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Occurrence and Subtype Distribution of *Blastocystis* in Smallholder Dairy Cattle in Bangladesh

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Abstract

Blastocystis is a widespread protozoan known to induce digestive disorders in humans and animals, including cattle globally. The parasite exhibits a significant amount of genetic variability and is divided into 42 recognized subtypes (STs) in mammals and birds. Sixteen of these subtypes are identified in cattle and 14 are zoonotic. This study explored the distribution and genetic variations of *Blastocystis* and its zoonotic potential in dairy cattle. Two hundred fresh fecal samples were collected from smallholder dairy calves (aged <6 months) in two milk pocket areas of Bangladesh: Sirajganj (n=100) and Pabna (n=100). A molecular study based on PCR assay targeting the small subunit ribosomal RNA (SSU rRNA) gene was employed to screen and subtype the parasite in the fecal samples. Analyses revealed *Blastocystis* among 10% of the cattle, with 8% positive cases in samples from Sirajganj and 12% in samples from Pabna. Various factors including sex, age, breed groups, and fecal consistency were considered, although these were found to be statistically insignificant. Out of the 20 positive *Blastocystis* isolates, only three subtypes, namely ST10, ST21, and ST26, were identified where subtype ST10 was the most prevalent. Notably, no zoonotic subtypes were detected in the fecal samples, suggesting a lack of zoonotic significance. The findings provide insights into the molecular epidemiology of *Blastocystis* infection in cattle, indicating its low genetic diversity in the study areas. Further research is needed to determine the exact frequency and genetic composition of *Blastocystis* and its zoonotic potential in cattle in Bangladesh.

Keywords: *Blastocystis*, Epidemiology, Subtype, Smallholder Dairy Cattle, Bangladesh.

Introduction

Blastocystis is a unicellular protozoan inhabiting the digestive tract of both humans and animals, including cattle (Sharifi *et al.*, 2020). The actual pathogenesis of *Blastocystis* is still unknown and assumed to be linked with the parasite's subtype (ST) and the host's immunological state (Elwakil and Hewedi, 2010). While asymptomatic transmission is widespread, this parasite is responsible for a variety of intestinal disorders, including nausea, vomiting, diarrhea, stomach ache, and irritable bowel syndrome in humans (Andersen and Stensvold, 2016). Severe infections may occur in immunocompromised individuals, especially those with AIDS and cancer (Lepczyńska *et al.*, 2017). Traditionally *Blastocystis* transmission occurs through the fecal-oral route, through the ingestion of food and water contaminated with cysts (Leelayoova *et al.*, 2008). Humans who

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come into contact with animals are more likely to become infected with zoonotic subtypes of *Blastocystis* (Parkar *et al.*, 2010).

Blastocystis has been reported in cattle, pigs, dogs, and cats, and various wild animal species all over the world (Aynur *et al.*, 2019; Li *et al.*, 2019; Rauff-Adedotun *et al.*, 2020). Earlier, *Blastocystis* has been successfully identified and subtyped from fecal samples using PCR assay targeting the small subunit ribosomal RNA (SSU rRNA) gene (Wawrzyniak *et al.*, 2013). Over the years, molecular tools have unveiled significant genetic variations within the parasite, with isolates from diverse hosts being categorized into 42 known subtypes. In addition, several unnamed subtypes were reported in reptiles, amphibians, and insects (Andersen and Stensvold, 2016; Ramírez *et al.*, 2014; Stensvold and Clark, 2016). While 14 subtypes (ST1–ST10, ST12, ST14, ST16, and ST23) were found in humans, only four of them (ST1–ST4) were responsible for 95% of the human infections (Andersen and Stensvold, 2016). The four main subtypes found in humans are also prevalent in different animals, including primates, several hoofed mammals, rodents, and birds (Li *et al.*, 2019; Skotarczak, 2018). Notably, less common human subtypes (ST5–ST8) are more often found in hoofed animals, birds, and non-human primates (NHPs), indicating possible zoonotic origin (Stensvold and Clark, 2016). Transmission between humans and animals is indicated by the presence of specific subtypes like ST5 in piggery attendants (Wang et al., 2014) and ST3 and ST8 in zookeepers (Alfellani *et al.*, 2013; Parkar *et al.*, 2010). Meanwhile, 16 subtypes were found in cattle, among which ST10 was the most prevalent (Aynur *et al.*, 2019; Rauff-Adedotun *et al.*, 2020).

Several investigations on *Blastocystis* in humans and zoo animals have been conducted in Bangladesh (Yoshikawa *et al.*, 2004). Two subtypes, such as ST1 (7.69%) and ST3 (92.31%), were identified in the initial investigation involving 26 isolates from clinical and subclinical patients (Yoshikawa *et al.*, 2004). In another study, *Blastocystis* was found to be 14% prevalent in 5,679 stool samples from children living in slums, utilizing *in vitro* culture and direct microscopic tests (Barua *et al.*, 2015). Another study conducted in captive mammals at the Bangladesh National Zoo found a 15.5% (31/200) occurrence of *Blastocystis* with seven subtypes, *i.e.*, ST1–ST3, ST10, ST11, ST13, and ST14 (Li *et al.*, 2019). This study used PCR and sequencing to investigate the natural infection status, subtype diversity, and zoonotic potential of *Blastocystis*, along with its epidemiological risk factors in smallholder dairy cattle from two prominent milk pocket areas, Sirajganj and Pabna in Bangladesh.

Materials and Methods

Sampling

Data and samples of this study were collected from two districts, namely Sirajganj and Pabna. Calves less than six months of age were included in the study. Fresh fecal samples from 200 calves were collected from Sirajganj (n=100) and Pabna (n=100) districts. Sterile latex gloves were used to collect fecal samples (about 10 gm) from the calves' rectum or the ground soon after defecation, which were then put in labeled zipper bags. The data on demographic characteristics of the calves (age, sex, and breed) and the consistency of the feces (formed, soft, or watery) were obtained using a predefined questionnaire.

Sample Processing

Upon arrival at the laboratory of the Department of Medicine, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur, the fecal samples were carefully transferred into separate 50-mL centrifuge tubes containing distilled water. After sieving through a fine mesh strainer with a pore size of approximately 250 µm, centrifugation was performed at 3,000 rpm for 5 minutes. The supernatant was discarded and sediments were collected for preservation or DNA extraction. To preserve the samples for further analysis, two aliquots from each sample were placed in 2.5% potassium dichromate solution and kept at 4° C.

DNA Extraction

After removing the potassium dichromate by washing the preserved fecal samples with deionized water, genomic DNA extraction from each fecal sample was carried out utilizing the E.Z.N.A.® Stool DNA kit (Omega Bio-tek, Inc., Norcross, USA), following the guidelines provided by the manufacturer. Briefly, 200 mg of fecal sample was combined with an equal

amount of glass beads in a 2 mL centrifuge tube on ice. After adding 540 µL SLX-Mlus Buffer and vertexing for 10 minutes, 60 µL DS buffer and 20 µL Proteinase K were added and mixed thoroughly, followed by a 70° C incubation for 10 minutes. Following the addition of 200 µL Buffer SP2 and incubation on ice for 5 minutes, the sample was centrifuged at 15,000 × *g* for 5 minutes. 400 µL supernatant was transferred to a new tube, mixed with 200 µL cHTR reagent, and incubated at room temperature. After centrifugation, 250 µL supernatant was mixed with 250 µL BL Buffer and 250 µL ethanol. The mixture was transferred to a HiBind® DNA column, washed, and eluted with 200 µL preheated Elution Buffer at 60° C. The obtained DNA extract was then centrifuged at 15,000 × *g* for 1 minute to elute the DNA, after soaking at room temperature for 2 minutes. Until utilized in PCR, this eluted DNA was stored at −20° C.

PCR Amplification

The DNA samples were examined for *Blastocystis* with PCR targeting the 600 bp region of the SSU rRNA gene. The primers, RD5 (forward): ATCTGGTTGATCCTGCCAGT and BhRDr (reverse): GAGCTTTTTAACTGCAACAACG, were used (Scicluna *et al.*, 2006). The PCR assay was accomplished in a 25 µL volume utilizing TaKaRa Taq DNA Polymerase, and both positive and negative controls were used in each PCR experiment. The PCR amplification was conducted in an Applied Biosystems Thermal Cycler, commencing with an initial 5-minute denaturation at 94° C. A total of 35 cycles were run with denaturation at 94° C for 45 seconds, primer annealing at 55° C for 45 seconds, and strand extension at 72° C for 1 minute. After that, the final extension step lasted 10 minutes at 72° C, concluding with a cooling step at 4° C. The PCR products underwent electrophoresis in a 1.5% agarose gel that was stained with ethidium bromide. Subsequently, the results were visualized using a UV transilluminator.

Nucleotide Sequencing and Analysis

The PCR products with the expected band size were sent to Sangon Biotech Co., Ltd. (Shanghai, China) and sequenced in both forward and reverse directions using the same primers. The obtained sequences were viewed using Chromas program version 2.6.6 (Technelysium Pty Ltd, South Brisbane, Australia) and analyzed with ClustalX software. Sequences were aligned using MUSCLE in MEGA 11 (http://www.megasoftware.net/) and compared to GenBank entries via BLAST (http://www.ncbi.nlm.nih.gov/blast/) for consensus identification. The identification of *Blastocystis* subtypes was confirmed using the online platform: Blastocystis locus/sequence definitions database (https://pubmlst.org/bigsdb ?db=pubmlst_Blastocystis_seqdef).

Phylogenetic Analyses

For sequence analyses, 20 nucleotide sequences were aligned with 44 *Blastocystis* reference sequences retrieved from the GenBank database using BLAST and ClustalX software. Phylogenetic analysis was performed with Mega software (http://www.megasoftware.net/), employing Neighbor-Joining (NJ) analysis to support subtype classifications. Bootstrap resampling was applied with 1,000 replicates. The tree was constructed with *Proteromonas lacerate*, *Developayella elegans*, and *Labyrinthuloides haliotidis* serving as outgroups.

Statistical Analyses

SPSS software was used in the statistical analyses of this study. The correlation of *Blastocystis* occurrence with the study location, sex, age, and breed groups of calves was verified with a chi-square test. A threshold value for statistical significance was set with a *P*-value below 0.05.

Data Availability

The representative SSU rRNA gene sequences from this study were deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) under the accession numbers PP581309, PP581314, PP581318, PP581323, and PP581329.

Table 1. Demographic information of the calves

Characteristics	Calves (n=200)
Study site	
Sirajganj	100 (50.0%)
Pabna	100 (50.0%)
Sex	
Male	99 (49.5%)
Female	101 (50.5%)
Breed	
Local	36 (18.0%)
HFC	125 (62.5%)
JC	33 (16.5%)
BrC	6 (3.0%)
Age (month)	
Mean (range)	4.053 (0.33–8) months

HFC = Holstein-Friesian Cross, JC = Jersey Cross, BrC = Brahman Cross

Results and Discussion

Demographics of the Study Calves

Out of the 200 calves examined in this study, 36 (18.0%) were local breed, 125 (62.5%) were Holstein-Friesian Cross (HFC), 33 (16.5%) were Jersey Cross (JC), and 6 (3.0%) were Brahman Cross (BrC) calves. The calves were categorized into three age groups, including <1 month (4.5%), 1–3 months (38.0%), and >3 months (57.5%) (Table 1). There were 99 males (49.5%) and 101 females (50.5%). Most of the calves under study were in good health, except for a few cases of diarrhea.

Occurrence of Blastocystis

Blastocystis was observed in 20 (10%) of the 200 calf fecal samples from Sirajganj and Pabna. The occurrence of *Blastocystis* based on study areas, sexes, ages, and breed groups is presented in Table 2. The overall occurrence reported in this study aligns closely with the reported rate in China (10.3%) (Zhu *et al.*, 2017). However, varying prevalence rates of the parasite have been documented globally. A notably higher prevalence rate, even reaching 100%, has been reported in Indonesia (Susana *et al.*, 2019). In Colombia, two studies on *Blastocystis* reported prevalence rates of 80% and 77.58% (Higuera *et al.*, 2021; Ramírez *et al.*, 2014). The prevalence rates in Lebanon, Japan, and Turkey were also higher, reaching 63.4%, 54.1%, and 58.7%, respectively (Greige *et al.*, 2019; Masuda *et al.*, 2018; Tavur and Önder, 2022). However, one study suggested a lower prevalence of 6.7% in Korea (Lee *et al.*, 2018). The prevalence rates in other countries are as follows: 21.4% in Brazil (Moura *et al.*, 2018), 22.7% in UAE (AbuOdeh *et al.*, 2019), 15.4% in Nepal (Lee *et al.*, 2012), 32.1% in Spain (Abarca *et al.*, 2021), 34.5% and 43.8% in Malaysia (Hemalatha *et al.*, 2014; Kamaruddin *et al.*, 2020), 33% in Italy (Gabrielli *et al.*, 2021), 19.4% in Turkey (Onder *et al.*, 2021), and 9.6% in Iran (Badparva *et al.*, 2015).

Several risk factors were considered in this study, including location, sex, age, and breed. Calves of the local breed had a higher infection rate (11.1%) than those of the Holstein-Friesian Cross (9.6%), and Jersey Cross (12.1%), with no infection in Brahman Cross (Table 2). Calves older than three months had the highest infection rate (12.2%), similar to the findings of Lee *et al.* (2018), who observed a higher rate in calves aged between 3 and 12 months in Korea. Subsequently, adults aged between 1–3 months exhibited a prevalence rate of 7.9%, while no infection was observed in calves aged <1 month.

However, Kamaruddin *et al*. (2020) documented a higher occurrence in calves aged less than 3 months in Malaysia. The infection rate in females (10.9%) was slightly higher than that in males (9.1%). Considering sex-wise prevalence, Kamaruddin *et al*. (2020) reported higher rates in females, while Lee *et al*. (2018) observed higher rates in males. Geographical and environmental factors affect the prevalence of *Blastocystis* (Lee *et al*. 2018); however, the two districts under consideration appeared to have similar geographical factors and climatic conditions, with infection rates of 8% in Sirajganj and 12% in Pabna. However, all the correlations between the risk factors in this study on *Blastocystis* occurrence were not statistically significant. Further investigation is imperative to confirm the actual impact of other risk factors in the study region.

Subtypes of Blastocystis

The partial SSU rRNA gene sequences revealed limited genetic heterogeneity among *Blastocystis* isolates from calves in this study. Three subtypes, ST10 (n=13), ST21 (n=3), and ST26 (n=4) were identified among the 20 isolates. Notably, ST10 was the predominant subtype (65.0%). The subtype distribution based on the age, sex, and breed of the calves is shown in Table 2. The results from Shams *et al*. (2021), highlighting the identification of 16 distinct subtypes (ST1–ST7, ST10, ST12, ST14, ST17, ST21, ST23–ST26) in cattle globally, underscore that cattle serve as a significant reservoir for a diverse range of *Blastocystis* subtypes, including some with zoonotic potential. Among them, ST1–ST3, ST10, ST11, ST13, and ST14 have been identified in captive mammals at the Bangladesh National Zoo (Li *et al*., 2019). Similar to the report of Shams *et al*. (2021), ST10 was found as the predominant subtype in this study. According to Zhang *et al*. (2023), ST10 and ST14 are regarded as animal-specific subtypes with no proven zoonotic significance. A recent report by Khaled *et al*. (2020) detected ST10 and ST14 in the fecal samples of healthy school children in Senegal, but conclusive evidence regarding their zoonotic nature is still lacking.

Phylogenetic Relationship of Blastocystis Isolates

Phylogenetic analysis, incorporating 20 sequences from this study and 44 reference sequences, along with three outgroups, revealed that the sequences of the three identified subtypes (ST10, ST21, and ST26) were clustered with their respective reference subtypes (Figure 1). The constructed phylogenetic tree revealed strong bootstrap support, with isolates within the same subtype forming distinct and well-supported monophyletic groups. This affirms the independent and distinct nature of the three subtypes.

Table 2. Occurrence of *Blastocystis* in calves by location, sex, age, and breed group (n=200)

Parameters	Samples	Positive (%)	$\chi 2$	*P*-value	Subtypes (n)
Location					
Sirajganj	100	08 (8.0)	0.889	0.346	ST10 (6), ST21 (1), ST26 (1)
Pabna	100	12 (12.0)			ST10 (7), ST21 (2), ST26 (3)
Sex					
Male	99	09 (9.1)	0.180	0.671	ST10 (6), ST21 (1), ST26 (2)
Female	101	11 (10.9)			ST10 (7), ST21 (2), ST26 (2)
Age group					
<1 month	09	0	1.978	0.372	
1–3 months	76	06 (7.9)			ST10 (5), ST26 (1)
>3 months	115	14 (12.2)			ST10 (8), ST21 (3), ST26 (3)
Breed group					
Local	36	04 (11.1)	0.903	0.825	ST10 (2), ST21 (1), ST26 (1)
HFC	125	12 (9.6)			ST10 (8), ST21 (2), ST26 (2)
JC	33	04 (12.1)			ST10 (3), ST26 (1)
BrC	06	0			

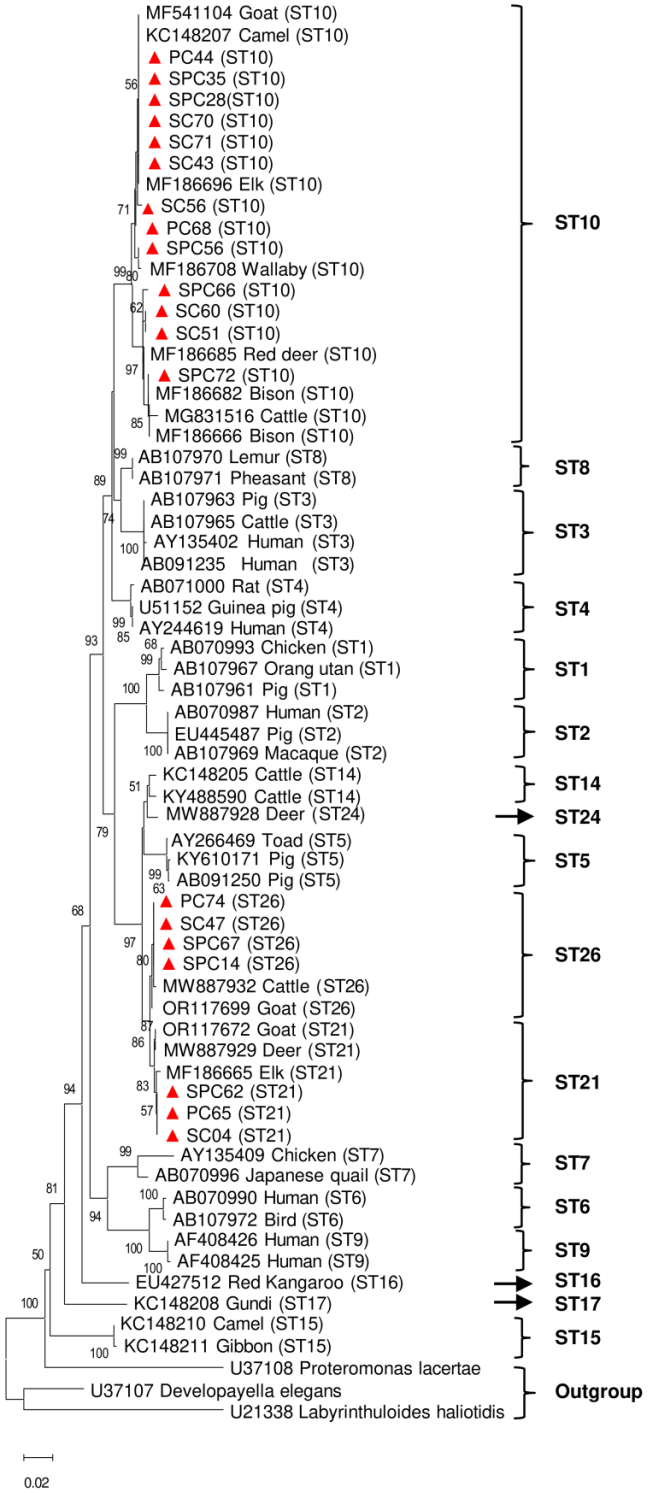

Figure 1. Phylogenetic analysis of *Blastocystis* SSU rRNA gene sequences using the Neighbor-joining (NJ) method. Bootstrap analysis with 1,000 replicates was performed, and values more than 50% were shown. The sequences from this study are represented by red-filled triangles.

Conclusions

This study reports the molecular epidemiology and genetic characterization of *Blastocystis* in smallholder dairy cattle in two milk pocket areas in Bangladesh. The study found a 10% prevalence of *Blastocystis* in cattle, with a rate of 8% in Sirajganj and 12% in Pabna. Only three subtypes were identified: ST10, ST21, and ST26, with ST10 being the dominant subtype. Notably, no zoonotic subtype was found suggesting that further extensive investigation is necessary to better understand the occurrence, genetic variation, and zoonotic potential of *Blastocystis* in Bangladesh.

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Decleration

The authors declare no conflicts of interest.

Authors' Contributions

MRK conceptualized and planned the study; MRK, JR, and ABH collected the data and fecal samples; MRK, JR, and SFS carried out the tests and analyzed the results; MRK provided reagents, laboratory supplies, and analysis tools; All authors contributed to writing, revising, and approving the final manuscript.

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