Addis Ababa University
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Zoonotic *Cryptosporidium* Species Infections in Humans and Sympatric Non-human Primates, *Chlorocebus aethiops* and *Colobus guereza* in Wurgissa and Hawassa, Ethiopia

BY

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Zoonotic *Cryptosporidium* Species Infection in Humans and Sympatric Non-human Primates *Chlorocebus aethiops* and *Colobus guereza* in Wurgissa and Hawassa, Ethiopia

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Declaration

I declare that the dissertation hereby submitted for the Degree of Doctor of Philosophy (PhD) in biomedical sciences to the School of Graduate Studies of Addis Ababa University, Department of Microbial, Cellular and Molecular Biology, is my own independent work and has not previously been submitted by me or anybody else at another University. The plagiarism checker report is also attached to this dissertation. The materials obtained from other sources have been duly acknowledged in the thesis.

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Abstract

Cryptosporidiosis has become a significant public and veterinary health concern worldwide. In Ethiopia, non-Human Primates (NHPs), *Chlorocebus aethiops* and *Colobus guereza* monkeys are now living in overlapping/adjacent geographical areas to human settlements and no studies in Ethiopia have assessed *Cryptosporidium* infection at species or genotypes level on NHPs. Therefore, this cross-sectional study was conducted to determine the prevalence of *Cryptosporidium* spp. infections, identify species and subtypes in both NHPs and humans in Ethiopia. Fecal samples were collected from 187 humans (94 from Wurgissa and 93 from Hawassa) and 185 NHPS (147 from Wurgissa and 38 from Hawassa) in Ethiopia. Humans who visited health centers (WHC and HHC) and volunteered to participate were interviewed and screened for the presence of *Cryptosporidium* spp. using modified Ziehl–Neelsen technique. All samples were submitted to Rouen University Hospital, France, for molecular analysis using DNA sequencing of the SSU 18S rRNA and Gp60 genes. 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 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. When 6 of the 8 *C. hominis* isolates were subtyped, subtypes IaA20 (n = 5), and IdA21 (n = 1) were identified. The overall prevalence of *Cryptosporidium* spp. infection in NHPs using molecular detection was 70.9 % (131/185). Of the 131 *Cryptosporidium* spp. infection in NHPs, 54 have been characterized and sequence analysis showed *C. parvum* (n=36), *C. hominis* (n=15), and C. cuniculus (n=3). *C. parvum* IIa subtype family (IIaA17G1R1) was the common subtype in NHPs. Also, multiple subtype families of *C. hominis* (Ia, Ib, Id, and Ie) were documented. Humans who had contact with NHPs and diarrheal person, family size and education status were major risk factors associated with *Cryptosporidium* spp. infection. The present study has provided strong evidence that NHPs in Ethiopia harbor *Cryptosporidium* spp. of public health importance and can be considered potential reservoirs of human cryptosporidiosis. Furthermore, the predominant occurrence of *C. parvum* in humans and NHPs and the existence of *C. hominis* are suggestive of zoonotic transmission. Thus, studies involving experimental cross infections of parasite isolates from both hosts and molecular characterization of the parasites can provide definitive evidence for the role of NHPs in the transmission of *Cryptosporidium* spp. to humans.

Key Words/phrases: Humans; Non-human primates; *Cryptosporidium* spp.; Zoonotic,

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Abbreviations / Acronyms

µm: Micrometer

18S rRNA gene: 18 Svedberg units of ribosomal Ribonucleic acid

AAU: Addis Ababa University

AIDS: Acquired immunodeficiency syndrome

AOR: adjusted odd ratio

BLAST: Basic Local Alignment Search Tool

bp: base pair

CHU: Centre hospitalier universitaire

CI: Confidence interval DMSO: Dimethyl Sulfoxide DNA: deoxyribonucleic acid

Exo-SAP: exonuclease shrimp Alkaline Phosphatase

GP60 gene: A 60-kilodalton glycoprotein gene

HHC: Hawassa Health Center

HIV: human immunodeficiency virus

NCBI: National Center for Biotechnology Information

NHPs: Non-Human Primates

PCR: Polymerase chain reaction

RNA: Ribonucleic acid

Spp.: species

SSU: small subunit

WHC: Wurgissa Health Center

1. Introduction

1.1. Global and regional prevalence of cryptosporidiosis

Cryptosporidiosis is a diarrheal disease that affects humans and animals across the world. The disease was estimated to be the fifth cause of diarrhea in children under the age of five, causing more than 48,000 deaths and 12.05 million disability-adjusted life-years lost in the year 2016 (Khalil *et al.*, 2018). In developing countries, cryptosporidiosis is the second leading etiologic agent (5–15%) of moderate-to-severe diarrhea in children younger than two years (Kotloff *et al.*, 2013). *Cryptosporidium* spp. is among the six diarrhea causing pathogens in children under 5 years in Africa and Asia (Vermeulen *et al.,* 2017). *Cryptosporidium* infections are associated with malnutrition and stunted growth in addition to deaths in children living in underdeveloped countries (Khalil *et al.*, 2018; Innes *et al.*, 2020). In some cases, infections are complicated with malnutrition and impaired physical fitness (Shirley *et al.*, 2012; Innes *et al.*, 2020). The disease has also been reported to cause waterborne diarrheal outbreaks in industrialized countries (Shirley *et al.,* 2012). However, the global burden of the disease is not adequately estimated due to the short-term nature of its diarrheal manifestation, lack of adequate diagnostic tools, less consideration given to its severity and long-term sequelae. Furthermore, many *Cryptosporidium* spp. infections are asymptomatic or mild and self-limiting and remain unrecognized (Shirley *et al.*, 2012; Khalil *et al.*, 2018).

Human invasion into natural ecosystems increased interactions between humans, domestic animals and wildlife populations that create conducive environment for the transmission of zoonotic diseases. Drinking water catchments and water reservoir areas have been easily contaminated by zoonotic waterborne pathogens. Thus, the epidemiology of zoonotic species of *Cryptosporidium* in free-ranging and captive wildlife became increasingly important (Zahedi *et al.,* 2016).

Cryptosporidium species infect a broad range of hosts including humans, domestic animals and wild animals worldwide causing asymptomatic or mild to severe gastrointestinal disease in their host species (Zahedi *et al.,* 2016).

Cryptosporidium spp. infections are asymptomatic or mild and self-limiting and often go under diagnosed in immunologically competent hosts. However, it causes moderate to severe diarrhea in immunocompromised hosts including malnourished children, AIDS patients, transplant recipients, cancer patients and patients infected with immunosuppressive pathogens (Wanyiri *et al.,* 2014; Shirley *et al.,* 2012). These spectra of clinical presentations are associated with intricate interplay of age, nutritional and immune status and probably by the infecting species and subtype (Cama *et al.,* 2008). Cryptosporidium is the cause of diarrhea in AIDS patients and globally, prevalence of *Cryptosporidium* infection in HIV-infected people was reported to be 14.0% (Wang *et al.,* 2018).

In developing counties, *Cryptosporidium* is predominantly transmitted through faecal-oral route, or through exposure to animal or through consumption of contaminated food or water (Shirley *et al.,* 2012; whereas in developed countries, transmission mostly occurs during waterborne epidemic due to contamination of drinking water, and recreational water by oocyst (Widerstrom *et al.,* 2014). Additionally, zoonotic transmission of *Cryptosporidium* occurs via contamination of crops and other agricultural products and contamination of surface water with feces from livestock and other animals (Shirley *et al.,* 2012). A multitude of factors play a role in the spread of *Cryptosporidium* in areas of poor sanitation and hygiene in both developed and developing countries. These factors include high number oocysts can be passed and they become infectious right away of their shedding, low doses of oocysts are infectious, oocysts remain infectious for up to 6 months in conducive environments and prolonged incubation period which sustain the transmission prior to outbreak recognized by relevant administrations (Chappell *et al.*, 1996; DuPont *et al.*, 1995)

Studies have investigated the occurrence of *Cryptosporidium* infection in areas where non-human primates (NHPs) interact frequently with humans' habitats. A study carried out in Western Uganda, where free-ranging NHPs make incursions to human habitats, showed 32.4 %, 11.1% and 2.2% of *Cryptosporidium* infection was detected in human, NHPs, and livestock, respectively (Salyer *et al.*, 2012). Likewise, a study conducted in Tanzania reported prevalence of 4.3%, 16.0%, and 9.6% in humans, NHPs, respectively (Parsons *et al.*, 2015). These transmissions of *Cryptosporidium* in humans and NHPs highlight epidemiological role of free-ranging primates to gain a better understanding of the dynamics of disease transmission and possible occurrence of anthropo-zoonotic transmission.

Ethiopia is one of the socio-economically disadvantaged countries susceptible to parasitic infections due to the prevailing environmental degradation and low living standard. These factors lead to the spread of intestinal parasites including *Cryptosporidium* infection and illnesses in the country. According to Raby (2019), parasitic infections are increasingly over-dispersed among people or primates belonging to a particular group and their distribution is often heterogeneous. In Ethiopia, *Cryptosporidium* species emerged as opportunistic protozoan parasites in HIV/AIDS patients in 1994 and 1998, which showed a prevalence of 39.7% (Mengesha, 1994) and 25.9% (Fisseha *et al.,* 1998), respectively. Subsequently, a plethora of studies have assessed the prevalence of cryptosporidiosis in HIV/AIDS, and the result have revealed prevalence ranging from 1.5% to 43.9% (Dufera *et al.*, 2008; Mariam *et al.*, 2008; Adamu and Petros, 2009; Getaneh *et al.,* 2010; Missaye *et al.*, 2013; Hailu, 2015). Also, the infection rate of *Cryptosporidium* in healthy and diarrheic children was found to be 8.1% (Tigabu *et al.*, 2010) and from 8.1 % to 9.4 % (Adamu *et al.*, 2006; Firdu *et al.*, 2014), respectively.

Furthermore, *Cryptosporidium* spp. has been detected from environmental water sources, as well as domestic and wild animals. A study conducted to investigate the occurrence of *Cryptosporidium* oocyst from the Addis Ababa municipal water supply showed *Cryptosporidium* oocysts from the source, highlighting the potential of water-borne transmission (Fikrie *et al.,* 2008). In addition, several studies reported *Cryptosporidium* in cattle with a prevalence ranging from 2.3% to 27.8% (Abebe *et al.,* 2008; Regassa *et al.,* 2013; Wegayehu *et al.,* 2013; Ayana and Alemu, 2015; Wegayehu *et al.*, 2016). Comparatively, few studies have reported the *Cryptosporidium* infection rate in NHPs in holiday resorts (Sodere, Wondo-Genet, Ethiopia) and Lake Hawassa (Legesse and Erko, 2004; Amenu *et al.*, 2015). These studies, as mentioned above, suggest multiple fecal-oral route transmission of Cryptosporidium following contact with *Cryptosporidium* oocysts via contaminated water and infected domestic and wild animals. However, studies conducted in Ethiopia used traditional diagnostic methods. Few studies have assessed the molecular epidemiology of *Cryptosporidium* infections in cattle and humans in Ethiopia (Adamu *et al.*, 2010; Manyazewal *et al.,* 2018; Wegayehu *et al.*,2016); but, the findings of these studies were inconsistent, probably due to the limitation of inclusion of other potential sources of infection, such as environmental sources and NHPs, thus further detailed studies are needed to understand transmission dynamics better.

Thus, the current study investigates whether cryptosporidiosis is an essential zoonotic infection within rural and urban recreational areas with increased monkey presence. It also focuses on identifying the genotypic diversity of *Cryptosporidium* that occurs in humans and monkeys at the interface and whether there is an indication for transmission between the two compartments. This work examines the occurrence of intestinal parasitic illnesses and assesses the zoonotic possibility of *Cryptosporidium* disease among sympatric humans and NHPs in Ethiopia.

1.2. Description of *Cryptosporidium* spp.

Cryptosporidium species are categorized under Phylum: Apicomplexa; Class: Gregarinomorphea; Subclass Cryptogregaria, and genus *Cryptosporidium* (Ryan *et al.,* 2016). About 38 species and 70 genotypes of *Cryptosporidium* have been recognized (Feng *et al.,* 2018; Zahedi *et al.,* 2016). In humans, *Cryptosporidium hominis* and *C. parvum* are the most frequent associates while *C. meleagridis, C. felis, C. canis, C. ubiquitum, C. muris* and *C. viatorum* being the less often detected species (Innes *et al.*, 2020). *Cryptosporidium* species show different morphological characteristics depending on the phase of the parasite's life cycle. These are sporozoites, trophozoites, merozoites, microgametocytes, macrogametocytes, and oocysts. The Oocyst stage of *Cryptosporidium* enters the environment from feces. Varied species of *Cryptosporidium* show various morphological features in their oocysts including shape and size. Oocysts are large in some species and small in other species. In addition, their forms appear spherical in some and oval in others. The morphological features depend on the various phases of the parasite (Borowski *et al.,* 2010). The oval-shaped oocysts are discharged into the surroundings and have smooth surfaces. (Borowski e*t al.,* 2010). It also has a thick wall that ensures its resistance while having a cleft on one side, which allows the release of sporozoites during excystation (Thompson *et al.,* 2005b). The measurement of the oocysts is around 5X7µm. On the contrary, sporozoites have a measure of approximately 5X0.5µm and have a rough surface, a pointed apical area, and a rounded posterior (Borowski *et al.,* 2010). Further, trophozoites have a length measuring 1 to 2.5µm, with a smooth surface and a shape like a hood.

Moreover, Type I and Type II meront have varied sizes but are characterized by an epicellular, smooth surface (Borowski *et al.,* 2010). Besides, the merozoites that they generate are increasingly smaller. It is essential to note that the Type I merozoites have a rod-like shape and a pointed apical area (Chalmers and Katzer, 2013). They also have a rough surface, distinguishing them from Type II merozoites. Further, microgametes are often formed from Type II merozoites and measure around 0.1um. They also have a spherical shape with a rough surface. Finally, macrogamont has a measurement of 4x5µm and an ovular shape with a rough surface.

A wide range of Cryptosporidium species and genotypes have been identified in drinking source water, storm water runoff, stream sediment, wastewater and seawater in various geographic locations including *C. hominis, C. parvum, C. Andersoni, C. muris, C. cuniculus, C. meleagridis* and *C. canis* as well as wildlife adapted genotypes and unidentified "environmental sequences" which probably represent yet unidentified wildlife genotypes and which also highlight the potential for contamination of water supplies by wildlife (Zahedi *et al.,* 2016). Although humans are the major hosts for C. hominis, it has been reported in many wildlife hosts including in non- human primates (Zahedi *et al.,* 2016).

1.3. Life cycle of *Cryptosporidium* Spp.

Cryptosporidium spp. has a monoxenous life cycle (Tandel *et al.,* 2019). Oocysts can be discharged into the surrounding with feces or different body excretions, including respiratory secretions. The oocysts usually stay within the environment until ingested by a host (Tandel *et al.,* 2019). When ingested by a host, the sporulated oocysts excyst within the gastrointestinal tract and discharge sporozoite which penetrates and infects the epithelial cells within the ileum and becomes enclosed by a thin cytoplasm layer (Tandel *et al.,* 2019). Further, a desmosome-like organelle is developed at the boundary, separating the pest and the host's cytoplasm cell, allowing the uptake of nutrients. In the cells, asexual reproduction leads to the development of Type I meronts, which rapture, releasing eight merozoites (Thompson *et al.*, 2005a). These free merozoites penetrate new cells in which they experience additional multiple fission to release new meronts (Tandel *et al.*, 2019). At this stage, the parasite should be controlled to prevent the cycle from progressing indefinitely. Type I merozoites are linked with the production of Type II meront (Tandel *et al.*, 2019). This form is characterized by four merozoites that mature to produce macrogametes and microgametes (Tandel *et al.*, 2019). When they are fully established, microgametes enter the macrogametes to produce the zygote. There is also the creation of the oocyst wall around the zygote while sporozoites develop via meiosis. Finally, the oocysts are released into the environment, allowing the cycle to progress (Tandel *et al.*, 2019). The life cycle of *Cryptosporidium* spp. is shown in the diagram (Figure 1).

In its life cycle, *Cryptosporidium* undergoes both sexual and asexual developmental stages. Infection begins with the ingestion by the host of the sporulated oocysts through contaminated water or food, directly via the faecal-oral route, or through inhalation of the oocysts. Each oocyst has four sporozoites which after excystation in the intestinal lumen or the respiratory tract, appear and invade the epithelial cells and develop into trophozoites. Trophozoites undergo asexual division (merogony) and form Type I Meronts consisting of 8 merozoites. Some of these merozoites form Type II meronts which have 4 merozoites and start the sexual phase of the life cycle. Macrogametocytes and microgametocytes are formed, fertilized, and produce zygote. Most of the zygotes develop into oocysts, the thick ones with a two-layered wall which are released to the environment, and the thin-walled oocysts which ease autoinfection. The prepatent period for *Cryptosporidium parvum* for example ranges from 7 to 21 days (Thompson *et al.*, 2005a).

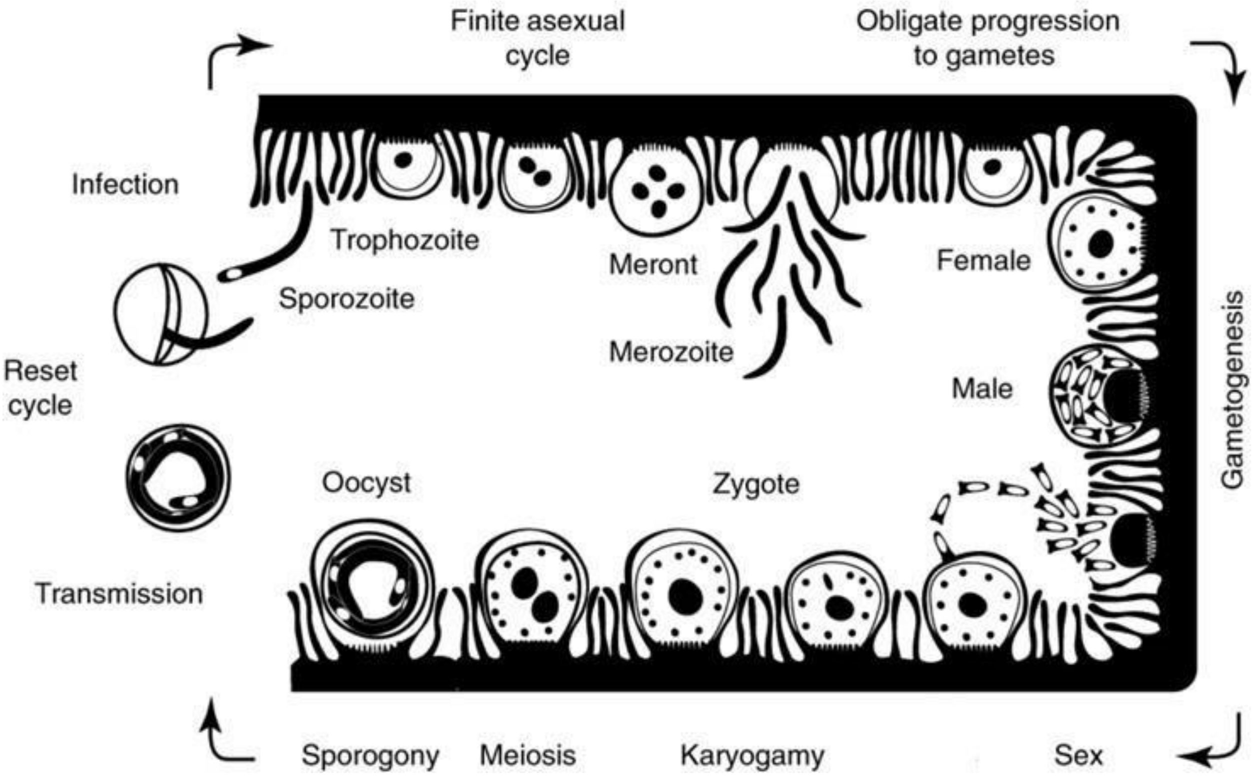

Figure 1: Schematic model of the *Cryptosporidium* life cycle

1.4. Pathogenesis

The first incidents of human cryptosporidiosis were reported during the mid-1970s in patients with severe watery diarrhea. The primary symptoms of the infection included diarrhea, vomiting, abdominal pain, low-grade fever, and nausea. The disease can also lead to myalgia, headache, malaise, and anorexia (Gerace *et al.,* 2019). Significantly, the severity of the illness usually depends on the type of *Cryptosporidium* spp. and different host factors, including age and HIV infection, which weaken the immune system. While immunocompetent people might not experience severe symptoms, immunocompromised HIV/AIDs patients have a high chance of being affected. The disease can lead to dehydration and wasting, which is highly life-threatening

(Gerace *et al.,* 2019). *Cryptosporidium* usually uses two forms of proteins, including mucin-like glycoproteins and thrombospondin-related adhesive proteins, which allow them to adhere to, attack, and establish themselves in the ileum's epithelial cells (Thompson *et al.*, 2005a). Here, they regenerate and reproduce to damage the cells by disrupting the cell junctions, lowering barrier function, and killing the cell. Researchers have increasingly linked the parasite to different molecules like hemolysins, phospholipases, and proteases in their pathogenetic processes (Certad *et al.,* 2017).

1.5. Immunity to cryptosporidiosis

Both innate and adaptive host responses are important in the control of *Cryptosporidium* spp. infection. However, crucial knowledge is still lacking concerning the mechanisms governing the protection and those responsible for the pathophysiology of infections in humans and large animals (Laurent and Lacroix-Lamande, 2017). Studies based mainly on mouse models have highlighted the fundamental role of intestinal epithelial cells in both parasite multiplication and the protective immune response (Ryan *et al.,* 2016). Due to this response, several cytokines such as γ-interferon (IFN-γ) and IL-12, IL-15, and IL-18, chemokines, antimicrobial peptides, and nitric oxide are secreted from the infected epithelial cells, preventing, or reducing the severity of infection (Laurent and Lacroix-Lamande, 2017). Also, other mechanisms are implicated in the host immunity during Cryptosporidium infection. MicroRNA (miRNA), which are small RNA molecules of 23 nucleotides that result in gene silencing via translational suppression or mRNA degradation, target Toll-like receptor 4 (TLR4) and regulate TLR4-mediated anti-*C. parvum* defense as well as alters *C. parvum* infection burden in vitro (Ryan *et al.,* 2016). Mannose-binding lectin (MBL), which is an evolutionarily conserved protein secreted by hepatocytes that functions in human innate immunity by binding to microbial surfaces and promoting opsonophagocytosis, is important in the protection against cryptosporidiosis, as children and HIV-infected adults with mannose- binding lectin deficiency have increased susceptibility to cryptosporidiosis and more severe diseases (Ryan *et al.,* 2016).

Regarding adaptive cellular immunity, CD4+ T cells seem to be essential for the eradication of the parasite (Lemieux *et al.,* 2017). Therefore, it has been shown that low counts of CD4+ T cells recorded in AIDS patients made them more vulnerable to *Cryptosporidium* infection (Lemieux *et al.,* 2017). The role of humoral immunity in the protection from cryptosporidiosis is vague (Ryan *et al.,* 2016; Lemieux *et al.,* 2017). However, the antibodies that have been linked to *Cryptosporidium* infection are serum Immunoglobulin M and Immunoglobulin G, as well as serum and secretory Immunoglobulin A. Antibodies may support protection as it has been shown in the case of bovine cryptosporidiosis, where hyperimmune bovine colostrum had prophylactic and therapeutic effects (Lemieux *et al.,* 2017).

1.6. Cryptosporidiosis in humans

Cryptosporidiosis is the cause of diarrhea in children under five years old (Lanata *et al.,* 2013). It mainly manifests profuse diarrhea of 1-14 days duration, abdominal cramps, vomiting, fever, and malaise (Cama *et al.,* 2008; Chalmers and Davies, 2010). High mortality and morbidity rate occurs among patients whose immune system is compromised (Wolska-Kusnierz *et al.,* 2007). In Ethiopia, the pattern of *Cryptosporidium* infection in HIV-infected Ethiopians showed a declining trend. However, it is shown that there is a shortcoming in running routine screening for *Cryptosporidium* in HIV-infected people (Mohebali *et al.,* 2020).

There is no vaccine, and treatments are inadequate or effectively treat cryptosporidiosis in humans. A few drugs have been evaluated, including Paromomycin, Mirazid with or without Paromomycin, Macrolides (such as Azithromycin and Spiramycin) & Albendazole, which are partially effective (Abdalla, 2014). In addition, Nitazoxanide is used to treat cryptosporidiosis in HIV-infected patients, and antiretroviral therapy (ART) significantly influences the outcome of cryptosporidiosis (Squire and Ryan, 2017). However, it can be prevented by boiling drinking water where access to clean water is obscured (WHO, 2008).

The reported prevalence of *Cryptosporidium* varies in the African continent. In Ethiopia, the recent meta-analysis study reported that the overall pooled prevalence estimate of *Cryptosporidium* infection among HIV patients in Ethiopia was 11% (Mohebali *et al.,* 2020), and 7.6% was found in diarrheic patients (Adamu *et al.,* 2010). In Uganda, a prevalence of 32.4% was detected in humans living in the region of Kibale National Park (Salyer *et al.,* 2012). A prevalence of 18.9% was seen in hospitalized patients in Equatorial Guinea, of which most of them were HIV-positive (Blanco *et al.*, 2009) and in Kenya, a prevalence study on *Cryptosporidium* was carried out among people with cattle contact and without in rainy and dry seasons, and among HIV-positive patients. Among the cattle contact group, 4% of them were infected during the dry season and 0.3% were during the rainy season; the non-contact group showed a prevalence of 5% and 0% were detected in the dry and rainy seasons respectively; the prevalence in HIV-positive patients was 5% in both seasons (Kange'the *et al.,* 2012). In South Africa, a study reported that 18% prevalence among school- children and hospital patients (Samie *et al.,* 2006). The lion-share-creating events of cryptosporidiosis were mainly taken by *C. parvum* and *C. hominis* followed by *C. meleagridis, C. felis, C. ubiquitum* and *C. canis* (Xiao, 2010). *C. andersoni,C. muris, C. suis,* and the *Cryptosporidium* cervine, skunk, and chipmunk I genotype rarely occurred in human cases (Xiao and Feng, 2008). *C. parvum* and *C. hominis* are both common in developed countries while *C. hominis* is most common in humans in developing regions (Xiao, 2010).

In developing countries, four typical *C. hominis* subtype families, namely Ia, Ib, Id, and Ie, are usually found in humans. In Ethiopia, Malawi, Madagascar, India, and Peru all four common subtype families were seen in children and HIV-positive adults (Peng *et al.,* 2003; Ajjampur *et al.,* 2007; Cama *et al.,* 2008; Adamu *et al.,* 2014), The highly complex nature of *C. hominis* in developing world could be an indicator of stable anthroponotic transmission in this region (Xiao, 2010).

1.7. *Cryptosporidium* spp. infection in nonhuman primates

Studies have been reporting on the occurrence of *Cryptosporidium* infections in NHPs. *Cryptosporidium* infection with oocysts has been described in squirrel monkeys (*Saimiri sciureus*), red-ruffed lemurs (*Varecia variegate rubra*), brown lemurs (*Lemur macuco- mayottensis*), marmosets (*Callithrix jacchus, Saguinus Oedipus*), macaques (*M. mulatta, Macaca nemestrina, Macaca fascicularis, Macaca fuscata*), spider monkeys (*Aetles belzebuth*), mangabeys (*Cercocebus torquatus lunulatus*), green monkeys (*Cercopithecus aethiops, Cercopithecus campbelli, Cercopithecus talapoin*), patas monkeys (*Erethrocebuspatas*), orangutan (*Pongo pygmaeus*), baboons (*Papioanubis*) (Kovatch and White, 1972; Heuschele *et al.,* 1986; Miller *et al.,* 1990; O'Donoghue, 1995; Hope *et al.,* 2004), and free-ranging mountain gorillas (*Gorilla gorillaberingei*) (Graczyk *et al.,* 2001).

Two *Cryptosporidium* spp. isolates originating from two human-habituated guerilla groups and two isolates from non-habituated gorillas (*G. gorilla beringei*) were found to be positive for *C. parvum* (Graczyk et al., 2001). The habituation of some populations of free-ranging mountain gorillas close to humans and the cross-transmissibility of *C. parvum* between humans and animals could contribute to the spread of zoonotic and anthroponotic transmission cycles (Graczyk *et al.,* 2001). Cryptosporidium infection was seen among 21% of park staff members who had frequent contact with gorillas in comparison with 3% of prevalence in the local community in Uganda (Nizeyi *et al.*, 2002). In a similar study, cattle that graze within the Bwindi Park were observed to be a source of C. parvum infection for gorillas (Nizeyi *et al.*, 2002).

In Ethiopia, *Cryptosporidium* oocysts were detected in 11.9% of 59 baboons (*Papio anubis*) and 29.3% of vervets (*Cercopithecus aethiops*) (Legesse and Erko, 2004). In these localities, primates were observed to use the same water sources as humans and range freely in human habitats, suggesting their role as potential sources of oocysts infectious to humans.

In Sri Lanka, a coprology survey of 125 monkeys (89 toque macaques, 21 gray langurs, and 15 purple-faced langurs) found *Cryptosporidium* infection among all the three monkey species that used water heavily contaminated by humans and livestock. Most macaques (96%) shedding *Cryptosporidium* oocysts were observed to be co-infected with other protozoan and helminthic parasites including *Enterobius* and *Strongyloides* species (Ekanayake *et al.*, 2006). These reports suggest that transmission of *Cryptosporidium* spp. might occur not only among primates in the wild but also between primates and humans, and possibly between primates and livestock. Therefore, understanding the role of nonhuman primates in the transmission dynamics of cryptosporidiosis could have an important contribution to the control of zoonotic cryptosporidiosis and improve the lives of both humans and animals.

1.8. Statement of the problem

Ethiopia is a country where co-habitation of rural communities and non- human primates such as *Chlorocebus aethiops* (vervet monkey) and *Colobus guereza* (*mantled gureza*) sharing the same areas for food and water (Amenu *et al.,* 2015). The close interaction among humans and NHPs including other wild animals creates a suitable situation for the circulation of zoonotic pathogens. In some regions, the close contact between vervet and human activities regarded vervet as a nuisance, particularly due to their crop-raiding activities (Dixon *et al.*, 2009; Yihune *et al.,* 2009). Vervet species have been implicated as wildlife reservoirs for zoonotic protozoa and helminths such as *Schistosoma* spp. *Trichura* spp., *A. lumbricoides*, hookworms, and *Strongyloides* spp. in the country (Legesse and Erko, 2004; Teklemariam *et al.,* 2018).

In this scenario, neglected zoonotic diseases, such as cryptosporidiosis, should be studied adequately whereby nonhuman primates live near human habitation and they use the same water sources as humans in rural areas and roam around urban residences, and range freely over human domestic habitat. As result, NHPS can harbor zoonotic species/genotypes/subtypes, and precise identification of the pathogens they harbor is essential. In Ethiopia, some reports are available on characterizing *Cryptosporidium* isolates from humans and an attempt to examine the source and transmission of *C. parvum* and its contribution to the occurrence of human cryptosporidiosis (Admau *et al.,* 2010). However, no study has been conducted on the potential transmission and distribution of Cryptosporidium spp. in NHPs, especially *Chlorocebus aethiops* and *Colobus guereza*, the two most common closer to human.

Nevertheless, it is unclear whether there is the transmission of these intestinal protozoans between humans and urban roaming vervet monkeys, and if so, how significant this is for public health and for the conservation of the Vervet. This study investigated the occurrence of *Cryptosporidium* spp.in human and vervet monkeys/colobus in rural (Wurgissa) and urban setting (Hawassa) in Ethiopia and conducted molecular characterization of isolates.

More specifically, insight into the molecular epidemiology of cryptosporidiosis at the interface of human and wildlife ecosystem will help to better understand the prevalence and interaction of such infections in the different host systems (NHPs and humans). Therefore, the following key questions were addressed.

- What species and subtypes of *Cryptosporidium* occur in humans in Hawassa and Wurgissa?
- What species and subtypes of *Cryptosporidium* occur in *Chlorocebus aethiops* and *Colobus guereza* in Hawassa and Wurgissa?
- What is the performance of microscopy, nested PCR and RT-PCR in the detection of *Cryptosporidium* species?
- What are the associated risk factors of human cryptosporidiosis in selected areas of Hawassa and Wurgissa?

Hypothesis of the study

There is possible transmission of *Cryptosporidium* spp. parasites among NHPs and humans in rural (Wurgissa) and urban (Hawassa) sites of human - NHPs interface.

2. Objective of the study

2.1. General Objective

To investigate molecular epidemiology of zoonotic *cryptosporidium* species in human and NHPs to estimate the potential of zoonotic transmission in Wurgissa and Hawassa, Ethiopia.

2.1.1. Specific Objectives

- To determine the prevalence and genotypes of *Cryptosporidium* spp. among humans in Wurgissa and Hawassa of Ethiopia.
- To determine the prevalence and genotypes of *Cryptosporidium* spp. among *Chlorocebus aethiops* and *Colobus guereza* in Wurgissa and Hawassa of Ethiopia.
- To examine the diagnostic performance of microscopy nested PCR and RT-PCR in the detection of *Cryptosporidium*.
- To determine the extent of contact between human and NHPs and risk factors of *Cryptosporidium* species transmission using community survey.

3. Materials and Methods

3.1. Description of study setting

A cross-sectional study was conducted at Hawassa and Wurgissa, Ethiopia from January to September 2018. Hawassa is the capital city of Sidma regional state. Wurgissa is a small rural village in North Wollo zone, Amhara Regional State (Figure 2). In Hawassa, colobus and vervet monkeys interact with visitors at recreational sites to get food from people. The description of the area is well elaborated on the earlier work of Amenu *et al* (2010). However, only vervet monkeys were found in the rural area and took part in this study. Vervet monkeys in the rural sites spend substantial amounts of time sleeping and feeding near or in human villages. A total of nine non-overlapping localities were surveyed for sampling nonhuman primate fecal samples (Table 1). Seven localities were in Wurgissa and two in Hawassa.

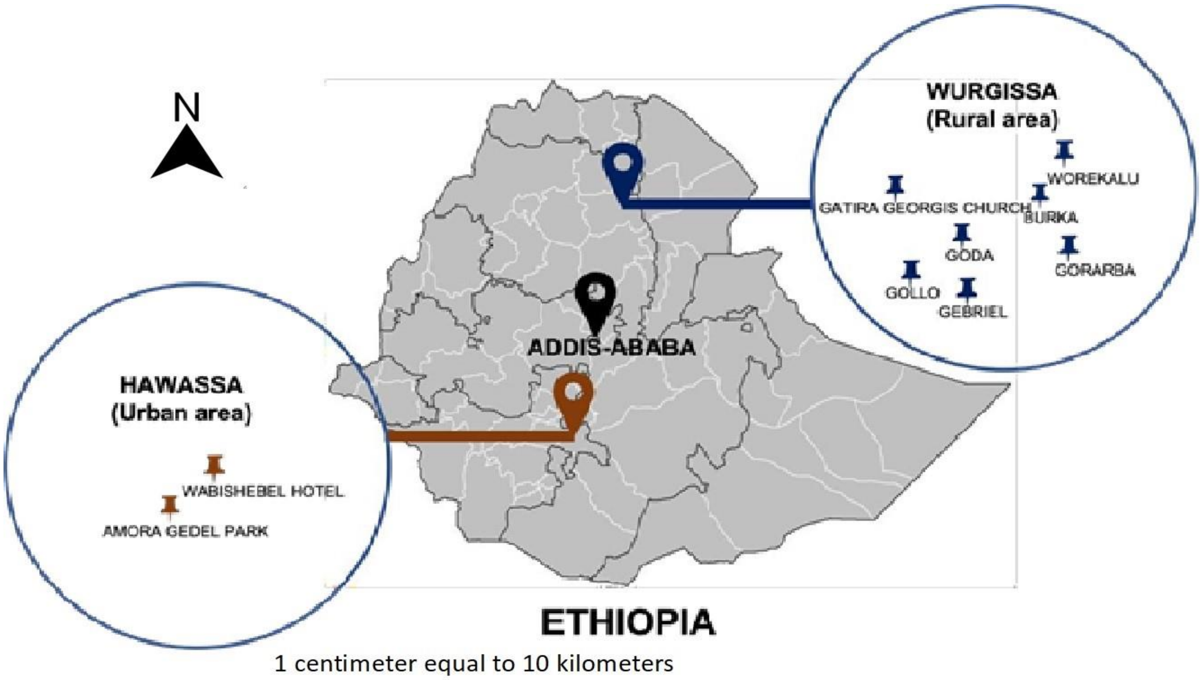

Figure 2: Study sites in Hawassa and Wurgissa, Ethiopia ET." Map. Google Maps. Google, 22, Apr. 2018.

3.2. Study population

Human population

A total of 94 passive treatment seeking volunteer patients who visited the outpatient department of Wurgissa Health Center (WHC) and 93 patients from Hawassa health center (HHC) participated in the study. A questionnaire was administered to each participant to collect data on socio-demographics, clinical information, medication histories, and potential risk factors for *Cryptosporidium* and other intestinal parasite infections. Diarrhea in other members of the household, HIV serostatus, presence of animals within the house, defecation sites, education, and drinking water supply sources using a pre-structured questionnaire at the time of sample collection was considered.

NHPs Study

Identification of monkey species and location was done to differentiate one herd from the other herd of the same species and then the total number of individuals in the herd was determined. Individuals were grouped by age (adult, infant) and sex. Identifiable individuals of an age/sex were followed until seen to defecate. Then, another individual was chosen, and those individuals followed until seen defecating or lost from the connection. In this manner, contact with a herd was maintained until all members were observed to defecate using a standard noninvasive sampling procedure (Gillespie *et al.*, 2008). A total of 185 fecal samples were collected from free-living nonhuman primates. In Wurgissa from Gollo site fecal matters of 19 vervet monkeys were sampled from a herd having 23 vervet monkeys; from Gatira Geowrgis 33 were sampled from a herd of 37 monkeys; in Gorareba site 17 were sampled from a herd having 23; in Wore Kalu 20 were sampled from a herd having 24 animals; in St. Mariam Church site 16 were sampled from a herd having 20 animals; in Goda site 19 were sampled among herd having 23 vervet animals and in Burka site 21 were sampled among 24 animals. A total of 40 monkeys (26 from Amora Gedel Park and 14 from Wabishabel Hotel) were sampled from Amora Gedel recreational park and the Wabishebel hotel near Hawassa Lake (Table 1).

Table 1: Geospatial location of nonhuman primate fecal sample collection sites in Hawassa and Wurgissa, Ethiopia, January –September 2018

Troop location		**GPS location**	**Number of animals in a troop**	**Number of animals Sampled**
Hawassa	Amora Gedel Park	7°02'37.4"N 38°27'24.1"E	26	26
	Wabishebel Hotel	7°02'55.6"N 38°27'34.7"E	14	14
Wurgissa	Gollo	11°32'22.1"N39°36'28.7"E	23	19
	Gorarba	11°32'10.1"N39°40'45.4"E	23	17
	Gatira Georgis church	11°33'10.2"N 39°36'51.6"E	37	33
	Goda	11°31'55.4"N 39°37'18.9"E	23	19
	Burka	11°33'21.6"N 39°39'39.6"E	24	21
	St. Mariam Church	11°32'43.8"N 39°37'13.9"E	20	16
	Worekalu	11°34'15.1"N39°40'03.2"E	24	20
				185

3.3. Fecal collection, transportation and processing

A single fresh fecal sample, approximately 2g, was collected with leak-proof, tightly cupped, and labeled sterile stool cup from each patient from both WHC and HHC. The fecal sample was transported to the biomedical science laboratory of Addis Ababa University (AAU) for microscopic diagnosis and about 1 g of fecal material was placed in an 8 mL aliquot of 2.5% (w/v) potassium dichromate, thoroughly mixed. The sample was transported to the Centre National de Reference Cryptosporidiosis (French National Reference Centre for Cryptosporidiosis) at Charles Nicole University Hospital, Rouen (France), kept at 4˚C for 2 weeks before DNA isolation and characterized using molecular techniques.

3.4. Microscopic techniques

Direct wet mount

A direct wet mount with normal saline (0.85% NaCl solution) was prepared for 372 samples on both study sites and observed for the presence of motile intestinal parasites and trophozoites under a light microscope at 10x and 40x magnification. Lugol's iodine staining was also used to observe cysts of intestinal parasites.

Formalin-Ether concentration Methods

The concentration technique was used for both samples. Briefly: after the removal of the preservative through washing, the removal of debris within the faecal pellet by centrifugation is done. Bypassing the homogenized sample through a fine sieve (125 µm mesh), to identify the parasites easily using a microscope. The debris trapped in the sieve was discarded. After adding 3 ml of diethyl ether to the mixture and shaking, the content was centrifuged at 500x g for 10 min. The supernatant was discarded, and iodine stain preparation was made using the sediment and examined under a microscope using 10x and 40x magnifications (Lindo *et al.*, 1998). Microscopic examinations were done independently by experienced clinical laboratory technicians and the investigator.

Modified Ziehl-Neelson method for *Cryptosporidium* spp.

A modified Ziehl-Neelson technique was used for the detection of *Cryptosporidium* spp. oocysts from concentrated of 372 samples. Briefly: a thin smear was prepared, air-dried, fixed with methanol for 5 minutes, and stained by modified Ziehl-Neelson technique (Henriksen and Pohlenz, 1981b). In this technique, the slides were stained with carbol-fuchsine (0.34% fuchsine and 4% w/v phenol) for 30 minutes and washed with distilled water. The slides were differentiated in 1% hydrochloric acid-alcohol (70%) for 1 minute and were counter-stained with 1% methylene blue for another 1 minute. Finally, the stained smears were microscopically analyzed using 1000x magnification. For estimation of the parasite load, 100 high-power fields were screened.

3.5. Molecular techniques

DNA extraction

Disruption of the oocysts is often performed to ensure that the DNA is available for extraction. Primarily freeze-thawing (Checkley *et al.,* 2015) techniques were performed to cross-confirm the kit-based DNA extraction. All the stool samples (185 Human + 187 NHPs) were processed using a QiAamp Power fecal DNA kit (Qiagen, France) at CHU. This was after the serial washing of the fecal sample aimed at removing the preservative. The protocols followed the manufacturer's instructions with slight modifications (annex 1). All the centrifugation steps were performed at room temperature (15-25oc). Solution (C1- C5) was used for the extraction and finally, DNA was eluted in 100 µl of C6 solution and stored at -20 °C, per the manufacturer's instructions.

Screening process for species identification and subtyping

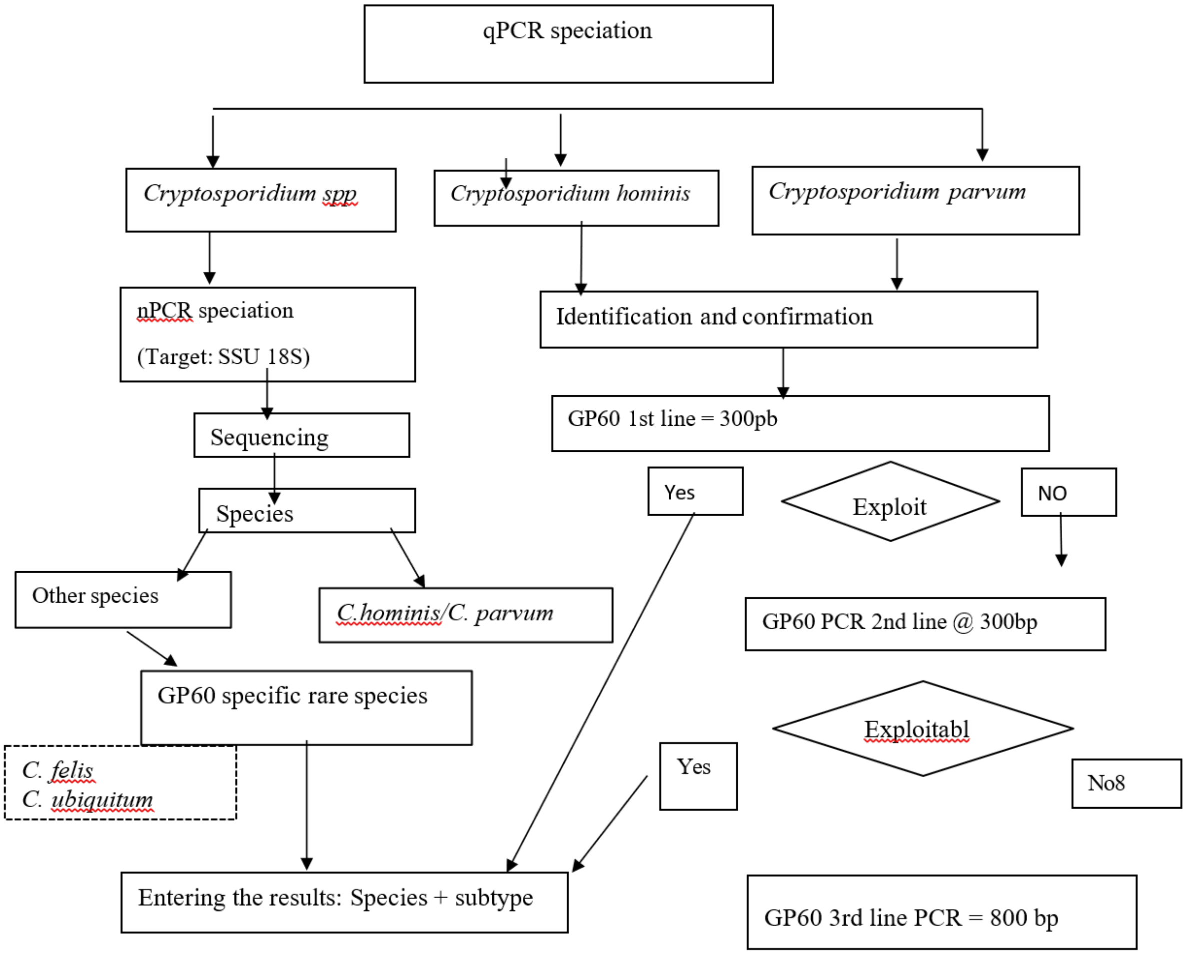

Figure 3: Process of screening species and subtypes of Cryptosporidium spp. In Cryptosporidium reference laboratory, Rouen, France from September – December 2018.

18s rRNA-based *Cryptosporidium* spp. identification

Cryptosporidium spp. were characterized using 18S rRNA gene real-time PCR, as described elsewhere (Hadfield *et al.*, 2011a). Briefly, PCR was conducted in duplicates and consisted of two duplex reactions: (i) a genus-specific PCR amplifying ∼300 bp of the *Cryptosporidium* 18S rRNA gene, duplexed with a *C. parvum*-specific PCR amplifying 166 bp of the LIB13 locus, and (ii) 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.

Alongside real-time PCR, genomic DNAs were subjected to PCR-based sequencing of 18s rRNA as described elsewhere (Koehler *et al.*, 2017), A two-step nested PCR protocol was used to amplify the 18S rRNA gene (215bp). For primary PCR, the cycling protocol was 94°C for 5 min (initial denaturation), followed by 30 cycles of 94°C for 45 sec (denaturation), 45°C for 2 min (annealing), and 72°C for 1.5 min (extension), with a final extension of 72°C for 10 min. For secondary PCR, the protocol was: 94°C for 5 min, followed by 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 30s, with a final extension of 72°C for 10 min. *C. hominis, C. parvum,* and no-template PCR controls were included in each run for each protocol.

Gp60 sequence amplification

Genotyping was performed by sequencing a fragment of the Gp60 gene (Figure 3). Primers AL3531 (ATA- GTC-TCC-GCT-GTA-TCC and AL3533 (GAG-ATA-TAT-CTT-GGT-GCG) were used in the primary PCR and primers AL3532 (TCC-GCT-GTA-TTC-TCA-GCC) and LX0029 (CGA-ACC-ACA-TTA-CAA-ATG-AAG-T) in the secondary PCR leading to amplification of a fragment of approximately 364 bp (Sulaiman *et al.*, 2005). Each PCR mixture (total volume, 50 µl) contained5 µl of 10X DreamTaq Buffer, each deoxynucleoside triphosphate at a concentration of 0.2 mM, each primer at a concentration of 100 nM, 2.5 U of DreamTaq polymerase, and 5µL of DNA template. Also, 1.25µL of DMSO (100%) was added to the mixture. A total of 40 cycles, each consisting of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, were performed. An initial hot start at 94°C for 3 min and a final extension step at 72°C for 7 min was also included. Each amplification run included a negative control (PCR water) and two positive controls (genomic DNA from C. parvum oocysts bought from Waterborne Inc., and C. hominis genomic DNA from a faecal specimen collected in Rouen University Hospital). Products were visualized in 2% agarose gels using ethidium bromide staining, and identification was confirmed by sequencing (Annex 5).

Positive samples were further genotyped by DNA sequencing of the Gp60 gene amplified by a nested PCR following the protocol described elsewhere (Sulaiman *et al.*, 2005). Positive samples were further genotyped by DNA sequencing of the GP60 gene amplified by a nested PCR following the protocol described (Annex-5).

Detection of amplification products using gel electrophoresis

Ethidium bromide was used to visualize DNA amplification in agarose gel electrophoresis (2%). Et. Br intercalates between the nitrogenous bases of DNA and fluoresces under UV light.

DNA sequence analysis

Sequencing was used to confirm *Cryptosporidium* species/genotypes from second-round PCR products (see annex 5). PCR amplicons were purified using Exonuclease I/Shrimp Alkaline Phosphatase (Exo-SAP-IT) (USB Corporation, Cleveland, USA). They were sequenced in both directions using the same PCR primers at 3.2 uM in 10 µl reactions (BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) in ABI 3500 sequence analyzer (Applied 229 Biosystems, CA, USA). Sequence chromatograms of each strand were examined with 4peaks software and compared with published sequences in the GenBank database using BLAST (www.ncbi.nlm.nih.gov/BLAST).

3.6. Phylogeny analysis

Evolutionary history was inferred using the Neighbor-Joining method according Saitou N. and Nei M. (1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. This analysis involved 39 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 187 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al.*, 2021).

3.7. Data analysis

SPSS software (version 26) was used for the data analysis. The prevalence of infection was compared across socio-demographic groups using the chi-square or fisher exact test (when the count for at least one cell was less than 5). Multiple logistic regression analysis was used to identify factors associated with *Cryptosporidium* spp. infections. P-values less than 0.05 and 95% confidence intervals were considered statistically significant associations between socio demographic factors and infection.

3.8. Ethical approval

This study was approved by the ethical clearance committee of the College of Natural and Computational Science at Addis Ababa University. All participants were briefed about the aims of the study protocol and verbal consent was obtained before sampling. As the procedure for obtaining stool samples from the study participants had minimal effect, the Institutional Review Board approved verbal consent, assent of the children and consent of their parent or guardian was looked for.

4. Results

4.1. Sociodemographic characteristics of study population

A total of 187 patients who had no contact directly or indirectly with monkeys before sampling participated in the study. Among the 187 participants, 108 (57.8%) were males, and 79 (42.2 %) were females. Among the total participants, 94 (50.3%) were from Wurgissa and 93 (49.7 %) were from Hawassa areas. from 94 Wurgissa participants. 53.2% and 46.8% were male and female, respectively, while among the 93 Hawassa participants, 62.37% and 37.63% were male and female, respectively (Figure 4).

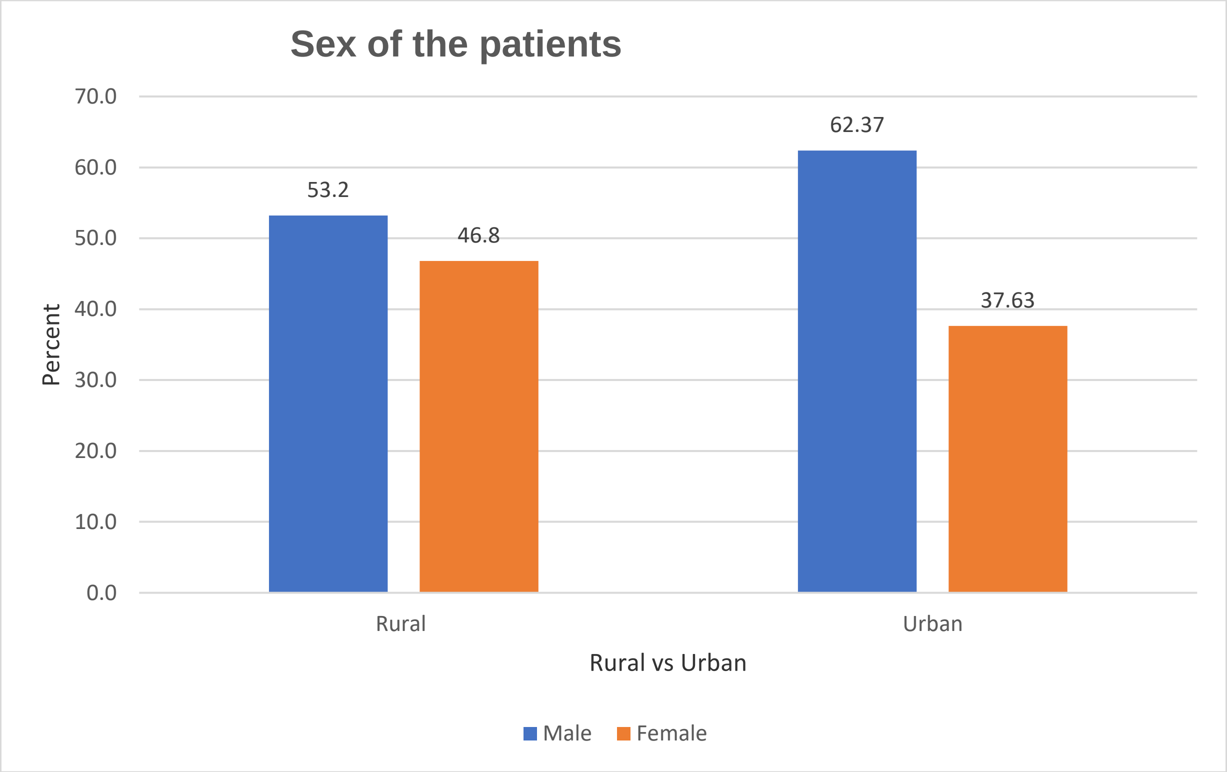

Figure 4: Sex of the study participants in Wurgissa and Hawassa, Ethiopia

Microscopic Detection of *Cryptosporidium* spp. and others intestinal parasite

The microscopic diagnosis resulted in fifty-one (27.3%) cases were found positive for protozoan parasites including *G. lambilia* (5.3%), Cryptosporidium species (17.1%), and Entamoeba species (4.8%). Among these six cases were observed to have *G. lamblia* and *Cryptosporidium* co-infection, three cases had Entamoeba spp. and *Cryptosporidium* spp. Co-infection, two *G. lamblia*, *Entamoeba* spp. and *Cryptosporidium* spp. co-existence, two cases of *G. duodenallis* and *Entamoeba* spp. Forty-two people (22.4 %) were infected with helminthic parasites such as *Hymnolepis nana* (8%) and hookworms (14.4%). Fifty-seven (30.5%) were also infected with Nematode *Strongyloids stercolaris* (9.1%), *Trichuris Tricuria* (21.4%) (Figure 5).

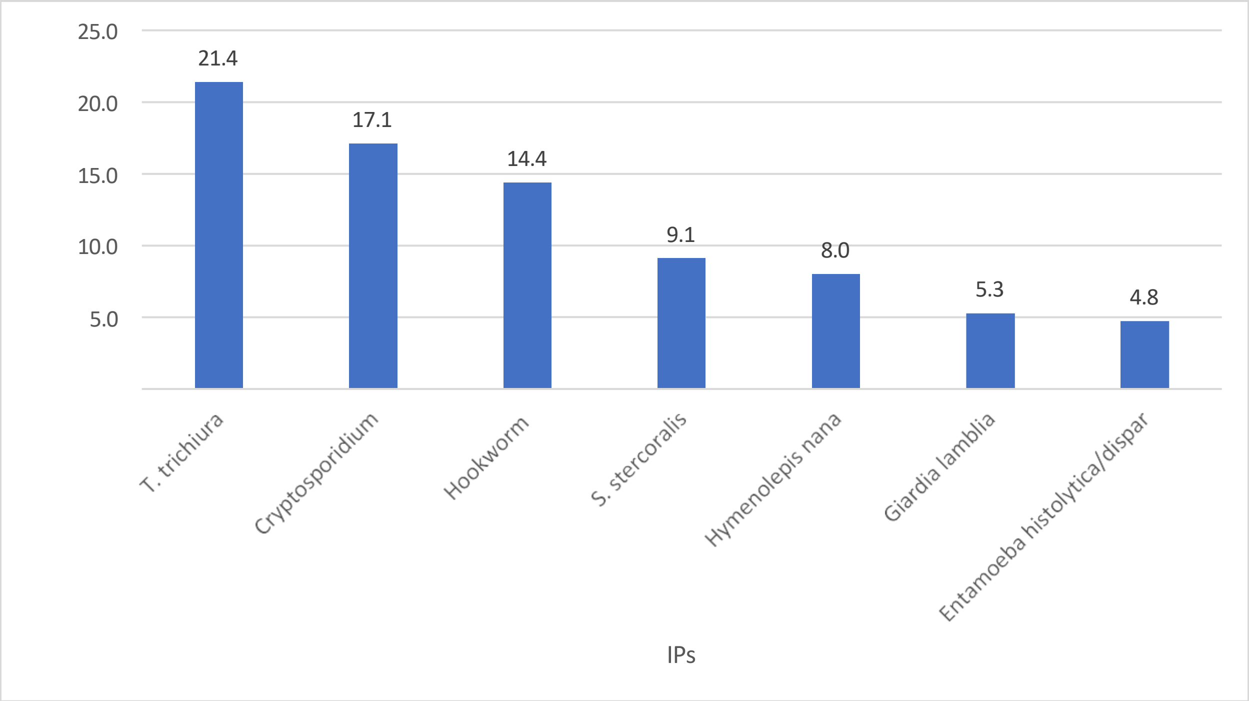

Figure 5: Intestinal parasite infection in humans in Hawassa and Wurgissa, Ethiopia, January – September 2018.

4.2. Prevalence of *Cryptosporidium* spp. in humans

The overall prevalence of infection was 46% (n = 86) by microscope and PCR. The prevalence was comparable between males and females. The difference in the prevalence of infection was also not significant across different age groups (Table 2). Of the 86 *Cryptosporidium* infections, 42.6% and 46.24% were identified in rural and urban areas, respectively (Figure 6).

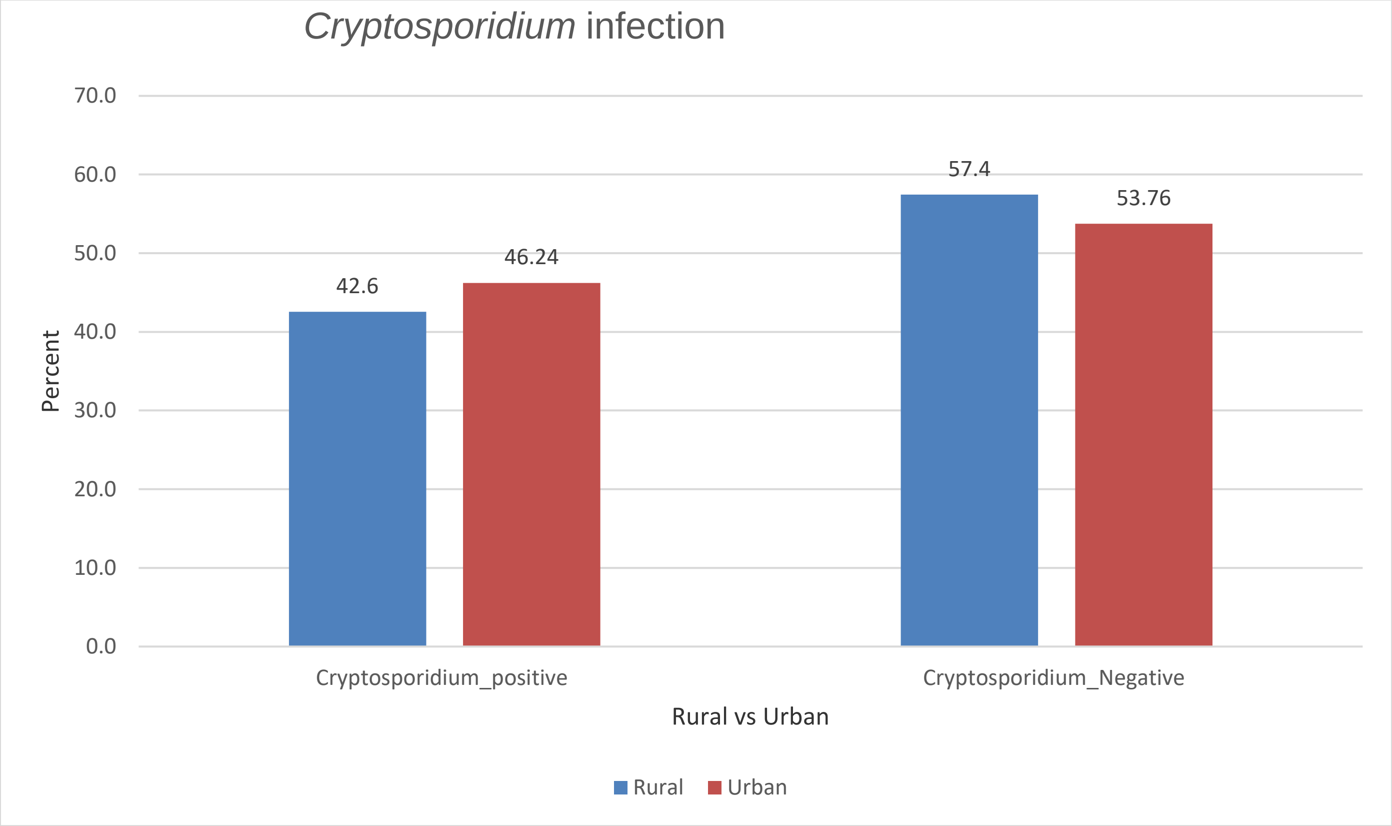

Figure 6: *Cryptosporidium* infections based rural and urban areas, Ethiopia.

Table 2: Prevalence of *Cryptosporidium* infection among humans in Hawassa and Wurgissa, Ethiopia, January - September 2018.

Characteristics	Categories	Number examined	Microscopy using Formalin/ether concentration (%)	Real time PCR n (%)	Nested PCR n (%)	Combined (%)
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
Location	Wurgissa	94	16.0%	40.4%	20.2%	42.6%
	Hawassa	93	18.3%	43.0%	29.0%	46.24%
p-value			0.70	0.76	0.18	0.99

4.2.1. Performance of the diagnostic tools

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 statistically significant difference ($p<0.01$) among microscopy, nested PCR, and real-time PCR in detecting *Cryptosporidium* spp.

All samples detected positive for *Cryptosporidium* spp. by microscopy were also positive by the nested PCR and real-time PCR. However, microscopy failed to diagnose 25 individual samples that were positive by the conventional PCR and 46 samples positive by real-time PCR. In addition, a total of 40 samples diagnosed 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.

Considering the combined results of the three methods as a 'true result', the sensitivity of the microscopy in detecting *Cryptosporidium* infection was 38.6%, nested PCR was 51.8% and real-time PCR was 94.0% (Table 3). The negative predictive value of microscopy was 67.1%, nested PCR 71.6%, and real-time PCR 95.4%. The specificity and positive predictive values of the 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 the results of Nested PCR and the results of the three methods combined in detecting infection was also moderate (0.51). The agreement between real-time PCR and the combined results was almost perfect (k=0.95).

Table 3. 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
Microscopy	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

4.3. *Cryptosporidium* spp. genotype and subtypes in humans

Cryptosporidium spp. genotype data were obtained for 48 of 86 positive PCR samples. Among the genotyped, 40 were *C. parvum* and 8 were *C. hominis* (Table 4). Subtype analysis was successfully carried out for 15 of the 40 *C. parvum* species and 6 of the 8 *C. hominis* species. Infections with *C. parvum* belonged to zoonotic subtype families IIa and IId. The zoonotic subtypes of the 15 *C. parvum* isolates were 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). Two subtype families were identified within *C. hominis* (Ia and Id). These subtypes were IaA20 (n=5) and IdA21 (n=1). Representative sequences were deposited in the NCBI database under accession numbers MW037825–MW037836.

Table 4: Cryptosporidium spp. and subtype in humans, Hawassa and Wurgissa, Ethiopia, January –September 2018

Identification		Collection sites		Total	
		Wurgissa	Hawassa		
		Count	Count	Count	%
Species	*C. hominis*	6	2	8	4.3%
	C. parvum	14	26	40	21.4%
Subtypes	IaA20	5	0	5	2.7%
	IdA21	0	1	1	0.5%
	IIaA14G1R1	1	0	1	0.5%
	IIaA15G2R1	0	1	1	0.5%
	IIaA16G1R1	2	0	2	1.1%
	IIaA16G3R1	0	2	2	1.1%
	IIaA17G1R1	1	0	1	0.5%
	IIaA19G1R1	1	0	1	0.5%
	IIaA20G1R1	0	3	3	1.6%
	IIaA22G1R1	1	0	1	0.5%
	IIaA22G2R1	0	1	1	0.5%
	IIdA23G1	1	0	1	0.5%
	IIdA24G1	1	0	1	0.5%

4.4. Risk factors for human infection with cryptosporidiosis

Contact with monkey (AOR =36.26, $p<0.01$), the occurrence of diarrhea in the household (adjusted odds ratio (AOR) =34.17, $p<0.01$), and household size (AOR=21.17, $p<0.01$) were positive factors for *Cryptosporidium* spp. infection (Table 5). 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* spp. 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* spp. 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* spp. infections.

Table 5: Factors associated with *Cryptosporidium* spp. infection in humans in Hawassa and Wurgissa, Ethiopia, January – September 2018.

Attribute	Categories	Unadjusted OR [95% CI]	Adjusted OR [95% CI]
Age	0-9	0	0
	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]
	>=50	4.28 [0.37-50.19]	29.52 [0.006-132218]
Gender	Male	0	0
	Female	0.97 [0.54-1.73]	0.75 [0.22-2.59]

Location	Wurgissa	0	0
	Hawassa	1.02 [0.57-1.81]	4.53 [1.00-20.52]
Education level	No Formal edu.	0	0
	Formal edu.	0.28 [0.14-0.55]	0.47 [0.13-1.60]
Family size	≤4	0	0
	>4	8.48 [3.32-21.64]	21.17 [2.89-155.12]
Contact with diarrhea patient	No	0	0
	Yes	8.07 [3.74-17.35]	34.17 [7.07-165.1]
Contact with Monkeys	No	0	0
	Yes	9.44 [4.27-20.88]	36.26 [6.02-218.3]
Source of drinking water	Tap water	0	0
	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	0	0
	Open field	0.80 [0.30-2.19]	3.36 [0.21-53.6]
	Near river	2.10 [0.86-5.13]	8.13 [0.59-1104]
Hand washing habit	No	0	0
	Yes	1.10 [0.62-1.96]	0.70 [0.22-2.24]
Presence of animal at home	No	0	0
	Yes	5.47 [2.60-11.5]	12.13 [2.34-62.93]
Presence of diarrhea	No	0	0
	Yes	2.35 [1.29-4.26]	1.92 [0.57-6.40]

4.5. Proportions of *Cryptosporidium* spp. in NHPs

A total of 185 fecal samples from NHPs were examined, samples in this study were collected from 147 NHPs located in Rural and 38 urban settings. Out of the 185 individual NHPs, as indicated in table 6, *Cryptosporidium* was present in 131 and 66 according to molecular test and microscopy examination respectively.

Table 6: Proportions of *Cryptosporidium* in NHPS based on RT-PCR and microscopy in Hawassa (N = 38) and Wurgissa (N = 147), Ethiopia, January to September 2018

Attributes	Category	Proportions of Cryptosporidium RT-PCR, % (n/N) [95% CI]	P-value	Proportions of Cryptosporidium microscopy/ MZN% (n/N) [95% CI]	P-value
Location	Wurgissa	66.6(98/147) [1.12-6.60]	<0.001	32.6(48/147) [0.92-2.84]	0.12
	Hawassa	86.8(33/38) [0.72-0.94]		47.3(18/38) [0.74-1.00]	
Sex	Male	35.9(47/131) [0.62-1.84]	0.74	34.6(24/66) [0.66-1.46]	0.94
	Female	64.1(84/131) [0.82-1.35]		63.6(42/66) [0.81-1.27]	
Age group	Infants	45 (59/131) [0.67-1.30]	0.75	40.9(27/66) [0.60-1.18]	0.35
	Adult	55(72/131) [0.79-1.40]		59.1(39/66) [0.88-1.50]	
consistency of the stool	Watery	14.5(9/131) [0.32-1.3]	0.13	10.6(7/66)) [0.23-1.10]	0.99
	Formed	85.5(112/131) [0.954-1.29]		89.4 (69/66)) [0.99-1.28]	
Overall Prevalence (n/N)		70.8 (131/185) [69.8 -71.8]		35.7(66/185) [34.7-36.7]	

N= Total positive by each of method of diagnosis n= rate of infection from each group

The rate of *Cryptosporidium* spp. infection between male and female NHPs by both methods has no variation and is statistically insignificant (P>0.05). NHPs with formed fecal samples during sample collection were an important predictor for the occurrence of *Cryptosporidium*. However, there was no significant association.

Microscopic detection of *Cryptosporidium* and others intestinal parasite

The prevalence of infection of Hookworm species, *Cryptosporidium*, and nematodes were higher among vervets in Wurgissa Area in northern Ethiopia than Hawassa (Figure 7).

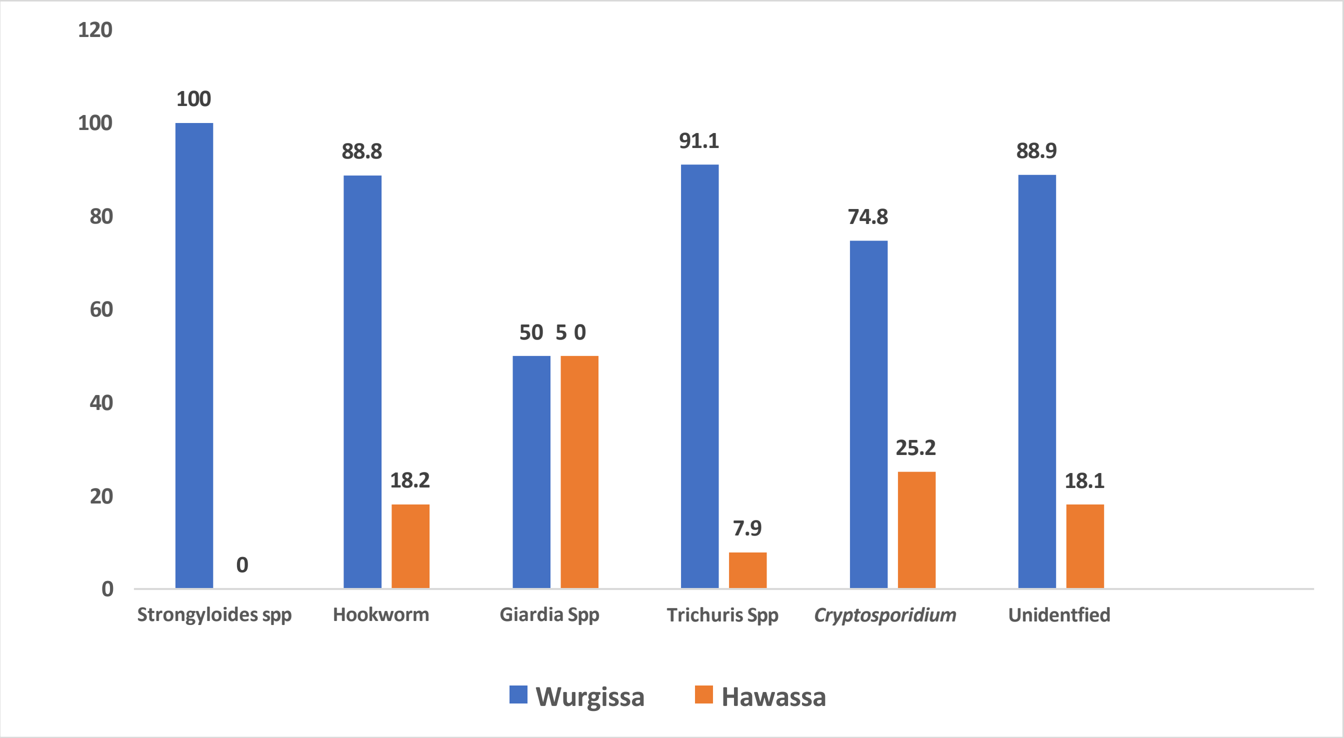

Figure 7: Distribution of Intestinal parasite among NHPS in Wurgissa and Hawassa, Ethiopia, January –September 2018.

4.6. *Cryptosporidium* spp. genotype and subtype in NHPs

Sequence analysis of GP60 and 18s RNA gene resulted in three species: *C. parvum* (n=36), *C. hominis* (n=15), and *C. cuniculus* (n=3) (Table 7). Subtyping analysis of the isolates at the gp60 gene locus identified four subtype families for *C. hominis* namely IaA20 (n=8), IaA26 (n=1); lbA10G2 (n=1), IdA21 (n=1); IeA11G3T3 (n=1), and a subtype family for *C. parvum* with IIaA19G2R1 (n=3), IIaA16G1R1 (n=1), IIaA17G1R1 (n=5), IIaA17G2R1 (n=1), IIaA15G2R1 (n=3), IIaA20G1R1 (n=1) in NHPs (Table 8). For the rest, sequence analysis was unsuccessful due to unreadable sequence (peaks unevenly spaced, nucleotide bases were not deciphered correctly, multiple overlapping traces after a point in the sequence, noisy and premature termination). *C. parvum* subtypes IIaA17G1R1 and *C. hominis* subtype IaA20 were detected particularly in *Chlorocebus aethiops* from rural Wurgissa. *C. parvum* subtypes (IIaA17G1R1, IIaA19G2R1) have been found circulating mainly in vervet species in rural Wurgissa. Representative sequences were deposited in the NCBI database under accession numbers MW037825- MW037836.

Table 7: Identification of *Cryptosporidium* genotypes and sub-genotypes among NHPS in Wurgissa and Hawassa, Ethiopia, January –September 2018.

Individually identified NHPs	NHP Spp.	No. Positive for genotype	18s RNA SSU	GP60	
				Seq-result	Sub-genotype
Mon6, Mon87, Mon3i, Mon29, Mon82, Mon80, Mon12h	Vervet	7		*C. hominis*	IaA20
Mon19	Colobus	1		*C. hominis*	IaA20
Mon86	Vervet	1		*C. hominis*	IaA26
Mon27, Mon830, Mon112	Vervet		*C. cuniculus*		
Mon68	Vervet	1		*C. parvum*	IIaA16G1R1
Mon100	Vervet	1		*C. parvum*	IIaA17G2R1
Mon113, Mon106, Mon7i, Mon826. Mon2014	Vervet	5		*C. parvum*	IIaA17G1R1
Mon814	Vervet	1		*C. parvum*	IIaA15G1R1
Mon96	Vervet	1		*C. hominis*	IaA21
Mon83, Mon024h	Vervet	2	*C. parvum*	*C. parvum*	IIaA15G2R1
Mon74	Vervet	1		*C. parvum*	IIaA16G2R1

Mon9	Vervet	1		*C. hominis*	
Mon103	Vervet	1		*C. parvum*	IIaA19G2R1
Mon123, Mon820	Vervet	2	*C. parvum*	*C. parvum*	IIaA19G2R1
Mon833, Mon9	Vervet	2	*C. hominis*		
Mon824	Vervet	1	*C. hominis*	*C. hominis*	IbA10G2
Mon26	Vervet	1	*C. hominis*	*C. hominis*	IeA11G3T3
Mon8h, Mon4h, Mon022h	Colobus	3	*C. parvum*		
Mon9h, Mon020h, Mon17h, Mon007h, Mon21h, Mon40ih, Mon21, Monk116, Mon120, Mon2, Mon4iih, Mon18h, Mon27h, Mon15h, Mon6i, Mon94, Mon14, Mon110, Mon105	Vervet	19	*C. parvum*		

Table 8: Distribution of species and sub-genotypes based on attributes of NHPs in Wurgissa and Hawassa, Ethiopia, January –September 2018.

Attributes		No. Examined	RT-PCR Positives (n)	*Cryptosporidium* species (N)	Subtype (N)
Location	Rural	147	98	*C. parvum* (21), *C. hominis* (13), *C. cuniculus* (3)	IIaA17G1R1(5), IaA20(6), IIaA16G1R1(1), IIaA17G2R1(1), IIaA15G2R1(2), IdA21(1), IIaA20G1R1(1), IIaA19G2R1(3), IIaA16G2R1(1), lbA10G2(1), IeA11G3T3(1), IaA26(1)
	Urban	38	33	*C. parvum* (15), *C. hominis* (2)	IaA20(2), IIaA15G2R1(1)
Age	Infants	85	59	*C. parvum* (12), *C. hominis* (7), *C. cuniculus* (2)	IaA20(3), IaA26(1), IIaA16G1R1(1) IIaA17G1R1(3), IIaA15G2R1(1), IIaA20G1R1(1), lbA10G2(1),
	Adult	100	72	*C. parvum* (24), *C. hominis* (8), *C. cuniculus* (1)	IaA20(5), IIaA17G1R1(3), IIaA17G2R1(2), IIaA15G2R1(2), IdA21(1), IIaA19G2R1(3), IeA11G3T3(1)
Sex	Male	68	47	*C. parvum* (21), *C. hominis* (13), *C. cuniculus* (3)	IIaA19G2R1(1), IIaA16G2R1(1), lbA10G2(1)
	Female	117	84	*C. parvum* (29), *C. hominis* (12), *C. cuniculus* (2)	IaA20(8), IIaA16G1R1(1) IIaA17G1R1(1), IIaA17G2R1(1), IdA21(1), IIaA19G2R1(2), IIaA15G2R1(3), IIaA20G1R1(1), IeA11G3T3(1), IaA26(1)
Stool	Formed	81	54	*C. parvum* (17), *C. hominis* (4), *C. cuniculus* (2)	IaA20(2), IIaA15G2R1(3), IIaA20G1R1(1), lbA10G2(1), IIaA19G2R1(2), IeA11G3T3(1)
	Loose	85	58	*C. parvum* (17), *C. hominis* (8), *C. cuniculus* (1)	IaA20(4), IaA26(1), IIaA17G1R1(5), IIaA17G2R1(1), IdA21(1), IIaA19G2R1(1), IIaA16G2R1(1)
	Liquid	19	19	*C. parvum* (2), *C. hominis* (3)	IaA20(2), IIaA16G1R1(1)

Colobus	Yes	8	7	*C. parvum* (3), *C. hominis* (1)	IaA20(1)
Vervet	Yes	177	124	*C. parvum* (33), *C. hominis* (14), *C. cuniculus* (3)	IaA20(7), IaA26(1), IIaA16G1R1(1), IIaA17G1R1(5), IIaA17G2R1(1), IIaA15G2R1(3), IIaA20G1R1(1), lbA10G2(1), IeA11G3T3(1), IIaA19G2R1(3), IdA21(1)

4.7. Phylogenetic tree analysis

Evolutionary history was inferred by using the Maximum Likelihood method. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Bootstrap values greater than 50% from 1000 replicates are shown. The sequence gp60 from *C. hominis* isolate CH2821 GP60 (GP60) gene partial cds was used as an outgroup.

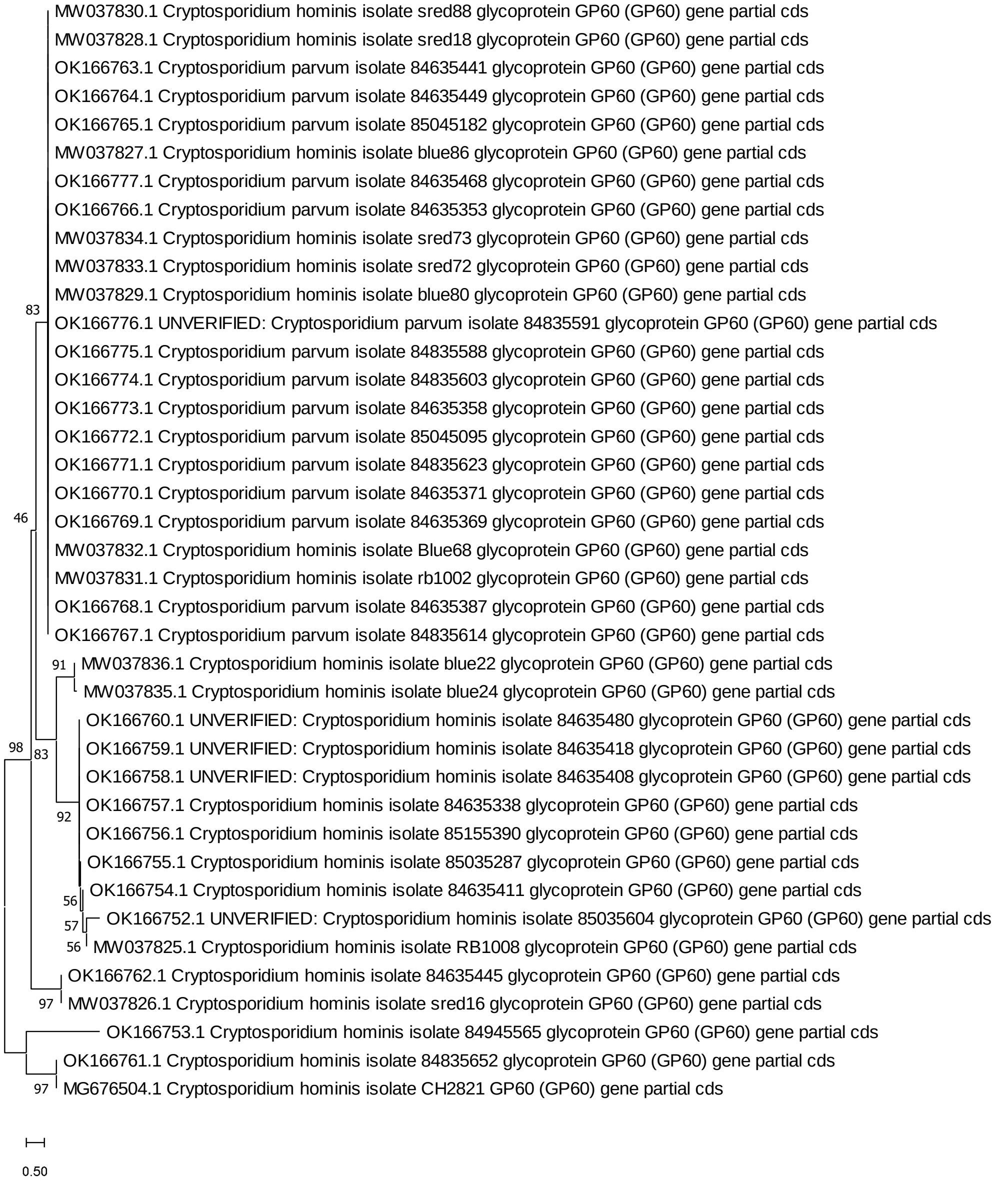

Figure 8: Phylogenetic relationship among *Cryptosporidium* sequences of the partial gp60

5. Discussion

The similarity and high prevalence of intestinal parasites in the two distant areas could be attributed to the commonality of factors such as favorable climate (hot, wet, and humid), socio-economic factors, and demographic factors. Besides, the findings indicated similarity in an inadequate supply of clean water, hygiene, and sanitation in the two study sites that play a crucial role in the spread of the parasites (Atabati *et al.,* 2020).

Cryptosporidium spp. specifically *C. parvum* was the most common species identified in this study. Its prevalence was high in Hawassa, which is an urban setting, compared to Wurgissa the rural setting. This result agreed with the study from Malawi (Morse *et al.*, 2007) and Tunisia (Essid *et al.,* 2018). The overall prevalence of *Cryptosporidium* spp. among patients in Wurgissa and Hawassa as substantially higher among patients having gastrointestinal symptoms in Oromia region in Ethiopia (1.1%) (Flecha *et al.*, 2015) than among HIV patients in Southern Ethiopia (13.2%). The *Cryptosporidium* prevalence among patients in Wurgissa and Hawassa in the current study was comparable with the prevalence reports among HIV/AIDS patients in Bahir Dar, Ethiopia (43.6%) (Alemu *et al.*, 2011) and Kenya (34%) (Wanyiri *et al.*, 2014).

The markedly higher prevalence of *Cryptosporidium* in this study might be attributed to suitable sources of infection, transmission mode, favorable soil, frequent contamination of environments, and the close interactions between humans and animals which might contribute to zoonotic transmission. The prevalence of *Cryptosporidium* among the HIV-negative, HIV-positive, and those whose serostatus unknown varied significantly. This result is comparable with the results of similar studies among HIV/AIDS patients in Addis Ababa (26.9%), southern Ethiopia (13.2%), and Northwest Ethiopia (43.6%) (Alemu *et al.,* 2011; Adamu *et al.,* 2014; Shimelis *et al.,* 2016).

The considerable occurrence of cryptosporidiosis among HIV/AIDS patients might be linked to the weakened immune status of the patients.

Education status, contact with NHPs, and gastrointestinal symptoms were observed to be statistically associated with *Cryptosporidium* infection. Educated people are less likely to be infected with intestinal parasites including *Cryptosporidium* owing to their better knowledge of the proper implementation of hygienic and sanitation practices. The positive relationship between contact with NHPs and cryptosporidiosis implicates the importance of NHPs as sources of human infection via contamination of drinking and recreational water, and edible fruits and vegetables. The significant association between gastrointestinal symptoms and *Cryptosporidium* infection could be attributed to the clinical manifestations of the infection that includes abdominal pain, fever, vomiting, malabsorption, and diarrhea (Chalmers and Davies, 2010; Bouzid *et al.,* 2013).

The *C. parvum* subtype IIaA20G1R1, detected relatively more frequently in the present study was rarely reported from humans but was seen in water buffalo in Brazil (Martins *et al.,* 2018) and in cattle in Serbia and Montenegro (Misic and Abe, 2007), Sweden (Insulander *et al.,* 2013), and Brazil (Toledo *et al.,* 2017). The second and third common subtypes of C. parvum, IIaA16G1R1, and IIaA16G3R1, respectively were reported in lambs, calves, and humans, as well as water sources in Romania (Vieira *et al.*, 2015), Estonia (Lassen *et al.*, 2014), and Slovakia (Kalinova *et al.,* 2018). Furthermore, the IIaA16G3R1 subtype was also seen in calves and goats in Spain, and England, and wild ponies in the Iberian Peninsula (Brook *et al.,* 2009; Couso-Perez *et al.,* 2020 and Díaz *et al.,* 2015). This shows a broad distribution of *C. parvum* subtypes globally.

A study reported a high prevalence of *C. parvum* subtypes that belong to the IIa and IId families in sheep and claves from Italy (Dessi *et al.*, 2020; Diaz *et al.*, 2018). The subtype IId has also been identified in human samples from Egypt, Ethiopia, and Malaysia (Adamu *et al.,* 2014; Ibrahim e*t al.,* 2016; Iqbal *et al.*, 2012; Lim *et al.,* 2011) and in a range of animal hosts from China, such as horses and donkeys, rodents, golden takins, yaks, sheep, and goats (Qi *et al.,* 2015, Zhao *et al.*, 2015). These findings suggest that diverse animals serve as reservoirs for C. parvum (Nader *et al.*, 2019; Feng *et al.,* 2018) and hence the zoonotic nature of the parasite.

Among the two subtypes of *C. hominis*, IaA20 (5/6) and IdA21 (1/6), recognized in the study, IdA21 was previously recorded in UK from a patient who had traveled to and returned from Africa (Chalmers *et al.,* 2013; Chalmers *et al.*, 2008). This suggests that *C. hominis* IaA20 could be the most widespread subtype in the study area with the potential zoonotic or anthroponotic transmission.

The NHPs hosted various kinds of intestinal parasites that included hookworms, *T. Trichura*, *Giardia lamblia*, and unidentified nematodes. The findings are comparable with earlier reports of primate parasites in southern Ethiopia. For instance, the prevalence of *Trichuris* spp. and *Strongyloides* spp. was 12.1 - 13.8 %, 36.6- 37.3 %, respectively in Sodore, Wondo-Genet, and Lake Hawassa (Legesse and Erko 2004; Amenu *et al.*, 2015).

The findings of the present study were like other studies in Vervets in South Africa, Uganda, and Tanzania (Bakuza, 2018; Gillespie *et al.,* 2004; Gaetano *et al.,* 2014) where *Trichuris* spp. and hookworms were common. The occurrence of common parasite infections among NHPs indicates their host-parasite co-evolutionary relationships (Nunn *et al.,* 2006). Vervets have possibly evolved to a range of parasites, particularly nematodes. The similarity in Vervet parasite fauna across their range may also reflect their common risk behaviors, such as omnivorous type of feeding (Hamilton *et al.*, 1978). This could possibly expose them to contaminated food and insects that may transmit the parasites. Their water contact habits and their tendency to adapt quickly to live near human settlements predispose them to parasites and diseases.

The discrepancy between polymerase Chain Reaction (PCR) based diagnosis and microscopy in parasite diagnosis might have resulted from the limited sensitivity of microscopic examination especially when there are low levels of infections. Even then, the infection prevalence detected in the present study is significantly higher than previous infection rates reported in vervet monkeys in Sodre (3.5%, 1/29) and Hawassa (9.5%, 6/63) (Legesse and Erko, 2004; Amenu *et al.,* 2015), perhaps indicating that human-NHP interactions have been increasing more intensely.

In addition, the prevalence in the present study was substantially higher than that reported from Uganda (4.0%), Rwanda (4.0%), Kenya (2.6%), Central African Republic (0.5%), Thailand (1.0%), and Indonesia (2.7%). It was also higher than the prevalence in macaques and gray langurs from the forests of Sri Lanka (27%), and the prevalence among NHPs in Tanzania (16%) (Graczyk *et al.,* 2001; Ekanayake *et al.,* 2006; Li *et al.,* 2011; Salyer *et al.,* 2012; Sak *et al.,* 2013; Parsons *et al.*, 2015; Mynarova *et al.,* 2016); each of which possibly relating to the diverse level of human-NHP interaction. This explanation is supported by the high (73%) prevalence from Ugandan gorillas habituated to humans (Nizeyi *et al.,* 1999).

The highest *Cryptosporidium* infection in the NHPs was due to *C. parvum* (67%; 36/54) followed by *C. hominis* (28%; 15/54) and *C. cuniculus* (5%; 3/54). *C. cuniculus* has been previously detected in rabbits, humans, and kangaroos (Chalmer *et al.,* 2011, Koehler *et al.,* 2014, Nolan *et al.,* 2010, Nolan *et al.,* 2013) but not in NHPs. This result agrees with previous studies conducted in Ethiopia (Adamu *et al.,* 2010; Manyazewal *et al.,* 2018). *C. hominis* was reported in NHPs from China and Kenya (Ye *et al.,* 2012, Li *et al.,* 2011; Chen *et al.,* 2019). The occurrence of *C. parvum* and *C.*

hominis in NHPs and humans indicates the zoonotic and anthropozonotic transmission potential of the parasites.

This study reported that free-ranging NHPs are infected with *C. parvum* IIa subtype family IIaA17G1R1 which is a common subtype. This report is in concurrence with previously reported from humans and cattle in the UK and Romania, respectively (Chalmers *et al.,* 2011; Vieira *et al.,* 2015). Also, the IIaA17G1R1 subtype was identified in Iranian diarrheic children (0.07%) and adults in the Netherlands (Sharbatkhori *et al.,* 2011; Wielinga *et al.,* 2008) it is suggesting zoonotic transmission. The high number of IIaA17G1R1 infections in the present study indicates that this subtype infects humans and free-living NHPs. The reproductive nature of *C. parvum* and its resistant infective stage that persist prolonged time in habitats where roaming monkeys share with livestock and humans increases the risk of transmission.

Multiple subtype families of *C. hominis*, Ia, Ib, Id, and Ie were documented as widespread intraspecific heterogeneity in *C. hominis* (Chen *et al.,* 2019, Nizeyi *et al.,* 2002). In Ethiopia, *C. hominis* subtype families Id, Ie, and Ib have been identified in humans; nevertheless, studies on NHPs are lacking (Adamu *et al.,* 2014). Reports from different countries have shown a diversity of *C. hominis* subtype families in NHPs (Ye *et al.,* 2012). A study carried out in China reported *C. hominis* subtype families Ia, Id, Ie, and If. A similar study undertaken in Kenya pointed out subtype families of *C. hominis*, Ib, If, and Ii (Li *et al.,* 2011). NHPs have closely related genetics to humans, and both could probably be susceptible to the same pathogens, including *C. hominis* (Widmer *et al.,* 2020). *C. hominis* is also described to have a wide range of subtype population structures (Liu *et al.*, 2015). The incursion behavior of NHPs to human habitats enhances the spread of the parasite through food and water contaminated with monkey feces.

The prevalence of NHPs *Cryptosporidium* infection in Wurgissa (74.8 %; 98/131) was higher than the prevalence in Hawassa (25.2 %; 33/131). Both *C. parvum* and *C. hominis* were detected in NHPs in rural areas of Wurgissa. The predominant isolate of *C. parvum* (IIaA17G1R1) was frequently detected in the rural Wurgissa area. Given the fecal-oral transmission of *Cryptosporidium* infection, relatively higher domestication of livestock, use of animal manure or excreta as a fertilizer, poor latrine use, and conducive temperature and rainfall pattern in the Wurgissa area could increase the infection rate. In addition, Ib, Id, and Ie subtype families of *C. hominis*, were detected in Wurgissa. However, the importance of local people or live stocks in transmitting the infection in the free-ranging NHPs remains unknown, which reinforces the need for multi-locus genotype analysis of the parasite including human, livestock, and water samples to properly elucidate the host population structure.

6. Conclusion and recommendation

6.1. Conclusion

Based on the findings of the study the following conclusions are drawn:

- *C. parvum, C. hominis* and *C. cuniculus* was detected in NHPs for the first time in Ethiopia.
- The predominance of *C. parvum* over *C. hominis* in humans was observed in both rural and urban areas.
- The presence of zoonotic IIa and IId gp60 subtype families of *C. parvum* in humans was associated with low education status and gastrointestinal symptoms.
- *Cryptosporidium parvum* (IIaA17G1R1) was most frequent in the rural Wurgissa area. This could be due to higher faecal-oral transmission due to relatively higher contact with domestic livestock, use of animal manure or excreta as a fertilizer, and poor latrine use
- *Cryptosporidium parvum* subtypes, IIaA15G2R1, IIaA16G1R1, IIa17G1R1, IIaA20G1R1, and *C. hominis* IdA21 subtypes identified in NHPs were also found in humans living in Wurgissa and Hawassa in Ethiopia. This suggests that local people or livestock or both could propagate the infection to free-ranging NHPs and vice versa.

6.2. Recommendation

Further studies should explore the hygiene, sanitation, and water purification practices employed in the localities where humans and NHPs sympatrically occupy the same habitat to prevent the possibility of zoonotic/anthroponotic transmission of *Cryptosporidium* spp.

- Additional studies involving NHPs' foraging behavior, characterization of parasites from humans, livestock and drinking and recreational water sources could help better understand the role of NHPs in the transmission of *Cryptosporidium* spp.to humans.
- Raising awareness of the community in the study areas will be necessary to prevent the possible zoonotic spread of cryptosporidiosis in humans.
- Cross-infection experiments of *Cryptosporidium* spp. parasite isolates may be necessary to provide definitive evidence for possible zoonotic/anthroponotic transmission.

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8. Annexes

Annex 1: QiAampPowerFecal Kit protocol for the purpose of extracting DNA from stool samples, Real Time PCR Process and GP60 amplification protocol

1. 0.25 g of stool was added to dry bead tube

2. 750 ul power bead solution was added to dry bead tube containing stool sample

3. The solution (C1) of 60 ul was added and the tube was inverted several times and vortexed briefly.

4. The tube was placed on the hot plate and heated for 10 min at 65oc

5. The tube was vortexed for 10 min.

6. The tube centrifuged (13000 xg) for 1 min

7. The supernatant was transferred to another 2ml tube

8. 250ul C2 solution was added and vortexed and incubated at 5oc for 5 min

9. The tube was centrifuged (13000xg) for 1 min

10. 600ul of the supernatant was transferred to the collection tube (2ml)

11. 200ul c3 solution was added to the tube, vortexed and incubated 5^{o}C for 5min

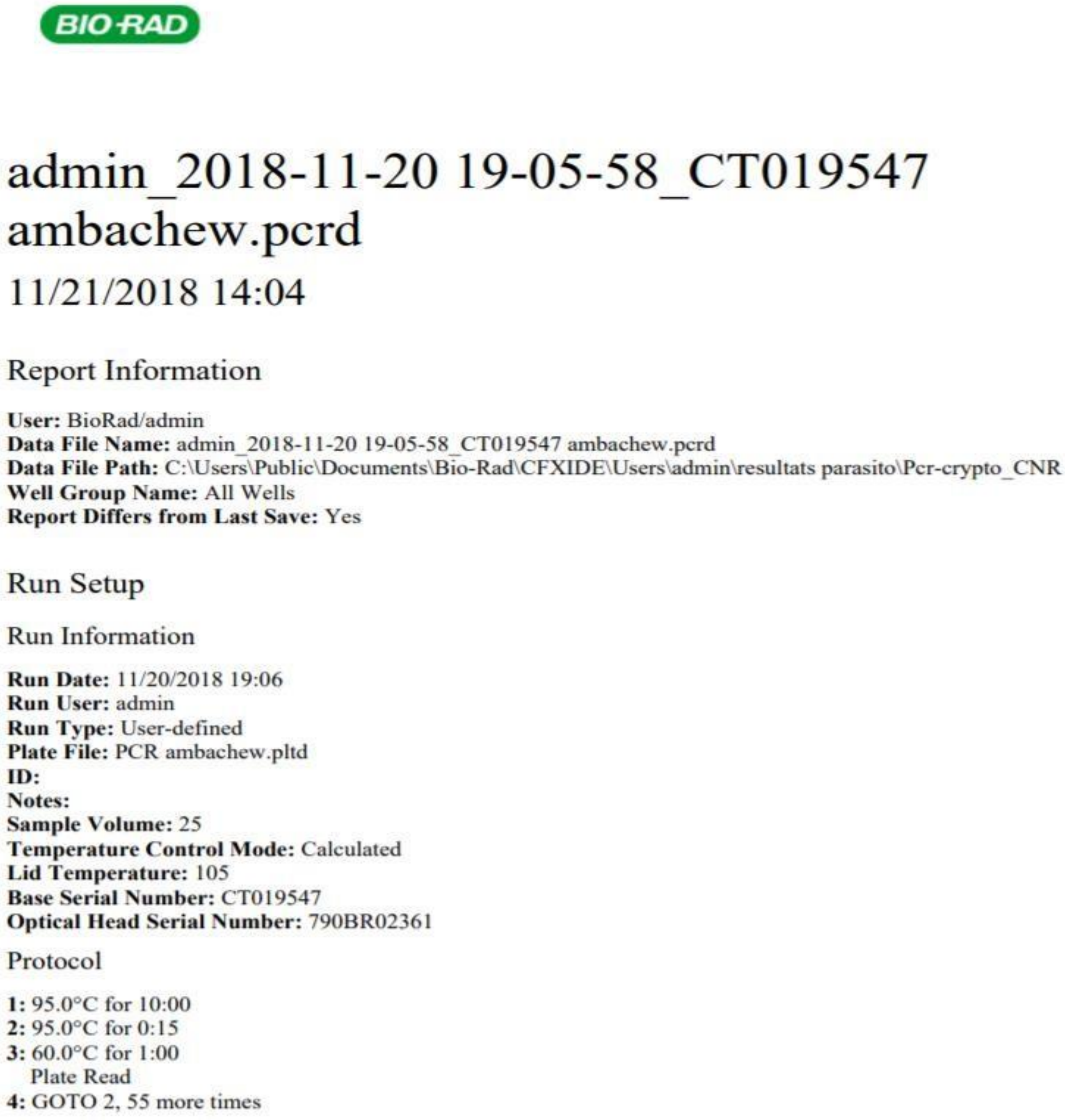
12. The tube was centrifuged (13000xg) for 1 min

13. The supernatant (750ul) was transferred to 2ml collection tube

14. C4 solution (1200ul) was added to the supernatant and vortexed for 5sec

15. The supernatant (650ul) was loaded onto a MB spin column (3 times) and centrifuged for 1 min
16. C5 solution (500ul) was added centrifuged (13000xg) for 1 min
17. The flow was discarded and centrifuged (13000xg) for 1 min
18. The MB spin column was placed on 2ml collection tube
19. C6 solution (100ul) was eluted on the center the tube
20. The tube was centrifuged (13000xg) for 1 min and the spin filter basket was discarded and the DNA is stored at -80oc.

Real Time PCR Process



admin_2018-11-20 19-05-58_CT019547 ambachew.pcrd

11/21/2018 14:04

Report Information

User: BioRad/admin
Data File Name: admin_2018-11-20 19-05-58_CT019547 ambachew.pcrd
Data File Path: C:\Users\Public\Documents\Bio-Rad\CFXIDE\Users\admin\resultats parasito\Pcr-crypto_CNR
Well Group Name: All Wells
Report Differs from Last Save: Yes

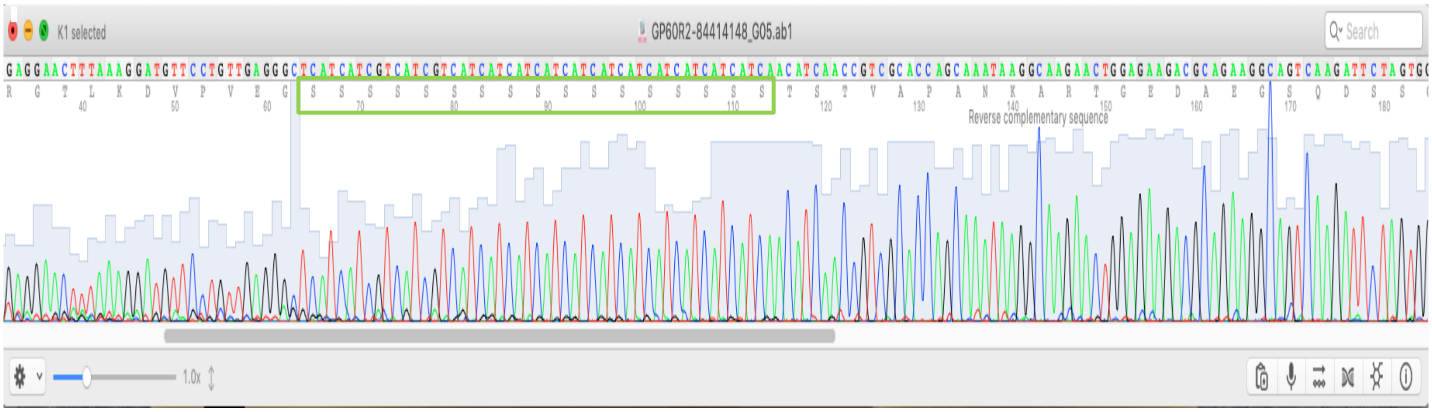
Run Setup

Run Information

Run Date: 11/20/2018 19:06
Run User: admin
Run Type: User-defined
Plate File: PCR ambachew.pltd
ID:
Notes:
Sample Volume: 25
Temperature Control Mode: Calculated
Lid Temperature: 105
Base Serial Number: CT019547
Optical Head Serial Number: 790BR02361

Protocol

1: 95.0°C for 10:00
2: 95.0°C for 0:15
3: 60.0°C for 1:00
  Plate Read
4: GOTO 2, 55 more times



Preparation of PCR Reagents

1. PCR

- Dilution of primers for PCR: Primers F / R at 10uM (1 / 10th of the stock solution): withdraw 10uL of the stock solution at 100uM and add 90uL of PCR water.

- Preparation of dNTPs at 25mM: Take 20uL of each dNTP (100mM stock solution) and add 240uL of PCR water.

- Preparation of dNTPs at 10mM (working solution): Withdraw 80uL of the 25mM dNTP solution and add 120uL of PCR water.

2. Sequence

- Dilution of primers for PCR sequencing: 3.2uM F / R Primers: Withdraw 64ul of 10uM primer solution and add 136uL of PCR water.

- Preparation of the Mix for PCR sequencing (for a final reaction volume of 10uL):

- Big Dye Buffer: 1.5uL
- Primer F or R: 1uL
- PCR water: 5.8uL
- Taq Big Dye: 0.7uL
- Template DNA: 1uL

1. Locus GP60 amplification protocol at *Cryptosporidium*

Preparation of PCR mix 1 (final volume of 50uL):

- Buffer Buffer: 5 uL
- DNTP: 1 uL
- Primer F1 GP60 ATGFmod: GAG-ATT-GTC-GCT-CGT-TAT-CG (2uL)
- Primer R1 GATR2: GAT-TGC-AAA-AAC-GGA-AGG (2ul)
- Dream Taq: 0.25 uL
- DMSO (5%): 2.5 uL
- Water: 34.75 uL
- Template DNA: 2.5 uL for PCR 1 and 5 uL for PCR2

Preparation of PCR mix 2 (final volume of 50uL):

- Buffer Buffer: 5 uL
- DNTP: 1 uL
- Primer F2: GP60F2: TCC-GCT-GTA-TTC-TCA-GCC (1uL)
- Primer F2': AL3531: ATAGTCTCCGCTGTATTC (1uL)
- Primer R2: GP15E: CCA-CAT-TAC-AAA-TGA-AGT-GCC-GC (1uL)
- Primer R2': GP60R2: CGA-ACC-ACA-TTA-CAA-ATG-AAG-T (1uL)
- Dream Taq: 0.25 uL
- DMSO (5%): 2.5 uL
- Water: 32.25 uL
- Template DNA: 5 µL for PCR2

Thermal cycler program:

- Initial denaturation: 94 ° C for 3 minutes
- Then 40 cycles:

o Denaturation: 94 ° C for 45 seconds

o Hybridization: 54 ° C for 45 seconds

o Elongation: 72 ° C for 1 minute

- Final elongation: 72 ° C for 7 minutes

2. Locus GP60 amplification protocol in *Cryptosporidium* (Soulaiman, *et al.* 2005)

Preparation of PCR mix 1 (final volume of 50uL):

- Buffer Buffer: 5 uL
- DNTP: 1 uL
- Primer F1 AL3531: ATA-GTC-TCC-GCT-GTA-TTC (2uL)
- Primer R1 AL3533: GAG-ATA-TAT-CTT-GGT-GCG (2ul)
- Dream Taq: 0.25 uL
- DMSO (5%): 2.5 uL
- Water: 34.75 uL
- Template DNA: 2.5 uL for PCR 1 and 5 uL for PCR2

Preparation of PCR mix 2 (final volume of 50uL):

- Buffer Buffer: 5 uL
- DNTP: 1 uL
- Primer F2: GP60F2 / AL3532: TCC-GCT-GTA-TTC-TCA-GCC (2uL)
- Primer R2: GP60R2 / LX0029: CGA-ACC-ACA-TTA-CAA-ATG-AAG-T (2uL)
- Dream Taq: 0.25 uL
- DMSO (5%): 2.5 uL
- Water: 32.25 uL

- Template DNA: 5 μL for PCR2

Thermal cycler program:

- Initial denaturation: 94 ° C for 3 minutes
- Then 40 cycles:

o Denaturation: 94 ° C for 45 seconds

o Hybridization: 54 ° C for 45 seconds

o Elongation: 72 ° C for 1 minute

- Final elongation: 72 ° C for 7 minutes

3. Locus GP60 amplification protocol in *Cryptosporidium* (alves, *et al.* 2003)

Preparation of PCR mix 1 (final volume of 50uL):

- Buffer Buffer: 5 uL
- DNTP: 1 uL
- Primer F1 AL3531: ATA-GTC-TCC-GCT-GTA-TTC (2uL)
- Primer R1 AL3535: GGA-AGG-AAC-GAT-GTA-TCT (2ul)
- Dream Taq: 0.25 uL
- DMSO (5%): 2.5 uL
- Water: 34.75 uL
- Template DNA: 2.5 uL for PCR 1 and 5 uL for PCR2

Preparation of PCR mix 2 (final volume of 50uL):

- Buffer Buffer: 5 uL
- DNTP: 1 uL
- Primer F2: GP60F2 / AL3532: TCC-GCT-GTA-TTC-TCA-GCC (2uL)
- Primer R2: AL3534: GCA-GAG-GAA-CCA-GCA-TC (2uL)
- Dream Taq: 0.25 uL
- DMSO (5%): 2.5 uL
- Water: 32.25 uL
- Template DNA: 5 µL for PCR2

Thermal cycler program:

- Initial denaturation: 94 ° C for 3 minutes
- Then 40 cycles:

o Denaturation: 94 ° C for 45 seconds

o Hybridization: 54 ° C for 45 seconds

o Elongation: 72 ° C for 1 minute

- Final elongation: 72 ° C for 7 minutes

Annex 2: Revelation on 2% Agarose Gel

Beforehand, reconstitute TAE: 20mL of TAE 50X and add distilled water (QSP 1L).

For a small gel:

- Weigh 2 g of agarose, place in the bottle provided. Add 150 mL of TAE, heat at maximum power in the microwave for 5min. 50 mL of TAE will be evaporated, leaving 100 mL of preparation
- Cool the bottle for a few moments under running water and add 5 uL of BET (CMR Caution) or 6 uL of midori for UV exposure. Gently homogenize.
- Put on a comb with the desired number of wells, and gently pour the gel. Watch out for air bubbles that must be removed with a cone.

For an average gel:

- Weigh 3 g of agarose, place in the bottle provided. Add 200 mL of TAE, heat at maximum power in the microwave for 5min. 50 mL of TAE will be evaporated, leaving 150 mL of preparation
- Cool the bottle for a few moments under running water and add 5 uL of BET (CMR Caution) or 9 uL of midori for UV exposure. Gently homogenize
- Put up to two combs (equidistant) including the number of wells required, and gently pour the gel. Watch out for air bubbles that must be removed with a cone.

For a large gel:

- Weigh 4 g of agarose, place in the bottle provided. Add 250 mL of TAE, heat at maximum power in the microwave for 5min. 50 mL of TAE will be evaporated, leaving 200 mL of preparation

- Cool the bottle for a few moments under running water and place 10 uL of BET (Caution CMR) or 12 uL of midori for UV exposure. Gently homogenize

- Put up to three combs (equidistant) including the number of wells needed, and gently pour the gel. Watch out for air bubbles that must be removed with a cone.

Filing procedure:

- Once the gel has set, carefully remove the combs and place it with its holder in the TAE buffer bath. Add TAE if necessary, to fill the wells with the gel.

- Add 10uL of PCR2 amplification products. If the PCR product is colorless, it will be necessary to stain it in a separate U-shaped well plate. Add 2uL of blue dye and mix with 10uL of PCR product. Place the colored mixture in a well.

- Make migrate 40 minutes at 120V

- Reveal in genetics under UV (be careful to turn off UV rays before opening the door). It is necessary to make the necessary adjustments in order to obtain the sharp focus of the image.

- Print the result obtained

Annex 3: Sequencing protocol for positive samples

Sequencing is indicated only after the revelation of PCR2 and if the bands obtained are of the desired size.

1. Clean Up of Amplification Products

Purpose: The purpose of this step is to purify the PCR amplification products and concentrate them for sequencing. This step eliminates the primers and the nucleotides not incorporated during the PCR reaction.

Two methods can be used.

1.1 Clean up and PCR plate enrichment (method 1) Steps:

1- Add to each well of the PCR plate, PCR water (DNAase, RNAase free) same volume (40uL remaining for each amplified well) as the content of the tubes (volume / volume).

2- Transfer the contents of each well into the multiscreen plate (Filter plate PCR µ96 # LSKMPCR50)

3- Aspirate the contents of the multiscreen plate with a vacuum pump

4- Plate washing with the same volume of water as the transfer volume

5- Vacuum pump suction

6- Collect the purified DNA by adding 20uL of PCR water (half of the water added in step 1). Recover DNA by aspirating / pushing back the pipette at the four corners of each well

7- Place the purified DNA on a clean and named PCR plate.

NB: This step is slow, tedious and requires suitable equipment and in good working order. Its advantage is that DNA is concentrated when amplification is low.

1.2 CLEAN UP PCR (method 2):

Use of ExoSAP-IT ™ PCR Product Cleanup Reagent (Ref 78200; Thermofisher). This method avoids losing DNA during the washing phases present in method 1. This step is faster and is routinely preferred over the first.

Steps: 1- Mix 5 µL of PCR product with 2 µL of EXO SAP IT in a clean PCR plate

2- Incubate at 37 ° C for 15 minutes

3- Incubate at 80 ° C for 15 minutes

2. PCR Sequence

Prepare the primers for each sample (3.2uM primer): take 64ul of the 10uM primer solution and add 136uL of PCR water. For GP60 sequencing, the GP60F2 primer is preferred for amplification protocols 1 and 3.

Prepare the mixes for each primer:

	Volume (µl)/well
Tampon 5X big dye	1,5
Enzyme taq big dye	0,7
Primer F andR	1
H2O	5,8
DNA	1

Distribute 9ul of the mix into each PCR tube then add 0.9ul to 1uL DNA (depending on the signal intensity revealed on the gel)

 Program Sequence: (Include 0.1 ° C / s ramping)

o Initial denaturation at 95 ° C for 2 minutes

o Denaturation at 96 ° C for 10 seconds

o Hybridization at 50 ° C for 5 seconds

o Elongation at 60 ° C for 2 minutes

o 40X repetitions

o Hold at 10 ° C

3. DNA PURIFICATION WITH SEPHADEX

- Make lines of Sephadex G-50 Fine DNA Grade (ref 17-0573-01 D. Dutscher)

- Transfer to plate (Multiscreen HV 96 well plate, Millipore # MAHVN4510)

- Reconstitute as many columns (8 wells each) of sephadex as necessary with 250 µl of water in the plate; vortex to remove any air bubbles

- Let the Sephadex stand for 2 hours

- Centrifugation at 2000g for 3min (program 1 in genetics)

- Add the amplification product (10µL plus 10µL of water), change the bottom plate for DNA recovery

- Centrifugation 2000g for 3min (program 1 in genetics)

- Proceed with sequencing. Parasito 500 program for GP60.

Note: 1 Run sequence = 8 wells if the number of samples is lower, fill the empty wells with 20ul of water

Supplements

Basic mix to amplify other loci (Final volume: 50uL): Buffer Buffer: 5uL

DNTP: 1uL

Primer Forward: 2uL Primer Reverse: 2uL

Dream Taq polymerase: 0.25uL DMSO: 1.25uL

PCR water: 33.50uL DNA template: 5uL

N.B.: the amount of water may vary as needed by adjusting the amounts of DNA starting!

Preparation of AmpliTaq Gold 360 PCR Mix (for a final reaction volume of 50uL): Amplitaq Gold mastermix: 25 uL

360 GC Enhancer: 1 uL - 10uL (depending on the type of primer !!!) Primer Forward: 1 uL

Primer Reverse: 1uL DMSO: 2.5uL

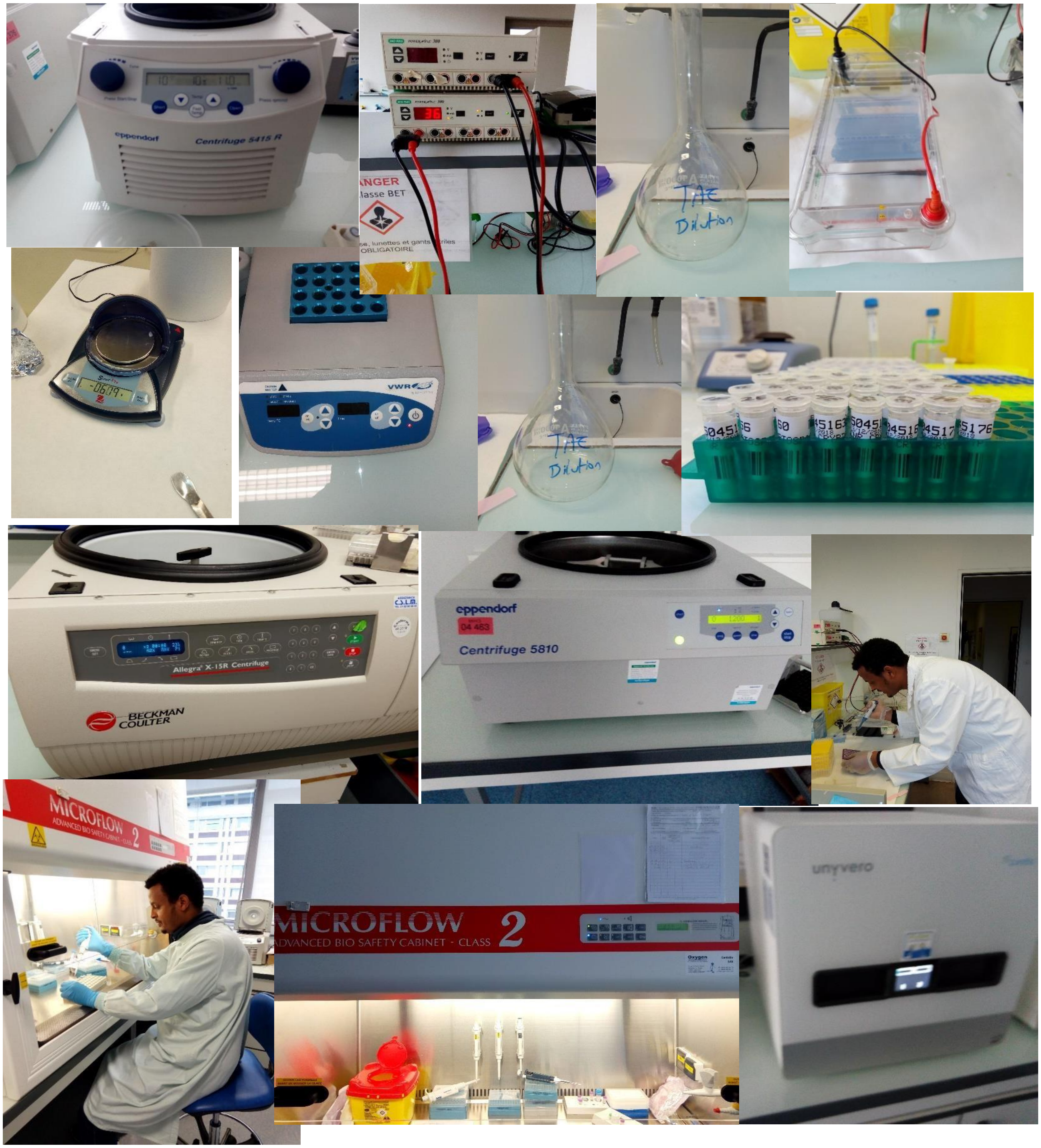
Water: 14.5 uL

N.B: This particular mix theoretically makes it possible to amplify difficult sequences rich in GC.

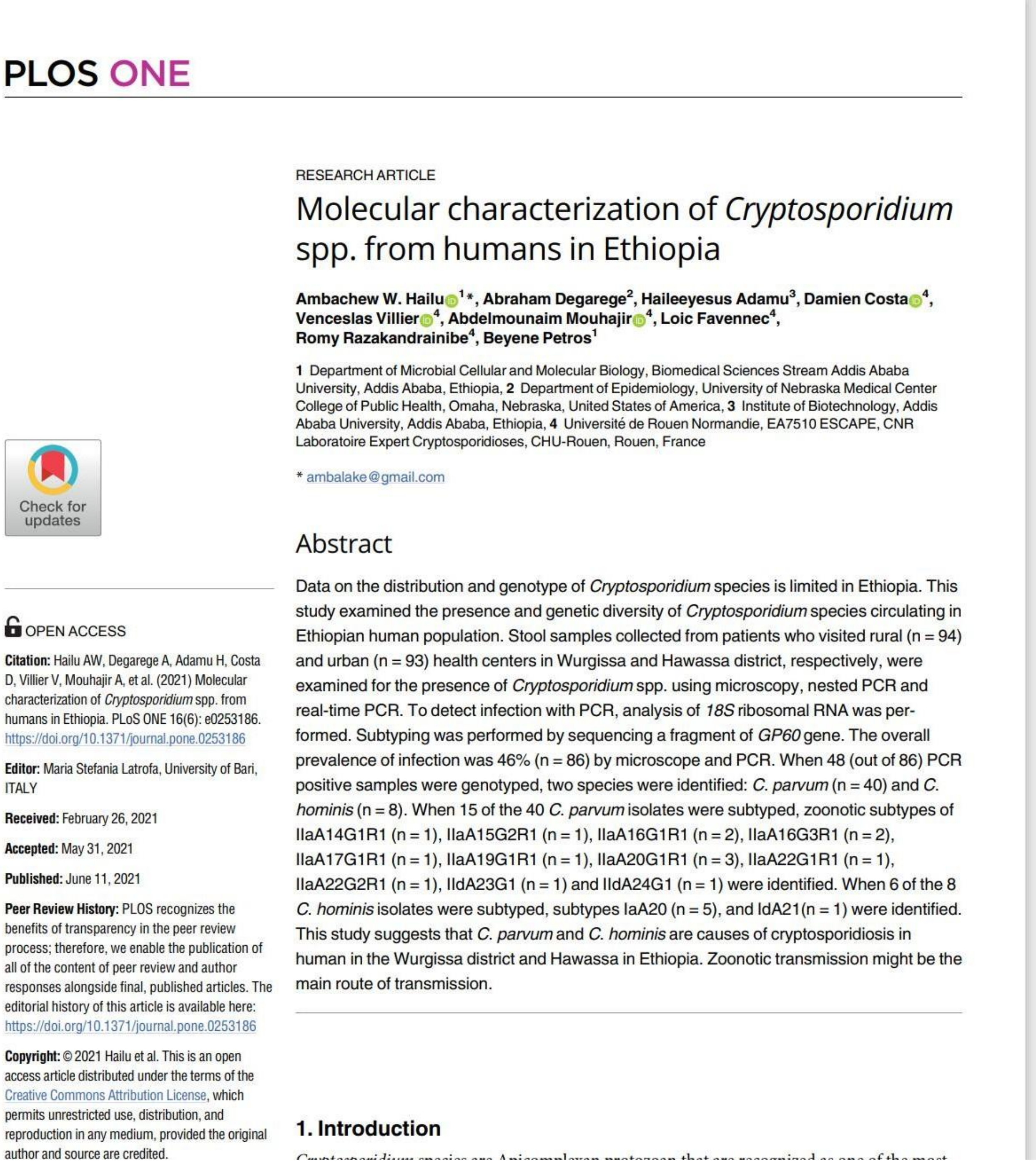
Annex 4: Field data collection, laboratory work in Hawassa and Wurgissa (Ethiopia) and Sequencing facilities in CHU (France)

Laboratory Setting and sample processing in AAU and Woldia Sp. Hospital

PCR, Sequencing facilities in CHU (France)



Annex 5: Research articles published



PLOS ONE

RESEARCH ARTICLE

Molecular characterization of *Cryptosporidium* spp. from humans in Ethiopia

Ambachew W. Hailu[1]*, **Abraham Degarege**[2], **Haileeyesus Adamu**[3], **Damien Costa**[4], **Venceslas Villier**[4], **Abdelmounaim Mouhajir**[4], **Loic Favennec**[4], **Romy Razakandrainibe**[4], **Beyene Petros**[1]

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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 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. When 6 of the 8 *C. hominis* isolates were subtyped, subtypes IaA20 (n = 5), and IdA21(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.

Citation: Hailu AW, Degarege A, Adamu H, Costa D, Villier V, Mouhajir A, et al. (2021) Molecular characterization of *Cryptosporidium* spp. from humans in Ethiopia. PLoS ONE 16(6): e0253186. https://doi.org/10.1371/journal.pone.0253186

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1. Introduction

PLOS ONE

RESEARCH ARTICLE

Genetic diversity of *Cryptosporidium* spp. in non-human primates in rural and urban areas of Ethiopia

Ambachew W. Hailu[1]*, Abraham Degarege[2], Beyene Petros[1], Damien Costa[3,4], Yonas Yimam Ayene[5], Ven-ceslas Villier[3], Abdelmounaim Mouhajir[3], Loic Favennec[3,4], Romy Razakandrainibe[3,4]◐, Haileeysus Adamu[6]◐

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Abstract

Non-Human Primates (NHPs) harbor *Cryptosporidium* genotypes that can infect humans and vice versa. NHPs *Chlorocebus aethiops* and *Colobus guereza* and humans have overlapping territories in some regions of Ethiopia, which may increase the risk of zoonotic transmission of *Cryptosporidium*. This cross-sectional study examined the molecular prevalence and subtypes of *Cryptosporidium spp*. from 185 fecal samples of *Chlorocebus aethiops* and *Colobus guereza* in rural and urban areas in Ethiopia. Samples were tested for *Cryptosporidium* infection using nested polymerase chain reaction (PCR), and subtypes were determined by sequencing a fragment of the 60-kDa glycoprotein gene (gp60). Of the 185 samples, fifty-one (27.56%) tested positive for *Cryptosporidium* infection. The species detected were *C. parvum* (n = 34), *C. hominis* (n = 12), and *C. cuniculus* (n = 3). Mixed infection with *C. parvum* and *C. hominis* were detected in 2 samples. Four *C. hominis* family subtypes (Ia, Ib, Id, and Ie) and one *C. parvum* family subtype (IIa) were identified. *C. hominis* IaA20 (n = 7) and *C. parvum* IIaA17G1R1 (n = 6) were the most prevalent subtypes detected. These results confirm that *Chlorocebus aethiops* and *Colobus guereza* can be infected with diverse *C. parvum* and *C. hominis* subtypes that can also potentially infect humans. Additional studies could help to understand the role of NHPs in the zoonotic transmission of *Cryptosporidium* in Ethiopia.

OPEN ACCESS

Citation: Hailu AW, Degarege A, Petros B, Costa D, Ayene YY, Villier V-c, et al. (2022) Genetic diversity of *Cryptosporidium* spp. in non-human primates in rural and urban areas of Ethiopia. PLoS ONE 17(4): e0267103. https://doi.org/10.1371/journal.pone.0267103

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Annex 6: Consent forms (Amharic and English version) and Questionnaire

የስምምነት ቅፅ (Amharic) ተራቁጥር------------------------

እኔ ከላይ በስም ተጠቃሹ ግለሰብ የአንጀት ጥገኛ ህዋስ በሽታ ሳይኖርብኝ እንደማይቀር ተነግሮኝ በሽታየን ለመመርመር የሰገራ ናሙና እንደሚያስፈልግ ተነግሮኛል።ይህም ምርመራ በሽታው ስለመኖሩ ለማወቅ የሚደረግ ምርመራ መሆኑን ተረድቻለሁ። በተጨማሪም ምርመራው የአንጀት ጥገኛ ህዋሱን በሽታ አምጭ ንዑስ ዝርያ ለመለየት እንደሚያገለግል ተረድቻለሁ። እኔም ስለጥናቱ ከተረዳሁ በሃላ አስፈላጊ ናቸው ብዮ ያመንኩባቸውን ጥያቄዎች ጠይቄ ስለጥናቱ ምንነት በሚገባ ተረድቻለሁ። በጥናቱም መጠይቅ የምሰጣቸው መረጃዎች እንዲሁም የስምምነት ቅፁ በምስጢር እደሚያዝ ከጥናት አድራጊው ጋር ስምምነት አድርገናል።

ስለራሴ የምጠየቀውን መረጃ ያለመስጠት ፡ ለጥናቱ ያለመተባበርና በየትኛውም ወቅት ከጥናቱ ራሴን የማግለል መብቴ የተጠበቀ መሆኑ የተገለጸልኝ ሲሆን ይህንንም በማድረጌ ምክኒያት በአጠቃለይ በሽታየን ከመታከምና ማንኛውንም የህክምና አገልግሎት ከማግኘቴ የሚያግደኝ የለም ማለት መሆኑን በሚገባ ተረድቻለሁ። ስለሆነም ጥናቱን ለሚያከናውነው ተመራማሪ የጥናቱ አካል እድሆን ፈቃዴን የሰጠሁት በሚገባ በመረዳቴነው። ከዚህ በተጨማሪም ለጥናቱ አስፈላጊ ሆነ ሲገኝ በምርመራው ወቅት የተገኘብኝን የአንጀት ተውሳክ መሰረት በማድረግ የሰጠሁት ናሙና ለተጨማሪ ጥናት ወደ ውጭ አገር ቢላክ ተቃውሞ የለኝም። እንዲሁም ከጥናቱ የተረፈው ናሙና በሌላ ጊዜ ለሚደረግ ተጨማሪ የላብራቶሪ ስራ ቢውል ደስተኛ መሆኔን አረጋግጣለሁ።

ፊርማ........................... ቀን............................

Consent form (English)

S/No.: --------------------------

I the above mentioned have been told that I may have intestinal parasitic disease and would like get my stool specimen for identification of parasites. The stool specimen is important to know the disease-causing parasite. Some portions of the stool specimen will be used to recover and extract Intestinal Parasite oocyst and ova for molecular genotyping and sub genotyping. I have asked questions relevant to the study and got satisfied answers with clarifications. Information and data in the survey questioner will be handled strictly confidential and used only for the specified study.

I have the right not to give any information, not cooperate and resign from this study and this will not affect my right from diagnosis and getting treatment. So, I understood, agreed, and signed this consent form. In addition to the above mentioned, I have no objection if part of the stool specimen is shipped to another country for further examination and characterization. I have also agreed to use this sample for the same experiment in the future.

Signature: -------------------------

Date: -----------------------------

Remarks

In a case of participants cannot read, before signing a consent and taking fingerprint, the researcher read and explained to them about the study, the details of information sheet and informed consent**.**

Questionnaire

1. Date of Sample Collection: ---------------------------------

2. Code No. ----------------------------

3. Age: ----------------------------   Sex: ------------------------------

4. Educational background: ----------------------------   (1 = Literate, 2 = Illiterate)

5. Income/ Economic status ---------------------   (1 = <500birr, 2 = 500-1000, 3 = >1000)

6. Abdominal health status: ------------------------   (1 = Health, 2 = Sick)

7. Stool consistency: -----------   (1 = Liquid/watery, 2 = Semi-liquid, 3 = Loose, 4 = soft)

8. Stool character ------------------------   (1 = Bloody mucoid, 2 = Mucoid)

9. Contact with person affected by diarrhea ------------------   (1 = Yes, 2 = No)

10. Presence of animals at home -------------------   (1 = Yes, 2 = No)

11. Are there NHPs near to your home: --------------------   (1 = Yes, 2 = No)

12. Where do you have contact with NHPs: ---------------- (1 = at farm, 2 = at different place)

13. Where do they share food: ----------------------- (1 = at home (cut and carry), 2 = at field)

14. Contact with NHPs or dung: ----------------------   (1 = yes, 2 = no contact)

15. Source of drinking water --------------   (1 = Tape water, 2 = River/unprotected 3. Water well)

16. Where do you defecate and dispose faeces? -------------- 1. near the streams 2. Open field

17. Do you wash your hands after defecation? ---------------- 1. Yes   2. No

18. Date of Diagnosis ---------------------------------

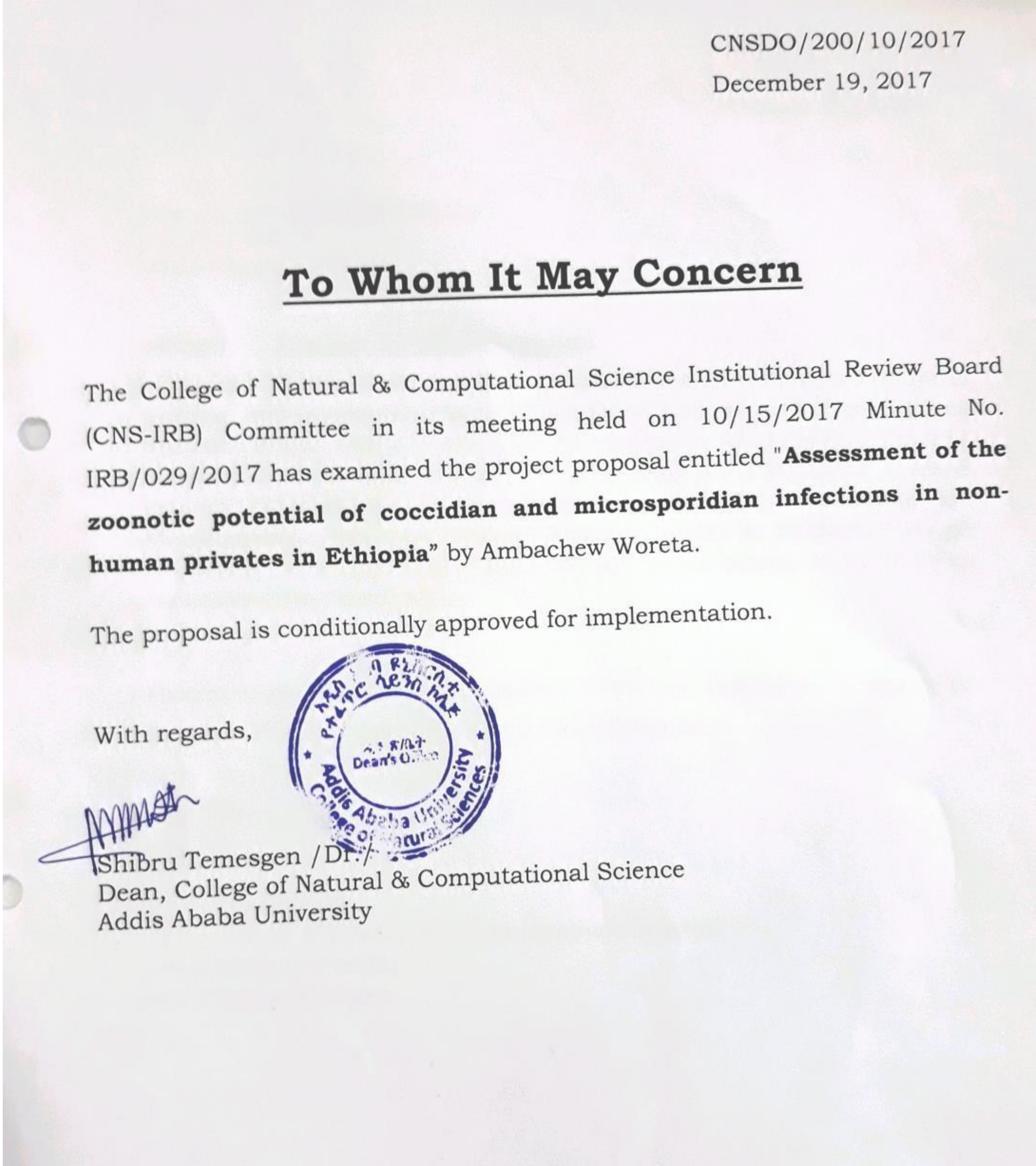
19. Other IP; Identification methods:

Direct Microscopy --------------------------------------

Concentration: ---

Diagnosis ---

Annex 7: Ethics approval letter and plagiarism checker reports



CNSDO/200/10/2017
December 19, 2017

To Whom It May Concern

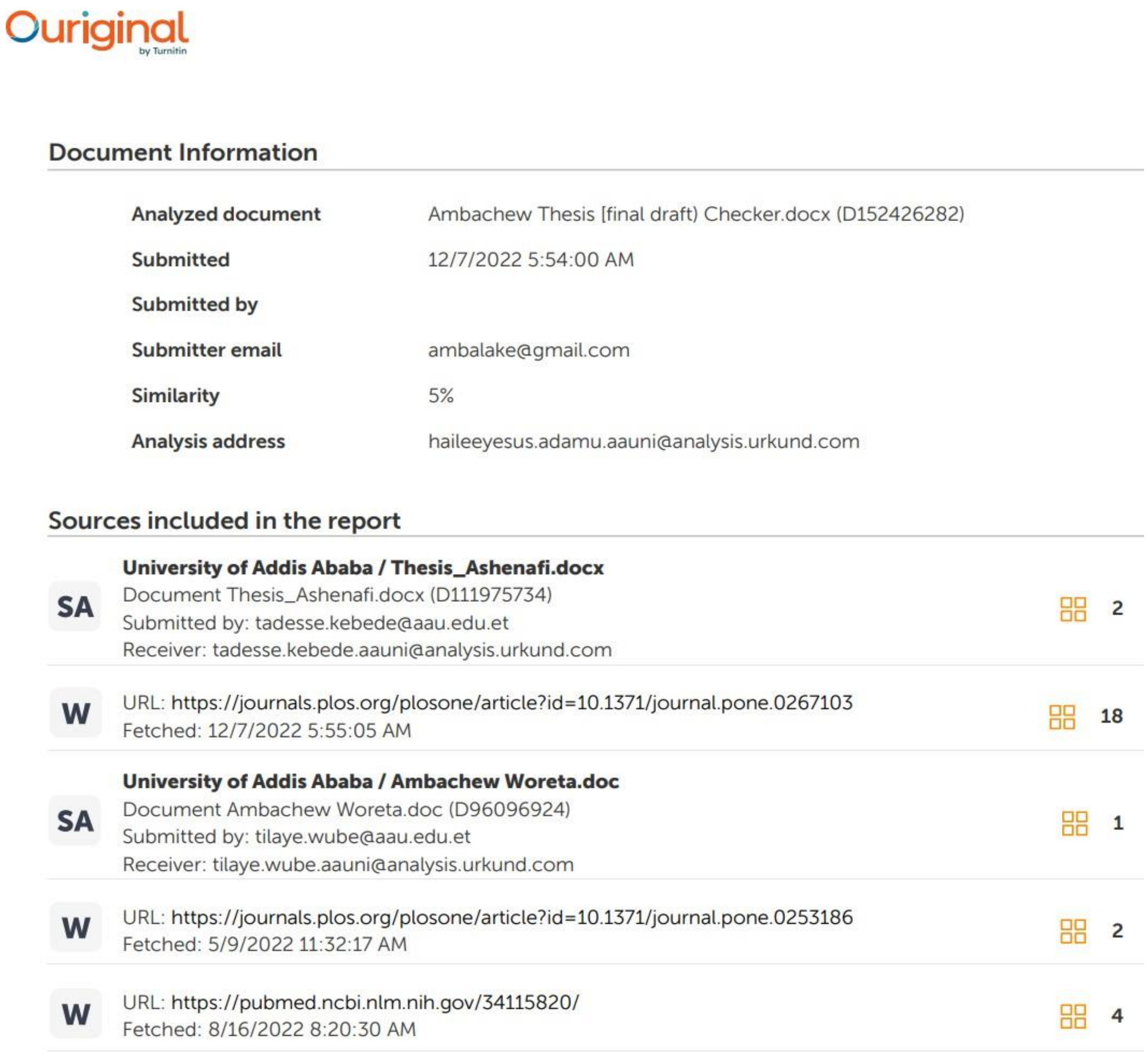
The College of Natural & Computational Science Institutional Review Board (CNS-IRB) Committee in its meeting held on 10/15/2017 Minute No. IRB/029/2017 has examined the project proposal entitled "**Assessment of the zoonotic potential of coccidian and microsporidian infections in non-human privates in Ethiopia**" by Ambachew Woreta.

The proposal is conditionally approved for implementation.

With regards,

Shibru Temesgen /Dr./
Dean, College of Natural & Computational Science
Addis Ababa University

Plagiarism checker report



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