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**THE PREVALENCE OF INTESTINAL PARASITES
AND MOLECULAR CHARACTERIZATION OF
CRYPTOSPORIDIUM SPECIES IN ETHIOPIA**

BY

HAILEEYESUS ADAMU

**A DISSERTATION PRESENTED TO THE SCHOOL OF GRADUATE
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THE REQUIREMENTS FOR THE DEGREE OF
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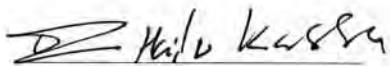
By

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Dedication

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Acronyms

AE	Elusion buffer
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral therapy
<i>Ase</i> I	Digestive enzyme
AW1 buffer	Washing buffer 1
AW2 buffer	Washing buffer 2
AZT	Zidovudine
bp	Base pair
BSA	Bovine serum albumin
COWP	<i>Cryptosporidium</i> oocyst wall protein gene
Cry-15	Reverse primer for COWP
Cry-9	Forward primer for COWP
DIC	differential interference contrast
DNA	Deoxyribo Nucleic Acid
dNTP	Nucleotide bases
EDTA	Ethylenediamine Tetracetic Acid
EHNRI	Ethiopian Health and Nutrition Research Institute
ELISA	Enzyme Linked Immunosorbent Assay
Eth	Ethiopia
GP60	Glycoprotein 60 gene
HAART	Highly Active Antiretroviral Therapy

HCl	Hydrochloric acid
HCT-8	Intestinal adenocarcinoma cell
HIV	Human Immunodeficiency Virus
ID	infective dose
IFN- γ	Interferon γ
IFT	Immunofluorescence test
IgA	Immunoglobulin A
KCl	Potassium chloride
M	Molecular Marker
mM	milli Mole
MOH	Ministry of Health
MZN	Modified Ziehl Neelsen
NaCl	Sodium Chloride
NEB	New England Biolabs
nm	nano meter
NNRTI	non-nucleoside reverse transcriptase inhibitors
OIP	Opportunistic Intestinal Parasites
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Pmol	Pico mole
PVPP	Polyvinylpolypyrrolidone
rb115	rabbit serum
RBC	Red Blood Cells
RFLP	Restriction fragment length polymorphism

RNA	Ribonucleic acid
rpm	Revolution per minute
<i>Rsa</i> I	Digestive enzyme
SAF	Sodium acetate-Acetic acid-Formaldehyde
SPSS	Statistical package for social sciences
<i>Ssp</i> I	Digestive enzyme
SSU-rRNA	small subunit ribosomal RNA
TBE	Tris buffer with Boric acid and EDTA
TCA	Thymine, Cytosine, Adenine
TCG	Thymine, Cytosine, Guanine
UNAIDS	Joint United Nations program on HIV/AIDS
UNICEF	United Nations Children's Fund
USA	United States of America
UV	Ultra Violet
WHO	World Health Organisation
μl	Microlitre
μm	Micrometre

Abstract

Cryptosporidium spp infections are recognized as important causes of diarrhoea in both immunocompromised and immunocompetent patients. Although a number of studies have been conducted on the prevalence of intestinal parasites in Ethiopia, none of them had indicated the importance of cattle as reservoirs for the prevalent human cryptosporidiosis in Ethiopia. In addition, no study had molecularly characterized *Cryptosporidium* spp isolates to determine the source (reservoir) of human cryptosporidiosis in Ethiopia. This study examined the prevalence and distribution of cryptosporidiosis in 9 different locations in Ethiopia. 1034 human faecal samples from patients with diarrhoea and 350 cattle dung were screened for *Cryptosporidium* spp oocysts by using modified Ziehl-Neelson staining method. 79 human stool samples (7.6%) and 8 cattle (2.3%) were positive for *Cryptosporidium* spp. The highest prevalence in humans (10.6%) was detected in the town of Awash 7 (Afar region) and the lowest prevalence (3.8%) in Bishoftu town (Oromia region). Molecular methods were used to determine genotypic and subgenotypic diversity of *Cryptosporidium* spp. isolates that infect humans in Ethiopia. DNA was extracted from all *Cryptosporidium* spp positive samples, PCR amplification of the *Cryptosporidium* spp oocyst wall protein gene (COWP), small sub-unit ribosomal ribonucleic acid (SSU-rRNA), and 60 kilo-Dalton glycoprotein (GP60) gene fragments were performed. Genotype analysis by PCR-RFLP based on COWP and the SSU-rRNA genes, and subgenotyping by DNA sequence analysis of GP60 gene fragments showed the importance of cattle reservoir for the high prevalence of cryptosporidiosis in humans. 52% of the 79 human stool samples and 75% of 8 cattle dung samples were positive in one or other of the three molecular characterization methods. Out of 79 human stool samples, 21(26.6 %) yielded a SSU-rRNA PCR product; 30 (38 %) were positive for

COWP and 30 (38 %) were positive for GP60. The majority of isolates (95%) were identified as *C. parvum*, while only 2.5% were *C. hominis* and another 2.5% mixed infections of the two species. Sequencing of the GP60 gene fragments of the 13 isolates resulted in three different subgenotypes of *C. parvum*, belonging to the zoonotic subgenotype family IIa, and one to the subgenotype of *C. hominis* (Ib). Phylogenetic analysis of the sequences showed *C. parvum* isolates to belong to three subgenotypes: 8 isolates typed as IIaA15G2R1; 3 isolates typed as IIaA16G2R1; and one isolate typed as IIaA16G1R1. The *C. hominis* genotype was typed as IbA9G3 subgenotype. The study has identified *C. parvum* as the major cause of human cryptosporidiosis in Ethiopia and has indicated the major source of cryptosporidiosis to be zoonotic with some (limited) anthroponotic transmission of *C. hominis*. In addition, it was determined that antiretroviral treatment in HIV/AIDS patients reduces infection with *Cryptosporidium* and other diarrheogenic protozoan parasites. Based on the findings of the present study, creation of a central national database on the prevalence of human cryptosporidiosis would be a useful step, so that information could be pooled from different regions of Ethiopia to better understand the epidemiology of the disease.

Key words: *Cryptosporidium*, Epidemiology, Genotyping, Sequencing, zoonotic, anthroponotic, Ethiopia

1. Introduction

Intestinal parasitic infections are among the most common infections world-wide. It is estimated that some 3.5 billion people are affected, and 450 million are ill as a result of these infections, the majority being children (WHO, 1998). Intestinal parasitic infections are caused either by protozoa or helminths or both and the main clinical manifestation of the disease caused by these parasites is diarrhoea (Chacon-Cruz, 2003).

Diarrhoea is one of the greatest causes of morbidity and mortality in the world. This is due to dehydration, which results from a rapid loss of fluid and electrolytes in diarrhoeal stools (WHO, 1984). In general, diarrhoeal diseases are significant causes of morbidity and mortality in all age groups, but paediatric patients and HIV positives experience more severe illnesses (Giannella, 1993). Moreover, nowadays diarrhoeal illnesses are becoming one of the most common clinically observable gastrointestinal manifestations in AIDS patients, occurring at late stages of HIV-1 infection; usually due to opportunistic infection (Fisseha *et al.*, 1999).

Based on the basis of clinical symptoms and pathogenesis; diarrhoeal diseases are classified as acute watery diarrhoea, acute dysenteric diarrhoea, persistent diarrhoea and chronic diarrhoea (WHO and UNICEF, 2000). Acute watery diarrhoea involves the small intestine and is characterized by the frequent passage of watery stools without blood, while acute dysenteric diarrhoea is mainly localized to the large intestine and the stool consists of blood and mucus. Persistent diarrhoea begins in acute form either of watery or dysentery, which lasts 14 days or longer. Chronic diarrhoea is characterized by an increased stool frequency and fluidity lasting for

longer than a month (Mathan, 1998). *Cryptosporidium* species are among the major diarrhoea causing intestinal parasites in humans.

The genus *Cryptosporidium* is composed of protozoan parasites that infect epithelial cells in the microvillus border of the intestinal tract of all classes of vertebrates. The parasite is found worldwide. Host specificity varies with the species of *Cryptosporidium*. Some species of *Cryptosporidium* infect many host species, whereas others appear restricted to groups such as rodents or ruminants, and still others are known to infect only one host species. Some species primarily infect the stomach, whereas others primarily infect the intestine. Some infections are acute, but self-limiting, whereas others are chronic. The severity and duration of infection with pathogenic species are also affected by the immune status of the infected host. Immunocompetent individuals might suffer mild, moderate, or severe acute illness, whereas immunocompromised individuals can suffer severe illnesses and even death.

At the present 20 *Cryptosporidium* species have been identified and named (Table 1), and nearly triple this number of unnamed *Cryptosporidium* has been identified only at the genus level. Most studies on biology, morphology, biochemistry, host preferences, immunology, pathogenicity, physiology, and prevalence of *Cryptosporidium* have been conducted on species of *C. parvum* and *C. hominis*. The reasons is that these species have veterinary and medical importance, they are geographically widespread and infects many host species, thus producing remarkable number of oocysts and making them more easily obtainable for scientific studies compared to other species of *Cryptosporidium*. Consequently, data derived from *C. parvum*, in some cases, have become generalized and extended to other members of the genus. However, each species and genotype has an individual characteristic that makes it different from other species and genotypes

and one has to keep in mind that differences exist between species and genotypes when considering the pathogenesis of *Cryptosporidium* species.

Ernest Edward Tyzzer was the first person to recognize, describe and publish an account of a parasite he frequently found in the gastric glands of the house mouse. In 1907, he described its asexual and sexual stages and discovered that each stage had an organelle resembling structure that was specialized for attachment to host cells. He also noted that oocysts were excreted in the feces of the house mouse. He identified the parasite as a sporozoan of uncertain taxonomic status and named it *Cryptosporidium muris* (Tyzzer, 1907).

In 1912, Tyzzer described another new species, *C. parvum* (Tyzzer, 1912). He demonstrated that *C. parvum* developed only in the small intestine of experimentally infected domestic laboratory mice and that the oocysts of *C. parvum* were smaller than those of *C. muris*. He remained ambiguous on the subject of whether stages were intracellular or extracellular and noted that stages similar to those of *C. parvum* in the mouse were present in the small intestine of the rabbit.

For 48 years after Tyzzer's first publication, because *Cryptosporidium* was not recognized to be of economic, medical, or veterinary importance, there were no subsequent studies and therefore no new information on the genus. Two new species were named, but these were misidentified organisms that were not even in the genus *Cryptosporidium*. In 1955, after the report of a valid new species in turkeys, *C. meleagridis*, the first species of *Cryptosporidium* associated with illness and death (Slavin, 1955), even the interest remained low. In the early 1970s, only slightly more interest was aroused when *Cryptosporidium* was reported to be associated with diarrhoea in cattle (Meutin *et al.*, 1974).

In 1970`s cryptosporidiosis was reported in four patients. Two were children and two were adults. Three patients had immune deficiency and one probably had exposure to animal source (Nime *et al.*, 1976; Meisel *et al.*, 1976; Lasser *et al.*, 1979; Weisburger *et al.*, 1979).

In 1993, interest again increased dramatically following a massive waterborne outbreak in Milwaukee, Wisconsin, involving an estimated 403,000 persons (MacKenzie *et al.*, 1994). In the mid-to-late 1990s, molecular methods for detection and identification emerged, were refined, and became the new cornerstone for taxonomic and epidemiologic studies. Genomic, proteomic and biochemical investigations followed.

Members of the genus *Cryptosporidium* are eukaryotic protozoan parasites. The genus *Cryptosporidium* is one of over 300 genera that include 4800 named species in the phylum Apicomplexa. All are parasitic, and some are important disease agents such as plasmodium and coccidia (including *Cryptosporidia*, *Isospora*, *Toxoplasma*, and *Sarcocystis*) of humans and animals (Figure 1). *Cryptosporidium* affects over 150 species of mammals including humans, as well as birds, reptiles, amphibians, and fish. Most apicomplexans have a complicated life cycle consisting of asexual and sexual stages resulting in sporogony (Soave and Armstrong, 1986).

Despite many morphological and life-cycle characteristics shared by *Cryptosporidium* species and all the aforementioned coccidia, some molecular analyses have suggested a closer relationship among *Cryptosporidium* spp. and others (Carreno *et al.*, 1999; Leander *et al.*, 2003).

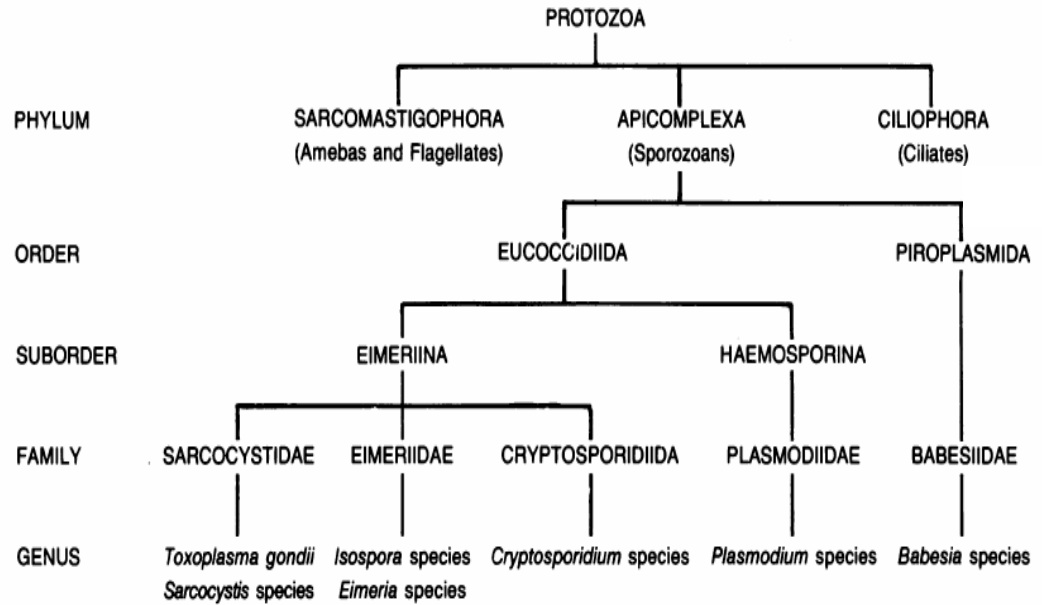


Figure 1: Taxonomic position of *Cryptosporidium*; simplified to show relationships with other medically important species (Soave and Armstrong, 1986).

There are 20 valid named species of *Cryptosporidium* (Table 1), and there are over 40 *Cryptosporidium* isolates referred to as genotypes (Fayer and Xiao, 2008; Plutzer and Karanis, 2009). These have been identified only as *Cryptosporidium* spp. followed by the genotype designation that reflects the host of origin for the oocyst stage. Some isolates, once identified as genotypes of *C. parvum*, have been elevated to species level, such as *C. hominis*, *C. bovis*, and *C. suis*. Other isolates of *C. parvum*, as well as those of *C. canis*, *C. galli*, and *C. muris*, have been differentiated at the molecular level and designated as genotypes or subgenotypes, and still others have been reported to vary in virulence (Fayer and Xiao, 2008).

Table 1: Valid species of genus *Cryptosporidium*, their major hosts, and oocyst measurements
(Modified from Fayer and Xiao, 2008; Plutzer and Karanis, 2009).

Species	Major Host	Location of infection	Mean Oocyst Size (μm)
<i>C. andersoni</i>	<i>Bos taurus</i> (domestic cattle)	Abomasum	7.4 x 5.5
<i>C. baileyi</i>	<i>Gallus gallus</i> (chicken)	Cloaca, bursa	6.2 x 4.6
<i>C. bovis</i>	<i>Bos taurus</i> (domestic cattle)	Small intestine	4.9 x 4.6
<i>C. canis</i>	<i>Canis familiaris</i> (domestic dog)	Small intestine	5.0 x 4.7
<i>C. felis</i>	<i>Felis catis</i> (domestic cat)	Small intestine	5.0 x 4.5
<i>C. galli</i>	<i>Gallus gallus</i> (chicken)	Proventriculus	8.3 x 6.3
<i>C. hominis</i>	<i>Homo sapiens</i> (human)	Small intestine	5.2 x 4.9
<i>C. meleagridis</i>	<i>Meleagris gallopavo</i> (turkey)	Intestine	5.2 x 4.6
<i>C. molnari</i>	<i>Sparus aurata</i> (gilthead seabream)	Stomach	4.7 x 4.5
<i>C. muris</i>	<i>Mus musculus</i> (house mouse)	Stomach	7 x 5
<i>C. parvum</i>	<i>Mus musculus</i> (house mouse), Cattle	Intestine	≥ 4.5
<i>C. scophthalmi</i>	<i>Scophthalmi maximus</i> (turbot fish)	Intestine	4.4 x 3.9
<i>C. serpentis</i>	<i>Elaphe guttata</i> (corn snake)	Intestine	2.8 x 3.6
<i>C. suis</i>	<i>Sus scrofa</i> (domestic pig)	Stomach	4.6 x 4.2
<i>C. varanii</i>	<i>Varanus prasinus</i> (Emerald monitor)	Intestinal	4.8 x 4.7

<i>C. wrairi</i>	<i>Cavia porcellus</i> (guinea pig)	Small intestine	5.4 × 4.6
<i>C. fayeri</i>	<i>Macropus rufus</i> (Kangaroo)	Small intestine	4.9 × 4.3
<i>C. fragile</i>	Blackspined toads	Stomach	6.2 × 5.5
<i>C. macropodum</i>	<i>Macropus giganteus</i> (Kangaroo)	Small intestine	4.9 × 5.4
<i>C. ryanae</i>	Cattle	Small intestine	3.16 × 3.73

A diagrammatic life cycle for *Cryptosporidium* spp. is shown in Figure 2. The primary site of infection with *C. hominis* and *C. parvum* is the small intestine. In some animals such as mice and calves, the ileum above the cecal junction is the favored location for *C. parvum*. *Cryptosporidium* has been found in extraintestinal sites in animals and in some severely immunocompromised humans. Other species such as *C. muris*, *C. andersoni*, and *C. serpentis* favor the gastric mucosa. *C. baileyi*, in chickens, favors the respiratory tree and the cloaca (Fleta *et al.*, 1995).

The sporulated oocyst, the exogenous stage, is excreted from the body of an infected host in the feces. It consists of a tough trilaminar wall that surrounds and maintains the viability of four internal sporozoites under adverse environmental conditions. Those sporozoites ultimately are the source of new infections.

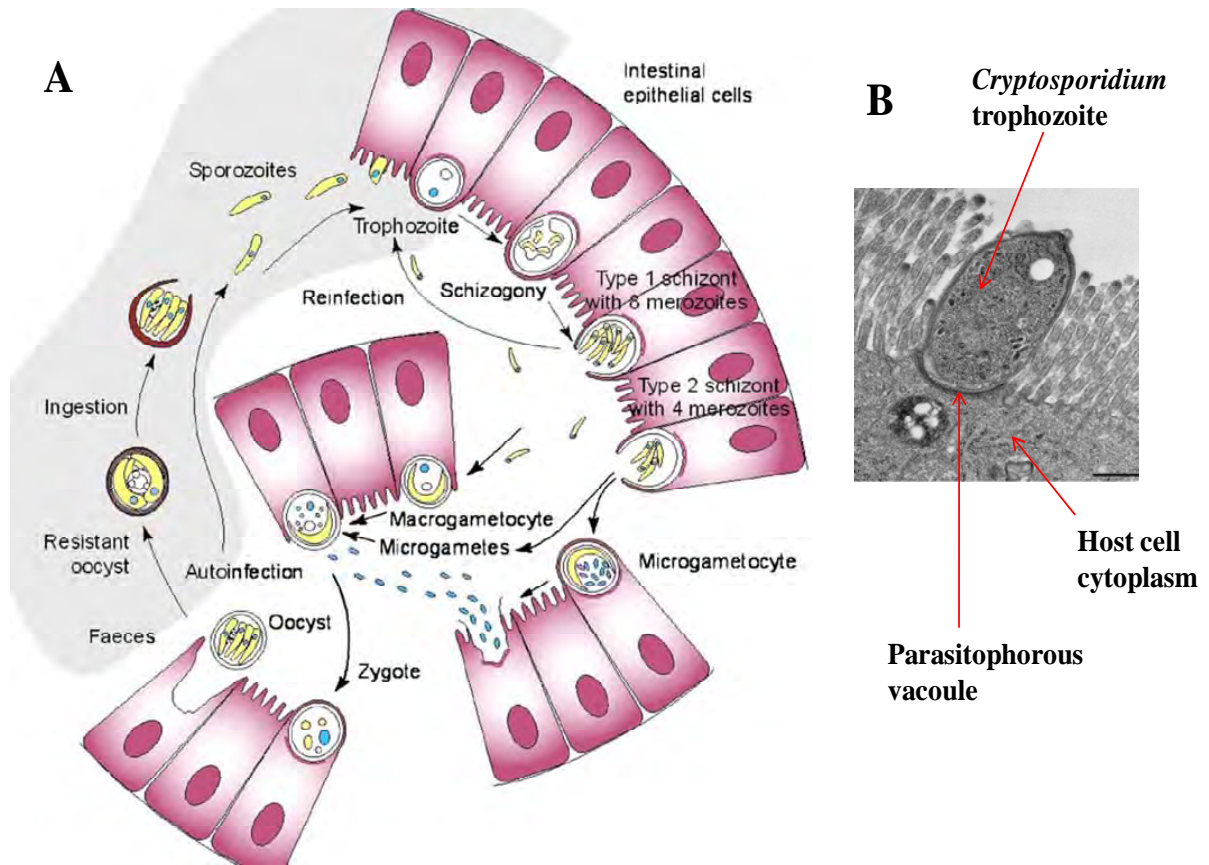


Figure 2: (A) Life cycle of *Cryptosporidium*. The sporozoites invade enterocytes to initiate the asexual cycle within a parasitophorous vacuole that is intracellular but extracytoplasmic. (B) Electron micrographs of gut section from a piglet experimentally infected with *C. parvum* showing ultrastructural view of the parasitophorous vacuole (Tzipori and Ward, 2002; Smith *et al.*, 2007).

1.1. The transmission cycle

Oocysts of *Cryptosporidium* spp. are small (from 3.1 by 3.73 μm to 6.3 by 3 μm) and contain 4 sporozoites (Figure 1). Their ubiquitousness, durability coupled with their small size, resistance to many disinfectants, as well as their ability to evade filtration provide easy transmission of the oocysts in municipal drinking water, with a potential to cause community-wide outbreaks (Robertson and Gjerde, 2007). The WHO's guideline for drinking water (WHO, 2006) classifies *Cryptosporidium* as a pathogen of significant public health importance, contributed in part by the organism's low infective dose (ID), ID₅₀ of 9 – 1042 oocysts (Okhuysen *et al.*, 1999; Smith *et al.*, 2005), and resistance to conventional water treatment such as chlorination.

C. parvum and *C. hominis* are the primary species infecting both immunocompetent and immunocompromised individuals worldwide (Xiao *et al.*, 2001). *C. parvum* infects cattle and other mammals in addition to humans, while *C. hominis* infects primarily humans. *Cryptosporidium* spp produce robust oocysts in the faeces of infected animals and transmitted directly through faecal/oral contact with infected persons or animals or indirectly by ingesting contaminated water or food. Water has been increasingly recognized as an important vehicle for *Cryptosporidium* infection (Smith *et al.*, 2006).

Molecular characterization of *Cryptosporidium* isolates reveals the complexity of cryptosporidiosis epidemiology. Geographic differences in the human incidence of *C. parvum* and *C. hominis* have been identified. The differences exist in the disease burdens attributable to these two species. In the United Kingdom, other parts of the Europe, and New Zealand, *C. parvum* is responsible for slightly more infections than *C. hominis*. In contrast, *C. hominis* is

responsible for far more infections than *C. parvum* in the United States, Australia, Japan, and developing countries where genotyping studies have been conducted (Joachim, 2004; Xiao and Ryan, 2008).

Cryptosporidiosis is caused by the oocyst stage through person-to-person contact with infected patients (family members, health care workers, users of communal swimming pools, travelers), animal-to-human contact (zoonotic) (veterinarians, farmers), or via indirect transmission through the environment (particularly by water) (Chen, 2002; Xiao and Ryan, 2008) (Figure 3).

The change in distribution of cases of cryptosporidiosis over time differs according to seasons. The seasonal increases in cases are linked to increased rainfall (Naumova *et al.*, 2005), and it is assumed that this reflects contamination of sources of drinking water by human and animal waste. There may also be thermal and daylight inactivation of oocysts in animal feces in the summer and freezing in the winter (Li *et al.*, 2005). *Cryptosporidium* infections were more common in the late summer in Canada (Laupland and Church, 2005). In the United States the peak incidence is in late summer (July–August) (Hlavsa *et al.*, 2005). The parasite survives winter season in the soil. It is resistant to freezing temperatures. In New Zealand *C. parvum* predominated in the spring and *C. hominis* in the autumn (Learmonth *et al.*, 2003). In Leipzig cases are more common in the late summer (Krause *et al.*, 1995).

A seasonal distribution of cryptosporidiosis in Gambian children was related to times of heavy rainfall and high humidity (Adegbola *et al.*, 1994). In slum children in Brazil the rate of faecal contamination with *Cryptosporidium* was higher in the rainy season than in the dry season (Newman *et al.*, 1993). In South Africa infections were more common in late summer and during

periods of high rainfall (Fripp *et al.*, 1991). Infections in Guinea- Bissau are at the beginning of the rainy season (Perch *et al.*, 2001). In Korea infection was more common in spring than at other times. In Kuwait in children 75% of all cases over a 2-year period occurred between January and March (Iqbal *et al.*, 2001).

1.1.1. Person-to-Person transmission

Transmission within families is common (Ribeiro and Palmer, 1986). In the United Kingdom cases occurring in family clusters are more likely to involve *C. hominis* than *C. parvum*. *Cryptosporidium* can be easily transmitted in nurseries, day care settings, and schools (Teresa, 2006). Changing diapers was a significant risk factor for *C. hominis* infection in the United Kingdom (Hunter, 2004b). HIV-positive individuals can become more severely ill with *Cryptosporidium* infection (Kim, 1998). Transmission of *Cryptosporidium* within the hospital environment has been reported (el Sibaei, 2003) and can be a problem in units that deal with immunocompromised patients (Martino, 1988).

1.1.2. Zoonotic transmission

Cryptosporidium can be transmitted from animals to humans through direct contact. This has occurred with veterinary workers (Preiser, 2003) and other people exposed to animals (Stantic-Pavlinic, 2003a), particularly farm workers (Mahdi and Ali, 2002), and in animal nursery fair (Ashbolt, 2003). Infections acquired from agricultural animals are predominantly *C. parvum*. A reduction in visits to the countryside due to an outbreak of foot-and-mouth disease was linked to a reduction in cases of cryptosporidiosis in the UK (Hunter, 2003).

1.1.3. Water-borne and food-borne cryptosporidiosis

In the last thirty years, there has been increasing epidemiological evidence linking *Cryptosporidium* with frequent outbreaks of waterborne disease (Fayer, 2004) (Figure 3). Over 40 waterborne outbreak of cryptosporidiosis have been reported in many countries around the world, with the majority being in the USA and in the UK (Slifko *et al.*, 2000). Most outbreaks have been implicated with contaminated surface water, but a significant number were also associated with unprotected groundwater. *Cryptosporidium* is also one of the most commonly recognised causes of recreational waterborne disease. Most outbreaks result from faecal accident in swimming pools. Faecal contamination coupled with oocyst resistance to chlorine, low infectious dose and high bather densities facilitate transmission (Fayer *et al.*, 2000).

Molecular studies have revealed the environment occurrence of *Cryptosporidium* species and genotypes. The most commonly found genotypes/species in contaminated water are *C. parvum* and *C. hominis*. However, many other species/genotypes that are not human pathogens are present in contaminated water. Mixed isolates were found in storm water samples, and sequence analysis indicated that most oocysts originated from wildlife (Xiao *et al.*, 2000). In waterborne cryptosporidiosis outbreaks where stools from infected individuals were analysed, *C. hominis* was responsible for more outbreaks than *C. parvum*, even in countries where the latter is the predominant human parasite, like in the UK (McLauchlin *et al.*, 2000; Pedraza-Diaz *et al.*, 2001). Mixed *C. hominis* and *C. parvum* infections were also detected, indicative of multiple contaminating sources or human sewage.

The role of food (Figure 3) in the epidemiology of cryptosporidiosis has been more difficult to investigate. Methods for detecting protozoan parasites on food matrices are modifications of those used for water, being based upon the examination of washings from food surfaces, and, therefore, may not be optimal as recoveries can be low and variable (1% to 85%) (Nichols and Smith, 2002). Some studies on the occurrence of oocysts on foodstuffs conducted in underdeveloped countries (Ortega *et al.*, 1997), showed that numerous raw vegetables such as basil, cabbage, celery, cilantro, green onions, leeks, lettuce, and parsley were found to be contaminated. Similar survey has demonstrated that contamination of food is not restricted to underdeveloped countries (Armon *et al.*, 2002).

In spite of these findings, reports of food-borne cryptosporidiosis are scarce, probably because of the similarity of the symptoms of cryptosporidiosis with other diarrhoeal diseases. Cryptosporidiosis has been attributed to ingestion of contaminated apple juice, chicken salad, milk, and food prepared by an infected food handler (Millar *et al.*, 2002). For the outbreaks where the species were identified, *C. parvum* was associated with apple juice outbreak, while *C. hominis* was associated with green onions, fruit or vegetables contaminated by food handlers (Quiroz *et al.*, 2000).

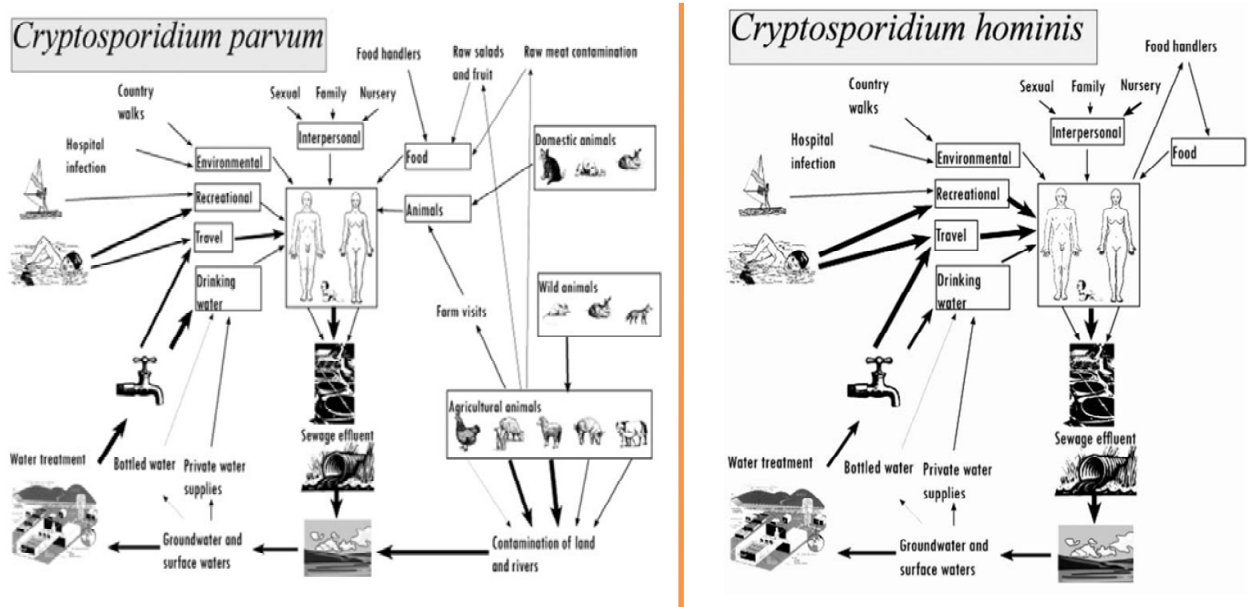


Figure 3: Transmission pathways for *C. parvum* and *C. hominis* (Rayan and Xiao, 2008).

1.2. Epidemiology of *Cryptosporidium* infection in the immunocompetent host

The application of PCR assays to identify *Cryptosporidium* species from stool samples has shown that *C. hominis* and *C. parvum* are the major causes of human cryptosporidiosis (Cacciò, 2005). Interestingly, the prevalence of these species varies in different regions of the world. Indeed, *C. hominis* is by far more prevalent in North and South America, Australia and Africa, while *C. parvum* causes more human infections in Europe, especially in the UK. Geographic variation occurs also within a country (McLauchlin *et al.*, 2000) and molecular epidemiological studies indicate that the proportion of *C. parvum* infections in humans is much higher in rural than in urban areas (Learmonth *et al.*, 2004).

Direct transmission of *C. parvum* from animals to humans is well documented; for example the outbreak of cryptosporidiosis among veterinary students in the US clearly demonstrates the potential for direct transmission (Current *et al.*, 1983). However, it is important to note that finding *C. parvum* in humans does not represent a conclusive evidence for zoonotic transmission. Indeed, subgenotyping analysis of human *C. parvum* isolates has revealed genetic variants that are very rarely found in other animals, suggesting that many *C. parvum* infections in particular areas may have originated from humans themselves (Xiao *et al.*, 2004).

Differences in the temporal pattern of these species have also been observed. In the UK, a marked bimodal seasonal pattern of disease has been described: one peak during spring and the second during late summer /early autumn (McLauchlin *et al.*, 2000). The spring peak is almost exclusively due to *C. parvum*, while both *C. parvum* and *C. hominis* occur in the late summer/early autumn peak. In the US, the peak onset of cryptosporidiosis occurred annually during early summer through early fall, and, as it coincides with the summer increased recreational water use, particularly by susceptible hosts like young children (Hlavsa *et al.*, 2005). Recently, risk factors for sporadic cryptosporidiosis have been investigated. A recent case-control study in the UK identified different risk factors for *C. hominis* and *C. parvum*: for the former, overseas travel and changing diapers of children less than 5 years of age were significant risk factors for infection, whereas for *C. parvum*, contact with animals was associated with infection (Hunter *et al.*, 2004a; Hunter *et al.*, 2004b).

It is now clear that infection with species other than *C. hominis* and *C. parvum* are not restricted to immunocompromised persons. Immunocompetent individuals have also been infected with other species *C. meleagridis*, *C. canis*, *C. felis*, *C. suis*, and *C. muris*, as well as with the cervine

and monkey genotypes of *C. parvum*. In particular, *C. meleagridis*, a parasite originally described in turkeys (Xiao *et al.*, 2004), is now recognized as an emerging human pathogen, being responsible for 1% of all infections in the UK (Pedraza-Diaz *et al.*, 2001), and about 10% in Peru, where its prevalence is as high as that of *C. parvum* (Cama *et al.*, 2003). The implication of these results is that farm animals and some wildlife are to be considered as important, but less frequent sources of infection for humans, especially, but not exclusively, for immunocompromised individuals.

1.3. Epidemiology of *Cryptosporidium* infection in the immunocompromised host

It is well known that cryptosporidiosis causes significant morbidity and mortality in immunocompromised individuals, in particular in those with HIV infection. There is strong evidence that the risk of faecal carriage, severity of illness and development of unusual complications of cryptosporidiosis are related to the CD4+ count (Pozio *et al.*, 1997). Indeed, patients with CD4 counts of less than 50 are at greatest risk for both the severity of the disease and prolonged carriage. With the introduction of the Highly Active Antiretroviral Therapy (HAART), the prevalence of opportunistic infections in HIV/AIDS infected individuals has decreased dramatically (Pozio and Morales, 2005). It appears that the protease inhibitors included in HAART not only restore cell-mediated immunity, but also have a direct inhibitory effect on the proteases of opportunistic protozoan, including *Cryptosporidium* spp (Pozio and Morales, 2005). However, cryptosporidiosis remains a major problem for HIV/AIDS infected patients not

responding to HAART, for those without access to HAART, and for severely malnourished children (Pozio *et al.*, 1997; Pozio and Morales, 2005).

From a review of about 600 HIV positive cases that investigated possible infection with *C. Parvum*, *C. hominis*, *C. meleagridis*, *C. canis*, *C. felis*, *C. suis*, and *C. muris* using molecular tools showed that *C. hominis* is the most prevalent species (57%), followed by *C. parvum* (23%), *C. meleagridis* (11%) and *C. felis* (6%). The distribution of *Cryptosporidium* species appears similar in both immunocompetent and immunocompromised individuals (Xiao *et al.*, 2004).

In addition HIV/AIDS infected patients, like patients with primary immunodeficiency diseases or with malignant neoplasms (Hunter and Nichols, 2002), and patients with solid organ transplant are also at risk of infection (Pozio *et al.*, 2004). In these patients, single and mixed species of *Cryptosporidium* have been identified (Pozio *et al.*, 2004).

1.4. Clinical features and pathogenesis

The natural history of the illness differs in immunocompetent and immunocompromised individuals. In the immunocompetent individual, *Cryptosporidium* commonly causes acute self-limiting gastroenteritis. Oocyst excretion occurs from less than 3 days to up to 30 days, coinciding with symptoms. In immunocompromised patients, symptoms include frequent episodes of watery diarrhoea, cramping, abdominal pain, weight loss, weakness, malaise, anorexia and low grade fever (Smith and Corcoran, 2004).

The molecular basis for pathogenicity is not understood and no specific virulence factors have been unequivocally shown to cause direct or indirect damage to host tissues. Diarrhoea in cryptosporidiosis could result from a combination of the following factors: 1) Loss of absorptive epithelium due to apoptosis and villus atrophy results in malabsorption, and release of inflammatory cell mediators stimulate electrolyte secretion and diarrhoea. 2) Increased intestinal secretion which may be partly mediated by enterotoxin-like activity produced by *C. parvum* as a result of endogenous secretory mediators such as prostaglandins which alter NaCl transport primarily by stimulating the enteric nervous system (Sears and Guerrant, 1994; Fayer, 2004). 3) Cytokines induced by infection may directly involve in the pathogenesis of diarrhoea as reviewed by Lean *et al.* (2002). Tumor necrosis factor- α (TNF- α) stimulates chloride secretion in the intestine via a prostaglandin mediator (Clark and Sears, 1996). Interferon (IFN- γ), besides conferring resistance to the parasite, has a well documented role in secretory diarrhoea. Exogenous IFN- γ inhibits both of the Na/H ion exchanger (Rocha *et al.*, 2001) and Na/K exchanger (Sugi *et al.*, 2001) resulting in increase in intracellular Na concentration which leads to an increase in cell volume, which in turn has been implicated in the decreased expression of a number of transport and barrier proteins (Sugi *et al.*, 2001). These events ultimately lead to a leaky and dysfunctional epithelium, manifested by diarrhoea.

1.5. Diagnosis

Laboratory techniques that are applied for identification of *Cryptosporidium* have been reviewed by Petry (2000). The need to identify the organism in diarrhoeal patients is useful to avoid misuse of antibiotics, to reduce the spread of the disease in a community as well as to follow the epidemiology of the disease in an area. Detection of *Cryptosporidium* oocysts has been

performed using: histological sections of small intestine (Meisel *et al.*, 1976); staining techniques to identify the oocysts in the feces (Petry, 2000); oocyst antigen detection via immunofluorescence, enzyme linked and agglutination immuno-assays (Petry, 2000); polymerase chain reaction (PCR) amplification of *Cryptosporidium* specific DNA targets (Sulaiman *et al.*, 1999). Serological diagnosis of *Cryptosporidium* specific antibodies has also been applied to detect wide range of time span post infection and also can be used as a marker for epidemiological surveys (Petry, 2000).

1.6. Prevention and control of cryptosporidiosis

All *Cryptosporidium* infections are contracted by the ingestion of oocysts. Therefore, effective control measures must aim to reduce or prevent oocyst transmission. Most oocysts are fully sporulated and infective when excreted from infected hosts and they are very resistant to a range of environmental conditions. *Cryptosporidium* oocysts stored in aqueous solutions have remained viable for up to 3 months at ambient temperature (15-20°C) and for up to one year when refrigerated (4-6°C).

Many recommendations have been made for the prevention and control of infections in specific locations; such at hospitals, laboratories, day care centers, households, zoos and farms. These recommendations have basically involved managerial practices designed to minimize host contact with sources of infection and the use of different disinfection procedures to destroy infective oocysts. Most precautionary measures are dictated by common sense and a basic understanding of the modes of transmissions or infections. High standards of hygiene, also applicable to other enteropathogens, should be observed. When practicable, infected individuals

should be identified and isolated to confine infections to particular areas which can then be regularly cleaned and disinfected (Robertson *et al.*, 1992). Self-limiting disease patients shed the oocysts after symptoms have stopped.

Susceptible individuals should avoid contact with contaminated areas or be removed to safe locations. Care should be exercised in the handling and disposal of biohazardous waste and suspect contaminated water should be boiled prior to consumption or use. Like many other coccidian oocysts, those of *Cryptosporidium* have proven remarkably resistant to chemical disinfection. Laboratory studies have shown that many commercial disinfectants such as aldehyde, ammonia, alcohol, chlorine or alkaline compounds are ineffective when used according to the manufacturers' instructions. In some cases, higher concentrations and longer exposure periods have been found to kill oocysts but the prolonged use of concentrated disinfectants in confined areas is inadvisable or impractical in many situations. Over 35 disinfectants have been tested (Fayer *et al.*, 1990) but only 5 have been found to be effective following relatively short exposure periods; 50% ammonia, 3% hydrogen peroxide, 10% formalin, Exspor and Oo-cide. Steam heat sterilization and fumigation with formaldehyde or ammonia gas have also been recommended as appropriate forms of decontamination (Campbell *et al.*, 1982).

Recent studies have indicated that most conventional methods of water treatment do not effectively remove or kill all *Cryptosporidium* oocysts from contaminated water. Laboratory simulation of different water treatment processes has shown that some oocysts can survive treatment with chlorine, monochloramine, chlorine dioxide, ozone, lime, polyelectrolytes, alum and ferric sulphate although treatment with higher than recommended levels of chlorine dioxide and ozone was found to be effective (Robertson *et al.*, 1992). The disinfection of water by

ultraviolet radiation was only effective following 150 min exposure or longer (Lorenzo-Lorenzo, *et al.*, 1993). Quantitative studies on the treatment of wastewater and sewage have shown that 74-84% of oocysts are removed by activated-sludge systems and 87-99% by subsequent sand filtration (Villacorta-Martinez *et al.*, 1992). Sand filters used to treat drinking water have been found to remove 91% of oocysts (Rose *et al.*, 1986). However, reliable and economical water treatment and disinfection procedures have yet to be established to prevent the transmission of *Cryptosporidium* oocysts.

Reducing oocyst contamination of public water sources are thought to offer the best protection from waterborne disease (Smith and Corcoran, 2004). Removal of the oocysts from drinking water by either boiling or by filtering the water through a filter with a pore size of <1 µm is also recommended for AIDS patients (Leav *et al.*, 2003). To date, there are no approved prophylactic means of preventing cryptosporidiosis formulated for either human or animal use.

1.7. Treatment of cryptosporidiosis

At present, there is no effective treatment available for cryptosporidiosis in humans or animals. Although many infections in immunocompetent hosts may be asymptomatic or only associated with mild clinical signs which resolve within 1-2 weeks of infection, some cases may be severe enough to warrant medical or veterinary intervention. Supportive care with oral or intravenous fluid and electrolyte replacement has been found to be beneficial primarily in alleviating the dehydration accompanying acute diarrhoea while awaiting spontaneous recovery. Parenteral nutrition may sometimes be deemed necessary to rest the gut and anti-diarrhoeal compounds may assist in controlling fluid loss. Nonetheless, both acute and chronic infections have been

associated with severe clinical disease and mortalities in various hosts, especially neonatal domestic animals and immunocompromised human patients. Thus, with the recognition that some form of effective treatment is urgently required, more than 120 different compounds have been tested against *Cryptosporidium* without marked success (Flanigan and Soave, 1993).

Most attempts at conventional chemotherapy with antiparasitic, antifungal, antibiotic or antiviral agents have been unsuccessful. Some anecdotal success has been reported in the treatment of immunocompromised patients with co-trimoxazole, diloxanide furoate, furazolidone, letrazuril, quinine plus clindamycin, paromomycin, spiramycin or interleukin-2 (Freid, 2005). These reports tentatively associated treatment with the clinical resolution or eradication of infections but most compounds have subsequently been found to be ineffective (Flanigan and Soave, 1993). Studies indicated that the macrolide antibiotic spiramycin may be effective but double-blind, placebo-controlled, clinical trials have shown that oral or intravenous treatment had little or no effect on infections in infants and in AIDS patients (Wittenberg *et al.*, 1989; Flanigan and Soave, 1993). High-dose spiramycin treatment has also been associated with acute intestinal injury in several patients (Flanigan and Soave, 1993). Some symptomatic improvement has also been reported in immunocompromised patients following treatment with various antidiarrhoeal agents, including dipheoxylate, morphine sulphate, procaine hydrochloride, octreotide and somatostatin. Treatment substantially reduced stool frequency and volume, although oocysts continued to be shed. Similar clinical improvement was noted in patients treated with the antibiotic erythromycin but treatment was discontinued due to adverse side effects.

A range of drugs have also been tested in experimentally infected animals, most being administered prophylactically. Although none have completely prevented infections, fewer and

lighter infections (compared to untreated controls) were detected in neonatal mice treated with alborixin, amprolium, arprinocid, cyclosporin A, dinitolmide, maduramycin, pentamidine, salinomycin or sulfaquinoxaline, in neonatal hamsters treated with arprinocid, in immunosuppressed mice treated with azithromycin and lasalocid, in immunosuppressed rats treated with azithromycin, clarithromycin, erythromycin, oleandomycin, lasalocid, mepacrine or sulfadimethoxine, in lambs treated with halofuginone, and in calves treated with lasalocid or halofuginone. Further studies are required to determine the therapeutic activities of these drugs at dose levels which do not produce any toxic side effects. The search for efficacious therapies for human and animal cryptosporidiosis has been, for the most part, unrewarding (Arrowood *et al.*, 1991; Ritchie and Becker, 1994).

Encouraging results have been obtained for the control of cryptosporidiosis by various forms of immunological intervention which act to restore or enhance host immune function or provide passive immunity (Stine *et al.*, 1985). Treatment with the antiretroviral agent azidothymidine (AZT or zidovudine) has also been associated with the resolution of opportunistic infections in several AIDS patients presumably through augmentation of their immune function has resulted in a decrease in chronic cryptosporidiosis in immunocompromised patients (Cacciò and Pozio, 2006). The role of HAART in immune reconstitution, in treatment and secondary prophylaxis has been shown when cryptosporidiosis is established (Chen *et al.*, 2002). With HAART therapy, the number of CD4⁺ T cells in the gastrointestinal mucosa, increases faster and to a higher level than in blood (Schmidt *et al.*, 2001). Hommer *et al.*, (2003) had reported that protease inhibitors included in HAART inhibited *C. parvum* development *in vitro*; and its inhibitory effect is significant when the protease inhibitors were used in combination with the aminoglycoside paromomycin. In non-AIDS related immunosuppression, such as in a post-renal transplantation

(Abdo *et al.*, 2003), reduction in immunosuppression was associated with oocyst clearance and resolution of the disease.

Passive immunotherapy using oral bovine serum concentrate improved symptoms and reduced oocyst shedding in calves with experimental cryptosporidiosis (Hunt *et al.*, 2002), but colostrums from cows hyperimmunized with *C. parvum* oocysts achieved limited success in both human and non-human hosts (Gomez-Morales and Pozio, 2002). A monoclonal antibody responsible for the circum-sporozoite precipitate-like reaction reduced, but did not eliminate, persistent murine *C. parvum* infection (Riggs, 2002).

Cryptosporidium genome sequencing has been completed; and therefore, further database mining is expected to contribute to the discovery of additional protective antigens and virulence factors (Abrahamsen *et al.*, 2004).

1.8. *Cryptosporidium* infection in Ethiopia

In 1996 the Federal Ministry of Health of Ethiopia reported that more than half a million outpatients visited hospital/clinic due to intestinal parasitic infections. However, this might be an underestimate, as most of the health institutions lack appropriate diagnostic methods to detect parasites with small detection limits. In addition, some of the diagnostic methods for specific intestinal parasites, especially for the newly emerging opportunistic intestinal parasites, were not available to most health institutions.

Among the common intestinal protozoan parasites *Giardia* and *Amoeba* are widely distributed in Ethiopia (McConnel and Armstrong, 1976). *Cryptosporidium* is now becoming a common opportunistic intestinal parasite in Ethiopia even though it is not diagnosed routinely. Reports from different parts of the country showed different prevalence rates of cryptosporidiosis. Recently a study conducted in Lege Dini, rural area in Dire-Dawa, showed the prevalence of cryptosporidiosis to be 12.2 % (Ayalew *et al.*, 2008). Another report indicated that the prevalence of *Cryptosporidium* among diarrhoeal patients referred to Ethiopian Health and Nutrition Research Institute (EHNRI) was 20.6 % (Endeshaw *et al.*, 2004). The prevalence of *Cryptosporidium* infection alone in children with diarrhoea ranged from 3.3 % in Jimma, 5.6 % in Addis Ababa, and 9 % in North-western Ethiopia (Mersha and Tiruneh, 1992; Assefa *et al.*, 1996; Gebru and Girma, 2000). Another study with special emphasis on opportunistic parasitic infections among paediatric diarrhoeal patients in visiting hospitals in Addis Ababa, showed that the rate of *Cryptosporidium* spp. infection among these patients to be 8.1% (Adamu *et al.*, 2006). The prevalence has been reported to range from 8.1% in children younger than five years to 25.9% in adult HIV/AIDS patients (Fisseha *et al.*, 1999; Adamu *et al.*, 2006). This is probably

because of the low standard of hygiene (Kloos and Yohanes, 1993), contamination sources of drinking water supplies (Fikrie *et al.*, 2008), and difference with immune status of study subjects, close contact with cattle, and living in overcrowded situations with many family members coupled with poor personal hygiene (Ayalew *et al.*, 2008).

1.9. Statement of the problem

In Ethiopia cattle and other domestic animals have close contact with people, to the extent of sharing the same residence. Furthermore, it is a routine practice that cattle manure is used as a fertilizer and the dried dung, for fuel. Thus, the day-to-day contact people have with domestic animals and their excreta would raise the possibility of acquiring infections with zoonotic pathogens such as members of the genus *Cryptosporidium*. Molecular analysis of *Cryptosporidium* spp. isolates has proved to be useful in determining the sources of infection (McLauchlin *et al.*, 2000). This allows determination of the genotypes and subgenotypes of *Cryptosporidium* spp. and hence enables one to distinguish human from nonhuman isolates. No work exists on the molecular epidemiology of cryptosporidiosis in Ethiopia, as a result of which the source and transmission of the disease remains undefined.

This study focuses on molecular characterization of *Cryptosporidium* spp. isolates from human and domestic animal resources with the view to determining the parasite species and the source of human cryptosporidiosis infection, that is, anthroponotic and/or zoonotic, in Ethiopia. In addition the prevalence of intestinal parasites among the study subjects will be determined.

Hypothesis: Cryptosporidiosis in Ethiopia is mainly a zoonosis.

2. Objective of the study

2.1. General Objective

The general objective of this study is to investigate the molecular epidemiology of cryptosporidiosis, and correlate it with genotypic and subgenotypic diversity of *Cryptosporidium* isolates with epidemiological features, and estimate the extent of anthroponotic and zoonotic transmission of *Cryptosporidium* spp. in Ethiopia.

2.2. Specific Objectives

The specific objectives of this study are as follows:

1. To examine the zoonotic and anthroponotic transmission potential of *Cryptosporidium* spp. infection and to characterize genotypes and subgenotypes *Cryptosporidium* spp. in Ethiopia using PCR restriction fragment length polymorphism (RFLP) of COWP and the SSU-rRNA genes and subgenotyping by DNA sequencing analysis of GP60 kD gene.
2. To determine the prevalence of cryptosporidiosis and genotypes and subgenotypes from humans and animals.
3. To determine the association between CD4⁺ T cell count and *Cryptosporidium* spp. infection in HIV/AIDS patients.

3. Materials and methods

3.1. The study areas

The study was carried out at different hospitals and health centers of Ethiopia including: Federal Police Hospital, Addis Ababa University Specialized Hospital (Tikur Anbessa Hospital), Addis Ababa; Bishoftu Hospital, Bishoftu; Adama Hospital, Adama; Awash Sebat Health center, Afar; Dil Chora Hospital, Dire-Dawa; Hiwot-Fana Hospital, Harar; Sebeta Health Center, Sebeta; Debre Markos Hospital, Debre Markos; Felege-Hiwot Hospital, Bahir-Dar (Figure 4), during September 2007 to August 2008. The study sites were selected by considering location, cost, time and other statistical elements.

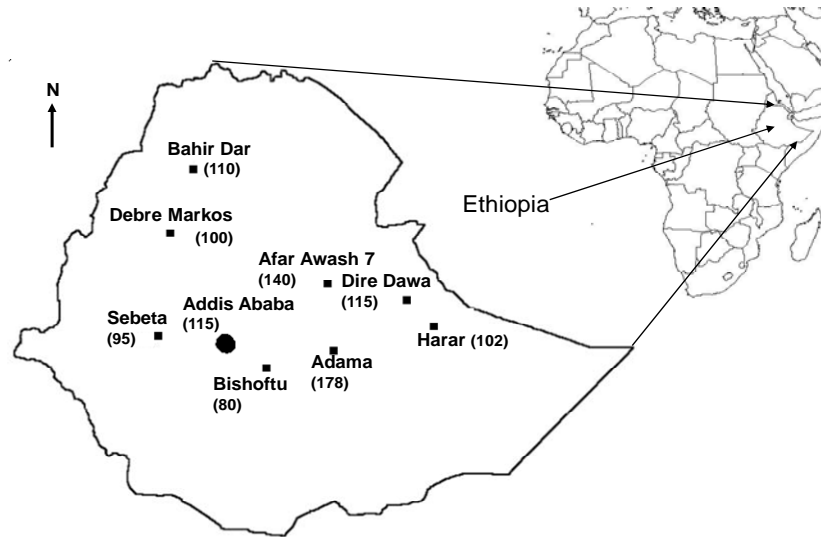


Figure 4: Study sites in Ethiopia (Numbers in parenthesis are number of study patients in each study site in Ethiopia (September 2007-August 2008).

3.2. The study population

The study subjects were humans (n=1034) (Table 3) with diarrhoea. The inclusion criterion diarrhoea, defined as passage of three or more loose or liquid stools per day, or more frequently than is normal for the individual (www.who.int/topics/diarrhoea/en/). As there were many diarrhoeal complaints in all the study sites, every third patient was sampled and if the third patient was not consenting the next patient was included in the study.

For humans, the study instrument used was a structured questionnaire. Socio-demographic and clinical information including diarrhoea and medication histories were obtained from the study subjects using survey questionnaire and their CD4⁺ T cell count was taken from their medical records at the time of sample collection. Selection criterion for cattle (n=350), had direct physical and indirect contact with humans were included in this study.

3.3. Stool collection and processing

A single fresh stool samples were collected with leak proof and tightly cupped and labeled sterile stool cup from consenting humans, and from cattle. The questionnaires concerning the prevalence study of all human study participants were filled by the investigator during sample collection. A portion of the stool was preserved with 2.5% potassium dichromate in a proportion of 1:1 for further molecular diagnosis, and the third portion was preserved with 10% formalin in a proportion of 1g of stool in 3ml of formalin. The remaining part was processed using the following methods (Ortega and Adam, 1997).

3.3.1. Direct Microscopy (Wet mount)

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

3.3.2. Formalin-Ether concentration

Using an applicator stick, about ~5g of preserved stool sample was placed in a clean 15 ml conical centrifuge tube containing 7 ml formalin. The sample was mixed thoroughly with the applicator stick. The resulting suspension was filtered through a sieve (cotton gauze) into a beaker and the filtrate was poured back into the same tube. The debris trapped on the sieve was discarded. After adding 3 ml of diethyl ether to the mixture and shaking, the content was centrifuged at 500 x 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.

Stool samples were screened for co-infection with other intestinal parasites i.e. *Trichuris trichiura*, *Entamoeba histolytica/ dispar*, *Giardia lamblia*, and *Blastocystis hominis* by microscopy. The remaining stool sample was preserved in 1:1 ratio with 2.5% potassium dichromate (w/v), transported from each sample site and stored at 4°C at Biomedical Sciences laboratory at Addis Ababa University. From a total of 1034 human study participants,

Cryptosporidium positive stool samples (n=79) and cattle (n=8) determined by modified Ziehl-Neelson staining method were shipped to the Institute of Medical Microbiology & Hygiene, University Medical Centre of the Johannes Gutenberg-University of Mainz, Germany for re-confirmation and molecular analysis.

3.3.3. Modified Ziehl-Neelson method

A modified Ziehl-Neelson method for detection of *Cryptosporidium* spp. oocysts was used. Fresh faecal samples were collected and thin smears were prepared, air-dried, fixed with methanol for 5 minutes and stained by modified Ziehl-Neelson technique (Henriksen & Pohlenz 1981). In this technique, the slides were stained with carbol-fuchsin (0.34% fuchsin 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 analysed using 1000x magnification. For estimation of the parasite load, 100 high power fields were screened.

3.4. *Cryptosporidium* oocyst detection by immunofluorescence test (IFT)

The faecal sample in potassium dichromate solution was homogenized using plastic Pasteur pipette and a vortexer in a laminar flow. After centrifugation for 10 minutes at 16,000 x g, in 4°C table top centrifuge, the supernatant was discarded (and disinfected); the pellet was mixed with an equal volume of 1xPBS and stored at 4°C or processed immediately. Faecal suspensions were homogenized with pipette tips, further diluted 1:5 in PBS and 5 µl aliquots were placed in duplicates on poly L-lysine coated slides (Adcell, Roth, Karlsruhe, Germany) to facilitate

adherence of the oocysts to the glass slides. After air-drying (for about 1 hr in laminar flow) and methanol fixation (by placing in a chemical fume hood for 10 min), slides were incubated for 1 h with a 1:500 dilution of anti-*C. parvum*-oocyst rabbit serum (rb115) (primary antibody) at room temperature, which has been generated using standard immunization protocols (Jakobi and Petry, 2006). During antibody incubation, drying of the slides was prevented by placing the slides in a rack containing moistened tissue paper and covered. After two washes, each for 5 min, in PBS with shaking, the slides were incubated with a 1:250 dilution (with PBS having 1 % BSA) of the secondary antibody (goat-anti-rabbit IgG-AlexaFluor 488; Molecular Probes, Invitrogen) for 1 hour in a wet and dark rack. This was followed by two washes as above, but in a dark condition to prevent bleaching of the labelling dye.

The slides were then covered with drops of glycerol, and with 24x60 mm cover slips. Air bubbles formed between the cover slide and the slide were removed using forceps. The slides were placed in dark till the cover slip is well-adhered to the slide and the edges of the slides were sealed with nail varnish to prevent drying out of the glycerol. The slides were dried in dark and examined under differential interference contrast (DIC) microscopy for oocyst detection using the oil immersion objective.

3.5. Molecular methods

3.5.1. DNA extraction

Cryptosporidium oocysts from stools were purified over saturated sodium chloride floatation procedure (Weber *et al.*, 1992), with modifications by increasing up to 5 ml of the stool sample preserved in 2.5% potassium dichromate. The sample was homogenized by vortexing and layered-over 6 ml of saturated sodium chloride solution in a 15 ml centrifuge tube using a disposable plastic bulb pipette and centrifuged at 500 x g for 10 min. This centrifugation step resulted in the following three layers: (from top to bottom) deionized water containing debris and *Cryptosporidium* oocysts, which were just above the surface of the sodium chloride layer; saturated sodium chloride; and a pellet containing the faecal debris. The uppermost 3.5 to 4 ml of the top layer was removed by using a disposable plastic pipette and discarded. The remainder of the top layer containing the oocyst and approximately 0.5 ml of the top portion of the sodium chloride layer were removed by plastic pipette and put in to a new 15ml centrifuge tube. The remaining interphase was discarded together with the centrifuge tube. The uppermost top layer (potassium dichromate with some faecal material) was removed using a disposable plastic pipette and discarded. The remaining interphase (containing *Cryptosporidium* oocysts) was removed with the pipette and washed twice in 10 ml of demineralized water by centrifugation at 500 x g for 10 min. Deionized water was taken-off and the sediment was ready for DNA extraction by DNA extraction kit.

Total DNA was extracted using a QIAmp DNA Stool Kit (QIAGEN, Hilden, Germany). Briefly: The total pellet resulted from saturated sodium chloride floatation method was dissolved in 2 ml

Buffer ASL from the QIAGEN kit in a 15ml centrifuge tube on ice box. The suspension was vortexed continuously for 1 minute or until the stool sample was thoroughly homogenized. One thousand and six hundred μ l of the stool lysate was pipeted into a 2 ml microcentrifuge tube (Roth, Karlsruhe, Germany) at room temperature (15–25°C).

The ends of the pipet tips were cut off to make pipetting viscous samples easier. The suspension was heated for 5 min at 80°C. The sample was vortexed for 15 seconds and centrifuged at full speed for 1 minute to pellet stool particles. One thousand and two hundred μ l of the supernatant was pipeted into a new 2 ml microcentrifuge tube and the pellet was disinfected and discarded. One InhibitEX Tablet for adsorbing PCR inhibitors was added to each sample and vortexed immediately and continuously for 1 minute or until the tablet is completely suspended. The suspension was incubated for 1 minute at room temperature to allow inhibitors adsorbed to the InhibitEX matrix. The sample sample was centrifuged at full speed for 3 minute to pellet stool particles and inhibitors bounded to InhibitEX matrix. All of the supernatant was pipeted into a new 1.5 ml microcentrifuge tube and the pellet discarded.

The sample was centrifuged at full speed for 3 minute. Fifteen μ l proteinase K for digestion of proteins was pipeted into a new 1.5 ml microcentrifuge tube. Two hundred μ l supernatant from the previous step was pipeted into the 1.5 ml microcentrifuge tube containing proteinase K. Two hundred μ l Buffer AL was added and vortexed for 15 seconds. The sample was incubated at 70°C for 10 minutes. The sample was centrifuged briefly to remove drops from the inside of the tube lid. Two hundred μ l of ethanol (99.9%) was added to the lysate, and mixed by vortexing. The lysate was centrifuged briefly to remove drops from the inside of the tube lid. The lid of a new QIAamp spin column was labeled and placed in a 2 ml collection tube. The complete lysate was

carefully applied from the previous step to the QIAamp spin column without moistening the rim. The cap was closed and centrifuged at full speed for 1 min. The QIAamp spin column was placed in a new 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 500 µl Buffer AW1 was added. The cap was closed and centrifuged at full speed for 1 minute. The QIAamp spin column was placed in a new 2 ml collection tube, and the collection tube containing the filtrate was discarded.

The QIAamp spin column was carefully opened and 500 µl Buffer AW2 was added. The cap was closed and centrifuged at full speed (13000rpm) for 3 minutes. The collection tube containing the filtrate was discarded. This step was repeated to get better DNA concentration and centrifuged at full speed for 1 minute. The QIAamp spin column was transferred into a new, labeled 1.5 ml microcentrifuge tube. The QIAamp spin column was carefully opened and 100 µl Buffer AE was pipeted directly onto the QIAamp membrane. The cap was closed and incubated for 1 minute at room temperature, then centrifuged at full speed for 1 minute to elute DNA. The elute was kept at -20°C until further use.

3.5.2. *Cryptosporidium* genotyping

3.5.2.1. Polymerase Chain Reaction (PCR)

Initially, all specimens were genotyped by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the SSU-rRNA and *Cryptosporidium* oocyst wall protein (COWP) genes. A fragment of the SSU-rRNA gene was amplified by nested PCR, as described previously (Xiao *et al.*, 1999). The primary PCR was performed with primers SSU-F2

(5'-TTCTAGAGCTAATACATGCG-3') and SSU-R2 (5'-CCCATTTCTTCGAAACAGGA-3') resulting in an approximately 1,300-bp fragment (Table 2). The primary PCR mixtures contained 10 µl of template DNA, 10x Taq buffer S (PeqLab, Erlangen, Germany), 10 mM deoxynucleoside triphosphate mix (dNTP) (Invitrogen, Karlsruhe, Germany), 20 pmol of each primer, 2.5 U of Taq DNA polymerase (PeqLab), and 0.5 µl of non-acetylated bovine serum albumin (BSA; 10 mg/ml) (New England Biolabs, Frankfurt, Germany) in a 50-µl reaction volume. Primary PCR cycling conditions consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 55°C for 45 sec and 72°C for 1 min, with a final extension of 72°C for 7 min and final 4°C cooling. The secondary PCR was performed with primers SSU-F3 (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') and SSU-R4 (5'-CTCATAAGGTGCTGAAGGAGTA-3'), resulting in an approximately 830-bp fragment (Table 2). The reaction conditions were similar to those described above for the primary PCR, with the exception that 5 µl of the primary PCR product was used as the template and no BSA being added. Cycling conditions for the secondary PCR consisted of 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 58°C for 45 sec, and 72°C for 1 min with a final extension of 72°C for 7 minutes and final cooling at 4°.

A fragment of the COWP gene was amplified by PCR, as described previously (Spano *et al.*, 1997). The PCR was performed with primers Cry-9 (5'-GGACTGAAATACAGGCATTATCTTG-3') and Cry-15 (5'-GTAGATAATGGAAGAGATTGTG-3') which amplify a 550-bp fragment (Table 2). The PCR mixture contained 10 µl of template DNA, 10x Taq buffer S, 10 mM dNTP, 20 pmol of each primer, 2.5 U of Taq DNA polymerase (PeqLab), and 0.5 µl of non-acetylated BSA (10 mg/ml) in a 50-µl reaction volume. PCR cycling conditions consisted of an initial denaturation at 94°C

for 3 min, followed by 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 min and final cooling at 4°C.

3.5.2.2. Restriction fragment length polymorphism (RFLP)

Restriction patterns of the SSU-rRNA amplicons was obtained by digestion of 10 µl of amplified products with 10 units of endonucleases *Ase* I (New England Biolabs) and 2 µl of 10 x NEBuffer 3 in a total volume of 20 µl, incubated at 37°C in a water bath for 2 hr under conditions recommended by the supplier. Ten µl of the COWP PCR were digested with 10 units of endonuclease *Rsa* I (New England Biolabs) and 2 µl of 10 x NEBuffer 4 in a total volume of 20 µl, incubated at 37°C for 2 hrs as above. As positive controls, a sample of *C. parvum* infected HCT-8 cells (Jakobi and Petry, 2006) and *C. hominis* DNA, from the collection at the Institute of Medical Microbiology & Hygiene in Mainz, Germany were used. As negative controls, HCT-8 cell DNA and DNase-free water was used. The digestion products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining.

3.5.3. Glycoprotein 60 (GP60) gene PCR amplification for *C. parvum* and *C. hominis* subgenotyping

A 60-kDa glycoprotein (GP60) gene fragment was amplified by nested PCR as described (Sulaiman *et al.*, 2005; Alves *et al.*, 2003; Xiao and Ryan 2008). The primary PCR was performed with primers GP60-AL3531 (5'-ATAGTCTCCGCTGTATTC-3') and GP60-AL3535 (5'-GGAAGGAACGATGTATCT-3') (Table 2). The primary PCR mixtures contained 10 µl of template DNA, 10x PCR buffer (Roche Diagnostics, Mannheim, Germany), 10 mM

deoxynucleoside triphosphate mix (dNTP) (Roche), 20 pmol of each primer, 2 U of Fast start Taq DNA polymerase (Roche) and 0.5 µl of BSA (10 mg/ml) (New England Biolabs) in a 50-µl reaction volume. Primary PCR cycling conditions consisted of an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 50°C for 45 sec and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes and final cooling at 4°C. The nested PCR was performed with primers GP60-AL3532 (5'-TCCGCTGTATTCTCAGCC-3') and GP60-AL3534 (5'-GCAGAGGAACCAGCATC-3') or GP60-LX0029 (5'-CGAACCACATTACAAATGAAGT-3') which amplifies an approximately 850 & 390-bp fragment respectively (Table 2). The reaction conditions were similar to those described above for the primary PCR; with the exception that 1 µl of the primary PCR product was used as the template and no BSA was added. Cycling conditions for the secondary PCR consisted of 95°C for 3 minutes, followed by 35 cycles of 95°C for 1 minute, 50°C for 45 sec, and 72°C for 1 minute with a final extension at 72°C for 7 minute and final cooling at 4°C.

The nested PCR protocol for amplification of the GP60 fragment described by Xiao and Ryan (2008) was optimized by increasing the annealing temperature of the primary reaction from 50°C to 54°C and of the secondary reaction from 50°C to 62°C. This resulted in fewer unspecific amplification products.

3.5.4. Agarose gel electrophoresis for analysis of DNA

Ten ml of each genomic DNA sample (originating from PCR) was mixed with 2µl of 6× DNA loading buffer (0.25% (W/V) bromophenol blue). Agarose gel electrophoresis was performed in 1× TBE buffer (0.89 M Tris, 0.89 M Boric acid, 0.02 M EDTA, pH 8.3) at 10 V/cm using 1.2 –

2% gels. A 100 bp DNA marker was run in parallel with the samples to determine the size of the DNA samples. Amplification products were stained with ethidium bromide (0.5 µg/ml) after electrophoresis for about 45 minutes, detected with a UV-illuminator and recorded with a digital camera (Kodak documentation and analysis system 120). Further processing of agarose gel images suitable for documentation and presentation was carried out using Adobe Photoshop® software (Adobe Systems Inc).

3.5.5. Extraction of DNA from Agarose Gels for Sequencing

DNA purification from agarose gel after electrophoresis was performed using the procedures of a commercial kit (QIAquick® Gel Extraction kit, Qiagen, Hilden, Germany). The principle for DNA purification after agarose gel electrophoresis was based on the spin-column technology and the binding properties of silica-gel membrane according to the manufacturer's instructions (Qiagen). Accordingly, the specific DNA fragment was excised from agarose gel under UV-illumination with a clean sharp scalpel. The gel slice in a colorless tube was weighed and 3 volumes of Buffer QG to 1 volume of gel (100 mg of slice ~ 100 µl of QG) was Added. For example, 300 µl of Buffer QG was added to each 100 mg of gel.

The gel slice was incubated at 50°C for 10 minute in a shaking cube until the gel slice has completely dissolved. After the lysis was completed the pH was adjusted with 3 M sodium acetate to facilitate the adsorption of DNA to the silica-gel membrane, which is efficient only at $\text{pH} \leq 7.5$. After the gel slice has dissolved completely, the color of the mixture was checked yellow (similar to Buffer QG without dissolved agarose). When the color of the mixture was orange or violet, 10 µl of 3 M sodium acetate, pH 5.0 was added and mixed. The color of the

mixture was turned to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at $\text{pH} \leq 7.5$. Buffer QG contains a pH indicator which is yellow at $\text{pH} \leq 7.5$ and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

One gel volume of isopropanol was added to the sample and mixed. For example, if the agarose gel slice was 100 mg, 100 μl isopropanol was added to increase the yield of DNA fragments. The sample at this stage was not centrifuged. QIAquick spin column in a provided 2 ml collection tube was placed. The sample was applied to the QIAquick column and centrifuged for 1 minute for binding DNA. The maximum capacity of the column is 800 μl . Flow-through was discarded and QIAquick column was placed back in the same collection tube (to reduce collection tubes wastage). Five hundred μl of Buffer QG was added to QIAquick column and centrifuged for 1 minute to remove traces of agarose. Seven hundred and fifty μl of Buffer PE was added to QIAquick column and centrifuging (the column stand 2–5 minutes after addition of Buffer PE and before centrifuging) for 1 minute. The flow-through was discarded and the QIAquick column centrifuged for an additional 1 minute at 13,000 rpm. QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. DNA was eluted by adding 30 μl of Buffer EB (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane and centrifuged (the column membrane was stood for 1 minute before centrifugation) the column for 1 minute. DNA concentration was measured spectrophotometrically and stored at -20°C until sequencing.

3.5.6. DNA sequencing

Secondary GP60 PCR products were purified using the procedures of a commercial kit (QIAquick[®] Gel Extraction kit, Qiagen, Hilden, Germany). Direct DNA sequencing of the gel-

purified PCR product was performed at Genterprise Genomics (Mainz, Germany). The primers used to sequence were GP60-AL3532 (5'-TCCGCTGTATTCTCAGCC-3'), GP60-AL3534 (5'-GCAGAGGAACCAGCATC-3') and GP60-LX0029 (5'-CGAACCACATTACAAATGAAGT-3') (Sulaiman *et al.*, 2005). DNA sequences were aligned with each other and with previously reported sequences using the BLAST program (<http://blast.ncbi.nlm.nih.gov/>) and ClustalX software (<ftp://ftp.ebi.ac.uk/pub/software/clustalw2/>) for identification of genotypes and subgenotypes.

Table 2: Primer sequences used for PCR and sequencing in this study

Target Gene	- Forward Primer sequence - Reverse	Length of PCR product (bp)	Annealing T _m (°C)	Reference
SSU-rRNA	SSU-F2 (5'-TTCTAGAGCTAATACATGCG-3') SSU-R2 (5'-CCCATTCCTTCGAAACAGGA-3')	1,300	55	Xiao & Ryan 2008; Xiao <i>et al.</i> , 1999
	SSU-F3 (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') SSU-R4 (5'- CTCATAAGGTGCTGAAGGAGTA-3')	830	58	
COWP	Cry-9 (5'-GGACTGAAATACAGGCATTATCTTG-3') Cry-15 (5'-GTAGATAATGGAAGAGATTGTG-3')	550	60	Spano <i>et al.</i> , 1997
GP60	GP60-AL3531 (5'-ATAGTCTCCGCTGTATTC-3') GP60-AL3535 (5'-GGAAGGAACGATGTATCT-3')		54	Xiao & Ryan 2008
	GP60-AL3532 (5'-TCCGCTGTATTCTCAGCC-3') GP60-AL3534 (5'-GCAGAGGAACCAGCATC-3')	850		
	GP60-LX0029 (5'-CGAACCACATTACAAATGAAGT-3')	390	62	Sulaiman <i>et al.</i> , 2005

3.5.7. Determination of nucleic acid concentration

For all kinds of nucleic acid preparation (genomic DNA and gel extracted DNA), concentration was determined spectrophotometrically (Nanodrop[®]ND, PeqLab, Erlangen, Germany) at 260 nm. The nucleic acid preparation was well mixed with sterile pipettes. The buffer used for eluting a given nucleic acid preparation was used as blank to adjust the spectrophotometer. Then, 2 µl of the nucleic acid was dropped on the lens of the instrument and concentration (in nanogram per µl) as well as the purity of the preparation (as 260/280 nm ratio) was determined by the spectrophotometer.

3.6. Data analysis

Statistical analysis was done using the SPSS version 15.0 statistical software. Data were summarized using frequency tables, and bar charts. Contingency tables were used and the strength of association was measured using the chi-square and its associated p-value. Values were considered to be statistically significant when the p-value obtained was less than 0.05. Softwares like Adobe Photoshop[®]CS2 Version 9.0.2 Adobe Systems Inc (California, USA), Kodak Digital Science 1D software (Kodak), NCBI: <http://www.ncbi.nlm.nih.gov/blast/> (Online public), NEBcutter V2.0 (tools.neb.com/NEBcutter2/index.php) (online), and Primer3 software (Online) (Rozen and Skaletsky, 2000) were used. DNA sequences were analysed with the BLAST (<http://blast.ncbi.nlm.nih.gov>) and ClustalX software (<ftp://ftp.ebi.ac.uk/pub/software/clustalw2/>).

3.7. Ethical Considerations

This research was conducted with the approval of the Ethical Review Committee of Research, Department of Biology, Faculty of Science, Addis Ababa University. Ethical considerations were addressed by treating patients with positive results with the appropriate treatment and drugs. Treatments were done by clinicians working at the study sites. Informed written consent was obtained from each study participant at the time of sample collection. Participants were also informed that they were free to withdraw consent at any time and their records and specimen was examined by authorized persons, and personal information on them have been treated strictly confidential.

4. Results

4.1. Socio-demographic description of study participants

Over a period of twelve months from September 2007 to August 2008, a total of 1034 faecal samples were collected from patients with diarrhoea who had and had not had contact with animals prior to sampling. Eight *Cryptosporidium* isolates from cattle were also studied, which had close contact with humans. Among the total human study participants 320 were HIV seropositive individuals (Table 3) and faecal samples were collected from patients with and without Antiretroviral therapy (ART) during the study period. Data on demographic and risk factors for *Cryptosporidium* infection were also collected for the study participants with and without diarrhoea.

Of the total study participants 539 (52.1%) were males and 495 (47.9%) were females, 265 (25.6%) lived in rural areas and 769 (74.4%) lived in urban areas, 100 (9.7%) were children, and 934 (90.3%) were adults. The mean age of the study participants was 33 years (ranged: 1 to 65 years) (Table 3). Socio-economic status based on income, and education showed that only 125 (12.1%) were from a high socio-economic group (monthly income >1000 birr), 245 (23.7%) from a middle income group (monthly income < 1000 birr) and the majority of cases 564 (54.5%) were from the low socio-economic status groups (farmers and pastoralists). The 100 (9.7%) were children.

Table 3: Socio-demographic characteristics of the study participants (n=1034) by age, sex, residence and HIV status from some parts of Ethiopia (September 2007-August 2008).

	Study sites	Age (Years) Mean (range)	Sex		Residence		HIV status			Grand total
			Male (n(%))	Female (n(%))	Urban (n(%))	Rural (n(%))	Negative (n(%))	Positive (n(%))	Unknown (n(%))	
1	Addis Ababa	21.5 (3-40)	46 (40.3)	68 (59.7)	114 (100)	0	69 (60.5)	21 (18.4)	24 (21.1)	114
2	Bishoftu	43.5 (22-65)	53 (66.2)	27 (33.8)	53 (66.2)	27(33.8)	53 (66.2)	27 (33.8)	0	80
3	Adama	28(12-44)	61 (34.3)	117 (65.7)	140 (78.6)	38 (21.4)	50 (28.1)	59 (33.1)	69 (38.8)	178
4	Afar (Awash 7)	18(2-34)	92 (65.7)	48 (34.3)	84 (60)	56 (40)	102 (72.8)	38 (27.2)	0	140
5	Dire Dawa	34(25-43)	51 (44.3)	64 (55.7)	63 (54.8)	52 (45.2)	10 (8.7)	83 (72.2)	22 (19.1)	115
6	Harar	35.5(28-43)	76 (74.5)	26 (25.5)	76 (74.5)	26 (25.5)	51 (50)	0	51 (50)	102
7	Sebeta	18(1-35)	38 (40)	57 (60)	80 (84.2)	15 (15.8)	76 (80)	19 (20)	0	95
8	Debre Markos	31.5(21-42)	50 (50)	50 (50)	75 (75)	25 (25)	50 (50)	25 (25)	25 (25)	100
9	Bahir Dar	21.5(2-41)	72 (65.5)	38 (34.5)	84 (76.4)	26 (23.6)	24 (21.8)	48 (43.6)	38 (34.5)	110
	Total		539 (52.1)	495 (47.9)	769 (74.4)	265 (25.6)	485 (46.9)	320 (30.9)	229 (22.2)	1034

HIV sero-status of the study patients is as follows: 485 (46.9%) were sero-negative, 320 (30.9%) were sero-positive, and for 229 (22.2%) their HIV sero-status was unknown (Table 3).

Of the 320 HIV sero-positive individuals, 176 (55%) were males, 144 (45%) were females. 228 (71.2%) were from rural areas bordering cities chosen for the study, and 92 (28.8%) were cities dwellers (Table 4). Among the 320 HIV sero-positives 59 (18.4%) were residents of Adama, 38 (11.8%) were residents of Afar, 83 (26%) were residents of Dire-Dawa and the remaining 140 (43.8%) were residents from other study sites (Table 4).

Table 4: Age, Sex and Area distribution of HIV sero-positive study participants (n=320) from Adama, Afar, Dire Dawa, and other study sites, Ethiopia (September 2007-August 2008).

	Study sites	Age range (in Years)	Sex		Residence		Grand total (n(%))
			Male (n(%))	Female (n(%))	Urban (n(%))	Rural (n(%))	
1	Adama	12-44	30(50.8)	29(49.2)	28(47.4)	31(52.6)	59 (18.4)
2	Afar (Awash 7)	2-34	20(52.6)	18(47.4)	19(50)	19(50)	38 (11.8)
3	Dire Dawa	25-43	50(60.2)	33(39.8)	33(39.8)	50(60.2)	83 (26)
4	Other sites*	1-65	76(54.2)	64(45.8)	12(8.5)	128(91.5)	140 (43.8)
	Total		176 (55)	144 (45)	92 (28.8)	228 (71.2)	320 (100)

* Addis Ababa, Bishoftu, Harar, Sebeta, Debre Markos, Bahir Dar

4.2. Prevalence of diarrhoea in the study population

Of the 934 patients with diarrhoea, 140 patients had acute diarrhoea. Ten patients had persistent diarrhoea for more than a month, 250 patients had three or more episodes of diarrhoea in the previous 6 months, and 534 patients had one episode of diarrhoea. Diarrhoea was associated with fever in 440 (42.5%) patients, with nausea in 356 (34.4%), and with nausea and vomiting in 138 (13.4%) patients. Stool consistency of the patients was recorded as follows: loose diarrhoea in 554 (59.3%) patients, watery diarrhoea in 219 (23.4%), bloody diarrhoea in 35 (3.74%) and the remaining 126 (13.5%) cases had diarrhoea with normal consistency.

Of the 320 HIV positives 130 (40.6%) were on anti-retroviral therapy (ART). The types of diarrhoea were associated with particular types of parasitic infections as follows: watery diarrhoea with *Cryptosporidium* spp, bloody diarrhoea with *E. histolytica*/*E. dispar* that engulfs Red blood cell, and mucoid diarrhoea with *G. lamblia*. Microscopic examinations of stools of HIV sero-positive patients with no known parasitic infections showed that many had pus cells, yeast cells, and red blood cells in their stools.

4.3. Prevalence of *Cryptosporidium* spp. and other intestinal parasites

Based on intestinal parasitological examinations of the stool specimens, 11 species of intestinal parasites were detected in 419 (40.5%) of the patients of which *Giardia lamblia* 132 (31.5%) and *Cryptosporidium* spp 79 (18.9%) make up the majority (Table 5, Figure 5).

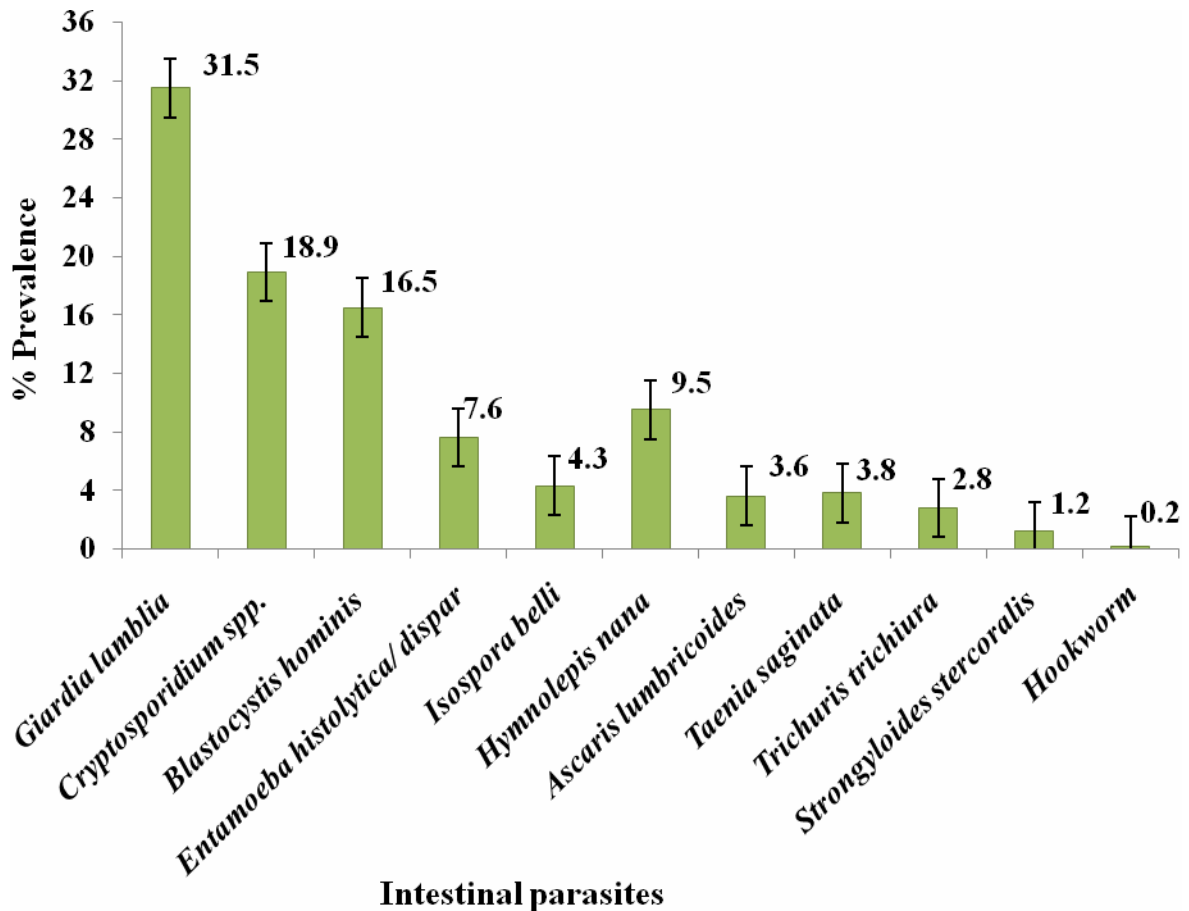


Figure 5: Relative distribution of *Cryptosporidium* and other intestinal parasite infections among parasite positive study participants (n=419) from different locations in Ethiopia (September 2007-August 2008).

Infections with various intestinal protozoan and helminthic parasites were common among the patients in the study. The prevalence of infection with different intestinal protozoan and helminthic parasites in the study patients were 330 (78.7%) and 89 (21.3%) respectively, indicating that protozoan parasites were the most common intestinal infectious agents among the 419 patients.

Table 5: *Cryptosporidium* and other intestinal parasites detected from patients from different locations in Ethiopia (September 2007-August 2008).

	<i>Cryptosporidium</i>	Other	All parasites
Regions	positive* n (%)	Intestinal parasites**	n (%)
1 Addis Ababa	10 (8.7)	32 (9.4)	42 (10)
2 Bishoftu	3 (3.7)	19 (5.5)	22 (5.2)
3 Adama	18 (10.1)	44 (12.9)	62 (14.7)
4 Afar (Awash 7)	15 (10.7)	34 (10)	49 (11.7)
5 Dire Dawa	11 (9.5)	42 (12.3)	56 (13.3)
6 Harar	4 (3.9)	47 (13.8)	51 (12.2)
7 Sebeta	5 (5.2)	31 (9.1)	36 (8.5)
8 Debre Markos	4 (4.0)	45 (13.2)	49 (11.7)
9 Bahir Dar	9 (8.1)	46 (13.5)	55 (13.1)
Total	79 (7.6)	340 (32.8)	419 (40.5)

* By Modified Ziehl-Neelson staining method

** Direct & Concentration methods Microscopy

From the total 320 HIV sero-positive individuals intestinal parasites were detected in 154 (48%) of the patients. Among the detected intestinal parasites *G. lamblia* was the most prevalent. *G. lamblia* 15 (9.7%), and *E. histolytica/ E. dispar* 14 (9.1%) were most prevalent in the Afar region. From the total of 154 parasite positive individuals *H. nana* and *B. hominis* were not detected in Adama and Dire-Dawa resident stool samples.

Of the 320 HIV sero-positive patients, 134 (87%) had positive parasitologic results show a significant association between the presence of parasites and diarrhoea ($P < 0.05$). The prevalence of *G. lamblia*, *Cryptosporidium* spp. and *I. belli* was positively and significantly associated with the presence of diarrhoea ($P = 0.0037$; $P < 0.001$ and $P = 0.0036$) respectively.

A. lumbricoides, *H. nana* and *B. hominis* were not detected in HIV sero-positive persons who are university graduates, but were detected in individuals with education levels ranging from grades 6-12. Similarly, *Cryptosporidium* spp. and *Isospora belli* were detected in individuals with the same level as in above. But, other types of parasite species were detected in people with lower education levels. The infections declined with increased educational level of the study subjects ($P < 0.05$). While college graduate HIV sero-positive individuals had fewer enteric parasites, non-college graduate, HIV sero-positive individuals had many enteric parasites. The main reason for the difference in infection level between the two groups is that non-college graduate individuals had relatively poor living conditions. Another protozoan coccidial parasite, *Cyclospora cayetanensis*, was not detected by modified Ziehl-Neelson staining method in this study.

There is a correlation between poor living conditions and presence of parasites. From the total 154 parasite positive individuals, 135 (87.6%) were living in “poor” conditions whereas 19 (12.3%) were living in “good” conditions. *G. lamblia* was the most common intestinal parasites identified in the study participants, which is environmental contaminant of the water and can be transmitted by drinking contaminated water (Table 6).

Table 6: Prevalence of *Cryptosporidium* and other intestinal parasite infections and living conditions of HIV sero-positive individuals (n=154) in selected ART centers, Ethiopia (September 2007-August 2008).

Parasites	Living condition*	
	Poor n(%)	Good n(%)
<i>G. lamblia</i>	36(81.9)**	8(18.1)
<i>E. histolytica/ dispar</i>	21(80.8) ^{§§}	5(19.2)
<i>Cryptosporidium</i> spp	25(84.6) ^{§§}	4(15.4)
<i>I. belli</i>	8(80) ^{§§}	2(20)
<i>A. lumbricoides</i>	9(100) [§]	0
<i>H. nana</i>	11(100) [§]	0
<i>B. hominis</i>	28(100) [§]	0
Total	135 (87.6)	19 (12.3)

* Living conditions were considered as good versus poor considering the degree of crowding, quality of water supply, disposal of excretion and type of floor (Tellez *et al.*, 1997).

** P<0.001; § P<0.01; §§ P<0.05

4.4. Association of *Cryptosporidium* spp and other intestinal parasites with ART and diarrhoea

From the total of 154 parasite positive HIV sero-positive individuals, 146 (94.8%) were infected with only one type of parasite, although there were five cases of polyparasitism; all patients with *G. lamblia* were also infected with *Cryptosporidium* spp, but only three individuals with *G. lamblia* infection were also infected with *E. histolytica/ E. dispar* out of the 320 HIV sero-positive individuals. Sixty-six out of 154(42.8%) of the study participants were on antiretroviral therapy (ART), 45% were females. Of the total patients on ART only four out of 64 (6.2%) were diagnosed with opportunistic parasites (*Cryptosporidium* spp, *I. belli*, and *B. hominis*) in this study (Table 7).

Table 7: Association between the presence of *Cryptosporidium* and other intestinal parasites among HIV positive individuals with ART (n=66) and without ART (n=88) in selected ART centers, Ethiopia (September 2007-August 2008).

Parasites	ART status		
	ART	Non-ART	p-value
<i>G. lamblia</i>	28(63.6)	16(36.4)	>0.05
<i>E. histolytica/ dispar</i>	14(54)	12(46)	
<i>Cryptosporidium</i> spp	4(15.4)	25(84.6)	< 0.05
<i>I. belli</i>	0	10(100)	
<i>A. lumbricoides</i>	9(100)	0	< 0.005
<i>H. nana</i>	11(100)	0	
<i>B. hominis</i>	0	28(100)	
Total	66 (42.8)	88 (57.2)	

Key:

ART: With Antiretroviral treatment

Non-ART: Without-Antiretroviral treatment

Of the three hundred and twenty, one hundred and thirty four (41.5%) of these patients were on antibiotics. Cotrimoxazole (bacterium) was the antibiotic most commonly used by the patients. Twenty five of these patients were both on antibiotics and ART. Of the 154 parasite positive HIV sero-positive individuals, eleven (7.1%) out of the total intestinal parasitic infected HIV Sero-

positives, were positive for *A. lumbricoides*, *G. lamblia*, and *E. histolytica/ E. dispar*. However, none of the patients on both ART and antibiotics as prescribed by clinicians were positive for any of the opportunistic parasites. Furthermore, *Cryptosporidium* spp and *I. belli* infections were significantly higher in HIV positives who were not enrolled in ART when compared with those enrolled in ART ($p < 0.05$) (Table 7). In Afar region, clients on ART had more opportunistic infections (61.9%) compared to patients on non-ART patients (38.1%). ART enrolled patients might be poorly adhering or improperly using ART.

From the 154 of HIV positive patients with intestinal parasites, 51/154 (33.1%) individuals had CD4⁺ T cell count $> 500/\mu\text{L}$, 29/154 (18.8%) had CD4⁺ T cell count between 200 - 499/ μL , and 74/154 (48.1%) had CD4⁺ T cell count $< 200/\mu\text{L}$. *Cryptosporidium* spp, *I. belli* and *B. hominis* were associated with lower CD4⁺ cell count $< 200/\mu\text{L}$ (20/26; 76.9%) (6/10; 60%), and (28/28; 100%) respectively with p value < 0.05 (Table 8).

Table 8: Prevalence of intestinal parasite infection among HIV sero-positive individuals and their CD4⁺ T cell count (n=154) in selected ART centers, Ethiopia (September 2007-August 2008).

Parasites	CD4 ⁺ Cell count		
	>500/ μ L n(%)	200-499/ μ L n(%)	<200/ μ L n(%)
<i>G. lamblia</i>	25(56.8)	8(18.2)	11(25)
<i>E. histolytica/ dispar</i>	5(19.2)	14(53.8)	7(27)
<i>Cryptosporidium</i> spp	2(7.7)	4(15.4)	23(76.9)*
<i>I. belli</i>	1(10)	3(30)	6(60)*
<i>A. lumbricoides</i>	7(77.8)	0	2(22.2)
<i>H. nana</i>	11(100)*	0	0
<i>B. hominis</i>	0	0	28(100)*
Total	51 (33.1)	29 (18.8)	74 (48.1)

*P<0.05

4.5. Risk factors for cryptosporidiosis

The risk factors for acquiring cryptosporidiosis were assessed in all the patients including occurrences of diarrhoea in other members of the household, defecation sites, drinking water supply sources and contact with animals. Four hundred and seven (39.3%) patients reported the occurrence of diarrhoea in other members of their households during the three weeks prior to their hospital visit. There was a significant association between presences of young children in

the household the occurrence of diarrhoea in other members of the household, and defecation sites with diarrhoea in the study patients. Tap water supply was used for drinking by 769 (74.4%) patients; covered well water supply was used for drinking by 109 (10.5%) patients; open well water supply was used for drinking by 150 (14.5%) patients; and river water supply was used for drinking by 6 (0.6%) patients were used. Occurrences of diarrhoea in other members of the household, defecation sites, drinking water supply sources and contact with animals showed significant association for *Cryptosporidium* infection ($P<0.05$).

Majority of the patients reported direct and indirect contact with domestic animals. 800 (77.3%) patients had close contact with cattle, and of these patients, 430 practiced mixed farming (animal husbandry and crop farming). The animal species contacted reported were mainly cows and calves in both urban and rural households; another 46 patients from rural households had contact with goats and sheep; and 54 patients from urban households reported the presence of chicken. A total of 154 patients with better socio-economic status from urban households reported the presence of dogs and cats. But, *Cryptosporidium* infection were not significantly associated with goats, sheep, chicken, dogs, and cats contacted, as molecular characterization could also clarified it.

4.6. *Cryptosporidium* spp prevalence and diagnosis

A total of 79 from 1034 human faecal samples (7.6%) and 8 of 350 cattle fecal samples were positive for *Cryptosporidium* by modified Ziehl-Neelson staining, Immunofluorescence test (IFT) and oocyst counting was performed using counting chamber under differential interference

contrast (DIC) microscopy (Figure 6). Oocyst counting was performed by screening 50 optical fields (x1000).

The size of the *Cryptosporidium* oocysts from all human and animal samples detected ranged from 4.5–5.5 μm (Figure 6). The measurement was done using computerized software which is suited to measure oocyst or cyst of parasites as reported by Fayer and Xiao (2008).

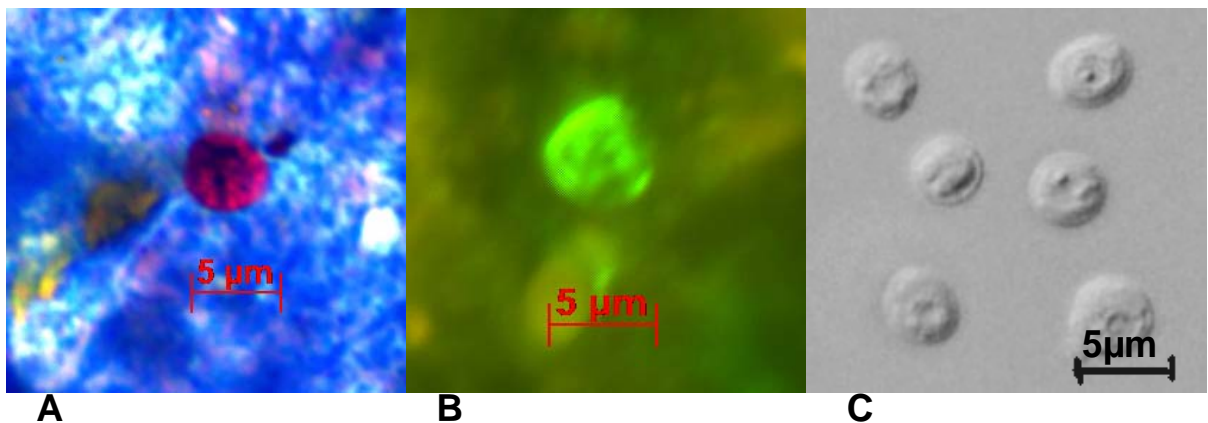


Figure 6: Red stained oocysts of *Cryptosporidium* by modified Ziehl-Neelson staining method (magnification x1000) (A); the green oval structure is *Cryptosporidium* oocyst by immunofluorescence test (IFT) observed under fluorescence microscope (magnification x1000) (B) and *Cryptosporidium* oocyst under differential interference contrast (DIC) microscopy (C).

Among the 79 *Cryptosporidium*-positive humans from 1034 (Figure 4 & 7), 50.6% were males and 49.4% were females. The study participants were 59 (74.7%) urban dwellers and 20 (25.3%) resided in rural areas. Of the 79 participants, 29 (36.8%) were HIV positive, 34 (43%) were HIV negative, and 16 (20.2%) were with unknown HIV status. Six of 29 (21%) HIV positive patients with cryptosporidiosis were receiving first-line regimen antiretroviral treatment (ART) (Table 9).

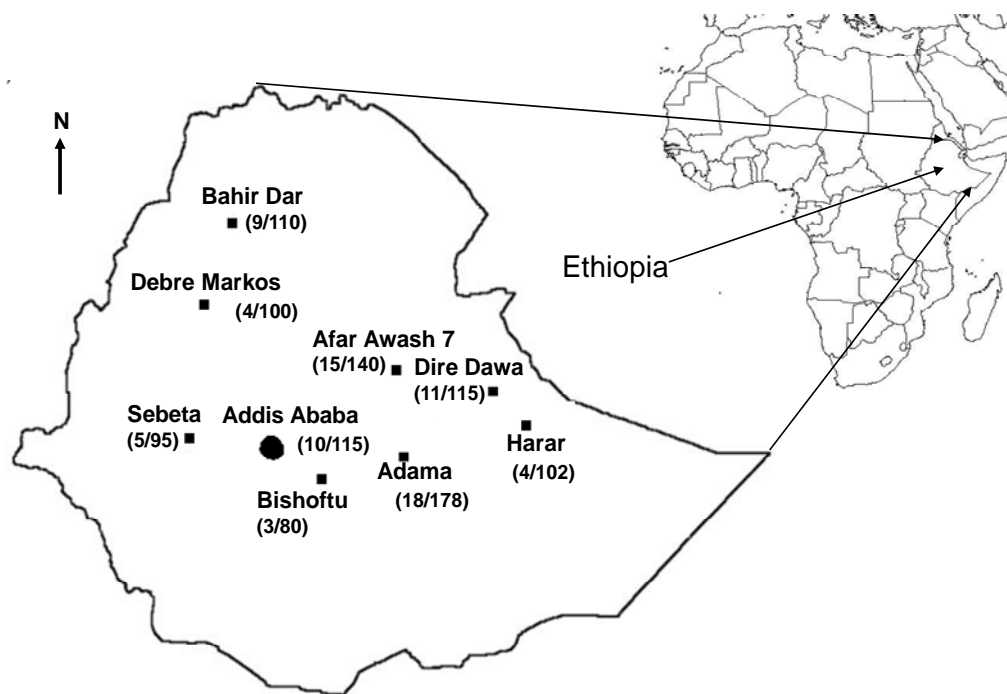


Figure 7: Study sites in Ethiopia. Numbers in parenthesis are *Cryptosporidium* positive specimens using modified Ziehl-Neelson staining method and immunofluorescence test (IFT) over total collected specimens and *Cryptosporidium* isolates are characterized molecularly.

Table 9: Demographic and parasitological data for human study participants with cryptosporidiosis from whom *Cryptosporidium* isolates were characterized molecularly from Ethiopia (n=41) (September 2007-August 2008).

Isolate Code	Region	Type of residence	Age (year)	Sex	HIV-status	Intestinal parasitic co-infection
Eth001	Afar (Awash 7)	Rural	4	M	Negative	<i>Blastocystis hominis</i>
Eth002	Afar (Awash 7)	Urban	2	M	Positive ^a	<i>Trichuris trichiura</i>
Eth003	Afar (Awash)	Urban	21	F	Positive	None
Eth004	Afar (Awash 7)	Urban	8	F	Negative	None
Eth005	Afar (Awash 7)	Urban	1	M	Negative	None
Eth007	Afar (Awash 7)	Urban	7	M	Negative	None
Eth015	Afar (Awash 7)	Rural	6	F	Negative	None
Eth016	Sebeta	Urban	24	F	Negative	None
Eth024	Debre Markos	Urban	29	F	Positive	None
Eth026	Bahir Dar	Rural	31	M	Negative	<i>B. hominis</i>
Eth027	Bahir Dar	Urban	33	F	Positive	None
Eth031	Bahir Dar	Urban	29	F	Negative	None
Eth032	Bahir Dar	Urban	2	M	Unknown	None
Eth033	Bahir Dar	Urban	25	M	Unknown	None
Eth035	Dire Dawa	Rural	43	M	Positive ^a	None
Eth036	Dire Dawa	Urban	30	F	Positive	None
Eth038	Dire Dawa	Urban	37	M	Unknown	None
Eth042	Dire Dawa	Rural	30	F	Positive	None

Eth043	Dire Dawa	Urban	34	F	Positive ^a	None
Eth044	Dire Dawa	Urban	29	F	Unknown	None
Eth045	Harar	Urban	28	M	Negative	<i>Giardia lamblia</i>
Eth046	Harar	Urban	30	M	Negative	None
Eth048	Harar	Urban	42	M	Unknown	None
Eth049	Adama	Urban	38	M	Unknown	None
Eth053	Adama	Urban	12	F	Unknown	None
Eth055	Adama	Rural	40	F	Unknown	None
Eth058	Adama	Urban	28	M	Positive	None
Eth059	Adama	Urban	23	F	Positive	None
Eth064	Adama	Urban	32	F	Negative	None
Eth065	Adama	Urban	26	M	Positive	None
Eth066	Adama	Urban	25	F	Positive	None
Eth067	Bishoftu	Urban	22	M	Negative	None
Eth068	Bishoftu	Rural	45	M	Negative	None
Eth069	Bishoftu	Urban	29	F	Positive ^a	None
Eth070	Addis Ababa	Urban	27	F	Unknown	None
Eth071	Addis Ababa	Urban	40	F	Negative	None
Eth073	Addis Ababa	Urban	32	F	Positive	None
Eth074	Addis Ababa	Urban	29	M	Positive	<i>Entamoeba histolytica / dispar</i>
Eth075	Addis Ababa	Urban	28	M	Negative	None
Eth077	Addis Ababa	Urban	3	M	Negative	<i>B. hominis</i>
Eth079	Addis Ababa	Urban	5	M	Negative	None

^a Patients receiving antiretroviral treatment (ART)

Cryptosporidium cases occurred throughout the year except in July, but the highest prevalence was seen in the month of March (29.1%) and higher prevalences were also seen in the months of February (11.4%), April (13.9%), and November (18.9%). The lower peaks were observed in the months of June, July to October and December with infections rates of 0–3.8 % (Figure 8).

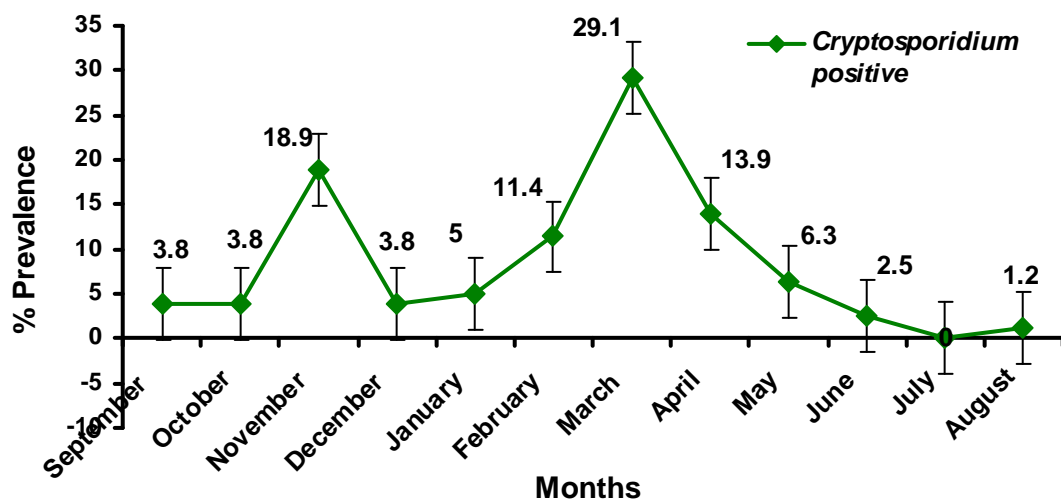


Figure 8: Cumulative isolation rates of *Cryptosporidium* spp. and variation between study sites in Ethiopia using modified Ziehl-Neelson staining method (September 2007-August 2008).

There has been no work done in Ethiopia on *Cryptosporidium* genotypes in human infection that can be demonstrated at molecular levels so that appropriate prevention and/or control measures may be taken to prevent human cryptosporidiosis, such as immunocompromised individuals and children. Therefore, molecular characterization of *Cryptosporidium* isolates was done to determine the genotypes and subgenotypes to investigate transmission and source of the infection.

4.7. Molecular characterization of *Cryptosporidium* spp. isolates

4.7.1. DNA concentration and purity of *Cryptosporidium* spp. isolates

DNA was extracted from all *Cryptosporidium*-positive faecal samples and amplification of the SSU-rRNA, COWP, and GP60 genes was performed (Figure 10). Using stools that had been spiked with *C. parvum* oocysts, we found that introduction of a saturated NaCl floatation step resulted in an increased recovery of *Cryptosporidium* DNA compared to the standard stool DNA preparation method (QIAGEN). Furthermore, up to 5 ml stool suspension could be processed.

DNA concentration from all samples purified for sequencing and unpurified DNA for PCR-RFLP was measured (Figure 9). The measurement was done using software loaded on computerized Nanodrop® ND-100 spectrophotometer. DNA yields were determined from the concentration of DNA in the elute, it was measured by absorbance at 260 nm. Purity was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an A_{260}/A_{280} ratio of 1.7–1.9. Absorbance readings were at 260 nm lied between 0.1 and 1.0 to be accurate. Sample dilution was adjusted accordingly.

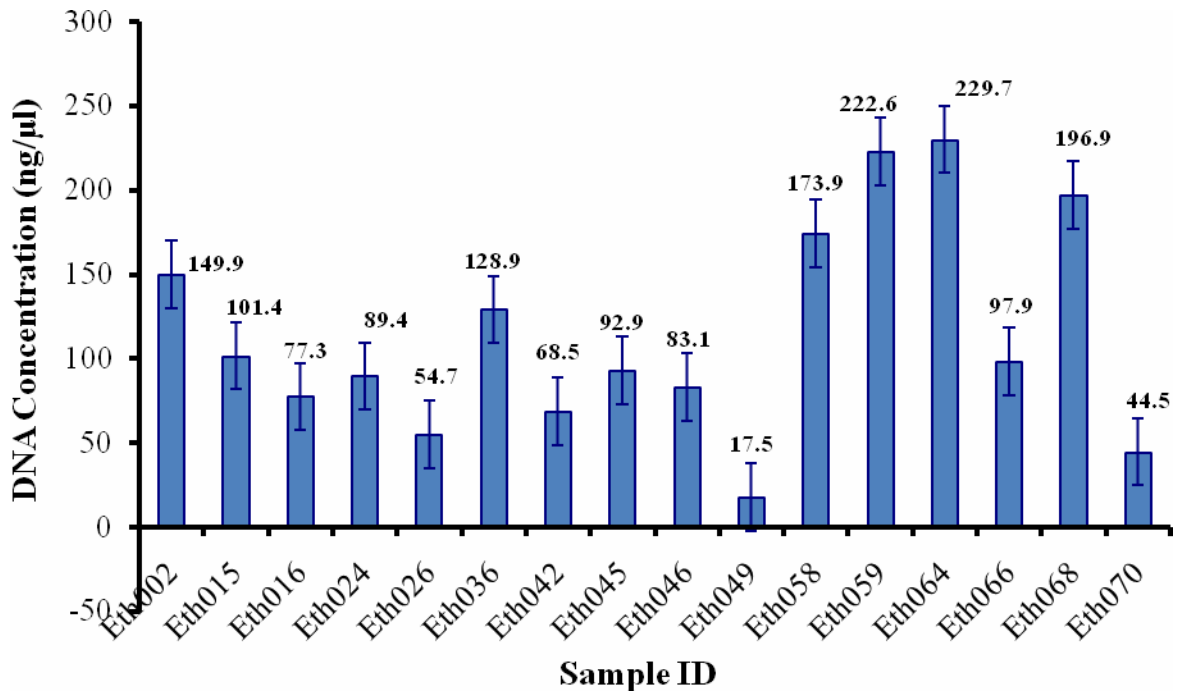


Figure 9: DNA concentration (ng/μl) of *Cryptosporidium* isolates in 2 μl sample from Ethiopia (September 2007-August 2008).

4.7.2. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of *Cryptosporidium* spp isolates for genotyping

From the 79 human and 6 cattle faecal samples on which PCR amplification and genotyping were done, *C. parvum* was detected in 39 (95%) human and all samples from cattle, *C. hominis* in 1 (2.5%), and *C. parvum* and *C. hominis* mixed in 1 (2.5%) human origin samples. Out of 79 samples, 21 yielded only in a SSU-rRNA PCR product, 30 were positive only for COWP and 27 were positive only for GP60. However, only 13 samples were positive in all the three PCR amplification methods (Table 13). Even though, no PCR protocol was significantly superior over the other two reactions. But, COWP gene amplification, 36 (76.5%) was better than the SSU-

rRNA 21 (44.6%) and GP60 27 (57.4%) amplifications. Overall, a total of 47 (54%) human and cattle samples were positive in any of the three typing methods.

A fragment of the SSU-rRNA gene was amplified by nested PCR, as described previously by Xiao *et al.*, (1999). Genotyping by a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the SSU-rRNA gene was performed as follows. The primary PCR was performed with primers SSU-F2 and SSU-R2 resulting in an approximately 1,300-bp fragment (Figure 10 A). The secondary PCR was performed with primers SSU-F3 and SSU-R4, resulting in an approximately 830-bp fragment (Figure 10 B). The genomic DNA of *Cryptosporidium* isolates of human and animal origin derived from different geographical areas (Figure 7) was amplified using primers cry-9 and cry-15. Thirty six samples yielded a COWP gene PCR product of approximately 550 bp that was digested with the endonuclease restriction enzyme *Rsa* I (Figure 10C).

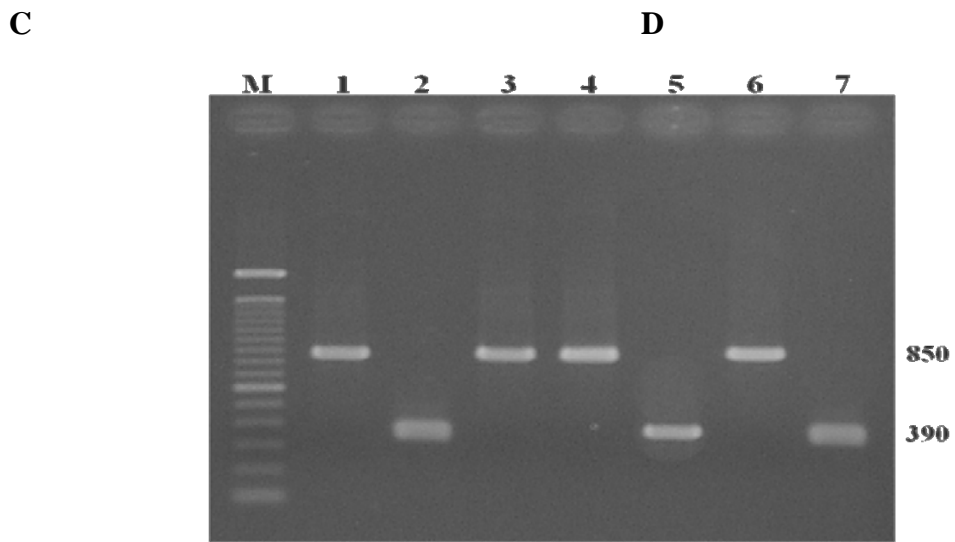
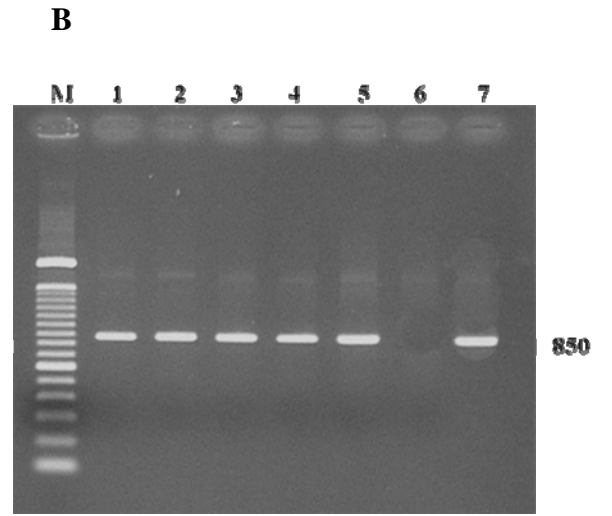
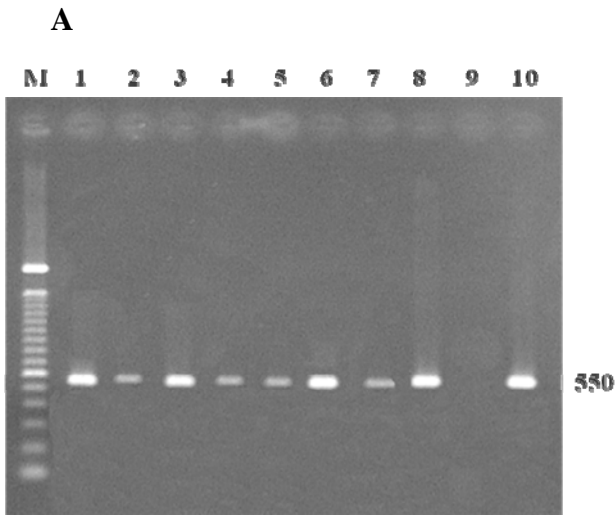
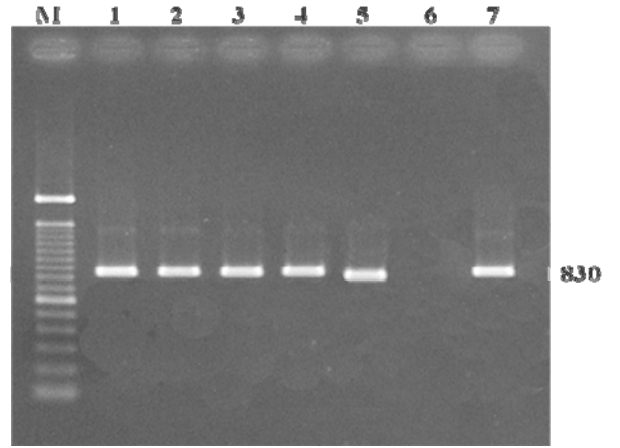
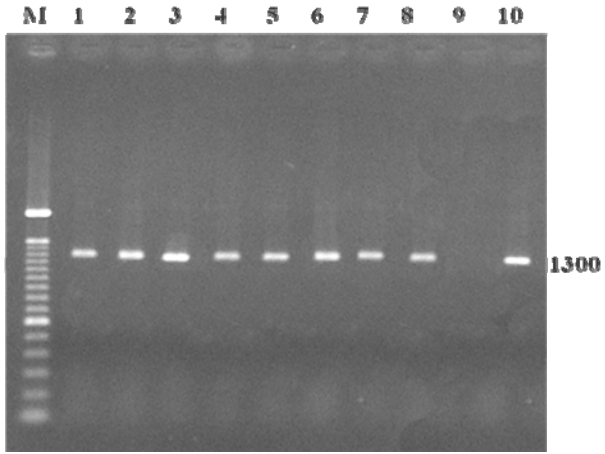


Figure 10: SSU-rRNA gene analysis of the PCR products; (**Plate A**= 1⁰ PCR products, size 1300 bp and **Plate B**= 2⁰ PCR products, size 830 bp) and were electrophoresed on a 1.2% agarose gel and visualized by ethidium bromide staining. **Plates A & C** Lanes: M=100bp molecular markers; *Cryptosporidium* sample DNA 1= Eth001, 2= Eth002, 3= Eth003, 4= Eth004, 5= Eth005, 6= Eth024, 7= Eth027, 8= Eth048, and 9= HCT-8 cells DNA negative control, and 10= *C. parvum* positive control. COWP gene analysis of the PCR products (**Plate C**, size 550 bp) and Glycoprotein 60 (GP 60) gene PCR product, **Plates D & E**; size 390 and 850 bp. **Plates B & D**, Lanes: M=100bp molecular marker; *Cryptosporidium* sample DNA 1= Eth070, 2= Eth046, 3= Eth036, 4= Eth027, 5= Eth024, and 6= HCT-8 cells DNA negative control, and 7= *C. parvum* positive control. **Plate E**, Lanes: M=100bp molecular marker; *Cryptosporidium* sample DNA 1= Eth070, 2= Eth069, 3= Eth058, 4= Eth031, 5= Eth038, 6= Eth016, and 7= Eth015.

As shown in Figure 11 A and B, *Rsa* I endonuclease digestion of COWP gene produced three related but clearly distinct digestion patterns. The molecular size of the digestion products reflected the position of the *Rsa* I cleavage sites identified in *C. parvum* was characterized by three band patterns of 410, 106 and 34 bp. The other one, comprising exclusively isolates of *C. hominis*, displayed four restriction fragments of 285, 125, 106 and 34 bp, indicating the presence of an additional polymorphic *Rsa* I restriction site splitting the 410 bp fragment characteristic of the *C. parvum*.

A total of twenty one nested SSU-rRNA gene PCR products were digested successfully with the endonuclease restriction enzyme *Ase* I and *Ssp* I. The molecular size of the digestion products

reflected the position of the *Ase* I as well as *Ssp* I cleavage sites identified in *C. parvum* was characterized by a three band patterns of 629, 115, 104 bp; and 450, 260 and 108 bp respectively (Figure 11 C & D). The other one, comprising exclusively isolates of *C. hominis*, displayed four restriction fragments of 561, 115, 104 and 71 bp for *Ase* I cleavage (Figure 11 C); and 450, 260 and 111 bp for *Ssp* I cleavage (Figure 11 D).

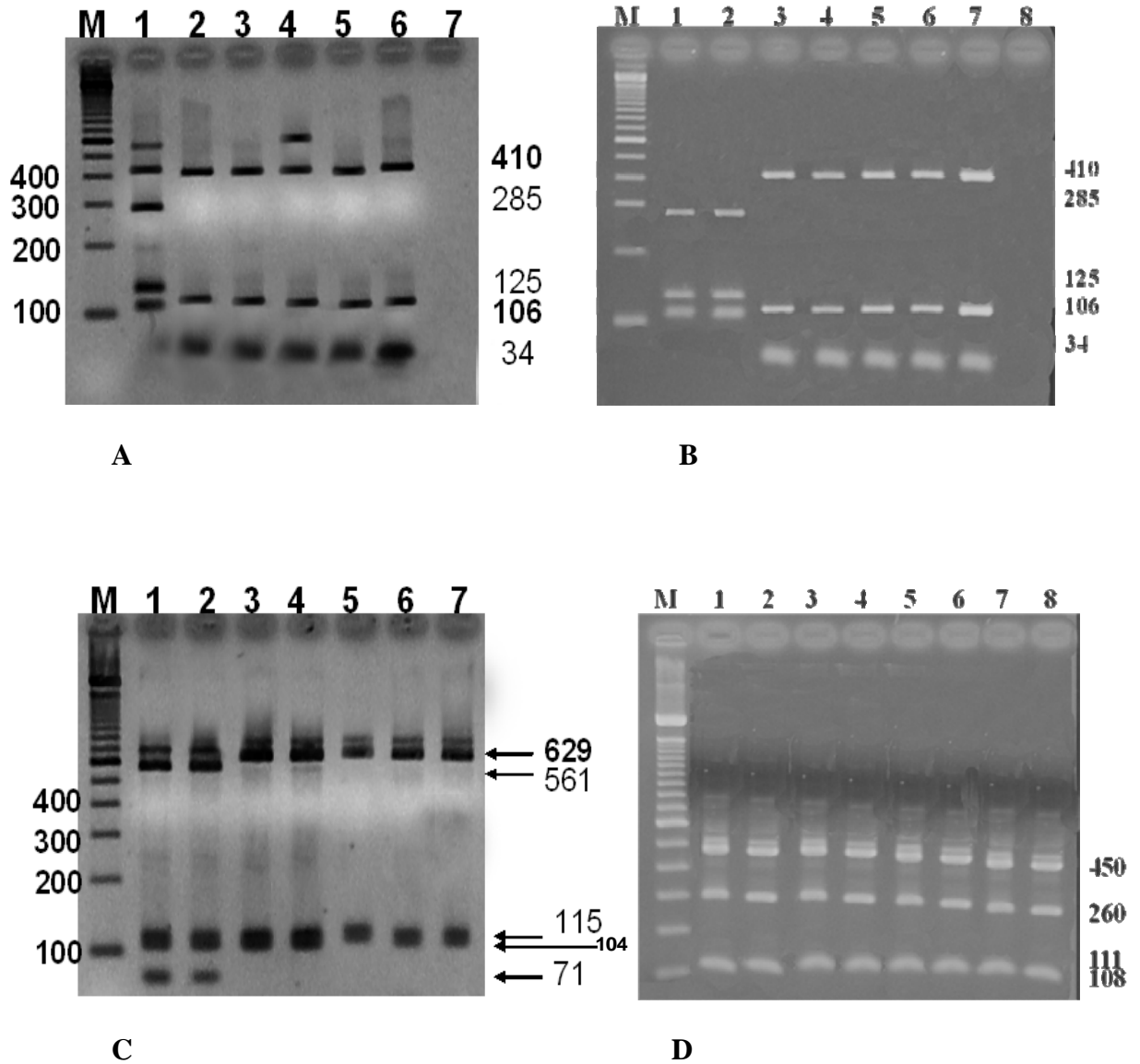


Figure 11: COWP RFLP and SSU-rRNA RFLP gene analysis of the PCR products. The *Rsa* I digested products of the COWP gene (**A & B**); *Ase* I digested products of SSU-rRNA gene (**C**), and *Ssp* I digested products of SSU-rRNA gene (**D**) were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. Lanes (**A**): M=100bp molecular marker; 1= *C. parvum* and *C. hominis* mixed infection (Eth007); 2, 3, 4, 5 are *C. parvum* (Eth070, 069, 058, 031 respectively); 6= *C. parvum* positive control,

and 7= HCT-8 cells DNA as a negative control. Lanes **(B)**: M=100bp molecular marker; 1= *C. hominis* (positive control); 2= *C. hominis* (Eth015); 3, 4, 5 and 6 are *C. parvum* (Eth001, 002, 003, and 004 respectively); 7= *C. parvum* positive control, and 8= HCT-8 cells DNA negative control. Lanes **(C)**: M=100bp molecular marker; 1= *C. hominis* (Eth016); 2= *C. hominis* (positive control); 3, 4, 5 and 6 are *C. parvum* (Eth001, 002, 003, and 004 respectively); and 7= *C. parvum* positive control. Lanes **(D)**: M=100bp molecular marker; 1, 2, 3, 4, 5, 6, and 7 are *C. parvum* (Eth026, 065, 073, 026, 069, 058, and 004 respectively); 8= *C. hominis* (Eth016).

RFLP analysis of PCR products revealed the presence of two species/genotypes of *Cryptosporidium* isolates from humans with cryptosporidiosis in Ethiopia. Of the total 41 human and 6 cattle *Cryptosporidium* isolates analyzed, 39 (95.8%) cases were due to *C. parvum* (Figure 11 A). In contrast, 1 (2.1%) case was due to *C. hominis* (Figure 11 B). One (2.1%) isolate was due to a mixed infection of *C. parvum* and *C. hominis*. The *C. hominis* genotype isolate was detected in an HIV negative patient from Afar (Awash 7) (Figure 7).

4.7.3. DNA sequence analysis of *Cryptosporidium* spp isolates and Subgenotyping

Phylogenetic analysis placed all *C. parvum* isolates into a single allelic group (allelic group IIa), while *C. hominis* was placed in a separate group (allelic group Ib) (Annex I). In this study, in the allele family IIa (Annex I), three subgenotypes were seen in Ethiopian isolates; one subgenotype had 15 copies of the TCA repeat and 2 copy of the TCG repeat, the second subgenotype had 16

copies of the TCA repeat and 1 copy of the TCG repeat; whereas the other subgenotype had 16 copies of the TCA repeat and 2 copies of the TCG repeat. Therefore, the three subtypes were designated IIAA15G2R1 (Table 10 B), IIAA16G1R1, and IIAA16G2R1, respectively (Table 13). In the subgenotype name IIAA15G2R1, IIA indicates that the subtype belongs to allele family IIA, A15 indicates that the subtype has 15 copies of the TCA repeat, and G2 indicates that the subtype has two copy of the TCG repeat (Table 10B).

In addition to the serine repeat, subgenotypes have one copy of the sequence ACATCA immediately after the trinucleotide repeats (TCA and/ or TCG repeat) whereas others have two copies of the sequence; R1 and R2 are used to differentiate these two types of sequences. Therefore, the only difference among the subgenotypes IIAA15G2R1, IIAA16G1R1, and IIAA16G2R1 in this study was the number of TCA and the number of TCG repeats; but, all had one copy of the repeat (R1). In contrast, the *C. hominis* subtype allele Ib seen in the study, which had 9 copies of the TCA repeat and in addition to 3 copies of the TCG repeat and was named IbA9G3 in this study (Table 13).

A representative from each study site and additionally *Cryptosporidium* isolates negative by COWP and SSU-rRNA PCR 13 DNA specimens were sequenced using GP60 gene. DNA from three specimens that failed to produce a product using GP60-AL3532 and AL3534 primers (product length approximately 850 bp; Figure 10 D & E) were amplified using GP60-AL3532 and GP60-LX0029 primers (product length approximately 390 bp; Figure 10 E). DNA sequences (Figure 12; Table 11 & 12) were aligned with sequences deposited at the NCBI nucleotide database using the BLAST programme.

Figure 12: *C. parvum* DNA sequence (Sample code Eth024); displayed by FinchTV (Signal Strength: A = 929 (green), C = 970 (blue), G = 928 (black), T = 1218 (red)).

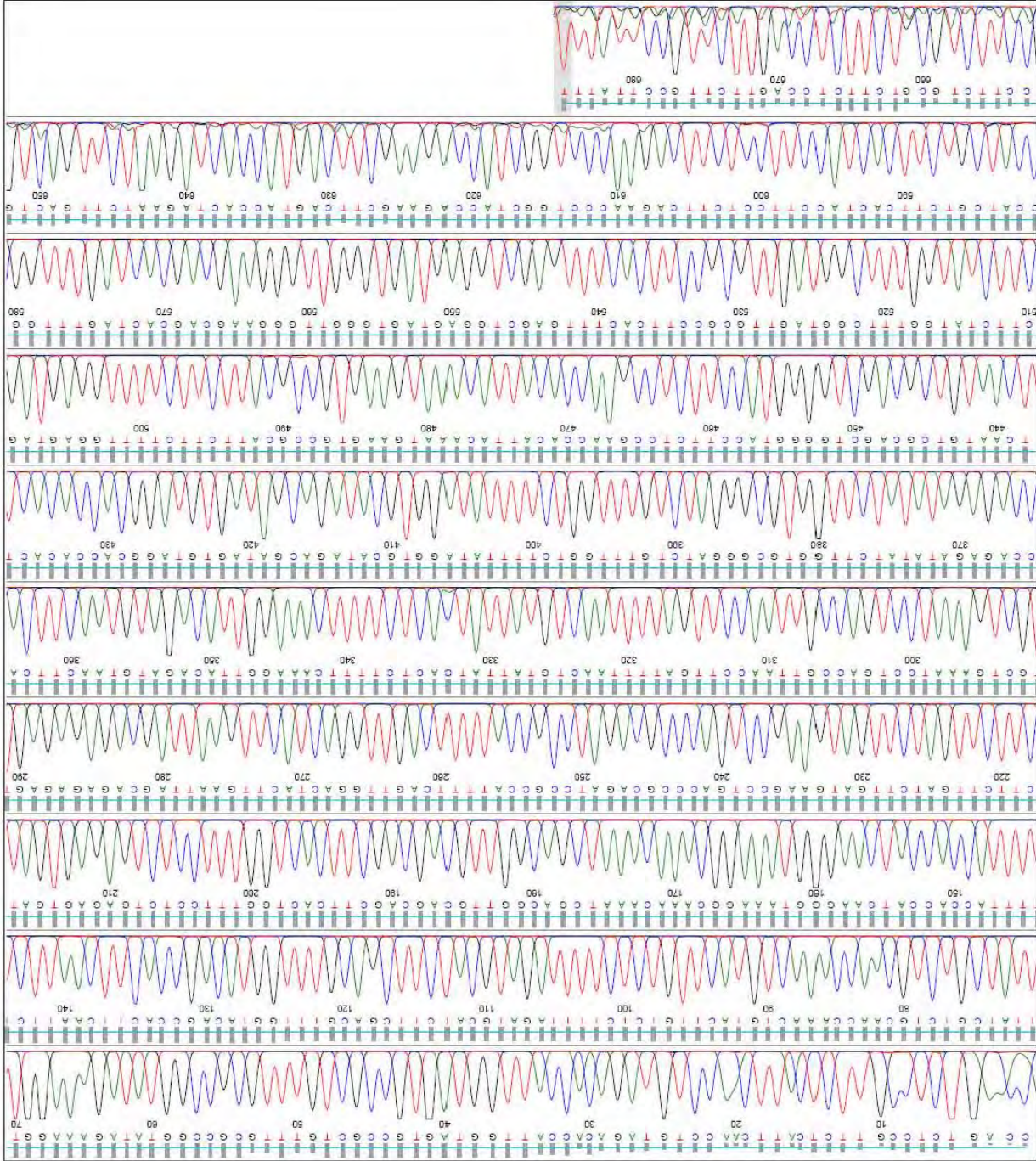


Table 10: *C. parvum* DNA sequence (Sample code Eth024); **(A)** Direct DNA sequence before BLAST; **(B)** After NCBI nucleotide database BLAST (IlaA15G2R1).

(A)

CCAGTCTCCGTTCTCATTCAACCTGTAGACACCATTGGTAGTGCCGCTGTTTGCGCCG
GTATAGAAAGGTTTATCGTCTGCAACCAAAGTACTTGTCTCTTTTAGATGCATCTT
CGACGTTTGGTACAGCCACTTCAATTCTTTACCACCATCAAGGGTAAAGGCAAACA
AATCGACGGTTGCAGCAGCTTCACTGGTTTCCTCTGAGAGTGATCTTCTTGATCTTGA
TGAAGCCTGACCCGCAGATCCGCCATTTTCAGTTGGACTACTTGAATTAGCAGAGAG
AGTGCTGAAATCCTGACCGTTAACCTTGATTTTAACTGTATTATCACTCTTTCAAAG
GTTACAGATGTAACTTCACCAGAGATATATCTTGGTGCGGGATCTGTTTGGTCTTTTA
TAGGTGCATAGACGATAGTGTAGGCACCACACTTCAATGTCGCAGCTGGGGTACCTT
CTCCGAACCACATTACAAATGAAGTGCCGCATTCTTCTTTTGGAGTAGCTTCTATGGT
TTCGGTAGTTGCGCCTTCACTTTGAGCTGGAGTAGTGGGTTGGGAAGCAGCACTAGT
TTGGCCATCGTCTTCACTACCTTCTTCTCAGAACCCTGGCTACCAGAAGCTTCAGTA
CCACTAGAATCTTGACTGCCTTCTGCGTCTTCTCCAGTTCTTGCCTTATTT

(B)

CTGTATTCTCAGCCCCAGCCGTTCCACTCAGAGGAACTTTAAAGGATGTTCTGTTGA
GGGCTCATCATCGTCATCGTCATCATCATCATCATCATCATCATCATCATCAACA
TCAACCGTCGCACCAGCAAATAAGGCAAGAACTGGAGAAGACGCAGAAGGCAGTCA
AGATTCTAGTGGTACTGAAGCTTCTGGTAGCCAGGGTTCTGAAGAGGAAGGTAGTGA
AGACGATGGCCAAACTAGTGCTGCTTCCCAACCCACTACTCCAGCTCAAAGTGAAGG
CGCAACTACCGAAACCATAGAAGCTA

Phylogenetic analysis of the sequences showed that *C. parvum* (Table 10 A) isolates belonged to three subgenotypes: 8 isolates were typed IIA15G2R1 (Table 10 B) (accession number AY738190; Sulaiman *et al.*, 2005), 3 isolates were IIA16G2R1 (accession number DQ640635; Feltus *et al.*, 2006), and one isolate was typed IIA16G1R1 (accession number DQ640633; Feltus *et al.*, 2006). The *C. hominis* genotype belonged to IB9G3 subgenotype (accession number DQ665688; Xiao and Ryan, 2008; Table 13).

Table 11: *C. parvum* genotype small subunit ribosomal RNA (SSU rRNA) gene complete sequence; molecular size=1,746 bp (Accession number = AF115377).

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1 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT
61 AAGTATAAAC TTTTATACGG TTAAACTGCG AATGGCTCAT TATAACAGTT ATAGTTTACT
121 TGATAATCTT TACTTACATG GATAACCGTG GTAATTCTAG AGCTAATACA TGCGAAAAAA
181 CCTAACTTTA TGGAAAGGTT GTATTTATTA GATAAAGAAC CAATATAATT TGGTGATTCA
241 TAATAACTTT ACGGATCACA TTTTAAATGT GACATATCAT TCAAGTTTCT GACCTATCAG
301 CTTTAGACGG TAGGGTATTG GCCTACCGTG GCAATGACGG GTAACGGGGA ATTAGGGTTC
361 GATTCCGGAG AGGGAGCCTG AGAAACGGCT ACCACATCTA AGGAAGGCAG CAGGCGCGCA
421 AATTACCCAA TCCTAATACA GGGAGGTAGT GACAAGAAAT AACAATACAG GACTTTTTTA
481 GTTTTGTAAT TGGAATGAGT TAAGTATAAA CCCCTTTACA AGTATCAATT GGAGGGCAAG
541 TCTGGTGCCA GCAGCCGCGG TAATTCCAGC TCCAATAGCG TATATTAAG TTGTTGCAGT
601 TAAAAAGCTC GTAGTTGGAT TTCTGTTAAT AATTTATATA TAATATTTTT AATATTTATA
661 TAATATTAAC ATAATTCATA TTACTATAAT TTTTATTAGT ATATGAAATT TTACTIONGAG
721 AAAATTAGAG TGCTTAAAGC AGGCATATGC CTTGAATACT CCAGCATGGA ATAATATAAA
781 AGATTTTTAT CTTTTTTATT GGTCTAAGA TAAAAATAAT GATTAATAGG GACAGTTGGG
841 GGCATTTGTA TTAAACAGTC AGAGGTGAAA TTCTTAGATT TGTTAAAGAC AACTIONGAGTGC
901 GAAAGCATTG GCCAAGGATG TTTTCATTAA TCAAGAACGA AAGTTAGGGG ATCGAAGACG
961 ATCAGATACC GTCGTAGTCT TAACCATAAA CTATGCCAAC TAGAGATTGG AGGTTGTTCC
1021 TTACTIONCTTC AGCACCTTAT GAGAAATCAA AGTTTTTGGG TTCTGGGGGG AGTATGGTCCG

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1081 CAAGGCTGAA ACTTAAAGGA ATTGACGGAA GGCACCACC AGGAGTGGAG CCTGCGGCTT
 1141 AATTTGACTC AACACGGGAA AACTCACCAG GTCCAGACAT AGGAAGGATT GACAGATTGA
 1201 TAGCTCTTTC TTGATTCTAT GGGTGGTGGT GCATGGCCGT TCTTAGTTGG TGGAGTGATT
 1261 TGTCTGGTTA ATTCCGTTAA CGAACGAGAC CTTAACCTGC TAAATAGACA TAAAAAATAT
 1321 TAATTTTTTA TTTGTCTTCT TAGAGGGACT TTGTATGTTT AATACAGGGA AGTTTTAGGC
 1381 AATAACAGGT CTGTGATGCC CTTAGATGTC CTGGGCCGCG CGCGCGCTAC ACTGATGCAT
 1441 CCATCAAGTA TATATTCCTA TTTCGAAGGA AATGGGTAAT CTTTTGAATA TGCATCGTGA
 1501 TGGGGATAGA TCATTGCAAT TATTGATCTT GAACGAGGAA TTCCTAGTAA GCGCAAGTCA
 1561 TCAGCTTGCG CTGATTACGT CCCTGCCCTT TGTACACACC GCCCGTCGCT CCTACCGATT
 1621 GAATGATCCG GTGAATTATT CGGACCATAC TTTGTAGCAA TACATGTAAG GAAAGTTTCG
 1681 TAAACCTTAT CATTTAGAGG AAGGAGAAGT CGTAACAAGG TTTCCGTAGG TGAACCTGCA
 1741 GGATCA

Table 12: *C. hominis* genotype small subunit ribosomal RNA (SSU-rRNA) gene complete sequence; molecular size=1,750 bp (Accession number= AF093489).

1 AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT
 61 AAGTATAAAC TTTTATACGG TTAAACTGCG AATGGCTCAT TATAACAGTT ATAGTTTACT
 121 TGATAATCTT TTACTTACAT GGATAACCGT GGTAATTCTA GAGCTAATAC ATGCGAAAAA
 181 ACTCGACTTT ATGGAAGGGT TGTATTTATT AGATAAAGAA CCAATATAAT TGGTGACTCA
 241 TAATAACTTT ACGGATCACA ATTAATGTGA CATATCATT C AAGTTTCTGA CCTATCAGCT
 301 TTAGACGGTA GGGTATTGGC CTACCGTGGC AATGACGGGT AACGGGGAAT TAGGGTTCGA
 361 TTCCGGAGAG GGAGCCTGAG AAACGGCTAC CACATCTAAG GAAGGCAGCA GGCGCGCAAA
 421 TTACCCAATC CTAATACAGG GAGGTAGTGA CAAGAAATAA CAATACAGGA CTTTTTGGTT
 481 TTGTAATTGG AATGAGTTAA GTATAAACCC CTTTACAAGT ATCAATTGGA GGGCAAGTCT
 541 GGTGCCAGCA GCCGCGGTAA TTCCAGCTCC AATAGCGTAT ATTAAAGTTG TTGCAGTTAA
 601 AAAGCTCGTA GTTGGATTTT TGTTAATAAT TTATATAAAA TATTTTTGATG AATATTTATA
 661 TAATATTAAC ATAATTCATA TTACTATTTT TTTTTTTAGT ATATGAAATT TTACTTTGAG
 721 AAAATTAGAG TGCTTAAAGC AGGCATATGC CTTGAATACT CCAGCATGGA ATAATATTAA
 781 AGATTTTTTAT CTTTTTTTATT GGTTCTAAGA TAAGAATAAT GATTAATAGG GACAGTTGGG

841 GGCATTTGTA TTTAACAGTC AGAGGTGAAA TTCTTAGATT TGTAAAGAC AAAC TAATGC
901 GAAAGCATTT GCCAAGGATG TTTTCATTA TCAAGAACGA AAGTTAGGGG ATCGAAGACG
961 ATCAGATACC GTCGTAGTCT TAACCATAAA CTATGCCAAC TAGAGATTGG AGGTTGTTCC
1021 TTAATCCTTC AGCACCTTAT GAGAAATCAA AGTCTTTGGG TTCTGGGGGG AGTATGGTCC
1081 CAAGGCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC AGGAGTGGAG CCTGCGGCTT
1141 AATTTGACTC AACACGGGAA AACTCACCAG GTCCAGACAT AGGAAGGATT GACAGATTGA
1201 TAGCTCTTTC TTGATTCTAT GGGTGGTGGT GCATGGCCGT TCTTAGTTGG TGGAGTGATT
1261 TGTCTGGTTA ATTCCGTTAA CGAACGAGAC CTTAACCTGC TAAATAGACA TAAGAAATAT
1321 TATATTTTTT ATCTGTCTTC TTAGAGGGAC TTTGTATGTT TAATACAGGG AAGTTTTAGG
1381 CAATAACAGG TCTGTGATGC CCTTAGATGT CCTGGGCCGC GCGCGCGCTA CACTGATGCA
1441 TCCATCAAGT ATATATTCCT GTTTCGAAGG AAATGGGTAA TCTTTTGAAT ATGCATCGTG
1501 ATGGGGATAG ATCATTGCAA TTATTGATCT TGAACGAGGA ATTCCTAGTA AGCGCAAGTC
1561 ATCAGCTTGC GCTGATTACG TCCCTGCCCT TTGTACACAC CGCCCGTCGC TCCTACCGAT
1621 TGAATGATCC GGTGAATTAT TCGGACCATA CTTTGTAGCA ATACATGTAA GGAAAGTTTC
1681 GTAAACCTTA TCATTTAGAG GAAGGAGAAG TCGTAACAAG GTTCCCGTAG GTGAACCTGC
1741 AGAAGGATCA

Table 13: *Cryptosporidium* spp. genotypes and subgenotypes from human (n=41) and animal (n=6) isolates, Ethiopia (2008).

Isolate code	COWP RFLP	SSU-rRNA RFLP	GP60 kDa gene		
			Positive [n]	Sequencing result	Allele/ subgenotype
Eth001	<i>C. parvum</i>	<i>C. parvum</i>	1	<i>C. parvum</i>	IIaA15G2R1
Eth024	<i>C. parvum</i>	<i>C. parvum</i>	1	<i>C. parvum</i>	IIaA15G2R1
Eth027	<i>C. parvum</i>	<i>C. parvum</i>	1	<i>C. parvum</i>	IIaA15G2R1
Eth036	<i>C. parvum</i>	<i>C. parvum</i>	1	<i>C. parvum</i>	IIaA15G2R1
Eth046	<i>C. parvum</i>	<i>C. parvum</i>	1	<i>C. parvum</i>	IIaA15G2R1
Eth077	<i>C. parvum</i>	<i>C. parvum</i>	1	<i>C. parvum</i>	IIaA15G2R1
Eth016	<i>C. hominis</i>	nd	1	<i>C. hominis</i>	IbA9G3
Eth015 ^a	<i>C. hominis</i>	nd	1	<i>C. parvum</i>	IIaA16G1R1
Eth031	nd	nd	1	<i>C. parvum</i>	IIaA15G2R1
Eth038	nd	nd	1	<i>C. parvum</i>	IIaA16G2R1
Eth058	nd	nd	1	<i>C. parvum</i>	IIaA16G2R1
Eth069	nd	nd	1	<i>C. parvum</i>	IIaA16G2R1
Eth070	nd	nd	1	<i>C. parvum</i>	IIaA15G2R1
Eth002, 003, 004, 005, 048, 071, 079	<i>C. parvum</i>	<i>C. parvum</i>	7	nd	nd
Eth032, 043, 055, 067	<i>C. parvum</i>	nd	4	nd	nd
Eth033, 049	<i>C. parvum</i>	<i>C. parvum</i>	0	nd	nd

Eth065, 073, 074	nd	<i>C. parvum</i>	3	nd	nd
Eth026, 045, 064	nd	<i>C. parvum</i>	0	nd	nd
Eth035, 042, 044, 053, 059, 066, 068, 075	<i>C. parvum</i>	nd	0	nd	nd
Eth007	Mixed ^b	nd	0	nd	nd
Eth081*,082*, 084*, 092*,093*,094*	<i>C. parvum</i>	nd	Nd	nd	nd
Subtotal	36*	21	27		
Total		47*			

Key:

nd, no data available

^a COWP RFLP showed *C. hominis* restriction profiles twice; SSU-rRNA RFLP failed twice but GP60 sequencing resulted in *C. parvum*.

^b mixed infection (*C. parvum* and *C. hominis*)

* 6 *Cryptosporidium* isolates were from cattle source

5. Discussion

The results of this study showed that the prevalence of intestinal parasitic infection are very high across the country in Ethiopia and *Cryptosporidium* spp. were among the most common enteric parasites associated with diarrhoea. However, since appropriate laboratory tests for *Cryptosporidium* parasites are not routinely done, the available prevalence data for the country may be considered an obvious underestimate.

This study has demonstrated that the prevalence of cryptosporidiosis in Ethiopia to be variable in different geographic regions. For example, its higher prevalence in the Afar region may be explained by the low per capita coverage of clean potable drinking water supply and the intense association of the pastoralist Afar population with domestic animals in comparison with Bishoftu, a modern urban center with potable water and limited contact with domestic animals. Other investigators have also reported that the prevalence of *Cryptosporidium* spp varies in different regions of the world and that species variability could occur in different geographic regions within a country (McLauchlin *et al.*, 2000).

The risk factors for acquiring cryptosporidiosis were determined to be diarrhoea among members of the household. This finding is in agreement with an earlier report from Ethiopia, whereby a case-control study Adamu *et al.* (2006) identified contact with diarrhoeic children less than 5 years of age was a significant risk factor for acquiring the disease. This study is also in agreement with studies from different parts of the world that supports the association of contact with cattle and diarrhoeic patients with *Cryptosporidium* spp infection (Roy *et al.*, 2004; Hunter

et al., 2004a). Furthermore, reports from UK (Hunter *et al.*, 2004b), USA (Roy *et al.*, 2004), and Australia (Robertson *et al.*, 2002) had shown contact with cattle to be a major risk factor for *Cryptosporidium* spp infection.

The overall prevalence of *Cryptosporidium* spp. infection detected in this study (7.6%) was closer to that reported from children with diarrhoea in Addis Ababa and North-western Ethiopia (5.6% to 9%) (Assefa *et al.*, 1996; Adamu *et al.*, 2006; Mersha and Tiruneh, 1992), and far lower than the rate detected in AIDS patients with chronic diarrhoea (25.9% to 39.7%) (Mengesha, 1994; Fisseha *et al.*, 1998 and Endeshaw *et al.*, 2004).

Unlike what was reported from tropical countries where the prevalence of cryptosporidiosis is highest in the rainy season. The highest prevalence in this study was in the dry months of November and March, which is similar to that reported from Kuwait where rainfall is scanty and the weather is dry (Sulaiman *et al.*, 2005).

The high intestinal parasite prevalence within the age group 18 – 35 years in males and in the females was similar to the findings of Fleming (1990). The higher prevalence observed in this age group may be partly explained by their frequent involvement of young males handle cattle and females in cleaning the homesteads and handling food staffs that might be contaminated with cysts and ova of parasites.

In addition, *I. belli* and *Blastocystis hominis* previously reported from HIV/AIDS adult patients (Fisseha *et al.*, 1999) and children with and without diarrhoea, in Addis Ababa (Adamu *et al.*, 2006) were also detected in this study.

This study on the whole showed that diarrhoea is an important manifestation of opportunistic parasites in HIV/AIDS patients, which is in agreement with the report of Lew *et al.* (1997) that showed more than 50% of the AIDS patients to be diagnosed with opportunistic enteroparasitosis. The association between cryptosporidiosis and diarrhoea may have been enhanced by environmental contamination of the drinking water supplies in Ethiopia (Fikrie *et al.*, 2008).

The opportunistic enteric coccidian parasite *I. belli* appears to be common in Ethiopian HIV/AIDS patients (Fisseha *et al.*, 1999; Awole *et al.*, 2003 and Hailemariam *et al.*, 2004). However, the actual prevalence of infection with *I. belli* in HIV positive individuals is likely to be underestimated for a number of reasons. The wide use of cotrimoxazole by patients may also decrease the prevalence of these parasites in this study.

According to Sorvillo *et al.* (1995), these include:

“a) asymptomatic persons may appear healthy but continue to shed oocysts; b) treatment with cotrimoxazole and other antibiotics for treating other infections may confer some protection against this parasite; c) the oocysts in faeces may have been easily overlooked in wet mounts because the thin oocyst wall is refractile and difficult to detect even in known positive specimens; and, d) the number of oocysts excreted is usually small”.

Cryptosporidium spp. and *I. belli* infections were significantly higher in HIV positives with CD4⁺ T cell count of <200/ μ L and receiving ART compared to HIV positive patients not receiving ART, indicating that the two parasites are less likely to cause serious infections in persons with high CD4⁺ T cell count (Foudraine *et al.*, 1998).

Cryptosporidium spp have become common parasites that cause chronic diarrhoea and wasting disease in HIV-infected patients with CD4⁺ T cell counts <100 cells/ μ L (Carr *et al.*, 1998), as antimicrobial agents have limited efficacy in preventing or eradicating them (Foudraine *et al.*, 1998). Although studies assessing the difference in the incidence of cryptosporidiosis in persons with high (normal) CD4⁺ T cell counts are lacking, it is known that diarrhoea due to *Cryptosporidium* spp infections resolve spontaneously with ART immune restoration among HIV-infected patients (Carr *et al.*, 1998; Foudraine *et al.*, 1998).

Although Mekonnen (2007) reported that the prevalence of intestinal helminth infections to be higher among HIV-positive individuals with CD4⁺ T cell count less than 200 cells/ μ L and receiving ART than in HIV-positive individuals with CD4⁺ T cell count more than 200 cells/ μ L, the prevalence of helminthes was not high in this study. It may be due to the fact that a large proportion of the study participants were drawn from semi-urban and urban dwellers where the prevalence of helminthes is lower than among rural dwellers where the prevalence of helminthes is normally higher.

It is known that cryptosporidiosis causes significant morbidity and mortality in immunocompromised individuals, particularly in those with HIV/AIDS infection (Caccio, 2005). Furthermore, severity of cryptosporidiosis is dependent on the level of CD4⁺ T cell count and those patients with lower (<200 cells/ μ L) CD4⁺ T cell counts are at greatest risk of the disease (Carr *et al.*, 1998). However, with the introduction of ART for patients with HIV/AIDS, the prevalence of this opportunistic infection has decreased dramatically. It appears that the protease inhibitors included in ART not only restore cell-mediated immunity, but also have a direct

inhibitory effect on the proteases of opportunistic protozoa, including *Cryptosporidium* (Pozio and Morales, 2005).

This study showed not only *Cryptosporidium* but also some other intestinal parasitic infections (*G. lamblia*, *Entamoeba histolytica/ E. dispar* and *I. belli*) to be common among HIV-positive patients; and that there is a relationship between the type of parasite and the CD4⁺ T cell count. This finding is similar to the study reported from HIV-positive patients in Cameroon (Sarfati *et al.*, 2006). The failure to detect some parasites frequently found in patients with diarrhoea was due to the inappropriateness of the detection method used in the study. Therefore, the actual prevalence of intestinal parasites among patients with HIV/AIDS might have been underestimated.

The prevalence of *Cryptosporidium* spp and other intestinal parasites in HIV-positive individuals was 48 %, which is comparable to the 52.6 % prevalence rate reported in a previous study from a teaching hospital in Jimma, Ethiopia (Hailemariam *et al.*, 2004) whereas, in this study, the prevalence of *Cryptosporidium* spp, *I. belli* and *B. hominis* was higher than the rate reported by Awole *et al.* (2003). It has been suggested that difference in the stage of HIV infection and the ART status of patients could probably explain the difference in the prevalence of opportunistic parasites in HIV-positive individuals in different parts of Ethiopia (Endeshaw *et al.*, 2006). The existence of such variation in prevalence may be explained by the difference in geographic location and general hygiene of the population as reported from elsewhere (Fleming, 1990).

Although *B. hominis* has not been clearly classified to be pathogenic, its detection in immunocompromised HIV/AIDS patients with diarrhoea is consistent with its possible implication as an opportunistic infection in Ethiopia (Endeshaw *et al.*, 2006).

It is known that opportunistic infections disproportionately affect persons with lower CD4⁺ T cell count due to HIV infection or due to other factors that affect the immune system. This is associated with enhanced establishment of opportunistic parasites. Thus, these parasites can serve as indicators/sentinels/ in assessing the health status of HIV infected diarrhoeal patients with and without ART treatment. The higher prevalence of *Cryptosporidium* spp. infection that was seen in HIV sero-positives who were not on ART can be an indicator of the lower CD4⁺ T cell count.

The widespread use of cotrimoxazole as a prophylactic measure against opportunistic infections in persons living with HIV/AIDS may have contributed to the lack of detection of *Cyclospora* spp. as it is highly susceptible to this antibiotic. In addition, the fact that *Cyclospora* is highly associated with wet seasons (Bern *et al.*, 2000; Ortega *et al.*, 1998) and that this study was conducted during shortage of rain in this particular year could be another explanation for its non-detection.

The higher prevalence of *Cryptosporidium* and other opportunistic parasite spp. in HIV/AIDS patients with lower socioeconomic conditions and their decline among patients with better education could be due to the latter's improved general socioeconomic and hygienic conditions.

The application of molecular characterization of *Cryptosporidium* isolates in field epidemiological studies is the most reliable approach for proper diagnosis and establishing of the source of cryptosporidiosis in human infections. This is because definitive identification of the

species of *Cryptosporidium* parasite has not been possible by using the routine diagnostic methods (Xiao and Ryan, 2008). Thus, the molecular characterization of *Cryptosporidium* spp isolates used in this study has enabled determination of the origin and distribution of *Cryptosporidium* genotypes and subgenotypes in human cryptosporidiosis in Ethiopia.

However, the fact that molecular characterization of the *Cryptosporidium* parasite isolates by PCR was successful in only 54% of faecal samples could be explained by the inappropriate storage temperature and extended transportation time from the sample sites to the laboratory in Addis Ababa and during shipment to Germany. These may have led to oocyst damage, resulting in the loss of DNA (Gelanew *et al.*, 2007) and the subsequent failure of PCR amplification. In addition, although the DNA isolation kit used was especially designed for DNA preparations from faeces, the presence of inhibitors in the faecal samples may have been an additional reason for the failure in some of the PCR amplifications.

In the present study, PCR amplification and genotyping of *Cryptosporidium* positive samples both from human and cattle sources showed *C. parvum* to be the predominant species (95.7%) in Ethiopia. Similar findings have been reported from other countries such as France (51%), the Netherlands (72%) and Italy (92.3%). On the other hand, *C. hominis* is the most frequently isolated species from humans in many countries such as Australia (76%), Canada (76%), USA (67%), Peru (79%), Thailand (83%), Japan (68%), and South Africa (82%) (Xiao and Ryan, 2008; McLauchlin *et al.*, 2000; Xiao *et al.*, 2001; Guyot *et al.*, 2001; Tiangtip and Jongwutiwes, 2002).

The results of PCR-RFLP in this study showed *C. parvum* and *C. hominis* to be the causes of human cryptosporidiosis in Ethiopia. This is in agreement with the findings based on molecular genotyping of isolates from many regions of the world (Xiao and Ryan, 2004). However, since human cryptosporidiosis is not limited to the two species and others such as *C. felis*, *C. meleagridis*, *C. muris*, and *C. canis* are also known to occasionally cause human infections (Morgan *et al.*, 2000), their relevance in Ethiopia cannot be discounted for certain before appropriate investigations are made .

Molecular epidemiological studies have indicated the proportion of *C. parvum* infections in humans to be much higher in rural than in urban areas (Learmonth *et al.*, 2004). This could be due to zoonotic transmission of *C. parvum* from animals which are more prevalent in rural areas. However, since the majority of study participants with cryptosporidiosis in this study were urban dwellers and had limited contact with domestic animals, drinking water which usually is drawn from rural sources may have been contaminated with cattle feces could serve as the source of infection. This is because municipality water treatment using chlorination did not seem to protect the population from *Cryptosporidium* infection even in big urban centers such as Addis Ababa and Adama (Fikrie *et al.*, 2008).

Subgenotyping is used to separate *C. hominis* from humans and *C. parvum* from humans and ruminants. One subgenotyping method is DNA sequence analysis of the 60 kDa glycoprotein. The GP60 gene is similar to a microsatellite sequence as it has tandem repeats of the serine-coding trinucleotides TCA, TCG or TCT at the 5' end. However, in addition to variations in the number of trinucleotide repeats, there are extensive sequence differences in the non-repeat regions, which categorize *C. parvum* and *C. hominis* to several subtype families. Within each

subtype family, the subgenotypes differ from each other mostly in the number of trinucleotide repeats (TCA, TCG or TCT microsatellite). The nomenclature of GP60 subtypes begins with the subtype family designation (Ia, Ib, Id, Ie, If, etc. for *C. hominis*, and IIa, IIb, IIc, IId, etc. for *C. parvum*) followed by the number of trinucleotide repeats of TCA (represented by the letter A), TCG (represented by the letter G), and TCT (represented by the letter T) (Sulaiman *et al.*, 2005).

Sequence analysis of GP60 gene is widely used in *Cryptosporidium* subgenotyping because of its sequence heterogeneity. Unlike other subgenotyping targets, such as double stranded RNA, internal transcribed spacer-2 and traditional microsatellites and minisatellites, which are generally considered non-functional, GP60 is located on the surface of apical region of invasive stages of the parasite, and is one of the dominant targets for neutralizing antibody responses in humans (O'Connor *et al.*, 2007). Thus, it is possible to link biologic characteristics of the parasites and clinical presentations with the subtype family identity. Some of the *C. parvum* subtype families, such as IIa and IId, are found in both human and ruminant isolates and are responsible for zoonotic cryptosporidiosis.

In areas with both IIa and IId, such as Spain, IIa subtypes preferentially infect calves whereas IId subtypes preferentially infect lambs and goat kids (Quilez *et al.*, 2008a). Other *C. parvum* subgenotypes, especially IIc (formerly known as Ic), have been so far found only in humans (Xiao and Feng, 2008).

Multiple subgenotypes were present in the *C. parvum* allele family of IIa in this study. Within each subgenotype family, subgenotypes differed from each other mostly in the number of trinucleotide repeats (TCA or TCG) coding for the amino acid serine (Sulaiman *et al.*, 2005; Xiao

and Ryan, 2008). In this study, except for one isolate, all GP60-DNA sequencing confirmed the results of the COWP and/or SSU-rRNA PCR-RFLP. One isolate that had been typed *C. hominis* on the basis of the COWP PCR-RFLP resulted in a GP60 gene sequence consistent with *C. parvum* IIAA16G1R1. These conflicting results could not be resolved by repetition of the COWP PCR-RFLP. However, as this isolate was the only IIAA16G1R1 subgenotype, a mix-up of samples can be ruled out. An attempt to type this isolate by SSU-rRNA PCR-RFLP failed, as this DNA preparation repetitively produced no SSU-rRNA PCR product. The molecular genotyping of isolates by SSU-rRNA PCR-RFLP was backed up by the other two methods (COWP PCR-RFLP and GP60 DNA sequence) except for Eth026, 045, 064, 065, 073, and 074. The patient with *C. parvum* and *C. hominis* mixed infection had close contact with cattle and lived in overcrowded family and unhygienic conditions. This is indicative of possible multiple contamination of sources to which humans were exposed resulting in mixed infections.

Eleven out of thirteen *C. parvum* isolates successfully sequenced by using GP60 gene locus were from humans and they belonged to IIA allele family. The fact that eight of the *C. parvum* genotypes had similar subgenotype sequences (IIAA15G2R1) identified in isolates from calves (O'Brien *et al.*, 2008) is a proof of zoonotic source in Ethiopia. Similar findings have been reported from other countries such as India (Gatei *et al.*, 2006), Kenya and Malawi (Gatei *et al.*, 2003), Peru (Xiao *et al.*, 2001), Portugal (Alves *et al.*, 2003), South Africa (Leav *et al.*, 2002), Thailand (Peng *et al.*, 1997), Uganda (Sulaiman *et al.*, 1998), the UK (Chalmers *et al.*, 2005; Leoni *et al.*, 2006) and USA (Peng *et al.*, 2003; Xiao *et al.*, 2003), which is indicative of the abilities of these strains in infecting humans and animals.

There were also similar findings from children in Kuwait where they were mainly infected with IIAA15G2R1 (Sulaiman *et al.*, 2005). Furthermore, a study of sporadic human cryptosporidiosis in Wisconsin (USA) showed the involvement of mainly IIAA15G2R1 and IIAA15G2R2 (Feltus *et al.*, 2006). The work of Xiao and Ryan (2008) has also demonstrated IIAA15G2R1 subgenotype of *C. parvum* to be a zoonotic infection to humans.

Although human-to-human transmission of the zoonotic allele is known to occur, this finding, and the fact that none of the *C. parvum* isolates from humans belonged to the anthroponotic allele IIc (Annex I), may imply that zoonotic *C. parvum* is the major source of human cryptosporidiosis in Ethiopia. Several cryptosporidiosis outbreaks in Europe and North America have been attributed to *C. parvum* allele IIA, including a waterborne outbreak in Minnesota, USA and two in Northern Ireland (Glberman *et al.*, 2002; Xiao and Ryan, 2004).

The molecular subgenotyping analysis of human *C. parvum* isolates has revealed the IIA subgenotype which is the same as the cattle subgenotype, suggesting that in Ethiopia *C. parvum* infections is of animal origin. Based on this molecular characterization of *Cryptosporidium* spp isolates, the zoonotic subgenotype is the major cause of human cryptosporidiosis in Ethiopia, suggesting that animal-to-human transmission may have led to the establishment of anthroponotic transmission.

6. Conclusions and Recommendations

1. Sequencing the GP60 gene fragments of different isolates resulted in three different subgenotypes of *C. parvum*, most belonging to the zoonotic allele family (IIa), and just one belonging to a subgenotype of *C. hominis* (Ib).
2. The majority of human *Cryptosporidium* spp isolates were identified as *C. parvum* of zoonotic origin with only one *C. hominis* isolate, and another one a mixed infection with *C. hominis* and *C. parvum*.
3. This study also confirmed earlier reports that antiretroviral treatment in HIV/AIDS patients would reduce infection with *Cryptosporidium* spp and other diarrhogenic protozoan parasites.

Based on the findings of the present study the following may be recommended:

1. Studies in typing of *C. hominis* genotypes would be necessary to have a comprehensive knowledge of the disease in Ethiopia.
2. Creation of a central national database on the prevalence of cryptosporidiosis would be a useful step so that information could be pooled from different regions of Ethiopia to better understand the epidemiology of the disease.

3. The reporting of cryptosporidiosis must be made mandatory by all clinical diagnostic laboratories by introducing the modified Ziehl-Neelson staining method in all Regional State Health departments in Ethiopia.

4. Further study on identification and molecular characterization of *Cryptosporidium* species from drinking water and animal sources in Ethiopia completes the present study.

5. Further study on characterization of *Cryptosporidium* species from immunodeficient patients such as malnourished children, congenital immunodeficient patients, organ transplant patients and others sources should be done in Ethiopia.

7. References

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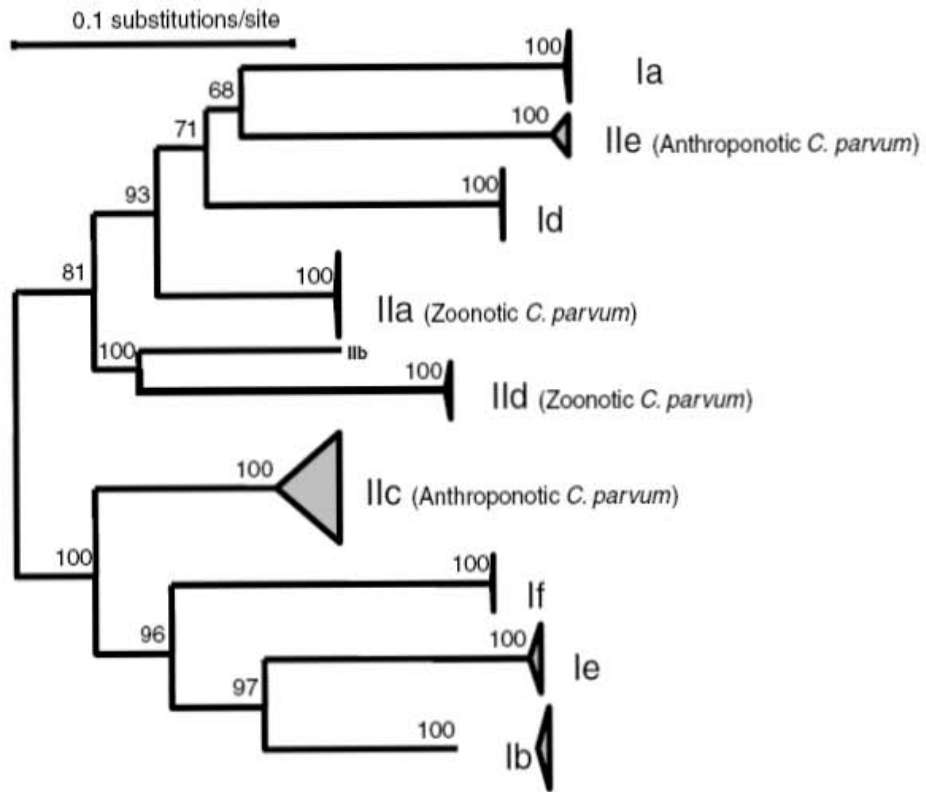
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Appendix I: Phylogenetic relationship among major subtype families of *C. parvum* (IIa, IIc, IId, and IIe) and *C. hominis* (Ia, Ib, Id, Ie, and If) based on a neighbour-joining analysis of the GP60 gene sequences (Xiao and Ryan, 2008).



Appendix II: የስምምነት ቅጽ (Amharic)

ተራ ቁጥር: _____

የካርድ ቁጥር: _____

ስም: _____

እኔ ከላይ በስም ተጠቃሽ የአንጀት ጥገኛ በሽታ (*Cryptosporidiosis*) ሳይኖርብኝ እንደማይቀር ተነግሮኝ በሽታየን ለመመርመር የሠገራ ናሙና (*Stool specimen*) አንደሚያስፈልገው ተነግሮኛል። ይህ ናሙና የበሽታዬን ምንነት ለማወቅ የሚያስችል ምርመራ የሚካሄድበት ነው። በተጨማሪም ከዚህ ናሙና የተወሰነው ክፍል በሽታውን አምጪ ለሆነው ጥገኛ ተወሳክ ዝርያ (*Cryptosporidium genotyping*) ለመለየት ለሚደረገው ጥናት ሥራ አላማ እንደሚውል ተገልጾልኛል። አኔም ስለጥናቱ በሚገባ ከተረዳሁ በኋላ አስፈላጊ ናቸው ብዬ ያሰብኳቸውን ጥያቄዎች ጠይቄ አስፈላጊውን ማብራሪያ አግኝቻለሁ። በጥናቱ መረጃ መሰብሰቢያ መጠይቅ ላይ የተዘረዘሩት መረጃዎች ሁሉ በሚስጥር የሚያዙ መሆናቸውንና የስምምነት ቅጹም በሚስጥር ተይዞ ጥናቱ አንደሚካሄድ ተገንዝቤአለሁ።

ስለራሴ የምጠየቀውን መረጃ ያለመስጠት፣ ለጥናቱ ያለመተባበርና በየትኛውም ወቅት ከጥናቱ እራሴን የማግለል መብቴ የተጠበቀ መሆኑ የተገለጸልኝ ሲሆን ይህንንም በማድረግ ምክንያት በአጠቃላይ በሽታዬን ከመታከም የሚያግደኝ ነገር የሌለ መሆኑን በሚገባ ተረድቻለሁ። ስለሆነም ለተመራማሪው መስማማቱን በፊርማዬ ያረጋገጥኩት ሁኔታውን በመረዳቴ ነው። በተጨማሪም አስፈላጊ ሆኖ ሲገኝ የሚወሰደው ናሙና ወይም የሚገኘው የአንጀት ተወሳክ ዝርያ ለጥናት ወደ ወጭ አገር ቢላክ ተቃዋሚ የለኝም። እነዚህም ከጥናቱ የተረፈው ናሙና ወደ ፊት ለሌላ የዚህ ተመሳሳይ የጥናት ተግባር ላይ ቢውል ፈቃደኛ ነኝ።

ፊርማ: _____

ቀን: _____

Appendix II: Consent form (English)

S/No.: _____

Card No.: _____

Full Name: _____

I the above mentioned _____ have been told that I may have intestinal parasitic disease known by cryptosporidiosis 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 *Cryptosporidium* oocyst for molecular genotyping and subgenotyping. I have asked questions relevant to the study and got satisfied answers with clarifications. Information and data in the survey questioner will be handled with 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 understand, agreed and signed this consent form. In addition to the above mentioned, I have no objection if part of the stool specimen will be shipped to other country for further examination and characterization. I have also agreed to use this sample for the same experiment in the future.

Signature: _____

Date: _____

Appendix IV: Epidemiological index card for *Cryptosporidium* spp

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. Patient living area _____ (1 = Urban, 2 = Rural)
7. Abdominal health status: _____ (1 = Health, 2 = Sick)
8. Stool consistency: _____ (1 = Liquid/watery, 2 = Semi-liquid, 3 = Loose, 4 = soft)
9. Stool character _____ (1 = Bloody mucoid, 2 = Mucoid)
10. Contact with person affected by diarrhoea _____ (1 = Yes, 2 = No)
11. Are there animals at your home: _____ (1 = Yes, 2 = No)
12. Which animal: _____ (1 = Cattle, 2 = Dog, 3 = Cat, 4 = other, specify)
13. Where do animals live: _____ (1 = at home, 2 = at different place)
14. Where do they graze: _____ (1 = at home (cut and carry), 2 = at field)
15. Contact with animals or dung: _____ (1 = yes, 2 = no contact)
16. Source of drinking water _____ (1 = Tape water, 2 = River/unprotected)
17. Date of Diagnosis _____
18. Other IP; Identification methods:
Direct Microscopy _____
Concentration: _____
19. *Cryptosporidium* _____

DECLARATION

I, the undersigned, declare that this PhD Dissertation is my own original work and has not been presented for a degree in any other university, and all sources of materials used for the Dissertation have been duly acknowledged.

Student: Haileeyesus Adamu

Signature: _____

Addis Ababa, Ethiopia

Supervisor: Professor Beyene Petros

Signature: _____

Addis Ababa, Ethiopia