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Research article

Phylogenetic analysis revealed the presence of *Babesia ovata* in cattle of Chattogram Division, Bangladesh

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A B S T R A C T

Bovine babesiosis is the most economically important haemoprotozoan disease caused by *Babesia* spp. The present study was conducted in selected hilly, coastal and plain areas of Bangladesh to determine the prevalence and identification of risk factors along with the molecular characterization of *Babesia* spp. Using traditional blood smear examination and Giemsa staining, 1.43% (n=1680) of cattle were was found positive for *Babesia* infection during this study. The prevalence of babesiosis was 1.17% (7 out of 600 cases) in the hilly areas; 1.67% (10 out of 600 cases) in the coastal areas and 1.46% (7 out of 480 cases) in the plain areas. The prevalence of *Babesia* spp. was found higher (P>0.05) in crossbred cattle compared to local or indigenous cattle. Seasonal variation of disease prevalence indicated that babesiosis was significantly higher (P<0.05) in the summer, followed by the rainy and winter months. Animals aged 6-18 months were found more susceptible to the infection based on collected samples from three different geographic areas of Bangladesh. Animals kept on the natural soil-type floor (mati floor) were found to be more affected by babesiosis than those kept on the cemented floor (Paka floor). No statistical difference was seen between male and female affected animals. Further molecular studies through PCR and DNA sequencing of all microscopically positive samples (by amplifying *the 18S rRNA* gene) confirmed the genus and their phylogenetic relationship. Interestingly, two distinct clusters were found through phylogenetic analyses of which one was closely related to *Babesia ovata*, and another cluster was closely related to *Babesia bigemina*. The knowledge will ultimately help develop effective control strategies, which mainly depend on vector control and farm management.

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1. INTRODUCTION

Bovine babesiosis is an economically important vector-borne disease of cattle caused by the blood protozoa of the genus *Babesia* (McCosker, 1981). The clinical form of the disease is characterized by anemia, fever, hemoglobinuria, jaundice, abortion in female animals and occasional nervous symptoms and

death. Among different species of *Babesia* that affect the cattle, *B. bovis* and *B. bigemina* are the most economically important species globally. The economic losses comprise mortality, loss of milk/meat production, and associated costs of control measures. However, the disease can significantly impact the international cattle trade for any country exporting meat and meat products (Bock et al., 2004). A reliable and accurate diagnosis is essential for effective monitoring, treatment and control of babesiosis (Bashir et al., 2009). However, routine diagnosis is usually accomplished by microscopy, while these techniques are suitable only for detecting acute and heavy infections (Aziz et al., 2014). Therefore, molecular tests such as PCR-based tools have been developed with high sensitivity and specificity (Almería et al., 2001). The technique has been proven to provide reliable results in detecting of *Babesia* spp in blood, particularly when the parasitemia is very low or not detectable in microscopic examination (Figueroa et al., 1992).

In Bangladesh, the prevalence of babesiosis in cattle has been reported in different areas by several investigators. Most published studies were based on the classical blood smear examination (Giemsa stain). Theprevalence was recorded as 1-14% with a variable sample size (Samad et al., 1989; Siddiki et al., 2010). These reports also investigated the possible risk factors associated with *Babesia* prevalence and their associations with host age, sex, climate and seasons. Here we attempted to investigate the prevalence of babesiosis at different locations in the Chattogram Division with particular emphasis on the molecular characterization of the organism for the first time in the country. The present study was conducted with the following specific objectives (a) to determine the prevalence and risk factors of babesiosis in cattle in selected areas of Bangladesh (b) Molecular identification and characterization of *Babesia* spp. along with the phylogenetic analyses.

2. MATERIALS AND METHODS

Study areas and seasons

The study was conducted in the hilly and coastal areas of 4 different southern districts of Chattogram Division, Bangladesh. These include Chattogram Metropolitan area (plain area), Bandarban sadar and Ruma upazila of Bandarban District, Rangamati sadar and Kawekhali upazila of Rangamati district (hilly areas); Noakhali sadar and Suburnochar upazila of Noakhali District and Lakshmipur sadar and Ramgoti upazila of Lakshmipur District (coastal areas). The study was conducted for a period of 12 months, and the total period was divided into three seasons; namely, i) winter (November to February), ii) summer (March to June) and iii) rainy (July to October).

Target animals

Holstein Friesian (HF) crossbred and local cattle (Red Chittagong /Indigenous/Non-descript) were selected as target animals. Cattle were classified into three subgroups to determine the age and breed susceptibility of different parasites. For HF crossbred cattle, it was calf (≤1 year), young (>1 – < 2.5 years) and adult (≥2.5 years), and for local cattle, it was almost the same but the age limit differs for young (>1-3.5 years) and adult cattle (≥3.5 years).

Study design and sample collection

A cross-sectional study with the random sample collection technique was considered to perform the current investigation. A standard questionnaire was used to collect demographic data such as breed, age, sex, animal identification, floor type (e.g. cemented or mud) and farmer's economic status. Farmer's economic status was categorized into (i) poor (ii) moderate and (iii) ultra-poor. Blood samples were collected at one point every month from each selected area. An individual animal was considered as a sampling unit. About 3-5 mL of blood was collected from the jugular vein in a blood collection tube containing EDTA. Samples were preserved at -25^0 freezer until further analyses. A total of 1680 whole blood samples (600 from each hilly and coastal area and 480 from plain areas) were collected from cattle randomly, irrespective of the clinical signs.

Microscopic examination:

During examining the samples, two thin blood smears were prepared and subsequently air-

dried and fixed with 100% methyl alcohol for 3-5 min. Next, the prepared thin blood smears were stained with Giemsa stain for 25-30 min following the previously described methods (Urquhart et al. 1996).

DNA extraction and PCR assay

Total genomic deoxyribonucleic acid (gDNA) was extracted from the whole blood samples using the PCI method (Barbaro et al., 2004). PCR amplification was carried out using a 2720 thermal cycler (Applied Biosystems, USA). The diluted DNA template was transferred into a PCR tube and the reaction volume composed of 12.5 ul of GoTaq® G2 Hot Start Green Master Mix (2X Green GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 4 mM $MgCl_2$), 1.5 ul (10 picomole) of each primer (Table 1), 2ul of template and 7.5ul of nuclease-free water. To identify the *Babesia* spp from DNA samples PCR amplifications were performed at the following thermal conditions: 94°C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 61 °C for 45 sec, 72 °C for 1 min and followed the final extension step at 72 °C for 10 min.

PCR amplicons were purified using a commercial PCR purification Kit (Favorgen, Taiwan). The purified DNA was then measured by fluorimeter for concentration (ug/ul) before sending for DNA sequencing through commercial sources. Purified PCR products were used for sequencing by a commercial supplier (Bioneer Corp, South Korea) for DNA sequencing. The sequences were initially checked for sequence similarity using a BLAST from the NCBI. The multiple alignment analysis was performed using the clustalw program, while the phylogenetic analysis was aided by the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distances were neighbor-joining method computed using the p-distance method using the MEGA software, version-5. The tree stability was estimated by a bootstrap analysis for 1,000 replications. The obtained information was imported, stored and coded accordingly using Microsoft Excel2003 to STATA/IC-11.0 (Stata Corporation College Station, TX, USA) for analysis. Significance was determined when $P<0.05$.

3. RESULTS AND DISCUSSION

Prevalence of babesiosis in cattle

Conventional microscopy was used for screening field samples before molecular tests were performed. Altogether, 1680 whole blood samples (600 from hilly areas, 600 from coastal areas and 480 from plain areas of Chattogram Division) were collected from cattle randomly without considering the clinical signs. Through microscopy (Giemsa stain), 1.43% of animals (n=1680) were positive for *Babesia* spp infection. As expected, substantial variation in the prevalence of babesiosis in different geographic areas was observed in this study (Fig 1). The prevalence of babesiosis was 1.17% (7 out of 600) in the animals from hilly areas; 1.67% (10 out of 600) in the coastal areas and 1.46% (7 out of 480) in the plain lands. However, these were not statistically significant.

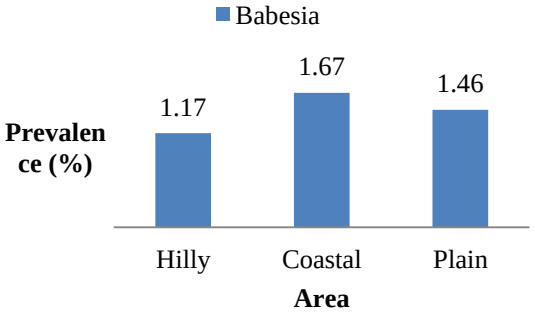

Figure 1. Area wise prevalence of *Babesia*. Higher prevalence was recorded in coastal area in compared to hilly and plain area.

Table 1. Details of the primers used for amplification of DNA of *Babesia* sp.

Target gene	Primer name	Sequence	Amplicon size (bp)	Reference
18S rRNA	Baf-F Baf-R	5' -TTTCTGMCCCATCAGCTTGAC -3' 5'- CAAGACAAAAGTCTGCTTGAAA -3'	422-440	Hilpertshauser et al., 2006

We investigated the association of different categorical variables such as seasons, breeds, age of the host, sex of the cattle, floor type at cattle sheds and economic condition of the animal owners with the prevalence of babesiosis (Table. 2). As predicted, crossbred cattle were highly susceptible to babesiosis compared to local or indigenous cattle. Animals were more affected in summer (P<0.05) followed by rainy and winter seasons. Cattle aged 6-18 months were more prone to suffer from the disease than other age groups (Figure 2). Animals kept on the soil-type floor (mati floor) were most (P>0.05) susceptible to babesiosis, followed by those on partially-cemented and cemented floors (Paka floor). No significant differences were found between male and female animals or the economic status of the animal owners with the relative prevalence of babesiosis.

Table 2. Association of different categorical variables (season, sex, breed, floor type) with blood parasite, *Babesia* by using Chi-square test

Variables	Categories (N)	Prevalence (%)
Season	Summer (560)	2.50 (14)***
	Rainy (561)	1.25 (7)
	Winter (559)	0.54 (3)
Sex	Male(400)	1.00 (4)
	Female (1280)	1.56 (20)
Economic condition	Poor (523)	1.72 (9)
	Moderate (1087)	1.38 (15)
	Ultra poor (70)	0.00 (0)
Floor	Paka (1011)	1.09 (11)
	Mati (669)	1.94 (13)
Breed	Cross (455)	1.53(7)
	Local (1225)	1.39 (17)

*** Significance at P<0.001.

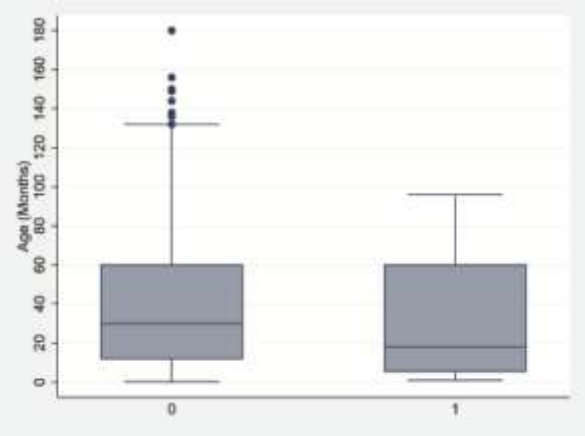

Figure 2. Box plot showing association between babesiosis with age, where "0" indicate negative and "1" indicate positive. The analyses indicate that 50% of infected animals were within the age group of 6-18 months.

Molecular identification of *Babesia*

Further molecular investigation of 24 positive samples (verified by microscopy) was achieved using PCR based on amplifying 18S rRNA gene fragments. All these amplicons produced characteristic bands of 421-440 bp on agar gel electrophoresis (Figure 3).

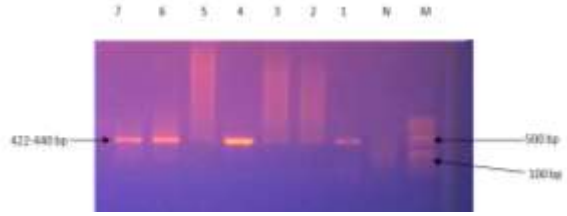

Figure 3. Amplification of the genomic DNA of *Babesia* sp. from blood of cattle by using *18SrRNA* gene. Lane M is for 100 bp DNA ladder; N is for negative control; Lanes 1-7 is suspected samples; Lanes 1, 4, 6 and 7 having amplicons of 422~440 bp indicated the positive cases of babesiosis.

Sequence data upload in NCBI

The six sequence data generated from this study were submitted to the NCBI database, and the following accession numbers were assigned: KU837251 (bab1). KU877881 (bab2). KU947081 (bab3), KU947082 (bab4), KX228228 (bab5) and KX228229 (bab6).

Sequence similarity and phylogeny

A phylogenetic tree inferred based on 18S rRNA gene sequences of *Babesia* spp isolates is shown in Figure 4. The 18S rRNA nucleotide sequences of *Babesia* spp were obtained from six randomly selected samples named as bab1, bab2, bab3, bab4, bab5 and bab6. The sequences (bab1, bab2, bab3, bab5 and bab6) were also compared with other known sequences published in GenBank and revealed 97% identities to *B. ovata* isolates from China (AY603400 and AY603403) and Korea (AY081192). On the other hand, sequence bab4 revealed 94% identities of *B. bigemina* isolates from India, China, Brazil and Australia.

4. Discussion

Babesiosis is an important protozoan disease causing a substantial economic loss in cattle in Bangladesh.. While few reports are published on this important protozoa, a variable prevalence rate (1-16.63%) has been reported in cattle in selected areas of the country (Siddiki et al., 2010; Mohanta and Mondal, 2013).

Conventional detection tools like microscopy are also insufficient for a reliable diagnosis of babesiosis. Therefore modern molecular tools were used to unravel the actual scenario of this important protozoa. The overall prevalence of babesiosis recorded during this study was 1.43% (n=1680). This observation was consistent with other investigators who have reported 1.52% to 2.29% prevalence in different parts of the country (Siddiki et al., 2010; Al Mahmud et al., 2015; Rahman et al., 2015). In Pakistan, this prevalence was recorded as 1.75% for *B. bigemina*, the most common species in cattle (Afridi and Ahmad, 2005). However, some reports involving Bangladeshi cattle have indicated a higher prevalence ranging from 3.28% (in subclinical cases) to 7.17% in some areas (Alim et al., 2012; Samad et al., 1989). Further higher prevalence of up to 16% was also reported in other reports where samples were collected from northern Districts of Bangladesh (such as Mymensingh and

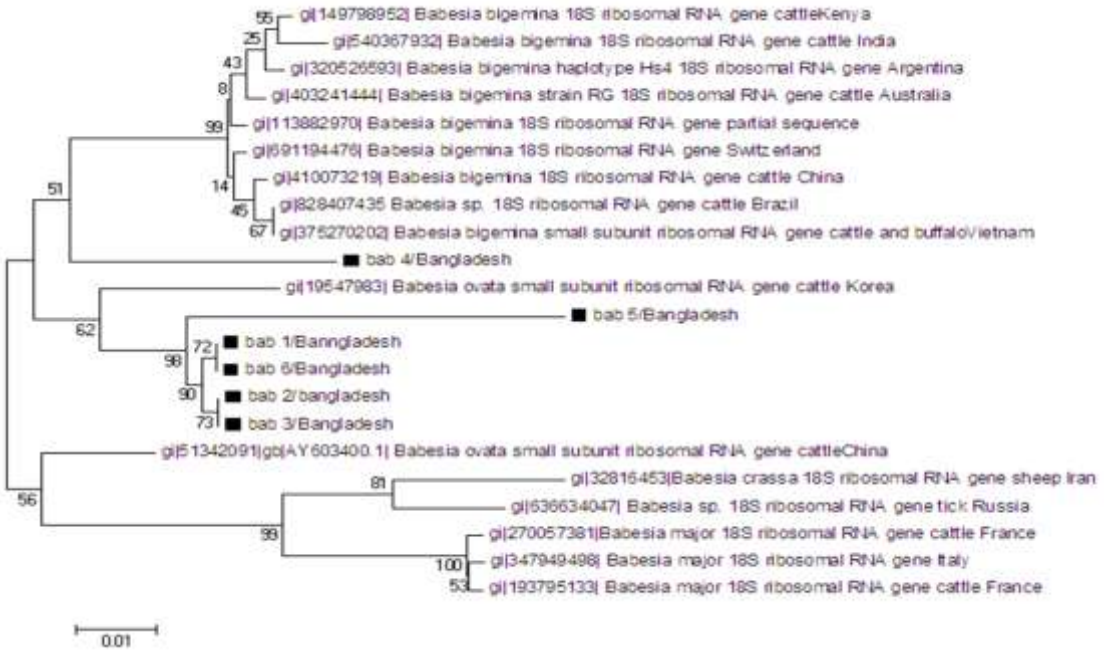

Figure 4. Phylogenetic tree generated from the six nucleotidic sequences of *Babesia* 18S gene and sequence fragments of this gene deposited in GenBank from different countries, the accession numbers and countries are shown with isolate names. The 18S sequences obtained in this study were indicated with black boxes.

Sylhet) (Banerjee et al., 1983; Nath and Bhuyian., 2013). One would assume that these variations in the prevalence of babesiosis may be due to climate-associated factors and the distribution of relevant vector ticks that might be active and available in different seasons.

During this study, the maximum prevalence of babesiosis was recorded in coastal areas (1.67%), followed by plain areas (1.46%) and hilly areas (1.17%). These were not consistent with other previous reports, which recorded as high as 16.67% prevalence in three hilly districts of Chittagong and Khagrachori hilly Districts in Chittagong (Alim et al., 2012; Mohanta and Mondal, 2013). Again, the prevalence of babesiosis in coastal areas was reported as high as 4.62% (in the Noakhali coastal area of Bangladesh) by previous investigators, which is inconsistent with this present study.

Seasonal occurrence is another crucial factor associated with the prevalence of blood parasitic diseases like babesiosis. During the present study, the prevalence of babesiosis was significantly higher in the summer season, followed by the rainy and winter seasons. A similar seasonal influence was observed by other researchers (Al Mahmud et al., 2015; Alim et al., 2012), who reported a higher prevalence in the summer season in some northern parts of Bangladesh. These seasonal influences could be linked to the variations in vector load, which depends on the humidity, rainfall and overall climatic condition (Radostits et al., 2006). Further analyses of meteorological data and the geographic information of the study areas in Bangladesh can provide important information which was not conducted during this study.

No significant differences were recorded among male and female animals with the incidence of babesiosis. A similar observation was reported earlier by other investigators (Al Mahmud et al., 2015; Alim et al., 2012; Kamani et al., 2010). Animals aged 6-18 months were found to be more susceptible compared to other age groups. Other researchers reported a similar observation that mentioned high prevalence in animals aged >1-2.5 years (Chakraborti, 2002; Chowdhury et al., 2006; Rahman et al., 2015). Calves generally resist acute infection because of innate immunity through colostrum. This colostral antibody confers protection to calves that may persist for up to 6 months for *B. bovis* and 3 to 4 months for *B. bigemina* (Wright, 1990).

The prevalence of babesiosis in this study was comparatively higher in crossbred cattle than in local cattle, although the observations were statistically insignificant. The findings were similar to the observation by previous investigators (Al Mahmud et al., 2015; Alim et al., 2012; Chowdhury et al., 2006). The zebu cattle *B. indicus* presents high innate resistance to *B. bovis* and *B. bigemina* infections compared to *B. taurus* and their crosses (Bock et al., 1997). The lower prevalence in local cattle might be linked to constant minimum exposure to infections, the development of passive immunity, and the genetic makeup of each cattle breed (Siddiki et al., 2010). When farm housing conditions were assessed, the prevalence of babesiosis was recorded higher in animals kept on the mati floor than on the paka floor. Similar finding was observed earlier and thought to be linked with higher vector load in muddy floor (Nath and Bhuyian, 2013).

Along with the classical Giemsa staining technique, PCR-based molecular techniques were applied during this study. All the positive samples (from microscopy) were successfully amplified by PCR assay using specific primers for the *Babesia* 18SrRNA gene. Further sequencing confirmed the genus and species of the protozoa, which were analysed for phylogenetics. The analyses revealed that five isolates are separately branched but clustered together with *Babesia* ovata, which was not previously reported in Bangladesh (Alim et al., 2012; Samad et al., 1989). Notable that all these reports in Bangladesh were based on morphological examination and accurate identification is not always possible based on microscopy. Earlier, *Babesia ovata* were reported in Japan (Minami and Ishihara, 1980), China (Bai et al., 1990), and South Korea (Suh, 1987) and the present report is the first record of this species in Bangladesh. All the five isolates from the present study are clustered together with *Babesia ovata* isolates recorded from Korea. The bab4 isolate is separately branched

in the phylogenetic tree and forms a cluster with *B. bigemina*, which also shows the highest nucleotide identity as found through BLASTn analyses. This bab4 isolates cluster together with the *B. bigemina* isolates of India, China and Vietnam with very short branch lengths indicating little genetic diversity among the species. The results from the present study indicate that both *B. bigemina* and *B. ovata* are present in Bangladeshi cattle sporadically. A further comprehensive study with a vast sample number and geographic areas is essential to understand the molecular epidemiology of this important hemoparasite.

5. CONCLUSION

The present study was aimed at determining the prevalence of babesiosis in Bangladeshi cattle along with identification of risk factors and subsequent molecular characterization of *Babesia* spp. Microscopic examination was complemented with molecular analyses, and babesiosis was detected from different geographic areas of Bangladesh. Different variables including age, sex, season, housing condition, were assessed with variable results. Seasonal incidence and prevalence were found to be significant, while the sex of the animal was not related to the occurrence of bovine babesiosis. Further analyses with PCR assay and subsequent sequence analyses and phylogenetics revealed novel species *B. ovata* as the causal agent in some animals. The other most common species found was *B. bigemina* in cattle in study areas and relevant tick vectors were available in these selected study areas. Further studies can be concentrated on identifying tick vectors along with molecular detection of organisms from both vectors and hosts from different country districts. This finding will develop a national database of vectors and hosts and help policymakers develop suitable control strategies to combat bovine babesiosis.

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