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Prevalence of bovine tropical theileriosis in sub-Himalyan region of northern India

ARUN RAJ M R[1], STUTI VATSYA[2] and RAJEEV RANJAN KUMAR[3]

Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263 145 India.

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Haemoprotozoan parasites like *Babesia*, *Theileria* and *Trypanosoma* often present a challenge to successful livestock farming. Bovine tropical theileriosis (BTT) caused by *Theileria annulata* occurs in different parts of India (Acharya *et al*. 2017, Sudan *et al.* 2017) including sub-Himalyan region of northern India (Rialch *et al*. 2013, Kohli *et al*. 2014a). In India, disease affects imported high grade/ crossbred cattle and young indigenous calves (Acharya *et al*. 2017) in which theileriosis is a severe and often lethal disease. Identification of carrier animals is of utmost importance in the epidemiological studies for inferring infection risk and for the implementation and monitoring of control programs. Field diagnosis is normally achieved by observing clinical signs and detection of piroplasms in the peripheral blood smear and schizont-infected leucocytes in Giemsa stained blood smear, and lymph node needle biopsy smear examination (Anonymous 2008). This method detects acute cases but have low sensitivity for the assessment of carrier animal (Almeria *et al*. 2001, Atlay *et al*. 2008). The molecular diagnostic based assays though quick yet require equipped laboratories, trained personnel besides elevated application costs. To overcome these constraints, a novel method called loop mediated isothermal amplification of DNA (LAMP) was developed (Notomi *et al.* 2000, Nagamine *et al.* 2002) and used in the successful diagnosis of bovine tropical theileriosis (Liu *et al*. 2012).

Considering the increasing reports of BTT, the present study was carried out to compare various diagnostic assays to detect *Theileria annulata* in different agro-climatic regions of sub-Himalyan region of northern India and also to determine latent infection of bovine tropical theileriosis using PCR and LAMP assays.

To know the prevalence of BTT in cattle and buffaloes, an epidemiological study was carried out in five districts for a period of one year from May 2013 to April 2014 in two geo-climatic zones, viz. tarai (300–1,000 m elevation) and hills (above 1,000 m elevation). District Udham Singh Nagar and Dehradun constituted the tarai zone while Nainital, Pithoragarh and Champawat constituted the hill zone. The sub-Himalyan region of northern India in which the study was conducted experiences summer (March to June), rainy (July to October) and winter (November to February). All these areas were approximately 100 km apart. Blood samples from cattle (334) and buffaloes (146) were screened for BTT. Thin blood smears were stained with Giemsa stain. Erythrocytes were searched for piroplasms (50 field × 400 RBC per field) (Aktas *et al.* 2006) and lymphocytes for Koch's Blue Bodies (KBB).

DNA from whole blood sample was extracted using modified protocol of Sambroo *et al.* (1989). *Tams1* gene was amplified from genomic DNA isolated from various blood samples of cattle and buffaloes as per Kirvar *et al.* (2000) using the following oligonucleotide primers: Forward primer—5'- ATGCTGCAAATGAGGAT-3'; reverse primer—5'-GGACTGATGAGAAGACG-ATGAG-3'.

Loop mediated isothermal amplification (LAMP) was conducted (Thekisoe *et al.* 2007) using primers: FIP: 5′-TGGGTTACGGGCTTCTTGGTTTCCTACGT-CGCATTCACTGAC-3′; BIP: 5′- ATTTTCGACGCCAA-GAGGCTCAAATGGCC-AGTGCTTCATGTC-3′; F3: 5′-GGAAACAGGACAACGCCG-3′; B3: 5′- CCGTTTGGT-CCGTTGGTAA-3′

Out of 480 blood samples, 53 (11.04%) were found positive for BTT, and 40 cattle (11.98%) and 13 buffaloes (8.9%) were positive for BTT. These included those showing clinical signs of theileriosis as well as those harbouring latent infection. Region-wise prevalence of bovine tropical theileriosis (Table 1) was maximum in tarai as compared to hilly region. In hill region, 6.40% of cattle were positive for bovine tropical theileriosis as against 3.13% of buffaloes. Overall prevalence of theileriosis was more in animals above 5 years followed by those between 1–5 years and minimum in animals less than 1 year of age (Table 2). Maximum occurrence of BTT was in summer (15.64%) followed by rainy season (10.53%) and winter (3.42%). Maximum prevalence was recorded during May (20%) and minimum in January (0%).

Bovine tropical theileriosis due to *T. annulata* in cattle and buffaloes has been reported from different parts of India (Gupta *et al.* 2006, Godara *et al.* 2009, Haque *et al.* 2010,

Present address: [1]MVSc Scholar (armr.krl@gmail.com), [2]Professor and Head (stutivatsya@gmail.com), [3]Assistant Professor (rajeevpara@gmail.com), Department of Parasitology, College of Veterinary and Animal Sciences.

Table 1. Overall per cent prevalence of bovine tropical theileriosis in different regions of sub-Himalyan part of northern India

Region	Place	Cattle	Buffalo	Total
Hill	Nainital	7.00	8.70	7.32
	Champawat	3.70	0.00	2.22
	Pithoragarh	6.67	0.00	4.41
	Total	6.40	3.13	5.51
Tarai	U.S. Nagar	19.42	16.67	18.62
	Dehradun	15.25	10.00	13.13
	Total	17.90	13.41	16.39
Overall		11.98	8.90	11.04

Table 2. Overall age wise per cent prevalence of bovine tropical theileriosis in different regions of sub-Himalyan part of northern India

Region	Place	Age (year)			Total
		<1	1–5	≥5	
Hill	Nainital	0	6.25	7.48	7.32
	Champawat	0	0	3.23	2.22
	Pithoragarh	0	0	5.36	4.41
	Total	0	2.38	6.19	5.51
Tarai	U.S. Nagar	16.67	16.67	20.95	18.62
	Dehradun	0	12.00	15.15	13.13
	Total	7.14	11.86	18.71	16.39
Overall		7.14	7.92	12.05	11.04

Kholi *et al.* 2014b, Acharya *et al.* 2017). Though there are few reports of *T. orientalis* from southern parts of India (George *et al.* 2015), no such cases are on record from the area under study. Vahora *et al.* (2012) reported 37% and 17% infection of haemoprotozoans in crossbred cattle and buffaloes, respectively. Vatsya *et al.* (2014) observed 4.48% cattle and 1.97% in buffaloes to be positive for BTT in a three year study in some parts of Uttarakhand. The high prevalence in cattle could be due to thinner skin and dry habitat of cattle that makes them more susceptible to tick infestation. Age-wise, the highest prevalence was observed in animals above 5 years of age. The findings are in consonance with those of Ananda *et al.* (2009) who also observed highest prevalence in animals between 4–6 years of age (63.15%) followed by animals between 1–2 years of age (21.05%) and least in animals below 6 months of age (15.79%). Seasonwise, the highest prevalence was recorded in summer followed by rainy season and winter. These results are in accordance with those of Panda *et al.* (2011) who also observed the maximum prevalence of bovine tropical theileriosis in summer (35%) followed by rainy season (23%) and then winter (22%) in coastal area of Odisha (central India) from 2002 to 2006.

The study is significant considering increasing cases of the disease in this part of the country especially in hilly areas from where it was hitherto not reported. Surprisingly, no *Hyalomma* ticks could be recovered from animals. Vatsya *et al.* (2008) also reported a 0.02% prevalence of *Hyalomma* ticks in Uttarakhand—a region of sub-Himalyan part of northern India and that too from transboundry areas with other states from where the ticks have been reported. The history of positive cases revealed that infective animals were purchased from neighbouring states from where the disease has been widely reported.

Eighteen blood samples (3.75%) were found positive for theileriosis; 12 (3.59%) blood samples from cattle were positive for theileriosis. Koch's Blue Bodies (KBB) were observed in 2 (0.6%), piroplasms in 6 (1.8%) and both KBB and piroplasms in 4 (1.2%) blood samples. Six (4.11%) blood samples of buffaloes were positive for theileriosis out of which 2 (1.37%) were found harbouring KBB; piroplasms in 1 (0.68%) sample and both KBB and piroplasms in 3 (2.05%) blood samples. Of the 480 blood samples screened for theileriosis, KBB were found in 4 (0.83%) blood samples, piroplasms in 7 (1.46%) samples and both piroplasm and KBB were found in 7 (1.46%) samples. In the present study, the most frequent encountered morphological appearance of theilerial piroplasms were annular forms and few rod shaped or dot forms with light staining cytoplasm.

The average A260:A280 ratio of extracted DNA from individual blood sample was 1.586–1.635. The concentration of DNA ranged from 2.734 to 4.362 µg/ml. Amplification of partial *Tams1* gene using AS-PCR assay yielded a partial *Tams1* gene of length 785 bp in all positive cases. Samples found positive with thin blood smear examination (TBE) were screened again with AS-PCR to find out false positive results. The screening of all the 480 samples with AS-PCR revealed that 32 (6.67%) samples were positive. In tarai region, PCR found 9.83% and TBE found 5.33% sample positive. In hill region, 3.39% samples were found positive with PCR and 2.12% with TBE. After screening of DNA samples with PCR, the samples were screened again using LAMP assay. A total of 53 (11.04%) samples were found positive with LAMP as against a total of 6.67% samples being positive with AS-PCR assay. LAMP method gave the highest level of sensitivity followed by PCR and then TBE. The results obtained after conducting LAMP assay was taken as the final prevalence rate of each region. Maximum positive samples were from tarai region.

Sudan *et al*. (2017) attempted a comparison of conventional and molecular assays to detect bovine tropical theileriosis. However, such comparative studies undertaken in India are few (Sudan *et al.* 2017, Acharya *et al.* 2017). TBE is the most common and routinely used method of detecting *T. annulata* in India. However, the present study indicated that it is associated with low sensitivity. PCR method was found more accurate and sensitive in diagnosing *T. annulata* organisms. In our study, PCR method was 1.77 times more accurate in diagnosing theileriosis infection as compared to TBE. Kirvar *et al.* (2000) reported PCR to be three times and Aktas *et al.* (2006) 2.78 times more accurate.

Lamp assay detected 2.94 and 1.65 times more *Theileria* positive cases as compared to TBE and PCR assay,

Table 3. Comparative diagnosis of bovine tropical theileriosis using blood smear examination, PCR and LAMP assay

Region	Place	NE	TBE (NE/NP)	PCR (NE/NP)	LAMP (NE/NP)	Total (%)
Hill	Nainital	123	123/3 (2.44)	123/6 (4.88)	123/9 (7.32)	7.32
	Champawat	45	45/1 (2.22)	45/1 (2.22)	45/1 (2.22)	2.22
	Pithoragarh	68	68/1 (1.47)	68/1 (1.47)	68/3 (4.41)	4.41
	Total	236	236/5 (2.12)	236/8 (3.39)	236/13 (5.51)	5.51
Tarai	US Nagar	145	145/9 (6.21)	145/17 (11.72)	145/27 (18.62)	18.62
	Dehradun	99	99/4 (4.04)	99/7 (7.07)	99/13 (13.13)	13.13
	Total	244	244/13 (5.33)	244/24 (9.83)	244/40 (16.39)	16.39
Overall		480	480/18 (3.75)	480/32 (6.67)	480/53 (11.04)	11.04

NE, Number of samples examined; NP, number of samples positive. Numbers in parenthesis indicate percentage

respectively. After screening of 480 blood samples of large ruminants for BTT using various diagnostic assays, 3.75% animals showing symptoms of tropical theileriosis were found positive in routine laboratory examination of blood samples (Giemsa staining). The rest 7.29% of blood samples which were found positive under molecular assays (AS-PCR and LAMP) were considered to be harbouring *T. annulata* in latent form. The findings of thin blood smear examination are in accordance with that of Vatsya *et al.* (2014) who also observed a prevalence of 3.72% during 2009–2011 in sub Himalyan region of northern India.

Identification of carrier animals is of utmost importance in the epidemiological studies for inferring infection risk, and for the implementation and monitoring of control programs. Latent cases of bovine tropical theileriosis as recorded in the present study are significant in its epidemiology as they serve as a source of infection for ticks. However, routine laboratory examination of blood/ lymph node aspirate can hardly detect them owing to low parasitemia. Out of the diagnostic assays (TBE, PCR and LAMP) employed to detect *T. annulata* in the present study, it is suggested that LAMP assay being more accurate can serve as a better diagnostic test in epidemiological studies especially carrier animals. The results recorded in this study would provide better understanding of this haemoprotozoan parasite and hence may help in devising timely control measures.

SUMMARY

The prevalence of bovine tropical theileriosis (BTT) caused by *Theileria annulata* was studied in large ruminants of sub-Himalyan region of northern India using thin blood smear examination (TBE), polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP) assays. About 11.04% blood samples were found positive for BTT. Maximum seasonal prevalence of BTT was observed in summer followed by rainy season and winter. Month-wise maximum prevalence was recorded in May and minimum in January. Laboratory examination with Giemsa staining for Koch's blue bodies (KBB) and piroplasms revealed that 3.75% samples were positive for theileriosis. LAMP method gave the highest level of sensitivity followed by AS-PCR and TBE. Hence, it is suggested that LAMP can be a better molecular diagnostic tool for large scale epidemiological studies of bovine tropical theileriosis. Our results provide better understanding of this haemoprotozoan parasite and hence may help in devising timely control measures.

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