congress, Cairo.

Anon. 2000. F.A.O., Production Year Book. Rome. **54:** 210-211.

Bhattacharya, P., S.N. Luktuke, A.S.P. Rao and S.K. De. 1954. Incidence of fertility under 5 various casual groups in buffalo cows in India. *Curr. Sci.*, **23:** 335.

Cohrs, P. 1967. *Special Pathological Anatomy of Domestic Animals*. First English ed. Pergamon Press Ltd., New York, London.

Damodaran, S. 1956. *Studies on genital pathology of the female buffalo* (*Bos bubalis*). M. V. Sc. Thesis, Madras.

Dawson, F.L.M. 1963. Uterine pathology in bovine infertility. *J. Reprod. Fertil.,* **5:** 397-407.

Elwishy, A.B. 1965. Some clinical studies on diagnosis of pregnancy and fertility in buffalo cows and possible treatment. *Vet. Med. J. Cairo.,* **11:** 7.

El-Sawaf, S.A. and Schmidt. 1963. Morphological changes in normal and abnormal ovaries of buffaloes with special reference to their function. *Vet. Med. J. Cairo.*, **8:** 249.

Garm, O. 1949. Investigation on cystic ovarian degeneration in the cow with special regard

to etiology and pathogenesis. *Cornell Vet.*, **39:** 52.

Gilmore, L.O. 1949. The inheritance of functional causes of reproductive efficiency. A review. *J. Dairy Sci.*, **32:** 71.

Hansen, H.J. 1970. Some traits of pathological anatomy of the genital system of the Egyptian buffalo cow. Lecture Notes. V.F.A.O./SIDA. International course on Vety. Pathology.

Jones, T.C. and R.D. Hunt. 1983. *Veterinary Pathology*, 5th ed. In proceedings of Lea and Febiger, Philadelphia. 358p.

Kaikini, A.S. 1992. Dimensions of infertility or sterility in cattle and buffaloes. *Indian J. Anim. Reprod.*, **13:** 10.

Khan, B.V. 1970. Incidence of various disorders of bovine ovary. *J. Remount Vet. Corp.*, **9:** 2.

Lagerlof, N. and H. Boyd. 1953. Ovarian hypoplasia and other abnormal condition in the sexual organs of cattle of Swedish Highland breed. Result of postmortem examination of over 6000 cows. *Cornell Vet.*, **43:** 64.

Lillie, R.D. 1954. *Histologic Technique and Practical Histochemistry*. The Blakistan div. McGraw Hills Book Company, Toronto.

Oshasi, O.M., V.R. Filho, W.G. Vale and J.S. De Sousa. 1984. Occurrence of genital lesions in buffalo cows slaughtered at the abattoir, I. Abnormalities of ovary and uterine tube. *Arq. Bras. Med. Vet. Zootec.,* **36:** 29-38.

Potekar, A.M. and B.B. Deshpande. 1981. Studies on pathological conditions of female genital organs in Indian buffaloes. *Indian Vet. Path.*, **5:** 91.

Rao, P.R. and B.S. Rajya. 1976. Pathoanatomy of the female genital tract of buffaloes. *Indian J. Anim. Sci.*, **46:** 125-130.

Saxena, S.C. 1966. Studies on some aspects of impaired infertility in bovines. A clinical approach. M. V. Sc. Thesis. Agra University, Agra.

Sharma, O.P., R.C. Bhalla and R.K. Soni. 1966. Abnormalities of cervics of buffalo cows. *Indian J. Anim. Hlth.*, **5:** 111.

A STUDY OF MANAGEMENTAL PRACTICES IN WATER BUFFALO (*BUBALUS BUBALIS*) IN INDIA

R.R. Ahirwar[1], Ashok Singh[2] and M.I. Qureshi[3]

ABSTRACT

Comparative evaluation of farmers of rural and urban areas of Indore district of Madhya Pradesh, India was undertaken in terms of various managemental practices followed on the basis of herd size among different classes of farmers, and their housing, feeding, breeding and health coverage practices in buffaloes. In rural areas, a significantly higher number (59.33%) of farmers had mud houses with mud floors, whereas in urban areas, 68% farmers had Kiln-dried brick houses with concrete floors. In rural areas, the space available per animal was adequate in all cases but in urban areas, 68% of the respondents had inadequate space per animal. Feeding of green fodder throughout year in both rural and urban areas was practiced. A significantly higher number (88%) of urban farmers offered balanced ration to their animals as compared to rural farmers. In rural areas, only 9.66% of the farmers bred their animals with A.I., and 90.33% preferred natural service. More urban farmers followed cross breeding and grading up as breed improvement practices as compared to rural farmers. The analysis revealed that the rural buffalo gives less profit in comparison to those in urban areas due to lack of scientific animal husbandry practice and the low price of milk in addition to poor fluid milk marketing.

Keywords: water buffaloes, managemenrtal practice, management, housing, feeding, breeding

INTRODUCTION

The role of dairy farming in the Indian rural economy is very outstanding. The significance of bovine economy is heightened by its massive contribution to livelihood of India's rural population. Over 73 percent of India's households have their own livestock. Tending, grazing, feeding and milking cows and buffaloes is one the largest sources of productive employment in rural India. In Madhya Pradesh state, especially in rural areas, the majority of buffalo owners are agriculture farmers and have not yet developed a commercial attitude towards dairy farming. The investigation on the managemental practices for dairy animals, especially buffaloes, is limited. The study has been carried out to determine the status of rural and urban farmers and the housing, feeding, breeding and health coverage practices in buffalo in urban and rural areas, to compare various managemental practices with respect to herd size among different classes of farmers, and to compare the cost of milk production in urban and rural areas.

[1]Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur (M.P.) India

[2]Department of Animal Breeding and Genetics, College of Veterinary Science and Animal Husbandry, MHOW (M.P.) India, E-mail: drashoksingh2006@yahoo.com

[3]Department of Livestock Management and Production, College of Veterinary Science and Animal Husbandry, MHOW (M.P.) India

MATERIALS AND METHODS

This study was conducted in Indore district of Madhya Pradesh in India. A total of 400 farmers, i.e. 100 farmers from urban and 300 farmers from rural areas were selected randomly. In the present study, general information viz. caste, categories of farmers, land holdings, education background, occupation, type of farming, size of dairy and financial support of the dairy by the government/or private agencies, and farmers themselves/ was obtained.

Managemental practices information was obtained on the following aspects: 1. Housing management - type of housing, type of floor, system of housing, space available per animal, light and ventilation, drainage system. 2. Feeding management - grazing of animal, feeding of green fodder, dry fodder, and concentrate, processing of roughages, feeding of common salt and mineral mixture, source of drinking water and maintenance of feed records. 3. Breeding programme - system of breeding, bull used for service, breed improvement practices, regularity in heat, and age at first calving, treatment of anoestrus. 4. Attitude of farmers towards health practices - treatment of animals, sick animals treated, deworming practices, control of ticks, and vaccination. 5. Milking practices and disposal of milk - Milking in clean separate place, washing the hind quarter, bathing of animal before evening milking, method of milking, frequencies of milking, milk production, disposal of milk, a Agency to which milk was sold and selling price. 6. Cost of milk production per litre - cost of fodder both green and dry, concentrate, cost of labour both hired and owned, cost of veterinary aid, cost of miscellaneous items such as mineral mixture, gur, salt, oil, rope and chain etc.

The farmers were interviewed and all managemental practices were observed personally. Frequencies were obtained for different managemental practices. Data were converted into percentage to draw inferences. The Chi-square test was used to determine the degree of association between different variables (Snedecor and Cochran, 1994).

RESULTS AND DISCUSSION

General information of dairy farmers

The present study revealed that the majority of the buffalo farmers in both rural and urban areas belonged to Other Backward Caste. Their main occupation was agriculture farming, and dairy farming was a subsidiary occupation. No significant difference was observed between rural and urban areas with regards to the education of farmers. Regarding landholding by farmers, the majority (59%) do not have any agriculture land; they engaged only in their dairy farming business. A similar trend was also reported by Malik and Nagpaul (1998). A significant ($P<0.01$) association was observed between urban and rural areas with regards to the size of buffalo herd. The majority of rural farmers had small size dairies (1-5 dairy animals) but in urban areas, there were medium to large size buffalo herds because of the fact that the majority of the urban farmers were commercial dairy farmers. Similar trends were observed by Malik and Patel (1987).

Housing management

A highly significant ($P<0.01$) difference was observed on the type of housing for buffaloes. The majority of the rural farmers (59.33%) had mud houses according to their economic status. In comparison to rural areas, a significantly ($P<0.01$) higher percentage of buffalo farmers had Kiln-dried brick (pacca) houses (42%) in urban areas.

The results of present finding revealed that significantly ($P<0.01$) higher number of animal sheds

had mud floors and improper drainage system in rural areas.

Regarding the housing system, a significantly higher (P<0.01) percentage of urban buffalo farmers had a two-row system (tail to tail or head to head) in comparison to rural farmers.

In rural areas, the availability of space per animal was adequate in compare to urban area. A significantly (P<0.01) higher number of respondents had inadequate light and ventilation (70.33%) in rural areas as compare to urban areas.

Feeding management

A majority of farmers in both urban and rural areas provided green fodder thoroughout the year. The majority of the farmers cultivated green fodder in rural areas and farmers provided green fodder to pregnant and productive animals. The traditional feeding system of buffalo is generally free grazing. The majority of farmers in rural (69.33%) areas sent their animals out for grazing. In the study it was observed that majority of the farmers in rural and in urban area provided their animals dry fodder on the basis of milk yield. The majority (70%) of the rural farmers did not provide a balanced ration to their animals, whereas a significantly higher percentage (88%) of urban farmers provided their animals balanced ration. Most of the farmers in rural areas (75.33%) provided home grown concentrate like wheat bran, gram / chunie etc. but urban farmers purchased concentrate from the market. A significant difference was observed in the feeding of common salt and mineral mixture to the buffaloes. Rural and urban buffalo farmers did not maintain any records of feed and fodder at the dairy farm. The majority of the rural farmers (80.33%) provided drinking water from a tube well, but in urban areas, 74% farmer supplied drinking water to their animals with a hand pump or by using tap water.

System of breeding

Both rural and urban buffalo farmers prefer natural service as system of breeding, but a significant (P<0.01) difference was observed in adoption of artificial insemination practice. A higher number of urban farmers prefer A.I. In urban areas, the farmers cross their animals from proven sire breeding bulls, but in rural areas, farmers prefer breeding through bull of the grazing herd. The breed improvement practice urban farmers followed were crossbreeding and grading up programmes. As we know, regularity in the oestrus cycle is a vital factor in determining the efficiency of animal production. The best buffalo is likely to produce a single calf per year, but this is only possible when the buffalo is regular in its oestrus cycle. A significantly higher number of buffaloes come in heat regularly in urban areas.

The age at first calving ranged from 50 to 55 months and from 42 to 45 months for rural and urban buffaloes, respectively. The majority of the farmers in rural and urban areas milk their buffaloes in the same shed in which they are kept. Farmers in urban areas wash the whole body of the animals prior to evening milking. Washing of hindquarters was not followed by either category of farmers. The washing of udder before milking was significantly different between urban and rural areas. A total 94% of the urban farmers adopted a hygienic method of milking. The majority of farmers of urban and rural area used the knuckling or thumb-in methods of milking, but the full hand method of milking is an ideal and scientific method. It was beliered by the urban farmers that the full hand method of milking causes pain to their hand, whereas the knuckling method was more comfortable but leads to udder injury and consequently mastitis. Farmers follows a twice-a-day milking schedule in both rural and urban areas. Similar observations were reported by Handa and Gill (1986).

Table 1. General information of farmers of buffalo herd.

S. No.	Description	Percentage	
		Rural Total Number = 300	Urban Total Number = 100
1	Caste wise distribution of dairy farmers		
	1.General	8 (24)	14
	2.Other Backward caste	78 (234)	79
	3. Schedule caste	3.33 (10)	5
	4. Schedule tribe	10.66 (32)	2
2	Occupation of dairy farmers		
	1. Dairy + Agriculture	68.33 (205)	36
	2. Dairy + Agriculture + Service	18 (54)	2
	3. Dairy + Agriculture + Business	8.33 (25)	6
	4. Dairy + Labour	2 (6)	18
	5. Dairy + Service	2.33 (7)	3
	6. Dairy alone	1 (3)	35
3	Educational Background of farmers		
	1. Illiterate	8.66 (26)	2
	2. Primary school	46.33 (139)	16
	3. Middle school	22.66 (68)	21
	4. Metric	17 (51)	42
	5. Graduate	4 (12)	10
	6. Post graduate	1.33 (4)	9
4	Land holding of dairy farmers		
	1. Landless	4.66 (14)	59
	2. Marginal farmers (<2.5 acres)	11.33 (34)	2
	3. Small farmers (2.5 to 5 acres)	54.66 (164)	28
	4. Large Farmers (> 5 acres)	29.33 (88)	11
5	Type of dairy farming		
	1. Dairy farming alone	3 (9)	56
	2. Mixed farming	97 (291)	44
6.	Size of dairy		
	1. Small (1-5 buffaloes)	27.66 (83)	2
	2. Medium (6-10 buffaloes)	48 (144)	58
	3. Large (> 10 buffaloes)	24.33 (73)	40
7.	Financial Support		
	1. Self finance	96.33 (289)	88
	2. Bank loan	2.66 (8)	12
	3. Government subsidy	1 (3)	-

Table 2. Housing management practices of buffalo herd.

S. No.	Description	Percentage	
		Rural Total Number = 300	**Urban Total Number = 100**
1	Type of Housing		
	1. Kiln-dried brick (Pacca) house	40.66 (122)	68**
	2. Mud house	59.33** (178)	27
	3. No housing	-	5
2	Type of floor		
	1. Concrete	31.33 (94)	82**
	2. Mud floor	65.33** (196_	13
	3. Brick floor	3.33 (10)	5*
3	Drainage system		
	1. Proper	24.66 (74)	79**
	2. Improper	75.33 (226)	21
4	System of housing		
	1. Single line	89.33 (268)	58
	2. Head to Head	3 (9)	12
	3. Tail to Tail	7.66 (23)	30**
5	Space available		
	1. Adequate	61 (183)	32
	2. Inadequate	39 (117)	68
6	Light and Ventilation		
	1. Adequate	29.66 (89)	86
	2. Inadequate	70.33** (211)	14

Table 3. Feeding management practices of buffalo herd.

S. No.	Description	Percentage	
		Rural Total Number = 300	Urban Total Number = 100
1	Green fodder		
	1. Yes	71.33 (214)	68
	2. No	28.66 (86)	32
2	Green fodder		
	1. Grown	81.66 (245)	18
	2. Not grown	18.33 (55)	82
3	Criteria for feeding green		
	1. Milk yield	100 (300)	100
	2. Body weight	-	-
4	Buffaloes sent for grazing		
	1. Yes	69.33 (208)	13
	2. No	30.66 (92)	87
5	Space available		
	1. Adequate	61 (183)	32
	2. Inadequate	39 (117)	68
6	Type of dry fodder provided to buffaloes		
	1. Soya bean straw	72 (216)	100
	2. Wheat Bhusa	8 (24)	-
	3. Wheat + gram Bhusa	20 (60)	-
7	Feeding balanced ration		
	1. Yes	30 (90)	88**
	2. No	70 (210)	12
8	Purchase of concentrate mixture		
	1. Home made	75.33 (226)	-
	2. Purchase	24.66 (74)	100
9	Feeding of common salt		
	1. Yes	6 (18)	41**
	2. No.	94** (282)	59
10	Feeding of mineral mixture		
	1. Yes	11 (267)	92**
	2. No	89 (33)	8
11	Feed Record keeping		
	1. Yes	-	18
	2. No	100 (300)	82
12	Source of drinking water		
	1. Well	13.33 (40)	-
	2. Pond	6.33 (19)	-
	3. Tube well	80.33 (241)	83
	4. Tap water	-	17

Table 4. Breeding management and milking practices of buffalo herd.

S. No.	Description	Percentage	
		Rural Total Number = 300	**Urban Total Number = 100**
1	System of breeding		
	1. Natural service	90.33 (271)	86
	2. Artificial Insemination	9.66 (29)	14*
2	Bull used for Natural Services		
	1. Non descript	73** (219)	5
	2. Breeding bull	27 (81)	95**
3	Breed Improvement practices		
	1. Crossbreeding	16 (48)	24**
	2. Grading up	-	5*
	3. None	84 (252)	71
4	Buffaloes send for grazing		
	1. Yes	69.33 (208)	13
	2. No	30.66 (92)	87
5	Regularity in estrus cycle		
	1. Yes	18.66 (56)	59**
	2. No	81.33** (244)	41
6	Treatment of anoestrus		
	1. Veterinary Treatment	71.66 (218)	89**
	2. Local (Deshi) Treatment	27.33** (82)	11
7	Milking of buffaloes in clean & separate Place		
	1. Yes	-	28**
	2. No	100 (300)	72
8	Bathing of buffaloes		
	1. Yes	15 (45)	66**
	2. No	85 (255)	34
9	Washing of Hind quarter before milking		
	1. Yes	5.66 (17)	22**
	2. No	94.33 (283)	78
10	Washing of Udder before milking		
	1. Yes	30.33 (91)	94**
	2. No	69.66** (209)	6
11	Method of milking		
	1. Full hand	11.33 (34)	8
	2. Knuckling method	88.66 (266)	92**
12	Frequency of milking		
	1. Once	-	-
	2. Twice	100 (300)	100

Table 5. Disposal of milk and disease control practices of buffalo herd.

S. No.	Description	Percentage	
		Rural Total Number = 300	**Urban Total Number = 100**
1	Quantity of milk consumed		
	1. At Home	22.33	11
	2. Sold in market	77.66	89
2	Agencies to whom milk was sold		
	1. Co operative society	20	17
	2. Milk Vender	6	2
	3. Direct to consumers	74	81
3	Treatment of sick buffaloes		
	1. Yes	100 (300)	100
	2. No	-	-
4	Sick buffaloes treated by		
	1. Self	5.66 (16)	4
	2. Assitant Veterinary Field Officer	65** (195)	18
	3. Veterinary Assistant Surgeon	29.33 (89)	78**
5	Regular Deworming		
	1. Yes	-	58**
	2. No	100 (300)	42
6	Control of Ticks of buffaloes		
	1. Yes	2.66 (8)	19**
	2. No	97.33 (292)	81
7	Vaccination		
	1. FMD	62 (186)	96**
	2. H.S	47 (141)	39
	3. B.Q.	34 (102)	21

Disposal of milk and disease control practices

The majority of the farmers consult veterinary staff for treatment of their buffaloes preferably the veterinary assistant surgeon and assistant veterinary field officer. Deworming and control of ectoparasites of animals is not a regular practice by either rural and urban farmers. The prophylactic practice results were very encouraging. The majority of the farmers in rural and urban areas had their animals vaccinated against H.S., B.Q. and F.M.D. The analysis revealed that the rural buffalo gives less profit in comparison to the urban buffalo due to scientific animal husbandry practice adopted by urban farmers and attractive price of buffalo milk and better fluid milk marketing.

REFERENCES

Handa, M.C. and R.S. Gill. 1986. Managemental practices adopted by different categories of farmers in Ludhiana district. *Indian J. Anim. Prod. Mgmt*., **2**: 78-88.

Malik, D.S. and P.K. Nagpaul. 1998. Studies on housing and feeding management practices of Murrah buffalo in home tract of Haryana. *Indian J. Anim. Prod. Mgmt*. **14**(3): 186-188.

Malik, Mahak and R.K. Pate. 1987. Status of dairying in rural areas of Nabha, Punjab. *Indian J. Anim. Prod. Mgmt*. **14**(3): 171-176.

Snedecor, G.W. and W.G. Cochran. 1994. *Statistical Methods*, 6th ed. Oxford and IBH Publishing Co., New Delhi.

COAGULASE GENE BASED MOLECULAR DETECTION OF *STAPHYLOCOCCUS AUREUS* DIRECTLY FROM MASTITIC MILK SAMPLES OF MURRAH BUFFALO

Neelesh Sindhu[1], Anshu Sharma[2] and V.K. Jain[1]

ABSTRACT

The study was conducted to detect *Staphylococcus aureus* directly in mastitic milk of Murrah buffaloes using coagulase gene based specific polymerase chain reaction assay. Out of 628 samples, a total of 140 samples were found positive with four amplified products of size 960 bp, 870 bp, 740 bp and 610 bp in 8.57 percent, 19.28 percent, 29.29 percent and 42.85 percent of the milk samples, respectively. On PCR examination of *Staphylococcus aureus* found positive by bacteriological examination and biochemical tests, similar amplified products were observed in 9.37 percent, 21.09 percent, 32.03 percent and 37.5 percent of culture isolates (n=128) respectively. Ubiquitous PCR assay with amplified product of size 108 bp was used as an internal control for detection of *Staphylococcus aureus.* By this assay, nonviable *Staphylococcus aureus* could also be detected in milk samples of animals treated with antibiotics The study revealed that several coagulase gene types are responsible for genetic heterogeneity among *Staphylococcus aureus* isolated from mastitis cases in buffaloes and predominance of these amplified products shows significant variation over time paving way for understanding of epidemiology of mastitis in a particular location.

Keywords: mastitis, *Staphylococcus aureus*, coa gene, Murrah

INTRODUCTION

Buffaloes constitute about 35 percent of the bovine population but contribute more than 55 percent to the total milk production in India (Kumar *et al.*, 2007). India has about 22 breeds of riverine buffaloes (Ahlawat *et al.*, 2006) of which the Murrah breed, found most abundantly in Haryana State, is capable of milk yields as high as 35 litres a day (http://www.haryana-online.com/murrah.htm). Despite intense research and control programs, bovine mastitis has remained a major economic problem of the dairy industry (Dua, 2001; Sasidhar *et al.*, 2002; Hillerton and Berry, 2005; Halasa *et al*., 2007; Huijps *et al*., 2008; Denis *et al.*, 2009). In India, a plethora of bacteria have been isolated and designated as etiological agents of mastitis in buffaloes, but *Staphylococcus aureus* has been reported as the major pathogen (Dang *et al*., 2007; Sahay *et al*., 2007; Sharma and Sindhu, 2007; Sindhu *et al.*, 2008). Polymerase chain reaction (PCR) amplification of the 3' end coding variable region of coagulase gene has been considered a candidate for DNA diagnostic assay for identification of *Staphylococcus aureus* in cow mastitis (Aarestrup *et al.*, 1995; Guler *et al*., 2005; Da Silva and Da

[1]Department of Veterinary Clinical Medicine, Ethics and Jurisprudence, College of Veterinary Sciences, CCSHAU, Hisar-125004, Haryana, India

[2]Veterinary College Central Laboratory, College of Veterinary Sciences, CCSHAU, Hisar-125004, Haryana, India

Silva, 2005; Vimercati *et al.*, 2006; Kalorey *et al.*, 2007) but there are very few reports of this technique being used in buffaloes (Vieira-da-Motta *et al.*, 2001). The study was carried out to detect *Staphylococcus aureus* directly from mastitic milk samples of Murrah buffaloes using coagulase gene based PCR assay and to compare the results with bacteriological examination.

MATERIALS AND METHODS

Collection of milk samples: A total of 628 milk samples collected from functional quarters of lactating Murrah buffaloes from organized farms and individual farmers brought from various parts of Haryana to the Veterinary College Central Laboratory, COVS, CCS Haryana Agricultural University, Hisar, were included in the present study.

Bacteriological examination: Ten microlitres of milk from each sample was streaked on five percent sheep blood agar plates and MacConkey's lactose agar plates separately and incubated for 24 h at 37°C. The resulting growth from respective plates of media was examined for colony characteristics, morphology, Gram's reaction and haemolysis patterns. All Gram positive, catalase positive and oxidase negative isolates were identified as *Staphylococcus* spp. Staphylococcal isolates were further classified as coagulase positive and coagulase negative on basis of standard citrate rabbit plasma coagulase test (Gibbs and Skinner, 1966). Coagulase positive isolates were further characterized biochemically by thermostable nuclease test (Faruki and Murray, 1986), latex agglutination test (Staph latex test kit, HiMedia, Mumbai) and mannitol fermentation.

DNA extraction directly from milk: DNA from milk samples was extracted by the method described by Phuektes *et al.* (2001) with some modifications. In brief, 1.5 ml of milk sample was centrifuged and the pellet suspended in 600 µl NTE buffer. After vortex, the suspension was treated with 100 µl of 24% sodium dodecyl sulphate and incubated in water-bath at 80°C for 10 minutes. The suspension was then digested using 12 µl of proteinase K (20 mg/ml, Fermentas, USA) and 2.5 µl of Ribonuclease A (Fermentas, USA) and incubated in a water-bath at 56°C for 2 h. 100 µl of 5M NaCl and 80 µl of CTAB-NaCl was then added and incubated in a water-bath at 65°C for 10 minutes. Then phenol:chloroform:isoamyl alcohol (PCI) and chloroform:isoamyl alcohol (CI) extraction was done until the interface was clear. The resultant aqueous phase was collected and one-tenth volume of 3M sodium acetate (pH 5.2) and two volumes of chilled 100% ethanol were added and kept at -20°C for one hour for precipitation of the DNA. After centrifugation at 15000 g for 15 minutes at 4°C, ethanol was removed and washing of DNA pellet was done twice with 70% ethanol and then it was air-dried. Finally, the DNA was dissolved in 50 l of TE buffer and stored at -20°C till further use. Purity and concentration of the DNA isolated was recorded using a biophotometer.

DNA extraction from bacterial culture isolates: For extraction of DNA from bacterial culture isolates and standard strains, the rapid boiling method was followed. In brief, a single colony from overnight grown culture was inoculated in 25 µl of TE buffer and boiled at 99°C for 15 minutes and then cooled immediately by putting on ice. The resultant template DNA was stored at -20°C and 5 µl of each sample was used for PCR analysis.

Polymerase chain reaction assay (PCR): PCR reactions were standardized using different

magnesium chloride concentrations, Taq DNA polymerase concentrations, primer concentrations, annealing temperature and number of cycles in thermocycler (Bio-Rad icycler,USA). 5'-3' sequences of oligonucleotide primers taken were F: ACCACAAGGTACTGAATCAACG and R: TGCTTTCGATTGTTCGATGC. These were taken from a report published earlier (Aarestrup *et al.*, 1995). For internal control, ubiquitous PCR assay as described by Martineau *et al.* (1998) was performed. 5'-3' sequences of primers taken for ubiquitous PCR assay were F: AATCTT TGTCGGTACACGATATTCTTCACG and R:CGTAATGAGATTTCAGTAGAT AATA CAACA. The PCR reaction was performed in a thermocycler with a reaction volume of 25 µl. DNA isolated from pure bacterial culture of *Staphylococcus aureus* ATCC 25923 was taken as positive control, nuclease free water was taken as negative control, and PCR mixture without template was taken as PCR control to check for the possibility of contamination.

Analysis of PCR Products: After amplification, five microlitres of amplified products were subjected to electrophoresis in 2% agarose gel prepared using 0.5X Tris Borate EDTA (Amresco) containing ethidium bromide at a concentration of 0.2 µg/ml. A 100 bp gene ruler (Fermentas) was used as marker. Electrophoresis was carried out at 6.5 V /cm of gel in 0.5X TBE running buffer in submarine electrophoresis apparatus, using a power supply (Amersham Pharmacia Biotech) for one hour. The gel was visualized using UV transilluminator (Biovis Gel V4). Sensitivity of PCR primers was evaluated by using different dilutions (CFU/ml) of bacteria. Specificity of PCR primers was checked with milk samples inoculated with *Streptococcus dysgalactiae, Streptococcus agalactiae, Streptococcus uberis* and *E. coli.*

RESULTS AND DISCUSSION

The optimized reaction mixture for *coa* gene based assay contained 200 µm dNTP mix, 1X PCR buffer (with 10 mM Tris-HCl, pH 8.8, 50 mM KCl and 0.8% Nonidet P 40), 1.5 mM $MgCl_2$, 20 pmol of each primer, 2.5 U Taq DNA polymerase, and 200 ng of DNA extracted from milk and DEPC treated nuclease free water added to make reaction mixture 25 µl. PCR amplification was done with initial denaturation at 95°C for 5 minutes, 36 cycles each of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute followed by final extension at 72°C for 10 minutes. The optimized PCR protocol for ubiquitous PCR assay was similar to *coa* gene based assay except that concentration of $MgCl_2$ was found to be 2.5 mM. None of the streptococcal and *E.coli* isolates were found positive with PCR when amplified with primers encoding *coa* gene, showing 100 percent specificity of primers.

On bacteriological examination, out of 628 milk samples, a total of 238 samples (37.89 percent) were found culturally positive. Further characterization on basis of colony characteristics, morphology, Gram's reaction and haemolysis patterns revealed 156 staphylococci (65.55 percent), 72 streptococci (46.15 percent) and *E. coli* (6.41 percent). Staphylococcal culture isolates were further differentiated into 128 coagulase positive (53.78 percent) and 28 coagulase negative staphylococci (11.76 percent) on basis of standard citrate rabbit plasma coagulase test. All coagulase positive isolates were identified biochemically as *Staphylococcus aureus,* and no isolate of other coagulase positive Staphylococci (viz. *Staphylococcus intermedius* and *Staphylococcus hyicus*) was found. When these *Staphylococcus aureus* culture isolates were screened by *coa* gene based PCR under optimized conditions, all isolates were found positive revealing 100 percent sensitivity.

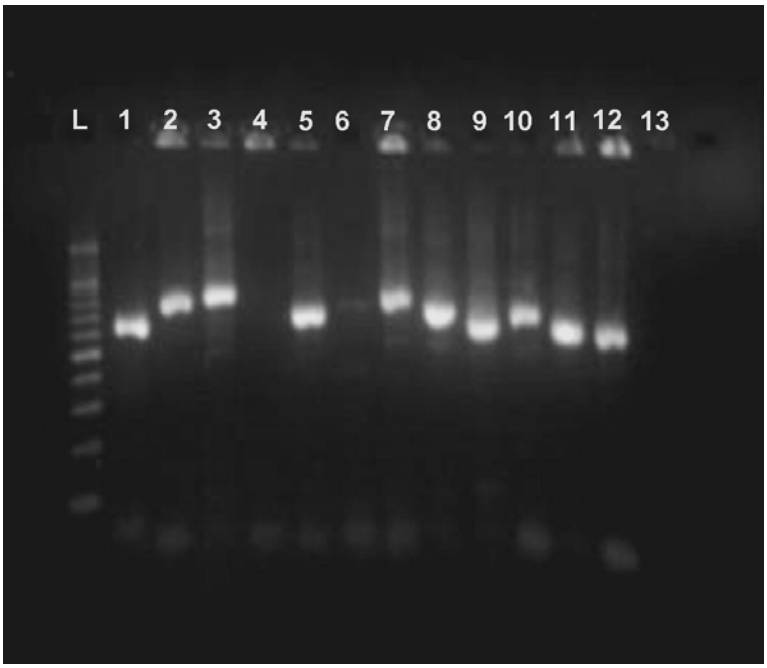

Figure 1. *Coa* gene amplification of *Staphylococcus aureus*.
Lane 1, 9, 11, 12: 610 bp; Lane 5, 6, 8, 10: 740bp;
Lane 2, 7: 870bp; Lane 3: 960 bp;
Lane 4: *coa* negative; Lane 13: Negative control;
Lane L: 100bp Ladder

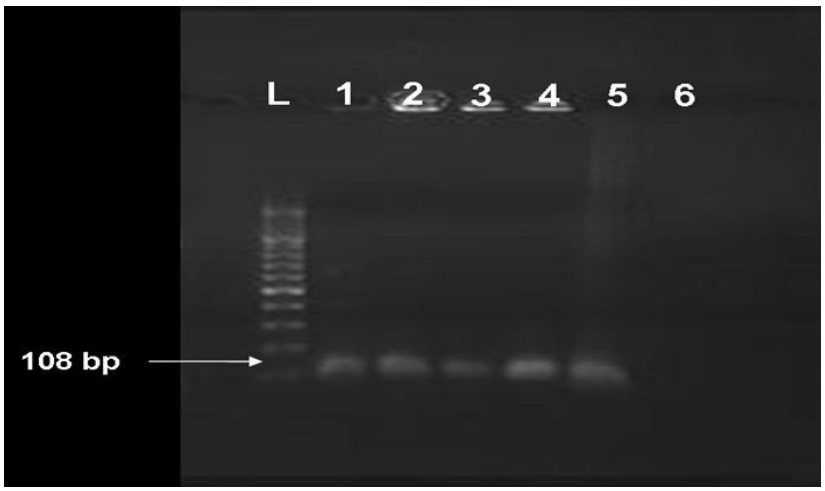

Figure 2. Ubiquitous PCR assay for *Staphylococcus aureus*.
Lane 1-4: *Staphylococcus aureus* Positive samples;
Lane 5: *Staphylococcus aureus* ATCC 25923;
Lane 6: Negative control with Nuclease free water;
Lane L: 100bp Ladder

Extensive polymorphism with four amplified products (Figure 1.) of size 960 bp, 870 bp, 740 bp and 610 bp was observed in 12 (9.37 percent), 27 (21.09 percent), 41 (32.03 percent) and 48 (37.5 percent) respectively. When these isolates were screened by ubiquitous PCR assay, all the *Staphylococcus aureus* isolates revealed amplified product of size 108 bp. (Figure 2).

When milk samples were subjected directly to PCR based on *coa* genes, a total of 140 (56 percent) samples were found to be positive with four amplified products of molecular weight 960 bp, 870 bp, 740 bp and 610 bp in 12 (8.57 percent), 27 (19.28 percent), 41 (29.29 percent) and 60 (42.85 percent) respectively All the culturally negative twelve samples which were tested positive by direct PCR with amplified product of 610 bp were found to be taken from buffaloes with histories of prior administration of antibiotics for treatment of mastitis, and this may have inhibited, the growth of bacteria. These "no-growth" samples pose a challenge for microbiological laboratories, veterinarians, and dairy producers. Failure of growth in as many as thirty percent of milk samples from clinical and subclinical bovine mastitis even after 48 h of conventional culture have been reported and identified by molecular analysis (Barlow *et al.*, 2008; Sharma *et al.*, 2009; Taponen *et al*., 2009).

The etiology of mastitis is diverse and varies significantly internationally as well as within regions within countries and between farms, and shows significant variation over time and between seasons on individual units (Bradley *et al.*, 2007). Analysis of coagulase-encoding *Staphylococcus aureus* DNA (*coa*) genes has demonstrated variable sequences in the 32 -end coding region. This region contains a polymorphic repeat region that can be used to differentiate *Staphylococcus aureus* isolates (Goh *et al*., 1992; Guler *et al.*, 2005). This genetic variability may contribute to the emergence of distinct epidemiological profiles which are dependent on predominant strains within a herd, suggesting the necessity to identify such strains or subtypes before applying specific measures of mastitis control (Zecconi and Piccinini, 1999).

On intensive review of literature, no study has been reported in India regarding PCR amplification of *coa* gene in Murrah buffaloes. In our study, four amplified products of sizes of approximately 610, 740, 870 and 960 bp were obtained, and this is in close agreement with the findings of Vieira-da-Motta *et al*. (2001) in buffaloes who reported similar fragment sizes of 612, 740, 870 and 964 bp in 8.6 percent, 29.7 percent, 19.5 percent and 42 percent samples, respectively. In a study in cows, Kalorey *et al*. (2007) obtained three different products of 627, 710 and 910 bp for 20, 10 and seven isolates respectively.

Studies carried out on PCR amplification of *coa* gene in different countries using the same primer pairs revealed extensive polymorphism with predominance of one or more of *coa* gene amplified products among *Staphylococcus aureus* responsible for mastitis in cows and buffaloes. Annemuller *et al*. (1999) obtained four PCR products of 990, 900, 800, and 740 bp, with 990 bp being the predominant product. Raimundo *et al*. (1999) reported 73.3 and 15.2 percent of isolates assigned to 1000- and 700- to 750- bp products, respectively. Lange *et al*. (1999) found seven PCR products ranging from 580 to 1060 bp. Schlegelova *et al*. (2003) identified three coagulase genotypes, and the 730 bp product was predominant in 83.3 percent of the isolates. Guler *et al*. (2005) obtained 1000-, 900-, 800-, and 700-bp PCR products in 60.8, 16.8, 12.8, and 9.6 percent of the isolates, respectively. Da Silva and Da Silva (2005) reported twenty-seven amplicons ranging from 579 bp to 1442 bp with 790, 759, 725 and 579 bp accounting for 52 percent of the isolates. Katsuda *et al.* (2005) found five types of amplified products ranging from 420 ± 20 bp to 820 ± 20 bp. Vimercati *et al.* (2006) observed amplified products of *coa*

gene ranging from 420 to 900 bp. Moon *et al.* (2007) reported amplified products between 620 to 809 bp in *Staphyloccoccus aureus* isolates. Saei *et al*. (2009) observed five different PCR products with molecular weight ranging from 490-850 bp in a study in nine dairy herds.

When pathogens of multiple genotypes infect a host, they compete for source of nutrition and transmission. In this condition, the genotype with greater virulence is competitive (Nowak and May, 1994). The predominance of one or more *coa* gene genotypes may be more beneficial in the control of *Staphylococcus aureus* mastitis since they were reported to be more resistant to neutrophil bactericidal activities than rare genotypes (Su *et al.*, 1999). It also suggests a common source, host to host transmission i.e. contagious transmission, host adaptation of subsets of the population of *Staphylococcus aureus* strains. Also, differences in distribution of coagulase gene variants in *Staphylococcus aureus* may reflect presence of virulence factors responsible for suppressing host defence mechanisms (Goh *et al*., 1992).

In conclusion, profile of *coa* gene based genotypic PCR assay can be used as additional suitable identification criterion to differentiate among coagulase staphylococci for understanding the epidemiology of mastitis associated with high milk yielding Murrah buffaloes since it enables rapid and accurate diagnosis of *Staphylococcus aureus* within hours and paves the way for new approaches aimed at improvement in mastitis diagnosis, treatment, prevention and control.

ACKNOWLEDGEMENTS

The technical help rendered by Sh. Bhupender Singh and Sh. Randhir Singh of the Veterinary College Central Laboratory, COVS, is gratefully acknowledged.

REFERENCES

Aarestrup, F.M., C.A. Dangler and L.M. Sordillo. 1995. Prevalence of coagulase gene polymorphism in *Staphylococcus aureus* isolates causing bovine mastitis. *Can. J. Vet. Res.*, **59:** 124-128.

Ahlawat, S.P.S., D.K. Sadana and P. Pandey. 2006. Buffalo genetic resources and their conservation in India. *Asian Buff. Mag.*, **3**(1): 18-29.

Annemuller, C., Ch. Lammler and M. Zschock. 1999. Genotyping of *Staphylococcus aureus* isolated from bovine mastitis. *Vet. Microbiol.*, **69:** 217-224.

Barlow, J., B. Rauch, F. Welcome, S.G. Kim, E. Dubovi and Y. Schukken. 2008. Association between Coxiella burnetii shedding in milk and subclinical mastitis in dairy cattle. *Vet. Res.*, **39:** 23.

Bradley, A.J., K.A. Leach, J.E. Breen, L.E. Green and M.J. Green. 2007. Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. *Vet. Rec*., **160:** 253-258.

Da Silva, E.R. and N. Da Silva. 2005. Coagulase gene typing of *Staphylococcus aureus* isolated from cows with mastitis in southeastern Brazil. *Can. J. Vet. Res.*, **69:** 260-264.

Dang, A.K., S. Kapila, P. Tomar and C. Singh. 2007. Relationship of blood and milk cell counts with mastitic pathogens in Murrah buffaloes. *Ital. J. Anim. Sci.*, **6**(Suppl. 2): 821-824.

Denis, M., D.N. Wedlock, S.J. Lacy-Hulbert, J.E. Hillerton and B.M. Bundle. 2009. Vaccines against bovine mastitis in New Zealand context: what is the best way forward. *N. Z. Vet. J.*, **57**(3): 132-140.

Dua, K. 2001. Studies on incidence, etiology and estimated economic losses due to mastitis in

Punjab and India -An update. *Indian Dairyman*, **53:** 41-48.

Faruki, H. and P. Murray. 1986. Medium dependence for rapid detection of thermonuclease activity in blood culture broths. *J. Clin. Microbiol.*, **24:** 482-483.

Gibbs, B.M. and F.A. Skinner. 1966. Identification methods for microbiologists part - A. *The Soc. Applied Bacterial.*, Tech. Sr. No. 1 Academic Press. London: New York.

Goh, S.H., S.K. Byrne, J.L. Zhang and A.W. Chow. 1992. Molecular typing of *Staphylococcus aureus* on the basis of coagulase gene polymorphisms. *J. Clin. Microbiol.*, **30:** 1642-1645.

Guler, L., U. Ok, K. Gunduz, Y. Gulcu and H.S. Hadimli. 2005. Antimicrobial susceptibility and coagulase gene typing of Staphylococcus aureus isolated from bovine clinical mastitis cases in Turkey. *J. Dairy Sci.*, **88:** 3149-3154.

Halasa, T., K. Huijps, O. Osteras and H. Hogeveen. 2007. Economic effects of bovine mastitis and mastitis management: A review. *Vet. Quart.*, **29:** 18-31.

Hillerton, J.E. and E.A. Berry. 2005. Treating mastitis in the cow- a tradition or an archaism. *J. Appl. Microbiol.*, **98:** 1250-1255.

Huijps, K., T.J. Lam and H. Hogeveen. 2008. Costs of mastitis: facts and perception, *J. Dairy Res.*, **75:** 113-120.

Kalorey, D.R., Y. Shanmugam, N.V. Kurkure, K.K. Chousalkar and S.B. Barbuddhe. 2007. PCR-based detection of genes encoding virulence determinants in *Staphylococcus aureus* from bovine subclinical mastitis cases. *J. Vet. Sci.*, **8:** 151-154.

Katsuda, K., E. Hata, H. Kobayashi, Mariko Kohmota, K. Kawashima, H. Tsunemitsu and M. Eguchi. 2005. Molecular typing of *Staphylococcus aureus* isolated from bovine mastitic milk on the basis of toxin genes and coagulase gene polymorphisms. *Vet. Microbiol.*, **1054:** 301-305.

Kumar, S., M. Nagarajan, J.S. Sandhu, N. Kumar and V. Behl. 2007. Phylogeography and domestication of Indian river buffalo. *BMC Evol. Biol.*, **7:** 186-193.

Lange, C., M. Cardoso, D. Senczek and S. Schwarz. 1999. Molecular subtyping of *Staphylococcus aureus* isolates from cases of bovine mastitis in Brazil. *Vet. Microbiol.*, **67:** 127-141.

Martineau, F., F.J. Picard, P.H. Roy, M. Ouellette and M.G. Bergeron. 1998. Species-specific and ubiquitous DNA based assay for rapid identification of *Staphylococcus aureus. J. Clin. Microbiol.*, **36:** 618-623

Moon, J.S., A.R. Lee, H.M. Kang, E.S. Lee, M.N. Kim, Y.H. Paik, Y.H. Park, Y.S. Joo and H.C. Koo. 2007. Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. *J. Dairy Sci.*, **90:** 1176-1185.

Nowak, M. and R. May. 1994. Superinfection and the evolution of parasite virulence. *Proc. R. Soc. Lond.*, **255:** 81-89.

Phuektes, P., P.D. Mansell and G.F. Browning. 2001. Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and streptococcal causes of bovine mastitis. *J. Dairy Sci.,* **84:** 1140-1148.

Raimundo, O., M. Deighton, J. Capstick and N. Gerraty. 1999. Molecular typing of *Staphylococcus aureus* of bovine origin by polymorphisms of the coagulase gene. *Vet. Microbiol.*, **66:** 275-284.

Saei, H.D., M. Ahmadi, K. Mardani and R.A. Batavani. 2009. Molecular typing of *Staphylococcus aureus* isolated from bovine mastitis based on polymorphism of the coagulase gene in the north west of Iran. *Vet. Microbiol.*, **137**(1-2): 202-206.

Sahay, S., B.P. Sinha and S.P. Verma. 2007. Incidence of sub clinical mastitis in dairy animals and sensitivity of isolated organisms. *Indian J. Vet. Med.*, **27**(1): 29-30.

Sasidhar, P.V.K, Y.R. Reddy and B.S. Rao. 2002. Economics of mastitis. *Indian J. Anim. Sci.*, **72**(6): 439-440.

Schlegelova, J., M. Dendis, J. Benedik, V. Babak and D. Rysanek. 2003. *Staphylococcus aureus* isolates from dairy cows and humans on a farm differ in coagulase genotype. *Vet. Microbiol.*, **92:** 327-334.

Sharma, A., N. Sindhu and V.K. Jain. 2009. 16S-23S rRNA intergenic spacer based molecular detection of *Staphylococcus aureus* directly from mastitic milk of crossbred cows. *Indian J. Anim. Sci.*, **79:** 350-352.

Sharma, A. and N. Sindhu. 2007. Occurrence of clinical and subclinical mastitis in buffaloes in state of Haryana (India). *Ital. J. Anim. Sci.*, **6**(Suppl. 2): 965-967.

Sindhu, N., A. Sharma and V.K. Jain. 2008. Phenotypic and genotypic characterization of *Staphylococcus aureus* isolated from mastitis cases of cows and buffaloes. *J. Immunol. Immunopathol.*, **10**(2): 119-123.

Su, C., C. Herbelin, N. Frieze, O. Skardova and L.M. Sordillo. 1999. Coagulase gene polymorphism of *Staphylococcus aureus* isolates from dairy cattle in different geographical areas. *Epidemiol. Infect.*, **122:** 329-336.

Taponen, S., L. Salmikivi, H. Simojoki, M.T. Koskinen and S. Pyorala. 2009. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *J. Dairy Sci.*, **92**(6): 2610-2617.

Vieira- Da- Motta, O., M.M. Folly and C.C.H. Sakyiama. 2001. Detection of different *Staphylococcus aureus* strains in bovine milk from subclinical mastitis using PCR and routine techniques. *Braz. J. Microbiol.*, **32:** 27-31.

Vimercati, C., P. Cremonesi, B. Castiglioni, G. Pisoni, P.J. Boettcher, A. Stella, G. Vicenzoni and P. Moroni. 2006. Molecular typing of *Staphylococcus aureus* isolated from cows, goats and sheep with intramammary infections on basis of gene polymorphisms and toxin genes. *J. Vet. Med.*, **53:** 423-428.

Zecconi, A. and R. Piccinini. 1999. Teoria e pratica de controle de mastite por *Staphylococcus aureus*. *NAPGAMA*., **5:** 4-11.

SPONTANEOUS EXTRUSION OF THE INTESTINES AND UTERUS AS A SEQUELAE TO VAGINAL PROLAPSE IN A BUFFALO HEIFER: A CASE REPORT

G. Veeraiah[1] and Manda Srinivas[2]

ABSTRACT

The present communication places on record clinical management of a case of spontaneous rupture of vagina with herniation of intestines and uterus as a sequelae to prolapse of vagina and abortion in a buffalo heifer.

Keywords: vaginal prolapse, vaginal rupture, extrusion of intestines, extrusion of uterus, abortion

INTRODUCTION

In bovines, the vaginal wall can rupture due to birth of a fetus with long extremities, maldispositions, sharp bony prominences of the fetus, perivaginal fat, emphysematous fetus and in a few cases spontaneous rupture can occur due to unknown reasons (Roberts, 1982). Extrusion of the intestine through the ruptured vaginal wall has been reported in sheep while reports in buffaloes are limited (Babu Rao and Veeraiah, 1998). The present communication reports a spontaneous rupture of vagina with herniation of intestines and uterus as a sequelae to prolapse of vagina and abortion in a buffalo heifer.

CASE HISTORY AND CLINICAL OBSERVATIONS

A primiparous buffalo at the eight month of gestation was brought to the Veterinary Dispensary with a history of recurrent vaginal prolapse over 2 days. The animal had been previously treated for mild vaginal prolapse during the sixth month of gestation. On clinical examination, the animal was dull and showed severe abdominal straining with prolapsed vagina about the size of a football. The physical examination of the prolapsed mass revealed a soiled, congested and abraded vaginal mucous membrane.

TREATMENTS AND DISCUSSION

Under epidural anesthesia, the prolapsed vagina was repositioned into the pelvic cavity as per the procedure described by Roberts (1982). Follow up therapy included administration of Inj. Enrofloxacin 15 ml intramuscularly, Inj. Chlorphenaramine maleate 10 ml intramuscularly, Inj. Dicycloamine 15 ml intramuscularly and supportive therapy with calcium and phosphorous supplementation. As the condition was prepartum retention of the prolapse was achieved by application of rope truss.

[1]Veterinary Assistant Surgeon, Veterinary Dispensary, PIPPARA - 534 197 West Godavari District, Andhra Pradesh, India

[2]Animal Reproduction, NTR College of Veterinary Science, Gannavaram 521 102, Andhra Pradesh, India, E-mail: mandasrinivas64@gmail.com

However, 6 h after therapy the animal showed severe abdominal straining that revealed prolapsed vagina and a fully dilated cervix with fetal parts. Under epidural anesthesia, the aborted fetus was removed by mild traction that resulted in straining and extrusion of intestines and the entire uterus through a longitudinal tear in the right dorso-lateral wall of the vagina (Figure 1). The extruded intestine and uterus were washed with normal saline and repositioned back into the abdominal cavity through the tear that was closed by continuous sutures with chromic catgut no. 2 under epidural anesthesia. Further, the occurrence of prolapse was prevented by application of Buhners sutures as per the procedure described by Noakes *et al.* (2001). Post-operative care included administering antibiotics, antihistaminics, antispasmodics, mineral supplementation for 7 days and the animal was managed on epidural anesthesia to minimize abdominal straining along with other managemental practices. The animal had uneventful recovery by the tenth day after initiation of the therapy.

In the present case, the traumatized and congested mucous membrane of the prolapsed vagina might have been responsible for excessive uterine and abdominal contractions which might have initiated the abortion (Noakes *et al.,* 2001). Other causes that might have been responsible for abortion include hormonal imbalances, urinary tract infections, excessive pelvic fat, lack of exercise, and genetic and some unknown factors. The spontaneous rupture of the vaginal wall and extrusion of intestines and uterus might have been due to strong abdominal contractions on a weak traumatized vaginal wall which might have been torn by the bony prominences of the fetus or dam (Roberts, 1982; Dhaliwal *et al.,* 1991).

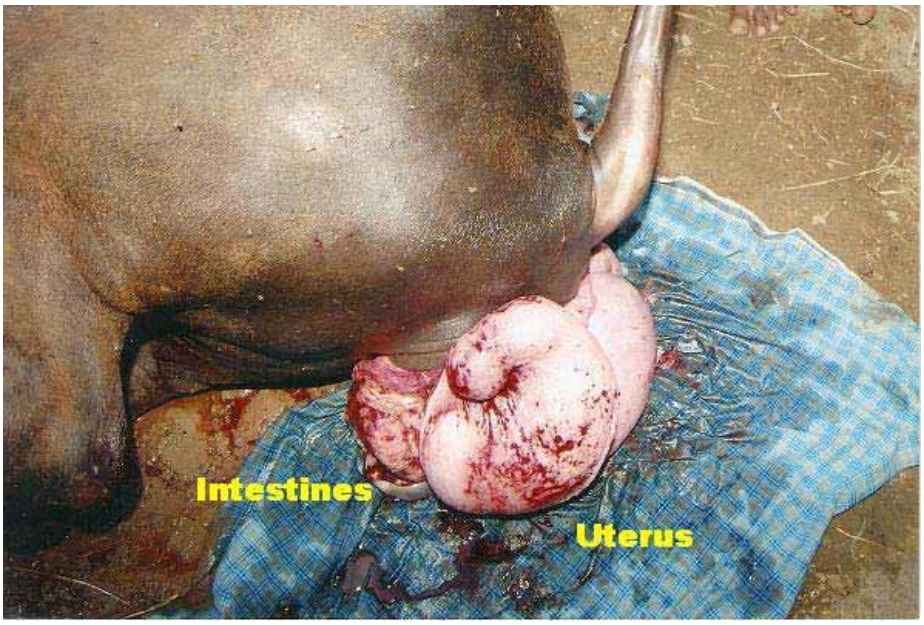

Figure 1. Showing the extrusion of intestines and uterus through the vaginal tear in an aborted buffalo heifer.

*Continued to page 64

A DIPROSOPUS BUFFALO NEONATE: A CASE REPORT

Amit Sharma, Subhash Sharma and N.K. Vasishta

ABSTRACT

A successful delievery of diprosopus monster through caesarean section is recorded.

Keywords: Diprosopus monster, buffalo, congenital anomaly

INTRODUCTION

A monster is a malformed fetus. Monstrosity is a disturbance of the development that involves sexual organs and causes great distortion of the individual (Vegad, 2007). Monstrosities are associated with either infectious disease or congenital defects (Arthur *et al*., 2001) and may or may not interfere with birth. Abnormal duplication of the germinal area in the fetus will give rise to congenital fetal abnormalities with partial duplication of body structures. Duplication of the cranial portion of the fetus is more common than that of the caudal portion (Robert, 2004). It is important to know various types of monsters in animals which usually cause dystocia, end which cannot be easily removed and so demand caesarean most of the time (Patil *et al*., 2004; Sharma, 2006).

CASE HISTORY AND CLINICAL EXAMINATION

An eight-year-old indigenous buffalo with normal gestation was brought to the Veterinary Hospital Berthin. The animal was presented with complaint that in spite of consistent straining for the previous eight hours after the expulsion of the first water bag, there was no progression to second stage of labor. Obstetrical examination revealed presence of abnormal fetus with two palpable heads joined at around 45^{o} to each other in anterior longitudinal presentation, dorso-sacral position with both forelimbs in birth canal. Since forced extraction was not possible, caesarean section was done.

TREATMENTS AND DISCUSSION

The paramedian laparohystrectomy was performed under local analgesia after restraining the animal in lateral recumbancy. The uterus was exteriorized to deliver a dead abnormal male fetus/ calf. Incision was closed in routine manner. The buffalo was treated with injection amoxycillin cloxacillin combination 5 mg/kg b.wt, injection Meloxicam 0.5 mg/ kg b.wt and supportive therapy for 7 days. The sutures were removed after 10 days.

Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Himachal Pradesh, India

The fetus had two heads on a single neck (Figure 1 and 2). One of the heads was better aligned with the spine. The pinnae of the medial ears were fused at the base. The neck, thorax, abdomen and limbs were grossly normal. These observations are in consonance with the earlier findings (Fisher *et al*., 1986). Dicephalus monsters have been reported in goats (Pandit *et al*., 1994), buffaloes (Chauhan and Verma, 1995; Raju *et al*., 2000; Bugalia *et al*., 2001; Srivastva *et al*., 2008) and cows (Chandarhasan *et al*., 2003; Patil *et al*., 2004; John Abrahan *et al*., 2007). The embryonic duplications are malformation due to abnormal duplication of the germinal area giving rise to fetuses whose body structures are partially duplicated. The embryonic disk starts to differentiate on the 13th day. If the split occurs after day 13, then the twins will share body parts in addition to sharing their chorion and amnion (Finberg, 1994). Conjoined twins may be caused by any number of factors, being influenced by genetic and environmental conditions. It is presently thought that these factors are responsible for the failure of twins to separate after the 13th day after fertilization (Srivastva *et al*., 2008). Jones and Hunt (1983) stated that the causes of many congenital anomalies are essentially unknown; however, the important known causes are prenatal infection with a virus, poisons ingested by mother, vitamin deficiency (A and folic acid), genetic factors and/or combination of these factors.

REFERENCES

Arthur, G.H., D.E. Noakes, H. Pearson and T.J. Parkinson. 2001. *Veterinary Reproduction and Obstetrics,* 8th ed. W.B. Saunders Co. Ltd., London. 118p.

Bugalia, N.S., R.K. Biswas and R.D. Sharma. 2001. Diplopagus sternopagus monster in an Indian water buffalo (*Bubalus bubalis*). *Indian J. Anim. Reprod.,* **22**(2): 102-104.

Chandrahasan, K., K. Kumar, M. Selvaraju, P.N. Richard, Jagatheesan and S.R. Kumar. 2003. Dystocia due to dicephalus montomus monster in a crossbred cow. *Indian J. Anim. Reprod.,* **24**(2): 175.

Chauhan, K.S. and H.K. Verma. 1995. A case of dystocia due to diplopagus monster in buffalo. *Indian J. Anim. Reprod.,* **16**(1): 75.

Finberg, H.J. 1994. *Ultrasound Evaluation in Multiple Gestation. In Callen's Ultrasonography in Obstetrics and Gynecology*, 3rd ed. Harcourt Publishers. 121p.

Figure 1. Diprosopus fetus head.

Figure 2. Diprosopus fetus.

Fisher, K.R.S., G.D. Partlow and A.F. Walker. 1986. Clinical and anatomical observations of a two-headed lamb. *Anat.Rec.*, **214**(4): 432-440.

John, Abrahan, S. Bihu, V.I. Raj and B. Lakshman. 2007. Dicephalic monstrosity in a heifer. *Indian J. Anim. Reprod.,* **28**(2): 109-111.

Jones, T.C. and R.D. Hunt. 1983. *Veterinary Pathology*, 5th ed. Lea and Febiger, Philadelphia. 115p.

Pandit, R.K., S.K. Pandey and R.G. Aggarwal. 1994. A case of dystocia due to diplopagus monster in goat. *Indian J. Anim. Reprod.,* **15**(1): 82.

Patil, A.D., N.M. Markandeya, V.B. Sarwade and S.D. Moregaonkar. 2004. Dicephalus monster in a non-descript cow - A Case report. *Indian J. Anim. Reprod.,* **25**(2): 161-162.

Raju, K.G.S., K.S. Rao, V.S.C. Reddy and G.P. Sharma. 2000. Dicephalus- biatlanticus monster in a buffalo. *Indian J. Anim. Reprod.,* **21**(1): 81.

Robert, S.J. 2004. *Veterinary Obstetrics and Genital Diseases*. Indian reprints 2004, CBS Publishers and distributors, Delhi-110032. 73-74p.

Sharma, A. 2006. Caesarean section in animals under field conditions: A retrospective study of 50 cases. *Indian Vet. J.*, **83**(5): 544-545.

Srivastava, S., A. Kumar, S.K. Maurya, A. Singh and V.K. Singh. 2008. A dicephalus monster in Murrah buffalo. *Buffalo Bull.,* **27**(3): 231-232.

Vegad, J.L. 2007. *Textbook of Veterinary General Pathology*, 2nd ed. International book distribution Company. 544p.

---

*Continued from page 61

REFERENCES

Babu Rao, K. and G. Veeraiah. 1998. Vaginal rupture associated with herniation of intestines in a pregnant buffalo - A case report. *Indian J. Anim. Reprod.,* **19**(2): 170.

Dhaliwal, G.S., S. Prabhakar, Jagir Singh and R.D. Sharma. 1991. Spontaneous rupture of vagina in a heifer. *Indian J. Anim. Reprod.,* **12**(1): 98-101.

Noakes, D.E., T.J. Parkinson, G.C.W. England and G.H. Arthur. 2001. Prolapse of the vagina and cervix. p. 145-153. *In Arthur's Veterinary Reproduction and Obstetrics*, 8th ed. Saunders, Philadelphia, USA. 864p.

Roberts, S.J. 1982. Diseases of puerperal period. p. 303-313. *In Veterinary Obstetrics and Genital Diseases*, First Indian Reprint. CBS Publishers and Distrubutors, Delhi, India.

CLINICAL MANAGEMENT OF SECOND DEGREE BURNS INA SHE BUFFALO: A CASE REPORT

P. Vidya Sagar, K. Rajesh, K. Lakshmi Kavitha and K. Suresh

ABSTRACT

This communication reports a case of second degree burns in a she buffalo which was successfully treated without any complications. Very few reports are available regarding the clinical management of burns (Archibald, 1974; Tyagi and Singh, 2002) in buffaloes.

Keywords: second degree burns, buffalo

INTRODUCTION

A burn is nothing but charring of tissue when it is exposed to dry heat. The burns are classified into first degree, second degree, third degree burns based on the extent of destruction to the tissue. Second degree burns are those which involve the epidermis and dermis layers of the skin. The burned area will be red and may show blisters and the skin is hot, painful to touch. In case of third degree burns all the layers of the skin are involved and the underlying structures such as nerve endings gets damaged. Hence in case of third degree burns there will be no pain. The first and second degree burns are together grouped as Group I (Davis, 1984). The outcome of the case mainly depends not only on the degree of burns but also on the extent or area involved.

Case history and observations

A she buffalo was presented to clinic with severe burns. The burns were due to the fact that the thatched housing under which the animal was kept caught fire. The buffalo was kept restrained so there was no way for the buffalo to escape. The case was immediately rushed to the hospital. Examination of the buffalo revealed that about 40% of the total surface area was involved. By the time the animal was brought to the clinic (50/minute), it was in recumbency and its responses to external stimuli were sluggish. The temperature was 104°F. The respiratory rate was 28/minute. The heart rate was 90/minute due to hypovolemia. The quality of pulse was weak

TREATMENTS AND DISCUSSION

The animal was put on comfortable bedding, and the treatment was started. As the quality of the pulse was very weak and the animal's response to external stimuli was sluggish, it was established that the animal was in a state of shock. The shock may have been due to fact that a large area of skin was involved, since this might have caused severe evaporative losses during and after the incident. (Tyagi and Singh, 2002) Dehydration was clearly evident with increased capillary refill time. In such cases, fluid therapy is primarily aimed to establishing the circulatory volume (Pierson, 1969). This can be achieved by giving the electrolyte solutions. But in such cases the quantity of the crystalloids need to

Veterinary Assistant Surgeon, Veterinary Polyclinic, Gudivada, A.P., India

Figure 1. 0th day- six hours post treatment- note the extensive burns involving 40% of the total surface area.

Figure 2. 0th day- six hours post treatment- note the extensive area involved on the face.

Figure 3. 8th day post treatment- note the marked improvement of the lesions.

be given are very high volumes. Hence, the colloid solution was considered initially (Archibald, 1974). This was done by giving Haemaccel [containing degraded gelatine (equivalent N2 content of 0.63 gm) 3.5 gm in 100 ml, Electrolytes in mmol/Litre are Na 145, K 5.1, Ca 6.25, Cl 145] 1 litre I/V followed by the crystalloid solution of DNS 3 litres I/V. Then hypertonic solution of 3 litres of 25% dextrose was given I/V. In order to save the life of the animal, dexamethasone was also given 0.1 mg/kg bwt I/V. To mitigate the pain ketoprofen 2.5 mg/kg bwt was also given. Silverex (silver sulfadiazine 1% w/v) ointment was prescribed which was applied topically in severely injured parts. A course of antibiotic was also started. The antibiotics metronidazole 10 mg/kg bwt I/V, benzithine penicillin 4,800,000 units on alternate days was considered. The combination was considered in order to achieve a broad spectrum of action. The treatment was continued for about ten days. Daily cleaning of the affected parts was done with potassium permanganate lotion 1:1000 dilution and the application of the silverex ointment on critically injured areas was done. The remaining areas were cleaned. After the third injection, benzithine penicillin was stopped, and an amoxycilllin and cloxacillin combination was started instead. Fluid therapy was done for three days by making use of Haemaccel 2 litres I/V, DNS 2 litres I/V, RL 1 litre I/V and 25% dextrose 2 litres I/V. From fourth day onwards DNS 3 litres, RL 1 litre and dextrose 3 litres were given intravenously. The intravenous administration of metranidazole was stopped on day 5. The amoxicillin and cloxacillin was given till the complete recovery, during the first 6 days the animal continuously received NSAID ketoprofen to alleviate pain and to make the animal comfortable. The lesions started healing from fourth day onwards. This was evident from the fact that there was a decrease in the pain, the charred skin started peeling off exposing the underlying tissue. Then slowly in less affected areas there is development of melanin pigment which showed the tendency to change to dark colour. From 8th day onwards the severely affected areas showed the development of leathery skin which continued to grow over affected areas. It took about 20 days for the complete healing. The treatment with antibiotics was stopped on the 12th day onwards, but topical application of antiseptic powder was

advised to prevent bacterial complications, which would cause delay in healing process.

The case of second degree burns presented to the clinic with poor prognosis was successfully treated by proper fluid therapy, broad spectrum antibiotic and good hygienic practices.

REFERENCES

Archibald, J. 1974. *Second Archibald Edition*. American Veterinary Publications Inc. Drawer KK, Santa Barabara, California.

Davis, L.E. 1984. Initial care of burns and electrical injury. Zaslow, I.M. Lea and Febiger (eds.) *Veterinary Trauma and Critical Care*. Philadelphia.

Pierson, R.E., K.A. Larson, D. Horton, K. Turbes and J.S. Palen. 1969. Treatment of second degree thermal burns in cattle. *Vet. Med*., **64:** 218.

Tyagi. R.P.S and Jit Singh. 2002. *Ruminant Surgery.* CBS Publishers & Distributors, New Delhi.

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
Office of University Library
Kasetsart University,
50 Pahonyothin Road, Chatuchak,
Bangkok 10900, Thailand
Tel. 66-2-942-8616

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

Buffalo Bulletin (March 2010) Vol.29 No.1

CONTENTS

Page

CONTENTS

Page

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