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Research Article

Molecular identification and characterization of *Trypanosoma* infection in cattle in Chittagong Metropolitan area, Bangladesh

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ABSTRACT

Trypanosomiasis is an important infectious disease of animals including cattle. The present study attempted to ascertain the prevalence of trypanosomiasis and relevant risk factors associated with the disease. All the samples for this study were collected from different dairy farms inside Chittagong Metropolitan Area (CMA) under Chittagong district of Bangladesh. The preliminary screening was aided by conventional thin smear technique (Giemsa stain) while modern molecular tools like PCR was used for accurate and reliable identification and characterization. Only three samples (out of 480 samples tested) were found positive through thick blood smear and PCR assay. All these three samples were from Holstein Friesian crossbred cow and none of the local indigenous breed was found positive. Data analyses revealed highest prevalence of trypanosomiasis in rainy season when compared to summer and winter season. Animals older than six years old were found to be infected by trypanosomiasis. PCR assay was applied to all three screened positive samples targetting *18S rRNA* gene fragment were successfully amplified. Further sequencing of PCR products and phylogenetic analyses revealed that the isolates recovered during this study were closely related to the isolates previously reported from Egypt, Taiwan, Thailand and Japan.

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1. INTRODUCTION

Trypanosomiasis is a chronic devastating diseases that affect human and all worm-blooded animals and caused by flagellated protozoans of the genus *Trypanosoma* (Laohasinnarong et al., 2011). It has great economic importance in the livestock industry due to high mortality and severe production losses in cattle with chronic trypanosomiasis. Trypanosomiasis not only caused the direct loss in the form of mortality, morbidity, infertility of the infected animals and costs of treatment or controlling the disease but also caused the indirect losses, through exclusion of livestock and animal power from the huge fertile tsetse infected areas (Kidane-Mariam, 2000). There are several species of trypanosomes in livestock which includes *Trypanosoma brucei, T. vivax, T. Congolense, T. evansiand T. theileri* (Garcia et al., 2011a; Nantulya, 1990; Sekoni et al., 1988). Several species of Tsetse fly play the major role in the the transmission of the disease (Ford and Katondo, 1977).

Different techniques are used for the detection of trypanosomes from the blood. Although parasitological approach remains the most appropriate method for the clinical diagnosis in field condition, it lacks sensitivity and specificity (Thumbi et al., 2008). Moreover, serological tests are not reliable for differentiating current or post treatment infections (Ahmed et al., 2013). This is replaced by modern molecular tools and polymerase chain reaction (PCR)

technique has been widely used in detecting trypanosomes because it has high sensitivity and specificity (Malele et al., 2003). For molecular analysis, various target sequences such as kinetoplast DNA, ribosomal DNA, internal transcribed spacer region and VSG genes are reliable targets for the detection of *Trypanosoma* spp. (Sengupta et al., 2010).

Though livestock contribute in national economy of Bangladesh, its development is hindered by different constraints. The most important constraints of livestock sectors are widespread diseases, including trypanosomiasis. Moreover the climatic condition of Bangladesh favor the growth and multiplication of vector and uncontrolled cattle trading systems also play role in disease production. Until now, a number of epidemiological and parasitological investigations were carried out on commonly available blood parasites in different regions of Bangladesh. However, no molecular investigation of haemoprotozoan diseases especially trypanosomes were reported. Earlier report on prevalence (5 out of 857 cattle) of *T. theileri* was the very first of its type where microscopic identification was performed (Rahman et al., 1982; Samad and Shahid-Ullah, 1985). These reports were interesting as several other parts excluding Chittagong was the study areas. The aim of the present study was to determine the prevalence, molecular identification and characterization of trypanosomiasis in cattle.

2. MATERIALS AND METHODS

2.1 Study area

The study was conducted in the selected farms located in Chittagong metropolitan area of Bangladesh. The survey was done to the randomly selected commercial dairy farms as well as individually reared animals.

2.2 Study periods

The study was conducted during January, 2013 till December, 2013 and the total period was divided into three different seasonal categories such as i) winter (November to February), ii) summer (March to June) and iii) rainy (July to October).

2.3 Target animals

Holstein Friesian (HF) crossbred and local cattle (Red Chittagong breed /Indigenous/Non-descript types of cattle) were selected as study animals. To determine the age and breed susceptibility of different parasites, cattle were classified into three subgroups. For HF crossbred cattle, it was calf (≤1 year), Young (>1 - < 2.5 year) and Adult (≥2.5 years) and for local cattle, it was somewhat different such as young groups (>1-3.5 years) and adult groups (≥3.5 years) (Sastrt et al., 2005).

2.4 Target sampling

In each month blood samples were collected from different selected areas at random basis. A questionnaire was used to record information like owner's name and address, animal Identification (ID), breed, age, sex, season, housing history and farmer's status etc. Farmer's status were categorized into viz i) Poor ii) Moderate and iii) ultra-poor. Housing history was treated as floor type and categorized into paka and mati floor.

2.5 Sample collection, preservation and examination

About 3-5ml of blood was collected from the jugular vein in an EDTA vial. After that the tube was kept in a cool box and then transferred in to the Parasitology laboratory, Chittagong Veterinary and Animal Sciences University (CVASU). Examination was done immediately after coming into the laboratory and rest of the blood samples were stored at -20^{0}C until required for molecular processing. Three wet smear and two thin smears were made. Wet blood smear were examined at low power (10X). To observe the Trypanosome, two thin blood smears were prepared and subsequently they were air dried and fixed by 100% methyl alcohol for 3-5 min. The prepared thin blood smears (Hendrix and Robinson, 2006) was stained with the Giemsa stain for 25-30 min. After rinsing with water, the stained blood smears were air dried and examined under binocular microscope (1000X) with immersion oil for the identification of blood parasites (Urquhart et al., 1996).

2.6 DNA extraction

The protocol for DNA extraction was followed as mentioned in the PCI method and the protocols have been briefly described in section 3.3.6 earlier.

2.7 PCR assay

The diluted DNA template was transferred into PCR tube and the reaction volume composes of 10µl master mix (Taq DNA polymerase, dNTPs, Mg Cl2 and reaction buffer, Promega®, catalog # M7122), 1µl (20 Pico mole) of each primer (Table 1), 2µl template and 6 µl deionized water. PCR amplification was carried out using a thermal cycler. The samples were programmed to a temperature step cycles at 94^{0}C for 4 min (min) initial denaturation followed by 40 cycles consisted of 60s at 94^{0}C, 90s at 58^{0}C and 120s at 72^{0}C. In case of semi nested PCR, 2µl of amplified products from the first run was added to the PCR tube as a template and other ingredients was same except for primer pair. The amplification programme was same to the first run of PCR except for 25 cycles.The mixture was examined for

the presence of DNA fragment by loading 5µl of PCR product into each well of 1.5% agar rose gel stained with Ethidium bromide. A 100 bp DNA ladder was included into the first well. The samples were run for 30 min at 130 volts and then finally washed under running tape water and visualized under UV illumination.

2.8 PCR products purification

The protocol for PCR product purification was followed as mentioned in the manufacturers instruction. The commercially available PCR purification kit (Favorgen, Korea®) was used for purification.

2.9 DNA Sequencing

Purified PCR products were sent for sequencing by commercial suppliers (Bioneer Corp, South Korea) by Sanger sequencing method.

2.10 Phylogenetic analyses

Nucleotide sequences were initially checked using a BLAST search hosted by the National Center for Biotechnology Information for the comparison with other known nucleotide sequences. By the BLASTN homology search the nucleotide sequences were determined that is corresponding with *Trypanosomasequences* published in GenBank. The multiple alignment analysis was performed using the Clustal W program (Thompson et al., 1994). Phylogenetic analysis was performed by neighbor-joining method. The evolutionary distances were computed using the p-distance method(Nei and Kumar, 2000) using the MEGA software, version 5 (Tamura et al., 2011). The tree stability was estimated by a bootstrap analysis for 1,000 replications (Felsenstein, 1985).

2.11 Statistical analyses

The obtained information was imported, stored and coded accordingly using Microsoft Excel-2003 to STATA/IC-11.0 (Stata Corporation College Station) for analysis. Descriptive statistics was expressed as proportion with Confidence Interval. The result were expressed in percentage with P-value for Chi-Square Test. Significance was determined when $P<0.05$.

3. RESULTS

3.1 Parasitological examination

After initial screening by microscopy, only three samples were found positive (n=480) which was further confirmed by molecular investigation by PCR and DNA sequencing. The characteristic morphological features of the trypanosomes were validated by taking photographs. Several organisms were found in each microscopic field, under high power objective (Fig.1). The organism appeared leaf-like with a single flagellum under oil immersion. Further data analyses also revealed epidemiological information which was not available in Bangladesh context earlier. No statistically significance differences between different age groups, however all three affected animals were older and more than six years of age (Fig. 2). The prevalence of trypanosomiasis was only recorded in crossbred cattle (Table 2). The prevalence of trypanosomiasis was recorded in animals that kept in paka (cemented floor) whereas no infections were recorded in animals that kept in mati floor (uncemented floor) significant. The management of the farm (from where positive cases were found) was not satisfactory as found by poor drainage system and high incidence of flies that might be responsible as vectors of the protozoa.

Table1: Details of the primers used for amplification of gene fragments of Trypanosoma sp.

Target gene	Primer name	Sequence	Amplicon size (bp)
18S rRNA	18 ST n F2	5'- CAACGATGACACCCATGAATTGGGGA-3'	700 - 800
	18 ST n R3	5'- TGCGCGACCAATAATTGCAATAC-3'	
18S rRNA	18 ST n F2	5'- CAACGATGACACCCATGAATTGGGGA-3'	600 - 700
	18 ST n R2	5'- GTGTCTTGTTCTCACTGACATTGTAGTG-3'	

Table1: Details of the primers used for amplification of gene fragments of *Trypanosoma* sp.

Table 2: Association of different categorical variables with the incidence trypanosomiasis (Chi-square test), all positive cases was detected in rainy season.

Factors	Factor level	Prevalence (%)
Breed	Cross	0.97
	Local	0.00
Season	Summer	0.00
	Rainy	1.88*
	Winter	0.00
Sex	Male	0.00
	Female	0.78
Floor category	Paka	0.76
	Mati	0.00
Farmers economic condition	Poor	0.00
	Moderate	1.10
	Ultra poor	0.00

*Significance at P<0.05

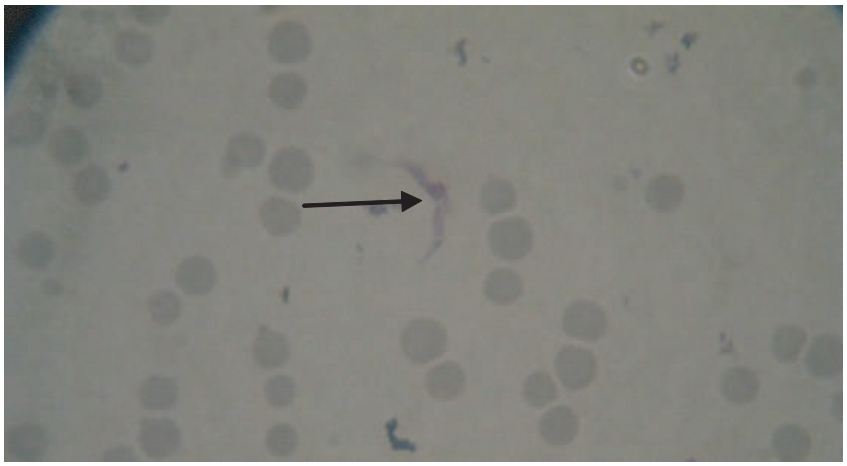

Fig 1: Snapshot from Giemsa stained thin blood smear indicating *Trypanosoma* sp. located outside the RBCs (Arrow) × 100

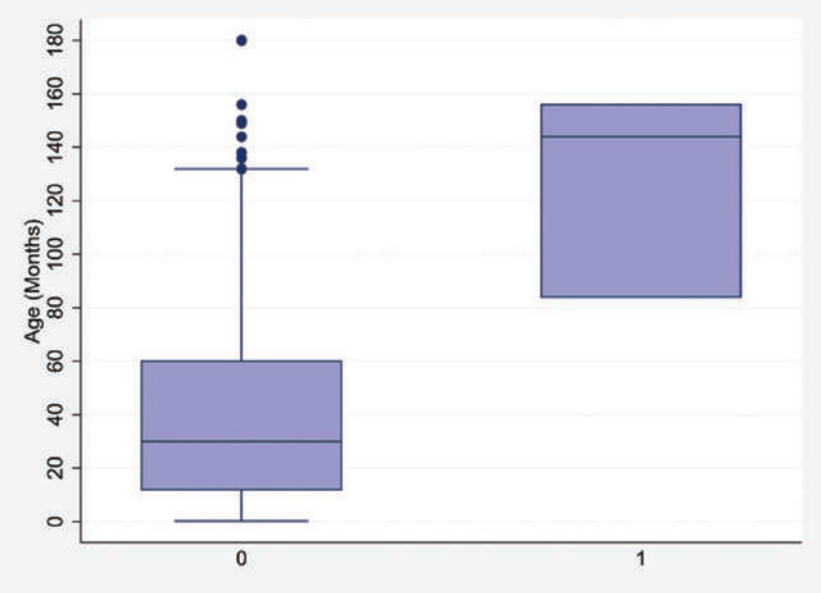

Fig 2: **Box plot showing association between trypanosomiasis with age,** where zero "0" indicate negative and "1" indicate positive to trypanosomiasis. Analyses indicate that 50% of infected animals were within the age group of 82-142 months.

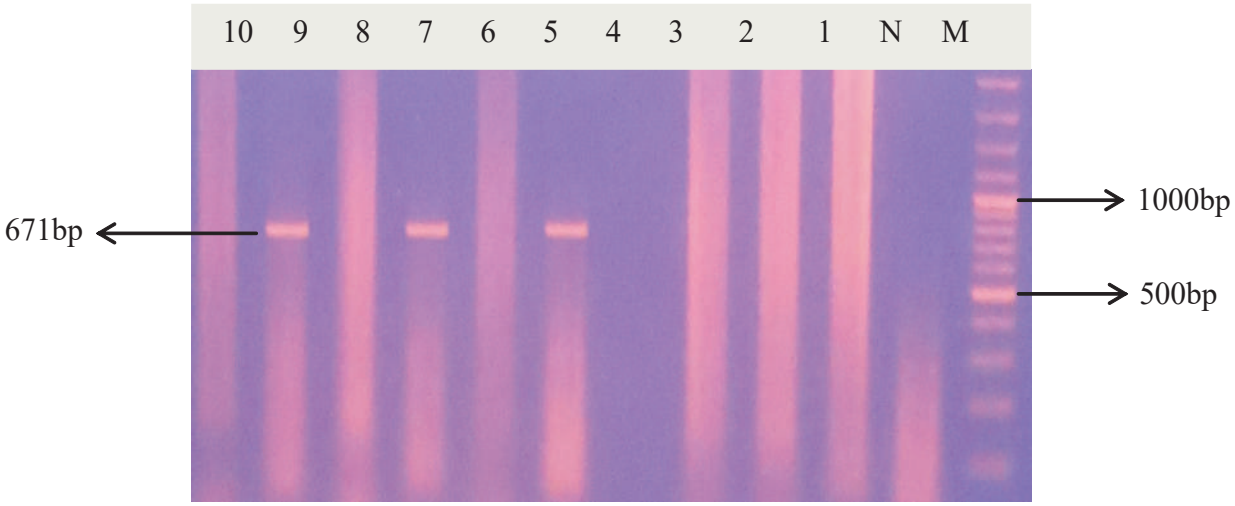

Fig. 3: **Amplification of the genomic DNA of *Trypanosoma* sp. from blood of cattle by using *18S rRNA* gene.** Lane M is for 100 bp DNA ladder; N is for negative control; Lanes 1-9 is suspected samples; Lanes 5, 7 and 9 having amplicons of 671 bp indicating the presence of *Trypanosoma specific* gene fragment.

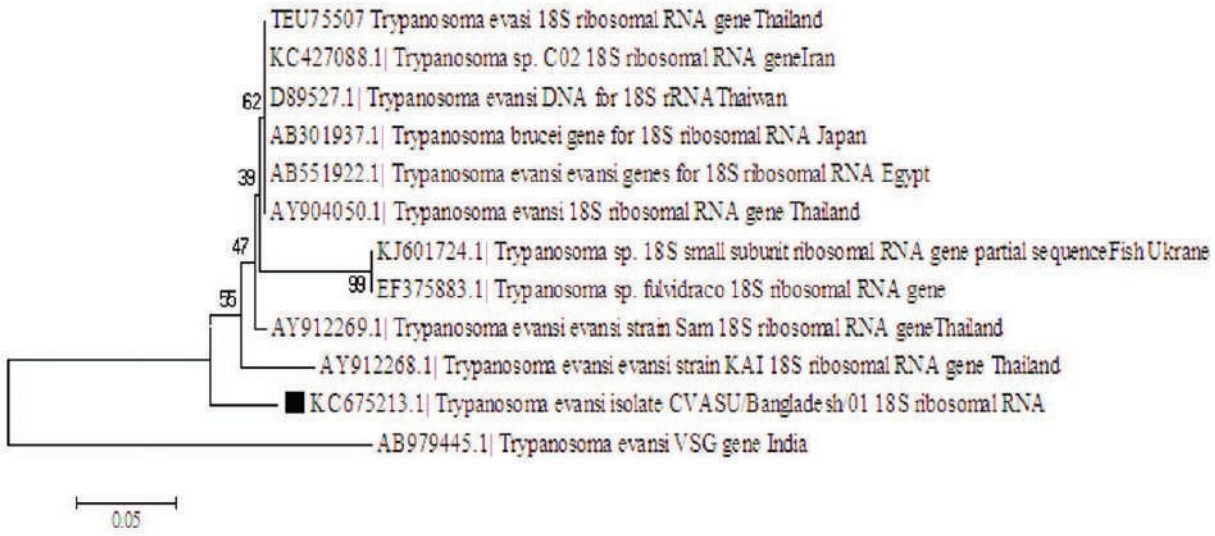

Fig. 4: Phylogenetic tree of the nucleotidic sequences of *Trypanosoma spp 18S rRNA* gene and all sequences of this gene deposited in GenBank from different countries, the accession numbers and countries are shown before isolate name. The 18SrRNAsequences obtained in this study were indicated with accession number KC 675213. The tree was inferred using the neighbor joining method of MEGA 5.2, bootstrap values are shown at each branch point. Number next to the branch demonstrates bootstrap support from 1000 replications. All sites of the alignment containing insertions-deletions, missing datawere eliminated from the analysis (option "complete deletion").

3.2 Molecular examination

The PCR assay confirmed the parasite as *Trypanosoma.* The PCR amplification using the first run primer pair gave trypanosome DNA tested a major product between 700 and 800 bp (Fig. 3), as predicted from the GenBankTM sequences. Second run amplification gave clear major amplicons between 600 and 700 bp with small size differences among most trypanosome species.

3.3 Phylogenetic analyses

A phylogenetic tree inferred based on *18S rRNA* gene sequences of *Trypanosoma* spp isolates are shown in Fig. 4. The 18SrRNA nucleotide sequences of *Trypanosoma* spp were compared with other known sequences published in GenBank, and revealing 99% identities to Thailand (AY912269) and 97% identities to *Trypanosoma* spp isolates from Egypt (AB551921 and AB551922), Thailand (AY904050), Taiwan (D89527) and Japan (AB301937). In the phylogenetic analysis, CVASU *Trypnosomaevansi* isolates (KC675213) showed a separate branch but cluster together with *Trypanosomaevansistrains* originating from Thailand, Egypt, Taiwan and Japan.

4. DISCUSSION

Trypanosomiasis is a devastating disease of cattle with potential zoonotic significance in some parts of the world. With little studies reported to date on trypanosomiasis in the country, present study was based on identification and characterization of trypanosome parasites using both these approaches. After initial screening by conventional microscopy samples were further analysed by molecular tools like PCR, DNA sequencing and phylogenetics etc. To our knowledge this is the first molecular level study of these important protozoa involving samples from Bangladesh. However several reports have already published in neighboring India and Pakistan where trypanosomiasis is reported more frequently. An overall prevalence of bovine trypanosomiasis in Chittagong region was recorded as 0.65%, during this study which was consistent with the previous findings by other researchers who reported 0.58% prevalence in north western part of the country (Rahman et al., 1982; Samad and Shahid-Ullah, 1985); The low prevalence of trypanosomes are thought to be due to variation of parasitic stages in peripheral blood as sample collection time could impact the microscopic test as a whole.

Another reason of low prevalence may be due to the availability and frequent use of trypanocidal drug such as Berenil® (Intervet), Babcop® (Square, Bangladesh) which is available throughout the country.

Although we didn't have enough focus on vectors in this but it was observed that tabanid flies were present in the farm areas where positive case were detected. It is generally known that the distribution of arthropod-borne diseases is greatly associated with the availability and abundance of its vector host. The arthropods fly involved in the transmission of *Trypanosomaevansi* in the Bangladesh are still not reported but this study indicated that it may be one of the vectors of trypanosomiasis of cattle in Bangladesh.

The three animals affected with trypanosomiasis were older than 6 years. This might not indicate any relationship of age of animals with the susceptibility. However similar findings were reported by other investigator elsewhere where cattle of 2-4 years of age were found affected (Baticados et al., 2012). Another study by other research group documented a high susceptibility of cattle to trypanosomes at the age of >5 years (Tasew and Duguma, 2012) One would assume that this age related susceptibility might be linked with lowered immunity of adult animals and other stressors such persistence of antibodies following treatment, chronic nature of infection and intermittent parasitaemia, stress, poor management, draught etc.

Infection was also recorded in cow during this study. This does not mean that male animals are less susceptible to trypanosomiasis. These variations may be linked with livestock management system in the farming animals, in which large numbers of males are removed and sold at any early age; the rest being used either for breeding. Another factors of higher prevalence in adult females might be due to pregnancy and lactation, which may reduce resistance in female camels and render them more susceptible to infection (Bhutto et al., 2010).

Climate associated factors are significant for transmission of trypanosomiasis. The prevalence was significantly higher in rainy season in compared to summer and winter season. Similar results were observed by many other investigators (Agarwal et al., 2003; Krishnappa et al., 2002; Rani et al., 2015). This is therefore essential to understand fly biology to efficiently control the infection to prevent further outbreak.

Although, erosion on different body parts of the animals are very common in cemented or partially cemented floor which subsequently attracting the flies and increasing the risk of the trypanosomiasis. But in this study we did not found any significant association between the floor type and the prevalence of the diseases.

Further sensitive molecular tools, PCR were applied for molecular detection of the organism. Although only positive case of microscopically examination was subjected to molecular techniques in this studies despite all three positive cases of microscopic screening techniques were also detected in molecular techniques. However PCR is sensitive enough to detect even one trypanosome per ml of blood (Masiga et al., 1996; Solano et al., 1999). Trypanosomes were basically identified by its morphological structure and host competency prior to the advent of modern genetic methods. Identification of different molecular markers and their uses for more precise identification approaches for this organism (Auty et al., 2012). Molecular tools including phylogenetic studies of trypanosomes and the integration of genetic information and morphological characters has helped to better our understanding of evolutionary and taxonomic relation- ships (Hamilton et al., 2004).

The amplified DNA from PCR analyses was subjected to sanger boned sequencing through commercial sources. The sequence data confirmed the presence of *Trypanosoma* spp. as inferred from sequence similarity based NCBI BLAST searching. Bioinformatics analyses revealed that the isolate of this study showed 97% identity with *T. evansi*. The phylogenetic tree was prepared based on the partial sequence of *18S rRNA* gene and by using neighbor joining methods indicating that the isolates reported from this experiment given a separate branch but clustered together with the isolates reported from Japan, Thailand and Taiwan. The phylogenetic tree showed the evolutionary relationship of the sequences in which the length of the horizontal line was proportional to the estimated genetic distance between the sequences. This suggests that considerable polymorphism occurred within the species although originating from different geographic regions. Again, only *T. theileri* has been recorded in Bangladesh by previous investigators through morphological studies (Rahman et al., 1982; Samad and Shahid-Ullah, 1985). We identified *T. evansi* during this study which is yet to be reported from cattle in Bangladesh.

5. CONCLUSION

Trypanosoma is one of the least known protozoa affecting domestic ruminants in Bangladesh. To our knowledge this is the first report of its incidence in Chittagong region of Bangladesh. Earlier two separate reports have been published although no molecular studies were employed.Only three positive cases were recorded during this study where all were cows aged more than 6 years. All cases were detected in rainy season and farms were located in areas frequented by cattles imported from neighboring India and Myanmar. The sequence analysis and subsequent phylogenetic investigation successfully identified the protozoa as *Trypanosomaevansi*. This was the first report of trypanosome from Chittagong region and third from Bangladesh. Further study regarding epidemiological aspects and use of sensitive detection techniques is highly recommended to determine the prevalence and risk factors of trypanosomiasis in the country. A complete understanding of the fly vectors, their distribution, farm biosecurity records etc. is essential to determine different associated risk factors.

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