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MANAGEMENT OF POST PARTUM COMPLETE UTERINE PROLAPSE IN A GIR COW AND A JAFFRABADI BUFFALO


ABSTRACT

Case reports of complete uterine prolapsed in a Gir cattle and a Jaffrabadi buffalo are presented. The complete uterine prolapsed cow and buffalo with inability to stand were treated. Applying gentle pushing and meticulous pressure on the prolapsed uterine mass repositioned the uterine horns normally in both, and both the animals were treated for prevention of uterine infection with antibiotics, anti-inflammatory and antihistaminic drugs. Both the animals recovered completely without any future reproductive complication.

Keywords: Prolapse, hypocalcaemia, uterus, reproductive complication

INTRODUCTION

Among the reproductive disorders, complete uterine prolapse is always an extremely serious condition in any farm animal. A prolapsed uterus is highly prone to mechanical injury and/or trauma and environmental contamination, and this may lead to increased maternal morbidity and even to the death of the animal owing to trauma, laceration, subsequent hemorrhage, tissue necrosis, bacterial contamination, some time urinary incontinence, hypocalcaemia, stress incontinence and shock (Jana and Ghose, 2004), and therefore, it requires early attention, prompt and efficient management and proper treatment to overcome further serious complications in reproductive performance of the animal in the future.

The present clinical case studies are on incidence of post partum complete uterine prolapse in an indigenous pluriparous post parturient cow and buffalo and its successful obstetrical and therapeutic management.

Case History And Observation

Case-I A seven-year-old gir cow in its 4th lactation attended (Cattle Breeding Farm, J.A.U., Junagadh) was treated for the correction of complete uterine prolapse and for its inability to stand, and for prevention of uterine infection.

Case-II A six-year-old Jaffraban buffalo in its 2nd lactation was treated for the correction of the uterine prolapsed, for its inability to stand and for prevention of uterine infection.

It was reported that both the animals cow and buffalo had given normal birth to healthy male and female calves, respectively, and both the animals expelled the placenta normally within 6-8 hr post calving, but after the placenta had been expelled, a uterine mass started protruding from the vulvar lips, and in the cow, during the night (16-18 h post calving), the whole uterus had prolapsed out whereas in the case of the buffalo, the whole uterus had prolapsed out in early morning (28-30
h post calving). The cow could not rise up even after mechanical supports were applied, while the buffalo was able to rise with assistance and keep standing in such a way that the hind quarters remained in upward position. In the case of the cow, gynecological examination led to the diagnosis of the condition as post parturient paresis and complete uterine prolapse, whereas in the buffalo, examination led to the diagnosis of the condition as complete uterine prolapse. Respiration and pulse were normal, but the animals were hypothermic (98°F in the cow and 98.5°F in the buffalo). Both the animals were alert, but were not taking food or water nor passing urine.

Treatment

The everted uteri were washed gently with 1% potassium permanganate solution with detachment of placenta and removal of all debris and dead tissues and prolapse masses were lifted upward to pass the urine. Gently application of 50 gm of nolapse powder (Virbac) on the prolapsed mass was made, followed by waiting for 10-15 minutes. With gentle pushing and meticulous pressure, the prolapse mass was pushed inside with half closed hand into the pelvic cavity and both the uterine horns were repositioned normally. The vulvar lips with were cleaned with 1% potassium permanganate solution and vaginal passaries viz., Furea (Intas Pharmaceutical) 2 boli were placed in each horn of the uterus in each animal. Horizontal metrees suture were taken at the vulvar lips in both the animals to overcome further complication.

As the rectal temperature of both the animals indicated hypothermic, hypocalcaemic, uterine inertia and loss of energy due to calving stress, immediate slow intravenous administration of 20 I.U. of oxytocine along with lactomag 450 ml (Intas Pharmaceuticals Limited) a combination of calcium gluconate, magnesium hypophosphite and anhydrous dextrose followed by intravenous administration of 450 ml of 25% dextrose (Wockstrose 25%, Wockhardt) to combat energy loss due to calving stress. A course of antibiotic i.e. two shots of long acting oxytetracycline hydrochloride (Terramycine L.A., pfizer) 5 mg/kg of body weight were injected intramuscularly at 48 h intervals and non steroidal anti inflammatory meloxicam (Zobid-M, Sarabhai Zydus) were administered intramuscularly 0.5 mg/kg of body weight for the 1st day followed by 0.2 mg/kg of body weight for the subsequent 3 days. The supportive treatment was given to both the animals with antihistaminic (Cadistin, Sarabhai Zydus) 10 ml total dose for 3 days and vitamin B-complex with liver extract (Balamyl, Sarabhai Zydus) 10 ml total dose for the subsequent five days.

RESULT AND DISCUSSION

Both the animals (the cow and the buffalo) rose after 3-4 h of treatment on their own and fed normally feeding. On the 5th day the sutures were removed. Both the animals recovered completely without any future reproductive complication.

Arthur et al. (1996) stated that uterine prolases are associated with the onset of uterine inertia during the 3rd stage of labor when a portion of detached afterbirth occupies the birth canal and protrudes from the vulva. In the present prolapses, intravenous administration of calcium and magnesium corrected the hypothermia due to hypocalcaemia and gave tonicity to perinial muscular tissue which helped to withhold the prolapse uterine mass, simultaneously oxytocin provided tonicity and slight contraction to the smooth uterine musculature which helped in
repositioning it normally and corrected uterine inertia. Parenteral as well as intrauterine antibiotic therapy were instituted to control and combat possible bacterial infection and to establish uterine hygiene for future reproduction and performance. Anti-inflammatories, analgesics and antihistaminic are helpful to correct pain and inflammation, and vitamin B-complex and liver tonic plays important role in correction of off feeding occurs due to long antibiotic therapy.

Both the cases (the cow and the buffalo) recovered successfully without further complication of prolapse and hypocalcaemic paresis for 3 months of post therapeutic observation.

Among reproductive disorders, prolapse of reproductive organs occurs as a common gestational accident. Post partum uterine prolapse is more common than the prepartum prolapse (Roberts, 1971) mainly because of increase in intra abdominal pressure, uterine inertia and loss of muscular tonicity. Post partum uterine prolapse most often occurs immediately after parturition and occasionally up to several hours afterwards (Arthur et al., 1996; Robert, 1971).

The relaxation of pelvic ligaments due to the perous nature of the cow along with straining and lack of uterine tonicity as described by Wani et al. (2000) and the increase in intra abdominal pressure, uterine inertia and loss of muscular tonicity as described by Arthur et al. (1996) might be the cause of uterine prolapse in this case.

REFERENCES


ABSTRACT

The present communication reports a case of uterine prolapse in a pluriparous Murrah buffalo and its successful treatment by reposition at proper anatomical site and treatment.

Keywords: uterine prolapse, Murrah buffalo, clinical management

INTRODUCTION

A prolapse of the uterus is effectively the whole of the uterus turning inside out and hanging out of the vulva of the buffalo (Roberts, 1971). Prolapse of the uterus is a common complication of the third stage of labour in the buffaloes (Joseph et al., 2001). Compared to the vaginal prolapse, the uterine prolapse is larger, longer (usually hanging down to the hocks when standing), more deep red in color and covered with the “buttons” where the placenta was attached (Figure 1) (Arthur et al., 1996). There seem to be two age groups that are susceptible to this condition, heifers and older buffaloes. When heifers suffer from a prolapse it is due to the uterine muscle becoming exhausted from the calving process. When the calf is finally expelled, the uterus flops out with the calf. In older buffaloes, it is often associated with milk fever.

The uterus like the other muscles in the body, requires calcium to contract. In a buffalo with milk fever, the uterus fails to contract as the calf is being passed and again flops out with the calf. To avoid problems with uterine prolapses, try to decrease the potential for the buffaloes being affected by the predisposing factor above.

A uterine prolapse is considered a medical emergency; therefore, this condition is life threatening. If the affected buffalo is not treated quickly, she could go into shock or die from blood loss. A veterinarian should be contacted for assistance with this procedure. If the uterus is pushed back improperly, it could result in internal bleeding and death of the buffalo.

CASE HISTORY AND OBSERVATION

A ten-year-old Murrah buffalo in fifth parity was presented to the College of Veterinary Science and Animal Husbandry, DUVASU, Mathura with a history of a normal parturition. A normal female calf had been born six hours before. The uterine horn was prolapsed along with the fetal membrane. The buffalo was healthy and in a standing position. The prolapsed mass was hanging from the vulva. The rectal temperature was recorded to be 101°F. Eye mucous membrane was congested. The placental cotyledons were attached to the maternal...
Figure 1. Showing uterine prolapse in buffalo.

Figure 2. Showing clinical management of uterine prolapse.

Figure 3. Showing clinical management of uterine prolapse.
coruncles. Severe bleeding was noticed. The newborn was apparently healthy and trying to suckle her mother.

**CLINICAL MANAGEMENT**

Considering the severity of the case and the owner’s agreement, the prolapsed mass was washed carefully with warm saline. Epidural anesthesia was given with 2% Xylocane to prevent straining. The fetal membranes were detached manually with fingertips from the maternal coruncles avoiding bleeding. Then the uterine mass was again washed with saline and finally with 1:1000 potassium permanganate solution. Then it was replaced to its normal anatomical position. To prevent further complications, intra-uterine antibiotic treatment was also done. Then the purse string suture with sterile shoe laces around anterior vagina was done (Figures 2 and 3). A truss was applied to prevent recurrence due to tenasmus. The animal was treated with antibiotic, anti-inflammatory, antihistaminic, analgesic i/m and i/v fluid therapy. The same treatment was followed for three days and vaginal suture was removed after one week. The animal became healthy with plenty of milk production and normal fertility.

**DISCUSSION**

The usual sequel of uterine prolapse is haemorrhage, shock, septic metritis, peritonitis, infertility or death. Sometimes in delayed cases, partial contraction of cervix interferes with proper repositioning, resulting in recurrence of prolapse. But in this case, after detaching the fetal membranes, the prolapsed mass became lighter and less voluminous, so it was easy to repositio as described by Bhoi and Parekar (2009) . Moreover we applied a truss, so even in the presence of a tenasmus, the recurrence was noticed. Uterine prolapse is predisposed to a violent tenasmus and retension of fetal membrane in this case as reported by Roberts (1971).

**REFERENCES**


ABSTRACT

Vegetative valvular endocarditis is one of the rare primary cardiac diseases in bovines. Among bovines, the incidence of this condition in Indian buffaloes is poorly reported. The present communication describes the gross and histopathological alterations in the case of vegetative valvular endocarditis observed in a 5-year-old she buffalo.

Keywords: buffalo, vegetative valvular endocarditis, pathological alterations

INTRODUCTION

Cardiac diseases in bovines are one of the easily overlooked veterinary cases (Maillard et al., 2007) and the diagnosis is normally made almost at the terminal stage with poor prognosis (Reef et al., 2008). Vegetative valvular endocarditis is rare (Healy, 1996) but one of the primary cardiac diseases that is usually caused by systemic or local infections by bacteria that colonize and injure the valvular endothelium. Although the left atrio-ventricular valves are most commonly affected in other species, in bovines this condition is frequently observed in the right atrio-ventricular valve (Jones et al., 1997). Since the incidence of this disease is very rare in buffaloes among bovines, the present communication will serve as a valuable record regarding the incidence and pathological alterations noted.

CASE HISTORY, GROSS AND HISTOPATHOLOGY

The animal had the clinical history of progressive emaciation, dullness, depression, reduced milk yield, exercise intolerance, dyspnea with grunt, chronic moderate pyrexia and death. On autopsy, the carcass had pin-point to ecchymotic hemorrhages on subcutaneous tissues and visceral surfaces. The heart was enlarged and the endocardium of the cardiac ventricles had suffusive hemorrhage. The cusps of right atrio-ventricular valves had irregular, yellow, loosely adhering, friable masses causing stenosis of the valvular space (Figure 1). The cortex of the kidney revealed, well demarcated, raised, round,
Figure 1. Photograph of heart shows vegetative valvular endocarditis in right-atrio ventricular valve. Arrow points cauliflower like vegetative growth.

Figure 2. Photograph of lung shows embolic pneumonia and purulent exudates on cut section (arrows).
Figure 3. Microphotograph of right-atrio ventricular valve shows thrombosis, highly basophilic clumps of bacterial colonies (arrows) and ongoing suppurative inflammation on the valve (arrow heads). ×400 H&E.

pin-point, miliary white foci bilaterally. The lung was wet, heavy and pale with multifocal irregular flat red foci and on cut surface showed purulent material on major airways of all lobes (Figure 1). On microscopical examination, the kidneys revealed, diffuse pyelonephritis bilaterally. Severe neutrophilic infiltration in interstitium and pelvis, tubular dilatation, focal to multifocal tubular necrosis, hemorrhage, interstitial edema and micro-cavities containing degenerating neutrophils (abscess) were observed in the cortex, medulla and pelvis of the kidneys. Severe pulmonary edema with suppurative inflammation in airways, obliteration of functional alveolar space with purulent exudates and thickening of alveolar walls was observed in lungs. Histopathology of the affected valve showed, severe suppurative inflammation and peri-vavular cuffing of thrombotic mass. The thrombotic mass was seeded with multifocal bacterial colonies surrounded by multiple concentric layers of eosinophilic fibrin material (Figure 3).

DIAGNOSIS AND DISCUSSION

Valvular endocarditis in animals can be caused by two ways: primary adhesion of the microorganisms to the valvular endocardium or their entrapment to the pre-developed valvular thrombi (Jones et al., 1997). In bovines, the causative organism includes Arcanobacter pyogenes - the most frequently isolated organism from these lesions (Vleet and Ferrans, 2007), Streptococcus sp., members of Enterobacteriaceae and Bartonella bovis (Maillard et al., 2007). The pathogenesis includes focal injury to the endothelial valve, turbulent cardiac blood flow admixed with the pathogenic bacteria, adherence and colonization of
the bacteria in the injured site, induction of acute inflammation and resultant hypercoagulability of the blood in that particular focus leading to the formation and growth of thrombus. Continuous proliferation of the microorganisms leads to further organization of fibrin that gives rise to cauliflower like growths around the cusps of valves. Thus this condition is called vegetative valvular endocarditis (Jones et al., 1997). In this present case the gross and histopathology of lung indicated, the presence of embolic pneumonia, which in bovines, usually results from the right side valvular endocarditis and dissemination of the septic emboli through hematogenous route (Vleet and Ferrans, 2007a). This type of dissemination also leads to formation of micro to large abscesses in multiple organs like kidney, spleen, liver etc., which is otherwise called as embolic shower.

The gross and histopathology findings observed in this case were in accordance with the description presented by previous workers and thus this present case is diagnosed as vegetative valvular endocarditis.

REFERENCES


ABSTRACT

In the present study, the antigenicity of caseinophosphopeptides (CPPs) enriched from the hydrolysis of buffalo casein with trypsin and neutrase was studied using a mouse model system. Relative levels of specific IgE and IgG made in response to intraperitoneal sensitization to CPPs enriched samples were determined using indirect ELISA. It was found that the CPPs enriched samples prepared from both the enzymes, i.e. trypsin and neutrase, were less antigenic as compared to intact protein, i.e. sodium caseinate. Among the CPPs enriched samples, the antigenticity of trypsin samples was less than that of neutrase samples. The results suggested that these CPPs enriched samples can be used as a food ingredient for food designed for hypoallergenic persons.

Keywords: buffaloes, Bubalus bubalis, caseinophosphopeptides, CPPs, antigenicity

INTRODUCTION

Food allergy is defined as an adverse clinical reaction due to an immune-mediated hypersensitivity response resulting from the ingestion of a food. Cow milk protein allergy represents the most frequent food allergy in infancy ranging between 2 and 7.5% of the infant population. Clinical challenges frequently reveal that patients who are allergic to milk react to the multiple protein fractions found in bovine milk (Savilahti and Kuitunen, 1992). Otani (1992) stated that minimum number of antigenic determinant sites for αs1-CN, β-CN and κ-CN were 6, 6 and 4 respectively. The milk proteins mainly responsible for the allergy are α- and β-casein, followed by β-lactoglobulin and α-lactalbumin to a lesser extent (Docena et al., 1996; Bernard et al., 1998; Jarvinen et al., 2001). Milk from other species, such as goat milk and sheep milk, has shown a high degree of cross reactivity (Spuergin et al., 1997; Bellioni-Businco et al., 1999). Milk from buffalo is a common drinking milk in many developing countries and is the main component of buffalo mozzarella eaten throughout the world. In vitro studies demonstrate a similar proteomic make-up and suggest antibody cross reactivity between cow milk and water buffalo milk proteins (Restani et al., 2002; D’Auria et al., 2005). Similarly, one human study showed a high degree of skin test positivity to water buffalo milk in patients with cow milk allergy (Katz et al., 2008). However, Sheehan and Phipatanakul (2004) described the first report of a child with cow milk allergy who is able to tolerate water buffalo milk.

Further the protein hydrolysates are widely used as milk substitutes for children with bovine...
milk allergy. Enzymatic hydrolysis is considered a desirable treatment for reducing the antigenicity of milk caseins. The allergenicity of casein hydrolysates that have been prepared using a single or a mixture of proteases that have specific cleavage sites have been reported (Boza et al., 1994; Nakamura et al., 1993; Mahmoud et al., 1993, Otani et al., 1990 and Takase et al., 1979). Cordle (1994) reported that the allergenicity declines with decreasing molecular weight, especially between 10000 and 2500 daltons. A number of biological active peptides have been identified in the casein hydrolysates. Among these the caseinophosphopeptides have been reported as mineral binding peptides which have a potential use as a food ingredient. Selective precipitation or enrichment of the phosphopeptides results in removal of potential antigens. Therefore, it is worth examining whether casein phosphopeptides are hypoallergenic. The main aim of the study was to compare the allergenicity of caseinophosphopeptides (CPPs) enriched products prepared from buffalo milk casein using membrane processing and selective precipitation method with two enzymes. The allergic potential of the proteins is generally evaluated as a function of serological responses i.e. IgG and IgE antibody production. The role of IgE in type I immediate hypersensitivity allergic reaction is well understood in the scientific literature. It is also strongly agreed in the scientific literature that allergic reactions may occur independent of antigenic specific IgE. High affinity receptors for IgG, on human mast cells and basophils are activated in immediate hypersensitivity reactions. IgG-mediated immediate hypersensitivity, also known as IgG-mediated anaphylaxis, is not a new concept in allergy research. So in the present study, the allergenic activity as a function of the ability of proteins to provoke IgG and IgE antibody response in a mouse model was studied by peritoneally injecting mice with sodium caseinate, phosphopeptides enriched ingredients and ova albumin with adjuvant (AlO3). A sandwich ELISA was used to determine levels of IgG and IgE in the blood.

MATERIALS AND METHODS

Preparation of samples

Sodium caseinate prepared from buffalo milk was hydrolysed by two commercial enzymes, i.e. trypsin and neutrase, under optimized conditions (Saini, 2012). After hydrolysis the pH of the solution was adjusted to 4.6 and supernatant was collected after centrifugation. Then the pH of supernatant was adjusted to neutral and the solution was incubated at room temperature after adding calcium chloride 1.1%. After incubation, CPPs was enriched from the solution by using two methods: ethanol precipitation and ultrafiltration. A total of three samples were prepared; samples prepared with trypsin hydrolysates were named as trypsin CPPs i.e. TC (ethanol precipitation), trypsin retentate i.e. TU (ultrafiltration) and the sample from neutrase hydrolysed was named as neutrase retentate i.e. NU (ultrafiltration).

Intraperitoneal immunization for allergic response

Swiss albino male mice weighing 25-30 g each were taken from the Small Animal House, NDRI, Karnal, India. They were maintained on standard pellet diet and had access to tap water ad libitum. They were housed in groups of eight mice per cage and kept in polipropylene cages with stainless steel wire-bar lids, using a rice husk as a bedding material, under a 12 h light/dark cycle at room temperature of 22-24°C. Mice were then
acclimatized to the environment for one-week prior to the experiment. Animals were randomly assigned to one of six groups of eight mice each as given below:

Group 1: Control group injected with adjuvant in phosphate buffer saline (PBS) (pH 7.4)
Group 2: Group injected intraperitonially with ovalbumin + adjuvant in PBS (AlO3)
Group 3: Group injected intraperitonially with TU + adjuvant in PBS (AlO3)
Group 4: Group injected intraperitonially with TC+ adjuvant in PBS (AlO3)
Group 5: Group injected intraperitonially with NU + adjuvant in PBS (AlO3)
Group 6: Group injected intraperitonially with Na caseinate + adjuvant in PBS (AlO3)

The mouse groups were immunized on the first day and a booster dose was given on the 15th day of the experiment. Seven days after the final injection, blood samples were collected aseptically by exsanguinations (cardiac puncture). Serum samples were prepared, diluted with PBS and used for antigen-specific IgE and IgG assay.

Immunoglobulin assay

The total IgE and IgG in serum was determined using an ELISA kit (Koma Biotech, Korea). The operating procedures were strictly followed as provided by the manufacturer. The principle of the ELISA kit was to employ the quantitative sandwich enzyme immunoassay technique. Polystyrene ELISA plates were coated overnight at 4°C with 100 μL of coating antibody (provided with kit) diluted in 50 mM carbonate-bicarbonate buffer, pH 9.6. After washing at 25°C with PBS containing 0.05% Tween 20, pH 7.4; the plates were post-coated with 200 μL bovine serum albumin (1%) in PBS, pH 7.4 for 1 h at 25°C. The plates were washed again with PBS containing 0.05% Tween 20, pH 7.4. Serum (100 μL) of optimal dilution (100-fold dilution for IgE, 2000-fold dilution for IgG) was added to each well, and plates were incubated for 1 hr at 25°C. After washing the plates as above, 100 μL of optimally diluted detection antibody specific for IgE and IgG (provided with kit) were added to each well and the plates were incubated for 1 h at 25°C. After incubation, the wells were aspirated to remove liquid and the plate was washed as above. Finally, 100 μL of colour development solution was added to each well and incubated at room temperature for a proper colour development. (5-10 minutes). After sufficient colour development, 100 μL of 3, 3′, 5, 5′ tetramethyldiamine benzidine was added to each well and the absorbance was measured at 450 nm using an ELISA plate recorder. Finally, 100 μL of 3, 3′, 5, 5′ tetramethyldiamine benzidine was added to each well and incubated at 25°C for a proper colour development. (5-20 minutes). After sufficient colour development, the reaction was stopped by addition of 100 μL of 2M H2SO4 to each well and the absorbance was measured at 450 nm using an ELISA plate recorder (TECAM InfiniteF200 Pro.).

RESULTS AND DISCUSSION

At 3 weeks postinjection, no significant difference (p≤0.05) was found in the level of serum IgE among the mouse groups injected with CPPs samples (TU, TC and NU) as compared to the control. Figure 1 shows the level of serum IgE towards peritoneally injected CPPs samples and ovalbumin. Whereas it is observed that serum IgE level in the group injected with Na caseinate (280.7±19.6 ng/ml) was significantly higher as compared to the control but significantly lower
than that of the ova+ve group (570.4±24.7 ng/ml). Among the CPPs samples the highest IgE level was observed in the NU sample (186.3±16.6 ng/ml) as compare to TU (170.6±12.9 ng/ml) and TC (178.5±11.8 ng/ml).

The level of serum IgG towards peritoneally injected CPPs samples and ovalbumin is given in Figure 2. At 3 weeks postinjection, IgG level in blood serum of mouse groups injected with CPPs samples did not differ significantly as compared to the control group (245.5±14.6 μg/ml). The serum IgG level of the group injected with Na caseinate (300.8±18.7 μg/ml) differed significantly from the control group and the ova+ve group (568.3±22.8 μg/ml). The highest value for IgG level was observed in the NU sample (266.5±15.6 μg/ml) as compared TU (255.6±10.8 μg/ml) and TC (247.2±12.7 μg/ml).

In this study, the levels of IgG in blood samples of mice was higher as compared to that of IgE induced by all the protein and peptides samples (Figures 1 and 2). There may be the possible explanation as reported by Wood and Wreghitt (1990) and Park and Allen (2000). The serum IgG may compete for antigen binding sites in the indirect ELISA assays or any IgE that was initially increased in the serum was suppressed by IgG, after circulating IgG secretion increased.

All the three caseinophosphopeptides enriched products i.e. TU, TC and NU showed significantly lower values for IgE and IgG as compared to that of Na caseinate and ovalbumin. The levels of IgE and IgG in all these three products remained similar to that of the control. This clearly indicates that the fragments of casein (CPPs) are significantly less or non immunogenic than sodium caseinates. The reduction in antigenicity of the caseinophosphopeptides may be due to removal of antigenic peptides from these products by ultrafiltration (TU, NU) and selective precipitation (TC). Similar findings were reported by Park and Allen (2000), that the tryptic αs-casein phosphopeptides induced significantly less IgG than the intact proteins or whole tryptic hydrolysates. They concluded that protein hydrolysis and subsequent purification of phosphopeptides using ultrafiltration might reduce the immune response.

Otani et al. (2000) observed that mice fed a caseinphosphopeptides added diet did not show enhanced IgG antibody level. Heddleson et al. (1997) also reported that specific antibody levels for the IgE in rats peritoneally injected with casein phosphopeptides rarely exceeded those of unimmunized rats, indicating that the allergenicity of casein phosphopeptides was inert when compared to native β-casein and skim milk proteins.

The present results suggest that the caseinophosphopeptides enriched products prepared from buffalo milk can be used in infant formulas and other functional foods to enhance mineral absorption and with reduced immunogenicity.

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Figure 1. Graphs showing the IgE antibody level in blood serum of the control mouse group and groups injected with ovalbumin, Na caseinate and different CPPs preparations (TU, TC and NU) (The values expressed as means ± SEM for eight mice per group. The values with different small letters superscripts differ significantly at 5% level of significance ($P \leq 0.05$), $CD= 18.7$).

Figure 2. Graphs showing the IgG antibody level in blood serum of the control mouse group and groups injected with ovalbumin, Na caseinate and different CPPs preparations (TU, TC and NU) (The values expressed as means ± SEM for eight mice per group. The values with different small letters superscripts differ significantly at 5% level of significance ($P \leq 0.05$), $CD= 23.34$).


ABSTRACT

During an outbreak of brucellosis in Chikhodra village of Gujarat, India, a study was performed to find the seroprevalence of brucellosis in two farms in the affected area. All animals (104) were screened using the rose bengal plate agglutination test. Thirty-four (32.69%) animals were found serologically positive for this test and positive samples were subjected to standard tube agglutination test for finding antibody titer against brucellosis. Twenty-eight animals had titer >320 IU; titer values of 160 and 80 IU were present in two animals each. Hematological values were compared statistically between seropositive and a control group. It was found that there was a statistical reduction of leukocytes, platelets and mean corpuscular volume in affected animals compared to control animals.

Keywords: brucellosis, rose bengal plate agglutination test, standard tube agglutination test, hematology, seroprevalence

INTRODUCTION

Brucellosis is named after Sir David Bruce, who in 1886 isolated the causative agent from a soldier in Malta, where the disease caused considerable morbidity and mortality among British military personnel. This disease occurs in cattle in most parts of the world. Brucellosis was first recognized in India in 1942. It occurs in cows, buffaloes, sheep, goats, pigs, dogs, and humans. Economic losses due to this disease are considerable in an agrarian country such as India. Outbreaks occur in heifers; older cows become infected but do not abort. Etiological agent is a facultative intracellular organism, and persistent infection is a characteristic feature of this disease. Since it is a zoonotic disease, prevalence of this infection among cattle is dangerous to human beings also. The important aim of this study was to find out the overall seroprevalence of brucellosis in two farms which were found positive for milk ring test.

MATERIALS AND METHODS

This study was carried out during an outbreak of brucellosis (July 2010) at Chikhodra village in Anand district of Gujarat, India. Milk samples from two farms where this study was carried out were already proved positive for the milk ring test. Blood samples were collected from all animals in these farms. Serum was separated from those samples and was subjected to heat treatment to avoid nonspecific reactions (56°C for 30 minutes).
Rose Bengal Plate Agglutination Test
All the procedures were carried out as described by Morgan et al. (1978). Before performing the test, the antigen and sera were brought to room temperature. One drop of serum (30 μl) was put on a glass slide using a micropipette. The antigen bottle was shaken well to ensure homogeneous suspension and one drop of antigen (30 μl) was added to the serum on the slide. The serum and antigen were then mixed quickly using separate spreader for each serum sample. The slide was held over a white surface and rocked gently from side to side for 4 to 5 minutes. Samples showing definite clumping were considered positive for brucellosis. In negative samples, the mixture remained homogenous without formation of any clumps.

Standard Tube Agglutination Test
The standard tube agglutination test (STAT) was performed according to Alton et al. (1975). All sera samples were tested as five dilutions. Five tubes were placed in a rack. Phenol saline 0.8 ml was taken in first tube and 0.5 ml in all other tubes. Serum 0.2 ml was added in the first tube and mixed well and 0.5 ml of diluted serum was transferred to the second tube and mixed thoroughly. Then, 0.5 ml from the second tube was transferred to the third tube. This process was continued up to the fifth tube, and 0.5 ml was discarded from the last tube after mixing. To each tube Brucella abortus antigen 0.5 ml was added and was mixed thoroughly. This provided a final dilution of 1:10, 1:20, 1:40, 1:80 and 1:160 etc. A control tube was set up to simulate 50 percent clearing by mixing 0.50 ml antigen with 1.50 ml of phenol saline in an agglutination tube. All the tubes were then incubated at 37°C for 20 h. Results were read after incubation. The reciprocal of the highest serum dilution showing 50 percent or more agglutination (50 % clearing) was taken as the titer of the serum. The titer so obtained was converted into International Unit (I.U.) of Brucella antibody activity by multiplying with 2 as recommended by the joint FAO/WHO expert committee on Brucellosis.

Hematology
Hematological parameters such as hemoglobin (Hb), total erythrocyte count (TEC), total leukocyte count (TLC), differential leukocyte count (DLC), packed cell volume (PCV), platelets count (PLT), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were measured from K3EDTA added blood samples using a Medonic CA 620 (Merck) blood auto analyzer.

Statistical analysis
Hematological values were analyzed by standard statistical procedure described by Snedecor and Cochran (1992) and were expressed as mean (± SE).

RESULTS AND DISCUSSION
Serological examinations
Out of 104 serum samples tested using the rose bengal plate agglutination test (RBPT), thirty-four were found positive. Those samples found positive for RBPT were subjected to STAT to find the titer of antibody against Brucella infection in the affected animals. Results of STAT are as shown in Table 1.

Antibody detection in paired serum samples is not recommended during an outbreak because of the length of time required to confirm a diagnosis (Radostits et al., 2006). The rose bengal test is an
inexpensive as well as quick test. It is an excellent test for large-scale screening of serum samples for brucellosis. Even though it gives highly sensitive results, it is not a highly specific test. So positive results of RBPT has to be confirmed using some other tests. In this study, positive results of RBPT were further confirmed using STAT. All samples found positive by RBPT were confirmed by STAT except one which was negative for this test. Combined results of RBPT and STAT are shown in Table 2.

**Hematology**

The mean hematological values of infected and control group are as shown on Table 3. Mean corpuscular volume, platelet count and total leukocyte count of the infected group were significantly decreased ($P<0.05$) compared to the control group. Figure1 depicts the percentage of animals suffering from different hematological conditions.

Even though hematological studies of brucellosis in human are adequate in number, there are very few in cattle. Results of this study showed that mean corpuscular volume, platelet count and total leukocyte count of the infected group significantly decreased ($P<0.05$) compared to control group. Similar observation has been reported in human brucellosis also (Crosby et al., 1984). Other nonsignificant conditions noted in the affected animals such as leukocytosis, anemia, lymphocytopenia and pancytopenia were also observed in human beings by many scientists (Crosby et al., 1984; Kadri et al., 2003). Lymphopenia has been recorded in Brucella infected camels also (El-Boshy et al., 2009).

Multiple possible mechanisms are responsible for thrombocytopenia and leukocytopenia in brucellosis (Crosby et al., 1984). Hemophagocytosis, disseminated intravascular coagulation, direct damage of bacteria to platelets, granulomatous lesions of the bone marrow, bone marrow suppression, hypersplenism, and immune-mediated damage are thought to be the major reasons for abnormal hematology. Krauss (2003) pointed hemophagocytic lymphohistiocytosis as the reason for leucopenia, thrombocytopenia and anemia. Increase and decrease of total leukocyte count in blood may depend on the stage of infection in animals. Pancytopenia in brucellosis is also multifactorial in origin and is attributed to hypersplenism and bone marrow involvement (Abdi-Liae et al., 2007). Marked pancytopenia or isolated deficits can be attributed to diffuse intravascular coagulation, hemophagocytosis, or immunologically mediated cellular destruction (Pappas et al., 2005). Bacteremia occurring while brucellosis explains granulocytosis noted in some affected animals. In the later stages of the infection, lymph nodes may develop chronic granulomatous lymphadenitis, leading to lymphoid depletion (Radostits et al., 2006). This may result in lymphocytopenia in many of the affected animals.

The herd characteristics and the results of the first herd test may be used as predictors of the potential presence or absence of *Brucella abortus* in herds with reactors to the tube agglutination test. The presence of only single suspicious reactor on the first test is a reliable predictor of lack of infection. The presence of one or more positive reactors on the first herd test is a reliable predictor of the presence of infection. Presence of more seropositive animals indicates high prevalence of brucellosis in these two farms.
Table 1. Results of the standard tube agglutination test.

<table>
<thead>
<tr>
<th>Titer (IU)</th>
<th>No. of animals</th>
<th>Interpretation of titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;320</td>
<td>28</td>
<td>Positive</td>
</tr>
<tr>
<td>160</td>
<td>2</td>
<td>Positive</td>
</tr>
<tr>
<td>80</td>
<td>2</td>
<td>Positive</td>
</tr>
<tr>
<td>40</td>
<td>1</td>
<td>Doubtful</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table 2. Combined results of the rose bengal plate agglutination test and the standard tube agglutination test.

<table>
<thead>
<tr>
<th>Test</th>
<th>Total</th>
<th>Serologically Positive</th>
<th>Doubtful</th>
<th>Serologically Negative</th>
<th>Percentage of Seroprevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBPT</td>
<td>104</td>
<td>34</td>
<td>-</td>
<td>-</td>
<td>32.69%</td>
</tr>
<tr>
<td>STAT</td>
<td>34</td>
<td>32</td>
<td>1</td>
<td>1</td>
<td>30.77%</td>
</tr>
</tbody>
</table>

Table 3. Hematological values of infected and control animals.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Brucellosis positive animals</th>
<th>Control animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEC (Millions / μl)</td>
<td>6.07 ± 0.31</td>
<td>6.10 ± 0.26</td>
</tr>
<tr>
<td>MCV (µμ l)</td>
<td>44.60 ± 0.85*</td>
<td>46.99 ± 0.75</td>
</tr>
<tr>
<td>PLT (Thousand / µl)</td>
<td>115.76 ± 13.13*</td>
<td>128.82 ± 16.63</td>
</tr>
<tr>
<td>TLC (Thousand / µl)</td>
<td>6.13 ± 0.70*</td>
<td>7.36 ± 0.72</td>
</tr>
<tr>
<td>Hb (g%)</td>
<td>9.41 ± 0.50</td>
<td>9.78 ± 0.34</td>
</tr>
<tr>
<td>MCH (µg)</td>
<td>15.50 ± 0.25</td>
<td>16.15 ± 0.25</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>34.81 ± 0.20</td>
<td>34.32 ± 0.20</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>43.73 ± 3.42</td>
<td>45.53 ± 5.11</td>
</tr>
<tr>
<td>Mid cells (%)</td>
<td>11.50 ± 0.95</td>
<td>10.32 ± 0.92</td>
</tr>
<tr>
<td>Granulocytes (%)</td>
<td>44.76 ± 3.53</td>
<td>43.56 ± 4.71</td>
</tr>
</tbody>
</table>

*P<0.05 Significant parameter.
REFERENCES


ABSTRACT

The present study was aimed to characterize the local field bovine herpesvirus 1 (BHV1) by cloning and sequencing of the glycoprotein C (gC) gene segment of BHV1, amplified by the polymerase chain reaction (PCR). The PCR product of 173 bp of a representative positive semen sample was cloned using the pTZ57R/T vector supplied with the InstAclone™ PCR product cloning kit. The cloned white colonies were confirmed by M13 PCR, which yielded an expected amplified product of 327 bp. The cloned PCR product of the field BHV1 gC gene was sequenced and then analysed by in silico analysis. The consensus sequence of 168 bp obtained was compared with the six published GenBank sequences, and it was found that the field BHV1 from Gujarat showed a range of homology (89% to 92%) with maximum identity (92%) with the BHV1 of three European and one North American isolates. The sequence obtained showed deletions at two positions i.e. 40 and 70 nucleotides and point mutations at seven different positions (2, 61, 116, 134, 143, 151 and 155) as compared to the six published sequences. Such alterations in the sequence indicated frameshift mutations in the gC gene region, with the corresponding alteration in amino acid (aa) sequence.

Keywords: buffalo bull, Bubalus bubalis, semen, cloning, bovine herpesvirus 1, BHV1

INTRODUCTION

Bovine herpesvirus 1 (BHV1) is associated with a variety of clinical diseases, including infectious bovine rhinotracheitis, conjunctivitis, reproductive disorders, encephalitis, and generalized systemic infections. BHV1 is a member of the genus Varicellovirus in the subfamily Alphaherpesvirinae, which belongs to the family Herpesviridae. It is an enveloped virus having an icosahedral nucleocapsid consisting of 162 capsomeres. The BHV1 genome is a single linear double stranded DNA having a total size of 135-140 kilobase pairs (kbp) (Mayfield et al., 1983).

BHV1 can establish a latent infection like other alphaherpesviruses (Gibbs and Rweyemamu, 1977) in the nerve ganglia of infected but clinically normal animals after the primary infection despite the development of neutralizing antibody (Pastoret et al., 1979). This kind of infection persists for the whole lifespan of an infected animal and therefore, such animals should be regarded as virus carriers and as a potential source of infection. Latent
infections are especially important in bulls, because these animals shed the virus in semen during both clinical and sub-clinical infections. Use of BHV1 infected bulls or their semen for AI can transfer the infection to the female population.

Schroeder and Moys recognized the IBR disease in the United States during the 1950s for the first time. In India, the first case of BHV1 infection was reported by Mehrotra et al. (1976) from cases of keratoconjunctivitis amongst crossbred calves at an organized cattle herd in Uttar Pradesh. Various workers have characterized BHV1 by sequencing the targeted genes. Sequencing provides an authentic tool to genetically characterize the target genes and the virus, thus providing valuable information on comparative variation among the viruses. The various glycoproteins present on the envelope of BHV1 play important role in the viral pathogenesis and the immunity. Some of these like gB and gD, are essential for viral replication while others are non-essential. BHV-1 codes for four non essential genes, viz. gC, gG, gI and gE (Kamiyoshi et al., 2008). Glycoprotein C (gC) has been found important for the attachment of the virus (Liang et al., 1991) and inducing neutralizing antibody response (Denis et al., 1994) in the host. It has been targeted earlier for various studies (Fitzpatrick et al., 1989; Brower et al., 2008; Anita et al. 2010; Lojkic et al., 2011). In the present study, the target gC gene was cloned and sequenced to characterize the Indian isolate of BHV1 from semen samples of breeding bulls.

MATERIALS AND METHODS

Cloning of gC gene in pTZ57R vector

The amplified PCR product (173 bp), using gC gene based primers described previously by Engelenburg et al. (1993), from a representative buffalo bull semen samples (of the 20 samples found positive by PCR out of the 52 buffalo semen samples processed) of an AI centre was purified using a Perfectprep® PCR cleanup kit (Eppendorf, Germany). The bulls kept on the AI centre had history of poor semen quality, and the females of the surrounding population had problem of repeat breeding and infertility. Purified PCR product was ligated in pTZ57R vector using an InsT/AClone™ cloning kit (MBI Fermentas) and the vector containing the insert was propagated in E.coli hosts (DH5-α). The vector carries an ampicillin resistance gene (bla) as selectable marker for transformed cells. Thirty-five μl of 2% X-gal solution and eight μl of 20% IPTG solution were pipetted on to a pre-made 90-mm LB agar plates containing Ampicillin (50 μg/ml). The solutions were spread with the help of a sterile bent glass rod. The plates were incubated at 37°C until all the fluid disappeared. Each of the individual colonies obtained following the transformation procedure were picked up and streaked in a regular pattern on the X-gal/IPTG plate. The plates were incubated at 37°C for 12-15 h. Growth on those sectors showing white colonies was selected for further screening and confirmation of recombinant clones carrying the correct insert was done by M13 PCR using M13 primers. The amplified PCR products were checked on 2% agarose gel, and clones which showed the amplification of 327 bp DNA fragment were considered as positive clones carrying the desired insert of the gC gene. The recombinants obtained were selected and grown in large quantity for plasmid extraction using a Fastplasmid® Mini Kit (Eppendorf, Germany).

Sequencing of gC Gene Segment

The concentration of the purified gC gene
PCR product using plasmid DNA was determined and was subjected to automated DNA sequencing on an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried out using a BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) following the manufacturer’s instructions. The sequences obtained in forward and reverse orientations were assembled by using BioEdit software. The consensus sequence of the gC gene obtained was aligned with known sequences of BHV1 available in GenBank using NCBI BLAST and CLUSTAL W (1.82) software. The PCR product used for sequencing was obtained directly from the field virus (named as Raj/ibr/Guj) without isolating the virus in cell culture.

RESULTS AND DISCUSSION

Cloning of gC gene of BHV1

Cloning of the targeted gene has been preferred prior to sequencing because cloning of the product facilitates sequencing allowing the use of primers in the vector, thus enabling us to determine the sequence of the whole insert without losing the sequence information of the initial stretch of the amplicon. Therefore, in this study, the PCR amplified product from a representative sample was purified and ligated to pTZ57R/T vector, having T overhang, supplied with the InSt/Aclone™ PCR product cloning kit. This vector has been reported to facilitate cloning of PCR product having A overhang (generated by Taq DNA polymerase). The ligated mixture was used to transform E. Coli (DH5-α) and the transformants were screened by blue white selection on X-gal/IPTG/LB agar plates containing ampicillin. After 12-15 h incubation, blue and white colonies were seen on the plates. All the white colonies which might contain the insert were streaked on a fresh plate for further analysis. The white colonies were screened for the presence of correct the PCR product by colony PCR using M13 primers. Colony PCR revealed the expected 327 bp product in the transformed colonies.

A similar approach had also been adapted by Rai et al. (2002) for cloning the PCR product of 520 bp sequence of gC gene of BHV1 using plasmid vector. Fuchs et al. (1999) cloned the PCR products obtained with strain LA of BHV1 as the template into the plasmid vector pCRII using the TA cloning kit.

Sequencing of gC gene of BHV1

The curated sequence obtained by using forward and reverse primers were assembled using the BioEdit software programme and a consensus sequence of 168 bp was obtained. This consensus sequence was then further used for alignment with the published sequence of BHV1 in GenBank using NCBI BLAST and CLUSTAL W (1.82) software.

Fuchs et al. (1999) used a similar approach for sequencing the gC gene of strain LA of BHV1 isolated from blood. They cloned the strain LA DNA and used the purified plasmid for DNA sequencing by automatic sequencing (model 377; Applied Biosystems, Weiterstadt, Germany) by using a dye terminator cycle sequencing kit (Applied Biosystems, Perkin-Elmer Corp., Weiterstadt, Germany). Sequence data were analyzed with the Wisconsin Package (version 9.1; Genetics Computer Group, Madison, Wis.) and BLAST.

Kholy and Abdelrahman (2006) directly sequenced the PCR amplicons of the Egyptian vaccinal Abu-Hammad strain of BHV1 gD gene in both directions with the same primers as those used to generate the PCR amplicons. Sequencing was carried out in an ABI PRISM system using
the dideoxy chain-termination method, which is based on the incorporation of fluorescent-labelled dideoxynucleotide terminators. The nucleotide sequences were aligned using the Clustal W (1.82) program from the European Bioinformatics Institute as done in the present study.

Brower et al. (2008) targeted and sequenced gC gene for the detection of BHV-1 in the brain tissues (an atypical manifestation of BHV-1) of aborted fetuses and differentiate it from BHV-5, which has 85% homology with BHV-1 throughout genome. But, these two differ considerably in some regions, viz. the thymidine kinase, gC and gE genes.

Lojkic et al. (2011) detected the BHV-1 infection in three dairy herds by gC gene based PCR and analysed the sequence of the gC gene of one isolate from each farm. On phylogenetic analysis based on gC gene sequence, the isolates were clustered with BHV-1.1.

The DNA sequence (168 bp) of the amplified gC region of BHV1 obtained in this study was deposited in GenBank under accession no. EU086706.

**Sequence analysis**

The nucleic acid sequences obtained were aligned with known sequences BHV1CGEN (Accession no. AJ004801), BVH1LFT31 (Accession no. Z54206), HSBGPG3A (Accession no. M27491), AY052397 (Accession no. AY052397), AF135441 (Accession no. AF135441) and BHV1GC (Accession no. Z49223) of BHV1 published in GenBank. The sequence alignment of the field BHV1 (named Raj/ibr/Guj) sample with the published sequences is presented in Figure 1.

Nucleotide (nt) sequence alignment of the gC region of the field BHV1 and published sequences showed variable percentages of homology (89% to 92%) (Table 1). The three European BHV1 isolates (BHV1CGEN, BVH1LFT31 and AF135441) and a North American isolate (HSBGPG3A) showed 92% homology with the field BHV1 (Raj/ibr/Guj), which was the maximum level of similarity. Lesser homology of 90% and 89% was observed with AY052397 (a South American isolate) and BHV1GC (a European isolate).

In contrast to present study, Fuchs et al. (1999) found homology of 100% between the amplified gC gene (LA strain) and the published sequence HSBGPG3A (accession no. M27491). Compared to the gC sequence of BHV1GC (accession no. Z49223), 10 base differences (leading to four different amino acids) accounted for 98.1% homology with the LA strain. Similarly, the homology was 89% due to 17 bp differences in the present study including deletions at two positions between the sequence obtained in the present study (Raj/ibr/Guj) and the BHV1GC (accession no. Z49223).

Comparison of the sequence of the amplified gC region of the field BHV1 with the published sequences showed deletions at 40 and 70 nucleotides, which indicate point deletion mutations at these places suggesting genetic variation in the gC gene of the field BHV1 of Gujarat. Out of the seven sequences subjected under multiple alignment, five sequences including the sequence (Raj/ibr/Guj) obtained in the present study [the other four being HSBGPG3A (Accession no. M27491), AF135441 (Accession no. AF135441), BHV1GC (Accession no. Z49223) and AY052397 (Accession no. AY052397)] revealed deletions from positions 90-93, 105-111 and 137-138. While these positions showed the properly aligned nucleotides in a conserved way among the two other sequences viz. BHV1CGEN (Accession no. AJ004801) and BVH1LFT31 (Accession no. Z54206). Moreover,
the sequence obtained in the present study revealed point mutations at seven positions, i.e. at positions 2, 61, 116, 143, 151 and 155, where A, C, C, C, G, T and C replaced G, A, A, G, C, G and A, which were found conserved in all the other six sequences. Such a situation of nt variations possibly indicates a frameshift mutation in the gene region, which can result in considerable amino acid (aa) variations.

The nt sequence obtained in the present study was translated into amino acid sequence by using Expasy software program. Out of three frames obtained, frame 1 was chosen for getting aa sequence as it yielded corresponding proper stretch of aa with proper alignment with the reference sequences, which was then further analyzed for alignment with the published aa sequences of BHV1 in GenBank using NCBI BLAST and CLUSTAL W (1.82) software.

The deduced aa sequence of the gC region of field BHV1 was compared with the published aa sequences obtained from the same six sequences BHV1CGEN (Accesion no. AJ004801), BVH1LFT31 (Accession no. Z54206), HSBGPG3A (Accession no. M27491), AF135441 (Accession no. AF135441), AY052397 (Accession no. AY052397) and BHV1GC (Accesion no. Z49223). The reference sequences BHV1CGEN, BVH1LFT31 and AF135441 of the European region and HSBGPG3A of the North American region scored the highest gC sequence identity (79%) with the aa sequence of the gC region of field BHV1, followed by the European isolate BHV1GC (76%) and the South American isolate AY052397 (75%). The deduced aa sequence alignment with the published aa sequences is presented in Figure 2. In spite of high gC nt sequence identity (92%) between the field BHV1 and reference BHV1CGEN, BVH1LFT31, HSBGPG3A and AF135441 sequences, a lower deduced aa homology (79%) was observed. Similarly, lower aa homology percentages were recorded between the field BHV1 and the other reference sequences studied. This could be attributed to the occurrence of possible frameshift mutations as discussed earlier. Both nt and deduced aa sequence alignments revealed variable degrees of sequence homology between the field BHV1 and published sequences.

In a similar study carried out by Kholy and Abdelrahman (2006), frameshift mutations were observed at nt 509 and 615 of the Egyptian BHV1 gD gene. Both nt and aa sequence alignments revealed variable degrees of sequence similarity with other alphaherpesviruses. Thus, the genetic variations, in the field BHV1, resulted due to possible frameshift mutations in the gC gene regions indicate antigenic differences between the Indian field virus and BHV1 strains from other parts of world.

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The authors are thankful to Professor and head, Department of Animal Biotechnology, College of Veterinary Science and A.H, Anand, for providing necessary facilities to carry out the present work.

REFERENCES


STUDIES ON THE TEMPERAMENT OF MURRAH BUFFALOES WITH VARIOUS UDDER AND TEAT SHAPES AND ITS EFFECT ON MILK YIELD

R.M.V. Prasad¹ and P. Jaya Laxmi²

ABSTRACT

A total of 200 buffaloes were utilized to study the temperament in Murrah buffaloes with various udder and teat shapes and its effect on the milk yield. Majority of the buffaloes with all udder shapes and with conical, pear-, cylindrical and funnel-shaped teats were with docile temperament. Slightly restless and restless temperaments were more frequent in buffaloes with bottle-shaped teats. However, there was no significant variation in the frequencies of buffaloes with different temperament scores among various udder teat shape categories. There were no significant differences among the temperament groups for various udder and teat measurements studied. The average milk yield in docile, slightly restless, restless, aggressive and nervous categories were 6.70 ± 0.15, 6.50 ± 0.34, 5.70 ± 0.26, 4.90 ± 0.30 and 4.60 ± 0.34 kg, respectively. It may be concluded that udder and teat morphology has no influence on the temperament but that temperament has an influence on milk yield and so buffaloes with docile temperament must be preferred for use in breeding programmes.

Keywords: temperament, udder shape, teat shape, Murrah buffaloes

INTRODUCTION

Temperament of buffaloes is important from the management point of view, and it also plays a role in influencing the milk yield. There were many studies on the temperament of cows but literature on temperament of buffaloes is very scant. Similarly, there are very few studies on the udder and teat morphology of buffaloes. Hence, the present study was taken up to understand the temperament of Murrah buffaloes in relation to udder and teat morphology and its effect on milk yield under rural conditions of India.

MATERIALS AND METHODS

The study was carried out in the Krishna and West Godavari districts of Andhra Pradesh. A total of 200 Murrah buffaloes were utilized for the study.

Description of Temperament Score

Subjective scoring was done on a 1-5 scale by the investigator through visual assessment of the animal’s reactivity during milking. Buffaloes were classified into five categories based on the

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milking temperament as docile (1), slightly restless (2), restless (3), aggressive (4) and nervous (5), respectively as per Tulloh (1961) and (Dogra 2002). The particulars observed for recording the temperament score of Murrah buffaloes are presented in Table 1.

Statistical analysis

Simple tabular technique of analysis using statistical tools such as frequencies and percentages was used to present the prevalence of various temperaments and udder and teat shapes in Murrah buffaloes. The chi-square test of contingency was used to study if there is any significant variation in the temperament of buffaloes according to the udder and teat shapes (that is to test if the frequency of animals with different temperament scores vary significantly among the udder and teat shape categories).

To test the significance of variation in milk yield among different temperament groups, analysis of variance with temperament as the source of variation was used (F- test). The means were compared for the significant difference (P<0.05) using Duncan’s multiple range test. The data obtained on various udder and teat parameters and milk yield were analyzed using an SPSS statistical package (version 15.0.1).

RESULTS AND DISCUSSION

Temperament in relation to udder and teat morphology

It was observed from the study that majority of the buffaloes with all udder shapes belonged to the docile category. The numbers of buffaloes in the nervous category were very few, indicating the attitude of the farmers and also the farm manager preferring animals with calm behaviour. It was further observed through chi-square test of contingency that there was no significant variation in the frequencies of animals with different temperament scores among different udder shape categories (Table 2).

Regarding the teat shape, majority of animals with conical-, pear-, cylindrical and funnel-shaped teats were of docile temperament. In the case of the bottle-shaped teat category, maximum percentage of buffaloes were in slightly restless and restless categories of temperament with no animals in the nervous group. However, the chi-square test of contingency revealed that there was no significant variation in the frequencies of animals with different temperament scores among various teat shape categories (Table 3).

Thus, it may be understood that temperament is not influenced by udder and teat shapes of buffaloes.

Regarding the mean udder measurements, udder width was lowest in docile buffaloes (49.16 ± 0.43 cm) followed by those in the nervous, aggressive, slightly restless and restless categories. The udder length was also lowest in docile buffaloes followed by those in the nervous, aggressive, restless and slightly restless categories. The udder depth was lowest in slightly restless animals (15.45 ± 0.23 cm) followed by buffaloes in the docile, nervous, restless and aggressive categories (Table 5). The average teat length and diameter were lowest in slightly restless animals (Table 6). However, in view of the non-significant differences among the temperament groups for the various udder and teat measurements studied, udder and teat dimensions did not appear to influence the temperament in Murrah buffaloes. Lack of literature on these aspects necessitates further studies in this regard to find out if there is any relationship between the
Table 1. Particulars observed for recording the temperament score in Murrah buffaloes during milking.

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Class</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very quiet; never gives any trouble, extremely docile during milking and preparation, the “ideal” milker</td>
<td>Docile</td>
<td>1</td>
</tr>
<tr>
<td>Stands quietly, not bothered by preparation or milking, but may move frequently, shifting weight from side to side, may flick tail occasionally, gives very little trouble.</td>
<td>Slightly restless</td>
<td>2</td>
</tr>
<tr>
<td>Generally quiet, but moves around a lot; may lift feet occasionally during preparation or milking, but does not kick, flicks tail frequently or appears restless occasionally.</td>
<td>Restless</td>
<td>3</td>
</tr>
<tr>
<td>Appears very restless during preparation or milking; kicks at handler occasionally; steps from side to side a great deal; quivers when a hand is placed on her.</td>
<td>Aggressive</td>
<td>4</td>
</tr>
<tr>
<td>Appears very restless during preparation or milking, kicks the handler and struggles violently, occasionally, quivers when hand is placed over the back</td>
<td>Nervous</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2. Frequency and percentage of different temperament scores in Murrah buffaloes with different udder shapes.

<table>
<thead>
<tr>
<th>Udder Shape</th>
<th>Temperament (Score)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Docile (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Slightly restless (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Restless (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aggressive (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nervous (5)</td>
<td></td>
</tr>
<tr>
<td>Bowl</td>
<td>59 (48.36%)</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>25 (20.49%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19 (15.57%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 (10.65%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (4.91%)</td>
<td></td>
</tr>
<tr>
<td>Globular</td>
<td>26 (76.47%)</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>1 (2.94%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 (14.70%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 (2.94%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 (2.94%)</td>
<td></td>
</tr>
<tr>
<td>Goaty</td>
<td>13 (72.22%)</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 (11.11%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 (11.11%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 (5.55%)</td>
<td></td>
</tr>
<tr>
<td>Pendulous</td>
<td>9 (34.61%)</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>6 (23.07%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 (19.23%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (11.53%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (11.53%)</td>
<td></td>
</tr>
<tr>
<td>overall</td>
<td>107 (53.50%)</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>32 (16.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31 (15.50%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19 (9.50%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11 (5.50%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Frequency and percentage of different temperament scores in Murrah buffaloes with different teat shapes.

<table>
<thead>
<tr>
<th>Teat shape</th>
<th>TEMPERAMENT (Score)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Docile (1)</td>
<td>Slightly restless (2)</td>
</tr>
<tr>
<td>Conical</td>
<td>13 (59.09)</td>
<td>3 (13.63)</td>
</tr>
<tr>
<td>Bottle</td>
<td>5 (22.72)</td>
<td>8 (36.36)</td>
</tr>
<tr>
<td>Pear</td>
<td>24 (60.00)</td>
<td>3 (7.50)</td>
</tr>
<tr>
<td>Cylindrical</td>
<td>55 (57.29)</td>
<td>15 (15.62)</td>
</tr>
<tr>
<td>Funnel</td>
<td>10 (50.00)</td>
<td>3 (15.00)</td>
</tr>
<tr>
<td>Total</td>
<td>107 (53.50)</td>
<td>32 (16.00)</td>
</tr>
</tbody>
</table>

Table 4. Average daily milk yield (kg) in Murrah buffaloes with different temperaments.

<table>
<thead>
<tr>
<th>Temperament</th>
<th>Docile</th>
<th>Slightly restless</th>
<th>Restless</th>
<th>Aggressive</th>
<th>Nervous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average daily milk yield* (Mean ± S.E)</td>
<td>6.70±0.15a</td>
<td>6.50±0.34a</td>
<td>5.70±0.26b</td>
<td>4.90±0.30c</td>
<td>4.60±0.34d</td>
</tr>
<tr>
<td>N</td>
<td>107</td>
<td>32</td>
<td>31</td>
<td>19</td>
<td>11</td>
</tr>
</tbody>
</table>

* Means with similar superscripts do not differ significantly.
Table 5. Mean udder dimensions and distance between the teats (cm) in Murrah buffaloes with different temperament scores.

<table>
<thead>
<tr>
<th>Temperament score</th>
<th>n</th>
<th>% of Total No.</th>
<th>Udder width</th>
<th>Udder length</th>
<th>Udder depth</th>
<th>Distance between front teats</th>
<th>Distance between hind teats</th>
<th>Distance between front and hind teats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Docile</td>
<td>107</td>
<td>53.5</td>
<td>49.16 ±0.43</td>
<td>53.40 ±0.47</td>
<td>15.52 ±0.13</td>
<td>8.70 ±0.22</td>
<td>8.69 ±0.24</td>
<td>8.71 ±0.21</td>
</tr>
<tr>
<td>Slightly restless</td>
<td>32</td>
<td>16.0</td>
<td>52.06 ±0.84</td>
<td>55.49 ±0.65</td>
<td>15.45 ±0.23</td>
<td>8.53 ±0.40</td>
<td>8.21 ±0.42</td>
<td>8.04 ±0.27</td>
</tr>
<tr>
<td>Restless</td>
<td>31</td>
<td>15.5</td>
<td>53.69 ±1.02</td>
<td>55.09 ±0.80</td>
<td>15.68 ±0.25</td>
<td>8.49 ±0.43</td>
<td>8.66 ±0.49</td>
<td>9.24 ±0.37</td>
</tr>
<tr>
<td>Aggressive</td>
<td>19</td>
<td>9.5</td>
<td>51.38 ±1.16</td>
<td>54.56 ±1.25</td>
<td>16.05 ±0.27</td>
<td>8.96 ±0.54</td>
<td>9.10 ±0.56</td>
<td>10.06 ±0.56</td>
</tr>
<tr>
<td>Nervous</td>
<td>11</td>
<td>5.5</td>
<td>50.80 ±1.58</td>
<td>54.45 ±1.69</td>
<td>15.60 ±0.41</td>
<td>7.84 ±0.51</td>
<td>8.25 ±0.50</td>
<td>9.33 ±0.65</td>
</tr>
<tr>
<td>Overall</td>
<td>200</td>
<td>100</td>
<td>50.63 ±0.36</td>
<td>54.16 ±0.33</td>
<td>15.59 ±0.09</td>
<td>8.62 ±0.16</td>
<td>8.62 ±0.17</td>
<td>8.85 ±0.15</td>
</tr>
</tbody>
</table>

Table 6. Mean teat dimensions (cm) in Murrah buffaloes with different temperament scores.

<table>
<thead>
<tr>
<th>Temperament score</th>
<th>Teat Length</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left fore</td>
<td>Right fore</td>
<td>Left hind</td>
<td>Right hind</td>
<td>Left fore</td>
<td>Right fore</td>
<td>Left hind</td>
<td>Right hind</td>
</tr>
<tr>
<td>Docile</td>
<td>7.13 ± 0.17</td>
<td>7.18 ± 0.18</td>
<td>7.81 ± 0.20</td>
<td>7.83 ± 0.19</td>
<td>7.48 ± 0.18</td>
<td>2.63 ± 0.03</td>
<td>2.71 ± 0.03</td>
<td>2.82 ± 0.04</td>
</tr>
<tr>
<td>Slightly restless</td>
<td>6.74 ± 0.35</td>
<td>7.04 ± 0.41</td>
<td>7.70 ± 0.44</td>
<td>7.55 ± 0.42</td>
<td>7.26 ± 0.39</td>
<td>2.64 ± 0.07</td>
<td>2.65 ± 0.09</td>
<td>2.70 ± 0.09</td>
</tr>
<tr>
<td>Restless</td>
<td>8.18 ± 0.43</td>
<td>8.60 ± 0.46</td>
<td>9.38 ± 0.57</td>
<td>9.05 ± 0.50</td>
<td>8.81 ± 0.47</td>
<td>2.65 ± 0.73</td>
<td>2.71 ± 0.08</td>
<td>2.78 ± 0.09</td>
</tr>
<tr>
<td>Aggressive</td>
<td>8.00 ± 0.56</td>
<td>8.09 ± 0.54</td>
<td>9.16 ± 0.69</td>
<td>8.74 ± 0.55</td>
<td>8.50 ± 0.56</td>
<td>2.76 ± 0.08</td>
<td>2.83 ± 0.09</td>
<td>2.90 ± 0.11</td>
</tr>
<tr>
<td>Nervous</td>
<td>8.33 ± 0.92</td>
<td>8.34 ± 0.90</td>
<td>9.03 ± 1.00</td>
<td>8.90 ± 0.94</td>
<td>8.65 ± 0.93</td>
<td>2.76 ± 0.12</td>
<td>2.71 ± 0.17</td>
<td>2.79 ± 0.15</td>
</tr>
<tr>
<td>Overall</td>
<td>7.37 ± 0.15</td>
<td>7.53 ± 0.16</td>
<td>8.23 ± 0.18</td>
<td>8.12 ± 0.17</td>
<td>7.81 ± 0.16</td>
<td>2.65 ± 0.02</td>
<td>2.71 ± 0.03</td>
<td>2.80 ± 0.03</td>
</tr>
</tbody>
</table>
temperament and udder and teat morphology in buffaloes.

Temperament in relation to the average daily milk yield

The average daily milk yields in the docile, slight restless, restless, aggressive and nervous categories were 6.70 ± 0.15, 6.50 ± 0.34, 5.70 ± 0.26, 4.90 ± 0.30 and 4.60 ± 0.34 kg, respectively (Table 4). Thus, the docile and slightly restless buffaloes had significantly higher daily milk yield when compared to the other categories. These findings were in conformity with findings of Mishra et al. (1975), Dash et al. (1976), Nayak and Mishra (1984), Gupta et al. (1985), Dogra et al. (2002), Bharadwaj et al. (2007) and Lallawmkimi and Mahendra Singh (2009).

The difference in the temperament of buffaloes observed could be because of the individual variability in an animal’s physical, hormonal and neuronal characteristics (Kilgour, 1975). The descending order of milk yield from docile to slightly restless, restless, aggressive and nervous cows could be because of the fact that under optimum conditions of milking the docile ones did not hold up any milk, while the other categories held up milk due to secretion of adrenalin. Thus a negative correlation existed between the temperament score and the average daily milk yield which is in conformity with Nayak and Mishra (1984).

The temperament of animals is generally considered innate and is the result of the animal’s physical and nervous organization (Dickson, 1970) but is also affected by external factors (Gangwar, 1982). Thus proper handling and management are important for temperament and higher milk yield. Rushen et al. (1999) also stressed that proper care and handling by people responsible for the cows are important to avoid fear in cows. It may therefore be understood that both genetic and managemental factors are important for the temperament of buffaloes.

It may be concluded from the study that temperament is not influenced by udder and teat morphology but that temperament has an influence on the milk yield of Murrah buffaloes and so animals with docile temperament must be preferred for use in breeding programmes.

REFERENCES


ABSTRACT

Disposition kinetics and urinary excretion of levofloxacin was investigated after a single intravenous dose of 3 mg.kg\(^{-1}\) in male buffalo calves. The drug concentration was estimated in plasma and urine by microbiological assay using *E. coli* as the test organism. Disposition kinetic parameters were determined using a two-compartment open model and an appropriate dosage schedule was computed. Drug levels above MIC in plasma were detected up to 10 h. Levofloxacin was rapidly distributed from blood to the tissue compartment as evidenced by high values of the distribution rate constant (7.46 ± 0.28 h\(^{-1}\)) and the micro rate constant of transfer of drug from the central to the peripheral compartment (4.88 ± 0.21 h\(^{-1}\)). The elimination half-life, AUC and volume of distribution were 2.56 ± 0.09 h, 10.5 ± 0.11 μg.h.ml\(^{-1}\) and 1.08 ± 0.05 L.kg\(^{-1}\), respectively. Approximately 25% of the microbiological activity of the administered levofloxacin was recovered in the urine of calves within 24 h. On the basis of disposition kinetic parameters, an appropriate intravenous dosage regimen for levofloxacin in buffalo calves would be 3.0 mg.kg\(^{-1}\) to be repeated at 12 h intervals. The observations on urinary excretion indicated that levofloxacin may be effective against urinary tract infections in buffalo species.

Keywords: buffalo calves, dosage, levofloxacin, disposition, urinary excretion

INTRODUCTION

Fluoroquinolone antibacterials are being increasingly employed in veterinary medicine for the treatment of mild to severe bacterial infections. Levofloxacin, a second generation fluoroquinolone, is very effective in the treatment of infections of upper and lower respiratory tract, genitourinary system, skin and soft tissue and possesses excellent activity against gram-positive, gram-negative and anaerobic bacteria. The pharmacokinetics of levofloxacin has been investigated in man (Chulavatnatol et al., 1999), rabbits (Destache et al., 2001) and calves (Dumka and Srivastava, 2007; Dumka et al., 2008). However, there is meager information available on the pharmacokinetics of levofloxacin in buffalo species barring two studies after extravascular administration (Ram et al., 2008, 2010).

In view of the marked species variation in the kinetic data of antimicrobial drugs, the present study was undertaken to determine the pharmacokinetics, urinary excretion and an appropriate dosage regimen of levofloxacin in buffalo calves following its single intravenous administration.
MATERIALS AND METHODS

The study was conducted on six healthy male buffalo calves of non-descript breed, ranging between 1-1.5 years of age and weighing 82-148 kg. The animals were kept in an animal shed under standard conditions and had access to green fodder and water ad libitum. The experimental protocol followed ethical guidelines on the proper care and use of animals and was approved by the institutional animal ethics committee. Levoflaxacin was administered at the dose of 3 mg.kg⁻¹ body weight into the left jugular vein.

For the disposition study, the animals were kept in metabolic stalls of standard size designed in such a way that whole amount of urine excreted naturally by the animals within a period is automatically collected without contamination or spillage in the containers placed beneath the stalls. Blood samples (5 ml) were withdrawn from the contralateral jugular vein into heparinized glass centrifuge tubes before and at 1, 2.5, 5, 10, 15, 30 minutes and 1, 2, 4, 6, 8, 10, 12, 16 and 24 h after administration of the drug. Plasma was separated by centrifugation at 1300 g. Urine samples were also collected simultaneously from the same animals at time intervals of 2, 4, 6, 8, 10, 12, 16 and 24 h after administration of the drug. The volume of total urine voided and collected in the container was measured for each animal, and after filtration, 10 ml samples were taken for analysis.

The concentration of levofloxacin in plasma and urine samples was estimated by a microbiological assay technique using Escherichia coli (ATCC 10536) as the test organism. This method estimated the level of drug and its active metabolites having antibacterial activity. The assay could detect a minimum of 0.1 μg.ml⁻¹ of levofloxacin (Figure 1). Disposition parameters were calculated manually by regression analysis.

RESULTS AND DISCUSSION

Evaluation of the results revealed that the disposition pattern of levofloxacin best fitted a two-compartment open model (Figure 1). Consistent to our findings, the disposition curve of levofloxacin in calves (Dumka and Srivastava, 2007) and other fluoroquinolones, danofloxacin in buffalo calves and ofloxacin in calves, followed a two-compartment open model after intravenous administration (Gaur et al., 2004; Gosal et al., 2008).

The disposition parameters of levofloxacin in buffalo calves are presented in Table 1. Levofloxacin was rapidly transferred from the central to the peripheral compartment in buffalo calves, as is evident from the short t₁/₂α and high value of K₁₂. In support of the present findings, a short t₁/₂α of 0.059 h and high value of K₁₂ (7.43 h⁻¹) has been reported after intravenous administration of levofloxacin in calves (Dumka and Srivastava, 2007). The high value of P/C ratio (4.58 ± 0.2) and apparent volume of distribution further suggested extensive drug distribution. High values of P/C ratio have also been reported for gatifloxacin (10.1) and danofloxacin (3.06) in buffalo calves and ofloxacin in calves (4.12) following their intravenous administration (Gaur et al., 2004; Gosal et al., 2008; Raipuria et al., 2007). The large Vdₐₑₜₐₐₑ cornerstone in this study (1.08 ± 0.05 L.kg⁻¹) is in agreement with the finding in cross bred calves (1.38 L.kg⁻¹) and man (0.94 L.kg⁻¹) following intravenous injection of levofloxacin (Langtry and Lamb 1998; Dumka et al., 2008). Further, large volume of distribution of another fluoroquinolone used in veterinary medicine, danofloxacin was reported to be 1.42 L.kg⁻¹ in goats and 4.35 L.kg⁻¹
Table 1. Disposition parameters of levofoxacin in buffalo calves following a single intravenous administration (n=6).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP₀</td>
<td>μg.ml⁻¹</td>
<td>15.9 ± 0.44</td>
</tr>
<tr>
<td>A</td>
<td>μg.ml⁻¹</td>
<td>13.5 ± 0.39</td>
</tr>
<tr>
<td>B</td>
<td>μg.ml⁻¹</td>
<td>2.29 ± 0.09</td>
</tr>
<tr>
<td>α</td>
<td>h⁻¹</td>
<td>7.46 ± 0.28</td>
</tr>
<tr>
<td>β</td>
<td>h</td>
<td>0.272 ± 0.009</td>
</tr>
<tr>
<td>t₁/₂α</td>
<td>h</td>
<td>0.093 ± 0.003</td>
</tr>
<tr>
<td>t₁/₂β</td>
<td>h</td>
<td>2.56 ± 0.09</td>
</tr>
<tr>
<td>K₁₂</td>
<td>h⁻¹</td>
<td>4.88 ± 0.21</td>
</tr>
<tr>
<td>K₂₁</td>
<td>h⁻¹</td>
<td>1.35 ± 0.07</td>
</tr>
<tr>
<td>AUC</td>
<td>μg.h.ml⁻¹</td>
<td>10.5 ± 0.11</td>
</tr>
<tr>
<td>Vd₁₂</td>
<td>L.kg⁻¹</td>
<td>1.08 ± 0.05</td>
</tr>
<tr>
<td>Clh</td>
<td>ml.kg⁻¹.h⁻¹</td>
<td>286.6 ± 2.74</td>
</tr>
<tr>
<td>K₁₂</td>
<td>h⁻¹</td>
<td>1.51 ± 0.03</td>
</tr>
<tr>
<td>MRT</td>
<td>h</td>
<td>3.07 ± 0.09</td>
</tr>
<tr>
<td>td</td>
<td>h</td>
<td>12.6 ± 0.41</td>
</tr>
<tr>
<td>P/C</td>
<td>ratio</td>
<td>4.58 ± 0.2</td>
</tr>
</tbody>
</table>

α and A = distribution rate constant from central to peripheral compartment and the zero time intercept of distribution phase, respectively; AUC = area under the plasma-concentration time curve; B and β = zero time intercept of the elimination phase and elimination rate constant, respectively; Clh = total body clearance of drug; CP₀ = plasma drug concentration at time zero after intravenous dose; K₁₂ and K₂₁ = micro rate constants of drug transfer from central to peripheral and from peripheral to central compartment, respectively; K₁₂ = rate constant for elimination of drug from central compartment; MRT = mean residence time; P/C = ratio of drug present in peripheral to central compartment; t₁/₂α = distribution half life; t₁/₂β = elimination half life; td = duration of therapeutic effect; Vd₁₂ = apparent volume of distribution.
Figure 1. Standard curve of levofloxacin in plasma of buffalo calves. Each point represents the mean of the results from 14 assays.

Figure 2. Semilogarithmic plot of plasma concentration-time profile of levofloxacin following its single intravenous injection of 3 mg.kg$^{-1}$ body weight in buffalo calves.
in buffalo calves after intravenous administration (Atef et al., 2001; Sappal et al., 2009). The high value of AUC (10.5 ± 0.11 μg.h⁻¹.ml⁻¹) was in agreement with the AUC of levofloxacin reported in man (55.3 μg.h⁻¹.ml⁻¹), rabbits (29.7±6.3 μg.h⁻¹.ml⁻¹) and calves (12.7 μg.h⁻¹.ml⁻¹) [2, 4, 5]. High values of AUC after iv administration have also been reported for other fluoroquinolones as 26.55 μg.h⁻¹.ml⁻¹ for ofloxacin in calves (Gaur et al., 2004), 17.1 μg.h⁻¹.ml⁻¹ for gatifloxacin in buffalo calves (Raipuria et al., 2007) and 29.6 μg.h⁻¹.ml⁻¹ for danofloxacin in goats (Atef et al., 2001).

The total body clearance of levofloxacin in the present study was 286.6 ± 2.74 ml.kg⁻¹.h⁻¹. This finding is in agreement with the values of ClB reported for levofloxacin (317.2 ml.kg⁻¹.h⁻¹) and ofloxacin (189.9 ml.kg⁻¹.h⁻¹) after iv administration in calves (Gaur et al., 2004; Dumka and Srivastava, 2007). The elimination half-life of levofloxacin in buffalo calves calculated in this study (2.56 ± 0.09 h) was longer than its corresponding value of 1.61 h in cross bred calves (Dumka and Srivastava, 2007); however, it was shorter than the t₁/₂β of 10.4 h reported for gatifloxacin in buffalo calves (Raipuria et al., 2007), 4.01 h, 4.24 h and 5.37 h for danofloxacin in goats, buffalo calves and camels, respectively (Atef et al., 2001; Aliabadi et al., 2003; Sappal et al., 2009) and 26.27 h for ofloxacin in calves (Gaur et al., 2004).

The concentration of levofloxacin-equivalent inhibitory units in the urine of buffalo calves was very high in this study. High urinary concentrations of danofloxacin (2.37 μg.ml⁻¹) and gatifloxacin (2.6 μg.ml⁻¹) have also been reported 24 h post intravenous dosing in buffalo calves (Raipuria et al., 2007; Sappal et al., 2009). Approximately 25% of the microbiological activity of the administered levofloxacin was recovered in the urine of buffalo calves within 24 h. This finding was less than the urinary recovery of 38.4% of the total dose of levofloxacin in cross bred calves (Dumka et al., 2008) but more than 19.7% of
gatifloxacin (Raipuria et al., 2007) and 4.53% of danofloxacin (Gosal et al., 2008) in buffalo calves within the first 24 h of intravenous administration.

The results indicated that levofloxacin may be effective against urinary tract infections in buffalo species. Under field conditions, for most bacteria sensitive to levofloxacin, an appropriate intravenous dosage regimen for levofloxacin, would be 3 mg.kg\(^{-1}\) repeated at 12 h intervals for the treatment of infections caused by levofloxacin susceptible bacteria in buffalo calves (Table 2).

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ABSTRACT

The present study was conducted on forty lactating Nili-Ravi buffaloes to compare the reproductive performance by treating with GnRH and PGF$_{2\alpha}$ for synchronization of estrus, ovulation and pregnancy per artificial insemination with or without supplemental estradiol. All buffaloes received 25 mg PGF$_{2\alpha}$ at 51 days in milk (DIM) for pre-synchronization of estrus cycles. After 13 days of PGF$_{2\alpha}$ injection, buffaloes were given one of four treatments; Group A (n=10) were given an intramuscular injection of 100 μg of GnRH followed by an injection of PGF$_{2\alpha}$ 7 days later and a final injection of GnRH at timed artificial insemination (AI) 48 h after the PGF$_{2\alpha}$ injection. Group B (n=10) were given the same treatment as that given to Group A buffaloes but with an injection of 1 mg of estradiol 24 h after the PGF$_{2\alpha}$ injection. Group C (n=10) were given an intramuscular injection of 100 μg of GnRH followed by an injection of PGF$_{2\alpha}$ 7 days later and a final injection of GnRH at timed artificial insemination (AI) 72 h after the PGF$_{2\alpha}$ injection. Group D (n=10) were given the same treatment as that given to Group C buffaloes but with an injection of 1 mg of estradiol 24 h after the PGF$_{2\alpha}$ injection. Blood samples (10 ml) were collected from all buffaloes 7 days before and again at the first GnRH of the BuffSynch protocols, at 48 and 72 h after the PGF$_{2\alpha}$ injection of the BuffSynch and at 7 days after the final GnRH of synchronization protocols. Estrus was observed in 65% (13/20) of the buffaloes after the BuffSynch72 (Groups C and D) compared with 45% (09/20) buffaloes in the BuffSynch48 (Groups A and B). Of the buffaloes which were treated with estradiol, 70% (14/20) displayed estrus as compared to 40% (08/20) of the buffaloes which were not treated with estradiol. Irrespective of treatments, a preponderance of buffaloes observed in estrus was identified 72 h after injection of PGF$_{2\alpha}$. It is obvious that estrus expression increased after estradiol treatment however merely at 72 h or 48 h after estradiol injection. Among different treatment groups, pregnancy per AI at 40 and 68 days after artificial insemination did not differ. At 40 days after artificial insemination, pregnancy per AI rates were 45.0% for BuffSynch48 (Groups A and B) and BuffSynch72 (Groups C and D), whereas 45.0% for buffaloes with (Groups B and D) or without (Groups A and C) receiving estradiol treatment. It was concluded that lactating buffaloes exposed to presynchronized timed artificial insemination program with variable interval of proestrus before GnRH, in which artificial insemination was done at the time of final GnRH injection, practiced

EFFECT OF TREATMENT WITH OR WITHOUT ESTRADIOL AFTER OVSYNCH PROTOCOLS AT TIMED AI ON THE PREGNANCY RATE IN LACTATING BUFFALOES

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analogous pregnancy per artificial insemination. It is important that prolonging the period of proestrus and treating buffaloes with estradiol show estrus mainly at 72 h after PGF$_{2\alpha}$ but did not increase fertility.

**Keywords:** buffalo, synchronization, GnRH, estradiol

**INTRODUCTION**

The reproductive efficiency of farm animals, which denotes the maximum effective use of their reproductive capacity, is of considerable practical and economic significance to farmers. Buffalo reproduction is an important issue, which has been the focus of many studies. Reproductive management of buffaloes has always been a challenge. Silent estrus is perhaps the most important factor leading to poor reproductive efficiency in buffaloes (Kanai and Shimizu, 1983). As the signs of estrus in buffaloes are less obvious than in cattle, the estrus detection accuracy is one of the major problems limiting the use of artificial insemination (AI) in this species.

Various estrus synchronization protocols have been tried among many other reproductive technologies for improving the fertility of buffaloes. A novel synchronization protocol named Ovysynch was developed in cows; this requires a three-injection schedule (GnRH-PGF$_{2\alpha}$-GnRH) for synchronization of ovulation. The technique was successfully carried out in cycling buffaloes (Paul and Prakash, 2005) for synchronization of ovulation and fixed timed artificial insemination (AI). Ovysynch is a timed artificial insemination (AI) program that reduces days open and increases pregnancy rates by allowing for control of first and subsequent inseinations. It has now been a trend to administer GnRH and/or PGF$_{2\alpha}$ in early postpartum cows and buffaloes in order to hasten early resumption of cyclic ovarian activity and thereby to increase the reproductive efficiency. The gonadotropin releasing hormone (GnRH) and prostaglandin (PGF$_{2\alpha}$) method of estrous synchronization has proven to be very successful in synchronizing estrus in cattle and buffaloes. Studies by Pursley et al. (1995) verified that, administration of GnRH after PGF$_{2\alpha}$ injection increases the rate of synchronized ovulation in cattle. It has been observed that when PGF$_{2\alpha}$ is administered on palpation of functional carpus leutum, about 60-70% of treated animals, were detected in estrus within 4 days post PGF$_{2\alpha}$ injection.

Synchronization of ovulation with 1 mg of estradiol cypionate increased pregnancy per artificial insemination (AI) compared with artificial insemination (AI) after a synchronized estrus (Cerri et al., 2004). It is practical to suggest that methods that increase estradiol concentrations and expression of estrus in timed artificial insemination (AI) protocols might improve fertility of dairy cows. This may be particularly important for the high-producing dairy cow, which seems to lack sufficient blood concentrations of estradiol to induce estrus, ovulation, and uterine priming because of inherently high metabolism and clearance of steroid hormones. No such studies have been undertaken in buffalo.

It is hypothesized that extending the period of proestrus and delaying the time of artificial insemination (AI) from 48 to 72 h after induced luteolysis would increase plasma concentrations of estradiol, expression of estrus, and fertility of buffalo. It is also hypothesized that supplemental estradiol would further enhance these responses, primarily in buffaloes receiving timed artificial
insemination (AI) at 48 h after induced luteolysis. The present study was therefore conducted to compare the reproductive performance of lactating Nili-Ravi buffalos treated with GnRH and PGF$_{2α}$ for synchronization of estrus, ovulation and pregnancy per artificial insemination with or without supplemental estradiol.

**MATERIALS AND METHODS**

The study was carried out on lactating Nili-Ravi buffaloes at the Buffalo Research Institute, Pattoki, District Kasur. Forty Nili-Ravi buffaloes of above 50 days post-partum were divided into four (n=10) groups (A, B, C, D). All buffaloes received 25 mg PGF$_{2α}$ at 51 days in milk (DIM) for pre-synchronization of estrus cycles. Thirteen days after the PGF$_{2α}$ injection, buffaloes were given one of four treatments; BuffSynch48

**Group A** (n=10) were given an intramuscular injection of 100 μg of GnRH followed by an injection of PGF$_{2α}$ 7 days later and a final injection of GnRH at timed artificial insemination (AI) 48 h after the PGF$_{2α}$ injection.

**Group B** (n=10) were given the same treatment as that given to Group A buffaloes but with an injection of 1 mg of estradiol 24 h after the PGF$_{2α}$ injection.

**BuffSynch72**

**Group C** (n=10) were given an intramuscular injection of 100 μg of GnRH followed by an injection of PGF$_{2α}$ 7 days later and a final injection of GnRH at timed artificial insemination (AI) 72 h after the PGF$_{2α}$ injection.

**Group D** (n=10) were given the same treatment as that given to Group C buffaloes but with an injection of 1 mg of estradiol 24 h after the PGF$_{2α}$ injection.

A buffalo which stood to be mounted by a penile deviated bull was considered to be in estrus. The artificial insemination (AI) was done once in the morning after the PGF$_{2α}$ injection.

**Blood Sampling for Progesterone Analysis**

Blood samples (10 ml) were collected from all buffaloes 7 days before and again at the first GnRH of the BuffSynch protocols, at 48 and 72 h after the PGF$_{2α}$ injection of the BuffSynch and at 7 days after the final GnRH of synchronization protocols, respectively. Blood samples were collected at 48 and 72 h after the PGF$_{2α}$ injection of the BuffSynch protocols (Group A, B, C, D), which corresponded to 24 and 48 h after treatment with estradiol, respectively. Samples were immediately transported to the laboratory within 1 h of collection. Progesterone was determined through ELISA by using a commercially available kit (BioCheck, Inc, Lot. RN-34859).

**Pregnancy Diagnosis**

All buffaloes were examined for pregnancy by rectal palpation of the uterus and its contents for detection of an embryonic vesicle at 40 ± 1 day after artificial insemination (AI), and pregnant buffaloes were reexamined 4 week later at 68 ± 1 days. Pregnancy per artificial insemination (AI) is defined as the number of pregnant buffaloes divided by the number of inseminated buffaloes within each treatment at 40 and 68 days after artificial insemination (AI).

**Statistical analysis**

The data thus obtained were analyzed statistically by using the Z-test. (Steel and Torrie,
RESULTS

By postponing the time of artificial insemination from 48 to 72 h, the estrus expression increased. Similarly by treating buffaloes with estradiol increased the estrus expression. Estrus was observed in 65% (13/20) of the buffaloes after the BuffSynch72 (Groups C and D) compared with 45% (09/20) the buffaloes in the BuffSynch48 (Groups A and B). Of the buffaloes which were treated with estradiol, 70% (14/20) displayed estrus as compared to 40% (08/20) of those which were not treated with estradiol. Irrespective of treatment, a preponderance of the buffaloes observed in estrus was identified 72 h after injection of PGF$_{2\alpha}$. It is obvious that estrus expression increased after estradiol treatment however merely at 72 h or 48 h after estradiol injection. Short-cycling buffaloes were not affected by BuffSynch or estradiol treatment (Table 1). Among different treatment groups, pregnancy per AI at 40 and 68 days after artificial insemination did not differ. At 40 days after artificial insemination, pregnancy per AI rates were 45.0% for BuffSynch48 (Groups A and B) and BuffSynch72 (Groups C and D), whereas pregnancy per AI rates were 45.0% for buffaloes receiving (Groups B and D) or not receiving (Groups A and C) estradiol treatment (Table 1). Between 40 and 68 days of gestation, none of the buffaloes lost pregnancy.

DISCUSSION

Scheduled artificial insemination programs are commonly used aimed at reproductive management of dairy cattle to confirm insemination of suitable cows (Caraviello et al., 2006). In lactating dairy cows, Pursley et al. (1998) evaluated the effect of the interval between GnRH and the time of artificial insemination. The pregnancy rate per artificial insemination was decreased in cows

Table 1. Effect of BuffSynch and estradiol treatment on reproductive responses of Nili-Ravi buffaloes.

<table>
<thead>
<tr>
<th>Items</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic</td>
<td>80.0 (8/10)</td>
<td>70.0 (7/10)</td>
<td>70.0 (7/10)</td>
<td>70.0 (7/10)</td>
</tr>
<tr>
<td>Detected estrus*</td>
<td>30.0 (3/10)</td>
<td>60.0 (6/10)</td>
<td>50.0 (5/10)</td>
<td>80.0 (8/10)</td>
</tr>
<tr>
<td>Short-cycling</td>
<td>5.8 (14/240)</td>
<td>6.6 (16/243)</td>
<td>3.3 (8/245)</td>
<td>5.4 (13/239)</td>
</tr>
<tr>
<td>Pregnancy per AI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(day 40)</td>
<td>40.0 (4/10)</td>
<td>50.0 (5/10)</td>
<td>50.0 (5/10)</td>
<td>40.0 (4/10)</td>
</tr>
<tr>
<td>Pregnancy per AI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(day 68)</td>
<td>40.0 (4/10)</td>
<td>50.0 (5/10)</td>
<td>50.0 (5/10)</td>
<td>50.0 (5/10)</td>
</tr>
<tr>
<td>AI interval(days)</td>
<td>25.6±1.1</td>
<td>25.2±1.1</td>
<td>27.0±1.1</td>
<td>27.0±1.0</td>
</tr>
</tbody>
</table>

*Buffaloes were classified as cyclic when progesterone concentration in plasma was $\geq$1 ng/ml in at least 1 of 2 sampling days at 57 and 64 days in milk.
inseminated about 32 h after GnRH administration while in the Ovsynch program, a high proportion of cows come to be pregnant and more calvings were attained once artificial insemination was completed 16 h after the final injection of GnRH (Pursley et al., 1998). Modifications in the Ovsynch program were evaluated, and it was observed that number of calvings increased when cows received the final GnRH injection and timed artificial insemination at 72 h as compared with GnRH and timed artificial insemination 48 h after the PGF2α (Portaluppi and Stevenson 2005). Portaluppi and Stevenson (2005) further found that pregnancy loss was reduced in cows which were given a GnRH injection and timed artificial insemination at 72 h in comparison with cows given GnRH and timed artificial insemination at 48 h. When dairy cows were exposed to a presynchronized estrus synchronization program and artificially inseminated at detected estrus, the estrus distribution after induced luteolysis gave rise to mean and median intervals of 3.2±1.0 and 3.0 days, respectively (Cerri et al., 2004). Out of 258 cows detected in estrus, the percentage of cows showing estrus on day 1, 2, 3, 4 and 5 after PGF2α injection were correspondingly 2.4, 12.2, 52.4, 22.8 and 7.1 % (Cerri et al., 2004). Cerri et al. (2004) further found that distribution of estrus at day 1, 2 and 3 after PGF2α injection was 7.1, 26.5 and 66.5 % in cows that were given estradiol cypionate injection 24 h after PGF2α. Thus, 3 days after the estrus synchronization program is the time when most of the animals are observed in estrus. Likewise, in our study, the majority of the buffaloes were observed in estrus at 72 h after PGF2α injection. Walker et al. (1996) reported that after a preliminary show of estrus, the time of ovulation is about 28 h, and this is comparable in those cows in which ovulation was induced by GnRH in a synchronization program (Pursley et al., 1995; Stevenson et al., 2004).

Our assumption was that prolonging the time of induced ovulation and artificial insemination from 48 to 72 h in a timed artificial insemination program will increase pregnancy per artificial insemination because of prolonged proestrus subsequently prolonged exposure to increased concentrations of estradiol and display of estrus. The findings of present study are in agreement with Portaluppi and Stevenson (2005). These authors reported similar pregnancy per artificial insemination in cows given GnRH injections and timed artificial insemination at either 48 or 72 h after luteolysis. As observed in the current study, many authors (Sterry et al., 2007; Brusveen et al., 2008) have reported non-existence of alteration in pregnancy per artificial insemination to first and following inseminations while cows were programmed for a 48 or 72 h synchronization protocol.

An important logic for prolonging proestrus was to enhance synchronization of ovulation with the anticipated time when the maximum number of buffaloes show estrus after luteolysis. This may be significant when buffaloes are induced to ovulate and inseminated at the same time. Peters and Pursley (2003) reported that in the Ovsynch program, restricting proestrus to below 36 h resulted in ovulation of smaller follicles and lower pregnancy per artificial insemination in dairy cows.

Earlier studies (Pancarci et al., 2002) indicated that in a timed artificial insemination program, no effect on pregnancy per artificial insemination was observed if the final GnRH injection is replaced by 1 mg estradiol cypionate. In contrast, Cerri et al. (2004) reported that when ovulation was induced with 1 mg estradiol cypionate, pregnancy per artificial insemination was increased in comparison with cows which were inseminated
after a synchronized estrus. Enhancements in pregnancy per artificial insemination were proposed by increased contact to estradiol during proestrus, which might have upgraded oocyte fertilization or well prepared the uterus for the succeeding luteal phase, which is significant for induction of endometrial progesterone receptors (Zelinsky and Stormshak, 1981). It was suggested that in high producing cows, there is a deficiency of adequate concentrations of estradiol in the blood to induce estrus, ovulation and uterine priming on account of their characteristically wide-ranging steroid metabolism and clearance (Wiltbank et al., 2006). Sartori et al. (2002) reported that heifers had advanced peak estradiol levels as compared to lactating cows, nevertheless cows had larger ovulatory follicles. Hence, it is likely that supplementation with estradiol during proestrus might benefit fertility of buffaloes, principally in synchronization programs which may restrict exposure to follicular estradiol.

Previously, Sellars et al. (2006) verified the assumption that supplemental estradiol might advantage fertility of dairy cows at timed artificial insemination programs. Cows were given 0.25 mg estradiol cypionate at the time of final GnRH injection in the Ovsynch program, and even though serum estradiol concentrations increased in cows treated with estradiol, pregnancy per artificial insemination persisted unchanged. In cows on the Ovsynch program given 0.5 mg estradiol cypionate 24 h before the final GnRH injection, a decrease in the percentages of oocytes and embryos was observed with no effect on fertilization (Cerri et al., 2004). Similarly, in buffaloes with 48 h of proestrus, it becomes doubtful that supplemental estradiol improves fertility. The findings of current study are in agreement with Sellars et al. (2006).

Actually, in the current study, it was observed that synchronized buffaloes and those having higher concentrations of progesterone at 7 days after artificial insemination were more likely to be pregnant at 40 and 68 days after artificial insemination, representing the significance of increased progesterone concentrations for pregnancy.

**CONCLUSION**

Lactating buffaloes exposed to presynchronized timed artificial insemination program with variable intervals of proestrus before GnRH, in which artificial insemination was done at the time of the final GnRH injection, practiced analogous pregnancy per artificial insemination. It is important that prolonging the period of proestrus and treating buffaloes with estradiol show estrus mainly at 72 h after PGF$_{2\alpha}$ but did not increase fertility. Buffaloes that showed estrus had higher concentrations of progesterone 7 days after the time artificial insemination. These interventions caused buffaloes which showed estrus are more likely to become pregnant than those not expressing estrus. The findings of current study established that timed artificial insemination protocols should be taken into consideration to increase displayed estrus either on the day of timed artificial insemination or after as it is related to increased fertility.

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ABSTRACT

A baseline survey for iodine status was conducted by measuring plasma inorganic iodine (PII) levels in 73 buffaloes from 67 dairy units located in the districts of Ludhiana, Jalandhar, Ferozepur and Hoshiarpur of Punjab. Concentrations of triiodothyronine (T₃), thyroxine (T₄), total cholesterol and free fatty acids (FFA) were compared between iodine deficient and normal buffaloes. Circulating T₄ and T₃ levels were determined before and after injection of ethiodised oil in animals with low PII levels. The prevalence rate of low iodine status was recorded to be 38.4 percent and varied between 26.3 to 50.0 percent among three age groups and 29.0 to 60.0 percent in different districts. Basal thyroxine (T₄) and triiodothyronine (T₃), T₄:T₃ ratio, total cholesterol and free fatty acids were not significantly affected by low iodine status. However, the activity of circulating T₄, T₃; T₄:T₃ ratio increased and that of T₃ decreased following iodine supplementation, thus suggesting that subclinical deficiency is prevalent. It may also be concluded from this study that elevation in T₄ and T₃; T₄:T₃ ratio in response to iodine supplementation is a more sensitive diagnostic test of iodine deficiency than a single basal T₄ and T₃ assay.

INTRODUCTION

Iodine exerts its physiological role as a component of thyroid hormones which exert global effects on body metabolism by virtue of control of cellular energy exchange, basal metabolic rate, tissue growth, reproduction and lactation. Recent figures from the surveys by the Government of India and state governments show that 82 percent of the districts surveyed (197/239) are endemic for iodine deficiency in the human population and no single state in the country is free from iodine deficiency disorders in people (Pandav and Anand, 1997). However, iodine deficiency disorders in animals have yet to receive the same attention as that of human beings. It is believed that due to ecology of iodine, the geographic pattern of dietary iodine availability to farm animals is similar to that of the human population. Based on this assumption, about 100 million animals (cattle, buffaloes, sheep and goats) have been estimated in iodine deficient zones of India (Bedi, 1997). This has also been reflected to some extent by few observations (Rajkumar,
1970; Raina and Pachauri, 1984) in small ruminants of these areas. In spite of this, no survey on iodine status has been done to determine iodine status of livestock, probably because clear signs are not commonly seen and marginal deficiency may be prevalent. However, reports are there that productivity is inevitably reduced in all animals even with marginal deficiency that does not cause clinical signs (Knights et al., 1979; Salakhutdinov, 1985; Sedina, 1987). Thus, it seemed necessary to determine iodine status and its effect on livestock health. Therefore, the objective of the present study was to assess iodine status of dairy buffaloes in Punjab state and to investigate the role of iodine injection in the control and prevention of iodine deficiency.

MATERIALS AND METHODS

Animals

Seventy-three buffaloes were selected randomly from 371 buffaloes in 67 dairy units located in four districts (Ludhiana, Jalandhar, Ferozepur, Hoshiarpur) of Punjab. They were classed by age (Table 1) as heifers (1 to 3 years), young buffaloes (3 to 6 years) and old buffaloes (>6 years). No iodophors or iodine-containing compounds for teat dipping or udder sanitation were used at any of these dairy units.

Plasma collection

Blood was collected by venipuncture in to acid washed heparinised vials during the months of July to September 1997. Plasma was separated by centrifugation and stored at -10°C for analysis within a month.

Biochemical assay

Plasma inorganic iodine (PII): Since PII is very sensitive to iodine intake in ruminants (Rogers, 1992), it was employed to assess iodine status. The concentration was measured by a chromatographic and colorimetric technique (Aumont and Tressol, 1987). On the basis of their PII values, the animals were classified as normal (>105 μg/L), marginal (51 to 104 μg/L) and low (<51 μg/L) (Rogers, 1992).

Cholesterol: Total plasma cholesterol was measured colorimetrically (Zak, 1957).

Free Fatty acids (FFA): The concentration of FFA was determined by the method of Lowry and Tinsley (1976).

Thyroxine (T₄): Activity was measured by radio-immunoassay using kits from Bhabha Atomic Centre (Radio-pharmaceuticals Operation Board of Radiation and Isotope Technology, BARC’s Vashi Complex, Navi Mumbai, India). Duplicate tubes of T₄ assay for blank, zero standard and four standard concentrations (3.22, 6.44, 12.80 and 25.70 nmol/L) and single tubes for two controls were prepared according to instructions provided with the kit. Single tubes were prepared for the samples by adding 100 μL each of diluted plasma (1:10 with assay buffer), radio-labelled T₄, T₄ antiserum and assay buffer. Polyethylene glycol solution (1 ml) was added after incubation (at 37°C for 30 minutes) to all tubes except the blank and were centrifuged at 2000 g for 20 minutes. Radioactivity of the precipitate was measured as counts per minute using a gamma counter (model LB2105; Berthold, Germany).

Triiodothyronine (T₃): Activity was measured by a radio-immunoassay (kit from Bhaba Atomic Research Centre). Duplicate tubes of T₃ assay for blank, zero standard, five standard concentrations (0.23, 0.46, 0.92, 1.84, 3.68 nmol/L) and two controls were prepared according to instructions. Single tubes were prepared for samples by adding 50 μL of plasma sample and
100 μL each of radio-labelled T₃, T₄ antiserum and 300 μL of assay buffer and incubated at 37°C for 45 minutes after mixing. The rest of the procedure was similar to the T₄ assay. All the samples were run in a single assay. The intra-assay variation was 5.90% for T₃ and 5.01% for T₄.

**Experimental Design**

A baseline survey was conducted to determine the PII concentration of all the animals. Concentration of plasma thyroxine (T₄), triiodothyronine (T₃), cholesterol and FFA were measured to assess their usefulness in the diagnosis of iodine deficiency. Plasma samples from 15 buffaloes with PII levels below 105 μg/L were compared with 15 animals with PII levels above 105 μg/L.

Since the herdsmen had not observed perinatal weakness, stillbirths or neonatal goitre in any of the herds, the low PII values in some animals suggested that a non-clinical deficiency might be prevalent (Rogers, 1992). Therefore, the effect of parenteral iodine supplementation on thyroid activity was examined in five iodine deficient cows. A single subcutaneous injection of 1 mL of 78% ethiodised oil was given to each of the cows. Levels of T₄ and T₃ were assayed before and 70 days after injection.

**RESULTS AND DISCUSSION**

Measurement of PII in this study showed that iodine intake was low in buffaloes of Ludhiana, Jalandhar, Ferozepur and Hoshiarpur districts of Punjab. The mean (±SEM) PII value of the sampled buffaloes from four districts of Punjab was 222.6 ± 24.4 μg/L (range 10.5 - 780 μg/L). Marginally low (83.9 ± 3.61 μg/L) and low (34.1 ± 3.19 μg/L) PII levels were recorded equally in 19.2 percent of the buffaloes (Table 1). In relation to age, prevalence rate was 27.8, 50.0 and 36.3 percent in heifers, young buffaloes (3-6 years) and mature buffaloes (>6 years), respectively (Table 1). Low PII levels were observed in 29, 47, 33.3 and 60 percent buffaloes of Ludhiana, Jalandhar, Ferozepur and Hoshiarpur districts. The district wise distribution of 24 dairy herds that had low PII levels was 8 of 36 in Ludhiana, 7 of 14 in Jalandhar, 4 of 11 in Ferozepur and 5 of 6 in Hoshiarpur.

However, Chhabra (2006) recorded lower mean PII levels of 43.4 and 49.1 mg/ml during summer and winter seasons, respectively, in buffaloes from Amritsar, Ludhiana, and Patiala districts. The prevalence rate was also higher (84.5%) compared to the present study. The difference might be due to soil type and area of study. Similar baseline surveys in sub-mountainous regions (Singh et al., 2006) and districts of Ludhiana, Hoisharpur, Jalandhar and Ferozepur (Randhawa and Randhawa, 2001) in cows demonstrated low iodine intake in 48.8% and 38.4% of crossbred cattle.

Ramakrishna and Prasad (1991) found that 41.5 percent of goat thyroids from Barielly abattoirs had goitrous lesions. Occurrence of visible goitre varied between 0.5-5.5 percent at a goat farm at Laxmipur (Rajkumar, 1970) and 0.16-5.66 percent in Terai region at Uttar Pradesh. Indirect evidence supporting our results is also provided by the low soil iodine content of the region and the occurrence of endemic goitre (assessed by thyroid palpation) in 11.6 and 46.7 percent school children from two different areas of Ludhiana district (Jain, 1990).

Perinatal weakness and stillbirths were observed only in only one of the sampled dairy herds. However, non-specific clinical signs viz. anoestrus, suboestrus and prolonged postpartum anestrus were observed in one of eight iodine deficient herds in Ludhiana, two of seven in Jalandhar, two
of four in Ferozepur and two of five in Hoshiarpur. Our findings on few characteristic clinical signs in iodine deficient buffaloes are supported by those of McCoy et al. (1997) who also demonstrated that dietary iodine deficiency (4-5 months) sufficient to produce clinical and pathological changes in thyroids of pregnant cattle may still allow normal births.

Plasma thyroid hormones, cholesterol and free fatty acids in iodine deficient buffaloes

The mean PII level of iodine deficient cows was 59.3±5.40 μg/L, which was well below the value considered normal (105 μg/L) by Rogers (1992) and was also lower to 297.0 μg/L in normal group. Concentrations of mean T₃, T₄, total cholesterol. A fall in plasma FFA levels showed that fat mobilisation was lower in buffaloes with low PII levels (Table 2). Non-observance of low T₃ and T₄ activities in cows having low PII values in this study concurred with the suggestion of Underwood (1981) that serum T₃ and T₄ are poorly related to thyroidal activity in domestic animals as had also been confirmed by Rogers (1992) in cows. In contrast, Baysu and Dundar (1984) measured lower iodothyronines concentrations in cattle having low milk production and fertility problems in areas where goitre was endemic in humans than those from non-goitrous areas. Pichaicharnarong et al. (1982), however, did not monitor clinical sign or iodine status of Swamp buffaloes, but recorded low T₃ (2.4 ± 0.07 vs. 2.20 ± 0.08 nmol/L) and T₄ activities (98.71 ± 3.94 vs. 57.40 ± 3.47 nmol/L) in human goitre endemic areas in comparison to non-goitrous areas in Thailand.

Diagnosis of severe iodine deficiency may be easily done from the clinical signs of goitre, perinatal weakness, hairlessness and stillbirths in neonates. However, less severe iodine deficiency is more difficult to diagnose (McDowell, 1992). Various indicators that have been used to assess iodine deficiency in cattle including thyroid function tests (T₃ and T₄), PBI, MI and total serum iodine (Allcroft et al., 1954; Puls, 1981), all have limitations.

Andrewartha et al. (1980) also concluded from their studies on iodine deficient sheep that single thyroxine assay provided little information on iodine status. Rogers (1992) examined several tests on cattle blood. He found that total iodine; protein bound iodine and thyroid hormones were difficult or impossible to interpret, and advocated the use of MI or PII. Aumont and Tressol (1987) had also shown that direct determination of PII was useful in diagnosis of dietary iodine imbalance.

Effect of iodine supplementation on thyroid activity of iodine deficient buffaloes

Since specific clinical signs of iodine deficiency were not apparent and the activities of thyroid hormone were not lower in the buffaloes in spite of low PII values, it appeared that deficiency might be non-clinical. However, after subcutaneous injection of ethiodised oil, there was a large increase (P<0.01) in mean PII concentration and elevation of T₄ (P<0.005) for more than 70 days post injection (Table 3). Simultaneously, plasma T₃ concentration declined (P<0.05) and T₄:T₃ ratio increased (P<0.005). These alterations suggested that thyroid hormonogenesis improved following iodine supplementation.

Walton and Humphrey (1979) have also recorded similar increase in plasma thyroxine level in clinically iodine deficient sheep (premature births, stillbirths and neonatal mortality) treated with iodised oil. Low T₄ levels, T₄:T₃ ratio and a remarkable production of T₃ had also been observed by Morinaga et al. (1990) in iodine-deficient
Table 1. Prevalence of iodine deficiency in buffaloes in Punjab.

<table>
<thead>
<tr>
<th>PII class (μg/L)</th>
<th>Heifers (n=18)</th>
<th>Young buffaloes (n=22)</th>
<th>Old buffaloes (n=33)</th>
<th>Total (n=73)</th>
<th>Percent of total buffaloes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (105)</td>
<td>13</td>
<td>11</td>
<td>21</td>
<td>45</td>
<td>61.6</td>
</tr>
<tr>
<td>Marginal (51-105)</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>14</td>
<td>19.2</td>
</tr>
<tr>
<td>Low (&gt;51)</td>
<td>2</td>
<td>7</td>
<td>5</td>
<td>14</td>
<td>19.2</td>
</tr>
<tr>
<td>% deficient (&lt;105)</td>
<td>27.8</td>
<td>50.0</td>
<td>36.3</td>
<td></td>
<td>38.4</td>
</tr>
</tbody>
</table>

Table 2. Effect of plasma iodine concentration on biochemical constituents involved in thyroid dysfunction in buffaloes.

<table>
<thead>
<tr>
<th>Class of buffaloes</th>
<th>PII (μg/L)</th>
<th>T₃ (nmol/L)</th>
<th>T₄ (nmol/L)</th>
<th>Cholesterol (nmol/L)</th>
<th>FFA (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>297.0ᵃ</td>
<td>1.73ᵃ</td>
<td>37.9ᵃ</td>
<td>1.88ᵃ</td>
<td>52.9ᵃ</td>
</tr>
<tr>
<td>Deficient</td>
<td>59.3ᵇ</td>
<td>1.55ᵇ</td>
<td>39.6ᵇ</td>
<td>1.85ᵇ</td>
<td>42.7ᵇ</td>
</tr>
</tbody>
</table>

Means with different superscripts in a column differ significantly at P < 0.05.

Table 3. Effect of iodised oil administration on PII and thyroidal activity of iodine deficient buffaloes (n=5).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Day after injection</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>PII (μg/L)</td>
<td>66.6 ± 18.0</td>
<td>285.2 ± 20.5**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₃ (nmol/L)</td>
<td>39.1 ± 1.28</td>
<td>63.3 ± 6.45***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₄ (nmol/L)</td>
<td>1.18 ± 0.15</td>
<td>0.72 ± 0.11*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₂: T₁</td>
<td>35.2 ± 4.82</td>
<td>92.1 ± 7.94***</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05  **P<0.01  ***P<0.005
goitre-affected calves. Therefore, improved thyroid hormonogenesis in response to treatment in our buffaloes showing low PII values suggest that sub-clinical deficiency may be prevalent, which, however, needs to be further confirmed by monitoring the effect on production.

The data in Table 3 further suggested that increase in PII and plasma T₄ concentration, T₄:T₃ ratio persisted for more than 70 days after iodine injection. It may, therefore, be inferred that 780 mg of I as ethiodised oil is a useful long-term iodine supplement for treatment and prevention of subclinical iodine deficiency in buffaloes. High milk iodine concentrations after iodised oil (400 mg of I) have been observed in ewes of goitre-affected flocks for more than two consecutive pregnancies (Statham and Koen, 1982; Azuolas and Caple, 1984). The results also revealed that thyroid provocation by iodine supplementation was more sensitive diagnostic test of low iodine intake than single basal T₄ and T₃ assay.

REFERENCES


Morinaga, Y., I.S. Osame, T. Sarashina and S. Ichigo.


ABSTRACT

In the Malwa region of Madhya Pradesh reproductive failure (anoestrus) is a major problem in buffaloes under field conditions due to under feeding and nonavailability of balanced ration. To find out the nutritional causes behind anoestrus, thirty anoestrus buffaloes {10 heifers (average body wt. 262.80 ± 22.51 kg) + 20 buffaloes (average body wt. 461 ± 10.83 kg, milk yield 7.62 ± 0.48 litre/h/d)} that had normal genitalia were selected randomly from ten villages of Indore district. Average daily feed intake of each animal was recorded and proximate principles, major (Ca and P) and trace elements (Fe, Zn, Mn, Cu and Co) in available feedstuffs were determined to find out nutrient availability. Deficiency of various nutrients was calculated by comparing with the standard requirements of the animals. A strategic nutrient supplement containing deficient nutrients was formulated on the basis of nutrient deficiency observed in rations of buffaloes of Indore district and a supplementation study was carried out in the same thirty affected anoestrus buffaloes by dividing them into two groups of 15 (five heifers + 10 buffaloes) each. One of them served as control (unsupplemented), while the other was supplemented with the strategic nutrient supplement (treatment). This supplementation study was continued for a period of two months, during which each individual animal was regularly examined for onset of estrus and artificially inseminated. After two months of the last artificial insemination, pregnancy diagnosis of each animal was carried out per rectally to find out the conception status. Onset of estrous (12 vs. 4) and conception rate (10 vs. 3) were higher in the animals of supplemented group and an additional advantage in the form of improvement (P<0.05) in the body weight of heifers of the treated group was also observed. On the basis of above findings it may be concluded that strategic nutrient supplementation not only improved the reproductive performance of the anestrous buffalo heifers and lactating buffaloes, but it also improved the growth performance of the heifers.

Keywords: nutrient, supplement, anoestrus, buffaloes, Malwa

INTRODUCTION

Reproductive failure of dairy animals is a major area of concern nowadays all over India, for it causes a huge economic loss to dairy owners. Among the various factors affecting it, nutrition is one of the most important though it receives less attention than it deserves. For normal development and activity of reproductive organs, feeding of a balanced ration is of utmost importance because
most field cases of reduced fertility or sterility are of nutritional origin. The interaction between nutrition and reproduction needs particular attention in India to overcome nutritional inadequacies either in terms of quantitative or qualitative nutrient deficiencies/imbalance.

In the Malwa region of Madhya Pradesh, buffaloes make a significant contribution as dairy animals. Nutritional deficiencies cause several infertility conditions in buffaloes and the highest (50.26%) prevalence was observed for anoestrus, while cases of repeat breeder, metritis, pyometra and prolapse were only about (25.69%) among the common field cases of Malwa region of Madhya Pradesh (Shukla et al., 2007). Thus anoestrus remains a major condition which constitutes about half of reproductive problems occurring in buffaloes. Farmers follow traditional feeding practices. Usually cotton seed cake is the only concentrate source fed to lactating animals along with straw (wheat/ masoor/ gram/ soybean), and mineral supplementation is rarely practiced by farmers (Mudgal et al., 2003). On the basis of these findings, a study was planned first to find out the nutritional status of the dairy buffaloes of Indore district affected with anestrous and after getting its deficiency picture, implementing strategic nutrient supplementation to overcome the problem.

**MATERIALS AND METHODS**

Thirty buffaloes {10 heifers (average body wt. 262.80 ± 22.51 g) + 20 buffaloes (average body wt. 461 ± 10.83 kg, milk yield 7.62 ± 0.48 litre/h/d)} with normal genitalia (by per rectal examination) and without any clinical infection, showing anoestrus were selected randomly from 10 villages (Borkhedi, Harsola, Kevti, Piplihamalhar, Umaria, Panda, Rau, Rangwasa, Sonvay and Bhaslai) around Veterinary College, Mhow of Indore District. Body weights (kg) of the animals were determined by recording the length (inch) and girth (inch) of each animal and then putting them in Shaeffer’s formula (Sastry et al., 1982). Feed offered and residue left of each animal was weighed with the help of spring balance at both feeding times (morning and evening) for three consecutive days. Then average feed intake of each animal was calculated. The representative samples of each feed were subjected to proximate analysis (AOAC, 1995), Ca and P (Talpatra et al., 1940) and trace mineral estimations by atomic absorption spectrophotometer.

Milk yield (litre) of each lactating animal was measured during milking (morning and evening) for three consecutive days. After that average milk yield was calculated. Availability of DM, DCP, TDN, major elements (Ca and P), trace elements (Fe, Cu, Mn, Zn and Co) for each animal was calculated on the basis of chemical composition of feedstuffs and their intake. Selenium, carotene and vitamin E intake were worked out using values given in the literature. Finally, the nutrient availability of individual animal was compared with the standard nutrient requirements calculated for the specific body weight and productivity of each individual animal with the help of feeding standards (Kearl, 1982) and thus the deficiencies/excess of each specific nutrient was worked out.

As per the deficiency status of the nutrients, a strategic nutrient supplement was prepared using SoyaDe Oiled Cake (for protein and energy), sodium dihydrogen orthophosphate dihydrate (for P), zinc oxide (for Zn), copper sulphate (for Cu) and vitamin supplement (for vitamin A and E) were used. The amounts of specific supplement added in the normal routine diet of heifers or buffaloes are...
presented in Table 3. Measured amounts of trace minerals were supplemented by placing them in gelatin capsules.

The supplementation study was carried out in same thirty anoestrus heifers and buffaloes by dividing them into two groups having five heifers and 10 buffaloes in each, the control animals were offered the same diet which was being routinely followed by the farmer, while each animal of the treatment group was supplemented with a specific amount of supplement (Table 3) in addition to the normal control diet. Deworming of each animal was done before starting the supplementation and other management practices remained same for both the groups like proper hygienic and ventilated housing with clean drinking water available ad-libitum. This supplementation study was continued for a period of two months, during which individual animal of both the groups were regularly examined for onset of oestrus and artificially inseminated. After two months of the last artificial insemination, pregnancy diagnosis of each animal was done per-rectally to find out the conception rate. The data were analyzed as per the standard statistical methods described by Snedecor and Cochran (1994) for mean, standard error and paired ‘t’ test.

**RESULTS AND DISCUSSION**

The chemical composition of specific feed ingredients being consumed by the animals has been presented in Table 1. The feeds being offered to the animals were mainly the agricultural by-products including wheat straw, gram straw, masoor straw, soybean straw and wheat bran, while the cake was only cotton seed cake, while at some places concentrate mixtures was also being used.

In Table 2, the availability of different nutrients to the animals was worked out and compared with the standard requirement and hence the excess or deficiency of each specific nutrient has been presented. As compared to the standard requirements (Kearl, 1982), availability of DM was about 4-6% less, which may be associated with the deficiency of TDN too. Similar findings was also been reported by Mudgal et al. (2003) and Tiwary et al. (2007). Between the groups deficiency of major nutrients was observed in heifers for digestible crude protein, which was only about 48% to that of the requirements, while buffaloes were observed with only limited deficiency (11%), which may be associated with the supply of concentrate to the lactating buffaloes. Similar observations were also recorded by earlier workers (Sinha, 1982; Sohal et al., 1982; Mudgal et al., 2003 and Tiwary et al., 2007). The lower levels of energy and /or protein may be associated with the ovarian inactivity and anoestrus (Wiltbank et al., 1965) as negative energy balance depresses the ovarian activity by inhibiting pulsatile LH release (Butler and Smith, 1989).

When the major minerals were compared, calcium was shown to be the element supplied in excess (33 to 283%) to the requirement, which may be due to supply of higher amounts of leguminous straws in their ration. Leguminous straw reduces the availability of phosphorus on one hand and over supplies calcium on other hand, as leguminous straws have a wider Ca: P ratio and a fair deficiency of element phosphorus. Phosphorus is necessary for normal energy and phospholipid metabolism as well as normal skeletal development and its severe deficiency may delay the onset of puberty, cause postpartum anoestrus, and increase incidence of cystic follicles because of inactive ovaries, and moderate and low conception rates (Pugh et al., 1985). Many other workers also have found lower levels of inorganic phosphorus in serum of anoestrus
Table 1. Macro and micro nutrient contents of feedstuffs (DM basis).

<table>
<thead>
<tr>
<th>Feedstuffs</th>
<th>Wheat straw</th>
<th>Gram straw</th>
<th>Masoor straw</th>
<th>Soybean straw</th>
<th>Wheat bran</th>
<th>Cotton Seed cake</th>
<th>Concentrate mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP (%)</td>
<td>3.95±0.22</td>
<td>6.24±0.24</td>
<td>6.52±0.32</td>
<td>6.14±0.34</td>
<td>13.99±0.52</td>
<td>22.60±0.42</td>
<td>16.16±2.70</td>
</tr>
<tr>
<td>EE (%)</td>
<td>0.99±0.06</td>
<td>0.63±0.50</td>
<td>1.50±0.11</td>
<td>0.80±0.06</td>
<td>3.45±0.11</td>
<td>10.22±0.64</td>
<td>3.50±0.18</td>
</tr>
<tr>
<td>CF (%)</td>
<td>33.08±0.69</td>
<td>39.16±0.74</td>
<td>36.91±0.82</td>
<td>41.77±1.48</td>
<td>9.99±0.92</td>
<td>27.15±1.72</td>
<td>15.19±1.36</td>
</tr>
<tr>
<td>NFE (%)</td>
<td>50.31±0.66</td>
<td>45.95±1.20</td>
<td>46.21±0.87</td>
<td>45.52±1.74</td>
<td>68.71±1.63</td>
<td>35.75±1.15</td>
<td>49.29±2.51</td>
</tr>
<tr>
<td>TA (%)</td>
<td>11.64±0.64</td>
<td>7.98±0.34</td>
<td>8.84±0.30</td>
<td>6.59±0.38</td>
<td>4.51±0.74</td>
<td>4.25±0.22</td>
<td>13.98±1.79</td>
</tr>
<tr>
<td>AIA (%)</td>
<td>5.84±0.16</td>
<td>2.44±0.15</td>
<td>4.17±0.23</td>
<td>0.69±0.06</td>
<td>0.48±0.10</td>
<td>0.18±0.02</td>
<td>6.08±1.48</td>
</tr>
<tr>
<td>Ca (%)</td>
<td>0.23±0.02</td>
<td>1.54±0.08</td>
<td>1.46±0.07</td>
<td>0.94±0.04</td>
<td>0.21±0.01</td>
<td>0.22±0.01</td>
<td>0.32±0.05</td>
</tr>
<tr>
<td>P (%)</td>
<td>0.06±0.01</td>
<td>0.04±0.00</td>
<td>0.05±0.00</td>
<td>0.24±0.015</td>
<td>0.61±0.05</td>
<td>0.51±0.02</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>Fe (ppm)</td>
<td>414.76±6.47</td>
<td>364.14±12.8</td>
<td>605.28±8.05</td>
<td>461.99±62.70</td>
<td>298.51±15.23</td>
<td>275.24±16.96</td>
<td>258.45±5.94</td>
</tr>
<tr>
<td>Zn (ppm)</td>
<td>13.48±1.12</td>
<td>8.41±0.28</td>
<td>23.24±1.92</td>
<td>26.43±1.37</td>
<td>63.40±8.19</td>
<td>41.64±3.03</td>
<td>28.83±2.49</td>
</tr>
<tr>
<td>Mn (ppm)</td>
<td>39.81±1.18</td>
<td>15.68±0.85</td>
<td>87.07±7.35</td>
<td>67.63±2.38</td>
<td>71.37±3.22</td>
<td>15.80±0.56</td>
<td>18.82±1.37</td>
</tr>
<tr>
<td>Cu (ppm)</td>
<td>7.91±0.36</td>
<td>4.36±0.29</td>
<td>4.86±0.41</td>
<td>10.01±0.70</td>
<td>11.68±1.16</td>
<td>10.12±0.80</td>
<td>2.66±0.32</td>
</tr>
<tr>
<td>Co (ppm)</td>
<td>0.16±0.03</td>
<td>0.72±0.06</td>
<td>0.71±0.06</td>
<td>0.18±0.02</td>
<td>0.69±0.17</td>
<td>0.55±0.05</td>
<td>0.86±0.05</td>
</tr>
</tbody>
</table>
Table 2. Daily requirements and availability of nutrients in anoestrus buffaloes.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Requirements for 300kg Body wt.</th>
<th>Availability for 262.80± 22.51 Body wt.</th>
<th>Deficiency / Excess (%)</th>
<th>Requirements for 500 kg Body Wt and 8 lits / day production (7% fat)</th>
<th>Availability For 461±10.83 kg Body weight</th>
<th>Deficiency / Excess (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMI (Kg)</td>
<td>5.99</td>
<td>5.75±0.43</td>
<td>4 (-)</td>
<td>12</td>
<td>11.33±0.47</td>
<td>6 (-)</td>
</tr>
<tr>
<td>DCP (g)</td>
<td>374</td>
<td>179.32±21.80</td>
<td>52 (-)</td>
<td>772</td>
<td>684.69±39.97</td>
<td>11 (-)</td>
</tr>
<tr>
<td>TDN Kg</td>
<td>3.55</td>
<td>2.83±0.21</td>
<td>20 (-)</td>
<td>7.28</td>
<td>6.06±0.26</td>
<td>17 (-)</td>
</tr>
<tr>
<td>Ca (g)</td>
<td>15</td>
<td>57.38±8.92</td>
<td>283 (+)</td>
<td>46.4</td>
<td>61.93±6.39</td>
<td>33 (+)</td>
</tr>
<tr>
<td>P (g)</td>
<td>12</td>
<td>8.16±1.28</td>
<td>32 (-)</td>
<td>35.8</td>
<td>24.41±2.15</td>
<td>31 (-)</td>
</tr>
<tr>
<td>Fe (mg)</td>
<td>299.5</td>
<td>2779.56±252.64</td>
<td>828 (+)</td>
<td>600</td>
<td>3801.73±239.87</td>
<td>534 (+)</td>
</tr>
<tr>
<td>Zn (mg)</td>
<td>179</td>
<td>118.24±13.74</td>
<td>34 (-)</td>
<td>480</td>
<td>294.24±21.01</td>
<td>39 (-)</td>
</tr>
<tr>
<td>Mn (mg)</td>
<td>239.6</td>
<td>353.96±38.74</td>
<td>48 (+)</td>
<td>480</td>
<td>396.24±24.83</td>
<td>17 (-)</td>
</tr>
<tr>
<td>Cu (mg)</td>
<td>59.9</td>
<td>44.61±3.22</td>
<td>26 (-)</td>
<td>120</td>
<td>91.40±4.09</td>
<td>24 (-)</td>
</tr>
<tr>
<td>Co (mg)</td>
<td>0.59</td>
<td>2.45±0.51</td>
<td>315 (+)</td>
<td>1.20</td>
<td>4.68±0.38</td>
<td>290 (+)</td>
</tr>
<tr>
<td>Se (mg)</td>
<td>0.6-1.8</td>
<td>0.78±0.06</td>
<td>Adequate</td>
<td>1.2-3.6</td>
<td>2.4</td>
<td>Adequate</td>
</tr>
<tr>
<td>Vit A (IU)</td>
<td>12000</td>
<td>5717.81±45</td>
<td>52 (-)</td>
<td>21000</td>
<td>11418.95±0.20</td>
<td>46 (-)</td>
</tr>
<tr>
<td>Vit E (IU)</td>
<td>15</td>
<td>9.49±1.54</td>
<td>38 (-)</td>
<td>15</td>
<td>57.96±4.98</td>
<td>286 (+)</td>
</tr>
</tbody>
</table>
heifers/buffaloes than cyclic animals (Naidu and Rao, 1982; Kumar et al., 1992 and Dutta et al., 2001).

Among trace elements, the supply of iron and cobalt remained on the plus side, while zinc and copper remained deficient, and Mn was deficient only in buffaloes but not in heifers. The presence of zinc is highly essential for certain enzymatic activities related to reproduction and indirectly it may act through the pituitary to influence the release of gonadotropic hormones or directly through complexing with specific ligands in gonads (Miller, 1979). Deficiency of copper may also be reflected on reproductive behavior as well as performance of animals. Inactive ovaries, delayed oestrus and early embryonic death have been reported to occur due to deficiency of copper (Hidiroglou, 1979 and Singh and Vadnere, 1987).

Due to practice of supply supplying little green feed by farmers, vitamin A remained the most deficient among the animals, and vitamin A is very important for maintaining the health status of epithelial tissue of the reproductive tract. The deficiency of vitamin E was only observed in heifers but not in lactating buffaloes and may be due to lack of concentrate in the ration of heifers. The negative impact of insufficient vitamin E has been observed on ovulation rates (Harrison et al., 1984) and postpartum activities (Arechiga et al., 1994) of animals.

On the basis of multiple deficiencies observed in buffalo heifers and lactating buffaloes, two separate strategic nutrient supplements were prepared (Table 3)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Heifers</th>
<th>Buffaloes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya De Oiled Cake</td>
<td>550 g</td>
<td>250 g</td>
</tr>
<tr>
<td>Sodium di hydrogen ortho phosphate di hydrate</td>
<td>-</td>
<td>50 g</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>60 mg</td>
<td>224 mg</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>10 mg</td>
<td>90 mg</td>
</tr>
<tr>
<td>Vitamin supplement*</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

* Each ml contains Vitamin A 12000 IU and vitamin E 48mg.

Table 4. Effect of strategic nutrient supplementation on reproductive performance of anoestrus buffaloes.

<table>
<thead>
<tr>
<th>Groups of animals</th>
<th>Total animals</th>
<th>No of animals exhibited estrus</th>
<th>Success (%)</th>
<th>No of animals conceived</th>
<th>Success (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heifers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>1</td>
<td>20</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>Treatment</td>
<td>5</td>
<td>4</td>
<td>80</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>Buffaloes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>3</td>
<td>30</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Treatment</td>
<td>10</td>
<td>8</td>
<td>80</td>
<td>7</td>
<td>70</td>
</tr>
</tbody>
</table>
and supplemented in the diet of affected animals. The effect of their supplementation has been presented in Table 4. After supplementation of strategic nutrients, 80% of the heifers and lactating buffaloes showed the signs of estrus, while 60% of the heifers and 70% of the lactating buffaloes conceived. The rate of onset of estrous for the control group remained 20% for heifers and 30% for buffaloes, while only 20% of the animals conceived. A significant improvement (P<0.05) in body weight of treated heifers was also observed as compared to the controls. Similar results have also been observed by several scientists (Baruah et al., 2000; Lall et al., 2000; Nayyar et al., 2003; Koley and Biswas 2004 and Sharma et al., 2007).

On the basis of above findings it may be concluded that strategic nutrient supplementation not only improved the reproductive performance of the anestrous buffalo heifers and lactating buffaloes, but it also improved the growth performance of the heifers.

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ABSTRACT

A two-year retrospective study was performed to determine the bacteriological aspects of coagulase positive Staphylococcal mastitis by bacterial culture and by polymerase chain reaction using specific primers in cattle and buffalo farms from three distinct geoclimatic zones of India. A total of 3934 quarters from 1022 cattle and buffaloes were screened from the Bareilly, Kumaon and Rajnanadgoan regions of India. Mastitis cases were detected from 754 quarter milk samples on the basis of CMT positive, SCC > 7 x 10^5 cells/ml and milk positive on bacterial culture. The overall prevalence of Staphylococcal mastitis (BSM) was 22.02% on cow basis and 8.64% on quarter basis in 1022 lactating cows with 3934 functional quarters. The intra-mammary infection (IMI) was higher to the extent of 45.54% in the Bareilly and 56.31% in the Rajnanadgoan regions as compared to the Mukteshwar region. The coagulase gene of *S. aureus* was amplified with a pair of primers using the polymerase chain reaction (PCR). PCR revealed the *cao* gene from 14.73%, 10.53% and 13.24% more mastitic milk samples as compared to the bacterial culture method. Amplification of the *coa* gene by PCR is an important technique for quick diagnosis of prevalent bacterial pathogens of a particular region. With the help of PCR, a large number of lactating dairy animals can be screened with accuracy in less time in the study area for implementing therapeutic and preventive measures.

**Keywords:** bovine mastitis, coagulase gene, geoclimatic zones, *Staphylococcus aureus*, polymerase chain reaction

INTRODUCTION

Mastitis is generally associated with intensive dairy farming systems. It causes great economic losses due to lower and poor quality of milk production and treatment cost (Ruegg, 2011). *Staphylococcus aureus* is the major contagious pathogen. It spreads rapidly in dairy herds during milking and generally leads to subclinical (SCM), chronic and sometimes clinical mastitis (Ott, 1999). The implementation of proper therapeutic and
preventive measures depends on the appropriate knowledge of clinical, epidemiological and bacteriological aspects of mastitis (Shpigel, 1998). Raw milk is a potential source of *S. aureus*. The gene encoding coagulase (*coa*) is an important virulence factor responsible for invasion of *S. aureus* into the mammary epithelium. *Coa* gene polymorphism is found suitable for epidemiological investigation of bovine *S. aureus* mastitis (Zecconi et al., 2006). The bacterial culturing of the milk samples is the standard procedure for mastitis testing, but the method is time consuming. About 25% of the mastitic milk samples do not yield bacterial growth with the conventional culturing method, and therefore a proper control program cannot be implemented on a particular farm (Makovec and Ruegg, 2003). The polymerase chain reaction (PCR) can identify the pathogen in the milk if present in traces (Bradley et al., 2007). The technique is simple and fast. In the present study, we collected milk samples from three distinct geoclimatic zone of India for detection of coagulase positive *S. aureus* by bacterial culture and PCR directly from the raw milk to note the sensitivity of these two techniques.

**MATERIALS AND METHODS**

A total of 1022 cattle and buffaloes were screened for udder health status in the Bareilly, Kumaon and Rajnandgoan regions. Udder health status was screened on the basis of CMT reaction, somatic cell count (SCC) and bacterial isolation. Milk samples positive for CMT and SCC > 0.6 to 3.2 million cells / ml of milk and positive for bacterial isolate were diagnosed as subclinical and clinical mastitis. Ten ml milk was collected from each teat, after cleaning the teat end with cotton soaked in 70% alcohol and after discarding 3-4 milk squirts. SCC of the milk samples were done as per the standard method (Schalm et al., 1971). Milk samples were collected for bacterial isolation and identification from 387, 95 and 272 quarters, found positive on CMT. The identification and biochemical characterization of causative organism in collected milk samples were carried as per (Balows et al., 1991).

**Amplification of the coagulase (coa) gene from raw milk samples**

DNA extraction was carried out as per the method described by Ahmadi et al. (2010). The DNA pellet was quantified in NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). The coagulase gene of *Staphylococcus aureus* from milk samples was amplified using the primer pair (Eurofins Genomics India Pvt Ltd, Bangalore, India) listed as below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Oligonucleotide sequence</th>
<th>Gene bank accession no.</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase</td>
<td>Forward</td>
<td>TGGTTATGCCGGCTTTGATC</td>
<td>AB436983.1</td>
<td>807</td>
</tr>
<tr>
<td><em>coa</em></td>
<td>Reverse</td>
<td>GCAGCAGCTTTCAATGACGGA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Primer pair used for amplification of in the study *coa* gene.
For PCR, each reaction mixture contained 2 μl of target DNA (approximately 260 ng/μl), 1 μl each of primers (20 pmol), 0.5 μl of a mix of deoxynucleotide triphosphate (200 μM each), 0.3 μl of Taq polymerase (5U) and 3 μl of PCR 10X buffer (Tris-HCl 100 mM; KCl 500 mM; Triton X-100 1% MgCl₂; pH 8.4). The volume was adjusted to 30 μl with nuclease free water. All the reactants were thoroughly mixed and flash spun in microcentrifuge (St.Louis, MO, USA). Amplification was carried out in a thermal cycler (MJ Mini Cycler Bio-Rad Laboratories, Hercules, California, USA) as follows: initial denaturation at 94°C for 4 minutes, 30 cycles of amplification (denaturation at 94°C for 1 minute, annealing at 56 ºC for 1 minute and extension at 72°C for 1 minute) and final extension at 72°C for 5 minutes. One positive control containing S. aureus reference strain MTCC No.96 (Microbial Type Culture Collection, Chandigarh, India) was included in each reaction. The PCR product was visualized by electrophoresis in 1.2% agarose gel containing 0.5 μg/ml ethidium bromide (Fermentas, Germany). The size of PCR product was determined by comparing with a standard molecular weight marker, and was photographed by gel documentation system (Alpha Imager™ 1220, Documentation and Analysis, Alpha Innotech Corporation, USA). The chi-square Test was used to assess association between the probability of developing Staphylococcal mastitis and region, CMT, SCC and bacterial examination.

RESULTS AND DISCUSSION

The prevalence of bovine Staphylococcal (coa gene positive) mastitis (BSM) was studied in three distinct geo-climatic regions of India i.e., the sub-tropical humid climate zone of the Bareilly region (Uttar Pradesh), the temperate and mountainous climatic zone of the Uttarakhand (Kumaoun) region and the tropical wet and dry climatic zone of the Rajnandgoan region (Chattisgarh). The prevalence of mastitis varied in these three distinct regions. The climatic conditions of Bareilly (U.P), Uttarakhand (Kumaon) and Rajnandgoan (Chattisgarh) are detailed in Table 2. One thousand twenty two (3934 functional quarters) cattle and buffaloes were screened for udder health status. In the present study, the prevalence of Staphylococcal mastitis (BSM) was 22.02% on animal basis and 8.64% on quarter basis in 1022 lactating dairy animals with 3934 functional quarters in three geo-climatic regions of India. The animal wise prevalence of BSM in Bareilly, Kumaoun and Rajnandgoan was 23.05%, 15.84% and 24.76% respectively. The prevalence of Staphylococcal intra-mammary infection (IMI) was higher to an extent of 45.54% in Bareilly and 56.31% in Rajnandgoan compared to the Kumaoun region.

Bacterial isolation and biochemical characterization in milk samples collected from Bareilly, Kumaoun and Rajnandgoan region of India

Out of 387 milk samples collected from mastitic quarters from Bareilly, 36 (9.30%) isolates were found coagulase positive Staphylococcus sp. (CPS) (Figure 1) and 110 (28.42%) isolates were coagulase negative Staphylococci (CNS). Whereas 6 (6.32%) and 22 (8.09%) milk samples from the Kumaoun and Rajnandgoa regions identified as CPS, while 38 (40.0%) and 89 (32.72%) were identified as CNS. The prevalence of CPS was 47.15% and 28.01% higher in the Bareilly and Rajnandgoan regions as compared to the Kumaoun region. The lowest prevalence (6.32%) of CPS was
recorded from the Kumaoun region.

**Prevalence of Coagulase Positive Staphylococci in quarter milk samples from bovine mastitis in three geo-climatic regions of India**

Amplification of the coagulase gene yielded a single PCR product of 807 bp in raw milk samples (Figure 2). The prevalence of CPS was higher to an extent of 39.89% and 25.74% in the Bareilly and the Rajnandgoan regions as compared to the Kumaoun region. The prevalence rate of CPS in quarter milk samples from mastitic cows and buffaloes varied significantly with the two different methods used for identification of the causitive organism i.e. by bacterial culture and PCR amplification of the coab gene. Mastitic milk samples revealed growth in 9.3%, 6.32% and 8.09%, positive CPS by conventional culturing method from the Bareilly, Kumaoun and Rajnandgoan regions, respectively. Whereas \textit{S. aureus} by PCR technique yielded the coab gene in 14.73%, 10.53% and 13.24% positive milk samples from the Bareilly, Kumaoun and Rajnandgoan regions, respectively (Table 3). The prevalence of coagulase positive \textit{S. aureus} by the PCR technique was found to be higher to an extent of 58.39%, 66.61% and 63.66% as compared to bacteriological examination in the Bareilly, Kumaoun and Rajnandgoan regions, respectively.

\textit{S. aureus} is the most common cause of contagious and sub-clinical mastitis in dairy herds (Mukherjee and Dash, 2003). For implementation of preventive and control measures for bovine mastitis on a dairy farm, somatic cell count and bacteriological aspects must be assessed at frequent time intervals. The coagulase gene is one of the virulence factors of \textit{S. aureus}; it determines the ability to coagulate mammalian plasma. Coa gene polymorphism is frequently applied for epidemiological investigations of bovine \textit{S. aureus}

mastitis (Zecconi \textit{et al.}, 2006). Mukherjee \textit{et al.}, (2004, 2010) recorded 14.28% and 36% positive \textit{S. aureus} mastitis in organized and unorganized dairy farms by bacterial and biochemical characterization. Moreover, the prevalence of CPS positive mastitic cases by isolating marker gene was reported from many countries (Su \textit{et al.}, 1999). Sindhu \textit{et al.} (2010) reported 56% of quarter milk samples from buffaloes were positive for \textit{S. aureus} based on detection of the coa gene in Haryana. Similarly, Ahmadi \textit{et al.} (2010) reported 21% of the milk samples from cow were positive for \textit{S. aureus} using amplification of the coa gene in industrial dairy herds in Iran. In conclusion, case occurrence of Staphylococcal mastitis varied with the geo-climatic conditions of the area, with the greatest number of Staphylococcal intramammary infections in the tropical, dry climate of Rajnandgaon followed by subtropical humid climatic zone of Bareilly and the fewest number of cases in the temperate Kumaoun zone. Amplification of the coa gene by PCR is an important technique for quick diagnosis of prevalent bacterial pathogens of a particular region. With the help of PCR, large numbers of lactating dairy animals can be screened with accuracy in less time in the study area.

**ACKNOWLEDGEMENT**

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Table 2. Geographical and meteorological parameters of three geo-climatic regions of India.

<table>
<thead>
<tr>
<th>Region</th>
<th>Specifications</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Air temp (degree celcius)</th>
<th>Average annual rainfall (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bareilly (Tropical &amp; humid climate)</td>
<td>Hot &amp; humid summer and cold winters, average three winter month</td>
<td>28°10’N</td>
<td>78°23’E</td>
<td>4.5-45.8</td>
<td>1032.0</td>
</tr>
<tr>
<td>Kumaoun (Temperate climate)</td>
<td>moderate summer and very cold winters, average six winter months</td>
<td>29°29’N</td>
<td>79°39’E</td>
<td>Summer-17-26 Winter-0 -14</td>
<td>1494.1</td>
</tr>
<tr>
<td>Rajnandgaon (Tropical climate and dry)</td>
<td>Hot and dry summer and moderate winters, average one winter months</td>
<td>20°70’ &amp; 22°29’ N</td>
<td>81°29’ &amp; 88°29’ E</td>
<td>8.4-46.6</td>
<td>1275.0</td>
</tr>
</tbody>
</table>

Table 3. Prevalence of coagulase positive staphylococci (CPS) (based on bacteriological and PCR method of identification) in quarter milk samples from bovine mastitis in three geo-climatic regions of India.

<table>
<thead>
<tr>
<th>Geo-climatic regions</th>
<th>Quarters screened</th>
<th>Positive for mastitis</th>
<th>Coagulase positive mastitis on the basis of bacterial culture</th>
<th>Coagulase positive mastitis on the basis of PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bareilly</td>
<td>1872</td>
<td>387</td>
<td>36 (9.3 %)</td>
<td>57 (14.73 %)</td>
</tr>
<tr>
<td>Kumaoun</td>
<td>856</td>
<td>95</td>
<td>6 (6.32 %)</td>
<td>10 (10.53%)</td>
</tr>
<tr>
<td>Rajnandgoan</td>
<td>1206</td>
<td>272</td>
<td>22 (8.09%)</td>
<td>36 (13.24 %)</td>
</tr>
</tbody>
</table>

Wald Chi-square (P value) = <0.0001, Intercept (P value) = <0.0001.
Paired T Test Results: DF = 2, t value = 13.36; Pr>ItI = 0.006.
Figure 1. Coagulation of rabbit plasma by pathogenic *Staphylococcus aureus* isolated from mastitic milk samples.

Figure 2. Amplification of Coagulase gene from mastitic milk samples.
Lane M – 1kbp molecular weight marker
Lane 1 - *S. aureus* reference strain MTCC 96
Lane 2 and 3 - *S. aureus* positive samples


ABSTRACT

Somatic cell count (SCC), total bacterial count (TBC), L selectin and CD 18 on milk leukocytes was studied in buffaloes inflicted with intramammary infection in response to *Curcuma longa* (*C. longa, CL*) and vitamin E plus selenium (group II), Enrofloxacin (group III) and sterile PBS (group IV). Significant reduction (P<0.05) in SCC, TBC was observed in post-treated buffalo cows. The mean fluorescent intensity (MFI) of L selectin increased significantly (P<0.05) in Group 2 post-treated cows; however, there was no reduction in CD 18 counts in this group. The results suggest that *Curcuma longa* (CL) possesses antibacterial, anti-inflammatory and immunomodulatory properties. In the present study the biological activity of the CL and vitamin E plus selenium at standardized dose against mastitis in buffalo cows is reported for the first time. Development of alternative therapy is an option for livestock farmers who are not allowed to use allopathic drugs under certain farming system.

Keywords: CD 18, *Curcuma longa*, L selectin, mastitis, vitamin E

INTRODUCTION

Considerable progress has been made through extensive research on bovine mastitis, still it continues to be the most frequent and costly disease of dairy animals (Ruegg, 2010). Leukocyte migration from the central pool into the infected udder depends on the activation of leukocyte adhesion molecules (LAM), however the expression of LAM like L selectin is generally lower during periparturient period and mastitis (Paape et al., 2002). Antibiotics are used for the treatment of mastitis, but it has got few limitations, like development of antimicrobial resistance and antibiotic residue in milk. Few antibiotics also reduce the functional activities of the immune cells. *Curcuma longa* (CL) or turmeric belongs to *Zingiberaceae* family, the rhizomes possess antimicrobial, anti-inflammatory, antioxidant and immunomodulatory properties (Menon and Sudhir, 2007). Antimicrobial activity of *Curcuma longa* against *S. aureus*, *S. epidermis*, *E. coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium* was observed by many researchers (Singh et al., 2002, Han yang, 2005). Micronutrients and vitamins like...
Selenium and Vitamin E helps in reducing severity and duration of clinical mastitis by alleviating inflammatory metabolites from the site of infection and by improving leukocyte activities (Politis et al., 2004; Smith et al., 1997, Mukherjee, 2008). There are no reports regarding the immunotherapeutic potential of CL against bovine mastitis. The present study, therefore, is an effort to evaluate the potential of CL along with selenium and vitamin E on the somatic cell count (SCC), total bacterial count (TBC) and milk LAM from the lactating buffalo cows inflicted with intramammary infection.

MATERIALS AND METHODS

Hydro-methanolic extract of *C. longa* (CL) was prepared as per (Peach and Tracy, 1956). The antibiogram and MIC of the extract was done as per standard method (NCCLS, 1997) against *Staphylococcus aureus*, CNS, *Streptococcus agalactiae*, *Streptococcus uberis* and *Coliform bacilli*. The dose was determined on the basis of mean MIC of the extract against the pathogens.

Twenty-four crossbred lactating buffaloes were divided in four equal groups. Buffalo cows of Group 1, selected where milk SCC < 0.5 million cells /ml of milk, served as control. Eighteen mastitic animals selected formed Groups 2, 3 and 4 positive for intramammary infection were taken for the drug trial, selection criteria being the SCC >0.7 million cells /ml of milk and milk secretions positive for pathogenic isolate. Buffalo cows of Group 2 received 150 mg of sterile CL extract per teat after reconstituting in 5 ml warm PBS by i/m route for 5 days or till clinical recovery. Group 4 animals received 5 ml of sterile PBS for 5 days by i/mam route. SCC and TBC of the milk samples were done as per the standard method (Schalm et al., 1971) (Balows et al., 1991). The identification and biochemical characterization of causative organism in collected milk samples was carried as per (Balows et al., 1991). SCC and TBC were done on day ‘0’ and thereafter on 2, 10 days PT. Milk leukocytes were isolated as per the method describe by Daley et al. (1991) for enumeration of CD 18 and L-Selectin in milk PMNs. About 100 μl of milk cell suspension was incubated in the dark with 1 μl of primary antibodies against CD 18 and L-Selectin for 30 minutes at room temperature. CD 18 and L-Selectin was done by using commercial kit (CD 18 ,Clone- BAQ30A, Isotype- IgG1, VMRD, Inc. Pullman, WA, USA and L-Selectin, Clone-DU1-29, Isotype- IgG1, VMRD, Inc. Pullman, WA, USA) as per method described by Soltys and Quinn (1999).

The data were analyzed applying one-way analysis of variance (ANOVA) to determine the level of significance between the groups, and Duncan’s multiple range test (DMRT) was applied to determine the level of significance within the group at different time intervals by using an SPSS 10.1 software package.

RESULTS AND DISCUSSION

There were no differences in SCC and TBC in the milk samples isolated from healthy cows at different time intervals of the study period. The SCC and TBC in Group 2 and Group 3 significantly decreased (P<0.05) on day 7 and day 15 (Table 1). Out of 14 milk samples collected
from diseased buffalo cows, the organisms isolated were *Staphylococcus aureus* (7%), CNS (56%), *Streptococcus agalactiae* (3%), *Streptococcus uberis* (9%), *Coliform bacilli* (14%), no growth (11%). Mean fluorescent intensity of L selectin was lower in mastitic cows (P<0.05) before treatment. However, the expression increased (37.42%) in Group 2 cows on day 7, whereas non-significant increased expression of L selectin was recorded in Group 3 posttreated animals (Table 2). Contrary to values of L selectin, the expression of CD18 was higher in the PMNs isolated from mastitic cows both before and after treatment (P<0.05). However, it reduced significantly (P<0.05) in Group 3 (42.26%) on day 7 as compared to pretreatment values and Group 2 (Table 2).

Mastitis is one of the major causes of economic loss in dairy cattle. Once established in the udder, it impairs alveolar function, reduces milk yield and has a deleterious effect on milk composition, including increased milk SCC and bacterial load. In the present study the SCC and TBC were higher in mastitic buffalo cows before treatment (P<0.05). Regulation of PMNs migration from the central pool to the infected site is of great importance for host defense against mastitis (Paape *et al.*, 2002). For appropriate elimination of pathogen the effectiveness of the drug and optimum functioning of the hosts’ immune system is required. In and around the calving period there is down-regulation of selectins, which affects migration of leukocytes into the udder parenchyma and its functional activities (Lee and Kehrli, 1998; Paape *et al.*, 2002). Leukocyte activities can be modulated by number of specific and non-specific mediators (Smith, 1994). Antibiotics are used to treat mastitis, but they are not effective in removing the inflammatory metabolites from the tissue milieu and also contaminate the milk (Erskine, 2000).

In the present drug trial significant reduction of SCC and TBC was observed in Group 2 treated with CL along with i.m. administration of vitamin E and selenium; however, the therapy enhanced the expression of L selectin and did not reduced the MFI of CD18 in post- treated buffalo cows. On the contrary, the expression of CD18 reduced significantly in Group 3 cows treated with enrofloxacin.

*C. longa*, commonly known as ‘turmeric’, is widely used as a spice in Indian cuisine. The rhizome possess several medicinal properties. Curcumin is the main phytochemical principal of CL attributing a wide array of biological activities such as antioxidant, anti-inflammatory, wound-healing, antifungal and antibacterial activity (Singh *et al.*, 2002). Singh *et al.* (2002) observed inhibition of growth of pathogenic *S aureus* with CL extract. Menon and Sudhir (2002) suggested that the therapeutic effect of CL could be due to its anti-inflammatory and antioxidant properties. Udder health in lactating animals is also influenced by the dietary status, supplementation of selenium and vitamin E in feed of lactating dairy cows was found to improve leukocyte migration, phagocytosis and intracellular killing respectively (Smith *et al.*, 1997). It has been observed that supplementation of micronutrients and vitamin E in feed helps in reducing severity and duration of clinical mastitis and helps in alleviating the acute phase response from the site of infection (Politis *et al.*, 2004).There are several reports on the therapeutic effect of vitamin E and selenium against mastitis; however, its synergistic effect with CL against mastitis, particularly the leukocyte activities, are undefined. In conclusion, this study represents an initial investigation into the synergistic effect of vitamin E plus selenium and CL on the activity of L selectin, CD 18, SCC and TBC in mastitic buffalo cows.
Table 1. Somatic cell count (SCC) 1x 10^5 cells/ml and total bacterial count (TBC) 1x 10^3 cells/ml of milk in response to the treatment with *C. longa* plus Vitamin E and selenium (Group 2) and Enrofloxacin (Group 3) and sterile PBS (Group 4) mastitic cows and in normal healthy cows (group I) (mean ± SE).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days post treatment</th>
<th>SCC</th>
<th>TBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
<td>Day 15</td>
</tr>
<tr>
<td>Gr I</td>
<td>2.79 ± 1.22^a</td>
<td>3.02 ± 1.26^x</td>
<td>3.09 ± 1.14^x</td>
</tr>
<tr>
<td>Gr II</td>
<td>8.87 ± 1.09^a,y</td>
<td>5.26 ± 1.04^b,y</td>
<td>5.09 ± 1.16^c,y</td>
</tr>
<tr>
<td>Gr III</td>
<td>9.07 ± 1.01^a,y</td>
<td>5.03 ± 1.17^b,z</td>
<td>5.07 ± 1.05^b,y</td>
</tr>
<tr>
<td>Gr IV</td>
<td>8.53 ± 1.33^a,y</td>
<td>8.12 ± 1.72^a,y</td>
<td>7.46 ± 1.96^a,y</td>
</tr>
<tr>
<td>TBC</td>
<td>0.27±1.11^a,w</td>
<td>0.26±1.23^a,w</td>
<td>0.27±1.21^a,w</td>
</tr>
<tr>
<td>Gr II</td>
<td>2.86±1.21^a,x</td>
<td>1.09±1.07^b,x</td>
<td>0.82±1.12^c,x</td>
</tr>
<tr>
<td>Gr III</td>
<td>3.06±1.73^a,x</td>
<td>0.98±1.05^b,y</td>
<td>0.58±1.79^c,y</td>
</tr>
<tr>
<td>Gr IV</td>
<td>2.75±1.66^a,x</td>
<td>2.53±1.95^a,x</td>
<td>2.21±1.59^a,x</td>
</tr>
</tbody>
</table>

*Values with different superscripts in each rows (a, b) and each column (w, x, y) differ significantly (P<0.05).

Table 2. Expression of L selectin and CD 18 in response to treatment with *C. longa* plus vitamin E and selenium (Group 2) and enrofloxacin (Group 3) and sterile PBS (Group 4) mastitic cows and in normal healthy cows (group I) (mean ± SE).

<table>
<thead>
<tr>
<th>Group of cows</th>
<th>L selectin on milk</th>
<th>PMNs</th>
<th>CD 18 in milk PMNs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 8</td>
<td>Day 0</td>
</tr>
<tr>
<td>Group 1</td>
<td>4.66±1.37^a</td>
<td>4.59±1.29^x</td>
<td>5.93±1.49^x</td>
</tr>
<tr>
<td>Group 2</td>
<td>1.99±1.52^a,c</td>
<td>3.18±1.16^b,c</td>
<td>8.63±1.68^a,y</td>
</tr>
<tr>
<td>Group 3</td>
<td>2.01±1.17^y</td>
<td>2.22±1.09^c</td>
<td>8.28±2.03^a,y</td>
</tr>
<tr>
<td>Group 4</td>
<td>2.26±1.32^y</td>
<td>2.15±1.44^d</td>
<td>8.35±2.03^a,y</td>
</tr>
</tbody>
</table>

*Values with different superscripts in each rows (a, b) and each column (x, y, z) differ significantly (P<0.05).
The results indicate the enhanced cellular defense of the diseased mammary gland and subsequently reduction in infection and inflammation.

ACKNOWLEDGEMENTS

The authors thank Dr. B. Sharma, National Professor for his expertise in FACS analysis. The junior authors also thank the Indian Council Agricultural Research, New Delhi for providing Junior Research Fellowship.

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veterinary medicine to mastitis therapy, World Buiatrics Congress, Santiago Chile.


ABSTRACT

In the present study, superovulatory response was determined in eight healthy matured Berari (Nagpuri) buffaloes which were divided in two groups (A and B). The superovulatory treatment was started in donor buffaloes from the 10th day after induced estrus. Animals in Group A and Group B were administered, respectively, with 500 mg and 560 mg FSH-P in descending doses (100, 75, 50, 25 mg and 100, 80, 60, 40 mg twice daily) for four consecutive days. PGF$_2\alpha$ (Lutalyse) 25 mg was injected to all the animals in both groups after 72 h of initiation of superovulatory treatment. Oestrus was recorded in all the buffaloes under study. Onset and length of superovulatoryoestrus in Groups A and B were recorded to be 20.00±0.00 h, 36.50±5.50 h and 19.00±0.00 h, 38.75±3.32 h, respectively. Per rectal assessment of superovulatory response on the 6th day after the last FSH-P injection revealed the average number of corpora lutea and unovulatory follicles per donor buffalo in Group A and Group B to the tune of 3.00±0.71, 1.00±0.00 and 2.75±0.48, 1.50±0.29, respectively. No significant difference was recorded in terms of superovulatory response in either group at 5 percent level (P < 3.185).

Keywords: buffalo, Berari (Nagpuri), superovulation

INTRODUCTION

Indian buffaloes constitute more than 50 percent of the world buffalo population. Region specific breeds of buffaloes are still reared in rural India with Murrah as an exception. The Berari (Nagpuri) buffalo is a native breed of the Vidarbha region of Maharashtra. This breed is common in Akola, Buldhana, Yeotmal, Washim, Amravati and Nagpur districts. Infertility is the most common cause of reproductive failure especially in buffaloes. Silent estrus coupled with low conception rate, seasonality of breeding, higher optimal age at first calving, anoestrus and repeat breeding are some of the major constraints associated with buffalo reproduction. Although, artificial insemination (AI) technique has significantly enhanced timely conception in milch animals, roblems of infertility and silent oestrus in buffaloes are still persistent. Oestrus synchronization and superovulation coupled with embryo transfer technology are proved to be promising tools in animal reproduction and has been found to be advantageous in national breeding.
programme. The process of superovulation means the increased capacity of the dam to produce a greater number of ova by injecting gonadotrophines. This technique is highly useful for the improvement and conservation of native germplasm and increasing number of offsprings from donor females at faster rate (Misra et al., 1990, Patel et al., 2010).

The superovulation of donor females has been traditionally done by the single intramuscular injection of pregnant mare serum gonadotropine (PMSG) but, nowadays injections of pituitary extract containing follicle stimulating hormone (FSH-P) are used. FSH is usually given within a period of 4 to 5 days at the mid luteal stage of the oestrus cycle through a series of injections so that follicles are recruited immediately prior to the lysis of the existing corpus luteum. The results of superovulation in buffalo are disappointing when compared with those in cattle (Lindsell et al., 1986). FSH-P preparations proved to be effective in superovulating cattle and produce no evidence of reduced embryo quality at high dosage (Wang et al., 1988; Mapletoft et al., 1988). Information about superovulation in Berari (Nagpuri) buffaloes is lacking in the available literature. Therefore, the present study was proposed with the objective of investigating the effect of two different doses of FSH-P (Folltropin-V) on superovulation in Berari buffaloes.

MATERIALS AND METHODS

Selection of animals: Eight (n=8) normal, healthy, cyclic Berari (Nagpuri) buffaloes free from any pathological and reproductive disorder were selected from the experimental animals of the ICAR - Embryo Transfer Technology project being implemented at the Department of Gynaecology, Dr. Panjabro Deshmukh Krishi Vidyapeeth, campus Akola. All the animals were maintained under uniform stall fed condition throughout the study.

Synchronization and Superovulation: All buffaloes showing regular cyclic activity for two consecutive cycles and those with an active corpus luteum were selected and oestrus was synchronized by administering 25 mg Lutalyse (prostaglandin F2α analogue) intramuscularly on day 0 and day 11. Synchronization of oestrus was determined on the basis of 1) efficacy of PGF2α for induction of synchronized oestrus, 2) time required for the onset of oestrus and 3) length of synchronized oestrus. Eight buffaloes, synchronized by injection PGF2α were divided into two groups (A and B). Folltropin-V (FSH-P) was used for superovulation. Superovulatory treatment commenced from the 10th day during the mid luteal stage of the oestrus cycle. The protocol for superovulation is shown in Table 1.

Experimental buffaloes were closely observed after treatment for induction of oestrus. The superovulatory response was estimated by per rectal palpation on day 6 after the last FSH-P injection.

Data analysis: The data collected were analyzed statistically and the difference in superovulatory response was tested using the student’s t-test.

RESULTS

a) Oestrus response and the time required for onset of oestrus:

The intramuscular injection of 25 mg Lutalyse was given to eight normal cyclic Berari (Nagpuri) buffaloes. Of the eight buffaloes, seven
(87.50 percent) exhibited oestrus, and on the 11\textsuperscript{th} day the second intramuscular injection at same dose was again given. All buffaloes (100.00 percent) exhibited oestrus to the 2\textsuperscript{nd} dose. The results of synchronization of oestrus are shown in Table 2.

b) Length of oestrus:

The length of synchronized oestrus after the first PGF$_{2\alpha}$ injection ranged from 22 to 28 h with an average of 20.80 ± 3.07 h. However, after the second injection of PGF$_{2\alpha}$, the length of oestrus was slightly higher (22-36 h) with an average of 26.50 ± 1.59 h. The mean time required for induction of oestrus after the first and the second injection of PGF$_{2\alpha}$ (Lutalyse) were 36.75 ± 6.10 h and 34.75 ± 2.74 h, respectively.

c) Onset and duration of superovulatory oestrus:

The time required for onset of oestrus after superovulatory treatment in Group A buffaloes was 20.00 ± 0.00 h and that in Group B was 19.00 ± 0.00 h. The aggregate average of onset of superovulatory oestrus in both the groups was 19.50 ± 0.18 h. The calculated ‘t’ value (2.00) for both groups elicited non-significant differences at 5 percent level of significance (3.182).

The length of oestrus during superovulatory treatment in Group A buffaloes was 36.50 ± 5.50 h and that in Group B buffaloes was 38.75 ± 3.32 h; however, the length of superovulatory oestrus in Group A was 26-46 h and while that in Group B was 29-44 h.

d) Superovulatory response: On the basis of number of palpable corpora lutea and unovulatory follicles on both the ovaries, the superovulatory response was graded manually. In the present studies, the number of corpora lutea in Group A buffaloes treated with 500 mg FSH-P ranged from two to five with an average of 3.00 ± 0.71, whereas in Group B buffaloes treated with 560 mg FSH-P, the number ranged from two to four with an average of 2.75 ± 0.48. Calculated ‘t’ value (0.245) elicited non-significant differences between the two groups (3.182) at 5\% level. The number of unovulatory follicles in Group A buffaloes ranged from zero to one with an average of 1.0 ± 0.00, whereas the number in Group B buffaloes ranged from one to two with an average of 1.5 ± 0.26. No significance difference was recorded between the two groups. Numbers of corpora lutea were nonsignificantly higher with 500 mg FSH-P dose as compared to 560 mg FSH-P.

DISCUSSION

In the present study, the time required for onset of oestrus was shorter; however, longer onset times have been recorded earlier by Pant and Singh (1980), Kamonpatana \textit{et al.} (1979), Rajeshwaran \textit{et al.} (1992) who recorded the time required for onset of oestrus to be 50.33 ± 9.46 h, 69.3 ± 5.6 h, 71.33 ± 6.38 h, respectively. The length of oestrus was longer with the second PGF$_{2\alpha}$ injection than with the first injection of PGF$_{2\alpha}$. The student’s’ test (1.81) elicited non-significant differences (2.365). The present findings are also in agreement with Rao \textit{et al.} (1982), Kaikini and Pargaonkar (1969) and Patil (2000) who recorded the average duration of oestrus to be 50.33 ± 9.46 h, 69.3 ± 5.6 h, and 71.33 ± 6.38 h, respectively. The length of oestrus was longer with the second PGF$_{2\alpha}$ injection than with the first injection of PGF$_{2\alpha}$. The student’s’ test (1.81) elicited non-significant differences (2.365). The present findings are also in agreement with Rao \textit{et al.} (1982), Kaikini and Pargaonkar (1969) and Patil (2000) who recorded the average duration of oestrus to be 50.33 ± 9.46 h, 69.3 ± 5.6 h, and 71.33 ± 6.38 h, respectively. Patil (1997) also recorded the average duration of oestrus to be 25.50 ± 1.79 h. In the studies of Chede (1990) and Chouhan \textit{et al.} (1992) it was respectively 28.61 ± 2.62 h and 28.40 ± 10.07 h, which are higher as compared to present findings.
Table 1. Treatment schedule of superovulation in Berari (Nagpuri) buffaloes.

<table>
<thead>
<tr>
<th>Days of oestrus cycle</th>
<th>Time</th>
<th>Donor buffalo treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Folltropin - V 500 mg (Group - A)</td>
</tr>
<tr>
<td>10th day</td>
<td>Morning</td>
<td>100 mg</td>
</tr>
<tr>
<td></td>
<td>Evening</td>
<td>100 mg</td>
</tr>
<tr>
<td>11th day</td>
<td>Morning</td>
<td>75 mg</td>
</tr>
<tr>
<td></td>
<td>Evening</td>
<td>75 mg</td>
</tr>
<tr>
<td>12th day</td>
<td>Morning</td>
<td>50 mg</td>
</tr>
<tr>
<td></td>
<td>Evening</td>
<td>50 mg + PGF₂α 25 mg</td>
</tr>
<tr>
<td>13th day</td>
<td>Morning</td>
<td>25 mg</td>
</tr>
<tr>
<td></td>
<td>Evening</td>
<td>25 mg</td>
</tr>
<tr>
<td>19th day</td>
<td></td>
<td>Per rectal estimation of superovulatory response</td>
</tr>
</tbody>
</table>

Table 2. Synchronization response and average time required for induction of oestrus in Berari (Nagpuri) buffaloes.

<table>
<thead>
<tr>
<th>PGF₂α analogue used for synchronization</th>
<th>Treatment</th>
<th>Response to the synchronization treatment</th>
<th>Average time required for onset of oestrus (h)</th>
<th>Range for time required (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of buffaloes treated</td>
<td>No. of buffaloes responded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dinoprost (Lutalyse)</td>
<td>First injection</td>
<td>8</td>
<td>7 (87.50 %)</td>
<td>36.75 ± 6.10</td>
</tr>
<tr>
<td></td>
<td>Second injection</td>
<td>8</td>
<td>8 (100.00%)</td>
<td>34.75 ± 2.74</td>
</tr>
</tbody>
</table>
The aggregate average of onset of superovulatory oestrus in both the groups was 19.50±0.18 h. The onset of superovulatory oestrus in both groups was shorter; however, some of researchers recorded average times required for onset of superovulatory oestrus in buffaloes ranging from 28 to 33 h (Taneja et al., 1995; Mathroo and Meharsingh, 1997; Sartape, 1999). Non-significant differences between the two groups at 5 percent level of significance (3.182) were also recorded (‘t’ value -0.508) in terms of length of oestrus during superovulatory treatment. These findings are corroborative with the findings of Sartape (1999) and Yadav et al. (1985) who recorded the average duration of oestrus to be 39.8±1.64 h and 41.3±1.25 h, respectively.

No significant difference was recorded with superovulatory response and presence of unovulatory follicles when ovaries were palpated manually. Numbers of unovulatory follicles were non-significantly lower with the 500 mg dose schedule. These findings are in close agreement with the studies of Mathroo and Meharsingh (1997), Taneja et al. (1995), Singla and Madan (1990). These results are also in accordance with those of Drost et al. (1986) and Singh and Narayana (1997) who recorded the average number of unovulatory follicle to be 1.50 and 1.4 ± 0.21, respectively.

Motwani (1986), Misra et al. (1991) and Beg et al. (1997) recorded slightly higher average numbers of unovulatory follicles viz. 2.2 ± 0.82; 2.67±0.71 and 3.00±1.05, respectively. A comparatively better superovulatory response was obtained in buffaloes injected with 400 mg of FSH than those injected with 600 mg in the studies by Patel et al. (2010). A non-significant increase in number of unovulatory follicles in 600 mg (1.90±0.67) compared to 400 mg (0.88±0.44) of FSH dose was observed. Such variation in results could be attributed to differences in location, breed, hormonal treatments and individual response to the treatment.

**CONCLUSION**

The response of synchronization of oestrus, time required for onset of oestrus and length of oestrus needs to be studied in a larger number of buffaloes in breeding and non-breeding seasons. Because of variable response to the two FSH-P treatment recorded in the individual buffaloes in the present studies, it is suggested that the trials on similar lines should be conducted in a larger number of donor buffaloes and in different seasons.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Groups</th>
<th>Number of animals</th>
<th>Average onset of oestrus (h)</th>
<th>Average length of oestrus (h) (with range in h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group-I FSH-P (500 mg treatment)</td>
<td>4</td>
<td>20.00 ± 0.00</td>
<td>36.50 ± 5.50 (26-46)</td>
</tr>
<tr>
<td>2</td>
<td>Group-II FSH-P (560 mg treatment)</td>
<td>4</td>
<td>19.00 ± 0.00</td>
<td>38.75 ± 3.32 (29 - 44)</td>
</tr>
<tr>
<td></td>
<td>Aggregate average (h)</td>
<td>8</td>
<td>19.50 ± 0.18</td>
<td>37.62 ± 3.01 (26-46)</td>
</tr>
</tbody>
</table>
of the year.

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Patil, M.S. 2000. *Comparative efficacy of two different prostaglandin F$_2$ alpha analogues: Dinoprostone (Lutalyse) and Luprostiol (Prosolvin) for inducing synchronized oestrus in Nagpuri buffaloes*. M.V.Sc. Thesis submitted to Dr. Panjabrao Deshmukh
Krishi Vidyapeeth, Akola.


ABSTRACT

The present research was carried out to study the effect of GnRH and PGF₂α administration on uterine involution and post-partum fertility in buffaloes. A total of 36 normally calved Murrah buffaloes were divided into three groups. The buffaloes of Group 1 were treated with GnRH (buserelin acetate) 0.020 mg while buffaloes of Group 2 with PGF₂α (cloprostenol sodium) 1.30 mg on day 14 post-calving intramuscularly while the buffaloes of Group 3 were kept as control. The average days required for involution of the uterus were 25.08±1.04, 22.75±0.91 and 30.33±1.31 while the average days required for exhibition of first post-partum oestrus were 37.5±5.16, 26.91±1.36 and 45.08±3.77 days in Groups 1, 2 and 3, respectively. The first service conception rate was highest 41.66% in Group 2 while the rates were 33.33 and 16% in Groups 1 and III, respectively.

Keywords: buffaloes, Bubalus bubalis, post-partum, fertility, GnRH, PGF₂α

INTRODUCTION

The buffalo occupies an important place in the livestock economy of Asia and India. Buffaloes are valued for milk, meat and draught power. Low reproductive efficiency in livestock in general and in buffaloes in particular remains a major economic problem globally, and its incidence is higher in India. The post-partum period is regarded as an important period in the reproductive life of bovines (Fonesca et al., 1983). Uterine involution begins and ovarian follicular waves resume soon after parturition due rising in FSH concentration (Schallengerger, 1985). However, the dominant follicle of these waves fails to ovulate due to failure to undergo final terminal maturation. Failure of post-partum dominant follicles to undergo final maturation is due to inadequate LH pulse frequency, which results in low androgen production in the follicle (Fortune, 1986) and inadequate oestriol positive feedback to induce an LH surge (Peters et al., 1985), which is perquisite for follicular terminal maturation prior to ovulation. Absence of LH pulses between days 15 to 30 post-partum is due to continued sensitivity of the hypotalamic GnRH pulse generator to the negative feedback effect of estradiol-17β, which results in an absence of GnRH pulses. The administration of GnRH will overcome the inadequate secretion of pituitary LH in the early post-partum period (Shah et al., 1990) and restore ovarian function earlier within the post-partum period and assists in cleansing the uterus, brining faster uterine involution and terminal maturation and ovulation of the dominant follicle (Takkar et al., 1999).
The reproductive cyclicity and its rhythm in terms of its reawakening during the early post-partum period has been linked to temporal changes of hormones, mainly prostaglandin (Perera et al., 1981). Leindell et al. (1980) reported that PG metabolites increased at the time of parturition and remained high for 8 to 16 days post-partum. So delay in involution of uterus was due to the short period of high prostaglandin F$_{2\alpha}$ metabolite release. It has also positive effect on the uterine musculature tone (Lindell and Kindahl, 1983). PGF$_{2\alpha}$ injection in the early post-partum period enhances the uterine involution and reproductive efficiency in normal calved buffaloes (Nazir et al., 1994). Considering these facts, the present research was carried out to evaluate the effect of GnRH and PGF$_{2\alpha}$ administration on day 14 post-calving on uterine involution and post-partum fertility in buffaloes.

**MATERIALS AND METHODS**

A total of 36 Murrah buffaloes in their second to seventh lactation which had normally calved were selected. The buffaloes were housed in a loose housing barn and fed chopped roughages like sugarcane, alfalfa, napier grass, green maize and jowar straw three times a day. A pre-calculated quantity of concentrate mixture was fed through an automatic concentrate feeding station (AFS) to each buffalo based on milk yield, body weight and pregnancy status. The selected buffaloes were divided into three groups and buffaloes of Group 1 were treated with GnRH (buserelin acetate) 0.020 mg intramuscularly while those of Group 2 with PGF$_{2\alpha}$ (cloprostenol sodium) 1.30 mg on day 14 post calving intramuscularly while the buffaloes of Group 3 were kept as control. The uterine involutions were monitored at weekly intervals on day 14, 21 and 28 post-partum. The finger width of left palm was pre-measured with a measuring tape and this was used as a scale for measuring internal biometry. The data pertaining to uterine involution, post-partum exhibition of oestrus, first service conception rate were analyzed by randomized block design described by Snedecor and Cochran (1989).

**RESULTS AND DISCUSSION**

**Uterine involution**

The average days required for involution of the uterus in these buffaloes were 25.08±1.04, 22.75 ±0.91 and 30.33±1.31 for Groups 1, 2 and 3, respectively. In Group 2, the least time was required for whereas Group 3 required more time

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Particulars</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group 1</td>
<td>25.08±1.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Group 2</td>
<td>22.75±0.91&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>Group 3</td>
<td>30.33±1.31&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 1. Average days required for involution of uterus in experimental buffaloes.

Column wise superscript (a, b and c) denoted the significant difference at P<0.05 and P<0.01 levels.
for involution of the uterus. The results were statistically significant at the \( P<0.05 \) and \( P<0.01 \) levels (Table 1).

The present findings regarding the effect of GnRH administration on the uterine involution is in close accordance with Barkawi et al. (1995) who reported 27.90±0.9 days while Takkar et al. (1999) recorded 31 days for uterine involution in GnRH treated buffaloes, which was slightly more than present findings. The results of the present study for the number of days required to complete uterine involution after PGF\(_2\alpha\) treatment were slightly less than those reported by Nazir et al. (1994) who observed 24.88±0.97 days required for uterine involution in Nili-Ravi buffaloes. Nasr et al. (1994) observed 29.9 days and Iqbal et al. (2003) observed 28.90±1.79 days for uterine involution in after PGF\(_2\alpha\) treatment. In the present study, the effect of PGF\(_2\alpha\) treatment on uterine involution was in close agreement with those of Malvi et al. (2004) who observed 22.7±1.75 days and Tiwari et al. (2004) who observed 18.20±1.47 days required for uterine involution in buffaloes.

The finding of days required for uterine involution in the control group was in accordance Nazir et al. (1994) who observed 29.75±0.75 days in Nili-Ravi buffaloes, Khasatiya et al. (2005) observed 30.00±1.36 and 33.75±1.65 days in post-partum fertile and infertile Surti buffaloes. The variation between values obtained by the above mentioned researcher and the present findings may be due to factors like difference in age, parity, nutrition of dams during late gestation, season of calving, sex and birth weight of the calf and the post-partum husbandry practices and hygiene of buffaloes.

### Days required for exhibition of first post-partum oestrus

The average days required for exhibition of first post-partum oestrus were 37.5±5.16, 26.91±1.36 and 45.08±3.77 days in Groups 1, 2 and 3, respectively. The results are significant at \( P<0.05 \) and \( P<0.01 \) (Table 2).

The average days required for first post-partum estrus exhibition after GnRH treatment in Group 1 were 37.5±5.16 days. The results are in concurrence with Barkawi et al. (1995) who reported 33.6±3.1 days in GnRH treated buffaloes. Fewer days required for PPE after GnRH treatment than present findings are reported by Takkar et al. (1999) who reported 24.0±2 and 27.3±4 days when GnRH was administered at doses of 8 \( \mu \)g and 12 \( \mu \)g, respectively in buffaloes.

The average days required for exhibition of first post-partum oestrus in Group 2 were 26.91±1.36. However, the days required for first post-partum exhibition after PGF\(_2\alpha\) treatment in the present study were less than those reported Nasr et al. (1994) 43.00 days and Tiwari et al. (2004) 54.00±3.19 days in buffaloes. In Group 3 (control group), the average days required for first

### Table 2. Average days required for exhibiting first PPE in experimental buffaloes.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Particulars</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group 1</td>
<td>37.5±5.16a</td>
</tr>
<tr>
<td>2</td>
<td>Group 2</td>
<td>26.91±1.36b</td>
</tr>
<tr>
<td>3</td>
<td>Group 3</td>
<td>45.08±3.77c</td>
</tr>
</tbody>
</table>

Column wise superscript (a, b and c) denote a significant difference at the \( P<0.05 \) and \( P<0.01 \) levels.
post-partum exhibition of estrus were 45.08±3.77. However, the days required for first post-partum exhibition of estrus in untreated buffaloes are less than reported by Suthar and Kavani (1992) 56.72±3.58 days in Meshana buffaloes and Tiwari and Pathak (1995) 57.7±5.61 days in suckled buffaloes. The variation between values obtained by the above mentioned research scientists and the present findings may be due to factors like difference in nutrition of dam during late gestation and the post-partum husbandry practices and hygiene, and estrus detection aids.

**First service conception rate**

The first service conception rate was highest 41.66% in Group 2 which is indicative of the fact that PGF$_{2\alpha}$ treatment was most beneficial. That the first service conception rate was 33.33% in Group 1 buffaloes indicates that GnRH treatment also yielded good results. Group 3 yielded the lowest results i.e. a 16.16% first service conception rate. The present results are low in comparison with Takkar et al. (1999) who reported a 50% conception rate in GnRH treated and a 38% rate in control buffaloes.

**CONCLUSION**

The buffaloes treated with PGF$_{2\alpha}$ showed the least time i.e. 22.75±0.91 days required for uterine involution and their first service conception rate was highest: 41.66%, which is indicative of the fact that PGF$_{2\alpha}$ treatment is beneficial for improving postpartum fertility in buffaloes.

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EFFECT OF PRE-PARTUM SUPPLEMENTATION OF VITAMIN E TO MURRAH BUFFALOES ON LYMPHOCYTE FUNCTION OF CALVES

A.K. Singh*, S. Pandita, R.C. Upadhyay and G. Chandra

ABSTRACT

The present study was undertaken in buffalo neonates born to vitamin E supplemented (treatment group) and non-supplemented (control group) Murrah buffaloes. Blood samples were collected into sterile vacutainer tubes from calves at day 0 (before colostrum feeding), 7, 28, and 126-post birth. The lymphocytes were separated and cultured in RPMI 1640 medium with mitogen phytohemagglutinin-P (PHA-P) for 36h at 37°C in a humidified CO₂ incubator (95% air and 5% CO₂). The lymphocyte responsiveness in vitro evaluated in response to different nitric oxide compounds viz. S-Nitroso-N-acetylpenicillamine (SNAP), Nω−Nitro-L-arginine methyl ester (L-NAME) and L-Nω−Monomethyl-arginine (NMMA). The blastogenic response between days in both groups varied significantly (P<0.01) but it was not found to be significant between different groups. The precolostrum stimulation index was significantly low (P<0.05) in all the calves. The mitogen induced lymphocyte blastogenesis was not affected by low levels (1.0 mM) of SNAP but significantly inhibited (P<0.001) at 10 mM in both the groups. At 10 mM of L-NAME the blastogenic response between days in both groups varied significantly (P<0.01) but it was not found to be significant at 10 mM of L-NMMA. Nitric oxide (NO) production by lymphocytes from all calves increased (P<0.01) progressively with age. The lymphocytes supplemented with SNAP (1.0 and 10 mM) significantly (P<0.001) produce higher amounts of NO in comparison to non-supplemented lymphocytes. At a low level (0.2 mM) of L-NAME and L-NMMA, the change in the NO production between days was significant in both the groups. However, at a higher level (10 mM) of L-NAME and L-NMMA, the differences were not found to be significant between days and between the two groups. The study demonstrates that buffalo calves lymphocytes have an active nitric oxide synthase (NOS) system. The present study also indicates that the immune competence of buffalo calf lymphocytes is not affected by prepartum supplementation of vitamin E to buffaloes.

Keyword: Murrah buffaloes, buffalo calf, cell mediated immunity, lymphocytes, nitric oxide

INTRODUCTION

The neonatal period represents a critical stage in the development of physiological and immunological functions. Metabolic instability during this period makes the neonate particularly sensitive to perinatal diseases that result in high mortality (Dwyer, 2008). The neonate has an

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immature immune system compared with that of an adult. The calf immune system is more susceptible to oxidative stress during the neonatal period due to a weak defense system against superoxide radicals (Inemani et al., 1999). The calves are lymphopenic at birth (Outteridge and Duffy, 1981; Manak, 1986). The lymphocyte concentration gradually increases with age (Osburn et al., 1974; Clover and Zarkower, 1980). Nagahata et al. (1991) found that antibody-producing activity of lymphocytes was lower in calves up to 3 weeks after birth. In addition, Tizard (2004) reported that antibody-producing activity in suckling calves is low at least up to 1 month after birth. Osburn et al. (1974) depicted a suppressed PHA-induced lymphocyte blastogenesis in the newborn as compared to bovine fetal lymphoid cells at 90 and 121 days of gestation (Osburn et al., 1974; Rensbaw et al., 1977). Clover and Zarkower (1980) reported that both PHA and poke weed mitogen (PWM)-induced blastogenic responses of peripheral blood lymphocytes from 6-hr old, colostrum fed calves were suppressed relative to those from 4-day old calves. Rajaraman et al. (1997) reported that peripheral blood mononuclear cells (PBMC) from 1-wk-old calves fed colostrum and milk were functionally hypo-responsive when compared to PBMC from adult cattle. Person et al. (1983) suggested that although the lymphocytes of neonate calves had the ability to respond to mitogens, individual variations are there in the lymphocyte reactivity. Compared with adult cattle, calves have a low percentage of E rosette-forming cells (Outteridge and Duffy, 1981) and their lymphocytes show less mitogen-induced proliferation (Manak, 1986). Yang and Shultz (1986) reported that lymphocytes from young piglets showed greater sensitivity to prednisolone than lymphocytes from 6-month old pigs. Other functional differences include the capacity of PBMC from young calves to produce inducible NOS, a component of bactericidal mechanisms of phagocytic leukocytes and interferon-γ (IFN), a pivotal cytokine in cell-mediated immunity, reduced secretion of other cytokines (Nonnecke et al., 2003), and reduced neutrophil function (Dore et al., 1991; Higuchi et al., 1997) have also been reported after birth.

Vitamin E has been reported to be an immunopotentiator, which not only provides additional immunity to dairy animals when fed peripartum but also has been reported to enhance calf viability (Amer and Hashem, 2008). Therefore, in order to improve the immune status of neonates, it is imperative to supplement pregnant animals with vitamin E to increase its level in the colostrum and thus transfer it to calves. Induction of the lymphocyte proliferative response induced by antigen/mitogen in vitro has been shown to be representative of cellular immunocompetence. This measure can potentially be used as an indicator of an individual’s ability to mount an immune response to a specific pathogen or immunomodulators. To date, no study describing the age-related changes in lymphocyte function in buffalo calves as influenced by antepartum supplementation of vitamin E to buffaloes is available. Furthermore, study on the in vitro effect of nitric oxide on lymphocyte function in the developing buffalo calf is unavailable. Therefore, the purpose of this study was to investigate the age-related changes in mitogen-induced lymphocyte proliferation and the effects of nitric oxide (NO) on in vitro immune competence in terms of the lymphocyte proliferative response in buffalo calves.
MATERIALS AND METHODS

The experiment was approved by the Institutional Animal Ethics Committee constituted as per the article number 13 of the CPCSEA rules, laid down by the Government of India.

Selection of animals

The experiment was conducted on twenty apparently healthy Murrah buffaloes in an advance state of pregnancy selected from the National Dairy Research Institute (NDRI), Karnal herd, from October 2009 until May 2010. These were randomly divided into two groups (10 in each group). The control group was fed the diet as practiced for pregnant buffaloes on the NDRI dairy farm. The treatment group was supplemented with 2000 IU vitamin E (dl-alpha-tocopherol acetate) mixed with moistened concentrate and fed individually in the morning each day with the normal diet. Vitamin E supplementation was initiated 30 days prior to the expected date of parturition and was continued until parturition. Calves born from these buffaloes (both the groups) were removed from their dams before colostrum ingestion and housed in a calf’s pen separately in two groups after weighing and ear tagging. The colostrum of the same dam was fed to the newborn within 2 h of birth, which was designated as 0 h, and subsequent feedings at an interval of 12 h for five days was followed.

Collection of blood and separation of lymphocytes

Approximately 15 ml of blood was drawn in sterile, heparinized vacutainer tubes (BD Franklin, USA) from each calf by jugular venipuncture on day 0 (before colostrums feeding), and on days 7, 28, and 126 post-birth at 6.00 AM in the morning. Blood was centrifuged at 3000 rpm for 40 min at 4°C; theuffy coat was harvested and resuspended in 1:1 v/v Dulbecco’s Phosphate Buffer Saline (DPBS). The total contents were carefully layered on lymphocyte separation medium (Histopaque 1077) in a ratio of 4:1 v/v in a sterile 15 ml polypropylene centrifuge tube and centrifuged at 1500 rpm for 40 minutes at room temperature. The lymphocyte rich layer was removed and washed (1200-1500 rpm; 10 minutes) twice with DPBS. The washed lymphocyte were resuspended in RPMI 1640 medium (3-4 ml) containing 25 mM HEPS buffer (Gibco Laboratories, Grand Island, NY) that was supplemented with 10% FCS (Hyclone), 2 mM L-glutamine, antibiotics (100 IU/ml of penicillin G and 100 μg/ml of streptomycin), antimycotics (5 μg/ml of amphotericin B) (all from Sigma Chemical Co., St. Louis, MO, USA). Cells were counted on a hemocytometer and cell suspensions were diluted with RPMI to 1 x 10^6 cells/ml. Viability of cells (>95%) was determined by trypan blue dye exclusion.

Lymphocyte proliferation in response to PHA-P and Nitric oxide

The lymphocytes (1x10^6 live lymphocyte/well) were cultured in 96 well flat-bottomed tissue culture plates. The cells were allowed to proliferate with and without mitogen (PHA-P) to determine the difference between cell proliferations. To the PHA-P positive wells, 20 μl PHA-P (5 μg/ml) was added. The effect of nitric oxide on the lymphocyte proliferation response was studied by using nitric oxide compounds viz. S-Nitroso-N-acetylpenicillamine (S-NAP) - a nitric oxide donor (0.1, 1.0, 10.0 mM/well) and nitric oxide synthase inhibitors i.e. Nω-Nitro-L-arginine methyl ester (L-NAME) and L-Nω-monomethyl-arginine (L-NMMA) (0, 0.2, 1.0, 5.0 and 10.0 mM/well). In all the cases, final culture volume was 200μl/well. The blank wells consisted of 200 μl of culture
media only. The culture plates were maintained at 37°C in a humidified atmosphere of 5% CO₂ for 36 h.

**MTT Assay**

The proliferative response of lymphocytes was estimated using the colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay according to the procedure given by Mosmann (1983). MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals, which are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent dimethyl sulfoxide (DMSO) results in the liberation of the crystals. The number of surviving cells is directly proportional to the level of the formazan product created. The plate was read at 450 nm with Microscan MS-5608A plate reader.

**Statistical Analysis**

All analysis was done using a Systat 12 software package. Data from different experiments are presented as mean ± SE. Analysis of variance of the data was done using RBD factorial design. The responses have been compared with respect to unstimulated cells for different experiments involving the mitogen and different immunomodulators (S-NAP, L-NAME and L-NMMA). Significance was considered at P<0.05 or is mentioned otherwise.

**RESULTS AND DISCUSSION**

In the present study, cell-mediated immunity was assessed by measuring the proliferation of mitogen-stimulated lymphocytes and nitric oxide (NO) produced by lymphocytes in buffalo calves under different experiments. The lymphocyte proliferation response was expressed as a stimulation index for mitogen stimulated and mitogen plus immunomodulator stimulated as a relative stimulation index. The blastogenic response between days in both groups varied significantly (P<0.01) but it was not found to be significant between different groups. The precolostrum stimulation index was significantly low (P<0.05) in all the calves. The values registered a significant rise on day 7 post birth followed by a decline subsequently. The ANOVA revealed that antepartum supplementation of vitamin E had a significant effect on the stimulation index (P<0.05) of mitogen-induced lymphocyte blastogenesis (Figure 1).

**Effect of nitric oxide donor (SNAP) on blastogenic response of lymphocytes**

Effect of different concentrations of SNAP on the blastogenic response of lymphocytes in vitro following the culture of lymphocytes in the presence of 1.0 and 10 mM of SNAP, the mitogen induced lymphocyte blastogenesis was not affected by low levels (1.0 mM) of SNAP. It was significantly inhibited (P<0.001) at 10 mM in both the groups. At the both SNAP concentrations (1.0 and 10 mM) the lymphocyte stimulation index was not significant between the different groups (Table 1).

**Effect of nitric oxide synthase (NOS) inhibitors (L-NAME and L-NMMA) on the blastogenic response of lymphocytes**

The blastogenic response of cultured lymphocytes was significantly reduced in the
presence of 0.2 and 10 mM of L-NAME and L-NMMA in all the animals of both groups. At 10 mM of L-NAME the blastogenic response between days in both groups varied significantly (P<0.01) but it was not found to be significant at 10 mM of L-NMMA. When the two groups were compared with respect to blastogenic response, the differences were not found to be significant between the two groups (Table 1).

The lymphocyte proliferation assay is a measure of immune activation/stimulation. This assay helps evaluate the immunostimulatory/immunosuppressive activity of a mitogen. Cell-mediated immunity requires a full repertoire of functional T cells. T cells, which derive from the bone marrow and fetal liver, mature in the thymus. In the blood circulation, they account 40% to 80% of the lymphocytes.

The results of the present study are in general agreement with Rajaraman et al. (1997) who reported that PBMC from 1-wk-old calves fed colostrum and milk were functionally hypo-responsive when compared to PBMC from adult cattle. These variations were also influenced by season and the age of the bovine (Soper et al., 1978). In piglet Hoskinson et al. (1990) found highest spontaneous proliferation in the newborn piglet at wk 0.5 but it decreased (P<0.008) over 75% by wk 1. Proliferation tended to decrease, but more gradually, through wk 6. The present study indicated that the animals in different groups exhibited different blastogenic responses to different levels of NOS inhibitors. Lymphocyte stimulation is widely used to measure (i) immune competence by stimulation of lymphocytes with phyto/mitogens (Douglas, 1972; Weigel et al., 1992); (ii) histocompatibility by mixed leukocyte culture (Thorsby, 1974) and (iii) exposure to infectious agents by stimulation of lymphocytes with specific antigens (Daguiliard, 1972).

In the present study, functional capacity of lymphocytes was further evaluated by nitric oxide (NO) production by lymphocytes and their responsiveness to immunomodulators. Secretion of nitric oxide (NO), which is essential for effective cell-mediated immunity, was also influenced by treatment with values lowering significantly in response to antepartum supplementation of vitamin E. The functional capacity of lymphocytes was further evaluated by adding different immunomodulators in culture. NO production by lymphocytes from all calves increased (P<0.01) progressively with age. However, mitogen stimulated cells produced less NO in culture until day 126 as compared to unstimulated cells of all the calves (Figure 2).

Nitric oxide is a small inorganic paramagnetic free radical gas, which acts as a biological messenger molecule transmitting signals within and between cells, exerting a number of biological actions including vasodilation, neurotransmission, cytotoxicity and modulator of inflammation and immunity. Nitric oxide is produced by lymphocytes, macrophage, endothelial cells and neutrophils. Although production of NO by leukocytes that are stimulated by pathogens is an important component of the innate immune system, excess production of this molecule can be damaging to the tissues of the host. Nitric oxide (NO) production is a component of the innate immune system that has not been well studied in neonates. Nitric oxide acts as an intracellular signaling molecule or as a neurotransmitter when produced in low quantities. When produced in higher quantities for extended periods, NO is involved in the killing of microorganisms and tumor cells (Nathan, 1995) and in hematopoiesis (Ouaaz, 1995). Chronic production of NO in
Figure 1. Age related *in vitro* changes in stimulation indices of buffalo calves in response to mitogen (PHA-P) stimulation.

Figure 2. Age related changes in NO production of buffalo calves in response to mitogen (PHA-P) stimulation.
Table 1. Age related changes in relative stimulation Indices of buffalo calves in response to different immunomodulators.

<table>
<thead>
<tr>
<th>Group</th>
<th>Days</th>
<th>SNAP 1 mM/well</th>
<th>L-NAME 0.2 mM/10 mM/well 10 mM/well</th>
<th>L-NMMA 0.2 mM/10 mM/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1.52±0.26</td>
<td>0.92±0.09</td>
<td>0.92±0.09</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.32±0.26</td>
<td>0.30±0.09</td>
<td>0.53±0.07</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>1.30±0.34</td>
<td>0.53±0.21</td>
<td>0.87±0.10</td>
</tr>
<tr>
<td></td>
<td>126</td>
<td>0.90±0.42</td>
<td>0.15±0.26</td>
<td>0.64±0.14</td>
</tr>
<tr>
<td>Treatment</td>
<td>0</td>
<td>0.98±0.26</td>
<td>0.81±0.09</td>
<td>0.85±0.07</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.34±0.28</td>
<td>0.96±0.10</td>
<td>0.76±0.08</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.95±0.28</td>
<td>0.75±0.10</td>
<td>0.92±0.08</td>
</tr>
<tr>
<td></td>
<td>126</td>
<td>0.89±0.28</td>
<td>0.78±0.10</td>
<td>0.98±0.08</td>
</tr>
</tbody>
</table>

Leukocytes from calves produced unusually high concentrations of NO when compared with those produced by cows, a possible indicator of the immaturity of the immune system of the neonatal calf. This reflected immature cell mediated immune functions in neonate buffaloes and could be explained on the basis of higher secretion of cytokines (interferon γ) by immune cells in the case of new born calves in response to any stress producing external stimuli (Nonnecke et al., 1993), thus exerting an inhibitory effect on NO production. Shoker et al. (1997) have also reported a relationship between IL-2 and NO in lymphocyte culture studies in humans. The higher the IL-2 production was, the lower the nitric oxide production. Rajaraman et al. (1998) showed that mitogen-stimulated PBMC from milk replacer fed calves produced more NO than parallel cultures of adult PBMC. They suggested that the age-related differences in NO secretion might be attributable to the immaturity of the neonatal immune system.

Effect of nitric oxide donor (SNAP) on nitric oxide (NO) production by lymphocytes

Effect of different concentrations of SNAP on in vitro nitric oxide (NO) secretion by lymphocytes following culture of lymphocytes in the presence of 1.0 and 10 mM of SNAP were evaluated. The lymphocytes supplemented with SNAP (1.0 and 10 mM) significantly (P<0.001) produced higher amounts of nitric oxide (NO) in comparison to non-supplemented lymphocytes. In both the groups, nitric oxide (NO) secretion in culture supernatants was significantly (P<0.001) increased at both the concentrations (1.0 and 10 mM). However, the increase in the nitric oxide production at higher levels (10 mM) was much higher than at low levels (1.0 mM) of SNAP (Table 2).

Animal experiments suggest that the synthesis of nitric oxide by L-arginine-NO biosynthetic pathway affects immunoregulation (Moncada et al., 1991). Hence, it was important to test in the first instance the maximum non-cytotoxic
Table 2. Age related changes in NO production of buffalo calves in response to mitogen and immunomodulators stimulation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Days</th>
<th>PHA-P 5 μg/ml</th>
<th>PHA-P + SNAP 1 mM/well</th>
<th>PHA-P + L-NAME 0.2 mM/well</th>
<th>PHA-P + L-NMMA 10 mM/well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>16.33±3.73</td>
<td>49.78±6.19</td>
<td>377.46±25.17</td>
<td>15.77±2.85</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>16.00±3.73</td>
<td>49.60±6.19</td>
<td>338.30±25.17</td>
<td>15.37±2.85</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>17.95±4.81</td>
<td>55.15±7.99</td>
<td>470.09±32.49</td>
<td>17.32±3.67</td>
</tr>
<tr>
<td></td>
<td>126</td>
<td>45.40±5.89</td>
<td>114.16±9.79</td>
<td>405.50±39.80</td>
<td>43.31±4.50</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>9.98±3.73</td>
<td>50.60±6.19</td>
<td>351.29±25.17</td>
<td>9.82±2.85</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>12.73±3.93</td>
<td>50.84±6.52</td>
<td>401.98±26.53</td>
<td>13.62±3.00</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>23.19±3.93</td>
<td>47.26±6.52</td>
<td>367.40±26.53</td>
<td>22.19±3.00</td>
</tr>
<tr>
<td></td>
<td>126</td>
<td>34.63±3.93</td>
<td>112.02±6.52</td>
<td>447.35±26.53</td>
<td>34.58±3.00</td>
</tr>
</tbody>
</table>
concentration of nitric oxide compounds. The maximum non-cytotoxic concentration of the nitric oxide donor (SNAP) using the trypan blue exclusion test was found to be 10 mM. At this concentration percent killing after exposing the lymphocytes for 24 h with SNAP was less than 5%. Shoker et al. (1997) using sodium nitroprusside (SNP) as a nitric oxide donor reported maximum inhibition at 2 mM concentration in human lymphocytes. SNAP decomposes at room temperature to produce nitric oxide. Nitric oxide in turn tends to affect the lymphocyte functions. Shoker et al. (1997) reported that human lymphocytes do not produce appreciable amounts of nitric oxide to affect lymphocyte mitogenesis. They further indicated that some of the nitric oxide generating compounds viz. sodium nitroprusside (SNP) and nitroglycerine (NG) had a potent but non-specific immuno inhibitory effect on human lymphocyte function by a mechanism other than nitric oxide production.

Effect of nitric oxide synthase (NOS) inhibitors (L-NAME and L-NMMA) on nitric oxide (NO) production by lymphocytes

The nitric oxide production in culture supernatants of lymphocyte was significantly inhibited in the presence of 0.2 and 10 mM of L-NAME and L-NMMA in all the animals of both groups. At 1.0 mM of L-NAME the nitric oxide production was not significant between different groups but at same concentration of L-NMMA, nitric oxide production was significantly (P<0.05) reduced in the treated group. At a low level (0.2 mM) of L-NAME and L-NMMA, the change in the nitric oxide production between days was significant in both the groups. However, at a higher level (10 mM) of L-NAME and L-NMMA, the differences were not found to be significant between days and between the two groups (Table 2).

L-NAME and L-NMMA are competitive inhibitors of the enzyme nitric oxide synthase (NOS). These work by preventing the availability of the substrate for NOS i.e. L-arginine to the enzyme resulting in reduced nitric oxide output. In the present experiment, the inhibitory effects of nitric oxide inhibitors (L-NAME, L-NMMA) at the lowest levels (0.2 mM) on lymphocyte proliferation further supported the contention that nitric oxide exerts a direct effect on lymphocyte mitogenesis.

CONCLUSIONS

In conclusion, these results indicate that the lymphocytes are responsive to nitric oxide compounds and produce significant amounts of nitric oxide to modify lymphocyte mitogenesis. Although production of NO by leukocytes that are stimulated by pathogens is an important component of the innate immune system, excess production of this molecule can be damaging to the tissues of the host. Production of high concentrations of nitric oxide is a possible indicator of the immaturity of the immune system of the buffalo calf. The immune competence of buffalo calf lymphocytes is not affected by vitamin E. Further studies are required to investigate the mechanisms and the pathways that affect mitogenesis in buffalo calves.

REFERENCES


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…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)

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