Parasitology International 104 (2025) 102967

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

A pilot study of intestinal protist detection in humans, animals, and the environment in a slum area in Mymensingh, Bangladesh

Sadiya Maxamhud [a], Md Shahiduzzaman [b], A.R.M. Beni Amin [b], Md. Zawad Hossain [b], Eleni Gentekaki [c], Anastasios D. Tsaousis [a,*]

[a] Laboratory of Molecular and Evolutionary Parasitology, RAPID Group, School of Biosciences, University of Kent, Canterbury, CT2 7NH Kent, UK
[b] Department of Parasitology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
[c] Department of Veterinary Medicine, University of Nicosia School of Veterinary Medicine, 2414 Nicosia, Cyprus

ARTICLE INFO

Keywords:
Cryptosporidium spp.
Giardia intestinalis
Blastocystis sp.
Entamoeba histolytica
One health
Molecular detection

ABSTRACT

The purpose of this study was to investigate the presence and transmission of *Cryptosporidium* spp., *Blastocystis* sp., *Giardia intestinalis* and *Entamoeba histolytica* between different hosts and their shared environment in a slum area of Bangladesh. A total of 102 samples were collected from Mymensingh, Bangladesh. This collection encompassed 16 human samples, 35 soil samples and 51 animal samples from various households within the region. The detection of *Cryptosporidium* spp., *Blastocystis* sp., *G. intestinalis*, and *E. histolytica* was carried out using nested PCR and/or quantitative PCR methodologies. Among the samples, 24 human, animal, and soil out of 102 were found positive for *Blastocystis* sp., spanning seven subtypes: ST2, ST3, ST7, ST10, ST23, ST24 and ST25. Additionally, eight samples (8/102) including human, animal and soil tested positive for *Cryptosporidium* spp., including *C. parvum, C. baileyi, C. bovis,* and *C. meleagridis*. Furthermore, one soil sample tested positive for *G. intestinalis* assemblage B, while no samples tested positive for *E. histolytica*. The detection of *Cryptosporidium* spp., *Blastocystis* sp., and *G. intestinalis* in this study has provided insights into their presence, extending beyond humans. Moreover, these findings highlight the importance of embracing a One Health perspective with an emphasis on specific parasitic microorganisms.

1. Introduction

Parasite-induced diarrhoea is a global public health issue. Moreover, diarrhoea-related mortality is concerning, especially in low- and middle-income countries, due to factors such as limited access to clean water and poor hygiene. The proximity of humans to animals, their shared living conditions, and limited access to suitable diagnosis and treatment comprise aggravating factors that exacerbate diarrhoea in humans due to increased transmission dynamics, ultimately leading to an increase in mortality [1]. Enteric protozoans have been recognized as important causes of diarrhoea in humans, with *Cryptosporidium* spp., *Giardia intestinalis (syn. G. lamblia, G. duodenalis),* and *Entamoeba histolytica* being commonly reported in relation to diarrhoea [2–4]. These three protozoan parasites are known to colonize the gut of a wide assortment of vertebrate hosts. Among these, *Cryptosporidium* spp. (mainly *C. parvum*) play a significant role in morbidity and mortality in humans, as well as livestock, while *G. intestinalis* and *E. histolytica* can be pathogenic for humans and can also colonize the gut of a variety of animals, though their veterinary pathogenic importance remains unknown [5–8]. In contrast, the unicellular eukaryote *Blastocystis* sp. is one of the most commonly found intestinal eukaryotic microorganisms in humans and other mammals [14]. However, its clinical and veterinary significance is not fully understood [9].

Cryptosporidium spp., *G. intestinalis, E. histolytica,* and *Blastocystis* sp. are transmitted through the faecal-oral route, either directly or indirectly including person-to-person, waterborne, or foodborne transmission [3,10–12]. The occurrence of these parasites across diverse communities worldwide underscores the need for One Health approach studies, in order to understand their cycling and transmission dynamics [13,14]. This becomes especially crucial in developing regions, where there is higher morbidity and mortality of diarrhoea-related illnesses [15].

Blastocystis sp. has a worldwide distribution in both developed and developing countries. Its prevalence, transmission routes and host specificity are continuously being investigated [16–18]. So far, at least 40 subtypes of *Blastocystis* sp. have been identified in various hosts based

* Corresponding author.
E-mail address: A.Tsaousis@kent.ac.uk (A.D. Tsaousis).

https://doi.org/10.1016/j.parint.2024.102967
Received 23 May 2024; Received in revised form 30 August 2024; Accepted 9 September 2024
Available online 11 September 2024

on the variation of the small subunit (SSU) rRNA gene, with new subtypes increasingly being discovered [9,11,19,20]. Comparatively, limited data is available on the molecular prevalence of *Blastocystis* sp. in Asia and specifically in Bangladesh [21,22]. *Cryptosporidium* has at least 44 validated species, among which *C. parvum* and *C. hominis* are the most prevalent and the ones that are more frequently associated with human cryptosporidiosis. *Cryptosporidium meleagridis, C. bovis, C. felis,* and *C. canis* are also known to cause cryptosporidiosis in humans, albeit sporadically [3]. *Giardia intestinalis* is a genetically diverse species that is divided in eight assemblages (A-H) [4,23] Among these, assemblages A and B are the only ones commonly known to infect humans. *Entamoeba histolytica* is the causative agent of amoebiasis, which is potentially fatal to humans [24].

Diarrhoea is responsible for 26 % of childhood deaths in South Asia [1]. In Bangladesh, diarrhoeal disease accounts for one-third of the total child death burden [25]. Specifically, *C. hominis and C. parvum* were identified as a common cause of diarrhoea in the country, along with *C. meleagridis* [26]. The former two *Cryptosporidium* spp. have been reported as the most prevalent in humans in Bangladesh [27]. *Giardia intestinalis* assemblage A has also contributed to the diarrhoea burden in humans residing within the country [28,29].

With a population density of 1330 people per km^2, Bangladesh is considered densely populated, with inhabitants distributed across both rural and urban areas [30]. A third of the urban population resides in overpopulated slums [31]. Untreated wastewater, routinely encountered in slums, is usually discharged into polluted rivers, creating multiple paths for pathogen circulation. This increases the likelihood of their transmission among humans, animals, and the environment [32,33]. Highlighting the occurrence of medical and/or veterinary important intestinal parasites is critical in understanding their circulation and transmission routes. Therefore, the primary objective of this pilot study was to investigate, for the first time, the occurrence of *Blastocystis* sp., *Cryptosporidium* spp., *G. intestinalis*, and *E. histolytica* in various hosts and the environment, namely soil, within the Railway Colony slum in Mymensingh, Bangladesh.

2. Materials and methods

2.1. Ethical approval

The Bangladesh Agricultural University ethics committee (approval no: AWEEC/BAU/2022) approved the research project. Consent was obtained from each household before the study.

A questionnaire was also distributed to the participants and filled out with the help of the local researchers to collect demographic data **(Supplementary Table S1).** Information on gastrointestinal-related symptoms, antibiotic usage, and sources of food and drinking water was obtained from the humans included in this study.

2.2. Study area

This study was conducted in the Railway Colony slum in Mymensingh, Bangladesh, which is located approximately 120 km north of the capital city Dhaka, (Fig. 1). The Railway Colony slum is situated on both sides of a railway and holds a population of approximately 5000 individuals. The slum is divided into compounds. Each household that was chosen for sample collection had running water indoors and a pit latrine used as a toilet, however, most of the population residing within the Railway Colony slum utilizes water from nearby ponds for bathing and general household washing tasks. A tube well is used to supply drinking water, which is taken directly from the ground and is neither treated nor filtered (Supplementary Fig. 1). Garbage disposal areas are often situated in close proximity to the ponds. A wide variety of animals including both free-ranging and confined livestock, are also known to frequent these water sources for drinking purposes. This includes the animals included in this study.

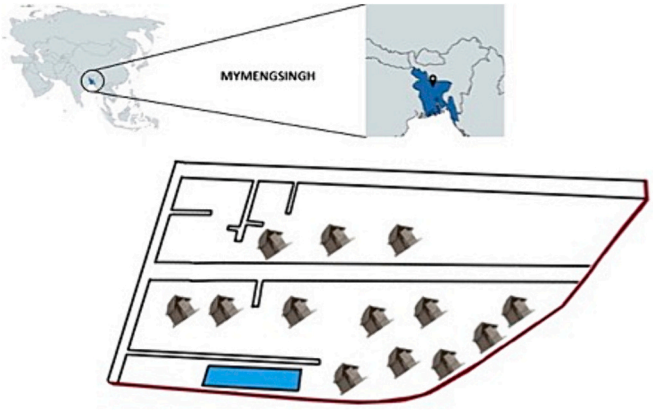

Fig. 1. The upper portion of the map shows the geographical location of the study area. The lower section of the map shows the studied slum area and houses included in this study, the road network, and nearby lake. The adjacent railway is highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Experimental

2.3.1. Sample collection and DNA extraction

A total of 102 samples were randomly collected from a compound within the Railway Colony Slum area in 2021. Of these, 16 were from human hosts residing in 15 different households. Fifty-one samples were obtained from animals, including 43 from free-ranging animals and eight from those reared in cages (pigeons and rabbits). Additionally, 35 samples were obtained from soil to have an idea of the environmental contamination from faecal material of human/animal origin. All 16 of the human participants had diarrhoea, which was an inclusion criterion. More specifically, ten had watery diarrhoea, five had mucoid diarrhoea, while one had bloody diarrhoea. All human participants in this study utilized tube well water. At the time of sampling, nine of the sixteen humans included in this study were undergoing antibiotic treatment. The animal samples consisted of cats ($n = 2$), dogs (n = 2), goats ($n = 9$), sheep ($n = 1$), rabbits (n = 2), cows ($n = 6$), and birds ($n = 29$). The birds consisted of cockatiels (*Nymphicus hollandicus*) ($n = 4$), pigeons (*Columba livia domestica*) (n = 6), hens (*Gallus domesticus*) ($n = 8$), ducks (*Anas platyrhynchos*) (n = 4), doves ($n = 3$), koels (*Eudynamys scolopaceus*) ($n = 2$), a titir (*Ortygornis pondicerianus*) ($n = 1$) and a bazrigar bird (*Melopsittacus undulatus*) (n = 1). The free-ranging animals roamed freely throughout the slum, with the potential to enter household compounds at any time. Cats, dogs, koels, titir and bazrigar were not owned. No animal showed symptoms of diarrhoea. Superficial soil samples were collected randomly from within the compound.

Samples were transported within 24 h after sample collection to the laboratory located at the Department of Parasitology at Bangladesh Agricultural University and kept at a temperature of −20 °C until DNA extraction.

DNA was extracted from 200 mg of stool and soil using the PureLink™ Microbiome DNA Purification Kit (Invitrogen, Carlsbad, California, USA) following the manufacturer's protocol that enables purification of microbial and host DNA from a wide variety of sample types. A total of 50 µl of the extracted DNA was subsequently stored at −20 °C until shipped to the laboratory at the University of Kent, United Kingdom, for further molecular analysis.

2.3.2. Molecular detection of Cryptosporidium spp

Cryptosporidium spp. was amplified using nested polymerase chain reaction (nPCR) targeting a 631 fragment of the SSU rRNA as previously described [34]. Using the primers CRY_SSU_F1 (5′-GATTAAGCCATGCATGTCTAA-3′) and CRY_SSU_R1 (5′-TTCCATGCTGGAGTATTCAAG-3′), the initial amplification included a denaturation step for 2 min at

94 °C, followed by 24 cycles of denaturation at 94 °C for 50 s, annealing at 53 °C for 50 s, and extension at 72 °C for 1 min. Lastly, a final extension step was carried out at 72 °C for 10 min. In the second PCR reaction, using the primers CRY_SSU_F2 (5′ -CAGTTATAGTTTACTTGATAATC- 3′) and the reverse primer CRY_SSU_R2 (5′-CCTGCTTTAAGCACTCTAATTTTC- 3′) were employed. Additionally, 1 μl of the PCR product obtained from the initial reaction was used as the template. The cycling conditions for the second PCR reaction differed from those of the initial reaction in two aspects: cycle number and annealing condition. The second reaction consisted of 30 cycles, compared to the previous publication [24]. Moreover, instead of an annealing temperature at 53 °C for 50 s, the second reaction employed an annealing temperature of 56 °C for 30 s. *Cryptosporidium parvum/hominis* were further genotyped by amplifying an 850 bp fragment of the 60 kDa - glycoprotein (*gp60*) gene, as previously described [34]. The *gp60* amplification with nested PCR (nPCR) involved the utilisation of primers AL3531 (5′ -ATAGTCTCCGCTGTATTC- 3′) and AL3535 (5′ -GGAAGGAACGATGTATCT- 3′) in the primary reaction, and primers AL3532 (5′-TCCGCTGTATTCTCAGCC- 3′) and AL3534 (5′ -GCAGAGGAACCAGCATC- 3′) in the secondary reaction. For both the first and second reaction, the cycling conditions consisted of an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 1 min. Lastly, a final extension step at 72 °C for 7 min concluded the process. When amplifying the targeted *gp60* locus, each sample was run in triplicates.

2.3.3. Molecular detection of Giardia intestinalis and Entamoeba histolytica

To detect *G. intestinalis* and *E. histolytica,* a probe-based quantitative PCR (qPCR) approach was employed, targeting the SSU rRNA gene [35,36]. Specifically, for detection of *E. histolytica* the primers Ehd-239F (5′-ATTGTCGTGGCATCCTAACTCA-3′) and Ehd-88R (5′-GCGGACGGCTCATTATAACA-3′) were employed, alongside an *E. histolytica*-specific probe (VIC-5′-TCATTGAATGAATTGGCCATTT-3′-NFQ). Additionally, PCR conditions comprised an initial denaturation phase at 95 °C for 2 min, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 54 °C for 30 s, and a final elongation step at 72 °C for 30 s. Each PCR run included positive DNA from an *E. histolytica* isolate as a positive control, along with PCR water serving as a negative control.

The detection of *G. intestinalis* was conducted through probe-based quantitative real-time PCR using the *G. intestinalis*-specific primers *Giardia*-80F (5′-GACGGCTCAGGACAACGGTT-3′) and *Giardia*-127R (5′-TTGCCAGCGGTGTCCG-3′), along with a *G. intestinalis*-specific probe (FAM-5′-CCCGCGGCGGTCCCTGCTAG-3′-black hole quencher). PCR conditions included an initial denaturation step at 95 °C for 2 min, followed by 50 cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for 30 s, and a final elongation step at 72 °C for 30 s. Each PCR run included positive DNA from a *G. intestinalis* isolate as a positive control, along with PCR water serving as a negative control.

Furthermore, in order to obtain assemblage information for the samples that were qPCR positive for *G. intestinalis,* genotyping through nested amplification were performed by targeting *glutamate dehydrogenase (gdh)* [37], *triosephosphate isomerase (tpi*) [38] and *β-giardin (bg)* [39] genes following the methodology as previously described [35]. Positive control consisting of *G. intestinalis* positive DNA sample as well as a negative control, consisting of PCR water, were included in each batch of samples analysed.

The secondary PCR products underwent electrophoresis on a 2 % agarose gel, after which the targeted band was cut from the gel. A total of 25 μl of the PCR reaction mixture was carefully loaded onto the gel for analysis. Following this, for samples that tested positive, a DNA purification process was carried out using the Thermo Scientific GeneJET Gel Extraction Kit (Thermo Fisher Scientific, CA, USA) and subsequently 10 μl of the gel extract were sent to Eurofins (Cologne, Germany) for bi-directional Sanger sequencing using 5 μl of the primers employed in the secondary PCR process.

2.3.4. Blastocystis species detection

For the detection of *Blastocystis* sp., the samples were analysed using nPCR targeting the barcoding fragment of the SSU rRNA gene. In the initial PCR reaction, the broad specificity primer pair RD3 (5′-GGGATCCTGATCCTTCCGCAGGTTCACCTAC-3′) and RD5 (5′-GGAAGCTTATCTGGTTGATCCTGCCAGTA-3′) was utilized [40]. The reaction conditions included an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min, and extension at 72 °C for 1 min. A final elongation step was performed at 72 °C for 10 min. Subsequently, a second nPCR was conducted to amplify the 650 bp barcoding fragment using the forward RD5f (5′-ATCTGGTTGATCCTGCCAGT-3′) and the reverse primer BhRDr (5′-GAGCTTTTTAACTGCAACAACG-3′) [41]. The PCR conditions for the second reaction involved an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. A final elongation step was performed at 72 °C for 10 min. Positive and negative controls were included in each batch of samples analysed. The secondary PCR products underwent electrophoresis on a 2 % agarose gel, after which the targeted band was cut from the gel. Following this, DNA purification was performed using the Thermo Scientific GeneJET Gel Extraction Kit (Thermo Fisher Scientific, CA, USA), and subsequently sent to Eurofins (Cologne, Germany) for sanger sequencing.

Additionally, SYBR Green qPCR was employed as an extra detection method for *Blastocystis* sp., encompassing all samples included in this study, to amplify a 330 bp segment of the SSU rRNA gene. [42]. The qPCR reaction mixture contained 10 μl mastermix volume consisting of 2 μl of water, 5 μl Luna Taq Universal MasterMix (New England Biolabs, USA), 0.5 μl of both forward (BL18SPPF1; 5′-AGTAGTCATACGCTCGTCTCAAA-3′) and reverse (BL18SR2PP; 5′-TCTTCGTTACCCGTTACTGC-3′) *Blastocystis*-specific primers and 2 μl of genomic DNA as previously described [13,43]. Reactions were run in 96-well plates in Quantstudio 3 (Thermo Scientific, USA). Positive and negative controls were used in each qPCR run.

The PCR positive products underwent electrophoresis on a 2 % agarose gel, after which the targeted band was cut from the gel. Following this, DNA purification was performed using the Thermo Scientific GeneJET Gel Extraction Kit (Thermo Fisher Scientific, CA, USA) and subsequently sent to Eurofins (Cologne, Germany) for sanger sequencing.

Human, animal, soil, and water samples were run in separate qPCR plates in order to reduce cross-contamination.

2.3.5. Cloning

Sequences that tested positive *Blastocystis* sp. and/or *Cryptosporidium* spp. that had overlapping peaks, and no clear single infection were cloned using the pGEM-T easy vector system I (Promega, Madison, WI, USA) using the methodology as previously described by Betts et al., 2018 [44]. A total of eleven nPCR-positive samples were cloned, with five of them, including three *Cryptosporidium*- and two *Blastocystis-positive* samples, being successful. Furthermore, nine samples that tested positive for *Blastocystis* sp. with qPCR were chosen for cloning; however, none of these samples were successfully cloned.

Five individual colonies were collected from each sample for analysis.

2.3.6. Blastocystis subtype identification

Subtypes were identified using the curated publicly accessible database pubMLST. (https://pubmlst.org/bigsdb?db=pubmlst_Blastocystis_seqdef).

3. Results

3.1. Occurrence of Blastocystis sp., Cryptosporidium spp., Giardia intestinalis and Entamoeba histolytica

A total of 23.5 % (24/102) of the samples were positive for *Blastocystis* sp. (Table 1). This included 19 % of the human samples (3/16) with two participants having watery diarrhoea and one having mucoid diarrhoea at the time of sample collection. Moreover, 41.2 % (21/51), and 2.9 % (1/35) of animal and soil specimens, respectively, were positive for *Blastocystis* sp. The organism was not detected in any of the carnivores (cats, dogs; 0 %, 0/4), while it occurred in 61 % of the herbivores (11/18) and 35 % of the avian hosts. Specifically, occurrence was 33 % (2/6) in cows, 100 % (9/9) in goats, 75 % (6/8) in hens, 33 % (2/6) in pigeons, 50 % (1/2) in koel birds and 100 % (1/1) in titir birds, while *Blastocystis* sp. was not detected in the sheep, rabbits, cockatiels, doves and bazrigars sampled in this study. Eleven samples tested positive for *Blastocystis* sp. when employing nPCR, while qPCR identified an additional eight. Six samples tested positive with both qPCR and nPCR.

Overall, seven *Blastocystis* subtypes were identified: ST2, ST3, ST7, ST10 ST23, ST24 and ST25. Of these, ST3 ($n = 2$) was the only subtype found in humans. One human-origin sequence could not be subtyped as it was of lesser quality and very short, but it was clearly Blastocystis due to a high percentage of identity similarities to reference *Blastocystis* sequences. Animals carried diverse subtypes as follows: ST23 were the most abundant of the five detected, followed by ST24 ($n = 4$), ST10 (n = 2), ST2 (n = 2), ST7 (n = 2), and ST25 ($n = 1$). A mixed infection of ST24 and ST23 was noted. A single ST24 sequence was detected in soil. Five subtypes (ST2, ST10, ST23, ST24, ST25) were detected in herbivores and four in birds (ST2, ST7, ST23, ST24). Combining subtype and host information, ST10 was the most widespread in two hosts (a cow and a hen) together with ST23 in two hosts [goats (n = 4), hen (n = 1)], ST2 detected in two hosts (a goat and a koel bird), ST7 in two (a titir bird and a hen), ST24 in two [goats ($n = 3$) and a hen], followed by ST25 in one (a cow).

Sequence comparison of a 289 bp fragment revealed identical sequences of *Blastocystis* species. Specifically, two ST23 sequences from a goat and a hen residing in the same household were identical along with three from free-ranging animals (hen, pigeon, and goat). Moreover, two identical *Blastocystis* ST24 sequences were detected in one hen and one goat. A total of 71 samples, comprising 13 humans, 31 soil, and 27 animal samples, were false positive for *Blastocystis* sp. when using nested PCR. Most of these were fungi. The high number of false positives is likely due to the use of the broadly specific primers used in the first round of amplification. No false positive was detected among the qPCR positive samples. Five samples of animal origin, namely hen ($n = 2$), goat (n = 2) and pigeon ($n = 1$), could not be subtyped due to the presence of mixed sequences. A total of eight samples (8/102) tested positive for *Cryptosporidium* spp. This included a human sample that tested positive for *Cryptosporidium parvum* subtype *IIaA17G2R2*. This host was not undergoing any antibiotic medical treatment at the time of sampling (Table 1). Notably, the presence of mucus in their stool was observed. Furthermore, six animal samples tested positive for *C. bovis* (n = 2), *C. parvum* (n = 2), *C. meleagridis* (n = 1) *and C. baileyi* (n = 1). This included goats ($n = 3$) and hens (n = 3). Out of these, five were freely roaming animals, consisting of three goats and two hens, along with one hen that was confined to a household. The household-owned hen and one of the free-ranging hens were infected with *C. parvum IIaA15G2R1*. One of the free-ranging hens tested positive for *C. meleagridis*. The remaining three free-ranging animals that tested positive for *Cryptosporidium* were all goats, of which two tested positive for *C. bovis*, while one had a mixed infection with *C. parvum* subtype *IIaA15G2R1*, and *C. baileyi*. One soil sample tested positive for *C. meleagridis*. The origin of this sample was from a location within a household compound where none of the samples collected from humans nor animals tested positive for *Cryptosporidium* spp.

The samples that tested positive for *C. meleagridis* were not further subtyped.

When the primers targeting *tpi, bg,* and *gdh* were used for *G. intestinalis* assemblage detection, only one soil sample tested positive for *G. intestinalis*. The genetic sequence of this particular soil sample was then analysed and subsequently classified as assemblage *B* using primers targeting the *tpi* gene.

None of the samples tested positive for *Entamoeba histolytica*.

A total of 18 different samples were positive for either *Blastocystis* sp. or *Cryptosporidium* spp. (Table 1). Additionally, five samples tested positive for the presence of *Cryptosporidium* spp. and *Blastocystis* sp. or *Blastocystis* sp. and *G. intestinalis* (Table 2). This included four samples, namely two goats and two hens, with a double infection of

Table 1
Infection data from human, various animal, and soil samples in this study were compiled, presenting a summary of the results regarding the prevalence of *Blastocystis* sp., *Cryptosporidium* spp., and *Giardia intestinalis*. Additional information gathered from the questionnaire used for humans in this study is also included.

Sample	*Cryptosporidium* species	*C. parvum* subtype	*G. intestinalis* subtype	*Blastocystis* subtype
Human 1	*Cryptosporidium parvum*	*C. parvum IIaA17G2R2*	–	–
Human 2	–	–	–	*ST3*
Human 3	–	–	–	*ST3*
Soil 1	*Cryptosporidium meleagridis*	–	*G. intestinalis B*	–
Soil 2	–	–	–	*ST24*
Goat 1	*Cryptosporidium bovis*	–	–	*ST23*
Goat 2	–	–	–	*ST23*
Goat 3	–	–	–	*ST24 + ST23*
Goat 4	–	–	–	*ST24*
Goat 5	*Cryptosporidium baileyi*	*C. parvum IIaA15G2R1*	–	*ST24*
Goat 6	*Cryptosporidium bovis*	–	–	–
Goat 7	–	–	–	*ST2*
Goat 8	–	–	–	*ST23*
Hen 1	*Cryptosporidium parvum*	*C. parvum IIaA15G2R1*	–	–
Hen 2	*Cryptosporidium parvum*	*C. parvum IIaA15G2R1*	–	*ST10*
Hen 3	*Cryptosporidium meleagridis*	–	–	*ST7*
Hen 4	–	–	–	*ST23*
Hen 5	–	–	–	*ST24*
Titir bird 1	–	–	–	*ST7*
Koel bird 1	–	–	–	*ST2*
Cow 1	–	–	–	*ST10*
Cow 2				*ST25*

Table 2
Summary of mixed infections in the samples used in this study.

Host	*Cryptosporidium species*	*C. parvum subtype*	*Giardia intestinalis subtype*	*Blastocystis ST*
Goat	*Cryptosporidium bovis*	NA	Negative	*Blastocystis ST23*
Goat	*Cryptosporidium baileyi*	*C. parvum IIaA15G2R1*	Negative	*Blastocystis ST24*
Hen	*Cryptosporidium parvum*	*C. parvum IIaA15G2R1*	Negative	*Blastocystis ST23*
Hen	*Cryptosporidium meleagridis*	NA	Negative	*Blastocystis* ST7
Soil	*Cryptosporidium meleagridis*	NA	Assemblage B	*Negative*

Cryptosporidium spp. and *Blastocystis* sp., and one soil sample with the presence of both *Cryptosporidium* and *G. intestinalis*.

GenBank accession numbers for the *SSU* rRNA sequences for *Cryptosporidium* are OR947712 – OR947716, for *Cryptosporidium parvum* PP067103- PP067106 and for the *tpi* sequences for *Giardia intestinalis* assemblage B PP481986.

4. Discussion

The study took place in a slum located near a major railway in Mymensingh, Bangladesh. Within the slum, 5000 people live and share the same space with animals (domesticated and stray), most of which roam free. Garbage disposal and sewage are in close proximity to water sources making the area an ideal environment for gastrointestinal parasites to thrive and associated diseases to manifest. Hence, this small-scale pilot study aimed to get an overview of certain microbial parasites circulating in the community.

Of the 16 human samples, three tested positive for *Blastocystis* sp. Of these, two were successfully subtyped and came from individuals residing in two different households. The two sequences were identical, suggesting a possible circulation of the same allele in the community. Notably, both human samples that tested positive for *Blastocystis* sp. exhibited watery stool. However, caution should be exercised in associating watery stool with *Blastocystis* sp., especially since all human study participants were diarrhoeic at the time of sampling and the presence of other microbes such as additional intestinal parasites, bacteria, and viruses was not analysed. In comparison to previous investigations in Bangladesh, the sample size and moreover the *Blastocystis* sp. carriage in humans was low in this study [45,46]. Given recent hypotheses that have come forth on *Blastocystis* sp. being part of the healthy gut microbiome, one possible explanation of the low *Blastocystis* sp. occurrence could be that the absence of the organism is due to unfavourable conditions in the gut ecosystem of participants. Alternatively, or perhaps additionally, subtype-level differences could be at play. However, as no subtyping was performed in previous slum-based studies, we cannot draw any conclusions.

Blastocystis carriage was much higher in non-human hosts, especially herbivores and birds. The organism was absent in the carnivores. Overall, six *Blastocystis* subtypes were detected in animal hosts. Subtype 23 was detected in four herbivores and one bird. Though the occurrence of ST23 is expected in ruminants, this is not a typical bird subtype [47–52].

Sequence analysis revealed that five animals (goats, hens, and a caged pigeon) shared identical ST23 sequences, suggesting cycling in these hosts and potentially others with multiple routes of transmission. Similarly, ST24, another ruminant subtype with identical sequences, was also found in a hen and a goat, suggesting cycling of these subtypes in these hosts and potentially others with multiple routes of transmission. The presence of ST23 in a pigeon is one of the few instances of this subtype in an avian species and the first instance in this bird [51]. *Blastocystis* ST2 was also detected in a koel bird. This subtype is more commonly detected in humans; however, it has also been detected in a wide range of animals, including monkeys, cattle, chickens, and pigs [53]. Several *Blastocystis* subtypes have previously been detected in a variety of birds, such as ST7, ST8, ST10 and ST14 [47,49,54]. However, to our knowledge, there have not been any previous reports of *Blastocystis* ST2 in koel birds. Earlier data pertaining to *Blastocystis* presence in Bangladesh, namely in a zoo of wildlife animals, identified a rich diversity of subtypes including ST1-ST3, ST10-ST11, and ST13-ST14 [21]. Herein, we add ST24 to the subtypes found in the country. Moreover, goats have previously been found to host a diverse array of *Blastocystis* species, encompassing ST1, ST3, ST6, ST7, and ST10 [50,51].

A total of 71 samples, including 13 from humans, 31 from soil, and 27 from animals, were false positives for *Blastocystis* sp. when utilising the nPCR approach. This occurrence is probably due to the nonspecific binding of the first set of primers, leading to the amplification of fungi in this instance.

In this study, a total of eight samples tested positive for *Cryptosporidium* spp. One of these was from a human host and corresponded to *C. parvum* genotype *IIaA17G2R2*. This host exhibited mucoid diarrhoea and was not undergoing antibiotic treatment during the time of sampling. This is a zoonotic subtype that has been sporadically reported in humans and cattle from the United States [52,55] and dogs from Brazil [56]. *Cryptosporidium bovis* was found in six animal hosts, of which two were goats. The third goat had a mixed infection with *C. baileyi* and *C. parvum*. The subtype of the latter was identified as *IIaA15G2R1*, which was also present in two hens. This is a cosmopolitan, hyper-transmissible subtype found mainly in calves and humans [57,58], responsible for previous outbreaks of human cryptosporidiosis [59,60]. Hence, the presence of *IIaA15G2R1* in two hosts suggests cycling of this subtype in the community and warrants further investigation (Fig. 2). The identification of *C. baileyi,* a bird-specific species [61], in a goat sample along with the overlap of the same *C. parvum* subtype in goats and hens, suggests possible transmission between the two hosts. Furthermore, it's crucial to consider that the presence of *C. baileyi* in a goat sample might result from incidental contamination. Therefore, it would be advisable to collect more samples from a broader range of sites for a comprehensive evaluation of infection.

One soil sample tested positive for both *C. meleagridis* and *G. intestinalis* assemblage B. The former was also present in a hen suggesting a possible environmental contamination of *C. meleagridis* from infected animals within the slum. A limited number of samples tested positive for *G. intestinalis* herein, highlighting the need for further investigations with a larger and more diverse sample. Exploring the diversity of various *Giardia* species in the same region would also be a valuable approach to comprehend their prevalence and distribution in the study area.

Our results demonstrate that hens have roles in spreading parasites in the community (Fig. 2). We are putting forth a testable hypothesis, whereby hens are the vehicles of transmission for *Cryptosporidium* and *Blastocystis* and potentially other parasites.

5. Conclusions

The proximity of humans and animals to water sources and particularly ponds used for activities like swimming, laundry, cattle bathing, and cleaning vegetables and utensils in slum regions raises concerns

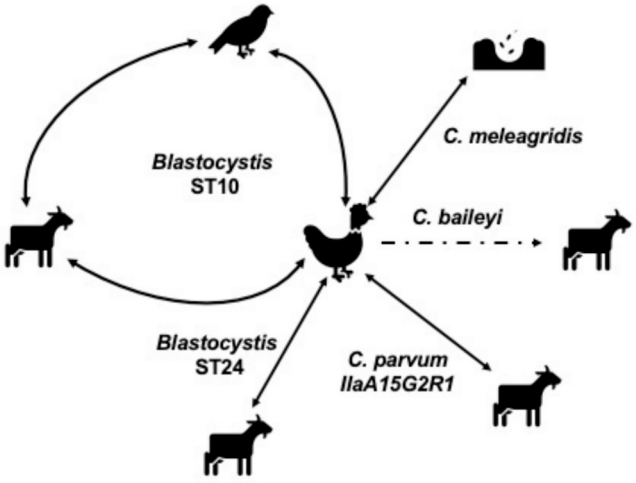

Fig. 2. Visual representation of identical *Blastocystis* species sequences revealed in this study, present in the host organisms such as goats, hens, and a pigeon for *Blastocystis* ST10 and a hen and a goat for *Blastocystis* ST24.This figure also visualizes the potential spread of diverse *Cryptosporidium* species between various animals and soil.

about potential parasite transmission via water and the surrounding environment. The detection of eukaryotic gut microbes in this study has provided insights into their occurrence not only in humans but also in various animals and soil in a slum in Bangladesh. This underscores the interconnectedness of humans, animals, and the environment and how all three must be considered for a comprehensive understanding and management of diarrhoea and associated intestinal parasitic diseases. Continuous investigations are important to enhance our knowledge of parasite transmission, especially in developing countries. Moreover, conducting future One Health studies in Bangladesh, encompassing larger populations, and expanding the research scope to include various study areas and sample types, such as different water sources, will yield valuable insight into intestinal parasites and their transmission dynamics.

5.1. Limitations

While the current study, serving as a pilot study, offers valuable insights into the presence of intestinal parasites within a specific context, its findings are subject to limitations. Primarily, the sample size, particularly the representation of human subjects and the diversity of environmental samples, is insufficient to comprehensively grasp the full spectrum of intestinal parasite prevalence and transmission dynamics. Including a wider array of locations and hosts would facilitate a better understanding of parasite distribution and transmission pathways. Thus, while this study serves as a valuable starting point, further research with expanded sample diversity is necessary to enhance our understanding of intestinal parasite dynamics.

5.2. Low incidence of Blastocystis sp. in humans

The notably low incidence of *Blastocystis* sp. observed in our study may be influenced by several factors that differ from previous research. Seasonal variations, which were not accounted for in earlier studies, could significantly affect parasite prevalence. Additionally, variations in hygiene practices, dietary habits, or health status (these were individuals with diarrhoea) among the populations studied may also play a critical role in influencing *Blastocystis* sp. prevalence. Furthermore, methodological differences in sample collection, storage, and processing between our study and previous reports could have impacted the detection rates. These factors should be considered when interpreting the lower prevalence found in our analysis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2024.102967.

Ethics statement

The Bangladesh Agricultural University ethics committee (approval no: AWEEC/BAU/2022[80]) approved the research project. Consent was obtained from each household before the study.

A questionnaire was also distributed to the participants and filled out with the help of the local researchers to collect demographic data **(Supplementary Table S1).** Information on gastrointestinal-related symptoms, antibiotic usage, and sources of food and drinking water was obtained from the humans included in this study.

Funding

SM was supported by a Ph.D. studentship from the Global Challenges Doctoral Centre at the University of Kent, United Kingdom.

CRediT authorship contribution statement

Sadiya Maxamhud: Writing – original draft, Methodology, Investigation, Formal analysis. **Md Shahiduzzaman:** Writing – review & editing, Supervision, Resources, Conceptualization. **A.R.M. Beni Amin:** Methodology. **Md. Zawad Hossain:** Methodology, Conceptualization. **Eleni Gentekaki:** Writing – review & editing, Validation, Supervision, Data curation. **Anastasios D. Tsaousis:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration.

Declaration of competing interest

All authors declared that there are no conflicts of interest. The funders had no role in the study's design; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Data availability

All data have been submitted to GenBank with accession numbers available. GenBank accession numbers for the SSU rRNA sequences for *Cryptosporidium* are OR947712 – OR947716. For *Cryptosporidium parvum* gp60 sequences, the accession numbers are PP067103-PP067106, and for *Giardia intestinalis* assemblage B *tpi* sequence, the accession number is PP481986.

Acknowledgments

We are expressing gratitude to all those in Mymensingh, Bangladesh, who generously dedicated their time and provided samples for this study, contributing to making it possible. We would like to thank Daisy Shaw for proofreading the manuscript.

References

[1] C.L. Fischer Walker, J. Perin, M.J. Aryee, C. Boschi-Pinto, R.E. Black, Diarrhea incidence in low- and middle-income countries in 1990 and 2010: a systematic review, BMC Public Health 12 (2012), https://doi.org/10.1186/1471-2458-12-220.

[2] B. Loftus, I. Anderson, R. Davies, U.C.M. Alsmark, J. Samuelson, P. Amedeo, P. Roncaglia, M. Berriman, R.P. Hirt, B.J. Mann, T. Nozaki, B. Suh, M. Pop, M. Duchene, J. Ackers, E. Tannich, M. Leippe, M. Hofer, I. Bruchhaus, U. Willhoeft, A. Bhattacharya, T. Chillingworth, C. Churcher, Z. Hance, B. Harris, D. Harris, K. Jagels, S. Moule, K. Mungall, D. Ormond, R. Squares, S. Whitehead, M.A. Quail, E. Rabbinowitsch, H. Norbertczak, C. Price, Z. Wang, N. Guillén, C. Gilchrist, S.E. Stroup, S. Bhattacharya, A. Lohia, P.G. Foster, T. Sicheritz-Ponten, C. Weber, U. Singh, C. Mukherjee, N.M. El-Sayed, W.A. Petri, C.G. Clark, T.M. Embley, B. Barrell, C.M. Fraser, N. Hall, The genome of the protist parasite *Entamoeba histolytica*, Nature 2005 433:865–868. doi:https://doi.org/10.1038/nature03291.

[3] U.M. Ryan, Y. Feng, R. Fayer, L. Xiao, Taxonomy and molecular epidemiology of *Cryptosporidium* and *Giardia* – a 50 year perspective (1971–2021), Int. J. Parasitol. 51 (2021) 1099–1119, https://doi.org/10.1016/j.ijpara.2021.08.007.

[4] U. Ryan, N. Hijjawi, Y. Feng, L. Xiao, *Giardia*: an under-reported foodborne parasite, Int. J. Parasitol. 49 (2019) 1–11, https://doi.org/10.1016/j.ijpara.2018.07.003.

[5] L.J. Robertson, *Giardia*sis in Animals - Digestive System - MSD Veterinary Manual, MSD Manual - Veterinary Manual. https://www.msdvetmanual.com/digestive-system/*Giardia*sis-*Giardia*/*Giardia*sis-in-animals, 2021 accessed February 5, 2023.

[6] B. Dadonaite, H. Ritchie, M. Roser, Diarrheal Diseases - Our World in Data, Our World in Data. https://ourworldindata.org/diarrheal-diseases#licence, 2019 accessed February 5, 2023.

[7] Y. Feng, L. Xiao, Zoonotic potential and molecular epidemiology of *Giardia* species and *Giardia*sis †, Clin. Microbiol. Rev. 24 (2011) 110–140, https://doi.org/10.1128/CMR.00033-10.

[8] L. Xiao, Molecular epidemiology of cryptosporidiosis: an update, Exp. Parasitol. 124 (2010) 80–89, https://doi.org/10.1016/J.EXPPARA.2009.03.018.

[9] J.G. Maloney, A. Molokin, R. Seguí, P. Maravilla, F. Martínez-Hernández, G. Villalobos, A.D. Tsaousis, E. Gentekaki, C. Muñoz-Antolí, D.R. Klisiowicz, C. Y. Oishi, R. Toledo, J.G. Esteban, P.C. Köster, A. de Lucio, A. Dashti, B. Bailo, R. Calero-Bernal, D. González-Barrio, D. Carmena, M. Santín, Identification and molecular characterization of four new *Blastocystis* subtypes designated ST35-ST38, Microorganisms 11 (2023), https://doi.org/10.3390/microorganisms11010046.

[10] S.M. Cacciò, R.C.A. Thompson, J. McLauchlin, H.V. Smith, Unravelling *Cryptosporidium* and *Giardia* epidemiology, Trends Parasitol. 21 (2005) 430–437, https://doi.org/10.1016/j.pt.2005.06.013.

[11] C.R. Stensvold, C.G. Clark, Current status of *Blastocystis*: a personal view, Parasitol. Int. 65 (2016) 763–771, https://doi.org/10.1016/j.parint.2016.05.015.

[12] K.S.W. Tan, New insights on classification, identification, and clinical relevance of *Blastocystis* spp, Clin. Microbiol. Rev. 21 (2008) 639–665, https://doi.org/10.1128/CMR.00022-08.

[13] V. Jinatham, S. Maxamhud, S. Popluechai, A.D. Tsaousis, E. Gentekaki, *Blastocystis* one health approach in a rural community of Northern Thailand: prevalence, subtypes and novel transmission routes, Front. Microbiol. 12 (2021), https://doi.org/10.3389/fmicb.2021.746340.
[14] S. Hoque, P. Pinto, C.A. Ribeiro, E. Canniere, Y. Daandels, M. Dellevoet, A. Bourgeois, O. Hammouma, P. Hunter, E. Gentekaki, M. Kváč, J. Follet, A. D. Tsaousis, Follow-up investigation into *Cryptosporidium* prevalence and transmission in Western European dairy farms, Vet. Parasitol. 318 (2023) 109920, https://doi.org/10.1016/j.vetpar.2023.109920.
[15] P.M. Rabinowitz, R. Kock, M. Kachani, R. Kunkel, J. Thomas, J. Gilbert, R. Wallace, C. Blackmore, D. Wong, W. Karesh, B. Natterson, R. Dugas, C. Rubin, Toward proof of concept of a one health approach to disease prediction and control, Emerg. Infect. Dis. 19 (2013), https://doi.org/10.3201/EID1912.130265.
[16] J.G. Maloney, J.E. Lombard, N.J. Urie, C.B. Shivley, M. Santin, Zoonotic and genetically diverse subtypes of *Blastocystis* in US pre-weaned dairy heifer calves, Parasitol. Res. 118 (2019) 575–582, https://doi.org/10.1007/s00436-018-6149-3.
[17] F. Eroglu, I.S. Koltas, Evaluation of the transmission mode of B. Hominis by using PCR method, Parasitol. Res. 107 (2010) 841–845, https://doi.org/10.1007/s00436-010-1937-4.
[18] M.A. Alfellani, D. Taner-Mulla, A.S. Jacob, C.A. Imeede, H. Yoshikawa, C. R. Stensvold, C.G. Clark, Genetic diversity of *Blastocystis* in livestock and zoo animals, Protist 164 (2013) 497–509, https://doi.org/10.1016/j.protis.2013.05.003.
[19] M. Santin, A. Figueiredo, A. Molokin, N.S. George, P.C. Köster, A. Dashti, D. González-Barrio, D. Carmena, J.G. Maloney, Division of *Blastocystis* ST10 into three new subtypes: ST42-ST44, J. Eukaryot. Microbiol. (2023), https://doi.org/10.1111/jeu.12998.
[20] L. Deng, L. Wojciech, C.W. Png, Y.Q.D. Kioh, G.C. Ng, E.C.Y. Chan, Y. Zhang, N.R. J. Gascoigne, K.S.W. Tan, Colonization with ubiquitous protist *Blastocystis* ST1 ameliorates DSS-induced colitis and promotes beneficial microbiota and immune outcomes, NPJ Biofilms Microbiomes 9 (2023), https://doi.org/10.1038/s41522-023-00389-1.
[21] J. Li, M.R. Karim, D. Li, S.M.M. Rahaman Sumon, S.H.M.F. Siddiki, F.I. Rume, R. Sun, Y. Jia, L. Zhang, Molecular characterization of *Blastocystis* sp. in captive wildlife in Bangladesh National zoo: non-human primates with high prevalence and zoonotic significance, Int. J. Parasitol. Parasites Wildl. 10 (2019) 314–320, https://doi.org/10.1016/j.ijppaw.2019.11.003.
[22] B. Zaman Wahid, M.A. Haque, M.A. Gazi, S.M. Fahim, A.S.G. Faruque, M. Mahfuz, T. Ahmed, Site-specific incidence rate of *Blastocystis hominis* and its association with childhood malnutrition: findings from a multi-country birth cohort study, Am. J. Trop. Med. Hyg. 108 (2023) 887–894, https://doi.org/10.4269/ajtmh.22-0662.
[23] Y. Feng, L. Xiao, Zoonotic potential and molecular epidemiology of *Giardia* species and *Giardia*sis, Clin. Microbiol. Rev. 24 (2011) 110–140, https://doi.org/10.1128/CMR.00033-10.
[24] M. Kantor, A. Abrantes, A. Estevez, A. Schiller, J. Torrent, J. Gascon, R. Hernandez, C. Ochner, *Entamoeba histolytica*: updates in clinical manifestation, pathogenesis, and vaccine development, Can. J. Gastroenterol. Hepatol. 2018 (2018), https://doi.org/10.1155/2018/4601420.
[25] C.G. Victora, S.R. Huttly, S. Fuchs, F. Barros, M. Garenne, O. Leroy, O. Fontaine, J. Beau, V. Fauveau, H. Chowdhury, M. Yunus, J. Chakraborty, A. Sarder, S. Kapoor, M. Bhan, L. Nath, J. Martines, International differences in clinical patterns of Diarrhoeal deaths: a comparison of children from Brazil, Senegal, Bangladesh, and India, J. Diarrhoeal Dis. Res. 11 (1993) 1993.
[26] R. Haque, D. Mondal, A. Karim, I.H. Molla, A. Rahim, A.S.G. Faruque, N. Ahmad, B. D. Kirkpatrick, E. Houpt, C. Snider, W.A. Petri, Prospective case-control study of the association between common enteric protozoal parasites and diarrhea in Bangladesh, Clin. Infect. Dis. 48 (2009) 1191–1197, https://doi.org/10.1086/597580.
[27] P.S. Korpe, C. Gilchrist, C. Burkey, M. Taniuchi, E. Ahmed, V. Madan, R. Castillo, S. Ahmed, T. Arju, M. Alam, M. Kabir, T. Ahmed, W.A. Petri, R. Haque, A.S. G. Faruque, P. Duggal, Case-control study of *Cryptosporidium* transmission in Bangladeshi households, Clin. Infect. Dis. 68 (2019) 1073–1079, https://doi.org/10.1093/cid/ciy593.
[28] R. Haque, D. Mondal, A. Karim, I. Hossain Molla, A. Rahim, A.S.G. Faruque, N. Ahmad, B.D. Kirkpatrick, E. Houpt, C. Snider, W.A. Petri, Prospective case-control study of the association between common enteric protozoal parasites and diarrhea in Bangladesh, Clin. Infect. Dis. 48 (2009) 1191–1197, https://doi.org/10.1086/597580.
[29] R. Haque, S. Roy, M. Kabir, S.E. Stroup, D. Mondal, E.R. Houpt, *Giardia* assemblage a infection and diarrhea in Bangladesh, J. Infect. Dis. 192 (2005) 2171–2173, https://doi.org/10.1086/498169.
[30] H. Ritchie, E. Mathieu, Which Countries Are most Densely Populated? - Our World in Data. https://ourworldindata.org/most-densely-populated-countries, 2019.
[31] United Nations Development Programme, Bridging the Urban Divide in Bangladesh. Event Report for the Joint Conversation Event of the Local Government Division, Ministry of Local Government, Rural Development and Cooperatives and the LCG Urban Sector. https://www.undp.org/bangladesh/publications/bridging-urban-divide-bangladesh, 2010 (accessed October 16, 2023).
[32] M.T. Rahman, M.A. Sobur, M.S. Islam, S. Ievy, M.J. Hossain, M.E.E. Zowalaty, A.M. M.T. Rahman, H.M. Ashour, Zoonotic diseases: etiology, impact, and control, Microorganisms 8 (2020) 1–34, https://doi.org/10.3390/microorganisms8091405.
[33] S.K. Karn, H. Harada, Surface water pollution in three urban territories of Nepal, India, and Bangladesh, Environ. Manag. 28 (2001) 483–496, https://doi.org/10.1007/s002670010238.
[34] P. Pinto, C.A. Ribeiro, S. Hoque, O. Hammouma, H. Leruste, S. Détriché, E. Canniere, Y. Daandels, M. Dellevoet, J. Roemen, A.B. Bourgeois, M. Kváč, J. Follet, A.D. Tsaousis, Cross-border investigations on the prevalence and transmission dynamics of *Cryptosporidium* species in dairy cattle farms in Western mainland Europe, Microorganisms 9 (2021) 2394, https://doi.org/10.3390/MICROORGANISMS9112394.
[35] S. Maxamhud, N. Reghaissia, A. Laatamna, H. Samari, N. Remdani, E. Gentekaki, A.D. Tsaousis, Molecular identification of *Cryptosporidium* spp., and *Giardia* duodenalis in dromedary camels (*Camelus dromedarius*) from the Algerian Sahara, Parasitologia 3 (2023) 151–159, https://doi.org/10.3390/parasitologia3020016.
[36] J.J. Verweij, R.A. Blangé, K. Templeton, J. Schinkel, E.A.T. Brienen, M.A.A. Van Rooyen, L. Van Lieshout, A.M. Polderman, Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR, J. Clin. Microbiol. 42 (2004) 1220–1223, https://doi.org/10.1128/JCM.42.3.1220-1223.2004.
[37] S.M. Cacciò, R. Beck, M. Lalle, A. Marinculic, E. Pozio, Multilocus genotyping of *Giardia duodenalis* reveals striking differences between assemblages a and B, Int. J. Parasitol. 38 (2008) 1523–1531, https://doi.org/10.1016/j.ijpara.2008.04.008.
[38] I.M. Sulaiman, R. Fayer, C.T. Bern, R.H. Gilman, J.M. Trout, P.M. Schantz, P. Das, A.A. Lal, L. Xiao, Triosephosphate Isomerase Gene Characterization and Potential Zoonotic Transmission of *Giardia duodenalis*, Emerg. Infect. Dis. 9 (2003) 1444–1452, https://doi.org/10.3201/eid0911.030084.
[39] S.M. Cacciò, M. De Giacomo, E. Pozio, Sequence Analysis of the b-Giardin Gene and Development of a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Assay to Genotype *Giardia Duodenalis* Cysts from Human Faecal Samples. www.parasitology-online.com, 2002.
[40] C.G. Clark, Extensive Genetic Diversity in *Blastocystis hominis*, Mol. Biochem. Parasitol. 87 (1) (1997) 79–83.
[41] S.M. Scicluna, B. Tawari, C.G. Clark, DNA barcoding of *Blastocystis*, Protist 157 (2006) 77–85, https://doi.org/10.1016/j.protis.2005.12.001.
[42] P. Poirier, I. Wawrzyniak, A. Albert, H. El Alaoui, F. Delbac, V. Livrelli, Development and evaluation of a real-time PCR assay for detection and quantification of *Blastocystis* parasites in human stool samples: prospective study of patients with hematological malignancies, J. Clin. Microbiol. 49 (2011) 975–983, https://doi.org/10.1128/JCM.01392-10.
[43] P. Poirier, I. Wawrzyniak, A. Albert, H. El Alaoui, F. Delbac, V. Livrelli, Development and evaluation of a real-time PCR assay for detection and quantification of *Blastocystis* parasites in human stool samples: prospective study of patients with hematological malignancies, J. Clin. Microbiol. 49 (2011) 975–983, https://doi.org/10.1128/JCM.01392-10.
[44] E.L. Betts, E. Gentekaki, A. Thomasz, V. Breakell, A.I. Carpenter, A.D. Tsaousis, Genetic diversity of *Blastocystis* in non-primate animals, Parasitology 145 (2018) 1228–1234, https://doi.org/10.1017/S0031182017002347.
[45] S.M. Fahim, M.A. Gazi, M.M. Hasan, M.A. Alam, S. Das, M. Mahfuz, M.M. Rahman, R. Haque, S.A. Sarker, T. Ahmed, Infection with *blastocystis* spp. and its association with enteric infections and environmental enteric dysfunction among slum-dwelling malnourished adults in Bangladesh, PLoS Negl. Trop. Dis. 15 (2021), https://doi.org/10.1371/journal.pntd.0009684.
[46] H. Yoshikawa, Z. Wu, I. Kimata, M. Iseki, I.K.M.D. Ali, M.B. Hossain, V. Zaman, R. Haque, Y. Takahashi, Polymerase chain reaction-based genotype classification among human *Blastocystis hominis* populations isolated from different countries, Parasitol. Res. 92 (2004) 22–29, https://doi.org/10.1007/s00436-003-0995-2.
[47] J. David Ramírez, L.V. Sánchez, D.C. Bautista, F. Corredor, A. Carolina Flórez, C. R. Stensvold, *Blastocystis* Subtypes Detected in Humans and Animals From Colombia, 2013, https://doi.org/10.1016/j.meegid.2013.07.020.
[48] J.G. Maloney, A. Molokin, M.J.R. da Cunha, M.C. Cury, M. Santin, *Blastocystis* subtype distribution in domestic and captive wild bird species from Brazil using next generation amplicon sequencing, Parasite Epidemiol. Control 9 (2020), https://doi.org/10.1016/j.parepi.2020.e00138.
[49] J. Wang, B. Gong, X. Liu, W. Zhao, T. Bu, W. Zhang, A. Liu, F. Yang, Distribution and genetic diversity of *Blastocystis* subtypes in various mammal and bird species in northeastern China, Parasit. Vectors 11 (2018), https://doi.org/10.1186/s13071-018-3106-z.
[50] Y. Chang, Y. Yan, H. Han, Y. Wu, J. Li, C. Ning, S. Zhang, L. Zhang, Prevalence of *Blastocystis* infection in free-range Tibetan sheep and Tibetan goats in the Qinghai-Tibetan plateau in China, One Health 13 (2021), https://doi.org/10.1016/j.onehlt.2021.100347.
[51] T.C. Tan, P.C. Tan, R. Sharma, S. Sugnaseelan, K.G. Suresh, Genetic diversity of caprine *Blastocystis* from peninsular Malaysia, Parasitol. Res. 112 (2013) 85–89, https://doi.org/10.1007/s00436-012-3107-3.
[52] G.R. Herges, G. Widmer, M.E. Clark, E. Khan, C.W. Giddings, M. Brewer, J. M. McEvoy, Evidence that *Cryptosporidium parvum* populations are Panmictic and unstructured in the upper Midwest of the United States, Appl. Environ. Microbiol. 78 (2012) 8096, https://doi.org/10.1128/AEM.02105-12.
[53] B. Skotarczak, Genetic diversity and pathogenicity of *Blastocystis*, Ann. Agric. Environ. Med. 25 (2018) 411–416, https://doi.org/10.26444/aaem/81315.
[54] J.G. Maloney, A. Molokin, M.J. Rodrigues Da Cunha, C. Cury, M. Santin, *Blastocystis* subtype distribution in domestic and captive wild bird species from Brazil using next generation amplicon sequencing, Parasite Epidemiol. Control 9 (2020), https://doi.org/10.1016/j.parepi.2020.e00138.
[55] D.C. Feltus, C.W. Giddings, B.L. Schneck, T. Monson, D. Warshauer, J.M. McEvoy, Evidence supporting zoonotic transmission of *Cryptosporidium* spp. in Wisconsin, J. Clin. Microbiol. 44 (2006) 4303, https://doi.org/10.1128/JCM.01067-06.
[56] A.G.L. De Oliveira, A.P. Sudre, T.C.B. Do Bomfim, H.L.C. Santos, Molecular characterization of *Cryptosporidium* spp. in dogs and cats in the city of Rio de

Janeiro, Brazil, reveals potentially zoonotic species and genotype, PLoS ONE 16 (2021) e0255087, https://doi.org/10.1371/JOURNAL.PONE.0255087.
[57] M. Mammeri, L. Cartou, A. Chevillot, M. Thomas, C. Julien, I. Vallée, B. Polack, J. Follet, K.T. Adjou, First identification of *Cryptosporidium parvum* zoonotic subtype IIaA15G2R1 in diarrheal lambs in France, Vet. Parasitol. Reg. Stud. Rep. 18 (2019) 100355, https://doi.org/10.1016/J.VPRSR.2019.100355.
[58] Y. Chen, J. Huang, H. Qin, L. Wang, J. Li, L. Zhang, *Cryptosporidium* parvum and gp60 genotype prevalence in dairy calves worldwide: a systematic review and meta-analysis, Acta Trop. 240 (2023), https://doi.org/10.1016/j.actatropica.2023.106843.
[59] T. Geurden, B. Levecke, S.M. Cacci, A. Visser, G. De Groote, S. Casaert, J. Vercruysse, E. Claerebout, Multilocus genotyping of *Cryptosporidium* and *Giardia* in non-outbreak related cases of diarrhoea in human patients in Belgium, Parasitology 136 (2009) 1161–1168, https://doi.org/10.1017/S0031182009990436.
[60] C.R. Stensvold, S. Ethelberg, L. Hansen, S. Sahar, M. Voldstedlund, M. Kemp, G. N. Hartmeyer, E. Otte, A.L. Engsbro, H.V. Nielsen, K. Mølbak, *Cryptosporidium* infections in Denmark, 2010-2014, Dan. Med. J. 62 (2015).
[61] A.A. Nakamura, M.V. Meireles, *Cryptosporidium* infections in birds - a review, electronic, Braz. J. Vet. Parasitol (2016) 253–267, https://doi.org/10.1590/S1984-29612015063.