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## Emergence of *Theileria luwenshuni* infection in goats of Assam, India

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### Abstract

Blood samples were collected by vein puncture from clinically affected goats (n=11), which showed pyrexia, anaemia, weakness and depression, and prepared blood smear for microscopic examination. Microscopic examination (ME) of blood smear stained with Giemsa's stain revealed occurrence of *Theileria* spp. 72.73% (8/11) based on morphological features, where a dot, ring, pyriform, tail, crescent, rod and comma shaped form were found to be noticed inside the erythrocytes. Genomic DNA extracted from all the blood samples were subjected to PCR, which amplified 18S ribosomal RNA (*18S rRNA*) gene of *Theileria* spp. that produced 1098 base pairs DNA fragment for 100% (11/11) samples. The present study indicated that false negative in microscopic examination showed positive result for theileriosis in PCR. Further sequencing analysis of the PCR product of *Theileria* spp. positive genomic DNA confirmed the presence of *Theileria luwenshuni*, which is reported in the present communication for the first time from north-eastern region of India.

**Keywords:** *Theileria luwenshuni*, goat, microscopic examination, PCR, 18S rRNA gene

### 1. Introduction

The traditional goat farming in Assam is profitable but some hindrance come to the farm and causes some diseases by bacteria, virus, fungus and parasites [6]. Among the parasitic diseases trematodes, cestodes, nematodes, haemoprotozoa, tissue protozoa reduces production and productivity of goats in Assam. So to get rid of the parasitic infection or infestations, some extensive works has been conducted on helminthosis [31, 12, 5], moneiziosis [9, 10], fasciolosis [7], amphistomiosis [12], haemonchosis [8, 11], schistosomosis [13] and coccidiosis [26]. *Babesia* and *Theileria*, the most important haemoprotozoa play a significant role causing clinical and subclinical form of diseases in goats. Caprine theileriosis, caused by *Theileria lestoquardi*, *T. luwenshuni*, *T. uilenbergi*, *T. separata*, *T. ovis* and *T. recondite* [32, 33, 24], the economically important disease of tropic and sub-tropic country [2, 18] has also been reported for the first time in India particularly from Karnataka [28], which was caused by *T. luwenshuni*. In Assam, there is no published information regarding the occurrence of theileriosis caused by *T. luwenshuni* due to lack of diagnostic facilities and interest of the researchers. Therefore, the present study was conducted to investigate the emergence of theileriosis using both conventional microscopic blood smear examination and amplification of genomic DNA extracted from blood samples.

### 2. Materials and method

#### 2.1 Clinical examination

All the infected Assam local goats (n=11) were clinically observed by physical examination individually for record of body temperature, palor of mucous membrane, anorexia, depression, dyspnoea, icterus, nasal discharge, lymphadenopathy, coloration of faeces / urine, mortality and presence of tick on the coat of the infected animals to detect abnormalities due to some protozoan diseases in and around Guwahati, Kamrup (Metro) district of Assam in 2015- 2016.

#### 2.2 Collection of blood samples

Blood samples from clinically infected Assam local goats (n=11) were obtained by vein puncture and collected in properly labeled vials containing Ethylene Diammine Tetraacetic Acid in the proportion of 1mg/ml of blood which were brought to the laboratory for parasitological and molecular diagnosis.

Parasitological examination was done on the same day of collection while anticoagulated blood samples were preserved in deep freeze -20 °C for molecular study.

### 2.3 Microscopic detection of haemoparasite

A thin blood smear was made from a drop of anticoagulated blood and stained with Giemsa stain as per standard method [29] followed by microscopic examination under oil immersion objective (100X) for the presence of both intracellular and extracellular haemoparasites. The parasites when present were identified on the basis of their morphology [22, 29].

### 2.4 Molecular study

#### 2.4.1 Genomic DNA isolation

The genomic DNA was extracted from blood using the DNeasy Blood and Tissue kit (Quiagen Kit, Catalogue No. 69504) as per manufacturer's protocol.

#### 2.4.2 Oligonucleotide design and PCR amplification

A set of oligonucleotide primers of each *Theileria* specific primer (989 F; 5'AGTTTCTGACCTATCAG-3'; 990 R; 5'-TTGCCTTAAACTTCCTTG-3') was used to amplify the 1098 bp sequence from *18S rRNA* gene of *T. luwenshuni* as described earlier by Allsopp *et al.* [31]. The PCR was performed in reaction volume of 25µl PCR mixture comprising of 5µl DNA template, 12.5 µl DyNAzyme II PCR mixture, 5.5µl Nuclease free water and 1 µl (10 pmol) of each *Theileria* specific primers. The reaction was performed with 30 cycles each at 94°C for 1 minute for denaturation, 56°C for 1 minute for annealing and 72°C for 1 minute for final extension and holding at 4°C in a Technee-5000 thermal cycler (Bibby Scientific).

#### 2.4.3 Electrophoresis and visualization

The PCR products obtained after DNA amplification were

subjected to electrophoresis in 1.5% agarose gel in TAE (1X) buffer containing Ethidium Bromide (0.5µg/ml) for 1 hour at constant voltage (60 V) and subsequent visualization done in gel documentation system (DNR Mini Lumi, Applied Bioimaging).

#### 2.4.4 Purification of PCR products and sequencing

The resultant PCR products (1098 bp DNA fragment size) were purified using QIAquick® PCR purified kit (Qiagen; Ct. No. 28104) sent to Molbiogen FAST-BASE, Malaysia for sequencing.

#### 2.4.5 Analysis of nucleotide sequence

The sequences without any error, obtained in sequencing analysis were subjected to NCBI BLAST analysis with other published sequences to know about homology with other database.

## 3. Results and Discussion

### 3.1 Clinical symptoms

Primarily the theileriosis caused by *T. luwenshuni* was diagnosed through clinical symptoms noticed in affected goats, where all the affected animals showed pyrexia (105 to 106.8 °C), pale mucous membranes, weakness and depression (Table 1), which are clinical symptoms of theileriosis as mentioned by Soulsby [29]. However, nasal discharge and dyspnea were observed in clinical cases of sheep in Great Britain [25] unlike our present study and also enlargement of superficial lymph glands, a common clinical sign due to *Theileria* infection observed by Tageldin *et al.* [30] was not observed in the present study. About 63.64% animals were also found to be lightly infested with ticks. In Pakistan, Naz *et al.* [23] reported haemoprotozoan infected small ruminants also infested with ticks akin to our annotations.

**Table 1:** Clinical symptoms observed in clinical cases of haemoparasitic infection

Parameters	No. of cases (n=11)	Observation %	Observation in goats with <i>Theileria luwenshuni</i>
Anorexia	11	100	++
Depression	11	100	++
Fever	11	100	++
Anaemia	9	81.18	++
Weakness	10	90.91	++
Dyspnoea	0	0.00	-
Nasal discharge	0	0.00	-
Lymphadenopathy	0	0.00	-
Coloration of urine	0	0.00	-
Coloration of faeces	0	0.00	-
Icterus	0	0.00	-
Diarrhoea	0	0.00	-
Mortality	0	0.00	-
Presence of tick on the body of animal	7	63.64	+

++ Moderate; + Mild

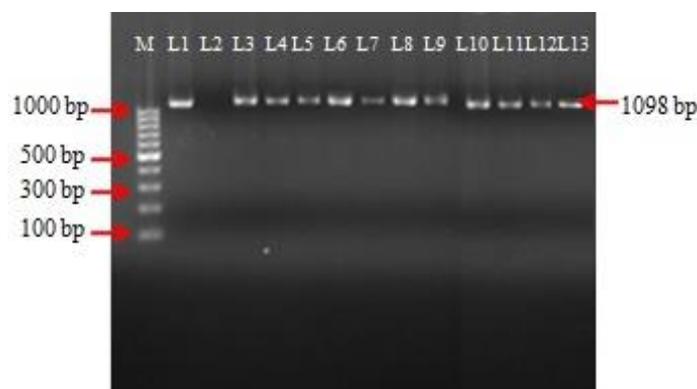
### 3.2 Microscopic examination

ME of blood smear showed a pyriform, ring, dot, tail, crescent, rod and comma shaped form of organism inside the erythrocytes indicative as *Theileria* spp. Different forms of *Theileria* spp. in the erythrocytes of goats were also reported by Yin *et al.* [35]. The study also revealed that out of 11 samples, 8 samples (72.73%) were found to be positive for *Theileria* spp. which are agreement with observations of Shruthi *et al.* [28], who recorded 68.08% goats were infected with *Theileria* spp. in Karnataka. Jadhao *et al.* [20] reported prevalence of theileriosis (0.4%) in goats from Vidarbha

region of Maharashtra, which was so less than our findings might be due to theileriosis caused by non-pathogenic species of *Theileria* spp. Simultaneously, Naz *et al.* [23] recorded only 8.20% goats were infected with theileriosis unlike our observations also might be due to different species were involved to do the disease. Phipps *et al.* [25] and Cao *et al.* [14] also observed higher prevalence rate of *T. luwenshuni* infections as compared to our findings which might be due to the genetic variation in both hosts and vectors, different geographical locations and dissimilar climatic conditions prevail in the study areas.

### 3.3 Molecular findings

Amplification of PCR of *18S rRNA* gene using *Theileria* genus specific primers showed 1098 bp DNA fragment in agarose gel (Fig.1) as observed by Allsopp *et al.* [4], which indicated for the presence of *Theileria* spp. Our findings are also corroborated with the observations of Ros-Garcia *et al.* [27], Jalali *et al.* [21] and Fatima *et al.* [16]. The PCR product of *Theileria* spp. positive genomic DNA was further sequenced and analyzed which confirmed the presence of *Theileria luwenshuni*, which is reported for the first time in north-eastern region of India. Cao *et al.* [14] detected *Theileria* spp. from the blood of the sheep by amplification of PCR followed by sequencing analysis confirmed the presence of *T. luwenshuni* as our present finding. In Britain, Phipps *et al.* [25] also distinguished *Theileria* spp. in the blood samples of sheep through amplification of PCR; sequencing analysis later confirmed only the presence of *T. luwenshuni*. All microscopically positive samples were confirmed by PCR. No *Theileria* piroplasms were seen on blood smears that were negative in PCR, which indicates that PCR is more sensitive and specific than microscopic blood smear examination. The alignment analysis of all the isolates (n=11) has shown that these sequences were highly homologous to each other with identities from 99.8% to 100%. The sequences, obtained blast against NCBI BLAST database indicated 100% homology with *Theileria luwenshuni*. Sequencing analysis confirmed the *Theileria* spp. as *Theileria luwenshuni*, as reported by Cao *et al.* [14] and Phipps *et al.* [25]. Our observations are also corroborated with the findings of Ge *et al.* [17] and Phipps *et al.* [25], who reported blast the sequences against NCBI database indicated 99% homology with *Theileria luwenshuni*.



**Fig 1:** Agarose gel electrophoresis showing PCR products of *18S rRNA* gene of *Theileria luwenshuni* in lane L3 to L13 positive samples; L1 and L2 indicates positive and negative control, respectively; lane M indicates 100 bp DNA size marker

### 3.4 Comparison of microscopy and PCR methods

The present study showed that 100.00% (11/11) clinical cases positive to *T. luwenshuni* in PCR, whereas 72.73% (8/11) cases could be diagnosed by ME of blood leaving only 27.27% (3/11) cases as false negative. The result of the present study showed PCR to be more efficient in detection of *Theileria* positive cases than the ME method. Various workers from different countries suggested PCR as the most efficient method in the detection of haemoparasite infections in the animals [1, 4, 15, 34]. Therefore our present investigation suggested to identify the role of each species in the epidemiology of theileriosis in goats, PCR, the sensitive and specific diagnostic, are required to be used, which has also been recommended by Aktas *et al.* [1], Atlay *et al.* [4] and Heidarpour Bami *et al.* [19].

### 4. Conclusion

With the most sensitive, specific and advance molecular technique we were able to diagnose theileriosis in goats for the first time in Assam caused by *Theileria luwenshuni* which is indicative of emerging diseases. Hence, PCR method that was designed in this study, can serve as beneficial diagnostic tool, especially in *T. luwenshuni* prevalent region. Further study need to be conducted on epidemiological factors of the disease and their transmission dynamic to formulate an effective control measures against this newly alarming disease.

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