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# Prevalence and molecular characterization of *Cryptosporidium* species in poultry in Bangladesh

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ABSTRACT

*Cryptosporidium* is an opportunistic parasite that has been reported in > 30 avian hosts worldwide, however, there is no information regarding *Cryptosporidium* spp. in poultry in Bangladesh. Accordingly, we investigated the prevalence of *Cryptosporidium* spp. in poultry at open live bird markets in Bangladesh. A total of 197 samples were randomly collected from poultry at open live bird markets in Bangladesh and screened for the detection of *Cryptosporidium.* Initial microscopic examination revealed *Cryptosporidium* spp. was observed in 19.8% (39/197) of the poultry specimens. Subsequent nested PCR targeting the 18S rRNA gene revealed that 15.7% (31/197) of the samples were *Cryptosporidium* positive. Of these 31 samples, 17 were *Cryptosporidium baileyi* (8.7%), 12 were *Cryptosporidium meleagridis* (6.0%), and 2 were *Cryptosporidium parvum* (1.0%). Nucleotide sequence analysis of the GP60 gene of the *C. meleagridis* revealed that two subtypes (IIIbA21G1R1 and IIIbA23G1R1), which were found in broiler, native and sonali chickens and a pigeon, matched those previously reported in humans and poultry. We identified two novel subtypes (IIIbA21G2R1 and IIIbA20G2R1) in sonali chickens, a broiler chicken and a layer chicken. We also amplified the GP60 gene of *C. parvum* and found two subtypes (IIaA11G2R1 and IIaA13G2R1) in a sonali and a broiler chicken that were previously reported in calf. These findings suggest that poultry can be a source of cryptosporidial infections for humans and animals in Bangladesh. This is the first molecular investigation of *Cryptosporidium* genotypes and subtypes in poultry at open live bird markets in Bangladesh.

## 1. Introduction

Cryptosporidiosis is a protozoan disease widely found in wild, domestic, and captive birds from several parts of the world [36]. *Cryptosporidium* has been recorded in > 30 avian species worldwide, including chickens, turkeys, ducks, geese, quails, pheasants, and peacocks [31]. So far, infections in birds are mainly caused by four species: *C. baileyi*, *C. galli*, *C. meleagridis,* and *C. avium* [29]. *Cryptosporidium* is transmitted through the fecal-oral route by environmentally resistant oocysts that are shed in the feces, contaminating soil and water, and thus providing multiple pathways into the food chain [32]. *C. meleagridis* is the only *Cryptosporidium* species known to infect both birds and mammals [5]. Therefore, humans can acquire cryptosporidiosis either by consumption of water and food contaminated with oocysts or direct contact with infected people or animals [49]. In 2010, *C. baileyi* was reported to be the dominant *Cryptosporidium* species found in all age groups of chickens [44], and in 2017, *C. parvum* was the most frequently observed species identified in poultry in Germany [14]. Cross-species transmission of *C. meleagridis* between birds and humans has been reported in Peru [43,45]. *Cryptosporidium meleagridis* is considered the third most common species of this genus emerging in humans [47]. *Cryptosporidium*-infected domestic pigeons have been reported in some regions of the world, such as in Thailand [19], China [23], and Brazil [30]. The markets where poultry is purchased are crowded with people, and it is possible for *Cryptosporidium* to spread to the surrounding environment due to close contact with other species of birds or mammals in the markets [4].

The poultry industry of Bangladesh is a promising sector for economic growth. In Bangladesh, documented studies of cryptosporidiosis

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in animals and poultry are limited. Moreover, no studies examining protozoan infections have been done in the open live bird markets of Bangladesh. A few studies of *Cryptosporidium* related to human disease have been done in Bangladesh, including reports of *C. parvum* and *C. meleagridis* in children in Bangladesh [15,20]. *C. meleagridis* was the predominant species in rural areas, whereas in urban areas *C. meleagridis* was the second most frequently identified species in Bangladesh after *C. parvum* [37].

In Bangladesh, open live bird markets offer wholesale poultry. Peoples gather to buy the poultry for consumption and sellers slaughter and process the poultry under unhygienic conditions. The poultry excrete droppings, which are not regularly cleaned up, creating a high risk for transmission of *Cryptosporidium* infection from poultry to humans. Therefore, the present study sought to determine the epidemiology of *Cryptosporidium* spp. in poultry at open live bird markets in Bangladesh and to molecularly characterize the *Cryptosporidium* spp. identified.

## 2. Materials and methods

### 2.1. Ethics statement

All samples were collected from live bird markets. Since samples were collected after slaughter of the poultry, no permission regarding laws on animal protection was required. We received permission from the poultry sellers to collect the samples.

### 2.2. Sample collection

A total of 197 intestinal colon samples from different poultry species including layer chickens ($n$ = 12), broiler chickens ($n$ = 80), sonali chickens ($n$ = 93), native chickens (n = 8) and pigeons ($n$ = 4) were obtained from 19 different live bird markets (LBM) in Dhaka, Bangladesh. The age of the poultry ranged from one month to two years. Poultry were kept together in cages for sale to customers. Fresh intestinal colon samples were collected after the slaughter of every bird from a cage, taking care to collect only the portion that did not have direct contact with the cage to avoid contamination. All relevant data such as breed and age were recorded for further analysis. Each sample was placed into an individual sterile polystyrene tube, labelled, and transferred in an isothermal box to the laboratory where it was kept at 4 ˚C until DNA extraction.

### 2.3. Microscopic examinations

The samples were examined for *Cryptosporidium* oocysts by using the sucrose flotation technique. Briefly, 1 g of colon contents from the sample was suspended in 9 ml of saturated sucrose solution (Specific gravity 1.2) in a tube and centrifuged at 1300*g* for 5 min. Then, sucrose solution was added up to the 15-ml tube, which was then left at room temperature for 30 min. Then, the tube was filled to create a meniscus by adding drops of solution and putting a coverslip over the tube for 5–10 min. After that, the coverslip was put on a glass slide and examined for oocysts under a light microscope [9]. Thereafter, oocysts of *Cryptosporidium*-positive samples were transferred from the surface of the suspension to a microscopic slide, air dried, and then fixed for 3–5 min in absolute methanol before they were stained with Ziehl–Neelsen carbol fuchsin for 20 min. After washing with tap water, smears were decolorized with 5% acid alcohol for 20–30 s, washed again with tap water and counterstained with 0.4% methylene blue for 2 min, then rinsed again with tap water and air dried. After drying, the stained smears were examined for oocysts under light microscope using an oil immersion objective lens (×100) [50]. The remaining samples were kept at 4 ˚C until DNA extraction [7].

### 2.4. DNA extraction

DNA was extracted directly from samples by using the QIAamp Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions with minor modifications. Modifications included the addition of 0.2 g of *E*-Z zirconia beads to 0.2 g of feces and 1.4 ml of lysis buffer [27]. Then, the mixture was heated at 95 ˚C for 10 min followed by vigorous shaking using the Biomedicals Fast Prep® instrument (1620 r/min for 150 s) to facilitate oocyst rupture. Nucleic acid was eluted in 100 µl of AE buffer to increase the quantity of DNA recovered. After the DNA was extracted, it was stored at −20 ˚C.

### 2.5. Nested PCR analysis of the 18S rRNA and GP60 genes

For the primary PCR, a PCR product of 1325 bp was first amplified using the primers (Table S1). The amplification was performed in a 25-µl volume with 2 µl of each DNA sample in 12.5 µl of 2 × PCR buffer, 5 µl of deoxynucleotide triphosphates (2 mM each), 0.25 µl of each primer (50 µM), 4.5 µl of double distilled water, and 0.5 µl of KOD- Fx Neo amplification enzyme (1 units/µl) (ToYoBo Co., Ltd., Osaka, Japan). Then, for the nested-PCR (nPCR), 2 µl of the primary PCR product was used with appropriate primers to amplify a ~ 830-bp fragment of the *Cryptosporidium* 18S rRNA gene as described previously [48]. The PCR reaction consisted of an initial heating at 94 ˚C for 2 min, and 35 cycles of 98 ˚C for 10 s, 55 ˚C for 60 s, and 68 ˚C for 30 s (primary PCR) or 98 ˚C for 10 s, 60 ˚C for 30 s, and 68 ˚C for 1 min 30 s (nested PCR). The 60-kDa glycoprotein (GP60; ~830 bp; [2,11,24]) was also amplified in positive samples on the basis of the results for the 18S rRNA. For subtyping of *C. meleagridis*, nPCR with a specific set of primers was performed to amplify a 1100-bp and a 900-bp fragment of the GP60 gene [38]. For subtyping of *C. parvum*, nPCR with a specific set of primers was performed to amplify an 850-bp and an 800-bp fragment of the GP60 gene [8]. Primary PCR was carried out in a total volume of 25 µl with 2 µl of each DNA sample in 12.5 µl of 2 × PCR buffer, 5 µl of deoxynucleotide triphosphates (2 mM each), 0.25 µl of each primer (50 µM), 4.5 µl of nuclease-free water, and 0.5 µl of KOD- Fx Neo amplification enzyme (1 units/µl). For the nested PCR, 2 µl of the primary PCR product was used. The PCR and cycling conditions were unique to the primary and nPCR and consisted of an initial denaturation at 94 ˚C for 2 min, and 35 cycles of 98 ˚C for 10 s, 50 ˚C for 30 s, and 68 ˚C for 60 s. As positive and negative controls, *C. parvum* genomic DNA and ultrapure water, respectively, were used instead of sample DNA. Known positive standards were used during each PCR run. Optimization of PCR was achieved and different temperature and PCR run conditions were followed as described previously by other investigators who have used similar primer sets [8] to identify and characterize *Cryptosporidium*. The amplified fragments were electrophoresed in 1.5% agarose, stained with GelRed® (Biotium), and visualized on an UV transilluminator by electrophoresis in the QIAxcel Advanced system (Qiagen, Valencia, California).

### 2.6. Sequencing

PCR products were purified from 1.5% agarose gel by using NucleoSpin® gel and a PCR clean-up kit (MACHEREY-NAGEL, Germany). Amplicons of 18S rRNA and GP60 genes were directly sequenced in both directions with the primers used for the secondary PCR by the ABI 3100 Genetic Analyzer and the BigDye Terminator v3.1 Cycle Sequencing Kit. DNA sequences were assembled with Codoncode Aligner version 7.1.1 software (CodonCode Corporation). The consensus sequences were assembled with homologous sequences published in GenBank using Clustal W [42] and BioEdit Sequence Alignment Editor [12]. The acquired sequences were submitted to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to initially define the species/genotypes and to confirm the high similarity and homology with other known sequences of *Cryptosporidium* spp. in GenBank. All sequences were multiple-aligned and analyzed by Bioedit and MEGA 7.0 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html and http://www.megasoftware.net/). Phylogenetic trees were generated using Maximum Likelihood (ML) analysis based on the

T92 + I model [40] for 18S rRNA gene in MEGA7 [21] using *Plasmodium falciparum* as an out-group. Phylogenetic trees were also generated using ML analysis based on the Tamura-Nei model [41] for the GP60 genes of the *Cryptosporidium* subtypes in MEGA7 [21]. To assess the reliability of this tree, bootstrap analysis was done with 1000 replicates.

### 2.7. Nucleotide sequence accession number

The partial 18S rRNA nucleotide sequences and GP60 sequences obtained in this study have been deposited in the GenBank database under accession numbers (MN133966 - MN133996 and MN192414 - MN192426).

### 2.8. Statistical analyses

Data analysis was performed using Microsoft Office Excel 2010, and results were considered to be statistically significant when *P*-values were ≤ 0.05. Prevalence rates with 95% confidence intervals were calculated by using Wilson (score) intervals [34] obtained in OpenEpi software (http://www. openepi.com/Menu/OE_Menu.htm).

## 3. Results

### 3.1. Positive rates of Cryptosporidium spp. in poultry

Microscopic examinations revealed the presence of *Cryptosporidium* oocysts in 19.8% (39 of the 197) poultry samples (Fig. 1). The modified Ziehl-Neelsen method indicated that *Cryptosporidium* was present in 50% (2/4) of the pigeon samples, 20.4% (19/93) of the sonali chicken samples, 18.8% (15/80) of the broiler chicken samples, 16.7% (2/12) of the layer chicken samples and 12.5% (1/8) of the native chicken samples. Not all microscope-positive samples were PCR-positive. The positive rate for *Cryptosporidium* was 15.7% (31/197) by nested-PCR. The highest rate (50%) of *Cryptosporidium* was found in pigeons both microscopically and by PCR, whereas the lowest rate was found in the native chicken samples (12.5%) by the microscopic method and in broiler chickens (11.3%) by nPCR (Table 1).

### 3.2. Detection of Cryptosporidium species in poultry

*C. baileyi*, *C. meleagridis*, and *C. parvum* were identified through sequencing; however, *C. baileyi* was the most predominant species. Of the 31 positive samples, 17 were for *C. baileyi* (8.7%), 12 for *C. meleagridis* (6.0%), and 2 for *C. parvum* (1.0%). *Cryptosporidium baileyi* was detected in 11.9% (11/93) of sonalis, 5% (4/80) of broilers, 8.4% (1/12) of layers, and 25% (1/4) of pigeons. *Cryptosporidium meleagridis* was also detected in this study in 5.4% of sonalis, 5% of broilers, 8.4% of layers, 12.5% of natives, and 25% of pigeons. Moreover, *Cryptosporidium parvum* was identified in 1% of sonali and 1.3% of broiler (Table 2). Significant statistical differences were not found in relation to the different species of poultry and the relationship between age and infection (Table S2).

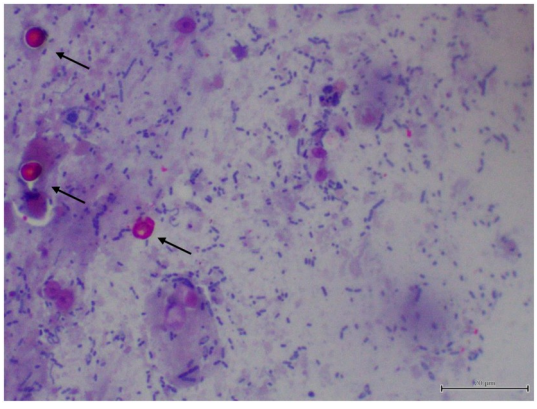

**Fig. 1.** Microscopic observation of *Cryptosporidium* oocysts. Oocysts were observed by using the Ziehl-Neelsen staining method under a microscope.

**Table 1**
Positive rates of *Cryptosporidium* species in poultry.

| Poultry spp. | No. sample | No. microscopy positive (%) | No. nested PCR positive (%) |
|---|---|---|---|
| Chicken (sonalis) | 93 | 19 (20.4) | 17 (18.3) |
| Chicken (Broilers) | 80 | 15 (18.8) | 9 (11.3) |
| Chicken (layers) | 12 | 2 (16.7) | 2 (16.7) |
| Chicken (natives) | 8 | 1 (12.5) | 1 (12.5) |
| Pigeon | 4 | 2 (50) | 2 (50) |
| Total | 197 | 39 (19.8) | 31 (15.7) |

### 3.3. Prevalence of Cryptosporidium spp. in different live bird markets (LBM)

*Cryptosporidium* spp. were found in most of the live bird markets in different areas of Dhaka, Bangladesh. The prevalence of *Cryptosporidium* spp. was high among chickens (10%–40%) and pigeons (50%) in Mirpur areas where LBM 4, 10, 12, and 13 were located and where *C. baileyi*, *C. meleagridis*, and *C. parvum* were detected. However, we found no *Cryptosporidium* in the areas of Kollyanpur, Mirpur new society, where LBM 6 and 11 were located (Table S3).

### 3.4. Sequence and phylogenetic analyses

All PCR-positive specimens were successfully sequenced and analysis of the nucleotide sequences of the 18S rRNA genes revealed the presence of three *Cryptosporidium* species: *C. baileyi*, *C. meleagridis*, and *C. parvum*. Direct sequencing of the 18S rRNA gene amplicons identified *C. baileyi* (8.7%; 17/197), *C. meleagridis* (6.0%; 12/197), and *C. parvum* (1.0%; 2/197). The sequences from *C. baileyi*, *C. meleagridis,* and *C. parvum* had 100% genetic similarity with sequences previously published in GenBank (JX548294, KT151550, KU744845, MK311146 HQ917077, KY448456, KY352486, MH062745, MF671870, MK491508 and LC270282).

ML analysis of 18S rRNA gene sequences revealed three distinct clusters among the isolates of cryptosporidia from poultry in the present study. Seventeen isolates clustered with *C. baileyi*, sharing 100% sequence identity with accession numbers (MN133967-MN133971, MN-133976- MN133977, MN133980, MN133981, MN133983, MN133986, MN133987, MN133989- MN133991, MN133993 and MN133995). Two isolates clustered with *C. parvum* sharing 100% sequence identity with accession numbers (MN133979 and MN133984). The remaining 12 isolates belonged to *C. meleagridis*, sharing 100% sequence identity with accession numbers (MN133966, MN133972- MN133975, MN133978, MN133982, MN133985, MN133988, MN133992, MN133994 and MN133996) (Fig. 2). All 12 *C. meleagridis*-positive specimens generated the expected GP60 PCR product. However, only 11 of the 12 isolates were successfully sequenced. Nucleotide sequence analysis of the GP60 gene revealed that all of the subtypes belonged to the most common subtype, the IIIb family of *C. meleagridis*. We detected two novel subtypes, IIIbA21G2R1, in sonali chickens ($n$ = 4) and a broiler chicken ($n$ = 1) and IIIbA20G2R1, in a layer chicken (n = 1), that had a different nucleotide sequence within the trinucleotide repeat region. We also identified two other subtypes (IIIbA21G1R1 and IIIbA23G1R1) found in broiler chickens ($n$ = 2), a native chicken (n = 1), a sonali chicken (n = 1) and a pigeon (n = 1). The GP60 gene of *C. parvum* was also amplified by specific sets of primers to reveal two subtypes (IIaA11G2R1 and IIaA13G2R1) identified in a sonali chicken (n = 1) and a broiler chicken (n = 1) that were previously reported in calf (Fig. 3). Therefore, these subtypes were named based on the established GP60 nomenclature [38,39].

**Table 2**
Detection of *Cryptosporidium* spp. in poultry.

| Poultry spp. | No. samples | No. positive (%) | | | Total positive (%) | 95% CI[a] |
|---|---|---|---|---|---|---|
| | | *C. baileyi* | *C. meleagridis* | *C. parvum* | | |
| Chicken (sonalis) | 93 | 11 (11.9) | 5 (5.4) | 1 (1) | 17 (18.3) | 11.8–27.4 |
| Chicken (broilers) | 80 | 4 (5) | 4 (5) | 1 (1.3) | 9 (11.3) | 6.03–20.02 |
| Chicken (layers) | 12 | 1 (8.4) | 1 (8.4) | 0 (0) | 2 (16.7) | 4.7–44.8 |
| Chicken (natives) | 8 | 0 (0) | 1 (12.5) | 0 (0) | 1 (12.5) | 2.3–47.1 |
| Pigeons | 4 | 1 (25) | 1 (25) | 0 (0) | 2 (50) | 15–85 |
| Total | 197 | 17 (8.7) | 12 (6.0) | 2 (1.0) | 31 (15.7) | 11.4–21.5 |

[a] Confidence interval.

## 4. Discussion

Few studies have genotyped and subtyped *Cryptosporidium* in poultry around the globe. This study represents, 19.7% of *Cryptosporidium* prevalence in different breeds of poultry based on microscopic results. In contrast, a report mentioned high frequency (about 37%) of *Cryptosporidium* in broiler flocks in Morocco by using microscopy [18]; however, in China, lower *Cryptosporidium* infection rates of 3.4% in broilers and 10.6% in layer chickens were identified from fecal samples by using bright-field microscopy [44]. Furthermore, only 0.5% of chickens at poultry slaughterhouses in Iran were reported to be *Cryptosporidium*-positive [13]. The quantity of oocysts was not evaluated in this study. However, in a positive sample, the sensitivity of the primer that we used, there should be at least $10^1$ oocysts found under microscope [17].

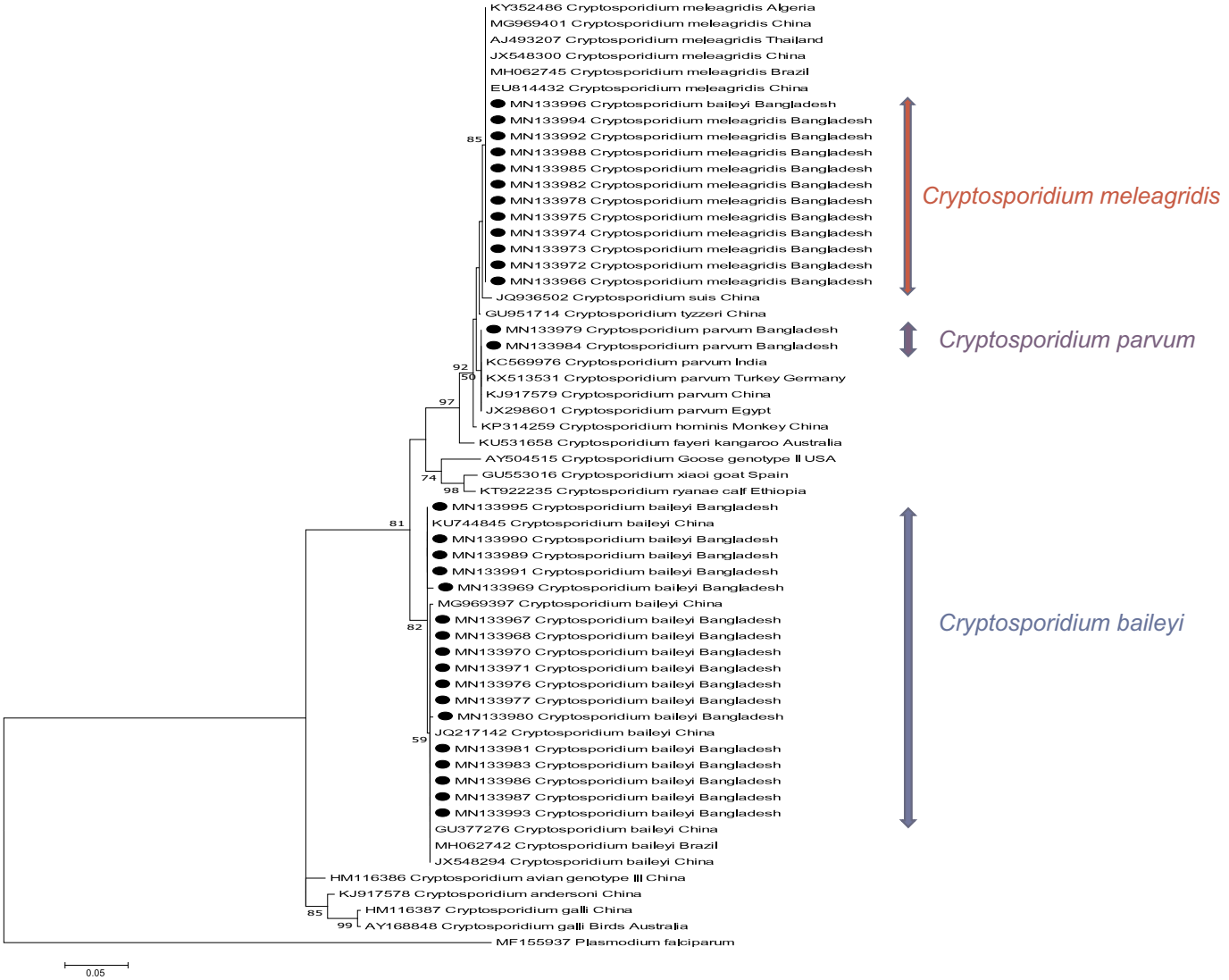


**Fig. 2.** Phylogenetic tree based on partial sequences of the 18S rRNA genes for *Cryptosporidium* spp. A Phylogenetic tree was constructed without nucleotide gaps using the Maximum Likelihood analysis with 1000 replicates based on the T92 + I model [40]. Species, host, region of identification, and GenBank accession number are included. Newly obtained sequences are bolded. The *Plasmodium falciparum* sequence was used as an out-group. Only bootstrap values > 50% from 1000 pseudo-replicates are shown. Evolutionary analyses were conducted in MEGA7 [21].

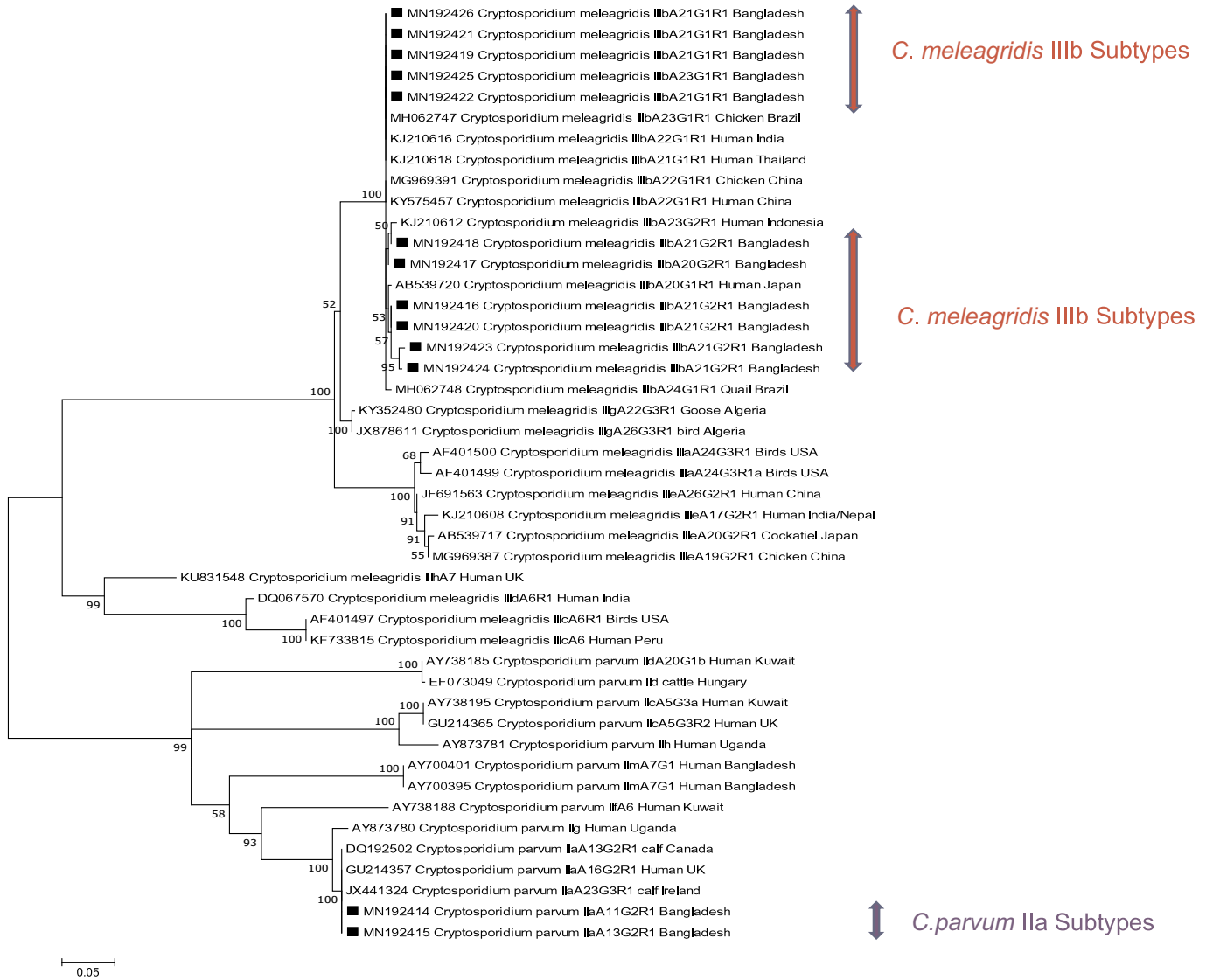


**Fig. 3.** Phylogenetic tree based on partial sequences of the GP60 genes for *Cryptosporidium* spp. Phylogenetic tree was constructed without nucleotide gaps using the Maximum Likelihood analysis with 1000 replicates based on the Tamura-Nei model [41]. Subtypes, host, region of identification, and GenBank accession number are included. Newly obtained sequences are bolded. *C. meleagridis and C. parvum* were subtyped in this study by use of reference sequences and observations. Only bootstrap values > 50% from 1000 pseudo-replicates are shown. Evolutionary analyses were conducted in MEGA7 [21].

In this study, the positive rates of *Cryptosporidium* obtained by using molecular techniques were comparatively higher than those obtained by other investigators, such as 7.03% found in Germany [14] and 10% found in China [43,45], although the rate of 14.8% found in Brazil is similar to our findings [6]. In general, molecular technique is more sensitive than microscopy to detect infection. However, sometimes, microscopy showed high detection rate. It might be due to presence of PCR inhibitors in feces including bilirubin, bile salts, and complex polysaccharides, and thus PCR can be inhibited [28].

In the present study, *Cryptosporidium baileyi* was detected in sonali, broiler, and layer chickens and also in pigeons. These findings are supported by the work of Baroudi et al., [3], who reported a similar prevalence (5.5%; 5/90) in broiler chickens in Algeria using molecular techniques. Moreover, similar findings have been reported in Germany in broilers (5.7%; 9/158) and layers (8.3%; 1/12) [14]. Broiler chickens might act as sources of infection due to shedding of oocysts and may be responsible for transmission and infection [35]. *Cryptosporidium baileyi* is considered the most common avian species of *Cryptosporidium* worldwide and has a wide host range [29,33]. In the present study, *C. baileyi* was the predominant species and was identified in all groups of chickens, although *C. meleagridis* was also observed. *C. baileyi* was detected in sonali, broiler, and layer chickens and in pigeons in this study, whereas *C. baileyi* has been reported frequently in chickens and pigeons worldwide [16,25]. *Cryptosporidium meleagridis* was also detected in sonali, broiler, layer, and native chickens as well as in pigeons. Similarly, *C. meleagridis* was previously reported in 3.2% of chickens [25], 9% of broilers in Algeria [22], 5.3% of broilers in China [43,45] and 10% of layer chickens in China [44]; however, some reports have shown infection rates of *C. meleagridis* as high as 28.9% in chickens [3]. *C. baileyi* was found in all age groups of chickens, whereas, *C. meleagridis* was identified in 31 to 120-day-old layer chickens [44]. Both *C. baileyi* and *C. meleagridis* were found in > 4 months of age of chicken [25]. The breeds of poultry in this study has different life span and genetic variation. We considered that young poultry were the risk group since their immune system is not developed. However, in the present study the infection of poultry was in adult group which might be due to stress factors for meat and egg production in poultry [14].

We identified two novel subtypes of *C. meleagridis* (IIIbA21G2R1 and IIIbA20G2R1) in sonali chickens, in one broiler chicken and in one layer chicken. The remaining 2 subtypes, IIIbA21G1R1 and IIIbA23G1R1, were

identified in chickens and pigeons, consistent with previous reports in humans and birds [1,10,38]. The presence of *C. meleagridis* in poultry in live bird markets in this study raises questions regarding potential zoonotic transmission from poultry to humans. *C. meleagridis* is a public health concern in that it has also been found in humans, associated with gastrointestinal symptoms in both immunocompetent and immunocompromised individuals and mammals [5]. In Peru, two subtypes of *C. meleagridis* identified from AIDS patients were shared by birds (chicken, pigeon, or duck) in the same location [43,45]. The subtype IIIbA22G1R1 was detected in people from Sweden who had traveled to India or Thailand prior to infection [38]. These differences in prevalence might be related to the different breeds of poultry has different susceptibility and immunity due to genetic variation, and the situation of location of LBM, and the improper hygienic condition in poultry cages of LBM. Environmental factors and differences in host species may also be responsible. Unhygienic conditions in cages, overpopulation, and keeping different birds together have contributed to the high infection rates of *Cryptosporidium* [4].

*C. parvum* also has zoonotic potential and is sporadically found in birds [29]. In Germany, the most frequent species found in chickens and turkeys was *C. parvum* [14], which raises the possibility that poultry could act as a source of infection and mechanical vector for other zoonotic *Cryptosporidium*, besides *C. meleagridis*. However, in our study, the sanitary conditions were characterized as poor due to the lack of periodic cage cleaning and overpopulation. Also, the presence of ruminants in LBMs, which could be infected with *C. parvum*, could have caused the spread of oocysts in the surroundings, favoring ingestion by poultry. In contrast, migratory birds would act as a source of transmission, mechanical passage of oocysts, and contamination of the environment, even though they have low-level infections [26].

Avian species have been identified as the natural reservoir for *C. meleagridis*, and zoonotic transmission has been described from domesticated chickens to humans [35]. In urban areas of Bangladesh, *C. meleagridis* was the second most frequently identified species occurring in 13%, and also *C. parvum* was identified in 2% infecting children without diarrhea. However, in rural areas, the predominant species was *C. meleagridis* (90%) while *C. parvum* was much less prevalent (4%) causing subclinical cryptosporidiosis [37]. *C. meleagridis* and *C. parvum* have been identified in infants in Mirpur, Bangladesh. This might be anthropozoonotic; transmitted from chickens kept in households [20]. Interestingly, our study also detected *C. meleagridis* and *C. parvum* in LBMs in Mirpur areas. It is not possible to say whether the *C. meleagridis* subtypes identified in this study are related to subtypes that have infected humans due to lack of identified GP60 gene subtyping data of *C. meleagridis* in humans in Bangladesh. The *C. parvum* subtype IIcA5G3R2 and subtype IImA7G1 were identified in children with diarrhea aged < 2 and 5 years, respectively, in Bangladesh. This subtype family has not been identified in any animal thus far and is generally considered “anthroponotic” [15,20]. In the present study, we detected two subtypes (IIaA11G2R1 and IIaA13G2R1) of *C. parvum* in broiler and sonali chickens that were previously reported as calf subtypes [46]. In the present study, *Cryptosporidium* was detected in pigeons; however, a detection rate of 7% was found in pigeons in Brazil and 25% in pigeons in Thailand [19,30]. Pigeons could be infected by contact with animals or their owners could passively spread oocysts in the surroundings. There is also a risk of the owners becoming infected due to contact with oocysts discharged by the pigeons [30]. The presence of *C. meleagridis* in domestic pigeons in China might lead to zoonotic transmission, especially to their handlers and the environment due to mice spreading the oocysts in water and food [23]. Market workers and customers were handling poultry in the markets could contaminate water, feed, and/or litter in poultry houses with oocysts of mammalian/human origin.

## 5. Conclusion

In conclusion, our research revealed that *Cryptosporidium* parasites are common among the live bird markets in Bangladesh. It is, therefore essential to pay attention to this pathogen. People and other animals could be infected with *C. meleagridis* and *C. parvum* via potential zoonotic transmission from poultry carrying the pathogen. Therefore, it is important to consider *Cryptosporidium* as a threat to public health as well as the economy.

## Authors' contributions

MHBK and KK conceived of the presented idea. MHBK wrote the manuscript with input from all authors. MHBK carried out the experiments and analyzed the data. MHBK, FM, SL, and ABN organized and sort out all data. FM, YH, SL, FR and ABN contributed to the interpretation of the results. XX and KK to investigate and supervised the findings of this work. All authors provided critical feedback, discussed the results and contributed to the final manuscript.

## Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2020.100122.

## References

[1] J.L. Abal-Fabeiro, X. Maside, X. Bello, J. Llovo, C. Bartolome, Multilocus patterns of genetic variation across *Cryptosporidium* species suggest balancing selection at the gp60 locus, Mol. Ecol. 22 (2013) 4723–4732.
[2] M. Alves, L. Xiao, I. Sulaiman, A.A. Lal, O. Matos, F. Antunes, Subgenotype analysis of *Cryptosporidium* isolates from humans, cattle, and zoo ruminants in Portugal, J. Clin. Microbiol. 41 (2003) 2744–2747.
[3] D. Baroudi, D. Khelef, R. Goucem, K.T. Adjou, H. Adamu, H. Zhang, L. Xiao, Common occurrence of zoonotic pathogen *Cryptosporidium meleagridis* in broiler chickens and turkeys in Algeria, Vet. Parasitol. 196 (2013) 334–340.
[4] T.C.B. Bomfim, R.S. Gomes, F. Huber, M.C.M. Couto, The importance of poultry in environmental dissemination of *Cryptosporidium* spp, Open Vet. Sci. J. 7 (2013) 12–17.
[5] C.L. Chappell, P.C. Okhuysen, R.C. Langer-Curry, D.E. Akiyoshi, G. Widmer, S. Tzipori, *Cryptosporidium meleagridis*: infectivity in healthy adult volunteers, Am. J. Trop. Med. Hyg. 85 (2011) 238–242.
[6] M.J.R. da Cunha, M.C. Cury, M. Santín, Molecular characterization of *Cryptosporidium* spp. in poultry from Brazil, Res. Vet. Sci. 118 (2018) 331–335.
[7] A. Elliot, U.M. Morgan, R.C.A. Thompsom, Improved staining method for detecting *Cryptosporidium* oocysts in stools using malachite green, J. Gen. Appl. Microbiol. 45 (1999) 139–142.
[8] Y. Feng, Y. Ortega, G. He, P. Das, M. Xu, X. Zhang, R. Fayer, W. Gatei, V. Cama, L. Xiao, Wide geographic distribution of *Cryptosporidium bovis* and the deer like genotype in bovines, Vet. Parasitol. 144 (2007) 1–9.
[9] T. Fujino, T. Matsuo, M. Okada, T. Matsui, Detection of a small number of *Cryptosporidium parvum* oocysts by sugar flotation and sugar centrifugation methods, J. Vet. Med. Sci. 68 (11) (2006) 1191–1193.
[10] S. Glaberman, I.M. Sulaiman, C. Bern, J. Limor, M.M. Peng, U. Morgan, R. Gilman, A.A. Lal, L. Xiao, A multilocus genotypic analysis of *Cryptosporidium meleagridis*, J. Eukaryot. Microbiol. Suppl (2001) 19S–22S.
[11] Y. Guo, E. Cebelinski, C. Matusevich, K.A. Alderisio, M. Lebbad, J. McEvoy, D.M. Roellig, C. Yang, Y. Feng, L. Xiao, Subtyping novel zoonotic pathogen *Cryptosporidium chipmunk* genotype I, J. Clin. Microbiol. 53 (2015) 1648–1654.
[12] T.A. Hall, BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT, Nucleic Acids Symp. Ser. 41 (1999) 95–98.
[13] H. Hamidinejat, M.H. Jalali, R.A. Jafari, K. Nourmohammadi, Molecular determination and genotyping of *Cryptosporidium* spp. in fecal and respiratory samples of

industrial poultry in Iran, Asian Pac J Trop Med 7 (2014) 517–520.
[14] Y.A. Helmy, J. Krücken, E.M. Abdelwhab, G. von Samson-Himmelstjerna, H.M. Hafez, Molecular diagnosis and characterization of *Cryptosporidium* spp. in turkeys and chickens in Germany reveals evidence for previously undetected parasite species, PLoS One 12 (6) (2017) e0177150.
[15] K.G. Hira, M.R. Mackay, A.D. Hempstead, S. Ahmed, M.M. Karim, R.M. O'Connor, P.L. Hibberd, S.B. Calderwood, E.T. Ryan, W.A. Khan, H.D. Ward, Genetic diversity of *Cryptosporidium* spp. from Bangladeshi children, J. Clin. Microbiol. 49 (6) (2011) 2307–2310.
[16] F. Huber, S. da Silva, T.C.B. Bomfim, K.R. Teixeira, A.R. Bello, Genotypic characterization and phylogenetic analysis of *Cryptosporidium* sp. from domestic animals in Brazil, Vet. Parasitol. 150 (2007) 65–74.
[17] Y. Jae-Ran, L. Soo-Ung, P. Woo-Yoon, Comparative sensitivity of PCR primer sets for detection of *Cryptosporidium parvum*, Korean J. Parasitol. 47 (3) (2009) 293–297.
[18] F. Kichou, F. Saghir, M. El Hamidi, Natural *Cryptosporidium* sp. infection in broiler chickens in Morocco, Avian. Pathol. 25 (1996) 103–111.
[19] K. Koompapong, H. Mori, N. Thammasonthijarern, R. Prasertbun, A.R. Pintong, S. Popruk, W. Rojekittikhun, K. Chaisiri, Y. Sukthana, A. Mahittikorn, Molecular identification of *Cryptosporidium* spp. in seagulls, pigeons, dogs, and cats in Thailand, Parasite 21 (2014) 52.
[20] 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 (7) (2019) 1073–1079.
[21] S. Kumar, G. Stecher, K. Tamura, Molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874.
[22] A.E. Laatamna, N. Holubova, B. Sak, M. Kvac, *Cryptosporidium meleagridis* and *C. baileyi* (Apicomplexa) in domestic and wild birds in Algeria, Folia Parasitol. 13 (2017) 64.
[23] J. Li, X. Lin, L. Zhang, N. Qi, S. Liao, M. Lv, C. Wu, M. Sun, Molecular characterization of *Cryptosporidium* spp. in domestic pigeons (*Columba livia domestica*) in Guangdong province, southern China, Parasitol. Res. 114 (2015) 2237–2241.
[24] N. Li, L. Xiao, K. Alderisio, K. Elwin, E. Cebelinski, R. Chalmers, M. Santin, R. Fayer, M. Kváč, U. Ryan, B. Sak, M. Stanko, Y. Guo, L. Wang, L. Zhang, J. Cai, D. Roellig, Y. Feng, Subtyping *Cryptosporidium ubiquitum*, a zoonotic pathogen emerging in humans, Emerg. Infect. Dis. 20 (2014) 217–224.
[25] C. Liao, T. Wang, A.V. Koehler, Y. Fan, M. Hu, R.B. Gasser, Molecular investigation of *Cryptosporidium* in farmed chickens in Hubei province, China, identifies 'zoonotic' subtypes of *C. meleagridis*, Parasit. Vectors 11 (1) (2018) 484.
[26] A.C. Majewska, T.K. Graczyk, A. Slodkowicz-Kowalska, L. Tamang, S. Jedrzejewski, P. Zduniak, The role of free-ranging, captive, and domestic birds of western Poland in environmental contamination with *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts, Parasitol. Res. 104 (2009) 1093–1099.
[27] J. McLauchlin, S. Pedraza-Díaz, C. Amar-Hoetzeneder, G.L. Nichols, Genetic characterization of *Cryptosporidium* strains from 218 patients with diarrhea diagnosed as having sporadic cryptosporidiosis, J. Clin. Microbiol. 37 (1999) 3153–3158.
[28] U.M. Morgan, L. Pallant, B.W. Dwyer, D.A. Forbes, G. Rich, R.C.A. Thompson, Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: clinical trial, J. Clin. Microbiol. 36 (4) (1998) 995–998.
[29] A.A. Nakamura, M.V. Meireles, *Cryptosporidium* infections in birds-a review, Rev. Bras. Parasitol. Vet. 24 (2015) 253–267.
[30] B.C.M. Oliveira, E.D. Ferrari, M.F.C. Panegossi, A.A. Nakamura, F.S. Corbucci, W.B. Nagata, B.M. Santos, J.F. Gomes, M.V. Meireles, G. Widmer, K.D.S. Brescian, First description of *Cryptosporidium parvum* in carrier pigeons (*Columba livia*), Vet. Parasitol. 243 (2017) 148–150.
[31] U. Ryan, *Cryptosporidium* in birds, fish and amphibians, Exp. Parasitol. 124 (2010) 113–120.
[32] M.A. Samad, Public health threat caused by zoonotic diseases in Bangladesh, Bangl. J. Vet. Med. 9 (2) (2011) 95–120.
[33] B.N. Santana, B. Kurahara, A.A. Nakamura, C.V. da Silva, E.D. Ferrari, G.S. da Silva, Detection and characterization of *Cryptosporidium* species and genotypes in three chicken production systems in Brazil using different molecular diagnosis protocols, Prev. Vet. Med. 151 (2018) 73–78.
[34] E.S.G. Sergeant, Epitools Epidemiological Calculators, Ausvet Pty Ltd, 2017 Available at http://epitools.ausvet.com.au (Accessed 20 June 2019).
[35] C. Silverlås, J.G. Mattsson, M. Insulander, M. Lebbad, Zoonotic transmission of *Cryptosporidium meleagridis* on an organic Swedish farm, Int. J. Parasitol. 42 (2012) 963–967.
[36] T. Sréter, G. Kovacs, A.J. Da Silva, N.J. Pieniazek, Z. Szell, M. Dobos-Kovacs, K. Marialigeti, I. Varga, Morphologic, host specificity, and molecular characterization of a Hungarian *Cryptosporidium meleagridis* isolate, Appl. Environ. Microbiol. 66 (2000) 735–738.
[37] K.L. Steiner, S. Ahmed, C. Gilchrist, Species of cryptosporidia causing subclinical infection associated with growth faltering in rural and urban Bangladesh: a birth cohort study, Clin. Infect. Dis. 67 (9) (2018) 1347–1355.
[38] C.R. Stensvold, J. Beser, C. Axén, M. Lebbad, High applicability of a novel method for gp60-based subtyping of *Cryptosporidium meleagridis*, J. Clin. Microbiol. 52 (2014) 2311–2319.
[39] I.M. Sulaiman, P.R. Hira, L. Zhou, F.M. Al-Ali, F.A. Al-Shelahi, H.M. Shweiki, J. Iqbal, N. Khalid, L. Xiao, Unique endemicity of cryptosporidiosis in children in Kuwait, J. Clin. Microbiol. 43 (2005) 2805–2809.
[40] K. Tamura, Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases, Mol. Biol. Evol. 9 (4) (1992) 678–687.
[41] K. Tamura, M. Nei, Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees, Mol. Biol. Evol. 10 (3) (1993) 512–526.
[42] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res. 24 (1997) 4876–4882.
[43] L. Wang, X. Xue, J. Li, Q. Zhou, Y. Yu, A. Du, Cryptosporidiosis in broiler chickens in Zhejiang province, China: molecular characterization of oocysts detected in fecal samples, Parasite 21 (2014) 36.
[44] R. Wang, F. Jian, Y. Sun, Q. Hu, J. Zhu, F. Wang, Large-scale survey of *Cryptosporidium* spp. in chickens and Pekin ducks (*Anas platyrhynchos*) in Henan, China: prevalence and molecular characterization, Avian. Pathol. 39 (2010) 447–451.
[45] Y. Wang, W. Yang, V. Cama, L. Wang, L. Cabrera, Y. Ortega, Population genetics of *Cryptosporidium meleagridis* in humans and birds: evidence for cross-species transmission, Int. J. Parasitol. 44 (2014) 515–521.
[46] P.R. Wielinga, A. de Vries, T.H. van der Goot, T. Mank, M.H. Mars, L.M. Kortbeek, J.W. van der Giessen, Molecular epidemiology of *Cryptosporidium* in humans and cattle in the Netherlands, Int. J. Parasitol. 38 (7) (2008) 809–817.
[47] L. Xiao, Molecular epidemiology of cryptosporidiosis: an update, Exp. Parasitol. 124 (2010) 80–89.
[48] L. Xiao, C. Bern, J. Limor, I. Sulaiman, J. Roberts, W. Checkley, L. Cabrera, R.H. Gilman, A.A. Lal, Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru, J. Infect. Dis. 183 (2001) 492–497.
[49] L. Xiao, U. Ryan, Cryptosporidium, in: L. Xiao, U. Ryan (Eds.), Biology of Foodborne Parasites, CRC Press, Boca Raton, 2015, pp. 77–95.
[50] D.A. Zaglool, A. Mohamed, Y.A. Khodari, M.U. Farooq, Crypto-Giardia antigen rapid test versus conventional modified Ziehl-Neelsen acid fast staining method for diagnosis of cryptosporidiosis, Asian Pac J Trop Med 6 (3) (2013) 212–215.