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Original Research

Molecular Detection and Characterization of Haemoprotozoan and Haemorickettsial Parasites of Cattle in the Indo-Bhutan Border Districts of Assam, India

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Abstract

Haemoprotozoan and haemorickettsial diseases like trypanosomosis, theileriosis, babesiosis, anaplasmosis and ehrlichiosis are important diseases of cattle and cause huge economic loss to the livestock industry. Cattle and other animals are being regularly traded across the porous Indo-Bhutan border districts of Assam. The present study was conducted to confirm the haemoparasites species of cattle recorded in 4 districts of Assam along the border. PCR analysis amplified Theileria orientalis DNA both from blood and eggs of Rhipicephalus (Boophilus). microplus tick and Babesia bigemina and Anaplasma marginale from blood. Phylogenetic analysis revealed T. orientalis isolate having 98.83% and 99.00% similarity with isolates reported from Vietnam and Myanmar, a new B. bigemina isolate and A. marginale isolate bearing 93.46% similarity with isolates from USA and Mexico.

Keywords: *Theileria orientalis, Babesia bigemina, Anaplasma marginale, Trypanosoma evansi*, PCR, *Rhipicephalus microplus*, Phylogenetic tree

Introduction

Livestock sector plays an integral role in Indian agricultural economy and its growing importance is dependent on dairying which accounts for more than 2/3rd of the livestock output in India. Assam situated in India's Northeast region owns 10.8 million cattle as the largest group as per 20th livestock census, 2019 (Directorate of Animal Husbandry & Veterinary, Assam). The per capita availability of milk is lowest in Assam which is only 81 g/capita/day and is characterized by low milk producing cattle with average productivity of 1.34 L/ day against the all-India average of 2.77 L/day. There was no local knowledge on prevalence of haemoparasite other than *Babesia* and *Anaplasma* in cattle of Assam till the report of predominance of *Rhipicephalus microplus* tick vector and incidence of *Theileria orientalis* in indigenous and crossbred cattle by Kakati (2013) and prevalence of *B. bigemina*, *A. marginale* and *T. orientalis* but no *T. annulata* in crossbred cattle. Despite widespread prevalence reports of *Trypanosoma evansi* in cattle from Northern India, this parasite is less reported from the North East. *T. evansi* was detected from clinical cases in crossbred cattle by Sarmah *et al.* (2015).

The present study was carried out for the first time in four border districts of Assam sharing boundary with Bhutan. Livestock are being regularly traded from both sides through the porous border causing risk for transmission of various diseases. Among diseases of cattle, intestinal worm infection, ticks and leech infestation and tick-borne diseases such as babesiosis, theileriosis and anaplasmosis are the major recognized problem in cattle of Bhutan (Phanchung *et al.*, 2012; Tshering and Dorji, 2013). The aim of the present investigation was molecular confirmation of the recorded haemoparasites species by PCR and their characterization.

Materials and Methods

Study Period

The study was carried out for one year during the period from April 2016 to March 2017 and samples were collected within this period.

Collection of Blood

A total of 533 anticoagulated blood samples were collected aseptically from individual cattle (456 indigenous, 77 crossbred) by jugular vein puncture in the study area comprising Kokrajhar, Chirang, Baksa and Udalguri districts and preserved individually in deep freeze at -20°C.

DNA Extraction from Blood

For molecular study, DNA was extracted from the blood using the DNeasy Blood and Tissue kit (Quiagen® Kit, Catalogue No. 69504) as per manufacturer's protocol. The test was conducted for identification and confirmation of tick transmitted species of *Theileria, Babesia, Anaplasma* and additionally *T. evansi*, a fly transmitted blood protozoan parasite using published standard primers (Table 1).

DNA Extraction from Eggs of *R.(B). microplus* and *Haemaphysalis bispinosa* Ticks for *Theileria orientalis*

Live *R. (B). microplus* engorged female ticks were collected from cattle exhibiting clinical illness and also having subclinical infection with *T. orientalis*. Similarly, *H. bispinosa* engorged ticks were collected from another cattle having parasitemia due to *T. orientalis*. Ticks were allowed to oviposit by keeping in desiccator in room temperature. All the eggs of *R. (B). microplus* and *H. bispinosa* were pooled together according to tick species for DNA extraction (Kakati, 2013). Eggs laid by individual tick species were monitored daily and allowed to develop for at least 10 days. About 10 mg of embryonated eggs in duplicate for each tick species were used. PCR assay was conducted in 10 DNA samples extracted from eggs of *R. (B). microplus* and 4 extracted from *H. bispinosa* eggs as per the procedure for detection of *T. orientalis* DNA.

PCR Assay for Detection of Haemoparasites

Techne-500 thermal cycler (Bibby Scientific) was used for amplification of DNA by PCR in a final reaction volume of 25 µl, consisting of 12.5µl PCR Master Mix, 1 µl (10pM) each of forward and reverse primer, 0.5 µl Taq

Polymerase, 5µl DNA template and 5 µl Nuclease free water. The thermocycling conditions followed were as per Kamau *et al.*, 2011; d'Oliveira *et al.*, 1995; Figueroa *et al.*, 1993; Laha *et al.*, 2012; Nutcha *et al.*, 2004; Kumar, 2016. The PCR products were subjected to electrophoresis in 1.5% agarose gel prestained with Ethidium bromide (0.5µg/ml) at 60 V for all species, except *A. marginale* (70V) for 90 minutes, except *A. marginale* (45 min). Visualization of the gel was done in gel documentation system (DNR Mini Lumi, Applied Bioimaging).

Table 1: Standard primers used for identification of *T. orientalis, T. annulata, A. marginale, B. bigemina, B. bovis* and *T. evansi*

Parasite	Primer sequence pair	Product Size	Reference
Theileria orientalis	Tor: F1:5'-CTT TGC CTA GGA TAC TTC CT-3'	776 bp	Kamau *et al.*, 2011
	Tor:R1:5'-ACG GCA AGT GGT GAG AAC T-3'		
Theileria annulata	TaF1:5'-GTA ACC TTT AAA AAC GT-3'	721 bp	d'Oliveira *et al.*, 1995
	TaR1:5'-GTT ACG AAC ATG GGT TT-3'		
Anaplasma marginale	Am: F1:5'-CAC ATT TCT TGG AGC TGG-3'	160bp	Figueroa *et al.*, 1993
	Am: R1:5'-TCT CTG GCA CTT TGA ACC-3'		
Babesia bigemina	Bbi: F1:5'-TGG CGG CGT TTA TTA GTT CG-3'	1124bp	Laha *et al.*, 2012
	Bbi: R1:5'-CCA CGC TTG AAG CAC AGG A-3'		
Babesia bovis	Bbo: F1:5'-GGG TTT ATA TAG TCG GTT TTG T-3'	711bp	Nutcha *et al.*, 2004
	Bbo: R1:5'-ACC ATT CTG GTA CTA TAT GC-3'		
Trypanosoma evansi	TRYPS-F: 5'-GAATATTAAACAATGCGCACG-3'	164bp	Kumar, 2016
	TRYPS-R:5'-CCATTTATTAGCTTTGTTGC-3'		

Sequencing and Phylogenetic Analysis of Haemoparasites

One representative positive PCR product (*T. orientalis*, *B. bigemina* and *A. marginale*) from present study area was selected for sequence analysis, purified using PCR purification kit (QIA quick PCR® Purification kit, Cat. No 28104, Germany) and outsourced to Mol Biogen, FAST-BASE Malaysia for determination of nucleotide sequence. Identity of nucleotide sequence of PCR products were confirmed by Basic Local Alignment Search Tool (BLAST) analysis and aligned using CLUSTAL W algorithm. Phylogenetic comparison was performed using MEGA7 software. Phylogenetic tree was constructed using UPGMA method along with the bootstrap consensus tree inferred from 500 replicates.

Results and Discussion

The result of prevalence of haemoparasites in crossbred and indigenous cattle from the Indo-Bhutan border districts of Assam is shown in Table 2.

Molecular Detection of Haemoprotozoan and Haemorickettsial Parasites

PCR employed for molecular detection of *Theileria orientalis* by amplification of MPSP gene showed clear band at 776 bp (Plate-I a), similar to works of Kamau *et al.* (2011) and Kakati (2013). 18S rRNA gene of *Babesia bigemina* positive samples revealed clear band at 1124 bp (Plate-I b), similar to work of Laha *et al.* (2012) who recorded a case report of *B. bigemina* in cattle from Meghalaya. Amplification of genomic parasite DNA gene of *Anaplasma marginale* positive samples showed clear band at 160bp (Plate –II a). PCR assay carried out to confirm the presence of *Trypanosoma* gene in 5 blood samples which were negative by blood smear examination and sero-positive by ELISA were found negative. However, the positive control showed clear band at 164bp (Plate- III). Presence of antibody in ELISA test might suggest that the animals had attained usual recovery from *T. evansi* infection (Singla *et al*., 2013) and may exhibit parasitaemia below the PCR detection level (1–20 trypanosomse/ml) (Desquesnes *et al*., 2009).

Table 2: Prevalence of haemoparasites in crossbred and indigenous cattle from Indo-Bhutan border districts of Assam

Haemoparasite species recorded	Kokrajhar		Chirang		Baksa		Udalguri		Total (533)	
	Crossbred (n=18)	Indigenous (n=110)	Crossbred (n=25)	Indigenous (n=114)	Crossbred (n=19)	Indigenous (127)	Crossbred (n=15)	Indigenous (n=105)	Crossbred (n=77)	Indigenous (n=456)
	No. positive (%)	No. positive (%)	No. positive (%)	No. positive (%)	No. positive (%)	No. positive (%)	No. positive (%)	No. positive (%)	No. positive (%)	No. positive (%)
Theileria orientalis	12 (66.67)	72 (65.45)	20 (80)	70 (61.4)	10 (52.63)	84 (66.14)	10 (66.67)	57 (54.28)	52 (67.53)	283 (62.85)
Babesia bigemina	2 (11.11)	2 (1.81)	0 (0)	1 (0.87)	0 (0)	2 (1.57)	1 (6.67)	2 (1.9)	3 (3.89)	7 (1.53)
Anaplasma marginale	0 (0)	2 (1.81)	0 (0)	8 (7.01)	1 (5.26)	1 (0.78)	2 (3.33)	0 (0)	3 (3.89)	11 (2.41)
Total	**14 (77.77)**	**76 (69.09)**	**20 (80.00)**	**79 (69.29)**	**11 (57.89)**	**87 (68.5)**	**13 (86.66)**	**59 (56.19)**	**58 (75.32)**	**301 (66.00)**
Overall prevalence	**90 (70.31)**		**99 (71.22)**		**98 (67.12)**		**72 (60.00)**		**359 (67.35)**	

Detection of *Theileria orientalis* DNA in Tick Eggs

PCR conducted in 10 DNA samples extracted from eggs of *R. (B). microplus* showed positive for *Theileria orientalis* DNA in 2 nos. Positive samples showed clear band at 776 bp (same as *T. orientalis*) confirming the presence of the parasite DNA in *R. (B). microplus* tick eggs (Plate II b). However, all the 4 samples of *H. bispinosa* were negative for *T. orientalis* DNA.

The *R.(B). microplus* tick could be suggested as the vector transmitting the parasite transovarially, thus confirming the findings of Kakati (2013) in cattle of Assam. Perusal of literature reveals *R (B). microplus* as the principal vector of *Babesia* and *Anaplasma* (Ghosh *et al*., 2007) in India. But epidemiology of *T. orientalis* and its vector relationship has not been adequately studied to compare with the present finding. However, Aparna *et al.* (2011) reported clinical cases of *T. orientalis* in adult crossbred cattle in Kerala identifying *H. bispinosa* on the body of the affected cattle. Epidemiological situation similar to the present study has also been reported by Altangerel *et al*. (2011) who recorded prevalence of *T. orientalis* in presence of *R.* (*B). microplus* as the only tick infesting cattle of Vietnam. Kakati (2013) also observed high prevalence of *T. orientalis* in cattle of Assam where *R.* (*B). microplus* was the predominant cattle tick.

Molecular Characterization of Haemoprotozoan and Haemorickettsial Parasites

Theileria orientalis

The phylogenetic tree analysis of the isolate identified in the present study bears 98.32% to 98.66% similarity with the isolate of *Theileria orientalis* reported from South India (HQ444179, JX648209), 98.16% to 98.83% similarity with Sri Lankan isolate (AB690871, AB701472, AB701462, AB701477, AB701470, AB701471, AB701437), 98.83% and 99.00% similarity with isolates reported from Vietnam (LC125432) and Myanmar (AB871368) respectively on the basis of percent nucleotide substitution (Fig. 1). However, highest sequence similarity of 99.00% was observed with the isolate Khonkan-2 strain (KT460094) from Thialand and Myitkyina-8 strain (AB871368) from Myanmar, which are of type 7. According to Khukhuu *et al.* (2011), *T. orientalis* piroplasms in cattle can generally be divided into the Ikeda group consisting of types 2 (Ikeda) and 7 and the Chitose group consisting of types 1(Chitose), 3, 4, 5, 8 and N3 based on MPSP sequence variations. Out of 8 genotypes of *T. orientalis* reported in Asia, reports from India have shown presence of 1, 3 and 7 types of parasites (Aparna *et al.,* 2011, 2013) while a recent report has shown types 1,3,5 and 7 in Sri Lanka which is one of the close neighbors of India (Sivakumar *et al.,* 2012). Kakati *et al.* (2015) reported type Ikeda in Assam by PCR analysis using Ikeda specific primer which supports the assumption of our finding to be of Ikeda type.

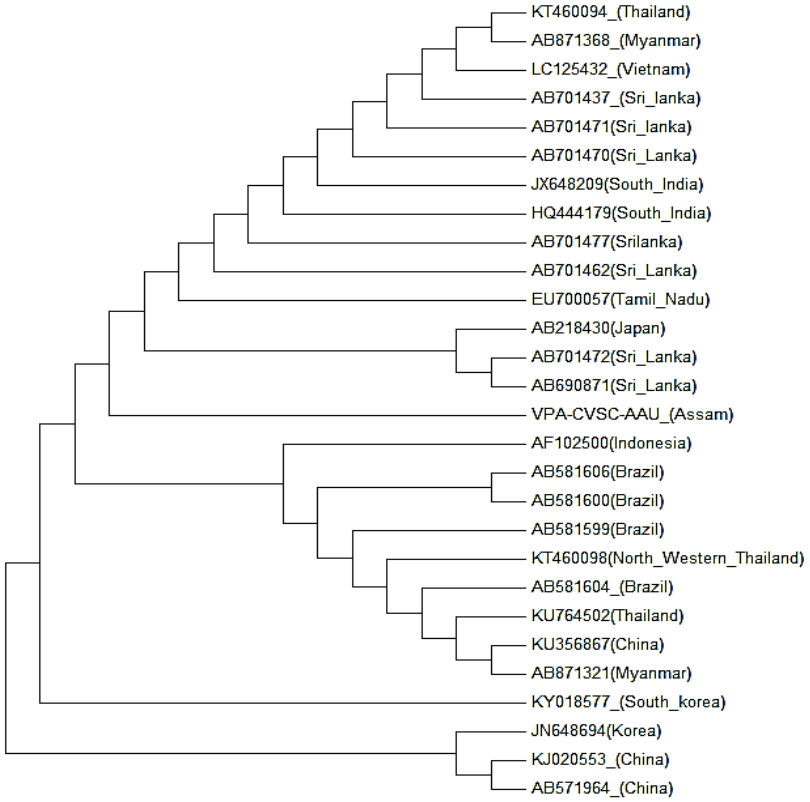
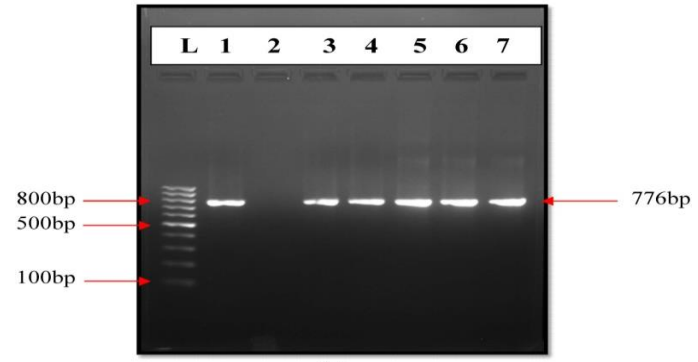
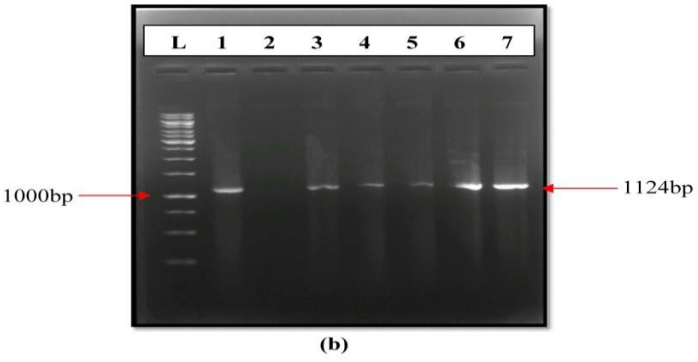


Figure 1: Phylogenetic analysis of local isolate (Assam) of *Theileria orientalis*



(a)

LANE L :Ladder 100 bp LANE 1 :Positive control
LANE 2 :Negative control LANE 3-7:Positive samples (776bp)



(b)

LANE L :Ladder 1000 bp LANE 1 :Positive control
LANE 2 :Negative control LANE 3-7:Positive samples (1124bp)

Plate I: Gel picture showing amplified PCR products (a) *Theileria orientalis*, 776 bp, (b) *Babesia bigemina*, 1124 bp

Babesia bigemina

The isolate of *Babesia bigemina* in the present study has been found to be very distantly related and having distinct molecular type with respect to 18S rRNA gene sequence of isolates reported from other parts of world (Fig. 2). The high level of unrelatedness could be due to lack of interaction between the present isolate and others submitted to the GenBank including that reported from Meghalaya by Laha *et al*. (2015). Thus, the present finding reports a new isolate of *B. bigemina* from the study area (Chirang district) without having any similarity with other isolates submitted to the GenBank.

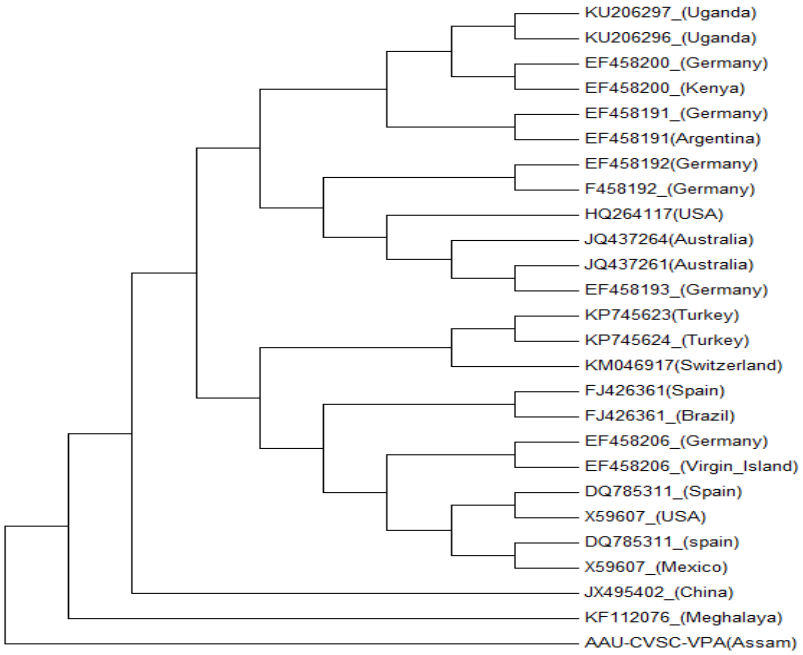
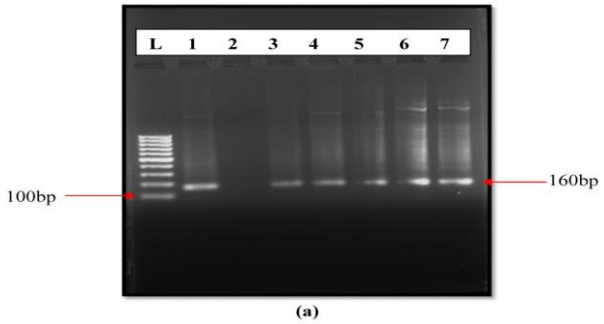
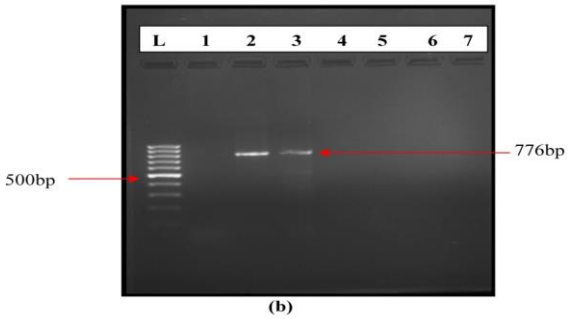


Figure 2: Phylogenetic analysis of local isolate (Assam) of *Babesia bigemina*



(a)

LANE L :Ladder 100 bp LANE 1 :Positive control
LANE 2 :Negative control LANE 3-7:Positive samples (160bp)



(b)

LANE L:Ladder 100 bp LANE 1:Negative control
LANE 2:Positive control LANE 3:Positive samples (776bp)

Plate II: Gel picture showing amplified PCR products (a) *Anaplasma marginale*, 160 bp (b) *Theileria orientalis* DNA in *Rhipicepahlus* (*Boophilus*) *microplus* tick egg, 776 bp

Anaplasma marginale

The phylogenetic tree analysis of *A. marginale* isolate in the present study was found to bear 93.46% similarity with isolates from USA (St. Maries: CP000030 and Florida: CP001079.1) and Mexico (Dawn: CP006847.1 and Gypsy: CP006846.1), Fig. 3. The present study indicates the first report of *A. marginale* isolate from Assam and North-East India.

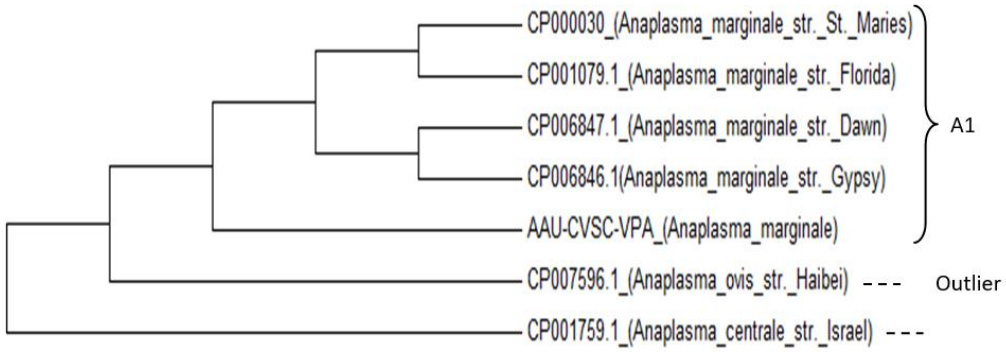
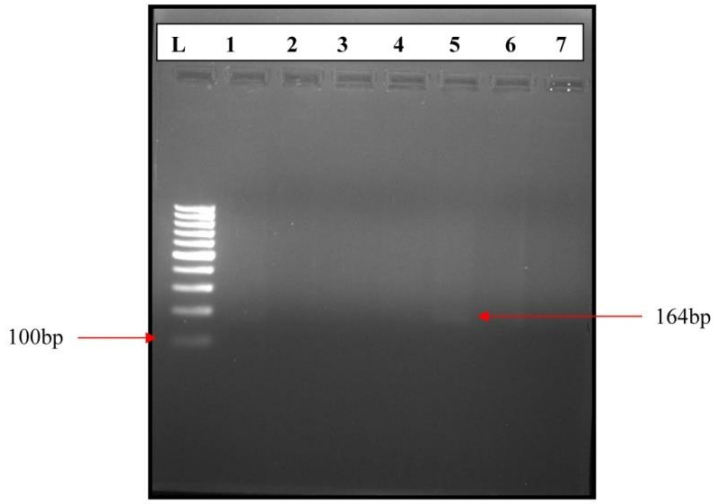


Figure 3: Phylogenetic analysis of local isolate (Assam) of *Anaplasma marginale*



LANE L:Ladder 100 bp
LANE 1:Negative control
LANE 5:Positive control
LANE 2,3,4,6,7:Negative samples.

Plate III: Gel picture showing PCR products of *Trypanosoma evansi*

Conclusion

It can be concluded that the haemoparasites recorded i.e., *B. bigemina*, *T. oreintalis* and *A. marginale*, from the four Indo-Bhutan border districts of Assam were also confirmed by molecular studies.

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Conflict of Interests

There is no conflict of interest.

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