Molecular characterization of Cryptosporidium spp. from humans in Ethiopia

Ambachew W. Hailu, Abraham Degarege, Haileyesus Adamu, Damien Costa, Venceslas Villier, Abdelmounaim Mouhajir, Loic Favennec, Romy Razakandrainibe, Beyene Petros

1 Department of Microbial Cellular and Molecular Biology, Biomedical Sciences Stream Addis Ababa University, Addis Ababa, Ethiopia, 2 Department of Epidemiology, University of Nebraska Medical Center College of Public Health, Omaha, Nebraska, United States of America, 3 Institute of Biotechnology, Addis Ababa University, Addis Ababa, Ethiopia, 4 Université de Rouen Normandie, EA7510 ESCAPE, CNR Laboratoire Expert Cryptosporidioses, CHU-Rouen, Rouen, France

*ambalake@gmail.com

Abstract

Data on the distribution and genotype of Cryptosporidium species is limited in Ethiopia. This study examined the presence and genetic diversity of Cryptosporidium species circulating in Ethiopian human population. Stool samples collected from patients who visited rural (n = 94) and urban (n = 93) health centers in Wurgissa and Hawassa district, respectively, were examined for the presence of Cryptosporidium spp. using microscopy, nested PCR and real-time PCR. To detect infection with PCR, analysis of 18S ribosomal RNA was performed. Subtyping was performed by sequencing a fragment of GP60 gene. The overall prevalence of infection was 46% (n = 86) by microscope and PCR. When 48 (out of 86) PCR positive samples were genotyped, two species were identified: C. parvum (n = 40) and C. hominis (n = 8). When 15 of the 40 C. parvum isolates were subtyped, zoonotic subtypes of IlaA14G1R1 (n = 1), IlaA15G2R1 (n = 1), IlaA16G1R1 (n = 2), IlaA16G3R1 (n = 2), IlaA17G1R1 (n = 1), IlaA19G1R1 (n = 1), IlaA20G1R1 (n = 3), IlaA22G1R1 (n = 1), IlaA22G2R1 (n = 1), IldA23G1 (n = 1) and IldA24G1 (n = 1) were identified. When 6 of the 8 C. hominis isolates were subtyped, subtypes IaA20 (n = 5), and IdaA21 (n = 1) were identified. This study suggests that C. parvum and C. hominis are causes of cryptosporidiosis in human in the Wurgissa district and Hawassa in Ethiopia. Zoonotic transmission might be the main route of transmission.

1. Introduction

Cryptosporidium species are Apicomplexan protozoan that are recognized as one of the most important diarrheal pathogens affecting people worldwide, particularly in Africa [1]. The two most common circulating Cryptosporidium species are Cryptosporidium hominis and Cryptosporidium parvum [2]. C. hominis is commonly associated with human infection while C. parvum is linked with infection in animals, especially young ruminants [3,4].
Water, residential surfaces, and food contaminated by Cryptosporidium spp. may serve as sources of infection, [5,6]. The prevailing risk factors to infection and the severity of the disease include young age, undernutrition, and impaired immunity [5]. Given the frequent and close contact between animals and human in rural areas with a possible zoonotic exposure [7–9], reports showed cryptosporidiosis is the leading cause of pediatric mortality and morbidity with anthroponotic transmission [3].

The existing routine diagnostic methods utilized for detecting Cryptosporidium parasites are the microscopic analysis of stool smears through staining methods such as Ziehl-Nelson [10,11]. The identification of species and subtypes of Cryptosporidium is dependent on molecular techniques [12]. In many parts of Africa, the infrastructure for molecular characterization is not yet evolved [3] and consequently studies on the distribution of Cryptosporidium species, genotypes, and transmission routes are scanty in the region.

Ethiopian’s population is growing rapidly (approximately 3% annually). The mixed crop-livestock system of Ethiopia carries more than 70% of the cattle population, which may increase anthroponotic and zoonotic transmission of Cryptosporidium [13]. In addition, dairy operations in densely populated urban and peri-urban settings, poor hygienic and sanitation conditions could create hot spots for zoonotic transmission. Indeed, a study reported Cryptosporidium parvum among 35 (87.5%) pre-weaned calves specimens examined in central Ethiopia [14]. However, due to the paucity of routine screening for Cryptosporidium spp., and absence of systematic investigation of cases by the health system, the prevalence of Cryptosporidium in infection and genetic diversity of the parasite in human population in Ethiopia remain uncertain. To our knowledge, there is only one study that reported genetic diversity of Cryptosporidium spp., (C. hominis and C. parvum) among HIV/AIDS patients in Ethiopia [15]. The prevalence and genetic characterization of Cryptosporidium spp. in the general population remain uncertain. The objective of this study was to determine the prevalence of Cryptosporidium infection and identify genetic diversity of the parasite circulating in human population living in Wurgissa district and Hawassa in Ethiopia.

2. Materials and methods

2.1. Study areas

The study was conducted in health centers located in Wurgissa and Hawassa districts from January to September 2018. Wurgessa district is located in the rural area of the Amhara region in northeast Ethiopia. Hawassa district is an urban city located in the Southern Nations, Nationalities, and Peoples’ Region.

2.2. Specimen collection and transport

Single fecal samples were collected from volunteer patients who visited Wurgessa Health Center (WHC) and Hawassa Health Center (HHC). Fresh stool samples were collected from 94 individuals at WHC and from 93 at HHC. The inclusion criterion for patients in this study was contact with domestic or wild animals. A questionnaire was administered to collect data on other potential risk factors for Cryptosporidium infection, such as diarrhea in other members of the household, HIV serostatus, presence of animals within the house, defecation sites, education, and drinking water supply sources. Approximately 2g of fecal sample was transported to the biomedical science laboratory of Addis Ababa University (AAU) for microscopy analysis. About 1 g of fecal material was placed in an 8 mL aliquot of 2.5% (w/v) potassium dichromate, thoroughly mixed, and transported to the Centre National de Reference Cryptosporidiosis (French National Reference Centre for Cryptosporidiosis) at Charles Nicole.
University Hospital, Rouen (France) for characterization. Samples were kept at 4˚C before DNA isolation.

2.3. Microscopic detection of Cryptosporidium spp. oocysts in fecal samples

After removal of the preservative through washing, the specimens were concentrated via formalin–ethyl acetate sedimentation [16], and a thin fecal smear was examined for each specimen after staining with modified Ziehl–Neelsen technique [17]. Briefly, slides were stained with carbol fuchsin and differentiated in 1% hydrochloric acid–alcohol (70%) for 1 min before counterstaining with 1% methylene blue for 1 min. The stained slides were examined using an oil immersion lens at 100× magnification, where oocysts stained pink to red or deep purple against a blue background. The presence or absence of Cryptosporidium was recorded for each stool sample examined.

2.4. DNA extraction, molecular detection, and subtyping

Nucleic acid was extracted from all fecal specimens using the QIAamp Power fecal DNA kit (Qia-gen, France) following the manufacturer’s protocol. To enable the rapid detection and identification of C. hominis and C. parvum, two major species that are associated with human cryptosporidiosis, samples were screened using 18S ribosomal RNA (rRNA) nested PCR and real-time PCR as described elsewhere [18]. Briefly, PCR was carried out in duplicate and consisted of two duplex reactions: a genus-specific PCR amplifying 300 bp of the Cryptosporidium 18S rRNA gene, duplexed (i) with a C. parvum-specific PCR amplifying 166 bp of the LIB13 locus, and (ii) with a C. hominis-specific PCR amplifying 169 bp of the LIB13 locus. Thermocycling conditions were as follows: 95˚C for 10 min, followed by 55 cycles of 95˚C for 15 s and 60˚C for 60 s. Data were collected from each probe channel during each 60˚C annealing/extension phase.

To correctly identify other species infecting human and to confirm results from the real-time PCR, genomic DNA extracts were subjected to a nested PCR-based sequencing protocol, targeting the 18S ribosomal RNA (rRNA) gene, as described elsewhere [19]. For the primary PCR, the cycling protocol was as follows: 94˚C for 5 min; followed by 40 cycles of 94˚C for 30 s, 58˚C for 45 s, and 72˚C for 1 min; with a final extension of 72˚C for 5 min. For the secondary PCR, the protocol was as follows: 94˚C for 5 min; followed by 40 cycles of 30 s, 58˚C for 45 s, and 72˚C for 45 s; with a final extension of 72˚C for 5 min. Products were visualized in 2% agarose gels using ethidium bromide staining. Positive samples were further subtyped by DNA sequencing of the GP60 gene.

Subtyping was performed by sequencing a fragment of the GP60 gene. Each sample was amplified at least three times by nested PCR. Primers AL3531 and AL3533 were used in primary PCR, and primers AL3532 and LX0029 were used in secondary PCR [20]. Reaction mixtures were prepared using 5 µL 10× DreamTaq Buffer, 0.2 mM of each deoxynucleoside triphosphate, 100 nM of each primer, 2.5 U DreamTaq polymerase, and 5 µL DNA template. Additionally, 1.25 µL of dimethyl sulfoxide was added to the mixture. Cycle conditions were as follows: one cycle of 94˚C for 3 min; 39 cycles of a denaturation step at 94˚C for 45 s, an annealing step at 54˚C (for both the first and the second rounds) for 45 s, and an extension step at 72˚C for 1 min; with a final extension for 10 min at 72˚C.

Each amplification run included a negative control (PCR water) and two positive controls (genomic DNA from C. parvum oocysts purchased from INRAE Centre Val de Loire-Nouzilly France, and C. hominis genomic DNA from a fecal specimen collected at Rouen University Hospital). Products were visualized in 2% agarose gels using ethidium bromide staining, and sequencing was used for identification and subtype confirmation. PCR amplicons were
of each strand were examined with 4Peaks software and compared with published sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov/BLAST).

2.5. Consent and ethical approval

This study was approved by the ethical clearance committee of the College of Science at Addis Ababa University. All participants were briefed about the aims of the study protocol and verbal consent obtained prior to sampling. As the procedure for obtaining stool sample from the study participants had minimal effect, the IRB approved verbal consent. Assent of the children and consent of their parent or guardian was sought.

2.6. Statistical analysis

SPSS Statistics (version 26) was used for the analysis. Prevalence of infection was compared across sociodemographic groups using chi-square or fisher exact test (when the count for at least one cell was less than 5). The combined results based on microscope, nested PCR and real-time PCR was used as a ‘gold standard’ (True result) to calculate the sensitivity, specificity, and predictive values of the three tests in detecting Cryptosporidium spp. infection. Multiple logistic regression analysis was used to identify factors associated with Cryptosporidium spp. infections. Kappa value was used to examine the agreement between the tests in detecting the presence of Cryptosporidium spp. infections. A kappa value greater than 0.81 was considered perfect agreement, and kappa value that fall between 0.61 and 0.80 were considered substantial agreement, while a value ranges between 0.41 and 0.60 was a moderate kappa agreement [21]. P-values less than 0.05 and 95% confidence intervals were considered statistically significant associations between sociodemographic factors and infection.

3. Results

3.1. Sociodemographic description of study participants

Of the 187 study participants, 108 (57.8%) were male, 94 (50.3%) lived in rural areas and 123 (65.8%) were illiterate. The mean age of the study participants was 31.7 years (range: 6–66 years). A total of 25 (13.4%) were children or adolescents and 162 (86.6%) were adults. Of the adults, 55.1% reported gastrointestinal symptoms with diarrhea perior to visiting the health center. From the children, 80% had a recent history of clinical signs related to the gastrointestinal tract.

3.2. Prevalence of Cryptosporidium infection

Based on the combined results applying microscope, nested PCR and real-time PCR, the prevalence Cryptosporidium spp. infections among the study participants was 46.0%. Cryptosporidium was more prevalent in patients with no formal education and those living with HIV (Table 1). The prevalence of infection was comparable between males and females. The difference in the prevalence of infection was also not significant across different age groups.

3.3. Performance of the nested PCR, real time PCR and microscope in detecting Cryptosporidium spp. infections

The prevalence of Cryptosporidium spp. infections was 17.1% (32/187), 24.6% (46/187) and 41.7% (78/187) using microscopy, nested PCR and real-time PCR, respectively. There was a
A statistically significant difference in the prevalence of Cryptosporidium spp. infection detected using microscopic, nested PCR and real-time PCR (p < 0.01).

All samples detected positive for Cryptosporidium spp. infections by microscopy were also positive with the nested PCR and real-time PCR. However, 25 individuals detected as positive by the nested PCR and 46 samples detected positive by real time PCR were negative by microscopy. A total of 40 samples determined positive by the real time PCR were negative by the nested PCR and 8 samples detected positive by nested PCR were negative by real time PCR.

Using the combined results based on the three methods as a 'true result', sensitivity of the microscopy, nested PCR and real-time PCR in detecting Cryptosporidium infection was 38.6%, 51.8% and 94.0% (Table 2). The corresponding negative predictive values for these tests were 67.1%, 71.6%, and 95.4%. The specificity and positive predictive values for nested PCR were 97.1% and 93.5%, respectively. However, the specificity and positive predictive values were 100% for microscope and real time PCR. The agreement between the microscope and the combined results using the three tests to detect infection was moderate (k = 0.41). The agreement between Nested PCR and the combined results using the three tests in detecting infection was also moderate (0.51) agreement. The agreement between real time PCR and the combined results was almost perfect (k = 0.95).

**Table 1. Prevalence of Cryptosporidium infection.**

| Characteristics | Categories        | Number examined | Microscope | Real time PCR | Nested PCR | Combined microscope, nested PCR and real time PCR |
|-----------------|-------------------|-----------------|------------|---------------|------------|--------------------------------------------------|
| Age             | 0–9               | 4               | 25.0%      | 25.0%         | -          | 25.0%                                            |
|                 | 10–19             | 21              | 9.5%       | 52.4%         | 42.9%      | 57.1%                                            |
|                 | 20–30             | 78              | 15.4%      | 35.9%         | 16.7%      | 41.0%                                            |
|                 | 31–40             | 43              | 11.6%      | 39.5%         | 18.6%      | 39.5%                                            |
|                 | 41–50             | 24              | 29.2%      | 50.0%         | 37.5%      | 58.3%                                            |
| ≥51             | 17                | 29.4%           | 47.1%      | 41.2%         | 58.8%      |
| p-value         |                   | 0.27            | 0.53       | 0.02          | 0.32       |
| Gender          | Female            | 79              | 15.2%      | 40.5%         | 20.3%      | 45.6%                                            |
|                 | Male              | 108             | 18.5%      | 42.6%         | 27.8%      | 46.3%                                            |
| p-value         |                   | 0.70            | 0.88       | 0.30          | 1.00       |
| Education level | No formal Education | 123          | 18.7%      | 51.2%         | 29.2%      | 56.1%                                            |
|                 | Formal Education  | 64              | 14.0%      | 23.43%        | 15.62%     | 26.56%                                           |
| p-value         |                   | 0.42            | 0.04       | 0.001         | 0.001      |
| Location        | Wurgissa          | 94              | 16.0%      | 40.4%         | 20.2%      | 45.7%                                            |
|                 | Hawasa            | 93              | 18.3%      | 43.0%         | 29.0%      | 46.2%                                            |
| p-value         |                   | 0.70            | 0.76       | 0.18          | 0.99       |
| HIV sero status | Positive          | 41              | 39.0%      | 80.5%         | 53.7%      | 87.8%                                            |
|                 | Negative          | 95              | 5.3%       | 20.0%         | 6.3%       | 24.2%                                            |
|                 | Unknown           | 51              | 21.6%      | 51.0%         | 35.3%      | 52.9%                                            |
| p-value         |                   | <0.001          | <0.001     | <0.001        | <0.001     |

**Table 2. Comparison of the performance of the nested PCR, real time PCR and microscope in detecting Cryptosporidium spp. infections.**

| Diagnostic methods | Prevalence of infection | Sensitivity | Specificity | Negative predictive value | Positive predictive value | Accuracy | Kappa |
|--------------------|-------------------------|-------------|-------------|---------------------------|---------------------------|----------|-------|
| Microscope         | 17.1%                   | 38.6%       | 100%        | 67.1%                     | 100%                      | 72.7     | 0.41  |
| Nested PCR         | 24.6%                   | 51.8%       | 97.1%       | 71.6%                     | 93.5%                     | 77.0     | 0.51  |
| Real time PCR      | 41.7%                   | 94.0%       | 100%        | 95.4%                     | 100%                      | 97.3     | 0.95  |
3.4. Cryptosporidium spp. and subtypes

Genotype data for Cryptosporidium spp. were obtained in 48 of 86 positive PCR samples. Among those genotyped, C. parvum (n = 40) was frequently detected. C. hominis was detected in 8 samples. Subtype analysis was successfully carried out for 15 of 40 infections with C. parvum and 6 of 8 infections with C. hominis. Infections with C. parvum belonged to zoonotic subtype families IIa and IId. When 15 of the 40 C. parvum isolates were subtyped, zoonotic subtypes of IIaA14G1R1 (n = 1), IIaA15G2R1 (n = 1), IIaA16G1R1 (n = 2), IIaA16G3R1 (n = 2), IIaA17G1R1 (n = 1), IIaA19G1R1 (n = 1), IIaA20G1R1 (n = 3), IIaA22G1R1 (n = 1), IIaA22G2R1 (n = 1), IIdA23G1 (n = 1), and IIdA24G1 (n = 1) were identified. Two subtype families were identified within C. hominis (Ia and Id). When 6 of the 8 C. hominis isolates were subtyped, subtypes IaA20 (n = 5), and Ida21 (n = 1) were identified. Representative sequences were deposited in the NCBI database under accession numbers MW037825–MW037836.

3.5 Risk factors for Cryptosporidiosis

The occurrence of diarrhea in other members of their households (adjusted odds ratio (AOR) = 34.17, p < 0.01) and the household size (AOR = 21.17, p < 0.01) were positive factors for Cryptosporidium infection (Table 3). A total of 131 (70.5%) patients had close contact with cattle, which were mainly cows and calves in both urban and rural households. In this context, the presence of animals was a positive predictor of Cryptosporidium infection (AOR = 12.13, p < 0.01). Many of the participants also had multifaceted contact with a non-human primate and this contact was a positive predictor of Cryptosporidium infection (AOR = 36.26, p < 0.01). In addition, urban recreational location (AOR = 4.53, p < 0.05) and HIV seropositivity (AOR = 168.22, p < 0.01) were significant factors in Cryptosporidium infection.

4. Discussion

In the present survey, the PCR based prevalence of cryptosporidiosis was 46% (86/187), which is comparable to earlier findings among HIV/AIDS patients in the northern part of Ethiopia (43.6%) [22]. However, this prevalence is considerably higher than that reported among patients with gastrointestinal symptoms (1.1%) [23], those living with HIV in southern Ethiopia (13.2%) [24], or schoolchildren in northwest Ethiopia (4.6%) [25]. The lower prevalence of cryptosporidiosis reported in the aforementioned studies in Ethiopia could be due to the less sensitivity of the microscopy procedures used for diagnosing Cryptosporidium infection [26]. On the other hand, the high prevalence infection observed in the present study could be due to increased animal contact, overcrowded living conditions, household diarrhea, open defecation and lack access to clean water which are significant risk factor for cryptosporidiosis infection [27]. Such condition might lead to a repeated exposure of the population to Cryptosporidium oocysts and the development of an immunity state and less symptoms of the infection [28]. In other study asymptomatic oocyst shedding has been noted in apparently healthy individuals [29] which can explain a passive transfer of oocysts in human digestive system. However, the perevalence of Cryptosporidium determined using a microscope was significantly lower than the estimate based on PCR. A microscope may miss oocysts when the intensity of infection is low [30]. In addition, through microscope, the oocysts my appear colorless, smooth, and spherical bodies increasing the chance of missing the infection [31].

Of the isolates of infected samples that were genotyped, C. parvum (n = 40) and C. hominis (n = 8) were the only detected species. C. parvum isolates demonstrated 15 subtypes belonging to two subtype families (Ia and IId) and C. hominis showed six subtypes that belong to two subtype families (Ia and Id). The most common detected C. parvum subtype was IlaA20G1R1. Reports of subtype IlaA20G1R1 in humans are rare. However, the IlaA20G1R1 subtype was
seen in water buffalo in Brazil [32] and in cattle in Serbia and Montenegro [33], Sweden [34], and Brazil [35]. The second most common subtypes of *C. parvum* identified in this study were IIaA16G1R1 and IIaA16G3R1. The IIaA16G1R1 subtype was reported in lamb, calves, and humans, as well as water sources in Romania [36], Estonia [37], and Slovakia [38]. The IIaA16G3R1 subtype was also seen in calves and goats in Spain, England and wild ponies on the Iberian Peninsula [39–41]. A study also reported a high prevalence of *C. parvum* subtypes that belong to the IIa and IId families in sheep and calves from Italy [42,43]. IId subtypes have also been identified in human samples from Egypt, Ethiopia, and Malaysia [15,44–46] and a range of animal hosts from China, such as horses and donkeys, rodents, golden takins, yaks, sheep, and goats [47,48]. Altogether, these findings may suggest human and animals as reservoirs for the *C. parvum* [49,50]. Thus, an integrated, transdisciplinary and multilevel one health approach strategies/intervention that target humans, animals and their shared environment/transmission routes would be necessarily to effectively control cryptosporidiosis in

Table 3. Factors associated with *Cryptosporidium* infection characteristics.

| Attribute                  | Categories | Unadjusted OR [95% CI] | Adjusted OR [95% CI] |
|----------------------------|------------|------------------------|----------------------|
| Age                        |            |                        |                      |
| 0–9                        | -          | -                      | -                    |
| 10–19                      | 4.00 [0.35–45.10] | 9.45 [0.002–32960]    |                      |
| 20–30                      | 2.08 [0.20–20.97] | 6.44 [0.002–19199]    |                      |
| 31–40                      | 1.96 [0.18–20.45] | 5.96 [0.017–20261]    |                      |
| 41–50                      | 4.19 [0.38–46.50] | 1.99 [0.0005–7293]    |                      |
| > = 51                     | 4.28 [0.37–50.19] | 29.52 [0.006–132218] |                      |
| Gender                     |            |                        |                      |
| Male                       | -          | -                      | -                    |
| Female                     | 0.97 [0.54–1.73] | 0.75 [0.22–2.59]      |                      |
| Location                   |            |                        |                      |
| Wurgissa                   | -          | -                      | -                    |
| Hawassa                    | 1.02 [0.57–1.81] | 4.53 [1.00–20.52]    |                      |
| Education level            |            |                        |                      |
| No Formal education        | -          | -                      | -                    |
| Formal education           | 0.28 [0.14–0.55] | 0.47 [0.13–1.60]      |                      |
| Family size                |            |                        |                      |
| ≤4                         | -          | -                      | -                    |
| >4                         | 8.48 [3.32–21.64] | 21.17 [2.89–155.12]  |                      |
| Contact with diarrhea patient |          |                        |                      |
| No                         | -          | -                      | -                    |
| Yes                        | 8.07 [3.74–17.35] | 34.17 [7.07–165.1]   |                      |
| Contact with apes           |            |                        |                      |
| No                         | -          | -                      | -                    |
| Yes                        | 9.44 [4.27–20.88] | 36.26 [6.02–218.3]  |                      |
| Source of drinking Water   |            |                        |                      |
| Tap water                  | -          | -                      | -                    |
| Open well water            | 1.57 [0.58–4.20] | 0.43 [0.03–4.97]   |                      |
| Stream water               | 1.26 [0.50–3.20] | 0.45 [0.026–7.78]   |                      |
| Defecation habit           |            |                        |                      |
| Toilet facility            | -          | -                      | -                    |
| Open field                 | 0.80 [0.30–2.19] | 3.36 [0.21–53.6]   |                      |
| Near To the river          | 2.10 [0.86–5.13] | 8.13 [0.59–1104]   |                      |
| Handwashing habit          |            |                        |                      |
| No                         | -          | -                      | -                    |
| Yes                        | 1.10 [0.62–1.96] | 0.70 [0.22–2.24]   |                      |
| Presence of animals at home|            |                        |                      |
| No                         | -          | -                      | -                    |
| Yes                        | 5.47 [2.60–11.5] | 12.13 [2.34–62.93] |                      |
| Presence of diarrhea       |            |                        |                      |
| No                         | -          | -                      | -                    |
| Yes                        | 2.35 [1.29–4.26] | 1.92 [0.57–6.40]   |                      |
| HIV Serostatus             |            |                        |                      |
| Negative                   | -          | -                      | -                    |
| Positive                   | 22.54 [7.91–64.19] | 168.22 [16.19–1747] |                      |
| Unknown                    | 3.52 [1.70–7.25] | 4.53 [2.89–155.12] |                      |

https://doi.org/10.1371/journal.pone.0253186.t003
regions endemic for *C. parvum* infection [51]. An integrated research involving veterinary, public health and environmental fields would help better understand the burden, risk factors, transmission routes of the zoonotic *C. parvum* infection and plan collaborative one health approach to treat or prevent infection in animals and humans, reduce environmental contamination and block transmission in endemic regions [51].

In this study, two subtypes of *C. hominis* were recorded: IaA20 (5/6) and IdA21 (1/6). The latter were previously recorded in travelers in the United Kingdom (UK) returning from Africa [52,53], with no prior identification in African studies. This may suggest that *C. hominis* IaA20 is the most widespread subtype for the study area. There could be potential for zoonotic or anthropoponotic transmission in the region. Further molecular studies from different hosts will be crucial to better understanding the epidemiology of cryptosporidiosis in Ethiopia.

In the present study, HIV infection, contact with animals, contact with non-human primates, household size (>4), and contact with diarrheal person were significantly associated with *Cryptosporidium* infection. The close proximity between human and non-human primates was found to be a positive predictor for *Cryptosporidium* infection. Though there is a dearth of epidemiological information on the association between humans and non-human primates (NHPs), studies in Uganda showed higher prevalence of *Cryptosporidium spp*. in human adapted NHPs [54] and similar subtypes recorded both in the community and NHPs [55]. In fact, wild animals (such as NHPs) are a potential source of infection as they can spread parasites to humans via direct contact or through contamination of drinking and recreational water, farms, and edible fruits and vegetables [56].

This study provides data on the genetic characterization of *Cryptosporidium spp*. in the general human population in rural and urban regions of Ethiopia where there is limited data. To our knowledge there is only one study that reported data on the genetic diversity of *Cryptosporidium spp* in human samples, focusing on HIV patients in Ethiopia [15]. However, the sample size for this study may not have enough power to test correlation of different sociodemographic factors and health condition with the risk of getting infection with *Cryptosporidium*. In addition, as genotyping was performed for small human samples, confirmation of zoonotic transmission was limited in this study.

5. Conclusions

This study suggests that *C. parvum* and *C. hominis* are causes of cryptosporidiosis in humans in the Wurgessa district and Hawassa in Ethiopia. The identification of the subtype IId family with high zoonotic potential in various hosts suggests that zoonotic transmission might be the main route of transmission in the study area. Studies with larger sample sizes, including animals, would be important to verify the current finding and understand the *Cryptosporidium* subtypes and possible routes of transmission in Ethiopia.

Acknowledgments

We thank all participants in this study, without whom this work could not have existed.

Author Contributions

Conceptualization: Ambachew W. Hailu, Haileyesus Adamu.

Data curation: Ambachew W. Hailu, Abraham Degarege, Haileyesus Adamu, Damien Costa, Abdelmounaim Mouhajir, Romy Razakandrainibe, Beyene Petros.

Formal analysis: Ambachew W. Hailu, Abraham Degarege, Damien Costa, Romy Razakandrainibe.
**Funding acquisition:** Loic Favennec, Beyene Petros.

**Investigation:** Ambachew W. Hailu, Haileyesus Adamu, Damien Costa, Venceslas Villier, Romy Razakandrainibe.

**Methodology:** Ambachew W. Hailu, Haileyesus Adamu, Damien Costa, Venceslas Villier, Romy Razakandrainibe.

**Project administration:** Ambachew W. Hailu, Loic Favennec, Romy Razakandrainibe, Beyene Petros.

**Resources:** Ambachew W. Hailu, Damien Costa, Loic Favennec.

**Software:** Ambachew W. Hailu, Damien Costa, Venceslas Villier, Abdelmounaim Mouhajir, Loic Favennec, Romy Razakandrainibe.

**Supervision:** Ambachew W. Hailu, Abraham Degarege, Haileeyesus Adamu, Loic Favennec, Romy Razakandrainibe, Beyene Petros.

**Validation:** Haileeyesus Adamu, Damien Costa, Abdelmounaim Mouhajir, Loic Favennec, Romy Razakandrainibe, Beyene Petros.

**Visualization:** Ambachew W. Hailu, Abraham Degarege, Damien Costa, Venceslas Villier, Abdelmounaim Mouhajir, Loic Favennec, Romy Razakandrainibe, Beyene Petros.

**Writing – original draft:** Ambachew W. Hailu, Abraham Degarege.

**Writing – review & editing:** Ambachew W. Hailu, Abraham Degarege, Romy Razakandrainibe, Beyene Petros.

**References**

1. Shirley DA, Moonah SN, Kotloff KL. Burden of disease from cryptosporidiosis. Opin. Infect. Dis. 2012; 25:555–563. https://doi.org/10.1097/QCO.0b013e328357e569 PMID: 22907279

2. Feng Y, Ryan UM, Xiao L. Genetic diversity and population structure of Cryptosporidium. Trends Parasitol. 2018; 34(11):997–1011. https://doi.org/10.1016/j.pt.2018.07.009 PMID: 30109020

3. Robertson LJ, Johansen ØH, Kifleyohannes T, Efnumshile AM, Terefe G. Cryptosporidium Infections in Africa-How Important Is Zoonotic Transmission? A Review of the Evidence. Front Vet Sci. 2020; 7:575881. https://doi.org/10.3389/fvets.2020.575881 PMID: 33195574

4. Fayer R. (2010). Taxonomy and species delimitation in Cryptosporidium. Exp Parasitol 124:90–97. https://doi.org/10.1016/j.exppara.2009.03.005 PMID: 19303009

5. Xiao L, Griffiths JK. Cryptosporidiosis. InHunter’s Tropical Medicine and Emerging Infectious Diseases 10th ed. Elsevier,USA, 2020; 712–718, https://doi.org/10.1016/B978-0-323-55512-8.00096-X

6. Pumipuntu N, Piratae S. Cryptosporidiosis: A zoonotic disease concern. Vet World. 2018; 11(5):681–686. https://doi.org/10.14202/vetworld.2018.681-686 PMID: 29915508

7. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchaligram S, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. The Lancet. 2013; 382:209–222. https://doi.org/10.1016/S0140-6736(13)60644-2 PMID: 23680352

8. Snelling W.J.; Xiao L.; Ortega-Piares G.; Lowery C.J.; Moore J.E.; Rao J.R.; et al. Cryptosporidiosis in developing countries. J. Infect. Dev. Cities. 2007; 1:242–256, https://doi.org/10.3855/jdcd.360 PMID: 19734601

9. Yu JR, Lee JK, Seo M, Kim SI, Sohn WM, Huh S, et al. Prevalence of cryptosporidiosis among the villagers and domestic animals in several rural areas of Korea. Korean J. Parasitol. 2004; 42, 1, https://doi.org/10.3347/kjp.2004.42.1.1 PMID: 15060334

10. Ahmed S.A; Karanis P. Comparison of current methods used to detect Cryptosporidium oocysts in stools. Int J Hyg Envir Heal. 2018, 221:743–63. https://doi.org/10.1016/j.ijheh.2018.04.006 PMID: 29776848
31. Smith H. Diagnostics. In: Fayer R, Xiao L, editors. Cryptosporidium and Cryptosporidiosis. 2nd ed. CRC Press; 2007. pp. 174–207.
32. Martins TA, Seixas M, Brito DR, Martins FD, Cardim ST, Melo P, et al. First identification of Cryptosporidium parvum subtype IIA20G1R1 in water buffalos (Bubalus bubalis). Res. Vet. Sci. 2018; 118, 181–183. https://doi.org/10.1016/j.rvsc.2018.02.002 PMID: 29514125

33. Misic Z, Abe N. Subtype analysis of Cryptosporidium parvum isolates from calves on farms around Belgrade, Serbia and Montenegro, using the 60 kDa glycoprotein gene sequences. Parasitology 2006; 134:351–358, https://doi.org/10.1017/S0031182006001508 PMID: 17076920

34. Insulander M, Silverlás C, Lebbad M, Karlsson L, Mattsson JG, Svenungsson B. Molecular epidemiology and clinical manifestations of human cryptosporidiosis in Sweden. Epidemiol. Infect. 2012; 141:1009–1020, https://doi.org/10.1017/S0031182012001665 PMID: 22877562

35. Toledo RD, Martins FD, Ferreira FP, de Almeida JC, Ogawa L, dos Santos HL, et al. Cryptosporidium spp. and Giardia spp. in feces and water and the associated exposure factors on dairy farms. PLoS ONE 2017; 12:e0175311, https://doi.org/10.1371/journal.pone.0175311 PMID: 28430147

36. Vieira PM, Mederle N, Lobo ML, Imre K, Mederle O, Xiao L, et al. Molecular characterisation of Cryptosporidium (Apicomplexa) in children and cattle in Romania. Folia Parasitol. 2015; 62(1):1–4., https://doi.org/10.14411/fp.2015.002 PMID: 25960546

37. Lassen B, Ståhl M, Enemark HL. Cryptosporidiosis—An occupational risk and a disregarded disease in Estonia. Acta Vet. Scand. 2014; 56:36, https://doi.org/10.1186/1751-0147-56-36 PMID: 24902957

38. Kalinová J, Valencakova A, Hatalová E, Danisova O, Trungelova M, Hromada R. Occurrence of Cryptosporidium in the water basins of nitra region, slovakia. Acta Trop. 2019; 183:36–38, https://doi.org/10.1016/j.actatropica.2017.12.019 PMID: 29274309

39. Couso-Pérez S, De Lima F.B.; Ares-Maza E, Gómez-Couso H. First report of zoonotic Cryptosporidium hominis. Parasitol. Res. 2019; 118:4099–4105, https://doi.org/10.1007/s00436-015-4639-0 PMID: 26212102

40. Brook EJ, Hart CA, French NP, Christley RM. Molecular epidemiology of Cryptosporidium subtypes in cattle. Vet. J. 2009; 179:378–82. https://doi.org/10.1016/j.tvjl.2007.10.023 PMID: 18083583

41. Díaz P, Quilez J, Prieto A, Navarro E, Pérez-Creo A, Diaz P, et al. Cryptosporidium species and subtype analysis in diarrhoeic pre-weaned lambs and goat kids from north-western Spain. Parasitol. Res. 2015, 114, 4099–4105, https://doi.org/10.1007/s00436-015-4639-0 PMID: 25960546

42. Dessi G, Tamponi C, Varcesia A, Sanna G, Pipia AP, Carta S, et al. Cryptosporidium infections in sheep farms from Italy. Parasitol. Res. 2020; 119(12):4211–8. https://doi.org/10.1007/s00436-020-06947-2 PMID: 33140165

43. Díaz P, Varcesia A, Pipia AP, Tamponi C, Sanna G, Prieto A, et al. Molecular characterisation and risk factor analysis of Cryptosporidium spp. in calves from Italy. Parasitol. Res. 2018; 117(10):3081–90. https://doi.org/10.1007/s00436-018-6600-x PMID: 30008134

44. Ibrahim MA, Abdel-Ghary AE, Abdel-Latef GK, Abdel-Aziz SA, Aboelhaid SM. Epidemiology and public health significance of Cryptosporidium isolated from cattle, buffaloes, and humans in Egypt. Parasitol. Res. 2016; 115:2439–2448. https://doi.org/10.1007/s00436-016-4996-3 PMID: 27044415

45. Iqbal A, Lim YA, Surin J, Sim BL. High diversity of Cryptosporidium subgenotypes identified in Malaysian HIV/AIDS patients targeting gp60 gene. PLoS one. 2012 Feb 8; 7(2):e31139. PLoS ONE 2012; 7: e31139. https://doi.org/10.1371/journal.pone.0031139 PMID: 22347442

46. Lim YA, Iqbal A, Surin J, Sim BL, Jex AR, Nolan MJ, et al. First genetic classification of Cryptosporidium and Giardia from HIV/AIDS patients in Malaysia. Infect. Genet. Evol. 2011; 11:968–974. https://doi.org/10.1016/j.meegid.2011.03.007 PMID: 21439404

47. Qi M, Zhou H, Wang H, Wang R, Xiao L, Arrowood MJ, et al. Molecular identification of Cryptosporidium spp. and Giardia duodenalis in grazing horses from Xinjiang, China. Vet. Parasitol. 2015; 209:169–172. https://doi.org/10.1016/j.vetpar.2015.02.030 PMID: 25794943

48. Zhao Z, Wang R, Zhao W, Qi M, Zhao J, Zhang L, et al. Genotyping and subtyping of Giardia and Cryptosporidium isolates from commensal rodents in China. Parasitology 2015; 142:800–806. https://doi.org/10.1017/S0031182014001929 PMID: 25579244

49. Nader JL, Mathers TC, Ward BJ, Pachebat JA, Swain MT, Robinson G, et al. Evolutionary genomics of anthropopism in Cryptosporidium. Nat. Microbiol. 2019; 4: 826–836, https://doi.org/10.1038/s41564-019-0377-x PMID: 30833731

50. Feng Y, Ryan UM, Xiao L. Genetic diversity and population structure of Cryptosporidium. Trends Parasitol. 2018; 34:997–1011, https://doi.org/10.1016/j.pt.2018.07.009 PMID: 30108020

51. Innes EA, Chalmers RM, Wells B, Pawlucic MC. A One Health Approach to Tackle Cryptosporidiosis. Trends Parasitol. 2020; 36(3):290–303. https://doi.org/10.1016/j.pt.2019.12.016 PMID: 31986309

52. Chalmers RM, Elwin K, Cheesbrough J, Hadfield SJ, Beeching NJ. Detection of IgG antibodies in sera from patients with Cryptosporidium parvum and Cryptosporidium hominis. J. Infect. 2013; 67:231–237. https://doi.org/10.1016/j.jinf.2013.04.019 PMID: 23644097
53. Chalmers RM, Hadfield SJ, Jackson CJ, Elwin K, Xiao L, Hunter P. Geographic linkage and variation in Cryptosporidium hominis. Emerg. Infect. Dis. 2008; 14: 496–498, https://doi.org/10.3201/eid1403.071320 PMID: 18325272

54. Nizeyi JB, Mwebe R, Nanteza A, Cranfield MR, Kalema GR, Graczyk TK. Cryptosporidium sp. and Giardia sp. infections in mountain gorillas (Gorilla gorilla beringei) of the Bwindi Impenetrable National Park, Uganda. J. Parasitol. 1999; 85: 1084–1088. PMID: 10647041.

55. Graczyk T, DaSilva A, Cranfield M, Nizeyi J, Kalema GR, Pieniazek N. Cryptosporidium parvum genotype 2 infections in free-ranging mountain gorillas (Gorilla gorilla beringei) of the Bwindi Impenetrable National Park, Uganda. Parasitol. Res. 2001; 87: 368–370. https://doi.org/10.1007/s004360000337 PMID: 11403378.

56. Bouzid M, Hunter PR, Chalmers RM, Tyler KM. Cryptosporidium Pathogenicity and Virulence. Clin. Microbiol. Rev. 2013; 26: 115–134, https://doi.org/10.1128/CMR.00076-12 PMID: 23297282