Haryana Vet. (April, 2021) **60(SI)**, 26-29

Research Article

AN OUTBREAK OF THEILERIOSIS IN A HERD OF CROSSBRED CATTLE AND MURRAH BUFFALOES AND ITS MANAGMENT

GAURAV CHARAYA*, NARESH JINDAL[1], VANDNA BHANOT[2], AMAN KUMAR[3]and PARVEEN GOEL[4]
Department of Veterinary Medicine,[1]Department of Veterinary Public Health and Epidemiology,
[2]D.I. Laboratory, Ambala,[3] Department of Animal Biotechnology;
[4]Director of Research, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125004, India

Received: 14.05.2020; Accepted: 17.07.2020

ABSTRACT

In the present study, the possible cause of ongoing mortality was investigated in small herd of 25 milch animals (11 cross bred cows and 14 Murrah buffaloes) in Ambala district of Haryana. Anamnesis revealed history of high grade fever, pale mucous membrane, inappetence, tremors, convulsion and corneal opacity in some diseased animals. On the basis of history and clinical signs, the animals were suspected to be suffering from haemoprotozoan disease. Hence, the blood samples were collected from seven animals (4 cows and 3 buffaloes) suspected to be diseased and were subjected to blood smear examination, molecular detection of haemoprotozoans (*Theileria annulata* and *Trypanosoma evansi*) and latex agglutination test for trypanosomosis. Microscopic examination revealed a characteristic ring shaped piroplasm in two blood smears (one each of cow and buffalo) suggestive of theileriosis whereas no smear was positive for *Trypanosoma evansi*. Molecular detection of *T. annulata* using PCR assay based on tams gene and cytochrome b gene in seven samples revealed a specific band of 721 bp and 312 bp in six (2 buffaloes and 4 cow) and two samples (1 cow and 1 buffalo), respectively. All the samples were found negative for trypanosomosis with latex agglutination test and PCR assay. Further the two PCR products obtained by targeting cytochrome b gene were purified, sequenced and phylogenetically analyzed to confirm *T. annulata*. All the animals were administered buparvaquone @ 2.5 mg/kg body wt. intramuscularly once along with oxytetracycline @ 10 mg/kg body weight, oral haematinics and vitamin C for 3-5 days. Finally all the clinically affected animals recovered and further no mortality was observed in the herd.

Keywords: Buffalo, PCR assay, Sequencing, Theileriosis

Tropical theileriosis is a tick borne haemoprotozoan disease caused by *Theileria annulata* causing huge economic loss estimated to the tune Rs. 8426.7 crore/ annum in India (Narladkar, 2018). Most of the cases of theileriosis have been reported in cattle; however, there are a few reports on clinical prevalence of theileriosis in buffaloes in India (Patil and Satibge, 2019). Most common clinical signs in early stages of theileriosis are weakness, weight loss, anorexia, high body temperature, petechiae on the conjunctival mucosa, swollen lymph nodes, anemia, and dyspnea (Constable *et al.*, 2017). Diagnosis is based on identification of parasite in Giemsa stained blood smear by microscopic examination. However, apart from difficulty in species differentiation, blood smear technique is not suitable for detection of infection with low parasitaemia (Nayel *et al.*, 2012; Charaya *et al.*, 2016). DNA based parasite identification assays, in general, are considered more specific, sensitive and objective from clinical point of view. The present study highlights an episode of clinical theileriosis in a dairy herd having crossbred cows and Murrah buffaloes, its identification by conventional and molecular diagnostics and its therapeutic management.

MATERIALS AND METHODS

Collection of samples: A dairy owner reported 20% mortality in a herd that had 25 milch animals (11 cows and 14 Murrah buffaloes) in Ambala district of Haryana. Anamnesis revealed history of recently purchased animals, high grade fever in 30% animals, in appetence in 35% animals, tremors in 10% animals, convulsions in 10% animals and corneal opacity in 25% animals. Corneal opacity was evident in both cattle and buffaloes whereas nervous signs were seen only in cattle. Detailed investigation was carried out and all the sick animals at the time of visit (n=7) were examined and rest (n=13) animals were apparently healthy. Physical and clinical examinations were undertaken in each of the affected animal including colour of mucous membrane, swelling of lymph node and rectal temperature. Blood samples from all seven sick animals were collected in EDTA vials for conventional parasitological diagnosis, haematological studies and molecular diagnosis. Thin blood smear was prepared on the spot and was fixed with methanol.

Microscopic examination: Fixed blood smears stained with Giemsa stain (Benjamin, 1978) were examined for intra-erythrocytic forms (signet ring, dot, or comma shaped) of *T. annulata* piroplasms for theileriosis and flagellate form of *Trypanosma evansi* for tryapnosomosis under100X objective magnification. About 20 microscopic fields per slide were observed to view the parasite. Smears were also screened for other haemoprotozoan parasites such as *Babesia* spp. and *Anaplasma* spp.

*Corresponding author: gcharaya9@gmail.com

Haematological examination: The blood samples collected in vials coated with K3EDTA were analyzed for complete haematological examination manually as per standard procedure (Jain, 1986). The parameters were: haemoglobin (Hb) in g/dl, total erythrocyte count (TEC) in 10^6/µl, total leucocyte count (TLC) in 10^3/µl, differential leukocyte count [lymphocytes (L%), monocytes (M%), neutrophils (N%), eosinophils (E%) and basophils (B%)]. Mean and standard error was calculated using SPSS 16.0 software and comparison of values was done with standard values available.

Serum latex agglutination test for trypanosomosis: Serum was separated and subjected to latex agglutination test for the diagnosis of trypanosomosisas suggested by Shyma *et al.* (2012).

Molecular detection of haemoprotozoan using simplex PCR assay: DNA was extracted from blood from all seven sick animals using DNA extraction kit as per the manufacturer instructions. For molecular detection of *T. annula* taprimer set {Forward: 5'- ATGAGGATGAAA AGAAAAAGGAGGAAAAAAAAGATGT-3'/ Reverse 5' GCGAAGACTGCA AGG GGG GAG AAC T-3'} (d'Oliveira *et al.*, 1995) targeting tams gene and primer set {Forward: 5'-ACT TTG GCC GTA ATG TTA AAC-3'/Reverse: 5'-CTC TGG ACC AAC TGT TTG G-3'}(Bilgic *et al.*, 2010) targeting cytochrome b gene was used to amplify a 721 bp and 312 bp region, respectively. For the detection of *T. evansi*, primers TR3 and TR4 (Forward: 5'- CGCGGATTCTTTGCAGACGA-3'/Reverse: 5'-TGCAGACACTGGAATGTTACT-3') were used to amplify a 257 bp region targeting repetitive nucleotide sequences (Wuyts *et al.*, 1994). Reaction mixture and PCR amplification conditions were same for both the parasites. PCR assay was carried out in 200 µl PCR tubes using ABI thermocycler. Each 12.5 µl reaction mixture comprised of 2 µl template DNA, 6.25 µl Dream Taq Green PCR Master Mix (×2; Fermentas), 0.5 µl each primer set (forward and reverse primer) and 3.25 µl nuclease free water. The PCR conditions include dinitial denaturation at 95° C for 2 min; followed by 30 cycles of 95° C for 30s (denaturation), 60° C for 30s (annealing), and 72° C for 1 min (extension); with a final extension step of 72° C for 10 min. Amplified PCR product was gel electrophoresed in 1.5% agarose gel. The images were captured and documented using gel documentation system (Bio Rad., USA).

Sequencing of PCR product: PCR products of two positive samples (one from cow and one from buffalo) obtained by targeting cyt b gene were gel purified using commercially available kit and subjected to sequencing using Sanger's method on an ABI 3130XL Genetic Analyser.

Treatment and control strategies: Anamnesis revealed administration of preliminary treatment at local level before the start of investigation. Before the start of investigation, all the affected animals were given with atropine sulphate and quinapyramine sulphate for the suspicious to be suffering either from poisoning or trypanosomosis; however, there was no response of the treatment. All the affected animals were administered oxytetracycline @ 10mg/kg body weight intravenously with normal saline solution twice daily alongwith ascorbic acid and antihistamines on clinical observation and tentatively diagnosis of haemoprotozoan involvement. On confirmation, all the animals were given buparvaquone @ 2.5mk/kg body weight deep intramuscularly once. The above treatment was continued for 5 days.

RESULTS AND DISCUSSION

Of the blood smears screened from seven animals, two (one cow and buffalo each) were found positive for theileriosis by microscopic examination showing characteristics piroplasms (Fig. 1). All seven serum samples were found negative for trypanosomosis based on *Trypanosoma evansi* latex agglutination test (TE-LAT) and PCR assay. Molecular detection using PCR assay based on tams gene and cytochrome b gene revealed a specific band of 721 bp and 312 bp suggesting the presence of *T. annulata* in six and two samples, respectively (Fig. 2 & 3). BLAST analysis of the aligned sequences further confirmed the presence of *T. annulata*. Partial sequence (cyt b) analysis revealed 96.33% identity with Rewa isolate and 100% identity with Shriwal, Pune *T. annulata* isolate sequences of Indian origin available in the GenBank databases. Phylogenetic analysis revealed that *T. annulata* detected in buffalo in this study was closely related to *T. annulata* isolate of Shriwal, Pune which was of crossbred cattle origin (Fig. 4). The haematological parameters were expressed as mean ± SE. Hb (8.21 ± 0.52 g/dl) and TEC ($5.00 \pm 0.59 \times 10^6$/µl) values were on the lower side of the normal range while lymphocyte revealed to be on higher side of normal range (65.0 ± 3.63%). TLC ($9.40 \pm 0.56 \times 10^3$/µl) values were found to be in normal range.

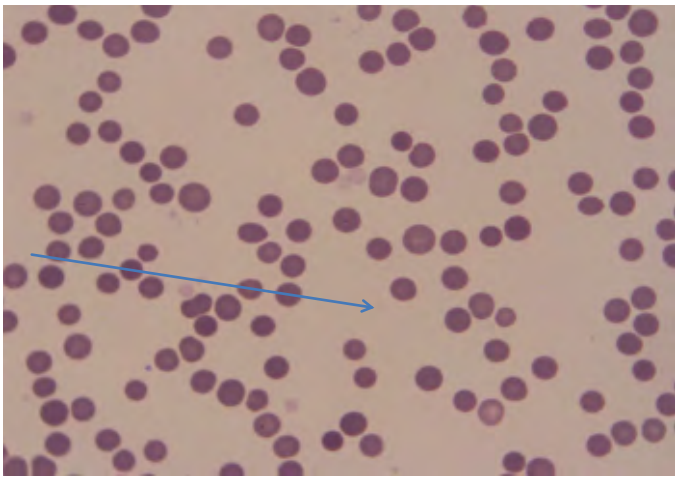

Fig.1. Giemsa stained blood smear of infected buffalo; Arrow showing characteristics ring shaped piroplasm of *Theileria anulata*

Therapeutic management was done using oxytetracycline before the confirmation of etiology which showed slight improvement in terms of clinical signs in all the sick animals. However, following confirmation of theileriosis, administration of buparvaquone led to marked recovery in all the sick animals. No further mortality was observed in the herd after the treatment.

In India, there are fewer reports on theileriosis in buffaloes. Recently, Patil and Satibge (2019) conducted a study on 79 buffaloes with signs of pyrexia, lymphadenopathy, pale mucous membrane, tick infestation, anorexia and detected 22.78% samples to be positive for theileriosis by PCR assay. In accordance with the present study, Patil and Satbige (2019) showed buparvaquone to be 100% effective in treatment of sick animals. In the present study, buparvaquone was given along with oxytetracycline to control the mortality. Salama and Al-Gabary (2007) and Hasanpour *et al.* (2008) reported the presence of *T. annulata* in water buffalo in Egypt and Iran with infected buffaloes having pyrexia, enlargement of superficial lymph nodes, slight nasal and ocular discharges, salivation, anaemia and respiratory distress. However, in the present study, the affected animals also had convulsions which could be attributed to intravascular and extra vascular aggregations of schizont-infected lymphocytes in brain causing thrombosis and ischemic necrosis. Normally, the disease is recorded in cattle with specific presentation, however, convulsions seen in the present study warrants buffaloes in endemic areas must be tested for the presence of haemoprotozoa infection. Only two blood smears were positive for piroplasms. Under field conditions, it is easier to prepare blood smears and is also less expensive. Inherent problem with blood smear examination lies with the fact that this technique is associated with false negative results and has low sensitivity in detecting the carrier status. The PCR, being more sensitive and specific as compared to blood smear examination, detected higher number of samples as positive for *Theileria annulata*.

In the present study it was noticed that animals were purchased and kept in herd without quarantine and testing of them for any disease. Furthermore, cow and buffaloes were kept together in herd which might increase the chances of transmission of parasite from sick cow to buffalo leading to disease. It seems that probably the parasite has adapted to new host, which otherwise never had the problem of theileriosis. To conclude, the study reveals theileriosis in buffaloes in clinical form that can be well treated with standard therapeutic regimen.

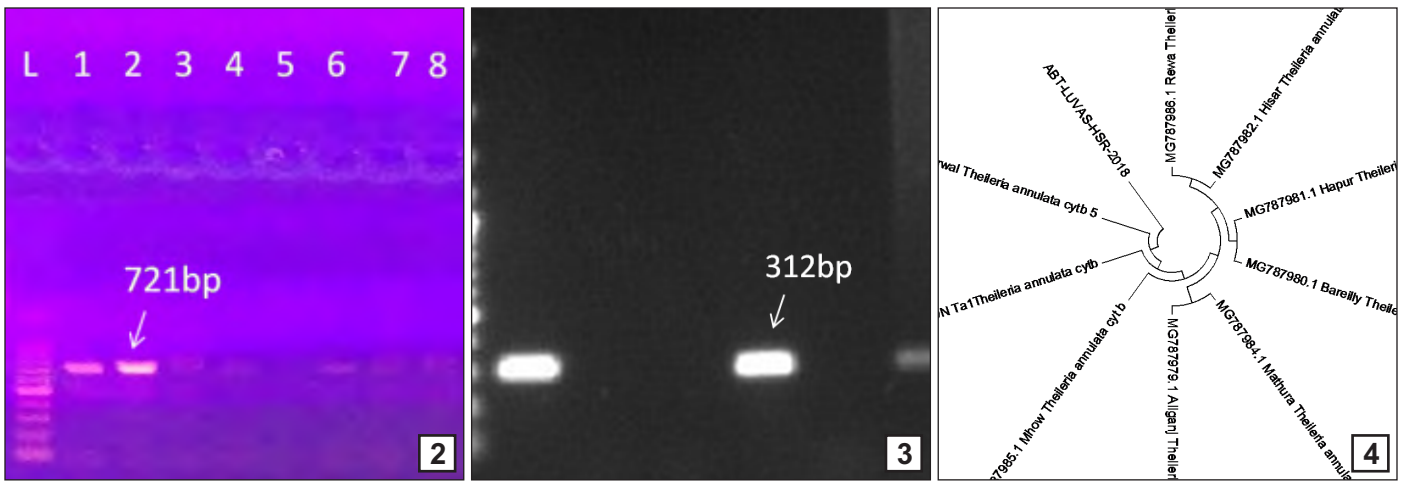

Fig. 2-4. (2) PCR assay for detection of *Theileria annulata* targeting tams gene: L-Ladder;1- Control positive; 2-strong positive samples; 3, 4, 6, 7, 8 - faint positive samples; 5-Negative samples; **(3)** PCR assay for detection of *Theileria annulata* targeting cyt b gene: L-Ladder; 1 - Control positive; 2 - Control negative; 3, 4, 6, 8 - Negative samples; 5, 7 - Positive samples; **(4)** Phylogenetic tree generated using the neighbor-joining algorithm based on the sequence alignment of the partial cytochrome b gene of *T. annulata* of Indian origin along with sequence generated (ABT-LUVAS-2018) in this study.

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