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Quantification of *Babesia bigemina* from cattle in Indo-Bhutan border district of Assam by real time PCR (RT-PCR)

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Abstract
Situated in the Northeast India, Assam with its four districts, namely Kokrajhar, Chirang, Baksa and Udalguri share a 267 km long international border with Bhutan. *Babesia bigemina* along with other haemoparasites were recorded in crossbred and indigenous cattle by blood smear examinations which were also confirmed by Polymerase chain reaction. The absolute quantification of *B. bigemina* was done with quantitative real time PCR (RT-PCR) using specific primers in 3 samples (2 from crossbred and 1 from indigenous cattle) using SYBR Green dye. The present analysis showed positive for *B. bigemina* DNA with mean C_T value 24.69 and standard deviation 1.77. The detection limit of the assay was assessed as 8.01 x 10^{11} DNA copies μl^{-1} of *B. bigemina*. Amplification of *B. bigemina* DNA by RT-PCR showed band at 88 bp.

Keywords: *Babesia bigemina*, PCR, phylogenesis, RT-PCR, SYBR Green dye, Agarose gel

Introduction
Livestock plays an important role in Indian agricultural economy. Sharing with the country's livestock population, Assam situated in India's Northeast region owns 8.2 million cattle as the largest group against 5.7 lakh buffalos in the livestock census conducted during 2009-10 [1]. Animal husbandry, which is an integral part of the farming system in the region, 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. Apart from the indigenous cattle, crossbreds are being produced by up-gradation of indigenous animals with Jersey and Holstein Friesian exotic germ plasms. Farmers are keeping cattle for milk, meat, wool, and hide production and also for various farm operations. However, these animals suffer from various diseases, mainly parasitic. In India, the damage caused by ticks and tick borne diseases (TTBDs) to livestock is considered very high [2]. The cost of control in animals has been estimated to be US $ 498.7 million per annum [3].There is a need to assess the true dimension of vector-borne haemoparasitic disease problems faced by the livestock farmers. Bovine babesiosis, caused by the intra-erythrocytic apicomplexan protozoa, mainly *Babesia bigemina*, is an economically important disease and is responsible for substantial mortality and morbidity, reduced milk and meat production and incurs huge economic losses in the livestock industry in tropical and subtropical regions. Infection by *B. bigemina* causes high rise of temperature, haemoglobinurea, anaemia, anorexia and diarrhoea in cattle [4]. Estimated a loss of about 51 litres milk in a month from a cross-bred cow having *B. bigemina* infection. Assam and neighboring states in the North East region of India have long been known to be endemic for tick borne haemoparasites.

Bhutan, known as the "Thunder Dragon Country" is a small independent country bordered in the south by the Indian state of Assam. The border trade between India and Bhutan takes place through several recognized passes or duars. Assam is the major state of which six districts such as Kokrajhar, Bongaigaon, Chirang, Baksa, Udalguri and Sonitpur covering approximately 1000 square miles area share boundary with Bhutan. Trading of livestock for milk production and draught purpose, free mixing of open grazed animals including the wild counterparts through the porous border are the important risk factors for the spread of new diseases in the border areas. Among diseases of cattle, babesiosis, theileriosis and anaplasmosis are the major recognized problem in Bhutan [5]. However, transboundary haemoparasitic diseases of livestock particularly cattle are poorly understood in the areas along

the Indo-Bhutan border. It was therefore pertinent to record the prevalence of haemoparasitic diseases of cattle in the four districts of Assam along the international border using conventional and molecular tools and develop an epidemiological base.

Materials and Methods

Study area

The present study was carried out in villages of 4 districts of Assam namely, Kokrajhar, Chirang, Baksa and Udalguri representing the Indo-Bhutan border areas in crossbred and indigenous cattle of both sex and belonging to different age groups. Blood samples were screened for haemoparasites by blood smear examination. Samples positive for *Babesia bigemina* were included for molecular analysis and quantification.

Molecular detection of haemoparasite

Blood samples from *Babesia* positive animals were taken for confirmation of parasite DNA by amplification of 18S rRNA gene of *Babesia bigemina* using Polymerase Chain Reaction (PCR) taking standard primers for *B. bigemina* (Bbi: F1:5'-TGG CGG CGT TTA TTA GTT CG-3'and Bbi:R1:5'-CCA CGC TTG AAG CAC AGG A-3') as per [4]. DNA extraction was done using the DNeasy Blood and Tissue kit (Quiagen® Kit, Catalogue No. 69504) as per manufacturer's protocol. PCR was carried out in Techne-500 thermal cycler (Bibby Scientific) in a 25 µl final reaction volume consisting of 12.5µl PCR Master mix, 1 µl each of forward and reverse primer, 0.5 µl Taq Polymerase, 5 µl Nuclease Free Water, 5µl DNA template. The thermocycling conditions included 40 cycles of initial template denaturation at 94°C/2min, denaturation at 94°C/30 sec, annealing at 55°C/30 sec, Primer extension at 72°C/1min, final extension at 72°C/5min and hold at 4 °C. The PCR products were subjected to electrophoresis in 1.5% agarose gel prestained with Ethidium Bromide (0.5µg/ml). Visualization of the gel was done in gel documentation system (DNR Mini Lumi, Applied Bioimaging).

Absolute Quantification of *Babesia bigemina* DNA

The absolute quantification of *B. bigemina* was done with quantitative real time PCR (RT-PCR)using specific primers of *B. bigemina* (**Table-1**) in 3 samples (2 from crossbred and 1 from indigenous cattle)by Stepone® software v2.2.3 (Applied Biosystem, 7500 Real time PCR System).SYBR Green dye was used as the active chemistry and ROX was used as passive. The test was carried out in triplicate each with three times repetition.

Table 1: Primer used for quantification of *Babesia bigemina* by Real- time PCR

Primer	Sequence (5'-3')	Product size	Reference
(F) cbisg-1 (R) cbisg-2	tgttccaggagatgttgattc agcatggaaataacgaagtgc	88bp	[12].

a) Optimization of assay

Initially, specificity of all assays were checked by conventional PCR using thermal cycler and product size of PCR amplicon was checked in agarose gel electrophoresis. If a single band was obtained then the optimization of real time PCR was performed. To exclude cross-reactivity between *Babesia bigemina* and other pathogens responsible for tick-borne diseases of cattle, the assay was evaluated for specificity by testing DNA extracts from blood samples positive to *Anaplasma marginale* and *Theileria orientalis*. Blood samples collected from uninfected cattle as well as sterile water were also included in the analysis as negative control. The optimal primer concentration for the primer pair was determined by performing triplicate real time PCR assay at different primer concentrations over the range from 5 pico-mole to 10 pico-mole in 10 µl reaction.

From the fluorescence data and melting curve analysis, the optimal primer concentration was obtained. The lowest CT value defined the optimal primer concentration. The increase in fluorescent signal was registered during the extension step of reaction and the data were analyzed with the appropriate sequence detector software (7500 System Software v.2.2.3). The growth of PCR product is proportional to an exponential increase in fluorescence (Δ Rn). The application software produces an amplification curve resulting from a plot of Δ Rn versus cycle number. The threshold cycle number (C_T) for each analyzed sample was regarded as the cycle number at which the amplification curve crossed the threshold. Lower C_T values corresponded to a greater amount of initial template and a negative result was considered to have a C_T value of 40 or more cycles.

b) Normalization of genes

The reactions were performed in 10µl volume reaction mixture and Non-Template Control (NTC) comprised of the following components listed in Table-2 and the thermal conditions in Table-3

Table 2: Components of Real - time PCR for experimental genes

Components	Reaction mixture	Non-Template control (NTC)
Fast SYBR Green Master Mix (2X)	5.0 µl	5.0 µl
Forward primer (10 pmol/µl)	0.5 µl	0.5 µl
Reverse primer (10 pmol/µl)	0.5 µl	0.5 µl
DNA	1.0 µl	-
Nuclease free water	3.0 µl	4.0 µl
Total	10.0 µl	10.0 µl

Table 3: Conditions for Real-time PCR reaction

Step	Temperature (°C)	Duration	Cycle
Initial Denaturation	95	10 min	35
Denaturation	95	15 sec	
Annealing	54	1sec	
Melt curve stage	95	15 sec	1
	60	1 min	
	95	30 sec	

Agarose gel electrophoresis of real time PCR product

The PCR products obtained were subjected to 1.5% agarose gel electrophoresis in 1X TAE buffer adding Ethidium bromide (0.5µg/ml) to final concentration. Samples were prepared on paraffin film by mixing 5µl of PCR amplicons and 2µl of 6X loading dye. A 100bp DNA ladder was run parallel to samples loaded in wells. Electrophoresis was carried out at 50 volts for 60 minutes. The Gel was then viewed under Gel documentation system (Bio-Imaging Systems Mini Lumi) and the image was captured under UV mode.

Results and Discussion

Three species of blood parasites were identified by microscopic examination of Giemsa stained blood smear in

the present study *viz*. *Theileria orientalis*, *Babesia bigemina* and *Anaplasma marginale* which were also confirmed through PCR analysis. Studies conducted in Assam [6] also revealed prevalence of *B. bigemina*, *A. marginale* and *T. orientalis* but no *T. annulata* in crossbred cattle.

Molecular detection of *Babesia bigemina*

PCR employed for amplification of18S rRNA gene of *Babesia bigemina* positive samples revealed clear band at 1124 bp similar to work of Laha *et al.* [4] who recorded a case report of *B. bigemina* from Meghalaya. Conventional diagnostic method, namely, giemsa stained blood smear examination revealed only 10 animals positive for babesiosis out of 533 animals examined. Light microscopy although is the gold standard but in chronic infection, it is unreliable and shows a low sensitivity and usually fails to detect carrier animals with low levels of parasitaemia exhibiting subclinical infection of babesiosis. Alternatively, PCR assays have been widely used for the detection of *Babesia* parasites owing to its high specificity and sensitivity over other tests [7, 8, 9, 10]. The limit of detection ranged from one parasite per 10^5 – 10^6 erythrocytes by microscopy while by PCR is up to one parasite per 10^9 erythrocytes [11].

Absolute Quantification of *Babesia bigemina* DNA

The present analysis showed positive for *B. bigemina* DNA with mean C_T value 24.69 and standard deviation 1.77. The detection limit of the assay was assessed as 8.01 x 10^{11} DNA copies μl^{-1} of *B. bigemina*. Representative amplification plot and melt curve of sample are shown in Fig-1. Amplification of *B. bigemina* DNA by RT-PCR showing band at 88 bp (Fig-2) confirms the finding.

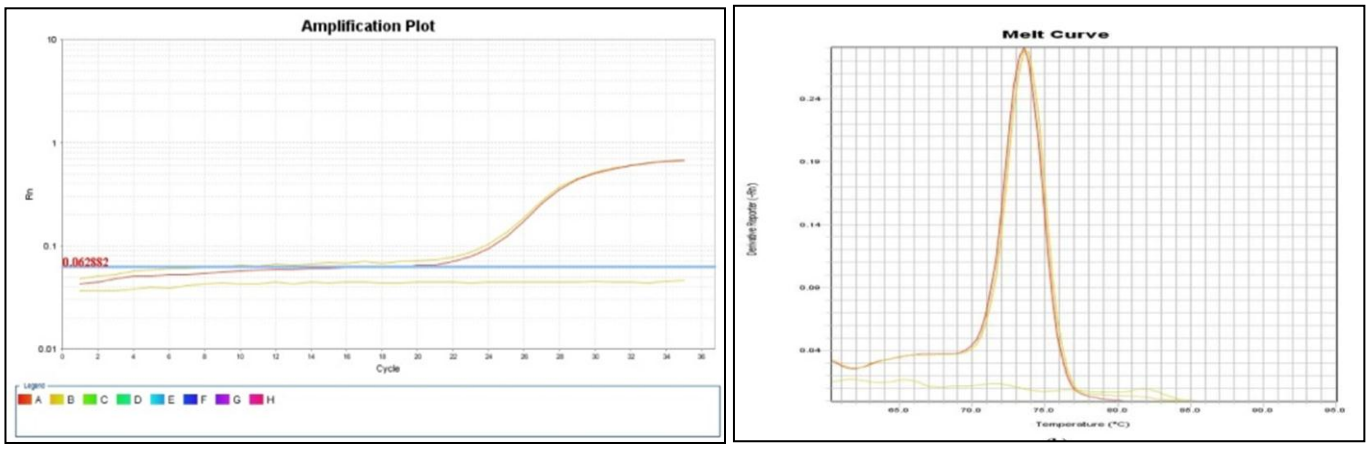

Fig 1: Amplification plot and Melt curve of *B. bigemina* quantification by RT-PCR

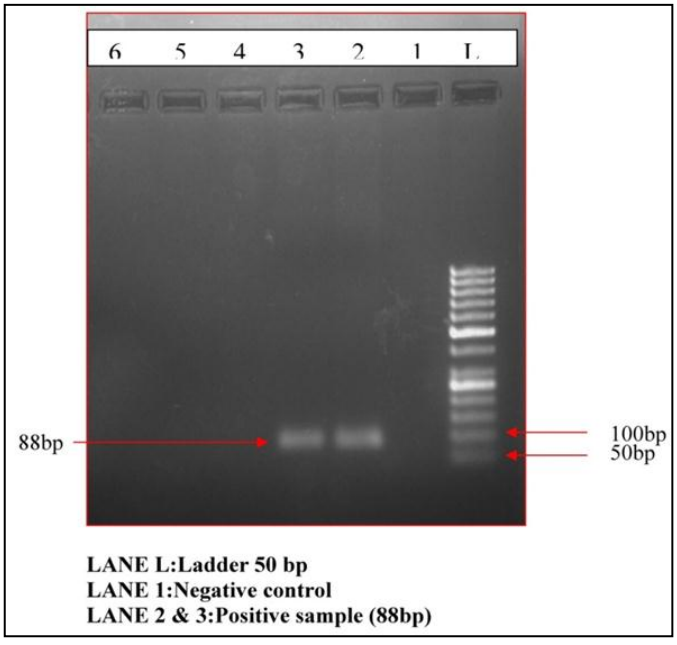

Fig 2: Gel picture showing RT amplicons of *Babesia bigemina*

Compared to other molecular methods (PCR, nested PCR etc), the real-time PCR assay described in the present study had several advantages such as increasing the laboratory throughput, shorter processing time, lower contamination risks because of the lack of post-PCR steps. These advantages make real-time PCR an attractive tool for the laboratory diagnosis of haemoparasites including *B. bigemina* infection and for a precise evaluation of the extent and duration of parasitemia in animals infected naturally. The assays performed confirmed the former finding of *B. bigemina* by [8, 12]. Two TaqMan-based real-time PCR assays were devised by [8] for the quantitative detection of bovine *Babesia* parasites, *Babesia bovis* and *B. bigemina*, and evaluated their diagnostic utility using cultured parasites and 92 field bovine blood samples collected from cattle in Brazil. The detection limits of both *B. bovis*- and *B. bigemina*–real time PCR assays were identical at the value of 2.5 parasites/_L of the infected blood. When compared with the results of nested-PCR assays, the *B. bigemina* real-time PCR showed a higher efficacy for parasite detection and also provided a rapid, sensitive, and quantitative way using continuous fluorescence monitoring of each *B. bovis* and *B. bigemina* DNA-specific dye from clinical blood samples. The quantification might also be useful for comparing different drug regimens and determine the prognostic value of the treatment of bovine babesiosis.

Conclusion

This is the first report of a method for quantifying *B. bigemina* DNA in cattle from Assam. This new and sensitive method may provide further insights into carrier status in domestic animals.

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