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Serum as clinical specimen in PCR for diagnosis of ovine brucellosis

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Brucellosis, a zoonotic disease of worldwide importance (Benkirane 2006), is characterized by abortions, still births and reproductive problems in animals (Garin-Bastuji *et al.* 2006). Primarily, the disease is associated with domestic and wild animals and man gets infected accidentally by contact with vaginal discharges, fetal fluids (Garin-Bastuji *et al.* 2006) or by ingestion of unpasteurized milk (Pappas *et al.* 2005).

Among 8 species of *Brucella*, 3 are known to be virulent for humans (*B. melitensis, B. abortus* and *B. suis*) (Fugier *et al.* 2007) with *B. melitensis* considered to be the most pathogenic for humans (*Benkirane* 2006) causing highest (up to 90%) number of human cases worldwide (Corbel 1997).

The most incontrovertible diagnosis is isolation of *Brucella* spp. (Alton *et al.* 1975). But, it is less sensitive, time consuming and labor intensive. As a result, the serological tests like rose Bengal plate test (RBPT), complement fixation test (CFT) and enzyme linked immuno sorbent assay (ELISA) besides many others are used for routine diagnosis as well as surveillance and/or eradication programmes. These tests have high sensitivity, but low specificity due to serological cross-reactions with other Gram-nagative bacteria (mainly *Yersinia enterocolitica* O:9).

Polymerase chain reaction (PCR), a sensitive, specific and rapid test for detecting a variety of pathogens (Yamakami *et al.* 1996) including *Brucella* (Serpe *et al.* 1999) was described. The PCR is diagnostic in recurrent and relapsed cases, too (Mitka *et al.* 2007). Using serum as clinical sample for PCR is a new approach (Zerva *et al.* 2001). In the present study, an attempt was made to use PCR in diagnosis of sheep brucellosis using serum as sample. The results of PCR were compared with those obtained in RBPT.

Serum samples (36) were collected aseptically from sheep of Karnataka state and stored at –20°C till use. The samples were tested by RBPT (Alton *et al.* 1975). Any degree of agglutination appearing within 4 min was taken as positive reaction.

For PCR, DNA was isolated from serum samples using the method described by Yamakami *et al.* (1996) with minor modifications. Briefly, 100 µl serum sample was mixed with 100 µl lysis buffer containing 100 mM KCl, 20 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.2 mg of gelatin per ml and 0.9% polysorbate 20. Proteinase K was added to a final concentration of 60 mg/ml and the mixture was incubated for 60 min at 55°C. Proteinase K was, inactivated by heating the mixture to 95°C for 10 min followed by centrifugation at 12 000× g for 10 min at 4°C. The supernatant was collected in fresh microcentrifuge tube to which 0.1 volume of sodium acetate (3 M) and 0.6 volume of isopropanol were added. The contents were mixed gently and kept on ice for 1 h and centrifuged at 8000 × g for 10 min. The pellet was washed with 70% alcohol twice and dried at 37°C finally pellet was suspended in 20–40 µl of MQ water/Tris-EDTA buffer and stored at –20°C till further use.

In PCR, BCSP-31 gene (Mayfield *et al.* 1988) of *Brucella* was targeted, with oligonucleotide primer (Serpe *et al.* 1999), which amplified 443 bp product. The sequence of primers used were 5'-GGGCAAGGTGGAAGATTT-3' (forward) and 5'-CGGCAAGGGTCGGGTGTTT-3' (reverse). The cyclic conditions used in PCR were–initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 45 s and extension at 72°C for 1 min with final extension at 72°C for 5 min. Positive and negative controls were always included in each run. A 443 bp band on electrophoresis in 1% agarose was observed in positive cases.

Out of 36 samples tested, 19 were positive for brucellosis by RBPT and 17 were negative. Of the 19 RBPT positive

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Table 1. Comparison of PCR on serum and RBPT in diagnosis of ovine brucellosis

Tests	PCR positive	PCR negative	Total field samples
RBPT positive	9	10	19
RBPT negative	4	13	17
Total field samples	13	23	36

samples, only 9 were detected as positive by PCR and rest of the 10 samples were negative. On the other hand, 4 of 17 RBPT negative sera samples were positive in PCR. Thus, PCR could detect 13 serum samples as positive and 23 as negative (Table 1).

Diagnosis of brucellosis had never been easy. In the present report, an attempt was made to use PCR using serum as a sample, which was reported by Zerva *et al.* (2001) to diagnose human brucellosis. We found only 13 samples as positive by PCR as against 19 by RBPT. The results indicate that serum could be used as sample for PCR in animals, too. The wide variation in number of samples detected as positive by RBPT (19) and PCR (13) could be because of many factors. PCR detects DNA, which may be in low quantity in serum samples even though the antibody titre is quite high. Alternatively, the titre of serum may be below detectable level but the amount of DNA may be sufficient enough to be detected by PCR as it has been reported that PCR could detect 5fg of DNA (Kaushik *et al.* 2006). Also, it is reported that standardization conditions of RBPT antigen suitable for diagnosing cattle infection are not adequate in sheep (Garin-Bastuji and Blasco 2004) and account for the low sensitivity of RBPT in small ruminants (Falade 1983). This is only a preliminary report which indicates that PCR can be a test to be applied for diagnosis of brucellosis in animals, too, using serum as samples. However, there is a need to test large number of serum sample from animals at different stages of infection with *Brucella*. A representative serum sample from sheep with bacteriological isolation (definitive case of brucellosis) need to be tested to arrive at a logistic estimation of sensitivity and specificity before the test could be brought into routine use in diagnosing brucellosis in sheep. This can even be extended to other species of animals in case the results are encouraging. The work in this direction is under progress.

SUMMARY

In this study, an attempt was made to use PCR in diagnosis of sheep brucellosis using serum as sample; and the results were compared with those obtained in RBPT. Out of 36 samples tested 19 were found positive for brucellosis by RBPT. PCR detected 13 samples as positive.

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Effect of cyproheptadine drugs on vascular permeability in the buffalo calf skin

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Cyproheptadine is an antagonist of 5–hydroxytryptamine. 5–hydroxytrytamine, a vasoactive amine like histamine, can induce smooth muscle contraction and increased capillary permeability (Levy 1974, Douglas 1975). The role of cyproheptadine on vascular permeability in buffalo calve is totally unexplored. Although, the present work is of fundamental nature and may help unravel mechanisms involved in anti-inflammatory reaction.

Healthy male buffalo calves (12), 3–6 month-old were divided equally into 2 group–bacterial and chemical inflammation. Each group had 6 calves for studies on increased vascular permeability. All calves were maintained under standard hygienic conditions. The site of the cutaneous reaction was prepared according to Zarrilli and Calhoun (1970).

Induction of inflammation: Six buffalo calves in bacterial inflammation were again divided into 2 subgroups i.e. control and experimental. Each subgroup has 3 calves. Each calf in subgroup 1A (control group) received 2 i/d injection of *Staphylosanthus epidermidis* suspension (0.1 ml) in normal saline at each time interval (0–2 min, 30 min, 1 h, 3 h and 6 h) on both sides (left and right) of thoraco-abdominal region. Each calf received in all 10 injections, 2 for each time interval and making 6 lesions per time interval. Evans blue was given i/v immediately after the last time interval of 0–2 min following the procedure of Awadhiya *et al.* (1981). The calves were euthanized by i/v injection of saturated solution of magnesium sulphate, 30 min after injection of Evans blue (Awadhiya *et al*. 1981). Skin pieces at site of intradermal injections were collected for assessing the vascular permeability by visual and quantitative method. The visual assessment of the increased vascular permeability was based on the size of the blue patch and the intensity of the blue colour on the ventral portion of the skin. Quantitative assessment of Evans blue exuded in the cutaneous lesion was made spectrophotometrically (Awadhiya *et al.* 1981).

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Each calf of subgroup IB (experimental group) was pretreated with cyproheptadine i/m, @ 1.0 mg/kg body weight 30 min prior to i/d injection of *Steph. epidermidis*, and it was repeated every 12 h. The rest of the procedure was the same as in control group IA. In chemical inflammation, total number of 6 buffalo calves were divided into control subgroup 2A and experimental group 2B, for estimation of increases in vascular permeability. Each group had 3 calves. In subgroup 2A, each calf received 2 i/d injections of turpentine (0.05 ml) at each time interval of 0–2 min, 30 min, 1 h, 3 h and 6 h. The rest of the procedure was same as in the control group 1A. Each calf of subgroup 2B was pretreated with cyproheptadine i/m, @ 1.0 mg/kg body weight 30 min prior to i/d injection of turpentine and it was repeated every 12 h. The rest of the procedure was the same as in control group 1A. The data was statistically analysed (Snedecor and Chochran 1969).

Bacterial inflammation

Control group 1A: The inflammatory reaction was induced by intradermal *Staph. epidermidis* suspension @ 0.1 ml per reaction in the calves. At 0–2 min (0.51±0.026 µg of dye) time interval blue patch was not seen visually and was indistinguishable from the uninjected skin site. A bluing patches gradually increased and maximal bluing was noticed at 3 h time interval (4.10±0.164). The permeability registered a sudden decrease at 6 h, quantitatively 1.50±0.162 µg of dye had exuded in lesion. Statistically the exudation of dye was significantly lower than 3 h time interval.

Experimental group 1B: The inflammatory reaction was less marked by intradermal injection of *Steph. epidermidis* suspension in the calves which were pretreated 30 min earlier with cyproheptadine as compared to the non-pretreated control. The vascular permeability were quantified as 0.46±0.011 µg at 0–2 min and gradually increased up to 3.55±0.107 µg at 3 h then again went lower 1.45±0.055 µg at 6 h but all were lower than control group. The suppression of permeability response was of 0–2 min (18.00%) to 6 h (3.33%). The maximum suppression was observed at 30 min, which was 30.00% as compared to that of non-pretreated control.

Chemical inflammation

Control group 2A: The inflammatory reaction was induced by i/d injection of turpentine @ of 0.05 ml/reaction in calve. At 0–2 min (20.90±0.873µg) time interval blueing was maximal. Statistically, the blueing was significantly highest at 0–2 min than the other intervals. However A bluing patches gradually decreased. The permeability registered a sudden decrease at 3 h, quantitatively 8.15±0.439 µg of dye had exuded in lesion. The blueing increased again. At 6 h blueing was quatitatively, 14.03±0.599 µg.

Experimental group 2B: Turpentine-induced lesion in calves pretreated with cyproheptadine revealed that blueing was maximal at 0–2 min time interval amongst the cyproheptadine pretreated lesion but lower as compared to the control of the same time interval. The increase in permeability was characterized by an exudation of 15.33±0.269 µg of Evans blue in the lesion. The suppression of permeability was 26.65% compared to the control. The blueing was gradually decreased up to 3 h. On quantitation, 7.33±0.305 µg of dye had exuded per lesion, which was minimal in cyproheptadine group. The suppression of dye exudation was 10.12%. However, maximal suppression 31.34% was observed at 1 h as compared to the control group. The vascular permeability again increased at 6 h but was less than control group. At this stage 3.27% of suppression of permeability was noticed, which was minimal of all stages.

The present suppression of bluing by cyproheptadine in the early phase indicated that bacterial and chemical injury also mediated its permeability through the release of 5–HT. The finding is also in agreement with Pande *et al*. (2003) and Gupta *et al*. (2008) in buffalo calf. Pande *et al.* (2003) have reported the promethazine hydrochloride suppress the participation of histamine during early stages of thermal injury in buffalo calf. Gupta *et al*. (2008) also reported the pretreatment with histamine antagonist promethazine hydrochloride produced suppression of the vascular permeability during initial stage up to 3 h time interval of *Steph. epidermidis*. The present finding also coincides with the report on turpentine-induced permeability response in reserpine pretreated chicken. Jain *et al.* (1995) also reported similar finding during inflammatory reaction induced by different stimuli in the chicken using reserpine as 5–HT antagonist. The present result indicates that like histamine, 5–HT also plays an ongoing role during early stages in the mediation of permeability response. Similar conclusions were drawn by Ito *et al*. (1989) from their studies on carrageenin-induced, and by Jain *et al*. (1995, 1997) from turpentine-induced acute inflammatory responses in the chicken.

SUMMARY

Effect of cyproheptadine drugs on vascular permeability in the buffalo calf skin was studied. Bacterial inflammation (2 i/d infection of *Staphylosanthus epidermidis* on both sides of thoraco-abdominal region) and chemical inflammation (2 i/d infections of turpentine (0.05 ml)) were caused in buffalo calves. The present suppression of bluing by cyproheptadine in early phase indicated that bacterial and chemical injury also mediated its permeability through the release of 5-hydroxytryptamine.

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Relationship between nitric oxide and luteinizing hormone concentration during non-breeding season in Mandya ewes

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Nitric oxide (Billar 1995) an endothelium derived relaxation factor plays an important role in the release of gonadotrophic hormones (Bredt and Snyder 1994) but its role in the regulation of onset of estrus, LH surge in ewes is not fully known. NO is responsible for the release of LHRH and for the increased circulatory changes that occur during the development of follicle and during onset of estrus in cycling animals. In this study we focus on the probable changes in the secretory pattern of nitric oxide with changes in the reproductive hormones LH and progesterone, during the transition from non-breeding to breeding season and to study the cohesive actions of these hormones during estrus cycle in Mandya sheep.

Blood samples from Mandya breed of ewes were collected at weekly intervals from January to April between 1100 h and 1300 h to look into the pattern of progesterone secretion. From May to June 12 ewes of the same reproductive status from day 3 of the estrous cycle were divided into 2 groups as treated group consisting of 6 multiparous ewes exposed to teaser ram for 3 h in the morning (from 6 AM to 9 AM), and 2 h in the evening from (4 PM to 6 PM) for 30 days during non-breeding (May-June) season and controls (6 ewes) were exposed to teaser ram for 15 min in the morning and evening to detect the estrus. Blood samples were collected at hourly intervals from the beginning of estrus and thereafter at daily intervals from both the groups. Plasma was separated and stored at —20°C till the analysis of samples for LH surge, progesterone, estradiol and NO. Progesterone and estradiol–17ß in the plasma were analyzed using radioimmunoassay kits. Plasma samples and tracer were added to the precoated tubes and the tubes were incubated at 18°C to 24°C with shaking at 3 000 rpm for 45 min. The tubes are decanted and radioactivity calculated in gamma counter. Luteinizing hormone was analyzed by radioimmunoassay (Niswender *et al*. 1969). Intra- assay coefficient of variation was 4.99% and inter- coefficient of variation for LH was 9.45%. Nitric oxide was estimated in the serum as nitrite/nitrate using a modified Griess method (Sastry *et al*. 2002). The data were subjected to one-way analysis of variance and correlation using Microsoft statistical package.

During luteal phase of estrous cycle, both the groups, showed elevated levels of progesterone concentration. Ewes exposed to ram in the treated group came to estrus by around 18 days as against 28 days in controls. Estradiol–17 ß (Fig. 2), LH and NO (Fig. 1) were significantly higher in treated ewes with an advancement of LH surge by 8 h (Fig. 3) with an estrous cycle of 18 days after the ram introduction. For the first time, we examined the changes in circulating NO, LH and steroids in response to exposure of Mandya ewes to ram during non-breeding season. Ewes exposed to ram increased circulating NO, and that there is a positive correlation between circulating LH and NO in ewes. This result differs from several ram effect studies, showing that a positive correlation between LH surges and its duration, estradiol, progesterone and NO levels in the plasma samples

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Fig. 1. Mean±SE of nitric oxide (NO_2/NO_3) mcmol/litre, LH (ng/ml) and progesterone concentration in control and ewes exposed to ram.

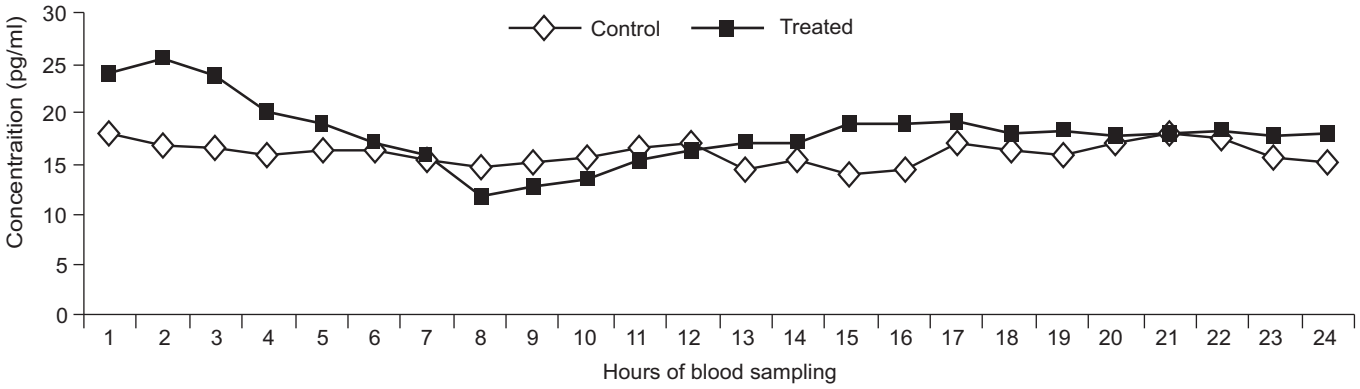

Fig. 2. Mean±SE plsma estradiol concentration (pg/ml) in control and ewes exposed to ram during non-breeding season.

Fig. 3. Mean±SE, min LH (ng/ml) concentration in control and treated ewes (n=6 each). Treated ewes showed significant increase in LH concentration during 6 h of blood collection. LH intervals of 24h ($P<0.01$) and control had >27h.

during estrus at hourly intervals in ewes. Until now, NO-mediated, reproductive activity in ewes in this type of model is not reported. One mechanism by which ram effect exerts its positive effects on the reproductive activity has been attributed to up-regulation of NO. Studies addressing to NO fluctuations during the estrous cycle have produced conflicting results depending on the method used for its measurement (Van Cleeff *et al*. 1998) in normal ewes. Furthermore, ram effect on ewes during estrus increased circulating NO, advanced the LH surges by about 6 h, advanced the estrous cycle by around 10 days compared to controls which may be due to NO crucial role in reproduction at every level in the organism. In the brain, it activates the release of luteinizing hormone-releasing hormone (LHRH). The axons of the LHRH neurons project to the mating centers in the brain stem and by efferent pathways evoke the estrus with increase in LH pulses and amplitude. It also activates the release of LH from pituitary and activates the release of gonadotropins by activating neural NO synthase (NOS) in the pituitary gland (Gelez and Fabre-Nys 2006). In controls, though ewes were exposed to ram, profile of plasma LH, estradiol–17 ß did not increase within the group and within the individual ewes**.** In contrast, treated ewes exposed to ram showed an increase in serum NO, suggesting a clear relationship between advancing the LH surge, estrus and plasma NO. This is may be due to release of some neurotransmitters at the hypothalamo- hypophyseal portal system by NO thereby increasing concentration of LH and advancing the peak LH surges within 6 hr after the onset of estrus. Estrus symptoms were more pronounced in treated animals compared to controls (Goodman *et al*. 1981) may be due to significant increase in estradiol–17 ß concentration; however progesterone levels were fluctuated between 0.5 ng/ml to 1 ng/ml in treated ewes (Fig. 1). Duration of estrus was 2 days in treated ewes as compared to controls with 12–13 h, which is in conformity with elevated estrogen synthesis, LH duration and stimulation of sensory stimulation such as olfactory, retinal and presence of a ram. However, administration, and the concomitant use of medroxy-progesterone acetate in the other studies (Cavender and Murdoch 1988) though improved the synchrony of estrus, but not as observed in this study. In sheep, exposure of seasonally anestrous females to the male results in activation of luteinizing hormone (LH) secretion and synchronized ovulation, which shows the involvement of the olfactory system's critical role in detection and integration of male odour. Our results showed that female responses depend on ram and thereby reproductive activity. The LH response to

ram could result from the above process (Gelez and Fabre-Nys 2006). The positive correlation between serum LH, E2 and NO may be due to a positive feedback effect of NO on NOS activity or expression in target sites such as endometrium, thereby increasing NO synthesis. Another explanation to our findings is that NO is ubiquitously distributed, and duration of ram effect, may influence NOS activity or expression differently in different tissues including the plasma. This hypothesis is supported by our data showing that NO altered LH surges as evidenced by its measurement in hourly samples in plasma. NO during estrous cycle fluctuates in control animals (Roselli *et al*. 1994) both during luteal and follicular phases. Based on the higher levels of NO during the follicular phase it would be expected that steroid hormones may up regulate NOS activity in the endothelium and other potential target sites resulting in greater secretion of NO into the circulation. It is concluded that, chronic exposure to rams increases circulating NO possibly via a positive feedback of NO on LH concentration. During the estrous cycle, NO levels were fluctuated during the luteal phase. Furthermore, ram effect influences the secretion of NO in a target-specific manner with effects in the ovarian compartment. Our data suggest that NO is a local physiological mediator of LH surges and its actions and efforts to study the role of this molecule should be focused at assessing NO dynamics in local microenvironments or the microvascular system. The patterns of NO, LH, and estradiol–17ß concentrations may be used as indicators of activity of the reproductive axis and to characterize estrus. The ram effect is effective in inducing estrous synchronization in ewes during the non -breeding season. We found an association between the circulating levels of NO immediately preceding the LH surge and the timing of the first estrus as animals transitioned from the non-breeding to the breeding season, i.e., higher NO levels in the ewes exposed to the ram to advance the timing of their first estrus. The use of the ram effect, even without hormonal priming, seems to be an effective technique for out-of-season estrous induction in Mandya ewes, which have high commercial value in the national and international market because of its quality meat with high nutritional value that can be incorporated in Indian livestock production systems.

SUMMARY

This study examined the reproductive activity, synchrony of estrus, its relationship between nitric oxide (NO), LH surges during non-breeding season. Multiparous female Mandya ewes (12) were divided into control group (6 ewes) and treated group (6 ewes). Ewes in treated group were exposed to ram for 3 h in the morning (from 6 AM to 9 AM) and 2 h in the evening (4 PM to 6 PM) for 30 days during non-breeding (May-June) season. Controls were exposed to ram for 15 min in the morning and evening to detect the estrus. Ram introduction advanced the onset of seasonal ovarian activity in the treated ewes compared to controls. Mean occurrence of estrus in treated ewes is 100%; compared to controls with higher incidences of estrus during morning hours than the evening hours after the ram introduction. Profiles of NO, LH, P4 and E2 varied between the animals in both the groups. LH surge occurred 8 h after the onset of estrus with high NO and E2ß in treated ewes as against the controls with LH surge of 17 h after the onset of estrus. It is concluded that, NO plays a positive role on LH surges thereby shortening the estrous cycle, improving the occurrence of estrus for breeding of Mandya ewes in non-breeding season.

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