Molecular identification and phylogeny of Tick-borne haemoparasites of cattle

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Abstract

In the present investigation, the overall incidence of Tick-borne haemoparasites (TBHs) was found 22.12%, out of which significantly (P<0.01) highest incidence was found for *Theileria* sp. (14.74%). The sex, age and breed wise study revealed statistical significance of these epidemiological factors on the TBHs infection in cattle in West Bengal (WB) with highest incidence in female, more than 3 years age group and in cross breed cattle, respectively. Molecular detection and phylogeny analysis of TBHs was done by PCR amplification and nucleotide sequencing of the respective 18S rRNA gene fragments. High degree of similarity has been observed for both *T. annulata* and *B. bigemina* isolates to the respective isolates from neighbouring states. The present study suggests high incidence of TBHs in WB which warrants immediate large-scale screening of cattle population to reduce the extent of spread of the organisms.

Key words: Molecular detection, Phylogeny, Tick-borne haemoparasites, Cattle, West Bengal.

Introduction

Tick and TBHs *viz. Theileria* spp., *Babesia* spp. and *Anaplasma* spp. cause substantial losses to the cattle husbandry and pose a big threat to the worldwide dairy industry including India. TBHs cause significant economic losses to livestock in terms of morbidity, production losses and mortality. They have been reported from developing nations (Farhang, 2017), various parts of India viz. Haryana (Chaudhri et al., 2013), Tamil Nadu (Velusamy et al., 2014), Rajasthan (Bhatnagar et al., 2015) and Assam (Mushahary et al., 2021) including WB (Debbarma et al., 2020, Shit et al., 2023). All these studies used conventional microscopy of blood smear, serology or post mortem analysis, which have considerable limitations (Pradeep et al., 2019). There are only few prior researches on the molecular characterization and identification of bovine TBHs. Accurate identification of TBHs is crucial for effective disease surveillance and control. Compared with conventional microscopic examination, molecular techniques provide greater sensitivity and specificity, allowing reliable detection of subclinical infections and precise species identification (El Damaty et al., 2021). In addition, molecular characterization enables the assessment of genetic diversity and evolutionary relationships among parasite isolates (George et al., 2015). Phylogenetic analysis of nucleotide sequences from different geographical regions can reveal patterns of genetic relatedness, with closely related isolates suggesting possible common ancestry or livestock movement, while genetic differences may indicate local adaptation or ongoing parasite evolution (Ullah et al., 2021). Such information contributes to a better understanding of parasite epidemiology, transmission dynamics, and the geographical distribution of circulating strains (Ntesang et al., 2024) which can aid in the development of new tools and the adoption of international TBHs infection control protocols. WB, a state of eastern India has a cattle population of 19 million and has climatic condition conducive for the survival and breeding of tick population which increases the risk of TBHDs (Debbarma et al., 2018). Except a few reports on prevalence, there is no systemic report or availability of any information on the genetic diversity and molecular characterization of TBHs in this state. Hence, the objective of the study was molecular identification of TBHs circulating in cattle of WB and to determine their genetic relationship with isolates reported from other geographical regions using the 18S rRNA gene fragment.

Material and methods

For the present study, whole blood samples from jugular vein were collected from 95 apparently healthy cattle of different age (less than 1 year, 1 to 3 years and more than 3 years), sex and breed (indigenous and crossbred) during November, 2021 to February, 2022 from different districts of WB. The animal experimentations were carried out strictly as per the guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, Government of India after due approval by the Department of Veterinary Parasitology, F/O- Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal, India. Thin blood smears were prepared according to Taylor et al. (2016) and were screened using Giemsa's stain to identify the presence of haemoparasites by light microscopy with oil immersion objective (100X). The indentifications were done on the basis of keys and description given by Soulsby (1982). Failure in detection of any haemoparasite in the blood smear after examination of at least 50 oil immersion fields was declared as microscopically negative sample.

The blood samples which were found positive for *Theileria* spp. and *Babesia* spp. in microscopic examination were subjected to molecular studies. Genomic DNA was extracted using commercial kit (HiPura™ Blood Genomic DNA Miniprep Purification Kit, HIMEDIA, Catalogue No. MB504-50PR) following manufacturer's protocol. Recovery and purity of each DNA sample were evaluated by using spectrophotometer (NanoDrop®ND-1000, PeqLab, Erlangen, Germany). For PCR amplification of DNA, oligonucleotide primers targeting the 18s rRNA of *Theileria annulata* and *Babesia bigemina* and the thermal conditions described by Allsopp et al. (1993) and Laha et al. (2015), respectively were used. The PCR reactions were conducted using a final reaction volume of 50 µl in a 200 µl PCR tubes consisting of 25 µl PCR master mixture (2x) (EmeraldAMP® GT PCR Master Mix, Takara), 2.5 µl of each forward and reverse primers (25pM), 5 µl Template DNA and 15 µl Nuclease-free water. The PCR products were resolved in a 1.2% agarose gel containing ethidium bromide (0.5µg/mL), visualized under UV light using a gel documentation system and were compared with 1kb DNA ladder (Thermo scientific gene ruler 1 kb).

The selected independently generated PCR amplified products were subjected to nucleotide sequencing commercially using Sanger's dideoxy nucleotide chain termination method with both the forward and reverse primers. The obtained DNA sequences were put to further analysis using bioinformatics tools including similarity search using the BLASTN program of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Following the homology/NCBI-BLAST study, the obtained 18S rRNA sequences were submitted to NCBI-GenBank for the assignment of the accession number. Further, the sequences were subjected for the construction of phylogenetic tree using MEGA X program (Kumar et al., 2018). The evolutionary history was depicted using the neighbor-joining method. The 18S rRNA gene segment sequence data of *Theileria annulata* and *Babesia bigemina* that have a detailed history of collection were retrieved from the NCBI database

and were used for the phylogenetic analysis. Results were expressed as the percentage. A difference with value $p<0.01$ was considered statistically significant. Chi-square test was performed to determine presence or absence of significant difference in parameters among the different groups using the Statistical Package for Social Sciences, Version 17.0.1 software (SPSS Inc., Chicago, IL, USA).

Results and discussion

Out of 95 blood samples, 21(22.12%) were found positive for presence of one or more haemoparasites. The total incidence rate of *Theileria* spp. [14 (14.74%)] was found significantly ($P<0.01$) higher followed by *Anaplasma* spp. [6 (6.32%)], *Babesia* spp. [3 (3.16%)] and mixed infection [2 (2.11%)] (Table 1). Similar pattern of incidence of tick-borne haemoparasitc infection were reported from different states of India and abroad by various researchers (Chaudhri et al., 2013; Velusamy et al., 2014; Debbarma et al., 2020). However, difference in infection rates may be due to variations in climatic condition, geographical location of the study areas, and variation in the animal husbandry practices and methodology adopted for the study. Sex wise, infection of haemoparasites was found more in female cattle which may be attributed to stress related to pregnancy, lactation and production, high levels of prolactin and progesterone hormone which make them more prone to any infection and also due to higher susceptibility to tick infestation than male. Highest incidence of haemoparasites was found in above 3 years of age. In above 3 years of age, most of the animals remain in peak lactating stage. The weakening of immunity during high milk yielding stage in addition to genetic makeup and seasonal stress in monsoon and summer could be reason for high susceptibility to this haemoprotozoan parasite in the cattle of above 3 years of age (Velusamy et al., 2014). The rate of infection was more in cross breed than indigenous cattle. It could be attributed to different genetic makeup and /or constant exposure to infections and development of immunity against such infections in indigenous cattle (Rathera et al,. 2015). On the contrary, better management and thus less pre-exposure of vectors and development of no or less immunity in crossbred cattle might have resulted frequent occurrence of such diseases (Alim et al., 2012).

The PCR amplification of the 18S rRNA gene fragment yielded product of 1098 bp and 1124 bp for *Theileria annulata* and *Babesia bigemina*, respectively. BLAST analysis confirmed the obtained sequences were from 18S rRNA gene of *T. annulata* and *B. bigemina*. The sequences were submitted to GenBank database under the accession number ON724327 and ON724328 for *T. annulata* and ON142383 for *B. bigemina*.

The present isolates of *T. anuulata* demonstrated 98.5%- 99.7% identities with the isolates of Bihar, Telengana, Uttar Pradesh (UP), Haryana, Andhra Pradesh, Maharashtra and Punjab etc. Phylogenetic study indicated that two study isolates of *T. annulata* were clustered in close proximity. Both the isolates (ON724327 & ON724328) formed a sub clade with the Bihar isolates. Isolates of other states obtained from NCBI database namely; Telengana, Uttar Pradesh, Haryana, Andhra Pradesh, Maharashtra, Punjab and even previous isolates from WB formed separate clades away from the studied isolates (Fig. 1). On other hand, *B. bigemina* isolate (ON142383) also formed a sub-clad with a Bihar isolate. The study isolate exhibited 95.9%- 99.9% sequence homology with other published sequences, included as representative of other Indian states *viz*. Meghalaya, UP, Mizoram and abroad *viz.* China, Kenya, Turkey, Argentina and Bangladesh etc (Fig. 2).

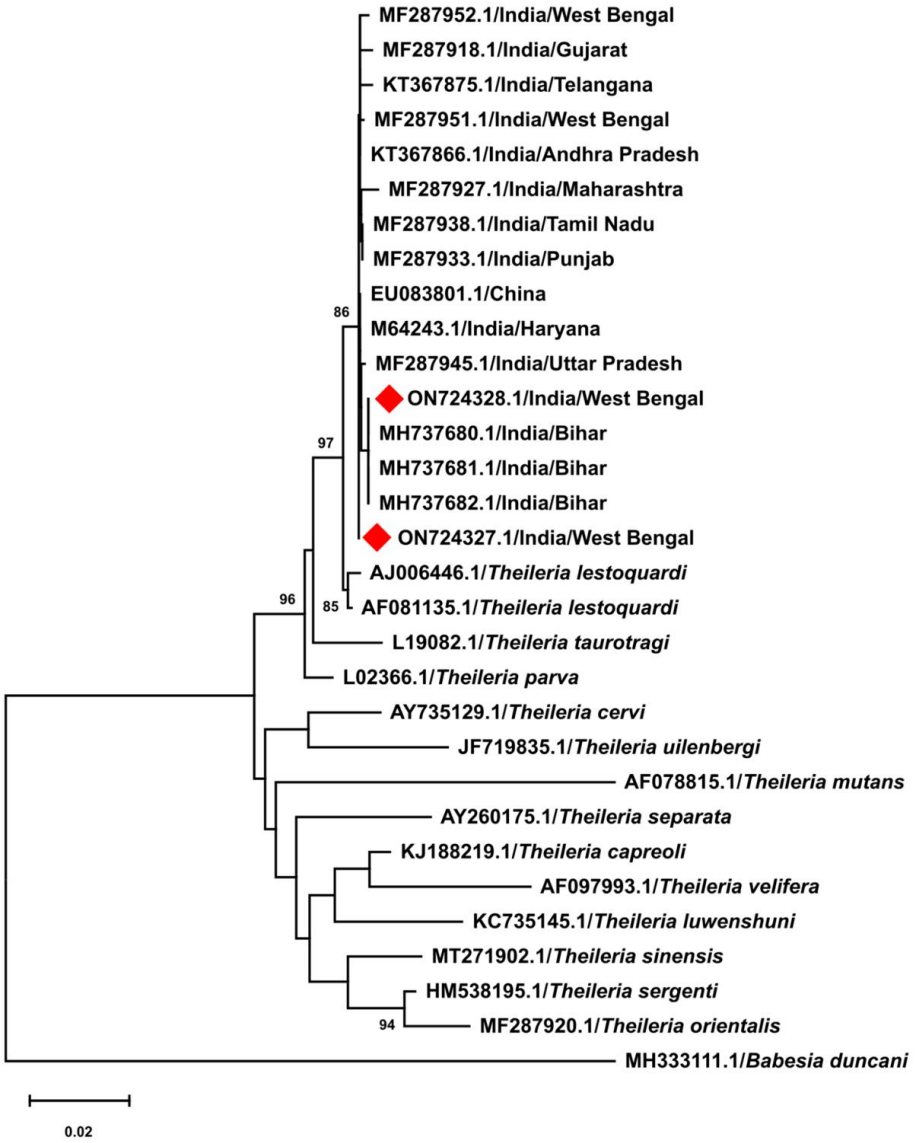
To date, the small subunit ribosomal RNA (18S rRNA) gene is widely used for detection and phylogenetic analysis of protozoan organisms due to its high level of conservation (Sudan et al., 2017). In this study, very high similarity at nucleotide level has been observed for both *T. annulata* and *B. bigemina* isolates with that of respective Bihar isolates. WB and Bihar is neighbouring states and shares common land border. As a result, frequent inter-state movement of farmer and livestock traders along with livestock and vectors commonly occur which is the probable reason of the nucleotides similarity observed in this study. The significant genetic resemblance observed across countries might also be attributed to increasing globalization, illegal animal trade, and cross border animal and vector movement, which facilitate the spread of haemoprotozoan genotypes (Perveen et al., 2021). These factors highlight the need for ongoing surveillance and more molecular studies to better understand the genetic diversity and transmission dynamics of haemoparasites in WB.

Table 1: Incidence of tick- borne haemoparasites in cattle of West Bengal according to different epidemiological factors

Epidemiological factor	No of cattle screened	No of cattle infected	Incidence (%)	No. (%) positive for haemoparasites			Mixed infection
				Theileria sp.	*Babesia* sp.	*Anaplasma* sp.	
Male	41	5	(12.19%)^{y}	5 (12.19%) ^{ya}	0 (0%)^{yd}	1 (2.44%)^{yb}	1(2.22%)^{xc}
Female	54	16	29.62%)^{x}	9 (16.67%)^{xa}	3 (5.55%)^{xc}	5 (9.26%)^{xb}	1(1.86%)^{yd}
Indigenous	39	6	(15.38%)^{y}	3(7.69%)^{yb}	1 (2.56%)^{yd}	4 (10.27%)^{xa}	2(5.13%)^{xc}
Cross Breed	56	15	(26.79)^{x}	11(19.64%)^{xa}	2 (3.57%)^{xb}	2 (3.57%)^{yb}	0 (0%)^{yc}
<1 year	13	3	(23.08%)^{y}	3 (23.08%)^{xa}	0 (0%)^{yb}	0 (0%)^{zb}	0 (0%)^{yb}
1-3 year	35	5	(14.29%)^{z}	5 (14.29%)^{ya}	0 (0%)^{yc}	2 (5.71%)^{yb}	2 (5.71%)^{xb}
>3 year	47	13	(27.66%)^{x}	6 (12.77%)^{za}	3 (6.38%)^{xc}	4 (8.51%)^{xb}	0 (0%)^{yd}
Total	95	21	22.11%	14 (14.74%)	3(3.16%)	6 (6.32%)	2 (2.11%)

N.B. Values bearing different superscripts viz., a, b, c… in a row and x, y.....in a column differ significantly at 1% level ($p<0.01$).

Fig. 1: Phylogenetic tree using partial 18S rRNA gene sequences showing the relationship between the *Theileria annulata isolates* from cattle of West Bengal and NCBI submissions from different states of India and abroad.



In the present study, examination of 95 thin blood smears revealed 14.74%, 6.32% and 3.16% samples were positive for theileriosis, anaplasmosis and babesiosis, respectively. Significant correlation has been observed for age, sex and breed on the incidence of TBHs in cattle of WB. Molecular study confirmed that causative agents of bovine tropical theileriosis (*T. annulata*) and bovine babesiosis (*B. bigemina*) are circulating in cattle of West Bengal. Further, phylogenetic study showed very close relationship between 18S rRNA of *T. annulata* and *B. bigemina* from cattle of WB to that of Bihar. The present finding warrants immediate attention to prevent the spread of the TBHs in the cattle population of WB including the vigilance on animal movement from the neighbouring states.

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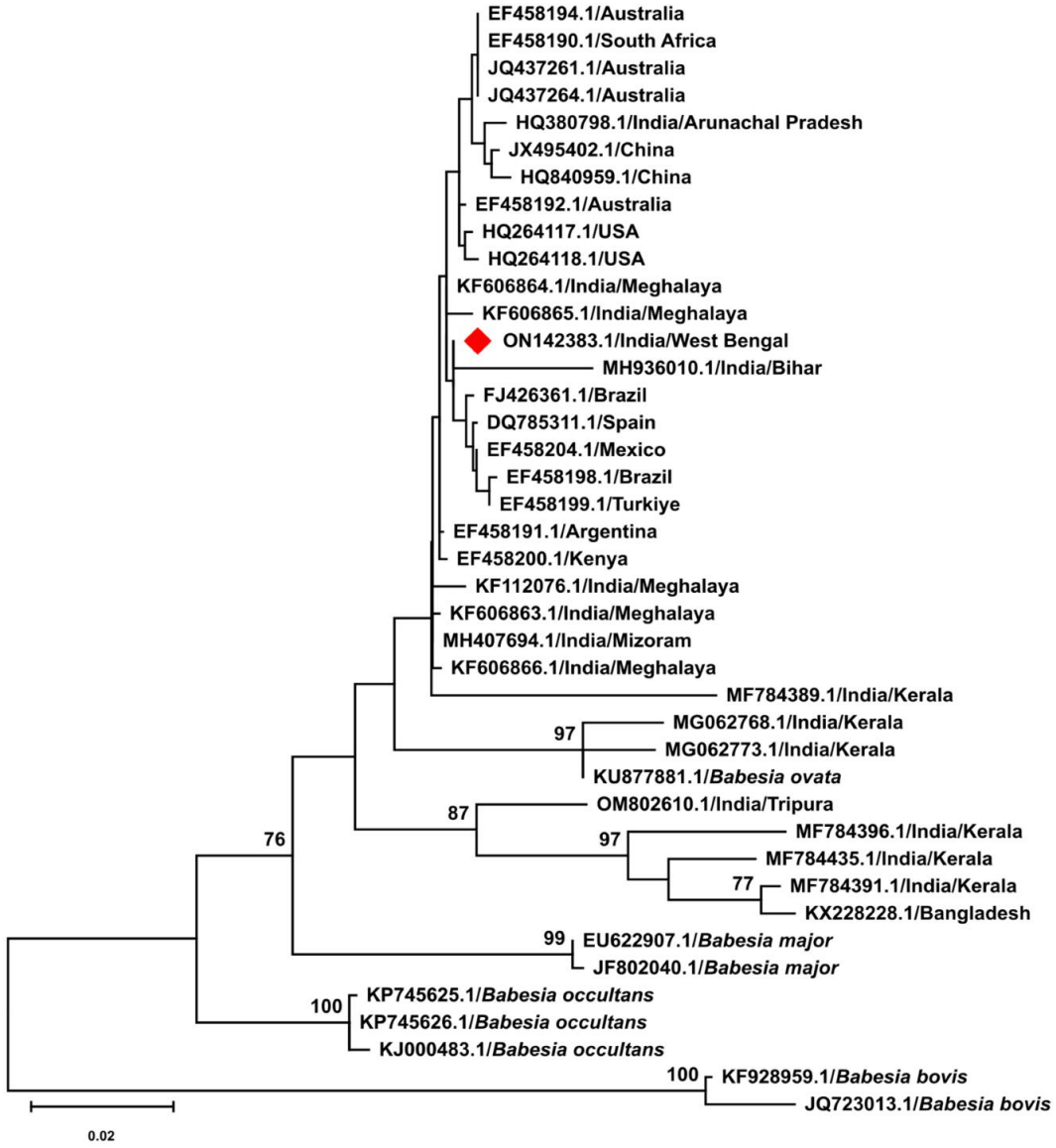
Conflict of interest

The authors declare no conflict of interest.

Fig.2: Phylogenetic tree using partial 18S rRNA gene sequences showing the relationship between the *Babesia bigemina isolates* from cattle of West Bengal and NCBI submissions from different states of India and abroad.



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