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Thirtha Kumar AR
Department of Veterinary Medicine, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Kshama MA
Department of Veterinary Medicine, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Ramesh PT
Department Of Veterinary Medicine, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Narayana Bhat M
Department of Veterinary Medicine, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Narayanaswamy HD
Department of Veterinary Medicine, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Rathnamma D
Department of Veterinary Medicine, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Mamatha GS
Department of Veterinary Medicine, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Corresponding Author:
Thirtha Kumar AR
Department of Veterinary Medicine, Veterinary College, KVAFSU, Bengaluru, Karnataka, India

Studies on the occurrence of *Theileria orientalis* in cattle in and around Bengaluru by PCR

Thirtha Kumar AR, Kshama MA, Ramesh PT, Narayana Bhat M, Narayanaswamy HD, Rathnamma D and Mamatha GS

Abstract
The present study was undertaken with the objectives of studying the occurrence of *Theileria orientalis* in cattle in and around Bengaluru by using Giemsa stained blood smear examination and Polymerase Chain Reaction (PCR) in cattle. Fifty clinical cases were screened based on history and clinical signs and blood was collected for a definitive diagnosis and evaluation of various hematological parameters. Diagnosis was made based on blood smear examination by using Giemsa stain and PCR. Of the fifty clinical cases, eighteen (36%) cases were positive for theileriosis through blood smear examination. On PCR, of the 50 samples, 34 (68%) were positive for theileria. Of the 34 positive samples, 26 (76.5%) were positive for *T. orientalis* by species specific primers.

Keywords: giemsa staining, PCR, species specific primers

Introduction
Blood parasitic infections are one of the major constraints for profitable dairy industry in tropical and subtropical countries including India. Among the various economically important bovine diseases, vector-borne haemoprotozoan infections such as theileriosis, babesiosis, anaplasmosis and trypanosomosis are recognized as a cause of severe clinical illness in cattle (Singh *et al.*, 2012) [9].

In tropical countries like India, ticks cause major havoc to the livestock as they affect the health of the animals and also act as vectors for transmission of blood parasitic infections (Aktas *et al.*, 2005) [1]. Theileriosis commonly called tropical thileriosis is caused by *Theileria annulata* (*T. annulata*), which is wide spread in North Africa, Southern Europe, India, the Middle East and Asia (Purnel, 1978) [6]. *T. orientalis* is a relatively benign species of theileria genus but it can cause symptoms that include fever, anemia and anorexia in infected cattle (Hitoshi *et al*., 2014) [2]. The various pathogens that cause theileriosis are often found together within a single host and clinical signs such as fever, anorexia, lymhodenopathy, weight loss, reduced milk production, anemia and icterus are commonly associated signs which are exhibited by the infected animals (Huseyn *et al*., 2007) [3].

Diagnosis of theileriosis is primarily based on clinical examination followed by laboratory diagnosis like peripheral blood smear examination for the presence of intra erythrocytic bodies by using Giemsa staining technique under oil immersion (100x) which is still considered the gold standard (Huseyn *et al*., 2007) [3]. Nowdays PCR is a highly sensitive and efficient molecular diagnostic technique which makes it an attractive tool for diagnosis of theileria infections and diagnosis upto the species level can be made which is not possible by blood smear examination. It can be used for accurate diagnosis blood parasite infections and for detection of carrier animals, which serve as potential sources of infection to the healthier groups through the infected vectors. (Aktas *et al*., 2005) [1].

Materials and Methods
The present study was undertaken to assess the occurrence of theileria in and around Bengaluru. Samples were collected from animals with clinical signs suggestive of blood parasite diseases, presented to Veterinary College Hospital, Bengaluru and also those cases reported from Veterinary Dispensaries in and around Bengaluru. Blood samples were collected from peripheral veins for blood smear examination and PCR and jugular vein for hematological examination from animals with clinical signs such as anorexia/ inappetence, high temperature, lymphadenopathy, dullness, pale mucous membranes, icterus, emaciation,

diarrhea, hemoglobunuria (Radostits, *et al*., 2007) [7] and a history of tick infestation and were subjected to further analysis.

Blood smear examination

Thin blood smears were prepared on the same day of collection of blood by using clean, grease free slides. The blood smear was fixed in methanol for 3 min and air dried. It was then placed on the staining rack and Giemsa's stain diluted to 1:5 with Phosphate Buffer Saline (pH 6.8- 7.2) was added and allowed to act for 30 min. Stained blood smears were then rinsed in distilled water and was drained in a drain rack and dried at room temperature. Prepared smears were examined under 100x oil immersion objective of a compound microscope for detection blood parasites.

Diagnosis by molecular method – PCR

DNA extraction was done using the QIAamp DNA minikit by Qiagen, Germany as per the protocol suggested by the manufacturer and the PCR was carried out as per standard protocol. The oligonucleotide primers synthesized and supplied in lyophilized form by Europhins Pvt. Ltd., Bengaluru were reconstituted to 100µmol/µl stocks in sterile Nuclease Free Water (NFW). These primers were then used at a working dilution of 20 pmol/ µl in sterile NFW. Each 25 µL of the PCR mixture comprised of about 5µl of DNA, 12.5µl of 2X Amplicon master mix, 1.5µl each of forward and reverse primers (20 pm concentration) and 4.5 µl NFW. The reaction conditions for theileria genus, *T. orientalis* and *T. annulata* was initial denaturation at 94 ºC for 1sec followed by denaturation at 94 ºC for 30 sec, annealing at 60 ºC for 30 sec, extension at 72 ºC for 30 sec, final extension at 72 ºC for 7 sec with 30 cycles.

Table 1: Genus and species specific primers used to amplify the small subunit ribosomal RNA gene sequences for theileria genus, *T. orientalis* and *T. annulate*

Primer	Primer Sequence	Expected amplification size	Source
Theileria 989 (F) 990 (R)	AGTTTCTGACCTATCAG TTGCCTTAAACTTTCTTG	1098 bp	(Oliviera *et al*., 1999)
T. orientalis IL246 (F) IL247 (R)	CACGCTATGTTGTTCAAGAG TGTGAGACTCAATGCGCCTA	876bp	(Tanaka *et al*.,1993)
T. annulata N516(F) N517(F)	GTAACCTTTAAAAACGT GTTACGAACATGGGTTT	721 bp	(Oliviera *et al*., 1999)

Results and Discussion

A total of 50 blood samples collected from the cattle suspected for blood parasitic infection were subjected to blood smear examination. Based on the morphology eighteen (36%) of the cases were identified as theileria organisms (Table 1). Huseyn *et al*. (2007) [3] reported that laboratory diagnosis in cattle was largely based on microscopic examination of peripheral blood smears for the presence of intra erythrocyrtic bodies in blood parasites. Nair *et al*. (2011) [4] was of the opinion that although microscopy was a widely accepted and a cost effective technique for the diagnosis of blood parasites, this technique lacked sensitivity, especially when the cattle was in carrier state, as the inclusion bodies cannot be detected in blood films. All the fifty samples were subjected to conventional PCR with genus specific and species specific primers after DNA extraction. Of the 50 samples, 34 (68%) yielded 1098 bp specific for theileria genus. These samples were further subjected to species specific primers for *T. orientalis* and *T.annulata* .Of the 34 positive samples 26 (76.5%) yielded 876 bp specific for *T. orientalis* and the overall per cent positive *T.orientalis* cases was 52% (26/50) (Table 2; Fig. 1&2). Findings similar to that of the present study was reported by Ramesh *et al*. (2003) [8] and it was reported that *T. orientalis* has worldwide distribution in bovines and is transmitted by haemophysalis ticks. Although it is known to cause a relatively benign disease, during high parasitemia it causes severe anaemia. PCR was found to be a highly sensitive and accurate technique for the diagnosis of blood parasite infections which helps to detect the subclinical infections and the animals in carrier state.

Thus the present study reports the occurrence of theileria organism and *T. orientalis* was found to be predominant species of theileria in and around Bengaluru and occurrence of *T. annulata* by PCR could not be detected.

Table 2: Results of blood smear examination and PCR

Sl. No.	Blood parasite	Blood smear Positive		PCR Positive	
		No' s	Per cent	No's	Per cent
1	Theileria	18	36	34	68
2	*T. orientalis*	-	-	26	76.5
3	*T. annulata*	-	-	-	-

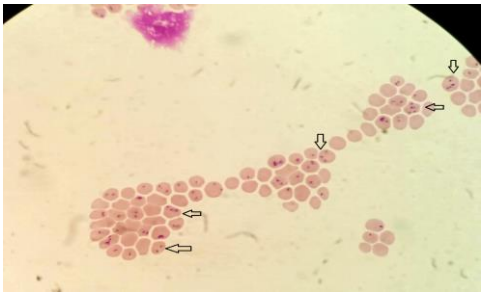

Plate 1: Blood smear exhibiting theileria organisms (Giemsa stain- 100X)

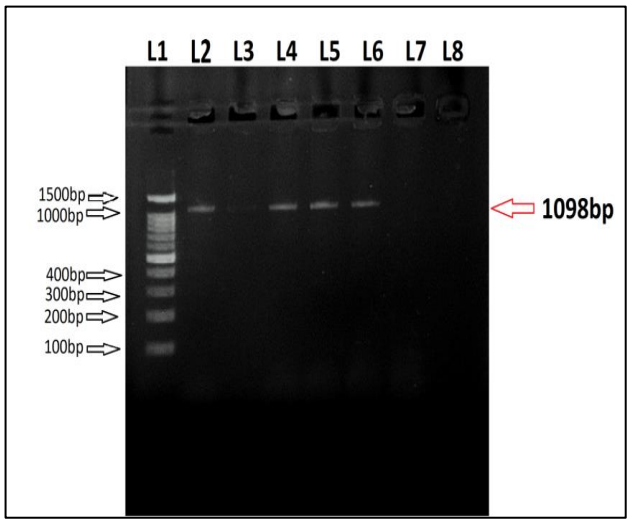

Plate 2: Analysis of amplified product (1098 bp) from blood samples with 989/990 primers set for theileria genus L1 - 1.5kb molecular DNA ladder, L2 - Positive control , L3 -Negative control, L4, L5, L6-- Positive for theileria genus

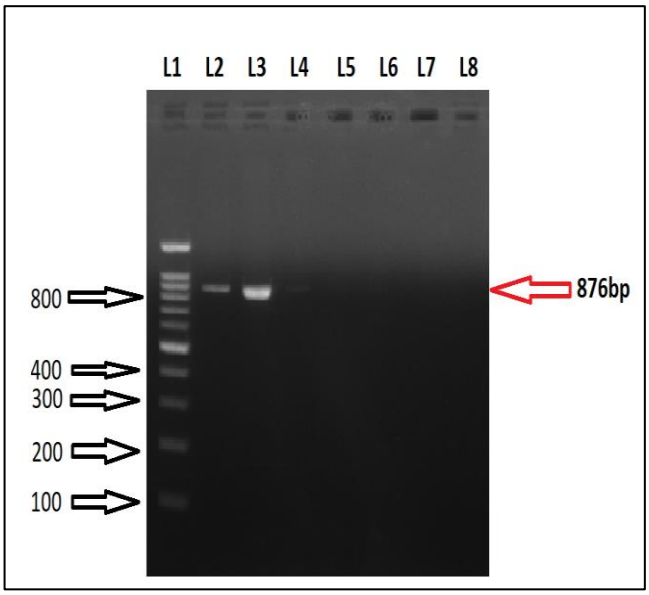

Plate 3: Analysis of amplified product (876 bp) from blood samples with IL246/ IL247 primer set for *Theileria orientalis* L1 - 1.5kb molecular DNA ladder, L2 - Positive control L3 and L4, - Positive for *Theileria orientalis,*

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