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Prevalence estimation and first molecular characterization of caprine *Theileria* species in West Bengal, India

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Abstract
Caprine theileriosis is a lymphoproliferative disease with serious impacts in terms of high morbidity, mortality and production loss. The present study was undertaken to determine the prevalence of *Theileria* sp. in goats of West Bengal from October 2019 to March 2020 by microscopic examination and polymerase chain reaction as well as for characterization of the species. A total of 135 whole blood samples along with thin blood smears were collected from randomly selected goats from five different areas of West Bengal. The Giemsa stained blood smears were screened under microscope for presence of *Theileria* sp. and thereafter genomic DNA was extracted from each of the whole blood samples. PCR was carried out to amplify the 1098 bp fragment of ssu-rRNA gene using published primer set. The diagnosis was based on demonstration of *Theileria* piroplasm on blood smear and positive PCR results. The estimated prevalence results were categorized according to the age, sex and area of the study. Out of 135, 11 samples (8.15%) were tested positive for *Theileria* sp. on microscopy compared to 17 (12.6%) on PCR. On the other hand; the sex wise prevalence was greater in females than males and age-wise prevalence was highest in <1 year old animals, followed by 1-2 years and >2 years old animals in both methods. The purified PCR product of one isolate was sequenced and analysed in DNASTAR software. The sequence was 98.21% homologous with two other published sequences of *Theileria luwenshuni* in NCBI BLAST. This is the first report of *Theileria luwenshuni* in goats of West Bengal.

Keywords: Goats, microscopy, PCR**,** *Theileria luwenshuni*, West Bengal

Introduction
Nicknamed as 'The Poor Man's Cow', goats are a very important livestock species, playing a pivotal role in employment generation as a subsidiary occupation amongst women and teenagers in rural Bengal (Nandi *et al*. 2011) [15]. In spite of being popular in West Bengal, goats of this region frequently suffer from variety of diseases due to the lack of modern husbandry practices and research attention. Various tick borne haemoparasitic diseases pose a serious threat to the goat husbandry of this region and caprine theileriosis is one amongst them. In majority of the cases, the etiological agents for small ruminants are *Theileria lestoquardi*, *T. ovis*, *T. separata* and the newly described *Theileria* sp. of China (Altay *et al*. 2007) [3]. Among these, *T. lestoquardi* is highly pathogenic and responsible for malignant ovine theileriosis, causing high mortality in mediterranean basin, west Asia and Indian subcontinent (Altay *et al*. 2007) [3]. The other species, i.e. *Theileria ovis* and *T. separata* are responsible for subclinical infections (Alani and Herbert, 1988) [1]. Recently, newer species like *T. luwenshuni* and *T. uilenbergi* are being identified as pathogenic for goat population causing clinical theileriosis (Yin *et al*. 2008; Phipps *et al*. 2016) [20, 18].
The clinical signs associated with *T. luwenshuni* are-fever, anorexia, weight loss, lymphadenopathy, respiratory signs, abortion in pregnant does, anaemia and icterus (Dhaygude *et al.* 2020) [6]. Incidence of *T. luwenshuni* has already been reported from Great Britain (Phipps *et al*. 2016) [18], Karnataka state (Mamatha *et al*. 2017) [12], Kerala state (Nagaraj *et al*. 2019) [14], Assam state (Begam *et al*. 2019) [5] and Maharashtra state (Dhaygude *et al.* 2020) [6] of India; with mortalities ranging from 8-60% (Mamatha *et al*. 2017; Dhaygude *et al.* 2020) [12, 6]. Goats can also carry subclinical infections, acting as potential vectors and active sources of infection to healthy animals (Nagaraj *et al*. 2019; Begam *et al*. 2019) [5, 14].
The diagnosis of small ruminant theileriosis is conventionally dependent on blood smear examination and clinical signs (Kirvar *et al*. 1998) [66].

The detection of piroplasms on light microscopy demands considerable expertise, and is neither sensitive to diagnose the carrier status of infection, nor suitable for determine the species causing the infection (Nagaraj *et al*. 2019) [14]. So, it is the PCR techniques that allow the accurate detection of theileriosis in low parasitaemia (A.Z. Durrani *et al*. 2011) [7].
In West Bengal, prevalence studies on caprine theileriosis reported so far are based on microscopic examination only (Patra *et al*. 2018; Halder 2019) [17, 9]. In this context, the present study was undertaken to establish, optimize and implement molecular diagnostics as a superior method to detect *Theileria*, obtain its prevalence and identify the species causing caprine theileriosis in West Bengal.

Materials and Methods

Study Area: Five different areas of West Bengal, namely- Burdwan, Payradanga, Hili, Bethuadahari and Jhargram were selected for the study (Fig. 1). All these areas contain high goat population (Fig. 2), playing an important role in local economy.

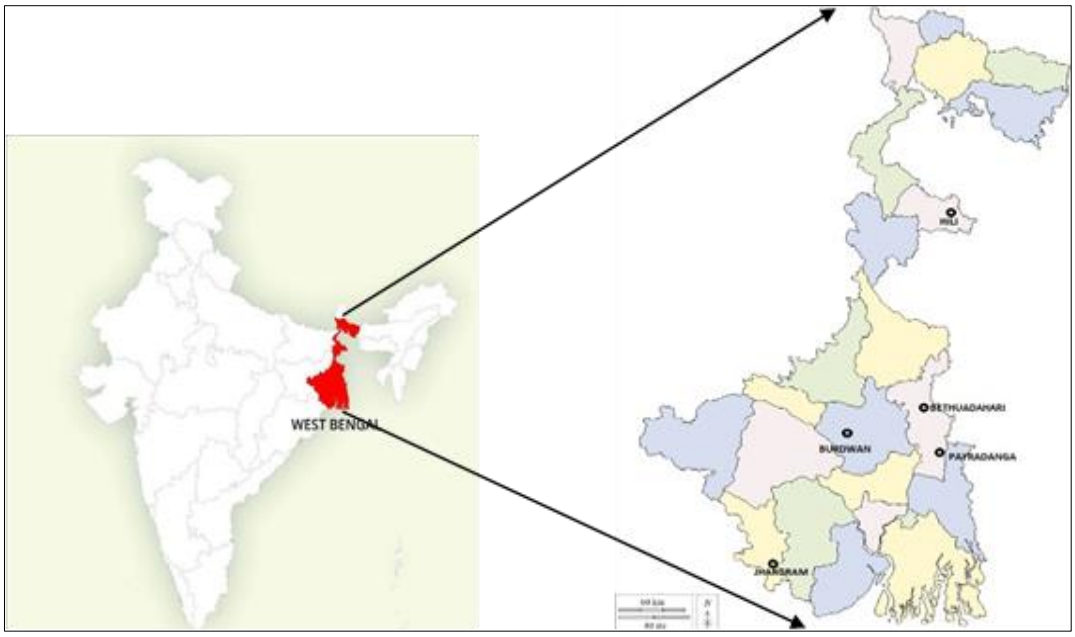

Fig 1: Showing Area of the Study

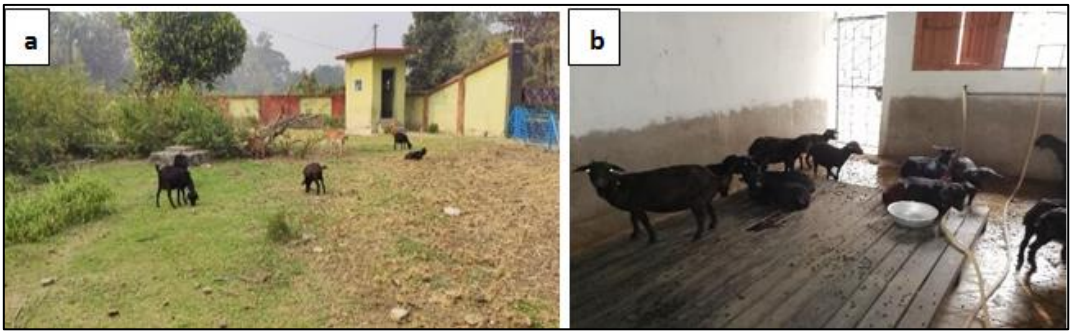

Fig 2: Different goat rearing systems in West Bengal. a Semi intensive Goat Rearing. b Intensive Goat Rearing

Animals of all ages and sexes were incorporated in this study. Sex wise, the animals were categorized into two groups- females and males and age wise, they were categorized into three groups- <1 year old, 1-2 years old and >3 years old.

Collection of Field Samples: In this study, a total 135 animals were randomly selected for sampling. 2 ml of whole blood samples were collected in EDTA vials from jugular veins and peripheral blood smears were prepared from ear vein from each animal. All blood slides and EDTA vials were appropriately labelled.

Microscopic Examination: Blood smears were fixed for 3 minutes in absolute methanol and stained with Giemsa's stain (Himedia Laboratories) as per protocol. Stained slides were screened under oil immersion lens of light microscope (Fig. 3).

Polymerase Chain Reaction

DNA Extraction: Total DNA was extracted from each of the collected whole blood samples using QIAamp Blood DNA Mini Kit (Qiagen®) according to the prescribed method and eluted in 25 µL elution buffer.

PCR Amplification: The reaction mixture was prepared in a total volume of 25 µL using genus specific published oligonucleotide primer sets (forward 5' AGTTTCTGACCTATCAG 3'; reverse 5' TTGCCTTAAACTTCCTTG 3'; Allsopp *et al*. 1993) [2] for amplifying 1098 bp fragment of ssu-rRNA gene of *Theileria* sp. with the following reagents- 2.5 µL 10X PCR buffer, 0.5 µL dNTP mix (10 mM), 0.5 µL of each primer (10 pmol/µL), 3 µL $MgCl_2$ (25 mM), 0.4 µL Taq Polymerase (5 U/µL), 5 µL genomic DNA and 12.6 µL Nuclease free water. Thermocycler programme was as follows: 94 °C for 5 min;

then 35 cycles consisting of 94 °C for 30s, 53 °C for 30s and 72 °C for 1 min, and final extension at 72 °C for 10 min. In each run, positive and negative controls were included. The positive control was taken from Dept. of Veterinary Epidemiology and Preventive Medicine, WBUAFS.

a. **Gel Electrophoresis:** Analysis of amplified product was done by 1% agarose gel electrophoresis using 100 bp DNA ladder (Fig. 4).
b. **Purification of PCR Products & Sequencing:** PCR products were purified using Nucleospin Gel and PCR Clean-up kit (MACHERY-NAGEL®) following manufacturer's protocol. Purified product of one isolate was sequenced from AgriGenome Labs, Kerala, India.
c. **Sequence Analysis:** The newly generated partial sequence of ssu-rRNA gene of *Theileria* sp., was analysed by DNASTAR software and compared with the published sequences of the nucleotide database of GenBank by BLAST programme of NCBI.
d. **Statistical Analysis:** The percent prevalence of *Theileria* sp. via both microscopic examination and PCR was estimated by the following formula (Thrusfield, 2007):
% Prevalence = (no. of animals tested positive ÷ total no. of animals) × 100.

The data regarding sex, area and age wise prevalence were subjected to analysis using chi-square test.

Results

The present study revealed that 11 animals (8.15%) out of 135 were positive for *Theileria* sp. (Table 1) on microscopy. Infection rate among the females (9.37%) was higher compared to males (7.14%, Table 2). In age wise study, highest prevalence was observed young animals ageing <1 year, followed by 1-2 years and >2 years old animals (Table 3). Area wise, the prevalence was significantly (P<0.05) higher in Hili, followed Bethuadahari, Jhargram, Burdwan and Payradanga (Table 4).

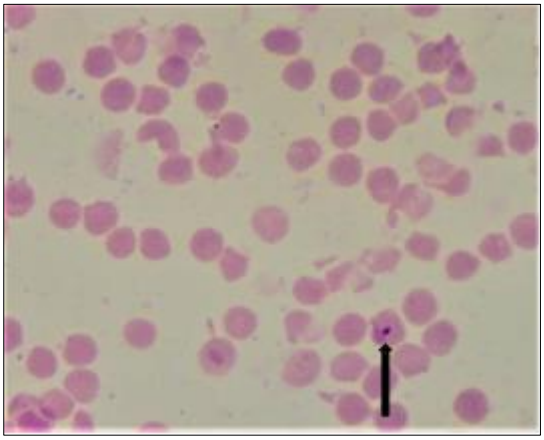

Fig 3: Intraerythrocytic Theileria Piroplasm

By PCR, 17 animals out of the 135 (12.6%) were tested positive for *Theileria* sp. (Table 1). Age, sex and area wise prevalence results were similar in both diagnostic methods. Females carried higher infection (14%) than males (11.27%, Table 1) and <1 year old animals showed higher prevalence rates than 1-2 years old and >2 years old animals (Table 2) respectively. Area wise, significantly (p<0.05) higher prevalence was observed in Hili, followed by Bethuadahari, Jhargram, Burdwan and Payradanga (Table 4).

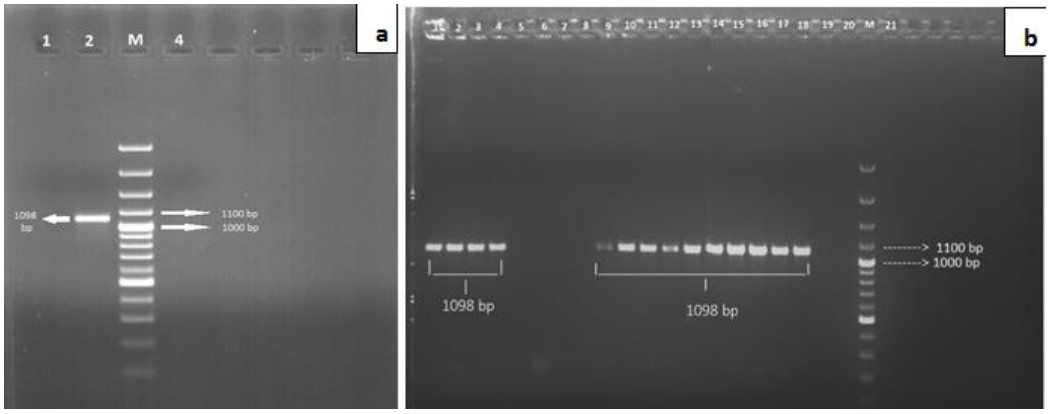

Fig. 4: Agarose gel electrophoresis of PCR products. **(a)** M = 100 bp ladder, 1&2 = goats' samples, 4= negative control. **(b)** M = 100 bp ladder, 1-20 = goats' samples, 4 = negative control

Table 1: Comparison of Overall Prevalence of *Theileria* sp. on Microscopy and PCR

No. of Goats Examined		No. of Goats Positive	% Prevalence
Microscopy	135	11	8.15
PCR	135	17	12.6

Table 2: Comparison of Sex-wise Prevalence of *Theileria* sp. on Microscopy and PCR

Sex Method	Females			Males		
	No. of Goats Examined	No. of Goats Positive	% Prevalence	No. of Goats Examined	No. of Goats Positive	% Prevalence
Microscopy	71	6	9.37	64	5	7.14
PCR	71	9	14	64	8	11.27

Table 3: Comparison of Age-wise Prevalence of *Theileria* sp. on Microscopy and PCR

Age / Method	<1 Year			1-2 Years			>2 Years		
	N	+	% Prevalence	n	+	% Prevalence	N	+	% Prevalence
Microscopy	23	3	13	65	5	7.69	47	3	6.38
PCR	23	5	21.74	65	8	12.3	47	4	8.51

n: Total no. of Goats Examined; +: No. of Goats Positive

Table 4: Comparison of Area-wise Prevalence of *Theileria* sp. on Microscopy and PCR

Area / Method	Burdwan			Payradanga			Hili			Bethuadahari			Jhargram		
	N	+	%	N	+	%	N	+	%	n	+	%	n	+	%
Microscopy	49	0	0	51	0	0	20	9	45	9	2	22.2	6	0	0
PCR	49	0	0	51	0	0	20	13	65	9	4	44.4	6	0	0

n: Total no. of Goats Examined; +: No. of Goats Positive; %: Percent Prevalence

The partial sequence of 1098 bp fragment of ssu-rRNA gene of *Theileria* sp., West Bengal isolate was submitted to GenBank (MW624380). Sequence similarity searches in BLAST revealed that the newly generated partial sequence was 98.21% identical with two published sequences of Theileria luwenshuni (KP407009 and LC326009). A closer comparison of all three sequences after alignment revealed that they bear 99.4-100% identity to each other and differ by 6 substitutions and 3 insertions/deletions.

Discussions

The present study was undertaken to estimate the prevalence of *Theileria* sp. among goats of West Bengal and to determine the species causing caprine theileriosis in this state as well. The results revealed that the prevalence of caprine theileriosis is 8.15% and 12.6% via microscopy and PCR respectively.

Various prevalence rates ranging from 2.88%-68% on microscopic examination are reported worldwide by numerous researchers, (Altay *et al*. 2007; Irshad *et al*. 2010; Zangana and Naqid 2011; Mohammed and Idoko 2012; Naz *et al*. 2012; Aydin *et al*. 2014; Patra *et al*. 2018; Halder 2019) [17, 9, 10, 21, 13, 16, 4, 3] confirming the endemicity of *Theileria* sp. throughout the global goat population.

On PCR, lower prevalence reports of 6.34% from southeast Anatolia and 6.17% from black sea region of Turkey are obtained on molecular diagnosis by Altay *et al*. (2007) [3] and Aydin *et al*. (2014) [4] respectively. In India, however, the reported rates are much higher, e.g. 95.7% in Karnataka state (Mamatha *et al*. 2017) [12] and 59.3% in Kerala state (Nagaraj *et al*. 2018) [14].

The differences in prevalence in different study regions may be subjected to variations in geo-climatic conditions, population and distribution of vectors, husbandry practices or the research methodology applied.

The higher infection rates in females and young animals ageing <1 year were observed via both methods. The contributing factors can be the relatively higher stress of production, gestation and parturition in females and the extensive grazing habits, causing greater tick burden amongst the younger animals.

When the two techniques, i.e. microscopy and PCR were compared, the later was found to be the superior in diagnosing caprine theileriosis since a significant proportion of the sample was tested false negative on microscopy. Hence molecular diagnosis should be the employed as a more reliable tool, especially in identifying the carrier status among asymptomatic individuals.

The isolate of *Theileria luwenshuni* obtained from the present study (MW624380) carries 98.21% homology with two previously reported isolates from China (KP407009) and Myanmar (LC326009). The differences revealed after aligning these 3 sequences of *T. luwenshuni* may have taken place due to antigenic variation occurring due to host factor or geo-climatic dissimilarities.

Theileria luwenshuni was already established for causing caprine theileriosis from three other states of India, i.e. Karnataka, Kerala and Assam recently (Mamatha *et al*. 2017, Nagaraj *et al*. 2018 and Begam *et al*. 2019) [14, 5, 12]. However, this is the first confirmed report of *Theileria luwenshuni* infection in goats of West Bengal state.

Conclusions

From the present study it can be concluded that caprine theileriosis is endemic in West Bengal and the species causing caprine theileriosis is *Theileria luwenshuni*. The organism affects females more than males. Among different age groups young animals ageing <1 year suffer most, followed by 1-2 years old and >2 years old ones. Goats from Hili and Bethuadahari areas carry higher infection rate. Also, between two methods, PCR is more sensitive diagnostic tool than microscopy.

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