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Research Article

Identification of Vector Borne Blood Protozoa in Cattle and Sheep in Bangladesh

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Abstract

Babesiosis, *Anaplasmosis* and *Theileriais* are the most common vector (Tick) borne blood protozoan diseases (TBDs) in Bangladesh. This study was conducted in cattle and sheep in a different area of Bangladesh. A total number of 1150 blood samples were randomly collected from Dhaka, Sirajganj and Nikhangsori for blood smear microscopy. However, co-infections, temperature, humidity, season, farming and prophylaxis were also under consideration. From the clinically positive sample PCR was done followed by gel electrophoresis. Prevalence of blood protozoa were 100% (55), 80% (n=320), 30% (n=120), 22% (n=44), 31% (n=22), 65% (n=16) in exotic sheep, intensive farming, milk-vita area, local cattle, hill tracts and native sheep respectively. The overall prevalence was 50.17% (n=577). Among the protozoa, *Anaplasma* spp. was 43%, *Babesia* spp. 19%, *Anaplasma* spp. with *Babesia* spp. 33%, *Theileria* spp 4% and *Anaplasma* spp. with *Babesia* and *Theileria* spp was 1%. The prevalence of blood protozoa in local breed ≥50%, up to 75% and above 75% cross or pure breed were 17.58% (n= 103), 31.91% (n=187) and 50.51% (n=296) respectively. Prevalence of blood protozoa during October to March was 16.041% (n= 94) and April to September was 83.959% (n=492). In PCR *Anaplasma marginale* showed positive band as 265 bp, *Babesia bovis* in 166 bp, and *Theileria annulata* in 312 bp, *Babesia ovis* in 422bp and *Babesia motasi* in 518bp respectively. Therefore, the tick is act as vector and high humidity and temperature is the main risk factor for vector borne diseases. In conclusion, blood protozoa are the silent emerging disease in livestock and need to improve the control strategy.

Keywords: *Anaplasma* spp; Bangladesh; PCR; Prevalence; Vector

Introduction

Anaplasma spp is a gram negative rickettsial protozoan whereas *Babesia* spp, and *Theileria* spp are apicomplexan parasite which infects red blood cell (RBC), indeed transmission occurs to animal through vector bite, notably Ixodes and usually know as tick borne diseases (TBDs) (Karim et. al., 2012) and also worldwide distributed [1, 2]. Parasitism one of the major hinders in livestock farming in Bangladesh and hot humid climatic condition greatly favours the development and survival of ecto and endo parasite that makes the violence of parasitism and knows as endemic disease [3]. Cross-breed animals were more susceptible than indigenous cattle and summer season was predominant for blood protozoa followed by winter and rainy season in tropical and sub-tropical countries. Adult and female were more susceptible than young and male [4]. The clinical sign showed that high fever (105-1070F), anaemia, profuse diarrhea, ascites, sometime bloody diarrhea, coffee color urine at last stage of in *Babesiosis* [5]. About 80% of the world cattle population is affected by TBDs [6]. The TBDS in Bangladesh predominated in forest and hilly areas, high humidity and temperature aggravate the outbreaks and cross or pure animal breed is more vulnerable to infection [7]. Humid and hot climatic condition favors the growth, multiplication and survival of tick and blood protozoa in RBC that causes an outbreak of TBDs [8]. It causes anaemia, hides damage, reduces milk production and poor reproductive performance, increased mortality and global economic losses estimated at US$ 18.7 billion [9]. In blood smear microscopy, *B*abesia spp. resemble as short and long loop formation in RBC (Piroplasmosis), *Anaplasma* marginale like as pointed round dot at periphery of RBC and in *Anaplasma* centrale pointed round dot inside of RBC. Where as in *Theileria* spp RBC was annular and, round, dot, rod shape was found [10]. Biting flies transmit the disease and multiplication is increased in sexual stages due to hot humid environment [11]. Usually recovered animal act as persistent carriers. Traditional impression smear staining is a routine test used to identify *Babesia*, *Theileria* and *Anaplasma* spp. Polymerase chain reaction (PCR) is now becoming a common tool for molecular detection [12]. Recovered animal act as carrier and notably, creating a potential source of infection [13]. The multiplex PCR with species specific primer gave positive bands at 166bp, 265bp and 312bp selective for B. *bovis*, A. *marginale* and T. *annulata* in cattle respectively [14]. However, *Anaplasma marginale*, *Anaplasma* centrale, *Babesia Ovis*, *Babesia motasi* and *Theileria annulata* are the causal agent for TBDs in sheep [15]. Early diagnosis and specific treatment along with vector control are necessary to prevent death and production

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losses [16]. Therefore, considering the importance of TBDs the research work was done for the identification and molecular detection of vector borne blood protozoan infection notably *Babesia* spp, *Anaplasma* spp and *Theileria* spp in cattle and sheep in Bangladesh with seasonal variation.

Materials and Methods

Study area

An epidemiological study was carried out in Parasitology Laboratory under Animal Health Research Division in Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka from July 2016 to June 2017.

Sample collection

About 2.5 ml of peripheral blood samples were collected from different cattle and sheep farm from several of Savar, Sirajganj Sadar, Shajadpur Upazila and Nikhangsori, Chottrogram within Ethylene Diamine Tetra Acetate (EDTA) tube with ice-cool storage and shifted to the Parasitology laboratory in BLRI. A total number of 1150 blood samples were collected randomly on questionnaire basis, among them 55 from Australian sheep (BLRI), 400 from a high yielding dairy farm of Savar, 400 from high yielding cattle from milk vita Bathan area (Baghabari), 200 from native cattle (Sirajganj), 70 from native hilly cattle of Nikhangsori, 25 from native sheep.

Laboratory identification of blood protozoa through Giemsa's stain Method

Samples were examined by Giemsa's stained blood smear (GMS) microscopy (FAO, 2016) and confirmatory diagnosis through Polymerase Chain reaction (PCR). The effect of topography, season, age and sex was remaining in consideration in this study. In GMS protocol thick and thin blood smear was done. After air dry absolute methanol fixation was done and stained with 10% Giemsa's stain. After washing, air dry and emulsification, magnification under 100x objectives. The haemoprotozoa were microscopically identified based on the characteristic morphology illustrated by Soulsby [17].

Molecular Identification and confirmation of tick borne protozoa (Blood Protozoa)

DNA extraction of blood protozoa

Blood protozoan DNA was extracted using a commercially available kit (Invitrogen Purelink Genomic DNA mini kit, Cat. no. K1820-01) from blood sample through chloroform method. At a glance, 200 µl whole blood was mixed with 200 µl of lysis buffer containing 20µg/ml proteinase K and incubation was done at 55 0C for 10 minutes. Thereafter, washing and centrifugation was done at 13,000x g for 3 minutes. Finally, the spin column was discarded and collecting the eppendorf tube containing extracted DNA and stored in -20 0C in the refrigerator. The purity of genomic was visualized by using spectrophotometry (260°A/280°A) with 1.5% gel electrophoresis (Sigma Aldrich, USA). The compaction of the DNA genome was adjusted to 100ng/µl nuclease free water.

Multiplex Polymerase Chain Reaction (PCR)

PCR was carried out in a final reaction volume of 25µl in the thin walled PCR tubes to amplify genomic DNA of *Babesia, Anaplasma* and *Theileria* species. The commercially available master mix kit (Thermo Scientific) was used to amplify fragments of genomic DNA in a programmable thermocycler (Eppendorf, Germany). Furthermore, after an initial enzyme activation step at 95°C for 5 min, the reaction mixture was subjected to 35 cycles each containing a denaturation step at 95°C for 30 sec, an annealing step at 68°C for 30 sec, and an extension step at 72°C for 1.5 min. After a final elongation step at 72°C for 5 min, PCR products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and then observed under UV light.

Oligonucleotide primers were used in the PCR amplification cycle (First BASE Laboratories sdnbhd, Malaysia). The PCR images were captured though computer software (Carl Zeiss, GmbH, Germany) and the positive samples were detected by specific band size of the PCR product (Table 1).

Results and Discussion

In blood smear microscopy prevalence of TBDs was 100% (n=55) in Australian sheep, 80% (n=320) in dairy farm, 30% (n=120) in Bathan area, 22% n= (44) in native cattle, 31% (n=22) in hilly cattle, and 65% (n=16) in native sheep, this findings strongly supported where he had found that prevalence of TBDs was significantly varied on area, season and breed (Table 2) [18].

The overall prevalence of TBDs was 50.17% (n=577) in cattle and sheep in which *Anaplasma* spp was 43%, Babesia spp 19%, *Anaplasma* spp. and *Babesia* spp 33%, *Theileria* spp 4% and *Anaplasma* spp. with *Babesia* and *Theileria* spp 1% of blood protozoa (Table 3).

This result was almost similar with where stated that in Turkey, the overall prevalence was 74.78%, *Anaplasma* spp and *Babesia* spp was 41.99%, and slightly higher from where over all prevalence was 38%, there was some variation due tropical and subtropical regions variation [19, 20]. In positive case the blood protozoa magnify slight purple color. In case of *Babesia* spp. short and long loop formation was found at the periphery of RBC (Piroplasmosis). In case of *Anaplasma marginale* pointed round dot at periphery of RBC and in *Anaplasma centrale* pointed round dot inside of RBC. In case of *Theileria* spp RBC was slight triangle in shape and ring form *Theileria* spp was found (annular), sometimes oval, round, dot, rod shape was found, this findings were notably similar with (Figure 1) [21].

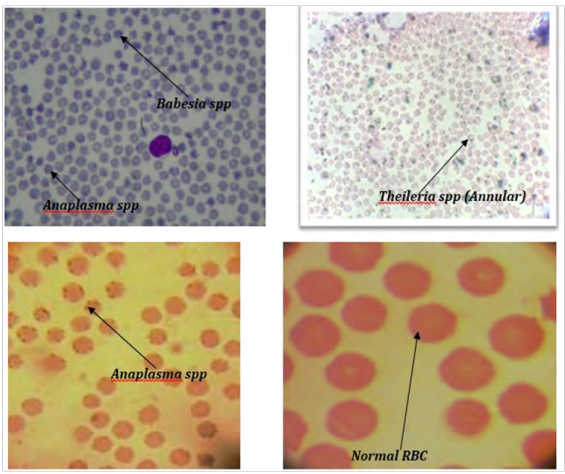

Figure 1: Blood smear microscopy of *Babesia* spp, *Anaplasma* spp and *Theileria* spp within RBC in 100x objectives.

SL No.	Primers Name	Sequence (5'-3')	Genes targeted/ Amplicon size	References
1	B. Ovis F1	[CCTGGGTAATGGTTAATAGGAACGG]	Multi-copy VESA- 1a/ 422bp	Bilgic et al., 2017
2	B. Ovis R1	[GCAGGTTAAGGTCTCGTTCGTTAAC]	Multi-copy VESA- 1a/ 422 bp	Bilgic et al., 2017
3	B. Motasi F1	[CTCTGGTACAATATGCATTGC]	Multi-copy VESA- 1a/ 518 bp	Bilgic et al., 2017
4	B. Motasi R1	[CTGGTTCCCAGATATGGTAGC]	Multi-copy VESA- 1a/ 518bp	Bilgic et al., 2017
5	A. Ovis F1	[CAGCCAGGCACTCTGCACCAC]	Multi-copy VESA- 1a/	Bilgic et al., 2017
6	A. Ovis R1	[CAACAATTGATGTGAGTGCGC]	Major surface protein-1b / 265bp	Bilgic et al., 2017
7	A. Margin F1	[GCTCTAGCAGGTTATGCGTC]	Major surface protein-1b / 265bp	Bilgic et al., 2013
8	A. Margin R1	[CTGCTTGGGAGAATGCACCT]	Major surface protein-1b / 265 bp	Bilgic et al., 2013
9	T. Anulata F	[ACTTTGGCCGTAATGTTAAAC]	Cytochrom b /312 bp	Bilgic et al., 2013
10	T. Anulata R	[CTCTGGACCAACTGTTTGG]	Cytochrom b /312 bp	Bilgic et al., 2013
11	B. Bovis F	[CAAGCATACAACCAGGTGG]	Multi-copy VESA- 1a/ 166bp	Bilgic et al., 2013
12	B. Bovis R	[ACCCCAGGCACATCCAGCTA]	Multi-copy VESA- 1a/ 166bp	Bilgic et al., 2013

Table 1: Positive samples were detected by specific band size of the PCR product.

Type of Sample area	No. of total sample	Positive Sample	Negative sample	% of Positive sample
Australian sheep/BLRI (Pure)	55	55	0	100%
High Yielding cattle (Cross), Milk Vita Baghabari.	400	120	280	30%
Native cattle, Sirajganj	200	44	156	22%
Native Hilly cattle, Nikhongchori	70	31	39	44%
On recognized Dairy Farm, Jamghora, Savar	400	320	80	80%
Native sheep, Savar	25	16	9	21.29%
Total	1150	586	564	50.96%

Table 2: Prevalence of Blood Protozoa on region basis.

Total Number of Positive Sample	Type of blood protozoa	Positive no. of spp. of blood protozoa	% of positive spp. of blood protozoa
586 (Out of 1150) blood sample)	*Anaplasma* spp	251	43%
	Babesia spp	113	19%
	Anaplasma spp + *Babesia* spp	193	33%
	Theileria spp.	23	4%
	Anaplasma spp. with *Babesia* and *Theileria* spp	6	1%

Table 3: Prevalence of Blood Protozoa on species basis.

However, *Anaplasma marginale* shown positive band as 265 bp, *Babesia bovis* in 166 bp, and *Theileria* annulata in 312 bp, in cattle blood whereas *Anaplasma marginale* in 265 bp *Babesia* ovis in 422bp, *Babesia* motasi in 518bp and *Theileria* annulata in 312 bp in sheep blood respectively, this finding was clearly significant with (Figure 2)[22].

However in seasonal study in Bangladesh it was observed that April to September environmental temperature is arise (above 30°C, sometimes 40°C) and humidity is above 70% (sometimes above 90%) that triggers the multiplication of tick biologically and also multiplication of TBDs protozoa both in tick and animal blood that progresses havoc of TBDs in high yielding animal and local animal act as carrier, this clarification also clearly justified the same findings, there he stated that infection in sheep 52% due to hot humid environmental condition [23].

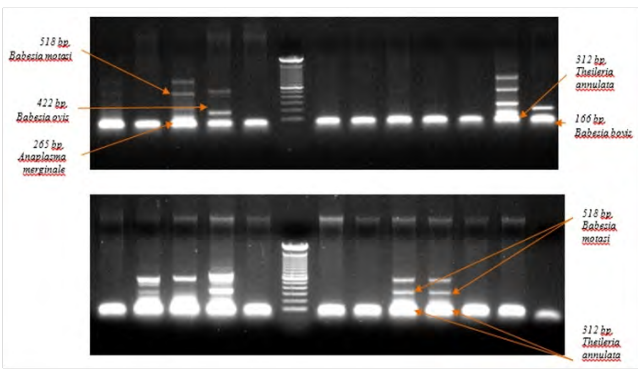

Figure 2: Molecular detection of *Anaplasma* spp, *Babesia spp* and *Theileria* spp. by multiplex PCR (Gel electrophoresis amplified DNA).

In addition, when environmental temperature is 30°C or below and humidity is below 70% notably during October to March animal act as carrier but not showing clinical sign, and prevalence of blood protozoa during October to march was 16.041% (n= 94) and April to September was 492 83.959% (n=492), this result was verified the findings (Table 4) [24].

Name of Month	Average temperature in BD	Average Humidity in BD	Prevalence of Blood protozoa
October to March	30°C or below	70% or below	94 (16.041%)
April to September	30°C to 40°C	70% to 90%	492 (83.959%)
Total number of positive sample (Out of 1150)			586

Table 4: Prevalence of Blood protozoa on the basis of temperature and humidity.

In case of high yielding animal (above 60% cross breed) and 100% pure breed show high clinical sign and even death in high percentage and response to treatment is low. In local breed or 50% crossed breed, upto 75% crossed breed and above 75% or even pure prevalence of blood protozoa was 17.58% (n= 103), 31.91% (n=187) and 50.51% (n=296) respectively (Table 5). The findings compared with where signifying that the crossbred are more susceptible to TBDs than local animal and consequently, strongly supported by where they were stated that, TBDs caused high morbidity, mortality and economic losses in high yielding animal than local breed of ruminants [25-27].

Type of breed (on the basis of farmers history)	Positive number of sample	Prevalence of blood protozoa
Local or Crossed up to 50%	103	17.58%
Crossed above 50% up to 75%	187	31.91%
Crossed above 75% to above or pure)	296	50.51%
Total number of positive sample 586 (out of 1150 sample)		

Table 5: Prevalence of blood protozoa on the basis of breed.

Conclusion

Tick borne blood protozoan disease (Babesiosis, Anaplasmosis, and Theileriosis) are now a days a crucial factor for livestock production in Bangladesh. Local animal act a as carrier but it indicating future havoc in livestock industry especially high yielding exotic animal (70 % to 100 % pure breed). Moreover, they are more susceptible to TBDs and it is very difficult to control because high temperature and humidity provoke the tick multiplication. To introduce high yielding animal in a farm strict biosecurity is essential for farming.

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Disclaimer

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