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**PREVALENCE AND MOLECULAR CHARACTERIZATION  
OF *CRYPTOSPORIDIUM* SPECIES. FROM HUMANS AND  
CATTLE IN ADDIS ABABA AND ITS ENVIRONS, ETHIOPIA**

**PHD DISSERTATION**

**BY**

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**DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC  
HEALTH PHD PROGRAM IN VETERINARY PUBLIC HEALTH**

**June, 2017**

**Bishoftu, Ethiopia**

**PREVALENCE AND MOLECULAR CHARACTERIZATION  
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CATTLE IN ADDIS ABABA AND ITS ENVIRONS, ETHIOPIA**

A dissertation submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Public Health

By

**Manyazewal Anberber Zeleke**

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Entitled: **Prevalence and molecular characterization of *Cryptosporidium* species from humans and cattle in Addis Ababa and its environs, Ethiopia**  
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## DEDICATION

This research work is dedicated to

Dr Girma Zewde, my former principal Advisor; Professor Fisha Gebreab, founder of the College and Professor Mosses Kyole, Instructor at the College, who departed during the course of this research, May their soul rest in peace

My Great-Grandfather *Kegnazmach* Ababulo Aredo, Grandfather Zeleke Ababulo and all heroes of the time who bravely fought for the sovereignty and freedom of their nation and sacrificed at *Welwel, Ogaden*, Ethiopia. May God rest their soul in peace

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## STATEMENT OF AUTHOR

First, the Author declares that this dissertation is his *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for a PhD degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College Library to be made available to borrowers under rules of the Library. The author solemnly declares that this thesis is not submitted to any other institution anywhere for the award of any academic Degree, Diploma, or Certificate.

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## LIST OF ABBEVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral therapy
bp	base pair
CDC	Center for Disease control and prevention
COWP	<i>Cryptosporidium</i> Oocyst Wall Protein
CSA	Central Statistics Agency
DFA	Direct Fluorescence Antibody
DIC	Differential Interference Contrast
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucleotide triphosphates
DWI	Drinking Water Inspectorate
EDTA	Ethylenediamine Tetracetic Acid
EIAs	Enzyme immunoassays
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Environmental Protection Agency
GCG	Genetics Computing Group
GP60	60-kDa glycoprotein
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
ID	Infective Dose
IFA	Indirect Fluorescent Antibody
IFN- $\gamma$	Interferon $\gamma$
IFT	Immunofluorescence Test
IgA	Immunoglobulin A
IMS	Immunomagnetic bead Separation
m.a.s.l	meters above sea level
mM	milli Mole
MOH	Ministry of Health
MZN	Modified Ziehl Neelsen

## LIST OF ABBREVIATIONS (*Continued*)

NTZ	Nitazoxanide
OPG	Ocysts per gram
PAHO	Pan American Health Organization
PCR	Polymerase Chain Reaction
Pmol	Pico mole
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
Rpm	Revolution per minute
rRNA	ribosomal Ribonucleic Acid
SAF	Sodium acetate-Acetic acid-Formaldehyde
SPSS	Statistical Package for Social Sciences
Ssp I	Restriction enzyme
SSU-rRNA	Small Subunit ribosomal RNA
UNAIDS	United Nations program on HIV/AIDS
WHO	World Health Organisation
$\chi^2$	Chi Square
$\mu$ l	Microlitre
$\mu$ m	Micrometre

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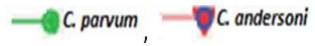
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## **Prevalence and molecular characterization of *cryptosporidium* species from humans and cattle in Addis Ababa and its environs, Ethiopia**

### **ABSTRACT**

Cryptosporidiosis causes significant diarrheal disease in humans and animals worldwide. In this study, conducted during June/2014 to June/2015, cross-sectional and longitudinal study designs were employed to determine the magnitude of *Cryptosporidium* infection in humans and cattle and to assess its public health significance in Addis Ababa and its surrounding area. The objectives of the study were to determine the prevalence, characterize species of the parasite and identify risk factors of the infection in humans and dairy cattle in the area. Faecal samples collected from 422 cattle and 300 humans were examined by the Modified Ziehl-Neelsen, PCR-RFLP and sequencing. Data on risk factors of the infection were collected using a pre-tested questionnaire and analysis was made by the SPSS software. The overall prevalence of *Cryptosporidium* in cattle and humans was 18.6% (95% CI: 14.8–22.5) and 9% (95% CI: 5.5–12.3), respectively. *Cryptosporidium andersoni* (73.3%) and *C. parvum* (26.7%) were identified in cattle, whereas *C. parvum* (81.5%) and *C. hominis* (18.5%) were detected in humans. In cattle, *Cryptosporidium* infection was significantly associated with absence of calving pen (OR=2.46, 95% CI: 1.08–5.61), absence of calf bedding (OR=10.55, 95% CI: 4.89–22.66), drinking river/stream water (OR=2.9, 95% CI: 1.5–5.5) and group penning of calves (OR=2.7, 95% CI: 1.0–7.0). While in humans, the infection showed significant association with drinking stream/well water (OR=3.3, 95% CI: 1.5–7.4) and contact with cattle and their faeces (OR=3.6, 95% CI: 1.4–9.5). *Cryptosporidium* infection occurred from week 1 to 3 months of age in calves with an overall prevalence of 40% (12/30), and pre-weaned and post-weaned calves were merely infected with *C. parvum* and *C. andersoni*, respectively. Phylogenetic analysis showed that the *C. parvum* human isolates belong to the bovine genotype family. In conclusion, the zoonotic *C. parvum* is prevalent and widely distributed in cattle and humans in the study area, pre-weaned calves are the potential risk group for human infection requiring special management attention to prevent transmission of the infection to humans and lessen contamination of the environment.

**Key words:** Cattle, *Cryptosporidium*, Ethiopia, Human, PCR-RFLP, Risk factors

## INTRODUCTION

Cryptosporidiosis is one of the most common causes of gastrointestinal diseases in a wide spectrum of vertebrate hosts, including humans (Fayer *et al.*, 2010; Chalmers and Katzer, 2013). *Cryptosporidium* parasites are very small intestinal protozoa dwelling in the stomach or in the small intestine of infected animals. *Cryptosporidium muris* was initially reported in the gastric glands of the common mouse (Tyzzer, 1907, 1910), and later on a related sporozoan from the intestine of mouse was identified and named as *C. parvum* (Tyzzer, 1912). However the impact of *Cryptosporidium* infection on animal and human health had not been recognized till the first cases were identified in calves (Panciera *et al.*, 1971) and in immunocompromized persons (Nime *et al.*, 1976). At present, 26 *Cryptosporidium* species and over 61 genotypes have been recognized; eight valid species were reported to infect humans among which *C. hominis* and *C. parvum* are the most important ones (Bouzid *et al.*, 2013; Lebbad *et al.*, 2013; Adamu *et al.*, 2014). The public health significance of the disease had been reported by a number of researchers around the world (Zaidah *et al.*, 2008; Szonyi *et al.*, 2010; Adamu *et al.*, 2014). In healthy individuals, the infection is usually self-limiting and resolves within 2–3 weeks of profuse, watery, non bloody diarrhoea, weight loss, abdominal pain, anorexia, fatigue and cramps (Warren and Guerrant, 2008). However, in immunocompromized persons such as AIDS and cancer patients the infection is more serious and can cause prolonged, debilitating, life-threatening illness (Chalmers and Davies, 2010). Owing to the lack of prophylactic and therapeutic measures against the disease, the mortality rate in humans is an emerging public health issue worldwide (Kothavade, 2011).

The prevalence of *Cryptosporidium* infection is higher in developing countries, 5.9- 17%, compared to developed countries, 0.1–2% (Mumtaz *et al.*, 2010). In developing countries, the infection rate is higher in infants and children less than 5 years of age (Chalmers and Davies, 2010). One of the main reasons for Cryptosporidiosis to gain importance was due to the epidemic of HIV/AIDS that created a pool of susceptible people during the 1980s. In cattle, the economic importance of *Cryptosporidium* and its role as a major cause of diarrhoea and gastrointestinal illness had been reported (Paul *et al.*, 2009; Szonyi *et al.*, 2010). The routes of transmission could be person to person through direct or indirect contact, animal to animal, animal to human, waterborne (drinking or recreational water) and food borne (Painter *et al.*, 2015). The

zoonotic *Cryptosporidium parvum* is known to occur widely in both source and drinking water and has caused waterborne outbreaks of gastroenteritis. Farm animals and human sewage discharges are generally considered the major sources of surface water contamination with *C. parvum* (Painter *et al.*, 2015). Since *Cryptosporidium* infection is common in wildlife, it is conceivable that wildlife can also be a source of *Cryptosporidium* oocysts in water (Robinson *et al.*, 2011). Understanding the spatial and temporal variation in the risk of *C. parvum* infection in dairy cattle is essential for designing cost-effective watershed management strategies to protect drinking water sources (Szonyi *et al.*, 2010).

In Ethiopia, studies conducted on HIV/AIDS patients showed prevalence of cryptosporidiosis ranging from 12.1% to 43.9% (Zelalem *et al.*, 2008; Adamu and Petros, 2009; Getaneh *et al.*, 2010). The prevalence in normal, non- diarrheic, children was reported to be between 8.1% and 12.2% (Ayalew *et al.*, 2008; Tigabu *et al.*, 2010) and that of diarrheic children and adults was between 5.6% and 9% (Adamu *et al.*, 2010), showing the significance of the disease in children and immunocompromized patients in the country. Although studies on dairy farms and drinking water sources are scarce in the country, the few studies conducted so far showed occurrence of *Cryptosporidium* oocysts in samples from both sources signifying their importance to human infections. A study conducted on twenty two drinking water sources in Addis Ababa and some nearby towns pointed out 100% positivity for *Cryptosporidium* oocysts (Fikrie *et al.*, 2008). Studies carried out on dairy farms in central and southern part of the country reported prevalence rates ranging from 2.3% to 27.8% (Abebe *et al.*, 2008; Wegayehu, 2009; Adamu, 2010; Alemayehu *et al.*, 2013; Dinka and Berhanu, 2015; Wegayehu *et al.* 2013, 2016). Most of the above studies employed conventional microscopy technique using the acid-fast stain; however this technique does not allow species identification, as the oocysts are morphologically indistinguishable (Chalmers and Katzer, 2013). Therefore, investigating *Cryptosporidium* infections in cattle and humans and characterizing the parasite to species/genotype level using molecular methods would help to assess the transmission dynamics of the parasite in the interface between human-cattle and the environment.

## **Statement of the problem**

Studies on human cryptosporidiosis in Ethiopia showed high prevalence of the disease in children and HIV/AIDS patients; however, the contribution of cattle for this high magnitude has not been well studied. Verifying the magnitude and distribution of *Cryptosporidium* species infecting dairy cattle and humans and assessment of their genetic relation is essential to design cost-effective management strategy to prevent human infection, losses in dairy cattle production and contamination of drinking water sources.

## **Hypothesis**

There is high degree of genetic relation between *Cryptosporidium* species/genotype infecting human and cattle in Addis Ababa and its surrounding areas, hence, high transmission of the parasite from cattle to human (and vice versa).

## **Objectives**

### **General Objective**

To investigate the prevalence of *Cryptosporidium* Infection and characterize species of the parasite in dairy cattle and humans in Addis Ababa, and its surrounding area.

### **Specific objectives**

1. To determine the prevalence of *Cryptosporidium* infection and identify species of the Parasite in dairy cattle in and around Addis Ababa.
2. To determine the prevalence of *Cryptosporidium* infection and identify species of the parasite in farm attendants and HIV/AIDS patients in and around Addis Ababa.
3. To investigate risk factors of *Cryptosporidium* infection in humans and dairy cattle in and around Addis Ababa
4. To determine the age related prevalence of *Cryptosporidium* infection in dairy cattle calves at two selected dairy farms in Holota town.

## 2. LITERATURE REVIEW

### 2.1. Taxonomy

Cryptosporidia have been recognized as ubiquitous and significant enteropathogens of humans and livestock, but the taxonomy of the genus *Cryptosporidium*, as is the case for many other protozoan parasites is still unsatisfactory and is undergoing major revisions in light of new developments in biochemical and genetic data (Plutzer and Karanis, 2009). *Cryptosporidium* had been classified as a coccidian parasite despite the many unique characteristics, such as the lack of host specificity, resistance to anticoccidial treatment, ability for autoinfection and its particular habitation within the host cell, however, phylogenetic analysis on the small-subunit rRNA locus indicated that *Cryptosporidium* is indeed not related to the coccidia. Cross-reaction of an anti-cryptosporidial monoclonal antibody with gregarines (Apicomplexa: Gregarinasina) suggested a close relation which was later confirmed by molecular phylogeny based on protein sequences, 28s rRNA and  $\alpha$ - and  $\beta$ -tubulin genes (Templeton *et al.*, 2010).

Subsequently, it was proposed that *Cryptosporidium* should be placed in a taxonomic group separate from the coccidians and close to the gregarine parasites (Plutzer and Karanis, 2009). Therefore, its taxonomic determination can be more accurately described as follows: *Cryptosporidium* spp. belong to the phylum Apicomplexa (=Sporozoa), whose members possess an apical complex; class Sporozoa, whose members reproduce by asexual and sexual cycles; subclass Coccidia, the life cycle of which involves merogony, gametogony, and sporogony; order Eucoccidiida (=Eucoccidiorida), in which schizogony occurs; suborder Eimeriina (=Eimeriorina), in which independent micro and macrogamy develop; and family Cryptosporidiidae, whose members have four naked sporozoites within their oocysts (Plutzer and Karanis, 2009). The clarification of *Cryptosporidium* taxonomy is useful for understanding the biology, assessing the public health significance of *Cryptosporidium* species in animals and the environment, characterizing transmission dynamics, and tracking infection and contamination sources (Xiao and Fayer, 2008).

## 2.2. Species and genotypes

The naming of *Cryptosporidium* species is undergoing rapid changes. The early classification of *Cryptosporidium* relying on host occurrence, lacking morphological characters to differentiate variants, created a huge debate on species organization. Moreover, it was not obvious to understand whether phenotypic differences were a consequence of genetic differences or a result of host or environmental induced changes. However, molecular characterization of *Cryptosporidium* helped to clarify the confusion in *Cryptosporidium* taxonomy (Xiao and Fayer, 2008). Lack of distinctive morphological characteristics of the exogenous oocyst stage for a long time did not permit a definite species classification. Characters such as: - morphology of the endogenous and exogenous developmental stages, predilection sites and host specificity served for species identification and naming of *Cryptosporidium* (Fayer *et al.*, 2010). Originally only one species: *Cryptosporidium parvum* was recognized divided into two genotypes that were host adapted: type 1 genotype, “human genotype” (H type) and type 2 genotype, “cattle genotype” (C type), these genotypes were later on separated as two different species, *C. hominis* (formerly type 1) and *C. parvum* (formerly type 2) (Xiao and Fayer, 2008).

*Cryptosporidium* taxonomy had been under continual review process and many of the host-adapted genotypes were given species status based on standardised guideline that includes:- morphometric data on oocysts, genetic characterisation, natural, and when feasible, experimental host specificity, and compliance with International Commission on Zoological Nomenclature rules (Ryan *et al.*, 2014). At present, 26 species have been taxonomically valid based on morphological, biological, and molecular features (Fayer and Santin, 2009; Elwin *et al.*, 2012; Lebbad *et al.*, 2013; Adamu *et al.*, 2014) having mammals, amphibians, reptiles and birds as hosts (Table 1). Human infections are mostly caused by the highly host adapted *C. hominis* or the more generalist and zoonotic *C. parvum* species. Less frequently, eight additional *Cryptosporidium* species (*C. baileyi*, *C. canis*, *C. felis*, *C. meleagridis*, *C. bovis*, *C. suis*, *C. andersoni* and *C. muris*) are known to infect humans (Xiao, 2010; Šlapeta, 2013, Ryan *et al.*, 2014). As biological and molecular data increases, many of the genotypes are expected to be named as valid species.

Table 1: Validated species of *Cryptosporidium*. (Adapted from Bamaiyi and Redhuan, 2016)

S/No	Species	Mean oocyst size ( $\mu\text{m}$ )	Major hosts	Zoonotic status	References
1	<i>C. andersoni</i>	7.4 × 5.5	Cattle	Yes	(Jiang <i>et al.</i> , 2014)
2	<i>C. baileyi</i>	6.2 × 4.6	Birds	No	(Blagburn <i>et al.</i> , 1991)
3	<i>C. bovis</i>	4.9 × 4.6	Cattle	Yes	(Murakoshi <i>et al.</i> , 2013)
4	<i>C. canis</i>	5.0 × 4.7	Dogs	Yes	(Xu <i>et al.</i> , 2016)
5	<i>C. cuniculus</i>	5.6 × 5.4	Rabbits	Yes	(Koehler <i>et al.</i> , 2014)
6	<i>C. erinacei</i>	4.9 × 4.4	Hedgehogs, horses	Yes	(Kv��e <i>et al.</i> , 2014)
7	<i>C. fayeri</i>	4.9 × 4.3	Marsupials	Yes	(Dowle <i>et al.</i> , 2014)
8	<i>C. felis</i>	4.6 × 4.0	Cats	Yes	(Scorza <i>et al.</i> , 2014)
9	<i>C. fragile</i>	6.2 × 5.5	Toads	No	(Xiao <i>et al.</i> , 2012)
10	<i>C. galli</i>	8.3 × 6.3	Birds	No	(Chelladurai <i>et al.</i> , 2016)
11	<i>C. hominis</i>	4.9 × 5.2	Humans	Yes	(Widerstr��m <i>et al.</i> , 2014)
12	<i>C. macropodum</i>	5.4 × 4.9	Marsupials	No	(Nolan <i>et al.</i> , 2013)
13	<i>C. meleagridis</i>	5.2 × 4.6	Birds, mammals	Yes	(Wang <i>et al.</i> , 2014)
14	<i>C. molnari</i>	4.7 × 4.5	Fish	No	(Koinari <i>et al.</i> , 2013)
15	<i>C. muris</i>	7.0 × 5.0	Rodents	Yes	(Tyzzer, 1910)
16	<i>C. parvum</i>	5.0 × 4.5	Ruminants	Yes	(DuPont <i>et al.</i> , 1995)
17	<i>C. ryanae</i>	3.7 × 3.2	Cattle	No	(Fayer <i>et al.</i> , 2008)
18	<i>C. scrofarum</i>	5.2 × 4.8	Pigs	Yes	(Kv��e <i>et al.</i> , 2013)
19	<i>C. serpentis</i>	6.2 × 5.3	Reptiles	No	(Cranfield & Graczyk 1994)
20	<i>C. suis</i>	4.6 × 4.2	Pigs	Yes	(Ryan <i>et al.</i> , 2004)
21	<i>C. tyzzeri</i>	4.6 × 4.2	Rodents	Yes	(Ren <i>et al.</i> , 2012)
22	<i>C. ubiquitum</i>	5.0 × 4.7	Primates & rodents	Yes	(Fayer <i>et al.</i> , 2010)
23	<i>C. varanii</i>	4.8 × 4.7	Reptiles	No	(Pavla��ek and Ryan, 2008)
24	<i>C. viatorum</i>	5.4 × 4.7	Humans	Yes	(Elwin <i>et al.</i> , 2012)
25	<i>C. wrairi</i>	5.4 × 4.6	Guinea pigs	No	(Vetterling <i>et al.</i> , 1971)
26	<i>C. xiaoi</i>	3.9 × 3.4	Sheep, goats	Yes	(Fayer and Sant��n <i>et al.</i> , 2009)

### 2.3. Life cycle

*Cryptosporidia* are protozoa with a complex monoxenous development life cycle which can be divided into an asexual (sporogony and schizogony/merogony) and a sexual (gamogony) phase.

The infective stage of the parasite, the oocyst, contains four small banana-shaped sporozoites. After ingestion of the oocyst, excystation processes is enhanced at 37°C and likely involve parasite derived enzymes (O'Hara and Chen, 2011). Zoite motility requires discharge of adhesive molecules from the apical complex and is temperature, Ca<sup>++</sup> ion, and parasite cytoskeleton dependent. Ultimately, the apical end of the parasite attaches to the epithelial cell and initiates the internalization process. The result of excystation, attachment, and internalization is a fully encapsulated parasite or trophozoite that undergoes cell divisions resulting in six to eight new banana-shaped parasites (merozoites) within a Type I meront (O'Hara and Chen, 2011) (Figure 1).

These merozoites can re-infect the epithelium and form either a type I meront, effectively escalating the infection, or a type II meront, destined for sexual reproduction. The merozoites derived from type II meronts re-infect the epithelium and differentiate into either a micro- or macrogamont. The microgamonts fertilize macrogamonts resulting in the zygote, the only diploid developmental stage of the parasite. The diploid zygote undergoes a process similar to meiosis and forms either a thin or thick walled oocyst. Most of the mature zygotes (around 80%), develop a tough outer cover measuring 2.5–5 µm in diameter, become infective oocysts and exit the host through faeces to contaminate the environment. The rest of the mature zygotes having only a thin outer membrane, excyst in the gut lumen and re-infects the host (O'Hara and Chen, 2011).

During the invasion of the free living stages, proliferation and differentiation take place within a unique parasitophorous vacuole under the host cell brush border, but outside the host cell cytoplasm (Leitch and He, 2012). Like Coccidia, *Cryptosporidium* thus attaches to the cell surface and undergoes gliding motility, a process by which the parasites move along the cell surface for a short time, before they start to enter the cell. However, unlike Coccidia, they do not invade the cell actively, but rather trigger the cell to embrace them with a host cell-derived membrane. As a result, *Cryptosporidia* do not fully invade the cell, but rather stay in an epicellular location. At the parasite-cell interface, *Cryptosporidium* forms an actin-rich disk, a feeder organelle that is thought to be a small channel funnelling into the host cell cytoplasm and responsible for intake of nutrition (Lendner and Dauschies, 2014).

The prepatent period, the interval between infection and oocyst shedding, of *Cryptosporidium* infection vary depending on the host and species of the parasite. The prepatent period for *C. parvum* infection is 3-14 days (Fayer *et al.*, 2008), and it is 10 days for *C. bovis* infection and 11-12 days for *C. ryanae* (Fayer *et al.*, 2005; Fayer *et al.*, 2008). Among *Cryptosporidium* species *C. andersoni* showed the utmost prepatent period ranging from 18-45 days (Kvac *et al.*, 2008). A study reported that *C. bovis* was identified in two 7-day-old calves showing that the prepatent period is shorter than the previously described 10 days by Fayer *et al.* (2005). The author highlighted that this prepatent period was determined based on experimental infection in two calves previously infected with *C. parvum*, thus the results indicate a shorter prepatent period in *Cryptosporidium*-naïve calves, perhaps due to a partial resistance to other species stimulated by an earlier *C. parvum* infection (Silverlås, 2010).

The same as the prepatent period, the patent period (the period from the first appearance of oocyst shedding until the host immune system cleared the infection) of *Cryptosporidium* infection can also vary depending on the host and species of the parasite. The patent period for *C. parvum* infection is 1 to 20 days, for *C. bovis* it is 18 days and that of *C. ryanae* is 15–17 days (Fayer *et al.*, 2005, 2008). From the epidemiological point of view, the patent period is very important since an infected calf can shed large number, about  $10^{10}$ , of infective oocysts (Silverlås, 2010) resulting in heavy environmental contamination.

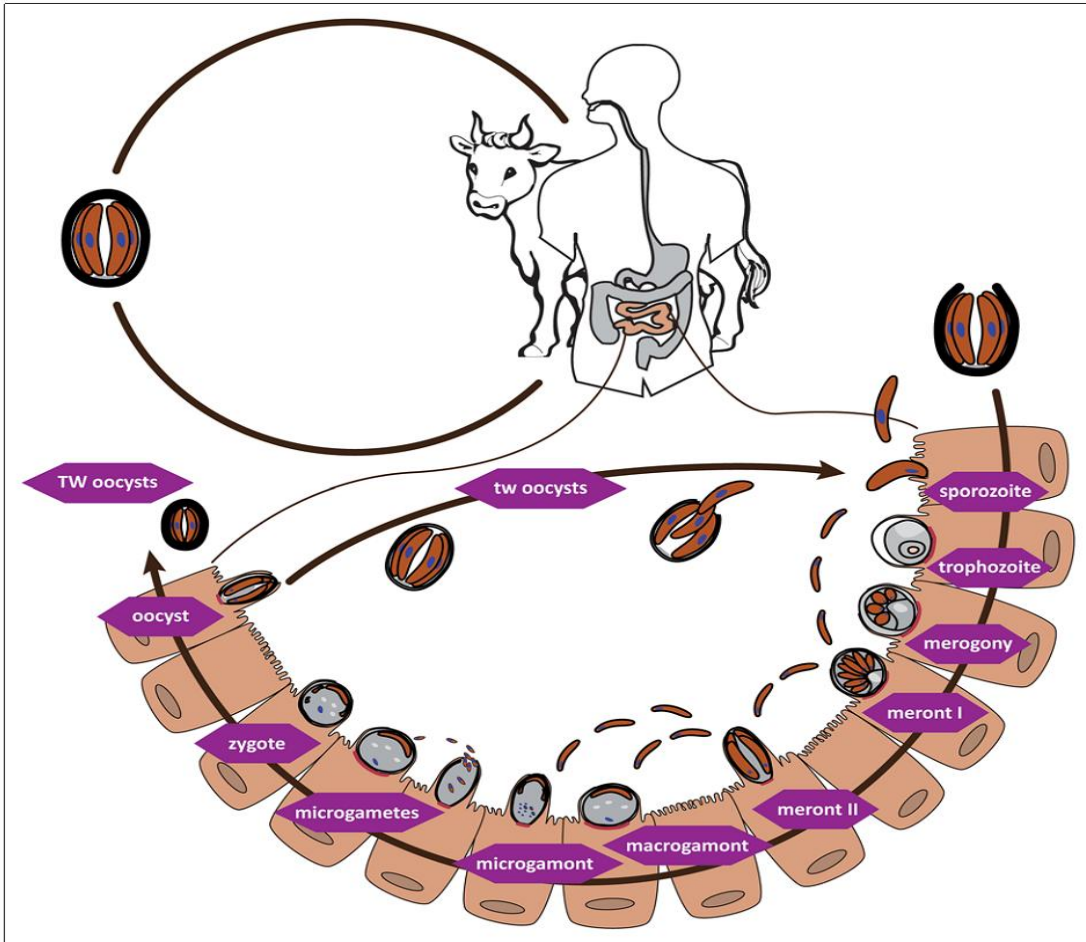


Figure 1: Life cycle of *C. parvum*

Source: Lendner and Dauschies, 2014

Sporulated oocysts are released with the faeces containing four infectious sporozoites. The sporozoites excyst in the small intestine and start to invade enterocytes. During invasion the sporozoite is embraced by protrusions of the host cell forming the parasitophorous vacuole. Underneath the forming trophozoite emerges a host cell-derived actin disc (shown in red). After asexual replication a meront type 1 develops that contains 6–8 merozoites. Type 1 merozoites infect adjacent epithelial cells either developing to another trophozoite or into a type 2 meront. Merozoites originating from a type 2 meront are thought to start the sexual replication cycle. Upon infection of another cell they either develop to macrogamonts or microgamonts. Microgamonts then release microgametes that fertilize a macrogamont. The resulting diploid zygote differentiates into four haploid sporozoites and subsequently the oocyst wall is formed around them. Two forms of oocysts are described. Thin-walled oocysts are described to lead to autoinfection whereas the thick-walled oocysts are released with the faeces to infect new hosts.

TW: thick-walled oocyst, tw: thin-walled oocyst.

## 2.4. Epidemiology of *Cryptosporidium*

Cryptosporidiosis is a worldwide distributed intestinal infection caused by parasites of the genus *Cryptosporidium*; the sporulated oocyst is the infectious stage emitted in the faeces of an infected host in large amounts. *Cryptosporidium* can be maintained in cycles involving livestock, especially cattle. Oocysts are transmitted from an infected host to a susceptible host by the faecal-oral route, the common transmission routes include a) person-to person through direct or indirect contact, where sexual activities may potentiate transmission, b) from animal-to-animal, c) animal-to-human, d) water-borne through drinking water and recreational water, and, e) food-borne (Bouzid *et al.*, 2013; Painter *et al.*, 2015). The infective dose of *Cryptosporidium* parasites, in human infections was calculated taking into account statistical data and experimental infection studies: the ID50 varies regarding the isolates, and it ranges from 9 to 1042 oocysts (Adam, 2001).

The features markedly influencing the epidemiology of the infections are: a) the infective dose is low b) oocysts are immediately infectious when excreted in faeces, and possess several transmission routes c) oocysts are very stable and can survive for weeks to months in the environment; d) water and food may become contaminated due to the environmental dispersal. The transmission of these infections, either direct or indirect, is favoured by several factors such as high population densities and close contact with infected hosts or contaminated water or food. These factors are dependent on the infecting species, either in zoonotic and anthroponotic transmissions. Studies suggested separated risks for *C. hominis* (such as travel abroad and contact with infected diarrheic individuals) and *C. parvum* (contact with cattle) (Bouzid *et al.*, 2013). In sporadic cryptosporidiosis, risk factors include the age of patients (children under five years of age), travelling, contact with infected individuals and contact with farm animals. Furthermore, swimming in public swimming pools or recreational areas represents a risk of infection (Lendner *et al.*, 2011). Although *Cryptosporidium* is transmitted through contaminated food, a small number of parasites in these samples may not induce infection with clinical symptoms but induces a protective immunity. *Cryptosporidium* is of particular concern for the following four reasons: (1) The oocyst is extremely resistant to disinfection and cannot be killed with routine water disinfection procedures (2) The disease is not treatable with antibiotics (3) The death from

infection in severely immunocompromized patients can be as high as 50-60% (4) Animal and human faecal wastes are associated with transmission of the disease to humans.

#### ***2.4.1. Epidemiology of human Cryptosporidium infection in Ethiopia***

The occurrence of *Cryptosporidium* infection in humans had been reported from different parts of Ethiopia (Table 2). Hospital based studies on HIV/AIDS patients without ART medication showed prevalence ranging from 15.2 to 40.3% (Zelalem *et al.*, 2008; Adamu and Petros, 2009; Getaneh *et al.*, 2010; Dawit *et al.*, 2014; Shimelis *et al.*, 2016), while the prevalence in HIV/AIDS patients on-ART medication varied from 0% to 28.4% (Dufera *et al.*, 2008; Adamu and Petros, 2009; Adamu *et al.*, 2013; Girma *et al.*, 2014; Shimelis *et al.*, 2016). Epidemiological studies involving apparently normal children showed magnitudes of *Cryptosporidium* infection rate varying between 7.3% and 12.2% (Ayalew *et al.*, 2008; Tigabu *et al.*, 2010; Wegayehu *et al.*, 2013) while hospital based studies on patient children reported prevalence ranging from 5.6-14.8% (Teshome *et al.*, 2014). These studies show significance of the disease in children and immunocompromized patients and reported contamination of drinking water, contact with calves and poor personal hygiene as risk factors of *Cryptosporidium* infection. Only two molecular based studies have been recognized so far in Ethiopia that characterized *Cryptosporidium* isolates of human infections (Adamu *et al.*, 2010; 2014). The studies reported *C. parvum* and *C. hominis* as the prevalent species infecting humans and highlighted the potential zoonotic importance of other species encountered in humans in Ethiopia (*C. viatorum*, *C. felis*, *C. meleagridis*, *C. canis*, and *C. xiaoi*).

Table 2: Overview of studies on human *Cryptosporidium* infection in Ethiopia

Study hospital/site	No of samples	Prev%	References
Nekemte Hospital	94 HIV+non-ART	25.5	Dufera <i>et al.</i> (2008)
	202 HIV+on-ART	8.4	
Southwest Ethiopia	160 HIV+	16	Zelalem <i>et al.</i> (2008)
Adama, Afar and Dire-Dawa	80 HIV+non-ART	17.5	Adamu and Petros (2009)
	120 HIV+on-ART	1.7	
Hawassa Hospital	214 HIV+	20.1	Assefa <i>et al.</i> (2009)
Fitche Hospital	164 HIV+non-ART	19.5	Adamu <i>et al.</i> (2013)
	214 HIV+on-ART	0.0	
Tikur Anbessa Hospital	520 HIV+	26.9%	Adamu <i>et al.</i> (2014)
Asella Hospital	384 HIV+	17.7	Dawit <i>et al.</i> (2014)
Yirgalem Hospital	134 HIV+non-ART	40.3	Girma <i>et al.</i> (2014)
	134 HIV+on-ART	28.4	
Hawassa Hospital	217 HIV+non-ART	15.2	Shimelis <i>et al.</i> (2016)
	274 HIV+on-ART	11.7	
EHNRI, Addis Ababa	152 patients	7.2	Endeshaw <i>et al.</i> (2007)
Lege Dini, East Ethiopia	655 norm* children	12.2	Ayalew <i>et al.</i> (2008)
Nine regions, Ethiopia	1034 different age	7.6	Adamu <i>et al.</i> (2010)
Pawi Special District	384 norm children	8.1	Tigabu <i>et al.</i> (2010)
North Shewa Zone	384 norm children	7.3	Wegayehu <i>et al.</i> (2013)
Yirgalem Hospital	230 children patients	14.8	Teshome <i>et al.</i> (2014)

\*norm= apparently normal

Prev%=prevalence in percent

#### 2.4.2. Epidemiology of *Cryptosporidium* infection in cattle in Ethiopia

Studies on cryptosporidiosis of dairy cattle in Ethiopia are scarce, however, the few studies conducted so far showed occurrence of *Cryptosporidium* infection in dairy farms at different parts of the country (Table 3). Most of the studies carried out in central region of the country reported

prevalence rates ranging from 7.2 to 15.8% (Abebe *et al.*, 2008; Dinka and Berhanu, 2015; Wegayehu *et al.* 2013, 2016). A wide-ranging study conducted in nine regions of the country reported an overall prevalence of 2.3% (Adamu, 2010) while a study in eastern region stated a prevalence of 27.8% (Alemayehu *et al.*, 2013). Molecular characterization of *Cryptosporidium* isolates from nine regions and central part of the country confirmed the existence of four species: *C. parvum*, *C. andersoni*, *C. bovis* and *C. ryanae* in the Ethiopian cattle (Adamu, 2010; Wegayehu *et al.*, 2016).

Table 3: Overview of studies on prevalence of *Cryptosporidium* infection in cattle in Ethiopia

Study site	No of samples	Prevalence (%)	References
Central Ethiopia	580 calves	17.6.	Abebe <i>et al.</i> (2008)
Nine regions	350 cattle	2.3	Adamu <i>et al.</i> (2010)
Eastern Ethiopia.	13 3 calves	27.8	Alemayehu <i>et al.</i> (2013)
North Shewa Zone	384 cattle	7.8	Wegayehu <i>et al.</i> (2013)
Bishoftu, Oromia	214 calves	13.6	Dinka and Berhanu (2015)
Central Ethiopia	449 calves	15.8	Wegayehu <i>et al.</i> (2016)

## 2.5. Human cryptosporidiosis

Human Cryptosporidiosis had been reported worldwide. The importance of *Cryptosporidium* as a wide-ranging public health problem began in 1993, when the world’s largest waterborne outbreak caused by *C. hominis* affected more than 400,000 residents in Milwaukee, Wisconsin, USA, due to consumption of contaminated drinking water (Thompson *et al.*, 2008). Relative to *Giardia* cysts, zoonotic and anthroponotic *Cryptosporidium* oocysts are more resistant to environmental conditions and disinfectants, making *Cryptosporidium* a major risk to public health (Pierce and Kirkpatrick, 2009). The potential risk of infection is high for both humans and animals (particularly pets) (BuduAmoako *et al.*, 2011, 2012). Immunocompromized individuals due to HIV/AIDS infection represent a serious risk group and the consequence of cryptosporidiosis as an opportunistic infection is well known (Adamu *et al.*, 2010; 2013; Putignani and Menichella, 2010). Observations showed that children in day-care centres, patients receiving chemotherapy for

cancer and patients with immunosuppressive infectious diseases represent another important group at risk (Davies *et al.*, 2009).

Molecular studies showed that *C. parvum* and *C. hominis* are the major species responsible for human cryptosporidiosis, *C. suis* and *C. muris* were reported to a lesser extent, and *C. meleagridis*, *C. felis*, *C. canis*, *C. xiaoi* and *C. viatorum* traditionally associated with animals, had been reported in AIDS patients (Xiao and Fayer, 2008; Adamu *et al.*, 2014). Geographic and disease burden differences were reported to *C. parvum* and *C. hominis* infections (Xiao and Ryan, 2008). In the UK *Cryptosporidium* is the commonest protozoal cause of gastroenteritis, with 3000-6000 annually confirmed laboratory cases *C. parvum* and *C. hominis* represent most of laboratory confirmed cases (Davies and Chalmers, 2009). In general, *C. hominis* is more prevalent than *C. parvum* in the USA, Canada, Australia, and Japan and in developing countries where molecular tools had been used to identify species (Xiao and Fayer, 2008).

### **2.5.1. Pathogenesis**

Infection with *Cryptosporidium* begins when the ingested oocysts release sporozoites, which subsequently attach to and invade the intestinal epithelial cells (IECs). The parasite has a particular predilection for the jejunum and terminal ileum and binds on the apical surface of the intestinal epithelium (Bouzid *et al.*, 2013). The Infectious dose vary depending on the species involved, for the species that commonly infect humans, *C. hominis* and *C. parvum* the lowest infectious dose has been calculated to be 10 oocysts, although in reality, one oocyst could be sufficient to cause infection in humans through direct or indirect routes of transmission (FDA, 2009).

The initial step in establishing infection is parasite attachment to host cells, *Cryptosporidium* establishes on the apical surface of the intestinal epithelium in a membrane-bound compartment promoting its reproduction causing direct injury to epithelial cells or indirect damage through the effect of inflammatory cells and cytokines recruited to the site of infection, leading to impairment in the absorptive and secretory functions of the gut (Okhuysen and Chappell, 2002; Bouzid *et al.*, 2013). Severity of *Cryptosporidium* infection can vary from an asymptomatic shedding of oocysts

to a severe and life-threatening disease. Patients who are at most risk are those with T cell immune deficiency, including patients with haematological malignancies (mainly children), HIV patients with low CD4<sup>+</sup> cell counts (particularly < 50), patients with primary T cell deficiencies such as SCID and CD40 ligand deficiency (hyper IgM syndrome) (Hunter and Nicholas, 2002). Individuals with neoplasm, severe combined immunodeficiency syndrome and acute leukemia are associated with increased risk of severe cryptosporidiosis disease. It was reported that patients on a tacrolimus based immunosuppressive regimen had a significantly higher risk of *Cryptosporidium* infection compared to patients on a cyclosporine based regimen (Bhadauria *et al.*, 2015). Not all forms of immune suppression lead to an increased severity of cryptosporidiosis but immunosuppressive disorders that impact T cell function, the most obvious being HIV/AIDS (Hunter and Nichols, 2002). In AIDS patients, CD4<sup>+</sup> lymphocytes are crucial for the resolution of infection, the most severe disease occurs in people with CD4<sup>+</sup> count <50 cells/mm<sup>3</sup>, patients with CD4<sup>+</sup> counts greater than 200 cells/mm<sup>3</sup> tend to have less severe disease (Huang and White, 2006).

Upon attachment to the cell and gliding around sporozoites of *Cryptosporidium* does not fully invade the cell, it resides in the extra-cytoplasmic area, for which histological and some molecular studies consider it as engulfment of the parasite and not as an active invasion, conversely, *Toxoplasma* and *Plasmodium* sporozoites force their way into the cell squeezing themselves into the nascent PV inside the host cell (Baum *et al.*, 2008; Sibley, 2010). The direct cell injury or activation of the immune system with release of pro inflammatory cytokines by the parasite remarkably affects intestinal cells with consequent alterations in absorptive and secretory functions (Bouzig *et al.*, 2013).

The paucity of information on fundamental questions about the specific molecules involved in host parasite interaction, cell-invasion and transmission was due to the inability to cultivate the organism in the laboratory (in vitro) and lack of suitable systems for genetic manipulation of the parasite. However, following the innovations of *C. parvum* and *C. hominis* genomes, substantial progress has been made in identifying over 25 putative virulence factors which are proposed to be involved in host-pathogen interactions from adhesion and locomotion to invasion and proliferation (Bouzig *et al.*, 2013). Progress has also been made in the contribution of host factors that are associated with variations in both the severity and risk of infection. Putative virulence factors of

*Cryptosporidium* have been identified as genes involved in the initial interaction processes of the oocysts and sporozoites with host epithelial cells, including excystation, gliding motility, attachment, invasion, parasitophorous vacuole formation, intracellular maintenance, and host cell damage (Wanyiri and Ward, 2006; Fayer, 2009).

Two classes of protein molecules, mucin-like glycoproteins and thrombospondin-related adhesive proteins, play a role in attachment of the parasite to host cells and to establish the infection (Wanyiri and Ward, 2006). CSL (circumsporozoite-like glycoprotein), released as a soluble glycoprotein, is associated with the apical complex of sporozoites and merozoites. CSL contains a ligand that binds specifically to a receptor on the surface of human and bovine intestinal epithelial cells (IECs) (Schaefer *et al.*, 2000). A 30 kDa Gal/GalNAc lectin (p30) has been identified that is predominantly located in the apical region of sporozoites and thought to play a substantial role in attachment, p30 is associated with gp40 and gp900, two glycoproteins involved in attachment, and therefore hypothesized that these molecules form an adhesion complex (Bhat *et al.*, 2007). Glycoprotein-900 (gp900) is a large mucin-like glycoprotein located in micronemes and at the surface of invasive merozoites and sporozoites, it is deposited in trails during gliding motility and is known to mediate invasion (Bonnin *et al.*, 2001). Prevention of gp900 binding to cells resulted in reduced infections, the structure and probably the attachment sites of gp900 as well seem to differ between *C. parvum* and *C. hominis*. Hence, gp900 might be a molecule through which *Cryptosporidium* defines host specificity. A sporozoite and merozoite cell surface protein, gp15/40/60 complex, present in the trails of gliding zoites have been shown to play a role in parasite attachment, invasion, and motility (Wanyiri and Ward, 2006; O'Connor *et al.*, 2007; Power *et al.*, 2009). TRAP-C1 (thrombospondin-related adhesive protein *Cryptosporidium* 1) is a protein localized on the apical pole of sporozoites and involved in parasite gliding, motility and cell penetration (Boulter-Bitzer *et al.*, 2007). Infection of human volunteers with *C. parvum* revealed that TRAP-C1 is an immunogenic protein (Okhuysen *et al.*, 2004).

All of the above proteins expressed on the surface of the invasive *Cryptosporidium* sporozoite and merozoite stages are shed in trails by gliding zoites and have common features with other apicomplexan proteins mediating host cell interactions (Tzipori and Ward, 2002). Cellular damage of enterocytes occur through the disruption of tight cell junctions, loss of barrier function, release

of lactate dehydrogenase, and increased rates of cell death. Several molecules can cause direct tissue damage, such as phospholipases, proteases, and hemolysins (Okhuysen and Chappell, 2002). Proteases have important functions in parasite's life cycle, such as mediating protein degradation, the invasion of host tissues, and the evasion of host immunity. Distinct protease activities have been identified for *Cryptosporidium* sporozoites: aminopeptidase, cysteine protease, and serine protease activities have been implicated in the excystation process. The identification of functional proteases in sporozoites during excystation and the prevention of infection in the presence of protease inhibitors suggest that proteases are important in the initial stages of *Cryptosporidium* infection (Okhuysen and Chappell, 2002). Hemolysin H4, identified in *C. parvum* has the ability to disrupt cell membranes suggests its role in cellular invasion and/or the disruption of vacuolar membranes, which would allow merozoites to exit the parasitophorous vacuole and spread to adjacent cells (Okhuysen and Chappell, 2002). *C. parvum* ATP-binding cassette (ABC) transporter gene (CpABC) localized in proximity to the electron-dense feeding organelle of the parasitophorous vacuole may well be associated with meeting key nutritional requirements.

Two Heat shock proteins (HSPs), HSP70 and HSP90, have been identified in *Cryptosporidium*. The HSP70 gene has considerable polymorphism and was used for genotyping purposes (Sulaiman *et al.*, 2000) the relationship between the level of HSP expression and *Cryptosporidium* virulence deserves further investigation. The *C. parvum*-specific protein (Cops-1) and the *C. hominis*-specific protein (Chos-1), appear to be distantly related and share similar characteristics, the proteins that they encode are predicted to be highly glycosylated, these characteristics suggested a possible role in host-parasite interactions (Bouzid *et al.*, 2010). The histopathological features of cryptosporidiosis include a minimal inflammatory infiltrate and blunting of the villus. More-pronounced inflammatory changes, such as disruption of the epithelial cell barrier and extensive infiltration of the lamina propria with inflammatory cells are seen in immunodeficient patients (Okhuysen and Chappell, 2002).

### 2.5.2. Immune response to cryptosporidiosis

Intestinal epithelial cells (IECs) provide barrier function to invading microbes through various physical and biochemical factors (Magalhaes *et al.*, 2007). IECs constitutively express pattern-recognition receptors, including Toll-like receptors (TLRs) and intracellular Nod-like receptors (NLR) that enable microbial recognition. Upon infection of the host IECs or ligation of TLRs or NLRs results in activation of innate immune responses by generating cytokines and up-regulating chemokines that attract and activate other immune cells (Fritz *et al.*, 2006; Mead, 2014). Subsequent to mucosal injury by the parasite, toll like receptors (TLR2 and TLR4) play an important part in initiating immune activation and establishing immunity to infection (Pantenburg *et al.*, 2008; McDonald *et al.*, 2013), via the NF- $\kappa$ B pathway or activation of the caspase-1 inflammasome, leading to induction of the expression of pro-inflammatory cytokines and chemokines that are essential components of the anti-pathogen response (Fritz *et al.*, 2006).

Because of the “minimally invasive” nature of *Cryptosporidium* infection, mucosal epithelial cells are critical to the host’s anti-*Cryptosporidium* immunity (Hu *et al.*, 2014). IECs also express major histocompatibility complex (MHC) class I and class II molecules and all the machinery required for antigen processing and presentation (Chen *et al.*, 2005). Nitric oxide produced through the induction of nitric oxide synthase (iNOS) of epithelial cells is significantly increased in *C. parvum* infection (Gookin *et al.*, 2004). Additionally, the production of antimicrobial peptides and type-1 interferons occur because of infection (Barakat *et al.*, 2009).

Complement plays a role in the innate immunity to *C. parvum*, although, the contribution of the complement system and the lectin pathway to the host defence against cryptosporidiosis may become apparent in situations of immunodeficiency such as HIV infections or in early childhood (Petry *et al.*, 2008). IFN- $\gamma$ -dependent responses in both human infections and animal studies are important in innate and protective immune responses. In humans, increased amounts of IFN- $\gamma$  are generated in response to cryptosporidial specific antigen (Preidis *et al.*, 2007). The treatment of IFN- $\gamma$  knockout mice or IL-12-deficient mice with anti-IL-18 antibodies increased parasite excretion, suggesting that the protective role of IL-18 is not totally dependent on IFN- $\gamma$  expression (Tessema *et al.*, 2009). In IFN- $\gamma$ <sup>-/-</sup> and IL-12<sup>-/-</sup> mice there was an increase in IL-4 and IL-13

expression in spleens of infected mice when treated with anti-IL18 antibody (Tessema *et al.*, 2009), supporting the role of IL-18 in polarization of T<sub>H</sub>1 response against the parasite. After prior exposure, a likely source of IFN- $\gamma$ -dependent responses was reported to be due to NK cells, however, depletion of NK-cells with anti-asialo-GM1 antibody treatment in mice or stimulation of NK-cells by IL-221 did not seem to affect infection. During the initial stages of infection, adaptive immunity is as important as innate immunity and is necessary to completely clear the parasites. Thus, while innate immunity is important in controlling the infection, elimination of the parasite demands response of adaptive immunity (McDonald *et al.*, 2013). Complex innate and adaptive immune response mechanisms are involved in clearing *C. parvum* infection in a redundant manner. The relevance of some of these components, especially the antibody response as well as the contribution of dendritic or natural killer cells, is still not clear (Petry *et al.*, 2010). Epithelial cells not only provide the first and rapid defence against *Cryptosporidium* infection, but also mobilize immune effector cells to the infection site to activate adaptive immunity. Protective cellular immunity to *C. parvum* was successfully transferred via primed IELs as well as intestinal and systemic CD4<sup>+</sup> T cells, indicating the importance of the adaptive immune response against *C. parvum* infection and strengthens the role of primed, pathogen-specific CD4<sup>+</sup> T-cell in resistance to the pathogen (Tessema *et al.*, 2009).

Attachment to the apical cell surface by *Cryptosporidium*, as well as molecules inserted into host cells after attachment, can activate host cell signal pathways and thereby alter cell function. Pathogen recognition receptors (e.g., Toll-like receptors) in epithelial cells recognize *Cryptosporidium* and initiate downstream signalling pathways (e.g., NF-kappa B) which trigger a series of antimicrobial responses and activate adaptive immunity. This is evident clinically in the immunocompromized individuals with more severe and potentially-life threatening disease such as HIV/ AIDS patients. In these patients, the CD4<sup>+</sup> lymphocytes are crucial for resolution of the infection; patients with CD4<sup>+</sup> counts greater than 200 cells/mm<sup>3</sup> tend to have less severe disease than those with less than 50 cells/ mm<sup>3</sup> (Mead, 2014). Unlike many other intracellular pathogens, CD4<sup>+</sup> T-cells play an important role in the immune response against the *Cryptosporidium* (Petry *et al.*, 2010). In the immunocompromized individuals, the spectrum and severity of cryptosporidiosis reflect the importance of the T-cell response since most severe diseases had been in individuals with defects in the T-cell response. As a result, immunocompromized adults and

children, especially those with AIDS, are at greatest risk (Desai *et al.*, 2012). Whereas much knowledge has been accumulated from studies in mouse infection models, the understanding of human cryptosporidiosis is still limited (Petry *et al.*, 2010).

### **2.5.3. Clinical manifestations in humans**

The severity and duration of *Cryptosporidium* infection is reliant on the infecting species, virulence of the parasite and the immune status of the host, and requires response of both Cell-mediated and humoral immune system for complete improvement (Chalmers and Davies, 2010; Bouzid *et al.*, 2013; McNair and Mead, 2013). The infectious dose is low, and as few as 10-30 oocysts can cause infection in healthy persons (Yoder and Beach, 2007). Persons infected with *Cryptosporidium* can excrete  $10^8$ - $10^9$  oocysts in a single bowel movement and that oocysts can be excreted for up to 50 days after cessation of diarrhoea, however, in most people, the shedding of oocysts stops within 2 weeks (Yoder and Beach, 2007), infected individuals can continue to shed the parasite and potentially transmit infection to other people after symptoms of illness have ended.

Immunocompetent individuals experience a transient self-limiting illness (up to 2 to 3 weeks). However, in immunocompromized patients, cryptosporidiosis causes a serious threat leading to chronic or fulminant disease, dehydration, wasting and death (Hunter and Nichols, 2002; Chen *et al.*, 2002; Mead, 2014). In addition to the above, *Cryptosporidium* infection can cause atypical extra-intestinal manifestations in immunocompromized patients, mainly AIDS such as biliary tract disease, cholangitis, respiratory tract disease, urinary tract infection and pancreatitis (Hunter and Nichols, 2002; Davies and Chalmers, 2009). Respiratory involvement is rare and is characterized by cough, dyspnoea, fever and thoracic pain whereas; biliary cryptosporidiosis is the most common extra-intestinal manifestation with clinical features of pain in the right upper quadrant, nausea, vomiting and fever. Patients with biliary symptoms have lower CD4+ T-cell counts and biliary reservoir may contribute to the chronic nature of the infection and the inability of therapy to eradicate the organism (Chen *et al.*, 2002). Other nonspecific symptoms described in immunocompetent and immunocompromized patients include, generalized weakness, myalgia, anorexia and headache (Bonatti *et al.*, 2012; Bouzid *et al.*, 2013). Some studies described acute

renal failure, most likely secondary to dehydration, hypotension and sometimes tacrolimus toxicity (Shirley *et al.*, 2012).

Although a number of *Cryptosporidium* species have been identified in humans, *C. hominis* and *C. parvum* account for more than 90% of the total cases (Bouزيد *et al.*, 2010; Chalmers *et al.*, 2011). With the exception of slight variations, the clinical presentations by the two species are greatly similar. In children and HIV-infected persons *C. hominis* had been associated with diarrhoea, nausea, vomiting, and malaise and *C. parvum* was associated only with diarrhoea (Cama *et al.*, 2007, 2008). A study on HIV/AIDS patients reported that diarrhoea was significantly associated with both *C. parvum* and *C. hominis* infections, and vomiting was associated with only *C. hominis* infections but not associated with *C. parvum* (Adamu *et al.*, 2014). In addition, it was shown that *C. hominis* infection was associated with non-intestinal sequelae (joint pain, eye pain, recurrent headache, and fatigue), which were not reported for people infected with *C. parvum* (Hunter *et al.*, 2004). Persistent vomiting and diarrhoea can lead to dehydration and wasting and have been associated with increased morbidity (Krause *et al.*, 2012). The frequent or chronic insults to the gastrointestinal system by enteric pathogens including *Cryptosporidium*, alone or by co-infection, are thought to result in a condition known as environmental enteropathy, which manifests itself in malnutrition, stunting, perturbation of the gut microbiome, impaired cognitive development, diminished oral vaccine efficacy and increased susceptibility to infections (Lang *et al.*, 2015).

#### **2.5.4. Treatment**

Despite the worldwide distribution of cryptosporidiosis and its rank among the most serious causes of global diarrheal illness, sufficient attention has not been paid by health agencies and the private sector to develop effective drug. This is mostly because of a perceived limited market for such drugs in developed countries and unaffordable cost of the drugs for developing countries where it causes deadly disease in malnourished or immune compromised individuals (Bamaiyi and Redhuan, 2016). *C. parvum* has proven to be a formidable foe, pharmaceutical industries lose interest in developing therapies against an organism that has been so intractable since the market for a specific therapy against the disease is relatively small and the financial rewards for developing a new product would probably be minuscule (Sterling, 2000). Consequently,

cryptosporidiosis was categorized by WHO under the list of globally “neglected diseases” which have a common link with poverty in most developing countries (Savioli *et al.*, 2006).

In addition, advances in developing drugs against *Cryptosporidium* have been hindered by limitations in laboratory propagation of the parasite, absence of an ideal cell culture method and the inability to genetically manipulate the organisms (Sonia, 2011). However, the breakthrough in achieving the complete genomic sequences of *C. hominis* and *C. parvum* and the ease of access of functional genomic data has provided researchers with new devices with which to explore unique metabolic pathways as targets for chemotherapy (Agüero *et al.*, 2008; Magariños *et al.*, 2012). Existing therapeutics for other Apicomplexan diseases are ineffective against *Cryptosporidium* infection, probably because of the unique intracellular, extracytoplasmic location of *Cryptosporidium* and the poorly understood host-parasite interface (Sonia, 2011).

#### 2.5.4.1. Specific anticryptosporidial drugs

Although a number of drugs and drug combinations, such as rifaximin, azithromycin, paromomycin and other antibiotics such as spiramycin, have been tried against cryptosporidiosis the results were unsatisfactory or inconsistent (Acikgoz *et al.*, 2012), a specific treatment has not yet been developed for cryptosporidiosis in man and animals. Mouse model based studies showed that parasite primed CD4+ T cells are competent to greatly reduce parasite development which could open theoretical opportunity to establish in vitro treatment for *C. parvum* infection (Tessema *et al.*, 2009). The broad-spectrum, anti-infective drug, Nitazoxanide (NTZ) (Alinia®, Romark Laboratories, Tampa, FL, USA), has shown the most promise against *Cryptosporidium* and was approved by the United States Food and Drug Administration (U.S. FDA) for use in immunocompetent patients older than 1 year (Amadi *et al.*, 2002; Rossignol *et al.*, 2006; Aly *et al.*, 2015). Efficacy of the drug may vary, with cure rates ranging from 56–96% in malnourished children and healthy adults (Amadi *et al.*, 2002; Rossignol *et al.*, 2006). In the UK, Nitazoxanide is not licensed but is available on a named patient basis (Davies and Chalmers, 2009). The drug has not proven to be the silver bullet for there is a paucity of information about NTZ’s use in children aged less than 12 months, despite the clinical vulnerability of this age group. A randomized, placebo-controlled trials of NTZ among immune compromised patients concluded that NTZ was

no more effective than placebo in resolving diarrhoea and achieving parasitological clearance in HIV-positive persons (Ryan *et al.*, 2014).

Nitazoxanide is not yet widely commercially available and awaits larger post marketing reports (Bamaiyi and Redhuan, 2016). It has been speculated that HIV-positive persons may benefit from longer duration regimens or higher doses of NTZ; however, in a compassionate-use program, a sustained clinical response was observed in only 59% of patients with HIV/AIDS who received off-label NTZ (Rossignol, 2006). Although refinement of the dosing regimen may improve clinical efficacy of NTZ, a prolonged therapeutic course will be impracticable in developing countries because of the expense and likely patient noncompliance (Checkley *et al.*, 2015). Given the seriousness of the outcomes of this infection in immunocompromized individuals and the potential to improve compliance by decreasing nausea and vomiting, it is worth considering using Nitazoxanide while clinicians await further evidence for its effectiveness in immunocompromized patients (Abubakar *et al.*, 2007). The use of fluid and electrolyte replacement and anti-motility agents may be the only option for the majority of immunocompromized patients.

In AIDS patients *Cryptosporidium* causes profuse and watery diarrhoea with loss of fluid over 10L/day (Arora and Arora, 2005). Fluid loss is especially prominent in debilitated individuals who are unable to drink; utmost attention needs to be paid to correct electrolyte abnormalities and prevent dehydration with intravenous or oral rehydration solutions (ORS) (De Hostos *et al.*, 2011). The main treatment approach is oral rehydration whenever possible; however, intravenous fluids that include sodium, potassium, glucose and bicarbonate may be required in severe cases (Florescu and Sandkovsky, 2016). ORS-based treatment is a highly efficacious and cost-effective way to counteract the effects and relieve some of the symptoms associated with acute secretory diarrhoeas such as that caused by *Cryptosporidium*, however, use of ORS in poor and rural areas remains quite low since treatment is labour-intensive and requires large volumes of ORS to maintain hydration (Zwisler *et al.*, 2013). Individuals should be encouraged to start ORS early to prevent even initial dehydration.

Optimum nutritional support should include a trial of oral nutritional supplements before considering total parenteral or intravenous hyper-alimentation. Nutritional supplements containing

medium chain fatty acids may be better absorbed in patients with small intestinal injury and malabsorption. A lactose free diet is recommended since *Cryptosporidium* destroys mature epithelial cells that are located in the villi resulting in loss of enzymes such as lactase (Florescu and Sandkovsky, 2016). Thus Milk and dairy products should be avoided since lactose intolerance is common.

#### 2.5.4.2. Antiretroviral agents

There is no effective therapy for cryptosporidiosis in HIV infected persons, thus the most effective strategy for managing *Cryptosporidium* appears to be immune reconstitution with Highly Active Anti Retroviral Therapy (HAART) (Miao, 2000). While HAART should increase patient's CD4+ above risk thresholds, concomitant target of the opportunistic infection remain important to prevent ongoing morbidity. Treatment of HIV consists of a combination of antiretroviral drugs, typically three drugs from two or more classes. Sometimes more than three drugs are used in patients who have been treated previously and are known or presumed to harbour viral strains with reduced susceptibility. In addition, some combination antiretroviral therapy (cART) regimens include a drug that increases or prolongs exposures of one or more drugs in the regimen because of an intentional drug interaction (CDC, 2014). The widespread use of HAART has a beneficial effect on recovery from cryptosporidiosis and on the frequency of infection in HIV positive patients (Adamu, 2010). A study reported that a severely immunodeficient AIDS patient with 45 CD4+ cells/ml of blood showed remarkable clinical improvement including resolution of diarrhoea and weight gain shortly after antiretroviral combination therapy, the patient showed no signs of cryptosporidiosis for more than two years, indicating eradication of the pathogen (Schmidt *et al.*, 2001). AIDS, as defined by the Centers for Disease Control and Prevention, is defined as the presence of HIV infection with a CD4+ cell count  $< 200 \text{ cells/mm}^3$  and/or the presence of an AIDS-defining clinical condition, which includes any number of opportunistic infections, malignancies, or other clinical syndromes (CDC, 2014).

HAART suppresses replication of human immunodeficiency virus (HIV) leading to an increase in circulating CD4+ T-lymphocytes, clinical recovery and eradication of cryptosporidiosis (Schmidt *et al.*, 2001). The goal of antiretroviral treatment is to indefinitely maintain suppression of plasma

HIV ribonucleic acid (RNA) levels (viral load) below the level of detection of sensitive HIV-RNA assays (CDC, 2014). In developed countries with low rates of environmental contamination and where potent antiretroviral therapy (ART) is widely available, cryptosporidiosis has decreased and occurs at an incidence of <1 case per 1000 person-years in patients with AIDS (Buchacz *et al.* 2010). However, cryptosporidiosis remains a common cause of chronic diarrhoea in AIDS patients in developing countries with prevalence rates ranging from 8-27% (Gupta *et al.*, 2008; Kurniawan *et al.*, 2009; Adamu *et al.*, 2014). No effect of CD4+ cell counts and HAART were observed on the occurrence of cryptosporidiosis in HIV/AIDS patients due to severe immunodeficiency in the study population (Adamu *et al.*, 2014). While improved antiretroviral regimens have significantly reduced the prevalence of AIDS and AIDS-related opportunistic infections, cryptosporidiosis remains among the most common causes of diarrhoea in patients with AIDS (Hunter and Nichols, 2002). Following treatment with HAART, HIV-RNA decreased below the limit of detection in blood and mucosa, CD4+ T cells in the mucosa increased to much faster and much higher levels than those circulating in the blood (Schmidt *et al.*, 2001).

Antiretroviral therapy, leading to recovery of the CD4+ count in AIDS patients, is a therapeutic intervention that has a dramatic effect on cryptosporidiosis (Maggi *et al.*, 2000; Miao, 2000). In a study of two patients with cryptosporidiosis, both were free from the parasite within 24 weeks after starting antiretroviral therapy. This finding was confirmed in another, larger study, where all patients taken antiretroviral agents showed clinical recovery (Maggi *et al.*, 2000). Two patients subsequently relapsed after the therapy was stopped. It was noted that resolution of the diarrhoea seemed to be related to an increased CD4+ cell count rather than a decrease in viral load. These findings give further support to the observation that cellular immunity is of paramount importance in clearing *Cryptosporidium* infection (Morpeth and Thielman, 2006).

### 2.5.7. Prevention

The absence of effective therapy for *Cryptosporidium* infection highlights the need to ensure that infection is avoided; unfortunately, evidence for the effectiveness and cost-effectiveness of preventive interventions is also lacking (Abubakar *et al.*, 2007). *Cryptosporidium* infections are contracted by the ingestion of oocysts, therefore effective control measures must aim to prevent intake of oocysts via direct or indirect transmission routes, though this remains largely elusive in sub-Saharan Africa and other developing regions. In the absence of vaccines; proper hygiene and consumption of food and water free from *Cryptosporidium* oocysts appear to be the only method at present, to prevent infection with cryptosporidiosis (Bamaiyi and Redhuan, 2016). Such measures may include extensive hand washing, avoiding direct contact with stool from animals or humans, avoiding the accidental ingestion of water used in recreational activities and taking measures to ensure the safety of the drinking water. It should be noted that the quality of local drinking water is regionally and seasonally variable.

*Cryptosporidium* oocysts can be removed from drinking water by either boiling the water for 1 min or by filtering through a filter of one micron (1 $\mu$ ) or smaller pore size (CDC, 2015). *Cryptosporidium* oocysts are resistant to many chemical disinfectants therefore, public health and municipal water authorities should provide specific information about the safety of the water supply and focus on alternative methods of inactivating waterborne oocysts such as UV irradiation and ozone (Tzipori and Giovanni, 2008; CDC, 2015). Public health measures to reduce contamination of water supplies and vigilant surveillance will reduce the risk to populations (Peletz *et al.*, 2013). Thus, increasing patient immune status and screening at least for those treatable parasites is important (Marcos and Gotuzzo, 2013).

Since chronic cryptosporidiosis occurs primarily in patients with advanced immunodeficiency should prevent the disease through initiation of appropriate ART combination treatment before the patient becomes severely immunosuppressed. HAART is the mainstay of treatment in the setting of severe immune suppression, it usually leads to immune restoration to a CD4+ count greater than 100 cells/ $\mu$ L and resolution of clinical cryptosporidiosis (Cabada and White, 2010) Thus, patients with cryptosporidiosis due to the risk of acquiring a life-threatening disease, should be

started on ART as part of the initial management of the infection and must take specific measures to help reduce the risk of waterborne cryptosporidiosis that includes: boiling drinking water for 1 minute or filtering through a filter of one micron (1 $\mu$ ) or smaller pore size, avoiding contact with young animals and avoiding swallowing water while swimming (CDC, 2015; John and Petri, 2006).

## 2.6. Cryptosporidiosis in animals

Cryptosporidiosis had been reported in various countries around the world infecting a variety of species of domestic and wild mammals, reptiles, fish and birds. In Cattle, four species: *C. parvum*, *C. andersoni*, *C. ryanae*, and *C. bovis*, are frequently reported though, *C. suis*, *C. hominis*, *C. serpentis*, *C. xiaoi*, *C. ubiquitum*, *C. meleagridis*, *C. muris*, and *C. felis*, have also been recognized (Gong *et al.*, 2017). *C. parvum* is reported to infect humans and ruminants Cattle, in particular young calves less than 2 months, are frequently infected by *C. parvum*. The prevalence in beef calves is often lower than in dairy calves (Kvac *et al.*, 2006). Prevalence rates of 16.3%, 27.2% and 57.9% were reported in calves in India, Pakistan and Chile, respectively (Maurya *et al.*, 2013; Muñoz *et al.*, 2014; Shafiq *et al.*, 2015). An overall prevalence of 14% was reported in cattle in Myanmar out of which calves less than 6 months shared a significantly higher proportion rate (Bawm *et al.*, 2014). About one third of the dairy cattle farms were contaminated by oocysts of the parasite in central Thailand (Jittapalapong *et al.*, 2006). *Cryptosporidia* species affecting cattle differ from country to country, *Cryptosporidium andersoni* infect mainly mature cattle, and *C. bovis* and the *Cryptosporidium* deer-like genotype infect older dairy calves (Fayer *et al.*, 2006; Feng *et al.*, 2007b; Langkjaer *et al.*, 2007). *C. bovis* and *C. ryanae* were encountered in cattle in Malaysia (Yap *et al.*, 2016), while a study in Turkey reported that *C. parvum* was the only species encountered in cattle (Arslan and Ekinci, 2012). A wide range of calf (0-59%) and farm (0-100%) prevalence estimates have been reported from different countries (Table 4). Although part of this variation is certainly due to substantial differences in prevalence between farms, the variation is also due to differences in study design, such as the number of animals or farms included or the age and breed of animals. The choice of diagnostic technique might also influence the prevalence estimate, since some techniques are more sensitive than others.

Sheep are mostly infected with *Cryptosporidium cervine* genotype and other genotypes (Santin *et al.*, 2007). Infection rates of 1.8% and 10.24% in lambs and 3.5% and 18.86% in goat kids had been reported from India and Iran, respectively (Khezri and Khezri, 2013; Maurya *et al.*, 2013). The zoonotic species, *C. parvum* and *C. ubiquitum*, were isolated from sheep in Australia and *C. xiaoi*, *C. parvum* and *C. ubiquitum* were identified in goats from china (Mi *et al.*, 2014; Yang *et al.*, 2015). Studies on *Cryptosporidiosis* of other animal species showed prevalence of 61% in camels in Iraq (Hussin *et al.*, 2015) and *C. andersoni* in horses (Liu *et al.*, 2015) and in camelids (Wang *et al.*, 2008).

*Cryptosporidium* infections in the nocturnal insectivorous lizard pet, geckos, due to *C. serpentis* lizard genotype and in the nocturnal insectivorous mammal pet, Hedgehog, due to *C. parvum*, *C. erinacei* (the hedgehog genotype) and *Cryptosporidium* horse genotype had been documented in Japan (Abe and Matsubara, 2015). *Cryptosporidium* is a primary pathogen in chickens, turkeys and quail, causing respiratory and/or intestinal disease, leading to morbidity and mortality. Three species infect poultry: *C. baileyi*, *C. meleagridis* and *C. galli*, (Ryan, 2010). The occurrence of *C. baileyi* and *C. meleagridis* in chicken (Wang *et al.*, 2010), In Nigeria, cryptosporidiosis has been reported in wild, exotic and local birds (Bamaiyi *et al.*, 2013), while in Thailand, the pigeon and seagull birds were reported being infected by *C. meleagridis* and *Cryptosporidium* avian genotype III, respectively (Koompapong *et al.*, 2014). *Cryptosporidium* infections in the Long-tailed Asian monkey, macaques, due to *Cryptosporidium* monkey genotype had been reported in Thailand (Koompapong *et al.*, 2014).

Studies demonstrated that zoonotic transmission may occur due to close contact between cattle and farm workers (Ng *et al.*, 2012; Ehsan *et al.*, 2015). A study in camels and camel herders in Iraq reported prevalence of 61% and 56%, respectively (Hussin *et al.*, 2015), indicating the high probability of cross-transmission between humans and camels. A few years back, it was thought that *C. parvum* could infect all mammals, although the genetic characterization showed absence of these species in wild mammals (Feng *et al.*, 2007a). Yet, later on, it was shown that the zoonotic *C. parvum* and *C. ryanae* are the most widely spread species identified in water buffaloes and Yaks; *C. parvum* was reported in water buffaloes in Thailand, Egypt and Australia (Amer *et al.*, 2013; Inpankaew *et al.*, 2014; Zahedi *et al.*, 2016) and in Yaks in china (Qi *et al.*, 2015) while, *C.*

*ryanae* was reported in water buffaloes in Thailand and Egypt, in roe deer in Spain (García-Preledo *et al.*, 2013) and in Yaks in china. *C. bovis* was the second common species reported in water buffalo in Australia and in Yaks in china, and *C. ubiquitum* was reported only in yaks. The prevalence of *Cryptosporidium* in water buffaloes vary among countries, it was 24.2% in buffalo calves in India (Maurya *et al.*, 2013), 5.7% in Thailand (Inpankaew *et al.*, 2014), 30% in farmed and 12% in wild water buffaloes in Australia (Zahedi *et al.*, 2016). The prevalence of *Cryptosporidium* in roe deer in Spain was 4.2% (García-Preledo *et al.*, 2013) and it was 4.0% in yaks in china (Qi *et al.*, 2015).

Studies on wild rats in Iran, china and Japan showed prevalence of 27.3%, 11.5% and 38% mostly attributed to the zoonotic *C. parvum* infections (Kimura *et al.*, 2007; Lv *et al.*, 2009; Bahrami *et al.*, 2012) while similar study in wild rats and mice in the Philippines showed an overall prevalence of 25.8% and the species identified were *C. parvum*, *C. muris*, *C. scrofarum*, rat genotypes I-IV and a *C. suis*-like genotype (Ng-Hublin *et al.*, 2013). Four of the *Cryptosporidium* species and genotypes identified in the above studies: *C. parvum*, *C. muris*, *C. suis*-like genotype and *C. scrofarum*, are known to cause human cryptosporidiosis (Kvac *et al.*, 2009; Xiao, 2010). These findings suggest that wild rats pose public and veterinary health risk since they can contaminate food stores and water sources with faecal droppings (Meerburg *et al.*, 2009; Singleton *et al.*, 2010). Companion animals, such as dogs and cats, are most often infected with host-specific *C. canis* and *C. felis* (Fayer *et al.*, 2006; Koompapong *et al.*, 2014). A study in Thailand identified *C. canis* and *C. parvum* in dogs with proportions of 72% and 28%, while in cats, *C. canis*, *C. parvum* and *C. felis* were identified with magnitude of 40%, 40% and 20%, respectively. About 30% - 40% of the infections were attributed to *C. parvum* suggesting the potential of zoonotic transmission from dogs and cats (Tangtrongsup, 2013). *C. canis* and *C. felis* infections were rarely reported for humans nonetheless, the role of these species has become significant, especially, in the immunocompromized subjects (Adamu *et al.*, 2014). The significance of *Cryptosporidium* infections in aquaculture and fish welfare is uncertain, although morbidity and mortality have been reported in hatcheries with high prevalence of infection (Garbor *et al.*, 2011).

Table 4: Overview of prevalence studies of *Cryptosporidium* in cattle in different countries (Modified after Geurden *et al.*, 2006)

Country	D	# C	# F	Age	PC%	PF%	Reference
Canada	M	500	51	7-21d	41	76	(Trotz-Williams <i>et al.</i> , 2007)
France	ELISA	2,068	196	<21d	23	-	(Lefay <i>et al.</i> , 2000)
USA	IFA	393	14	<2m	41	100	(Santin <i>et al.</i> , 2004)
Czech Republic	M	2,056	11	<2m	27	100	(Kvac <i>et al.</i> , 2006)
		367	11	<2m	1	27	
Belgium	PCR	234	100	<1m	45	-	(Geurden <i>et al.</i> , 2004)
		234	100	>1m	49	=	
Zambia	ELISA	250	37	<3m	43	76	(Geurden <i>et al.</i> , 2006)
		238	25	<3m	8	44	
USA	M	2,943	109	<6m	2	13	(Wade <i>et al.</i> , 2000)
Norway	IFA	1,386	136	<6m	12	53	(Hamnes <i>et al.</i> , 2006)
USA	IFA	447	14	3-11m	26	100	(Santin <i>et al.</i> , 2004)
Denmark	IFA	895	50	<12m	40	96	(Maddox <i>et al.</i> , 2006)
USA	PCR	571	14	1224m	12	93	(Fayer <i>et al.</i> , 2006)
Czech Republic	M	2,217	11	>3m	0	0	(Kvac <i>et al.</i> , 2006)
		2,381	11	>3m	0	0	

**Key:** Diagnostic technique (D); Number of calves (#C) Number of Farms (#F), Age of calves, prevalence in calves (PC%) prevalence in farms (PF%); Microscopy (M).

### 2.6.1. Pathogenesis and clinical symptoms in cattle

The intestinal *Cryptosporidium* species, *C. bovis* and *C. parvum*, complete their life cycle in the ileum, and occasionally in the colon, caecum and duodenum. The pathogenicity of *C. bovis* is believed to be limited, probably due to immunity in older calves (Santin *et al.*, 2004). In young calves, *C. parvum* is considered highly pathogenic and predominate other species. The invasion and colonisation of the epithelial surface by the different parasitic stages results in loss of epithelial cells and microvillus brush border. Furthermore, the epithelial tight junctions are disrupted leading to an increased epithelial permeability, decreased intestinal surface area, impaired nutrient and electrolyte transport as well as loss of membrane-bound digestive enzymes such as lactase. *Cryptosporidium* associated diarrhoea is caused by two pathogenic mechanisms. Malabsorptive diarrhoea is caused by loss of enterocytes and blunting of villi, which reduces the intestinal surface and presence of mature cells, leading to decreased nutrient and water absorption

(Foster and Smith, 2009; Klein *et al.*, 2008). Prostaglandins induce secretion of chloride and carbonate ions into the intestinal lumen, and decrease absorption of sodium chloride; this produces an osmotic pressure that forces water into the lumen, resulting in secretory diarrhoea (Foster and Smith, 2009).

Intestinal damage caused by massive infection may lead to reduced growth rates; however, intestinal absorption was restored three weeks post infection, indicating that no prolonged or permanent damage occurs (Klein *et al.*, 2008). In histopathological preparations villus shortening and fusion, as well as crypt hyperplasia and an increase in intra-epithelial lymphocytes can be observed (Robinson *et al.*, 2003). Clinical symptoms are most frequently observed in calves between the age of 5 days and 1 month, it includes malabsorptive and secretory diarrhoea which is usually self-limiting within 2 weeks. The diarrhoea can be mild to severe with pale to yellowish watery or mucoid faeces. Calves can be dehydrated, depressed and anorectic. The severity and duration of the clinical symptoms is highly variable, depending on concurrent viral, bacterial or parasitic infections, but also on host factors (Klein *et al.*, 2008). *Cryptosporidium andersoni* is a cattle specific species with a prepatent period of 18-45 days (Kvac *et al.*, 2008). This species invades the peptic and pyloric glands of the abomasum in weaned calves and older cattle causing glandular dilatation and hypertrophy of the gastric mucosa and thinning of the epithelial lining (Ralston *et al.*, 2003). Infection with *C. andersoni* does not usually result in pronounced diarrhoea, but mainly causes inhibition of protein digestion due to increased gastric pH and decreased gastric proteolytic activity. This results in maldigestion; moderate to severe impairment of weight gain, decreased feed efficiency and reduced milk production (Ralston *et al.*, 2003).

Mortality is variable and is most often observed in calves most of the infected calves recover spontaneously but a few may die. On necropsy, the small or large intestine or both may be distended with gas and contain watery yellow fluid. Enteritis and colitis may be apparent. Calves with severe cryptosporidiosis can take several weeks to fully recover, and there is certainly an initial negative impact on production due to weight loss or impaired weight gain, and due to treatment expenses. Whether cryptosporidial infections early in life have long-term detrimental effects is uncertain (Ralston *et al.*, 2003). Calves that recover from a *Cryptosporidium* associated diarrhoea usually do not have recurrent clinical infections.

### 2.6.2. Chemotherapy

Several chemotherapeutic agents have been tested for the treatment of bovine cryptosporidiosis, but none has resulted in a 100% reduction of oocyst excretion. Effective drug are lacking due to antimicrobial resistance, however, spiramycin, halofuginone and paromomycin may be of some value in reducing oocyst output and the severity of diarrhoea and/or mortality in infected animals (Fayer and Xiao, 2008). Although halofuginone could not completely prevent infections under field conditions, it is the only effective drug available for the treatment of bovine cryptosporidiosis. It is relatively toxic and requires accurate dosages according to given instructions (Joachim *et al.*, 2003).

Prophylactic halofuginone treatment had no effect on mortality but lowered infection and diarrhoeal prevalence as long as calves were treated. However, it is clear that infection or diarrhoea cannot be completely prevented even during treatment, and both infection prevalence and diarrhoeal prevalence increased in treated calves once treatment had stopped (Silverlås, 2010). The effect of halofuginone is cryptosporidiostatic, it depresses rather than kills the parasites (Silverlås, 2010) and it is toxic at twice the recommended dosage, thus precise dosage is required and it has to be administered to each calf individually which is time-consuming. Therefore, considering its cryptosporidiostatic effect and its narrow therapeutic window, this drug should be reserved for herds with severe problems and only be used in a transitional period in conjunction with improved management routines to decrease infection pressure (Silverlås, 2010). In Belgium and other European countries, halofuginone lactate is the only registered drug for treatment in calves, at a dose rate of 100µg/kg bodyweight per day during seven consecutive days. Toxicity can be observed at a dose rate of 500µg/kg. For preventive treatment, halofuginone should be administered within 48 hours after parturition, and for curative treatment, within 24 hours after the onset of the clinical symptoms (Joachim *et al.*, 2003; Jarvie *et al.*, 2005). Treatments of cryptosporidiosis consist of supportive (fluid and electrolyte) therapy. Keep affected animals warm, dry, well fed and at a constant ambient temperature, to minimize their energy requirements during the course of clinical disease. Treatment with halofuginone lactate reduces the occurrence of diarrhoea and postpones oocyst excretion and results were better in experimental settings than under natural conditions with a high environmental infection pressure (Jarvie *et al.*, 2005).

Although not registered for the treatment of cryptosporidiosis, prophylaxis with paromomycin sulphate is effective in preventing oocyst excretion and clinical signs and mortality in calves and goat kids, at a dose rate of 100mg/kg BW per day during 10 or 11 consecutive days. Similar to halofuginone, efficacy was higher under experimental compared to natural conditions (Grinberg *et al.*, 2002; Johnson *et al.*, 2000). In naturally infected lambs, curative treatment with paromomycin at a similar dose rate during 2-3 consecutive days resulted in a reduction of oocyst excretion and clinical symptoms (Viu *et al.*, 2000). Paromomycin or amanosidin is a broad-spectrum aminoglycoside antibiotic, with well-known efficacy against several protozoan parasites like *Cryptosporidium* in calves (Viu *et al.*, 2000; Grinberg *et al.*, 2002), Paromomycin is poorly absorbed from the gastrointestinal tract and is therefore well tolerated by calves (Grinberg *et al.*, 2002). Azithromycin is another antibiotic with therapeutic efficacy against *Cryptosporidium* (Elitok *et al.*, 2005), but is not registered for use in calves

### **2.6.3. Prevention and control**

Good hygienic measures are important in disease control and prevention. Because *Cryptosporidium* infections are initiated through ingestion of environmentally resistant oocysts, control of this stage is the single most important factor in limiting the spread of the disease. The logical approach of prevention is to minimize faecal-oral transmission between young animals, especially calves; healthy calves should be confined separately from sick calves. Use all-in, all-out management, with thorough cleaning and several weeks of drying between batches of calves. Pens and utensils should be cleaned with an ammonia solution and dried for several days before introduction of a new group of calves. Rats and mice should be controlled, because they probably are a reservoir for *C. parvum* infection of calves (Xiao *et al.*, 2004). Infected animals and humans will continue to contaminate the environment, and elimination of these sources is virtually impossible. The control of cryptosporidiosis in calves relies on a combination of animal treatment and appropriate hygienic measures and management. Due to the particular parasite characteristics, such as high oocyst excretion by infected calves, environmental resistance of excreted oocysts and presence of asymptomatic carriers, cryptosporidiosis should be considered as an endemic problem on infected farms. Hygienic measures must therefore aim to minimize the environmental infection pressure in order to prevent the spread of infection to susceptible calves and to break the

transmission cycle. Frequent removal of bedding and thorough cleaning combined with disinfection help to reduce the oocyst load in the environment (Castro-Hermida *et al.*, 2002a).

*Cryptosporidium* oocyst is extremely resistant to commonly used disinfectants, most of the effective disinfectants, ammonia, methyl bromide, ethylene oxide and ozone, are toxic thus emphasis should be given to cleaning and removal of faecal material rather than disinfection. Oocysts are susceptible to extreme temperatures and to desiccation (Li *et al.*, 2010), cleaning with hot water followed by drying is therefore the most effective practical means for killing oocysts or reducing infectivity (Xiao *et al.*, 2004). Good management practices include warm and dry individual calf facilities, avoiding high stocking densities and quarantine unit to isolate clinically affected calves (Castro-Hermida *et al.*, 2002a). Separate tools, boots and coveralls should always be used to prevent spread of the infection.

Since colostral antibodies protect calves from developing severe clinical symptoms by blocking parasite invasion and immobilisation of gut luminal parasitic forms, ensuring adequate and timely intake of colostrum by calves had been stressed to prevent *Cryptosporidium* infection. Timely feeding of colostrum is the simplest and most effective method to prevent diarrhoea in neonatal calves, while in post weaned calves, the daily appliance of high-pressure cleaning and use of straw bedding in calf pens had shown preventive effects against *Cryptosporidium* oocyst contamination (Zhang *et al.*, 2013). Delayed supply of colostrum was reported as a putative risk factor for cryptosporidiosis and subsequently, maternal milk was mentioned to having significant role in protecting neonates against *Cryptosporidium* infection in humans and animals (Trotz-Williams *et al.*, 2007; Bilenko *et al.*, 2008)

#### **2.6.4. Prevention and Control of Cryptosporidiosis in Source and Drinking Water**

*Cryptosporidium* oocysts are remarkably resistant to a wide range of environmental pressures and to most disinfectants and antiseptics. Most viruses, bacteria and protozoa, such as *Giardia*, are effectively inactivated by the commonly used water treatment method of chlorination or ultraviolet (UV) disinfection; however, *Cryptosporidium* is more resistant to environmental conditions and to chlorination. Oocysts of *Cryptosporidium* are generally removed from wastewater and drinking

water sources using membrane filtration, ozone oxidation or ultraviolet (UV) disinfection (Kothavade, 2012; Khan, 2013). Severely immunocompromized persons should avoid any contact with young animals, water in lakes and streams and should not drink such water. Routine testing of drinking water should be considered in all water-treatment plants since any accidental treatment failure may pose significant risk to public health (Kothavade, 2012).

## **2.7. Laboratory diagnostic methods**

Variety of confirmatory diagnostic techniques has been developed to identify *Cryptosporidium* oocysts or DNA in stool, intestinal fluid, tissue samples, biopsy specimens, or other biological samples, the methods having high positive predictive value (PPV) include direct fluorescent antibody (DFA), serological tests, polymerase chain reaction (PCR), enzyme immunoassay (EIA), or microscopy with tinctorial and fluorescent stains (CDC, 2012). Detection of *Cryptosporidium* antigen by screening test methods such as immunochromatographic card/rapid card test gives presumptive diagnosis of the infection. In-vitro propagation of the organisms is not possible. The immunofluorescence microscopy is used as the gold standard method of detecting *Cryptosporidium* oocysts at reference laboratories in USA and Europe (Checkley *et al.*, 2015), whereas, the modified Ziehl-Neelsen staining is considered as the gold standard method by others (Idzi and Marjan, 2010).

### **5.7.1. Histopathology**

Histopathological diagnosis of *Cryptosporidium* depends on the identification of the 4-6 µm intracellular spherical oocysts (oocyst components) within biopsy specimens of gastrointestinal mucosa (Suleiman *et al.*, 2001). In haematoxylin and eosin-stained sections, developmental stages of the parasite appear as small, spherical, basophilic bodies (2-5 µm) within the microvillous region of the intestinal mucosa. Transmission electron microscopy can be used to confirm diagnosis and reveal distinct life cycle forms, each within a parasitophorous vacuole confined to the microvillous region of the host cell (Fayer and Xiao, 2008).

### **2.7.2. Concentration methods**

Stool concentration techniques that are useful for identification of *C. parvum* oocysts include flotation of oocysts in Sheather's sugar solution, zinc sulphate (specific gravity 1.18 or 1.2) or saturated sodium chloride (specific gravity 1.27). Stool concentration techniques using sedimentation include formalin-ether and formalin-ethyl acetate. If one is using concentration methods to look for *C. parvum* oocysts in stool or other body fluid samples, it is advisable to centrifuge at greater than 500 x g for at least 10 min.

### **5.7.3. Staining of faecal specimens**

Diagnostic sensitivity of microscopic staining methods is often limited by the shedding of organisms intermittently or in low numbers. This sensitivity is also dependent on the skills of the microscope technicians. The differential staining methods for *Cryptosporidium* include: The modified acid fast Ziehl-Neelsen stain (Henricksen and Pohlenz, 1981), the negative staining technique of Heine, Safranin stain, trichrome stain and DMSO-carbol fuchsin (Pohjola *et al.*, 1985) which stain the parasite in red/ unstained/ bright reddish-orange and counterstain the background. Auramin erhodamine staining of stool sediment smears followed by modified Ziehl-Nielsen (acid-fast) confirmatory staining is a sensitive and specific approach for the identification of *Cryptosporidium* oocysts in stool. Since oocysts of *Cryptosporidium* species are indistinguishable from one another, molecular methods are essential for identification of the

species, genotype, and subtype of *Cryptosporidium* in order to specifically identify the organism responsible for the infection and the source and routes of transmission (Putignani and Menichella, 2010).

#### 5.7.3.1. *The modified acid fast Ziehl-Neelsen stain (Henricksen and Pohlenz, 1981)*

This Gold Standard stain for the detection of *Cryptosporidium* species is classically performed by staining a methanol fixed thin smear of faecal material with undiluted carbol-fuchsin solution for at least 15 minutes. Subsequently, the slide is rinsed in tap water and placed in an acid-alcohol solution to remove the stain, while acid-fast structures will resist to the acid-alcohol's destaining action. After rinsing again, the slide is placed for a short period in a counter-staining product, such as methylene blue, providing contrast between background material and acid-fast structures. The slide is rinsed once more and after the slide has been air-dried, it can be examined using x40 eyepieces and an oil-immersion objective of x100 magnification (Henricksen and Pohlenz, 1981). *Cryptosporidium* oocysts will appear as pink stained, round to oval structures of about 4 to 6 µm in diameter, containing distinct internal structures (Figure 2A).

The modified acid-fast staining is a time-consuming procedure (about 30 to 45 minutes) and good staining and visual skills are necessary. A common problem is distinguishing *Cryptosporidium* oocysts from other elements, such as moulds and yeast, these “pseudo-Cryptosporidia” can be ruled out based on their dimensions (Idzi and Marjan, 2010). Although the modified Ziehl-Neelsen staining remains the Gold Standard for the detection of *Cryptosporidium* species it is claimed to lack sensitivity and specificity (Idzi and Marjan, 2010). It has about 70% sensitivity compared with immunofluorescent antibody stains (Chalmers *et al.*, 2011). However, lack of specificity could be resolved by lowering the sensitivity of the test. For instance, a sample could be considered positive if five oocysts or more were observed, causing samples with low-level shedding of oocysts to be interpreted as negative. This extra loss of sensitivity in turn could be resolved by using repeated stool sample examinations on consecutive days (Brook *et al.*, 2008a). The modified Ziehl-Neelsen staining is a low cost technique and provides a permanent stain that makes it possible to send doubtful or scanty positive slides to a reference laboratory for confirmation (Paul *et al.*, 2009).

### 5.7.3.2. The negative staining technique of Heine

In this method a small amount of faecal matter is mixed with an equal amount of undiluted carbolfuchsin solution on a microscope slide. A thin smear is prepared, allowed to air dry and examined using x40 eyepieces and an oil-immersion objective of x100 magnification. *Cryptosporidium* oocysts appear as unstained, strongly refractive, round to oval structures of about 4 to 6  $\mu\text{m}$  in diameter. Internal structures are slightly visible as darker specks inside the oocyst (Figure 2B). The slides should be examined within 15 minutes after they have been air-dried. This time-lapse can be prolonged to 30 minutes by using samples which have been fixed in 10% formalin, prior to staining. If the slide is not examined within 15 - 30 minutes the oocysts will dry out and become less visible (Idzi and Marjan, 2010).

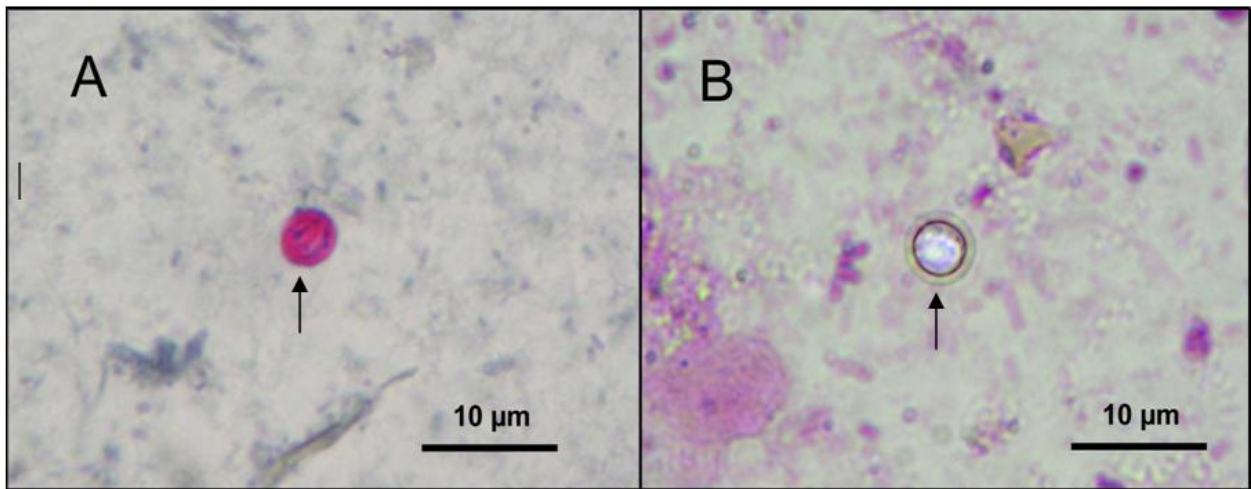


Figure 2: Oocyst of *Cryptosporidium* spp. stained by: (A) the modified Ziehl-Neelsen stain (B) The negative stain of Heine Source: (Potters and Van Esbroeck, 2010)

### 2.7.3.3. Safranin stain

Oocysts of *Cryptosporidium* often (but not always) stain a bright reddish-orange colour (Figure 3A). This method, advocated for *Cyclospora*, is not widely used for *Cryptosporidium* because *Cryptosporidium* oocysts may not always properly stain (CDC, 2015).

#### 2.7.3.4. Trichrome stain

Oocysts may be detected, but should not be confirmed, by this method. This staining method is inadequate for definitive diagnosis because all oocysts will appear unstained. Oocysts appear as small round structures measuring 4 to 6  $\mu\text{m}$  (Figure 3B) (CDC, 2015).

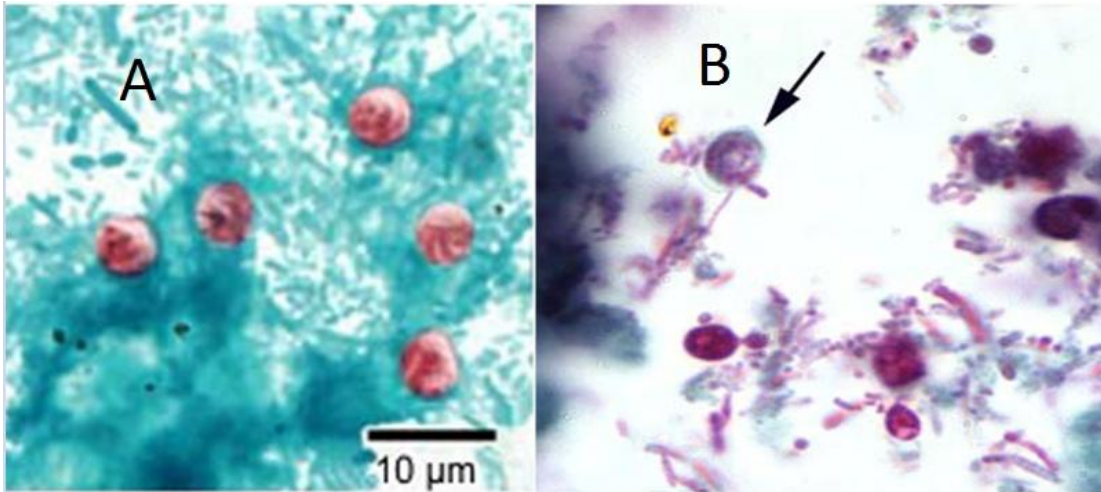


Figure 3: *Cryptosporidium* oocysts;(A) Safranin stain (B) Trichrome stain (CDC, 2015)

#### 2.7.3. Serological methods

Serological assays are important tools for epidemiological surveillance of *Cryptosporidium*, as specific antibody responses develop after symptomatic and asymptomatic infections, and are able to discriminate past, recent, and repetitive infections. Since Immunoglobulin A (IgA) responses are generally short-lived and IgG responses can persist for several months, Antibody to Cp23 appears to correlate with distant infection, responses to Cp17 (gp15) suggest recent infection and responses to P2 are associated with repeated infection (Priest *et al.*, 2006).

##### 5.7.3.1. Antigen detection methods

These tests have good sensitivity (70–100%) several commercially-available kits in enzyme immunoassay, immunofluorescence assay, and immunochromatography test formats. But the tests

are costly for resource-poor countries (Chalmers *et al.*, 2011). Direct and indirect fluorescent antibody detection using mAbs against *Cryptosporidium* oocyst wall antigens (C-mAbs) are specific and sensitive methods for detecting oocysts in faecal smears and in environmental samples (Kaushik *et al.*, 2008). Many mAbs recognizing epitopes on the oocyst surface have been developed and commercialized. This technique offers the highest combination of sensitivity and specificity, and considered as gold standard test by many laboratories (Checkley *et al.*, 2015).

A comparative study of fluorescent antibody detection technique and the conventional staining methods showed a great increase in sensitivity and specificity of the fluorescent antibody test (Chalmers *et al.*, 2011). The three criteria recommended for the identification of *Cryptosporidium* oocysts by immunofluorescence are (1) characteristic apple-green fluorescence delineating the oocyst wall, under the FITC filter set (2) round or slightly ovoid objects; and (3) a size of 4 to 6  $\mu\text{m}$  in diameter for most human pathogens. Some kits include Evans Blue, which reduces nonspecific fluorescence and generates red background fluorescence (Smith, 2008) (Figure 4A). The fluorescent antibody detection technique does not provide a stained slide that can be archived, requires fluorescence microscope and commercially available test kits.

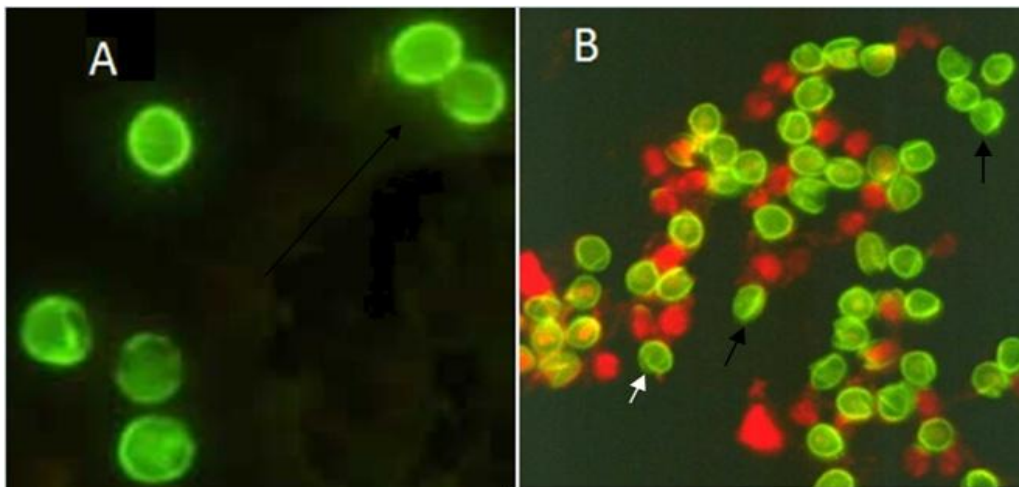


Figure 4: Oocysts of *Cryptosporidium parvum* by fluorescent antibody technique

Source: Fayer *et al.* (1997)

### 2.7.3.2. Antigen detection using antibodies labelled with enzyme reporters

Initially, enzyme immunoassay and enzyme-linked immunosorbent assay systems differed in assay design, but both techniques are based on the principles of separating bound from unbound reagents. EIAs are useful: (1) substances, such as antibodies or antigens, can be passively adsorbed to solid surfaces (2) one of the reactants is attached to the solid phase, the separation of bound and free reagents is easily undertaken (Smith, 2008). The reasons why the ELISA format predominates in infectious diseases diagnostics include: (1) passive adsorption to plastics in the form of 96-well microtiter plates (or 8 well strips) enables easy manipulation and dispensing of reagents, the use of small volumes of reagents, and the potential for handling large numbers of samples rapidly, and (2) the colour reaction can be read by eye, or automated using specially designed multichannel spectrophotometers, enabling electronic transfer of data that can be analyzed by statistical packages (Smith, 2008). *Cryptosporidium* antigens can be sought in faecal samples with or without oocyst concentration. ELISA and immunochromatographic (IC) formats are the applications used most for commercial kits.

### 2.7.3.3. Enzyme Immunoassays

Is an in vitro immunoassay for the qualitative determination of *Cryptosporidium* antigen in faeces, It is a double antibody (sandwich) ELISA using an anti-*Cryptosporidium* antibody to capture the antigen from the stool supernatant. A second anti-*Cryptosporidium* antibody is then added which sandwiches the captured antigen. This reaction is visualized by the addition of an anti-second antibody conjugated to peroxidase and the chromogen tetramethylbenzidine (TMB). The resulting blue colour development indicates the presence of *Cryptosporidium* antigens being bound by the anti-*Cryptosporidium* antibodies. This test, which has shown comparable sensitivity to experienced microscopic examinations, is simple to perform and do not require the observation of intact organisms (Smith, 2008). Limitations of the test includes: test results should be used as an aid in diagnosis and should not be interpreted as diagnostic by itself, assay will not give accurate results on a concentrated sample, a negative result can occur from an antigen level lower than the detection limits of this assay and multiple samples over time may be indicated for those patients that are suspected of being positive for *Cryptosporidium*.

#### 2.7.3.4. *Immunochromatographic Assays*

In lateral flow immunochromatography, all fluids are drawn by wicking action through a membrane enclosed in a cassette. Soluble *Cryptosporidium* antigens in the test sample are drawn through the membrane, to meet with, and bind to immobilized antibodies, which dramatically increases the speed of antigen–antibody interaction. Positive reactions are qualitative and are seen as a band of colour at a specific location on the membrane, normally identified by a line on the cassette (Smith, 2008). Assay format can vary between kits. Immunochromatographic assays provide diagnostic laboratories with a convenient alternative method for performing antigen detection assays for *Cryptosporidium* on stool samples. Specificity of the test was reported to be high (98–100%) (Smith, 2008), whereas reduced sensitivity (Weitzel *et al.*, 2006) had been reported compared to conventional staining methods.

#### 2.7.4. *Molecular detection methods*

Molecular methods for detecting *Cryptosporidium* in clinical specimens have been shown to be more sensitive than conventional microscopy (Chalmers and Katzer 2013; Yang *et al.*, 2013). Different types of molecular techniques have been used to differentiate *Cryptosporidium* species/genotypes, with the *SSU* rRNA-based tools being the most employed, especially PCR-RFLP (Xiao, 2010).

##### 2.7.4.1. *Polymerase Chain Reaction (PCR)*

DNA extracted from oocysts can be amplified by one of several PCR protocols: standard PCR, nested PCR, Reverse Transcription PCR (RT-PCR), real-time PCR, Random Amplified Polymorphic DNA PCR (RAPD-PCR) and Arbitrary Primed PCR (AP-PCR) (Egyed *et al.*, 2002). The nested PCR, PCR-restriction fragment length polymorphism (RFLP) and real-time PCR are the most commonly used methods for detection of *Cryptosporidium* and species identification (Xiao, 2010). In standard PCR, one pair of primers is used to amplify a gene in the forward (5'-) and reverse (3'-) directions, whereas in nested PCR two sets of primers are used, of which the first

(external) primer pair targets the gene of interest and the second (internal) primer pair amplifies a shorter (internal) segment of the amplicons produced by the primary PCR.

#### 2.7.4.2. Genotyping and sub-typing of *Cryptosporidium*

The gene encoding for 18S ribosomal subunit, the COWP gene, encoding a protein of the oocyst wall, the *hsp70* gene, which encodes a heat shock protein, internal transcribed spacer (ITS)-1 and ITS-2, the TRAP gene, and the gene encoding the glycoprotein GP60 and GP40 are the genetic markers used in *Cryptosporidium* (Xiao, 2010, Navarro-i-Martinez *et al.*, 2011, Galván *et al.*, 2014). A number of highly preserved genes of *Cryptosporidium* have been used as genetic markers and targeted for Genotyping of *Cryptosporidium* spp., these include the small subunit rRNA (18S rRNA), the 70 kilo Dalton (kDa) heat shock protein (HSP70), the COWP gene, encoding a protein of the oocyst wall, the 60 kDa glycoprotein (GP60) and the internal transcribed spacer (ITS)-1 and ITS-2, the TRAP gene and the actin genes (Spano *et al.*, 1997; Nichols *et al.*, 2010; Xiao, 2010; Valenzuela *et al.*, 2014).

The heat shock protein 70 kDa (HSP70) gene is a good target for sub-typing and multilocus study of *Cryptosporidium* isolates. This gene has a high level of heterogeneity spread over the entire sequence of a variety of *Cryptosporidium* isolates from human and animal hosts (Sulaiman *et al.*, 2000). The 18S rRNA gene is useful because in addition to regions that vary between species, it contains several regions that are conserved within the *Cryptosporidium* genus. This makes it easy to develop primers that target most species.

PCR and sequence analysis of the 60 kDa glycoprotein (GP60) gene has been frequently used for sub-typing of various *Cryptosporidium* isolates (Jex and Gasser, 2010). The GP60 gene has a highly polymorphic region of microsatellites in the 5' end, consisting of trinucleotide repeats (TCA, TCG, TCT), all coding for the amino acid serine. *Cryptosporidium* subtypes are named according to the number of each repeat; some subtypes have other short repetitive sequences (R) immediately after the trinucleotide repeats. *C. hominis* subtype families have prefixes Ia, b, and d-g, whereas *C. parvum* subtype families have prefixes IIa-IIk (Jex and Gasser, 2010; Xiao, 2010). About 14 subtype families of *C. parvum* (IIa to IIo) have been identified based on partial sequence

analysis of the GP60 gene. The IIc family has been found merely in humans, but other families, including IIa and IIc, are found in both humans and ruminants, and are responsible for zoonotic cryptosporidiosis. The IIa family is the most frequently reported subtype family in cattle worldwide (Ryan *et al.*, 2014). Nevertheless, GP60 subtyping is usually based on conventional Sanger sequencing, which is not effective in detecting the presence of mixed infections, and has mostly been used as a single-locus method, which is unreliable for investigating the population structure of sexually reproducing organisms (Widmer and Sullivan, 2012)

#### 2.7.4.3. PCR-RFLP

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) of the 18S rRNA gene is a technique largely used in *Cryptosporidium* genotyping. This gene is highly polymorphic within the genus and is useful as a target for the identification and differentiation of *Cryptosporidium* species and genotypes (Xiao, 2010). PCR-RFLP combines the PCR amplification with the digestion of the amplicons, or PCR amplified fragment. The digestion is performed by the use of a restriction enzyme. Regarding the translation of RNA to proteins, differences in the DNA sequence may cause two scenarios: a) the mutation does not cause a change in the amino acid (synonymous mutation), or b) the mutation may change the codon changing the amino acid (non synonymous mutation). Even if the mutation is synonymous, it may introduce a new restriction site for a restriction enzyme or delete it. If these mutations or polymorphisms exist in different species and strains, the PCR-RFLP takes advantage of this fact and shows different restriction patterns in agarose gel for different species. The usefulness of the SSU rRNA-based PCR-RFLP technique to differentiate *Cryptosporidium* species and the *C. parvum* genotype and for tracking *Cryptosporidium* contamination sources in water samples had been reported (Xiao, 2010). One disadvantage of PCR-RFLP technique is that it uses endonuclease (s) that only recognize a small number of variable sites, and such approach do not detect all of the length and sequence variation within or among amplicons during analysis. PCR and PCR- RFLP of the COWP gene, a single copy gene encoding major constituent of the inner layer of *Cryptosporidium* oocysts wall protein, is an alternative genotyping tool frequently used in molecular study of *Cryptosporidium* (Spano *et al.*, 1997).

#### 2.7.4.4. PCR and direct sequencing

The direct sequencing of an amplified gene or gene portion remains the “gold standard” approach for detecting genetic variation or polymorphisms and, consecutively, accurate species assignment. It is the most common technique used throughout the world regarding genotyping of *Cryptosporidium*. This technique has been used in the assignment of genotypes inferred by a neighbour-joining analysis of the partial SSU rRNA gene. It can be applied to single-copy and multi-copy genes in several other organisms, such as *Toxoplasma*, *Echinococcus*, or *Escherichia coli* (Xiao *et al.*, 2004). With the use of the PCR direct sequencing more information is obtained from the markers.

### 3. MATERIAL AND METHODS

#### 3.1. Study area

This study was conducted in Addis Ababa and its surrounding Oromiya districts (Akaki, Sebeta, Barak, Walmara and Sululta). The study area is located between latitudes of  $8^{\circ} 46' 34''$  and  $9^{\circ} 23' 00''$  N and longitude of  $38^{\circ} 32' 27''$  and  $39^{\circ} 14' 43''$  E and situated at an altitudinal range of 1950 to 2600 m.a.s.l. (Figure 5). It has around 5,627,934 inhabitants (CSA, 2007) and about 5200 large, medium and small scale dairy farms with 58,500 cattle (almost 50 percent crossbred) (Tegegne *et al.*, 2002). Thirty thousand peoples directly depend on incomes earned from the dairy subsector. The total annual milk production is estimated to 44 million litres about 79% of which comes from urban producers (Tegegne *et al.*, 2002).

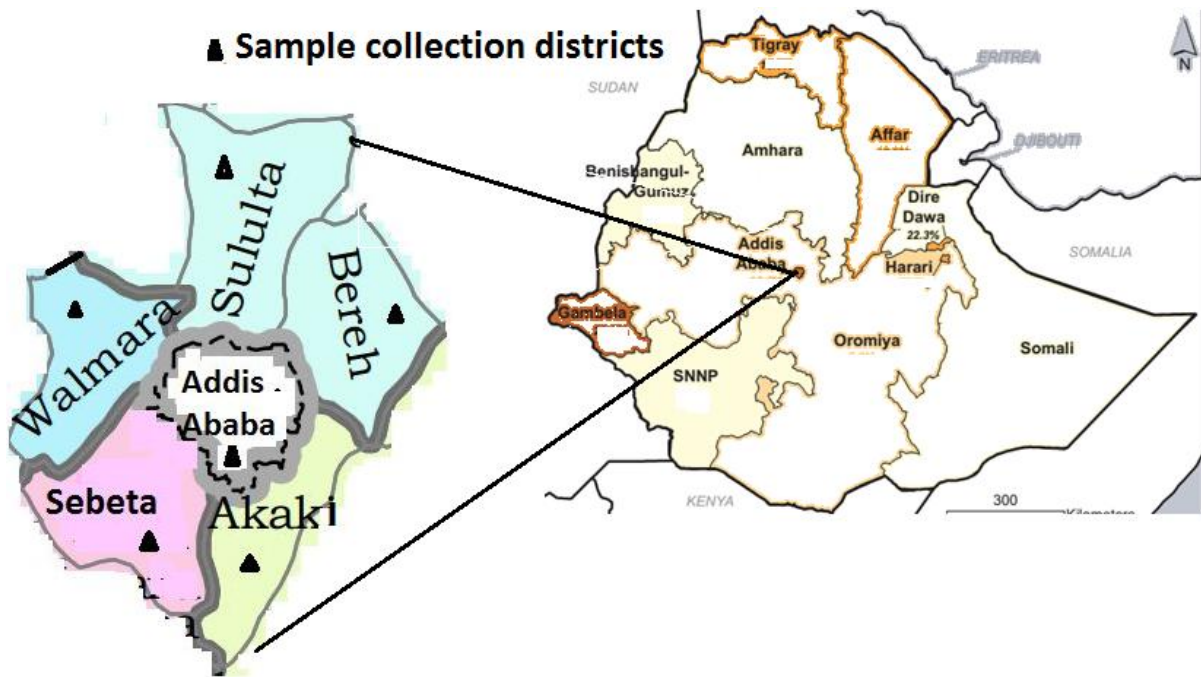


Figure 5: Location of sampling districts in Addis Ababa and its environs (June/2014-June/ 2015)

### ***3.1.1. Livestock production system***

Three major dairy production systems are distinguished in the study area:-

**The highland smallholder milk production:** is predominantly subsistence smallholder mixed farming, with crop and livestock husbandry typically practised within the same management unit. The majority of milking cows are indigenous animals which have low production performance (Tegegne *et al.*, 2002). But also a very small number of crossbred animals are milked to provide the family with fresh milk butter and cheese. Surpluses are sold, usually by women, who use the regular cash income to buy household necessities or to save for festival occasions (Tegegne *et al.*, 2002). All feed requirement is derived from native pasture and a balance comes from crop residues and stub grazing.

**Urban and peri-urban milk production:** is commonly found in and around major cities and towns which have high demand for milk. It is based on cross breed dairy stock, mainly Friesian x Zebu and comprises small and medium size dairy farms (Zegeye, 2003). The main feeds sources are agro-industrial by products (Oil Seed Cakes, Bran, etc) and purchased roughage, farmers also use all or part of their land for home grown feeds. The primary aim of this production system is to sale milk as a means of additional cash income.

**Intensive Dairy Farming:** is a more specialized dairy farming based on exotic pure bred stock. It is practised by individuals and some state sector on commercial basis. The urban, peri-urban and intensive dairy farms produce 2% of the total milk production of the country (Zegeye, 2003).

### ***3.1.2. Water supply and sanitation***

The water supply of Addis Ababa originates mainly from three surface water reservoirs and one well system situated in rural landscapes. Legadadi and Dire Dams located at a distance of 30 km Northeast and Gafersa Dam located 20 km Northwest of Addis Ababa supply 80% of the total water volume while the Akaki wells located at a distance of 10 km South of the city cover the remaining 20% water supply (Van Rooijen and Taddesse, 2009) (Figure 6). Wastewater treatment capacity is very small in Addis Ababa (NEDECO, 2002). Therefore, wastewater is discharged

directly into natural water courses of the Akaki River, which eventually joins the Awash River. The Akaki River is an important source of water for small scale farmers in and around Addis who are producing vegetables and fodder for livestock. The registered Wastewater irrigated land is estimated to 400 ha, but the real figure is assumed much higher (Van Rooijen and Tadesse, 2009).

Less than 10 % of Addis Ababa, mainly the central area, is sewerred, while 75% of the households make use of pit latrine that disposes their wastewater in the storm water drainage network (AAWSA, 2008). The remaining quarter relies on a flush toilet (17%) or uses a field/forest (6%) or another way (2%). Addis Ababa has two sewage treatment plants. The Kality treatment plant, runs under its designed capacity of 7,600 m<sup>3</sup>/day or 200,000 population equivalents, while it treats on average 5,200 m<sup>3</sup>. The other treatment plant, called Kotebe treatment plant, receives only sludge from vacuum trucks that empty septic tanks, with an estimated annual volume of 85,000m<sup>3</sup> (NEDECO, 2002).

The great majority of the rural community and their livestock water supply rely on groundwater through shallow wells, deep wells and springs. People who have no access to improved supply usually obtain water from rivers, unprotected springs and hand-dug wells. Wells, rivers and springs can be contaminated and cause waterborne diseases (Seleshi *et al.*, 2007). However, between 1990 and 2008 the country's water coverage has increased at a promising rate with an increment of 38% in improved water supply (98% for urban and 26% for rural areas) and 12% in improved sanitation (29% in urban and 8% in rural areas) (JMP, 2010). Despite the progress seen in the country, 43% of the population does not have access to an improved water source and 28% practice open defecation.

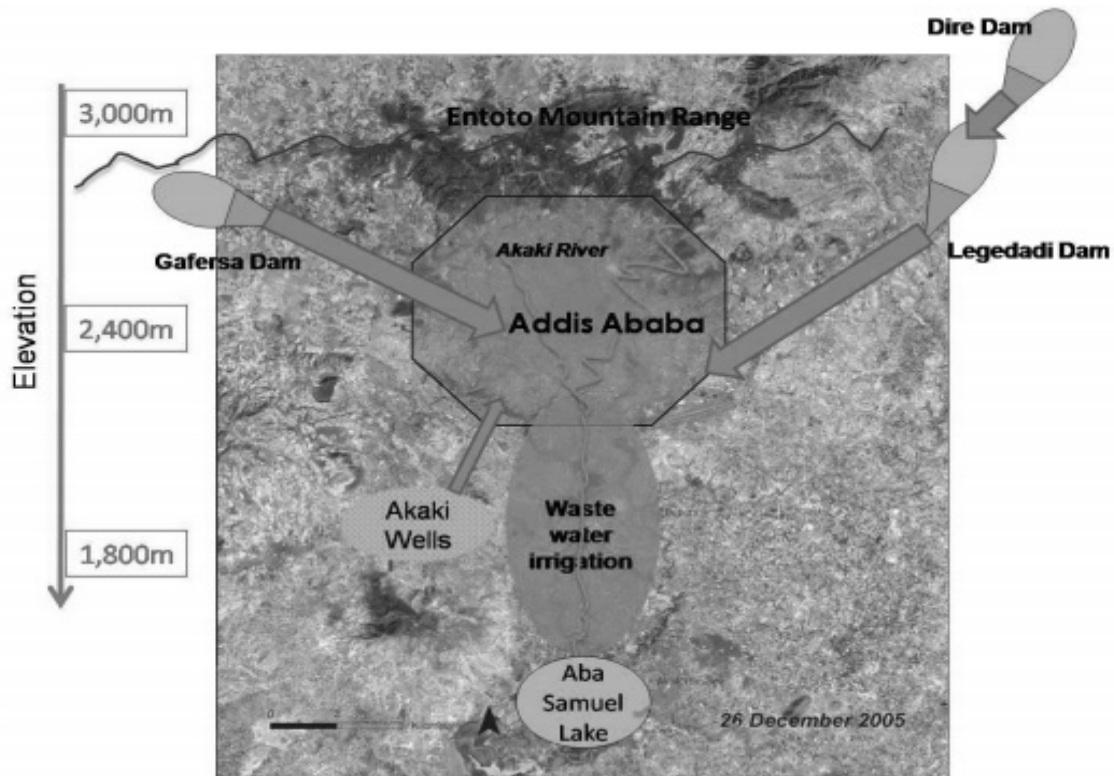


Figure 6: Satellite image of Water links of Addis Ababa with surrounding rural area

Source: Van Rooijen and Taddesse (2009)

### 3.2. Study design and sample size

Cross-sectional and longitudinal study designs were used in this study, the cross-sectional to determine the prevalence of *Cryptosporidium* in cattle and human inhabitants, and the longitudinal to determine the age-related distribution of *Cryptosporidium* in dairy cattle calves. The sample sizes for the cross-sectional studies were determined using the formula by Thrusfield (2005) at a precision level of 5% and confidence interval of 95%. Based on previous reports in the country prevalence of 15 % for cattle (Abebe *et al.*, 2008) and 11 % for human (Shimelis *et al.*, 2016) were used in the formula to calculate the sample size for the studies on cattle and human, respectively.

$$n = \frac{1.96^2 P_{\text{exp}} (1 - P_{\text{exp}})}{d^2}$$

Where: n = required sample size

$P_{\text{exp}}$  = expected prevalence

d = desired absolute precision

Accordingly, the calculated sample size to estimate prevalence in simple random sampling was 196 for cattle and 150 for humans. However, in order to adjust the sample size required for the present multistage random sampling method and to make the prevalence estimates more precise, the sample sizes were inflated twice and set to 392 for the study on cattle and to 300 for the study on humans.

### **3.3. Study population and sampling**

The study population for the cross-sectional studies on humans and cattle were comprised of human and cattle population in the study area, whereas for the longitudinal study, it contained calves born at two selected dairy farms (Holeta Agricultural Research Centre (HARC) and Holeta Cattle Genetic Improvement Centre (HCGIC)) during February.-August/ 2014.

#### ***3.3.1. Animal population***

##### *3.3.1.1. Cross-sectional study*

The study animals were selected by a two-stage proportional stratified random sampling method whereby dairy farms and Peasant Associations (PAs) were selected first and the sample animals were then selected later. The systematic random sampling method was used throughout the study to select farm size and age group strata and finally the sample animals. Stratification was made twice to include large farms (> 100 cows) medium farms (30 -100 cows) and small farms (< 30 cows) as well as three age groups (< 2 months, 2-6 months and > 6 months). A total of 45 dairy farms and 24 PAs were selected from 2000 dairy farms and 300 PAs found in six districts (Kality, Akaki, Sebeta, Barak, Walmara and Sululta) of the study area. After selection of dairy farms and PAs, the sample animals were then selected from each age group at all dairy farms and PAs.

Accordingly, 392 animals were selected and single faecal sample was collected from each animal during the study.

### *3.3.1.2. Longitudinal study*

The longitudinal study was conducted at two government owned dairy cattle farms namely: Holeta Agricultural Research Centre (HARC) and Holeta Cattle Genetic Improvement Centre (HCGIC) found in Holeta town, 45 kms west of Addis Ababa. Thirty calves born to these farms during Feb.-Aug/ 2014 were followed up till three months of their age for the study. Nine faecal samples were collected from each calf, at a weekly interval till one month of their age and at a bi-weekly interval then after (day 1, day 7, day 15, day 21, day 30, day 45, day 60, day 75 and day 90).

### *3.3.2. Human population*

Dairy farm communities (farm owners, attendants and their children) of the selected dairy farms/PAs plus HIV seropositive people registered at health centers in Addis Ababa and its surrounding districts were the study population for this study. A total of 2000 dairy farm community members were listed of which 120 were chosen by the systematic random sampling method. Nine of the 32 ART centres found in the study area were selected by the simple random sampling method and 180 HIV seropositive patients, new comers and regularly visitors of these centres, were selected (by the systematic random sampling method) to be enrolled in the study. Hence 120 dairy farm community members and 180 HIV seropositive patients, 120 on-ART and 60 non-ART, were selected and used as the study participants for this study. A single stool sample and questionnaire data was collected from all study participants and analysed. In case of failure of getting consent or insufficient stool amount the next sample unit was considered for replacement.

### **3.4. Questionnaire and medical record data**

#### ***3.4.1. Questionnaire data***

A pre-tested questionnaire was used to collect relevant data on the study animals and human participants. Dairy farm owners, farm attendants, PA members and HIV seropositive study participants were respondents to the questionnaire. The questionnaire was designed to comprise mostly close ended (categorical) questions to ease data processing, minimize variation, and improve precision of responses. Data on risk factors of *Cryptosporidium* infection such as: - dairy farm location (urban, peri-urban, or rural), age, type of pen floor (concrete, kraal/stone, wooden), level of floor hygiene (clean, medium, dirty), source of drinking water (tap, well, river, spring) presence of diarrhoea and infectious diseases (Foot and Mouth Disease, Pasteurellosis) and disposal of farm waste water were collected thoroughly. Data from human participants include socio-demographic, age, sex, contact with animals, source of drinking water (tap, well, river, spring) and water treatment habits.

#### ***3.4.2. Data from medical record***

Medication history of the HIV positive study participants, ART medication status (pre-ART or on-ART) and period of ART medication were gathered from the medical records of their respective health stations, whereas the CD4<sup>+</sup> count of the participants (120-800 cell/ $\mu$ l) was collected on the sampling dates.

### **3.5. Sample collection and preparation**

Approximately 1-2 gram of faecal specimens were collected directly from the rectum of animals using sterile gloves, and the same amount of stool samples were collected from the human participants by the individuals themselves. Collected faecal /stool specimens were then kept in to sterile stool cups and conveyed to the laboratory in ice boxes.

### **3.6. Laboratory examinations**

In the laboratory, each faecal/stool specimen was portioned in to two halves immediately on the sampling day, the first portion was processed by the modified Ziehl-Neelsen acid-fast staining method and the remaining portion was preserved in 2.5% potassium dichromate (w/v) solution in 1:1 ratio and kept at 4<sup>0</sup>C for molecular studies.

#### **3.6.1. Microscopy**

##### *3.6.1.1. The modified Ziehl-Neelson method*

Thin slide smears of faecal/stool samples were stained by the modified Ziehl-Neelsen staining method (Henricksen and Pohlenz, 1981). A small amount of faeces was spread over the surface of a clean slide on an area of approximately 2cm x 1cm, the smears were allowed to dry and then fixed in absolute methanol for 5 minutes, in the case of human participants positive for HIV/AIDS, smears were fixed in formalin (37% Formaldehyde solution) vapour for 15 minutes by placing the slides in a petridish containing cotton wool ball soaked in formalin. The slides were then flood with carbol fuchsin stain (3% fuchsine and 4% phenol) for 5 minutes, washed off with water and flood with acid-ethanol solution (conc HCL and 95% ethanol) to decolorize until faint pink. Subsequent to washing in water, the slides were then counter-stained with 0.33% malachite green solution for 2 minutes, washed with water and placed in a slide rack to drain and dry. The dried slides were examined under the x100 magnification objective of the microscope. Oocysts of *Cryptosporidium* species stained by this method show a variety of stain reactions from pale pink to deep red. Oocysts measure 4–6µm and the sporozoites within the oocysts have an outer rim of deep stained material with a pale centre. This differentiates oocysts from some yeast that may stain red but have a homogeneous smooth appearance.

A sample was considered positive for *Cryptosporidium* spp. if an oocyst of correct morphology: optical properties, internal structure, size and shape was detected as described by Fayer *et al.* (1997). When *Cryptosporidium* oocysts were identified microscopically the positive results were recorded and the corresponding potassium dichromate preserved specimens were identified and

labelled to be used for molecular study. The intensity of infection was estimated semi-quantitatively according to the average number of oocysts in 20 randomly selected fields observed at 1000x magnification, following the criteria used by Castro-Hermida *et al.* (2002b): 0 (0 oocyst); I (1 oocyst); II (2–5 oocysts); III (6–10 oocysts); IV (>10 oocysts).

### **3.6.2. Molecular techniques**

#### *3.6.2.1. Extraction of genomic DNA*

DNA from faecal and stool samples was extracted using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, USA) following the manufacturer's suggested procedures. one ml InhibitEX Buffer was added in to 2 ml microcentrifuge tube containing 180–220 mg (200 µl If liquid) stool sample placed on ice, the solution was then vortexed continuously for 1 min, thoroughly homogenized and centrifuged at 20,000 x g (14,000 rpm) for 1 min to pellet stool particles. 600 µl of the supernatant was pipetted in to a new 2 ml microcentrifuge tube containing 25 µl proteinase K in to which 600 µl Buffer AL was added, vortexed for 15s and incubated at 70°C for 10 minutes. 600 µl of ethanol (96–100%) was added to lysate, and mixed by vortexing. 600 µl of the lysate was then carefully applied to the QIAamp spin column and centrifuged at 20,000 x g (14,000 rpm) for 1 minute. The QIAamp spin column was placed in to a new 2 ml collection tube and the tube containing the filtrate was discarded. This centrifugation step was repeated (average-2x) until the QIAamp spin column becomes empty and all of the lysate has been loaded on the column. The QIAamp spin column was carefully opened, 500 µl Buffer AW1 was add and centrifuged at 20,000 x g (14,000 rpm) for 1 min and the collection tube containing the filtrate was discarded. The QIAamp spin column was placed in to a new 2 ml collection tube, carefully opened, 500 µl Buffer AW2 added, centrifuged at 20,000 x g (14,000 rpm) for 3 min and the collection tube containing the filtrate was discarded; the QIAamp spin column was placed in to a new 2 ml collection tube, centrifuged at 20,000 x g (14,000 rpm) for 3 min and transferred into a new 1.5 ml microcentrifuge tube. Finally, 200 µl buffer ATE was pipetted directly in to the QIAamp membrane, incubated for 1 min at room temperature and centrifuged at 20,000 x g (14,000 rpm) for 1 min to elute DNA. The concentration of eluted DNA was measured spectrophotometrically

on a Nanodrop 2000 (Thermo Fisher Scientific inc.USA) and stored at -20<sup>0</sup>C before it was used for molecular analysis.

### 3.6.2.2. Nested PCR

A two-step nested PCR protocol was used to amplify a fragment of the SSU rRNA gene of *Cryptosporidium* species oocyst (840 bp) as described previously (Fayer and Xiao, 2008). In the primary PCR, a PCR product of 1325 bp was amplified using the forward and reverse primers SSU-F2 (5'-TTCTAGAGCTAATACATGCG-3') and SSU-R2 (5'-CCCATTTTCCTTCGAAACAGGA-3'), respectively. The primary, 25 µl PCR mixture, consisted of 1x PCR buffer, 6 mM MgCl<sub>2</sub>, 200 mM of each of the four deoxyribonucleotide triphosphates (dNTPs), 10 pmol of each primer, 2.5 units of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), 5 µl of template DNA and non-acetylated bovine serum albumin (BSA) (New England Biolabs, Beverly, MA, USA). The cycling conditions for the primary PCR were: an initial denaturation at 94<sup>0</sup>C for 3 min followed by 35 cycles (94<sup>0</sup>C for 45 s, 55<sup>0</sup>C for 45 s, 72<sup>0</sup>C for 60 s) and a final extension at 72<sup>0</sup>C for 7 minute. In the secondary PCR, a product size of 819-825 bp was amplified using the forward primer SSU-F3: 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and the reverse primer SSU-R4: 5'-CTCATAAGGTGCTGAAGGAGTA-3' (Fayer and Xiao, 2008).

The secondary PCR reaction mixture consisted of 1x PCR buffer, 3 mM MgCl<sub>2</sub>, 200 mM of each dNTPs, 10 pmol of each primer, 2.5 units of Taq DNA polymerase and 2 µl of the primary PCR product in a final volume of 25 µl. The cycling conditions for the secondary PCR were the same as the primary PCR. Secondary products of the nested PCR reactions were analyzed by 1.5% agarose gel electrophoresis and visualised on Gel red staining. Each specimen was analyzed at least twice using reagent water as the negative control and DNA of *C. baileyi* as the positive control to reduce the likelihood of PCR contamination by any of the bovine species. All PCR reactions were run on a Thermocycler (PCR-Gene Amp PCR System 9700, Applied Biosystems)

#### 3.6.2.3. Purification of nested PCR product

Prior to using for genotyping and sequencing, the 18S rRNA gene nested PCR product was purified using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instruction. Five volumes of Buffer PB was added to 1 volume of the nested product and mixed, the sample mixture was then applied to the QIAquick column placed in a 2 ml collection tube and centrifuged for 30–60 s to bind DNA, the flow-through was discarded and the QIAquick column was placed back in the same tube, then 0.75 ml Buffer PE was added to the QIAquick column and centrifuged for 30–60s to wash the sample, the flow-through was discarded and the QIAquick column was placed back in the same tube. The QIAquick column was centrifuged once again in the 2 ml collection tube for 1 min to remove residual wash buffer and the column was placed in a clean 1.5 ml microcentrifuge tube. Finally 30 µl of water (pH 7.0–8.5) was added to the centre of the QIAquick membrane and the column was let to stand for 1 min and centrifuged for 1 min to elute DNA.

#### 3.6.2.4. Restriction fragment length polymorphism (RFLP)

The purified nested products were digested using the *SspI*, *AseI* (*VspI*) or *MboII* (New England BioLabs Inc.USA) restriction enzymes (Xiao *et al.*, 1999a, 1999b, 2001a; Feng *et al.*, 2007a). Briefly, 10 µl of the purified secondary PCR product was digested with 5 units of each enzyme and 2 µl of the corresponding 10x buffer in a final volume of 20 µl. All restriction digestions were carried out at 37 °C overnight, fractionated on 2% agarose gel and visualised after Gel red staining.

#### 3.6.2.5. Sequencing

The sequencing reaction of purified secondary nested PCR products was performed in both directions using the secondary PCR primers and the Big Dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems, CA, U.S.A.) on an ABI 3730- 48 Capillary Genetic Analyzer (Applied Biosystems Sequencer, Foster City, CA). The obtained sequences were analyzed using the CLC main workbench (CLC version 7.6.4, QIAGEN Aarhus) and compared with the Gene Bank

sequences of *Cryptosporidium* using BLAST (Basic Local Alignment Search Tool, NCBI <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) to identify species and determine homology percent. Nucleotide sequence data reported in this work are available in the GenBank database under the accession numbers KX264360 to KX264365 and KX855997 to KX856004. Sequencing was performed at the Segolip unit of the Biosciences Eastern and Central Africa-International Livestock Research Institute Hub (BecA-ILRI Hub), Nairobi, Kenya.

### **3.6.3. Phylogenetic analysis**

Thirty nucleotide sequences (14 from this study and 16 GeneBank references) were involved in the phylogenetic analysis. Sequences from this study: KX264363, KX264364, KX264365, KX855997, KX855998, KX855999, KX856003 and KX856004 for *C. parvum*; KX856000, KX856001 and KX856002 for *C. hominis*; and KX264360, KX264361 and KX264362 for *C. andersoni* were aligned with each other and with sequences retrieved from the GeneBank. The GeneBank retrieved references include S40330, X64341, AB513881, AF040725, EU660038, AB513880, AY204238, KM870599 and AB089290 for *C. parvum*; JQ313989, EU186156 and KM085019 for *C. hominis* and KJ917578, KT175424 and KF826314 for *C. andersoni*. Sequence alignment was performed using CLUSTAL X version 2.0 Software (Larkin *et al.*, 2007, University College Dublin, Ireland). The phylogenetic tree was constructed by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach. The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. The tree was anchored by using *Eimeria tenella* (AF026388) as the out-group. Phylogenetic and molecular evolutionary analyses were done using the MEGA version 6 software (Tamura *et al.*, 2013).

### **3.7. Statistical analysis**

Data were analyzed by the SPSS statistical software package (SPSS ver. 20.0 for Windows, SPSS Inc, Chicago, IL). The Chi-Square test was used to evaluate the association between hypothesized risk factors and *Cryptosporidium* infection. Further analysis of the association was made by the multivariable logistic regression, the adjusted odds ratio (OR), computed as the exponent of the respective regression coefficients, was used to quantify the effect of risk factors on the likelihood of *Cryptosporidium* infection. Confidence level was held at 95% and  $P < 0.05$  was set for significance level.

### **3.8. Ethical considerations**

Ethical clearance for the study on human subjects was obtained from the Ethical Review Committee of Research, Addis Ababa City Administration Health Bureau (Ref. No: 4842/227). Informed written consent was obtained from the study participants at the time of sample collection and they were informed that their specimen and records will be examined by authorized persons, personal information are treated strictly confidential and that they are free to withdraw the consent at any time. Ethical clearance for the study on animals was obtained from the College of Veterinary Medicine and Agriculture, Addis Ababa University. The aim of the study was explained and permissions were obtained from farm owners before collection of samples and data.

## 4. RESULTS

### 4.1. Cross-sectional study of cattle

#### 4.1.1. Prevalence of *Cryptosporidium*

The infection was observed at all of the six surveyed districts with a range of 4.3% to 57.2% (Figure 7). Of 392 faecal samples examined using MZN and molecular methods *Cryptosporidium* infection was detected in 73 animals with an overall prevalence of 18.6% (95% CI: 14.8–22.5). The farm prevalence was 37.7% with a range of 7.4 – 100%. The prevalence obtained in cattle managed under the intensive production system, 21.4%, was significantly higher than the 11.2% prevalence in cattle managed under the extensive system ( $p = 0.021$ ). Dairy farms sited in urban area showed significantly higher prevalence (41.8 %) ( $p = 0.000$ ) than farms located in rural area (12.8 %). Prevalence of the infection was similar across the age groups ( $p = 0.749$ ) and between the sexes ( $p = 0.062$ ) (Table 5).

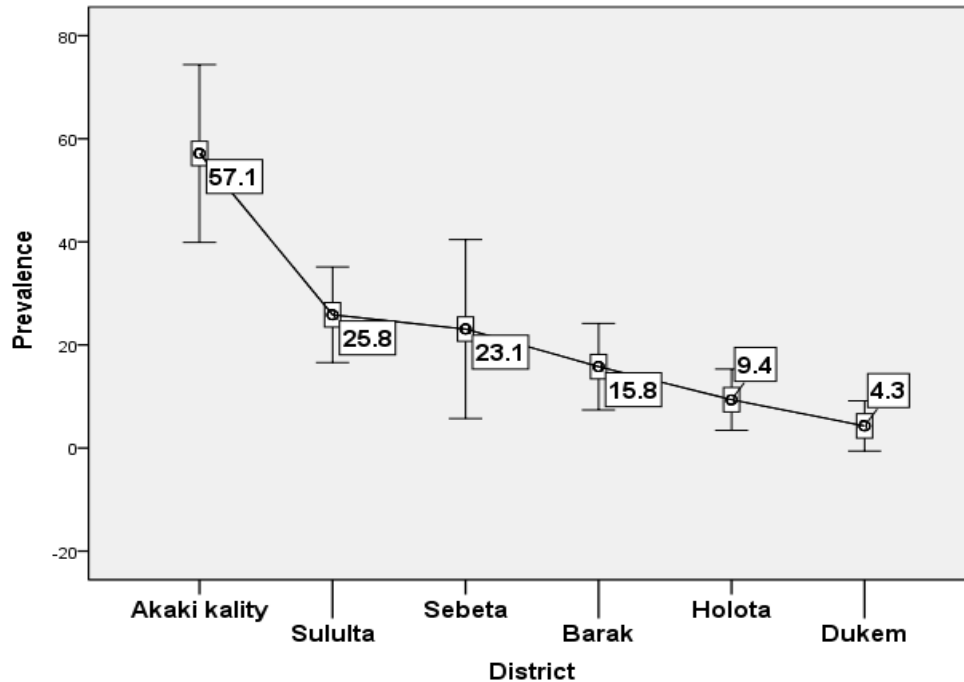


Figure 7: District-wise prevalence of *Cryptosporidium* infection in cattle(June/2014–June/2015)

Table 5: Prevalence of *Cryptosporidium* infection in cattle by Age, sex and management system (June/2014 - June/2015) (N=392)

Variables	Group	<sup>1</sup> No	Prevalence		P value
			percent	95% CI <sup>2</sup>	
Age group	< 2 months	104	17.3	9.8–24.8	0.749
	2-6 months	209	18.2	12.9–23.5	
	> 6 months	79	21.5	2.9–30.2	
Sex	Female	253	21.3	16.5–26.1	0.062
	Male	139	13.7	7.2–20.1	
Management system	Extensive	107	11.2	3.8–18.6	0.021
	Intensive	285	21.4	16.9–25.9	
Farm location	Rural	313	12.8	8.6–16.9	0.000
	Urban	79	41.8	33.5–50.0	
Overall		392	18.6	14.75 – 22.49	

<sup>1</sup> = Number of samples      <sup>2</sup> = Confidence Interval

#### 4.1.2. Oocyst morphology

Oocysts of *Cryptosporidium* were pinkish or red colored clusters of tiny oval to circular bodies measuring 4-6 µm. The sporozoites are visible in the center of oocysts (Figure 8).

#### 4.1.3. Intensity of infection

Out of 30 semi-quantitatively examined samples 12 showed an average of > 10 oocysts, 5 showed 6-10 oocysts, 6 showed 2-5 oocysts and 7 samples showed an average of 1 oocyst. All of the highest intensity of infections was in calves less than 3 months of age while adult cattle showed the least intensity of infection (Figure 9).

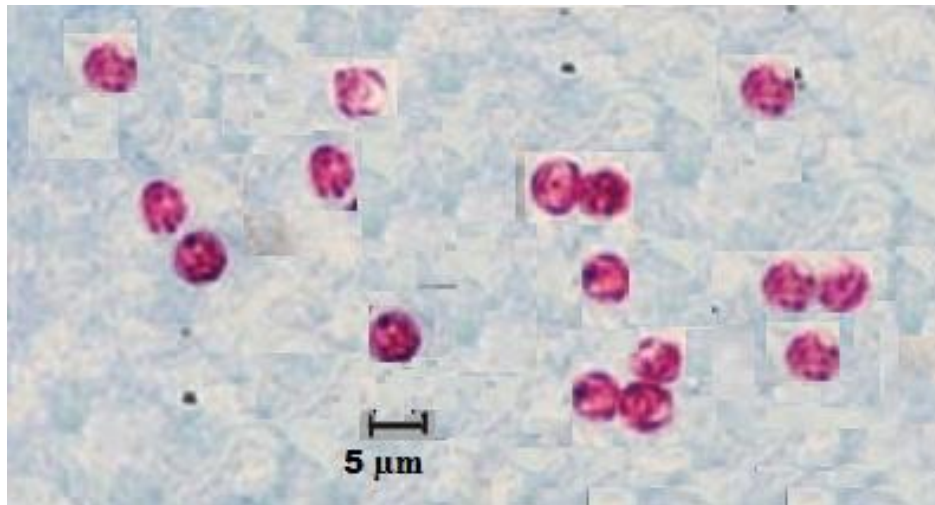


Figure 8: MZN stained oocysts of *Cryptosporidium* Species (100x mag.)  
(June/2014 - June/2015)

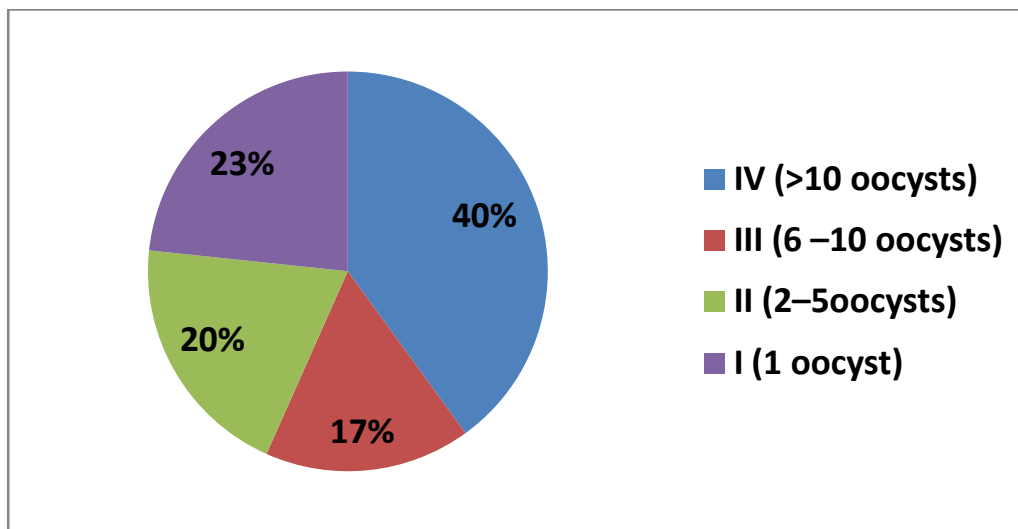


Figure 9: Intensity of *Cryptosporidium* infection in cattle in the study area  
June/2014- June/2015.

#### 4.1.4. Risk factors

Risk factors of *Cryptosporidium* were generally categorized into two groups:- factors related to farm location and management and factors related to water supply and sanitation. Among factors

related to farm location and management: intensive farming, urban location of farms, medium herd size, absence of calving pen, absence of calf bedding, dam suckling and weaning age  $\geq$  6 months showed significant association with increased infection rate (Table 6). Whereas, among factors related to water supply and sanitation, river/stream water sources, limited access to drinking water, disposal of farm waste water to wells, occurrence of other diseases (Foot and Mouth Disease and Pasteurellosis), group penning, unclean pens and unclean tail, hindquarter and flank of animals were significantly associated with increased prevalence of *Cryptosporidium* (Table 7). Animals under intensive farms and animals in farms sited in urban area were about two (OR=2.16, 95% CI: 1.11–4.18) and five (OR=4.91, 95% CI: 2.81–8.54) times more likely to acquire cryptosporidiosis as compared to animals under extensive management system and animals from farms in rural area, respectively. Animals from farms without calving pens and animals from farms where calf bedding was not practiced were 2.5 (OR=2.46, 95% CI: 1.08–5.61) and 10.5 (OR=10.55, 95% CI: 4.89–22.66) times more likely to be infected by *Cryptosporidium* as compared to animals from farms with calving pens and animals from farms practicing calf bedding, respectively, (Table 5).

Farms holding medium herd size were about 7 times (OR=6.99, 95% CI: 3.32–14.71) and those holding small herd size were about 3 times (OR=2.93, 95% CI: 1.40–6.13) more likely to acquire *Cryptosporidium* compared to farms consisting larger herd size. Farms practicing hand feeding of colostrum and weaning age of less than six months were 2 and 3.5 times less likely to be affected by the parasite when compared to farms practicing dam suckling and weaning age of 6 months and above, respectively, (Table 6). Cleanness of pens and hindquarters of animals has reduced the infection rate by two folds (OR=2.0, 95% CI: 1.13–4.93) (Table 6). Farms using well water sources were 2.4 times (OR=2.4, 95% CI: 1.2 - 4.9) and those using river/stream water were about 3 times (OR=2.9, 95% CI: 1.5–5.5) more likely to acquire *Cryptosporidium* infection compared to farms using tap water sources. Farms where animals have free access to drinking water were 2 times less likely (OR=2.11, 95% CI: 1.13–3.96) to acquire *Cryptosporidium* compared to farms where access to drinking water was limited. Farms at which Pasteurellosis and foot and mouth disease had been documented showed significantly higher prevalence (OR=5.10, 95% CI: 2.25–11.55) as compared to farms without any record of these diseases. Disposal of farm waste to field has decreased the occurrence of *Cryptosporidium* by more than 2.5 times (OR=2.79, 95% CI:

1.62–4.91) when compared to disposal of farm waste to nearby wells. The infection occurred 2.5 times more likely (OR=2.66, 95% CI: 1.01–7.04) in farms practicing group housing of calves compared to farms practicing individual pens (Table 7).

On the other hand, results of the study showed absence of statistically significant association between infection and type of floor (concrete/soil/stone), method of floor cleaning (dry/wet), experience of calf attendants ( $\leq 5$  years/ $> 5$  years), consistency of faeces (liquid/ soft/solid), colour of faeces (brown / creamy orange / creamy brown ), presence or absence of blood and mucus in the faeces, history of diarrhoea in the farm, age of farms (since establishment) (1-5yrs, 6-10yrs,  $> 10$  yrs), breed type (local zebu /cross breed (Holstein Friesian x zebu) and method of supplying water (group / Individual).

Table 6: Risk factors of *Cryptosporidium* related to general management system in cattle in Addis Ababa and its environs, June 2014- June/2015(N=392)

Risk factors	Label	<sup>1</sup> Prev	<sup>2</sup> $\chi^2$	Adjusted OR		
				<sup>3</sup> OR	95%CI <sup>4</sup>	p value
Management system	Extensive	11.2	5.78	2.16	1.11–4.18	0.016
	Intensive	21.4				
Farm location	Rural	12.8	30.26	4.89	2.81–8.54	0.000
	Urban	41.8				
Presence of calving pen	Yes	9.6	5.48	2.46	1.08–5.61	0.019
	No	20.7				
Method of colostrum feeding	Hand feeding	11.8	6.86	2.15	1.18–3.91	0.009
	Suckling	22.3				
Presence of bedding	Yes	4.26	55.37	10.55	4.89–22.66	0.000
	No	31.7				
Weaning age	<6 months	17.5	12.10	3.45	1.75–6.79	0.001
	≥6 months	42.2				
Herd size	>100	7.4	30.88			0.000
	<30	18.9		2.93	1.40–6.13	
	30-100	35.8		6.99	3.32–14.71	

<sup>1</sup> = Prevalence in percent    <sup>2</sup> = Chi-square value

<sup>3</sup> = Odds Ratio                      <sup>4</sup> = Confidence Interval

Table 7: Risk factors of *Cryptosporidium* related to water and sanitation in cattle in Addis Ababa and its environs, June/ 2014 – June/ 2015 (N=392)

Risk Factors	Category	Prevalence (%)	Adjusted OR		
			OR	95%CI <sup>4</sup>	p value
Source of drinking water	Pipe	10.3			
	Well	21.4	2.36	1.2–4.9	0.003
	River/stream	24.8	2.86	1.5–5.5	
Disposal of farm waste water	To a field	14.6	2.79	1.6–4.9	
	To well	32.2			
Access to water	Free access	14.3	2.11	1.1–4.0	0.018
	Limited	26.0			
Presence of other diseases	No	16.4	5.10	2.3–11.6	0.000
	Yes	50.0			
Pen cleanness	Clean	12.4	2.37	1.1–4.9	0.014
	Medium/unclean	25.0			
Cleanliness of hindquarter	Clean	13.1	1.96	1.1–3.4	0.013
	Medium/unclean	22.8			
Pen type	Individual pen	10.4	2.66	1.0–7.0	0.030
	Group pen	23.6			

<sup>1</sup> = Odds Ratio      <sup>2</sup> = Confidence Interval

#### 4.1.5. Molecular results

Fifty six microscopy positive and 144 randomly selected microscopy negative genomic DNA samples were examined by the Nested PCR targeting the SSU rRNA gene of *Cryptosporidium*. All microscopy positive (56) and 17 of the microscopy negative samples generated the expected PCR product of *Cryptosporidium* species, approximately 830-bp (Figure 10). *SspI* and *MboII* -RFLP restriction of 73 purified secondary PCR products indicated *C. parvum* in 19 (26.0%) specimens, and *C. andersoni* in 54 (74.0%) specimens. Electrophoresis of *SspI* digested products showed

restriction patterns of *C parvum* with three visible bands at the level of 449, 267 and 108 bp while *C. andersoni* generated two bands at the level of 448 and 397 bp as described by Feng *et al.* (2007b). *MboII* digested secondary PCR products showed restriction patterns with two visible bands of 771 and 76 bp for *C parvum*, and bands of 769 and 76 bp for *C. andersoni* (Figure 11). *Cryptosporidium parvum* infections were exclusively detected in neonates and calves less than two months of age while *C. andersoni* infections were noticed in heifers, adult cows and calves older than three months of age, indicating age related distribution pattern of the infection in the study herds. Mixed species infection was not detected in this study.

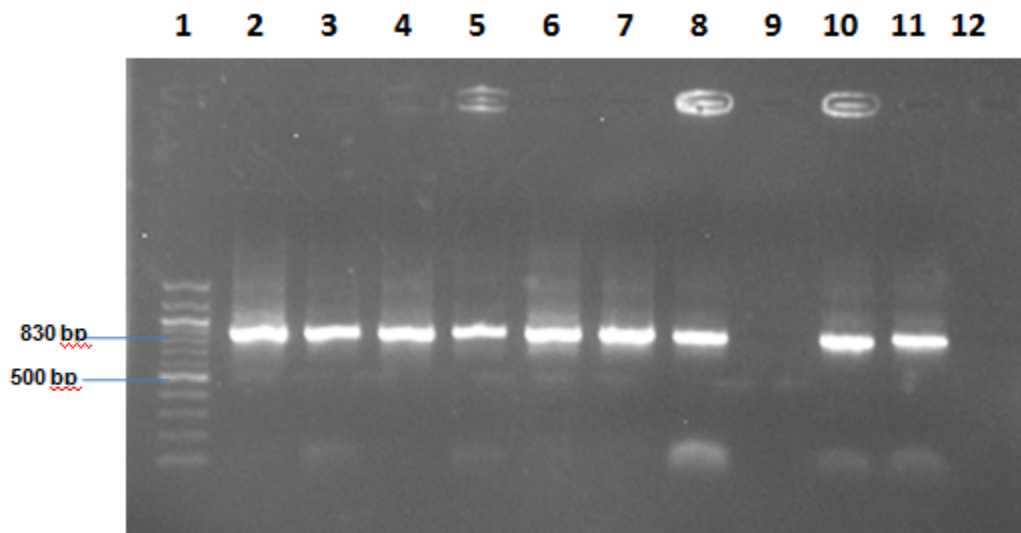


Figure 10: Nested PCR products of amplified SSU rRNA gene of *Cryptosporidium*. (June/2014 - June/2015)

Lane 1:100 bp ladder, lanes 2-8 & 10 positive samples, lane 9: negative sample, lane 11: positive control, lane 12: negative control.

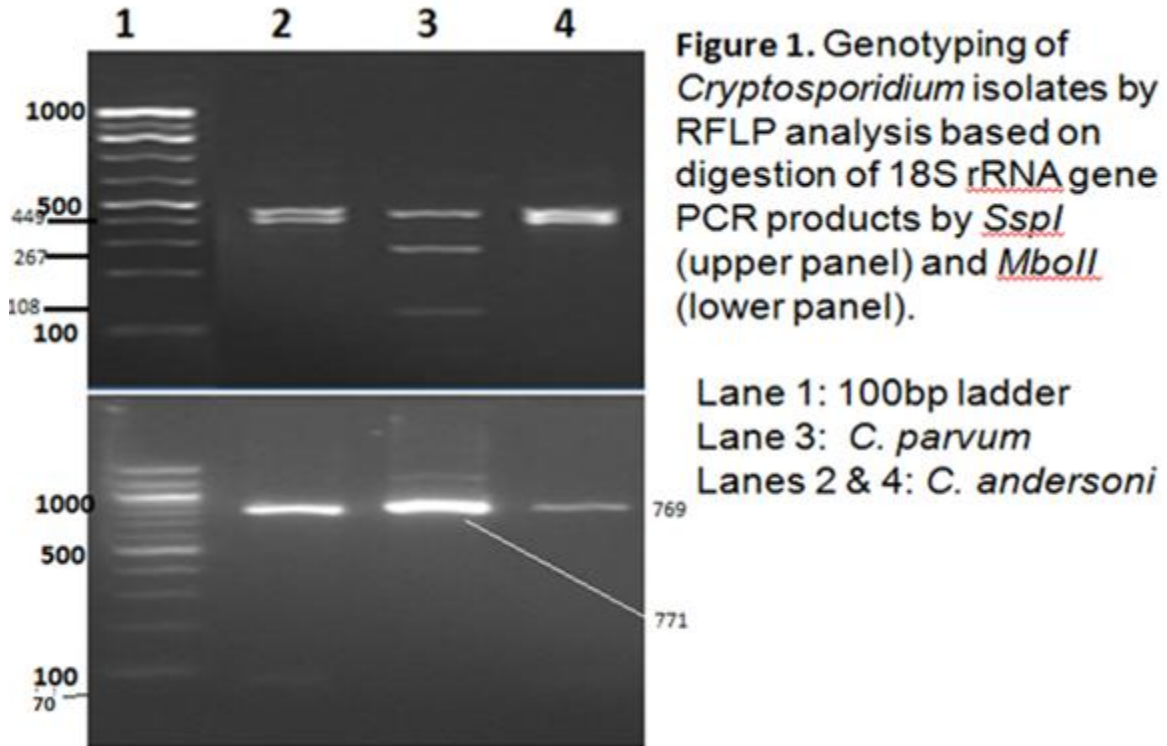


Figure 11: Genotyping of *Cryptosporidium* isolates by RFLP analysis based on digestion of the 18s rRNA gene by *SspI* and *MbolI* enzymes (upper and lower panel)

#### 4.1.6. Sequence analysis

Secondary nested PCR products of 45 specimens were successfully sequenced and analysed. Homology search in the GenBank characterized 12 of the 45 sequences (26.7%) as *C. parvum* isolates and 33 (73.3%) sequences were identified as *C. andersoni* isolates. These results confirmed the identification of *Cryptosporidium* species previously carried out by RFLP analysis. BLAST (Basic Local Alignment Search Tool) searches of the partial SSU rRNA gene sequences had shown 99% similarity to reference sequences in the GenBank for *C. parvum* (KP004206, X64340, S40330 and X64341) and 100% similarity to reference sequences in the GenBank for *C. andersoni* (KJ917578, AB777193, AB513856, AB089285, HM002493, FJ608606, KM199850, KF826311). (<http://www.ncbi.nlm.nih.gov/blast/>)

## 4.2. Cross-sectional study of human *Cryptosporidium* infection

### 4.2.1. Prevalence of *Cryptosporidium*

Three hundred stool samples of human study participants collected from 13 dairy farms and 9 health centers found at ten districts in Addis Ababa and its environs were examined by MZN microscopy and molecular methods. *Cryptosporidium* infections were detected at eight of the health stations (88.9%) with prevalence range of 3.8% - 15% and eight of the dairy farm communities surveyed (61.5%), range 4.3% - 18.8%. (Table 8). The overall prevalence of *Cryptosporidium* in human participants was 9% (27/300), the prevalence in dairy farm community, 10.8% was not different ( $p = 0.369$ ) from that of HIV/AIDS patients, 7.8% (Table 9). Prevalence of the infection in Addis Ababa, 8.2%, and Oromia, 9.4%, were statistically similar ( $p = 0.725$ ), and there was no statistically significant difference among the ten surveyed districts ( $p = 0.619$ ). The prevalence of *Cryptosporidium* in female and male subjects 9.4% and 8.5%, respectively, was not significantly different ( $p = 0.780$ ), and likewise, no statistically significant difference obtained among the age groups ( $p = 0.128$ ) (table 9). Among farm community participants of the study, high prevalence of the infection ranging from 6-25% was detected in persons from farms with high prevalence of the infection in cattle. 85% of the infection in the farm community was in individuals from infected dairy farms while only 15 % was in individuals from farms at which no infection was detected in cattle.

Table 8: Prevalence of *Cryptosporidium* infection in humans by sampling districts in Addis Ababa and its vicinity, June/2014-June/2015

District	Study subjects	No of samples	species		Total positive	Prevalence%
			<i>C. parvum</i>	<i>C. hominis</i>		
Kality	<sup>1</sup> farm com	17	3	0	3	17.6
	<sup>2</sup> HIV/AIDS	-	-	-	-	-
Barak	Farm com	19	3	0	3	15.8
	HIV+	20	1	1	2	10.0
Bole	Farm com	-	-	-	-	-
	HIV+	23	1	1	2	8.7
Akaki	farm com	23	1	0	1	4.3
	HIV+	20	1	1	2	10.0
Walmara	farm com	21	1	0	1	4.8
	HIV+	20	2	1	3	15.0
Kolfe	farm com	-	-	-	-	-
	HIV+	16	0	0	0	0.0
Nifas silk lafto	farm com	-	-	-	-	-
	HIV+	16	2	0	2	12.5
Sebeta	farm com	16	3	0	3	18.8
	HIV+	20	1	0	1	5.0
Sululta	farm com	24	2	0	2	8.3
	HIV+	19	1	0	1	5.3
Yeka	farm com	-	-	-	-	-
	HIV+	26	0	1	1	3.8
Total		300	22	5	27	9
Prevalence of species%			7.3	1.7	9	

<sup>1</sup>= Farm community members (owners, attendants, children)

<sup>2</sup>= HIV sero-positive study participants

Table 9: Prevalence of *Cryptosporidium* infection in humans by demographic characters in Addis Ababa and its vicinity, June/2014 - June/2015.

Demographic characters		Number examined	Prevalence %	95% CI <sup>3</sup>	P value
Study area	Addis Ababa	98	8.2	2.5 – 13.9	0.722
	Oromia	202	9.4	5.4 – 13.4	
Study group	Farm community	120	10.8	5.7 – 16.0	0.369
	HIV patients	180	7.8	3.6 – 12.0	
Age group	1-10	65	15.4	8.4 – 22.4	0.128
	11-20	54	7.4	0.2 – 15.1	
	21-50	168	7.7	3.4 – 12.1	
	>50	13	0.0	00	
Sex	Male	141	8.5	3.8 – 13.3	0.780
	Female	159	9.4	5.0 – 14.0	
Overall		300	9.0		

#### 4.2.2. Intensity of *Cryptosporidium*

Twenty seven samples were examined semi-quantitatively, of which, 7 showed an average of > 10 oocysts, 5 showed 6-10 oocysts, 7 showed 2-5 oocysts and 8 showed an average of 1 oocyst (Figure 13). The highest intensity was observed in children and pre-ART group.

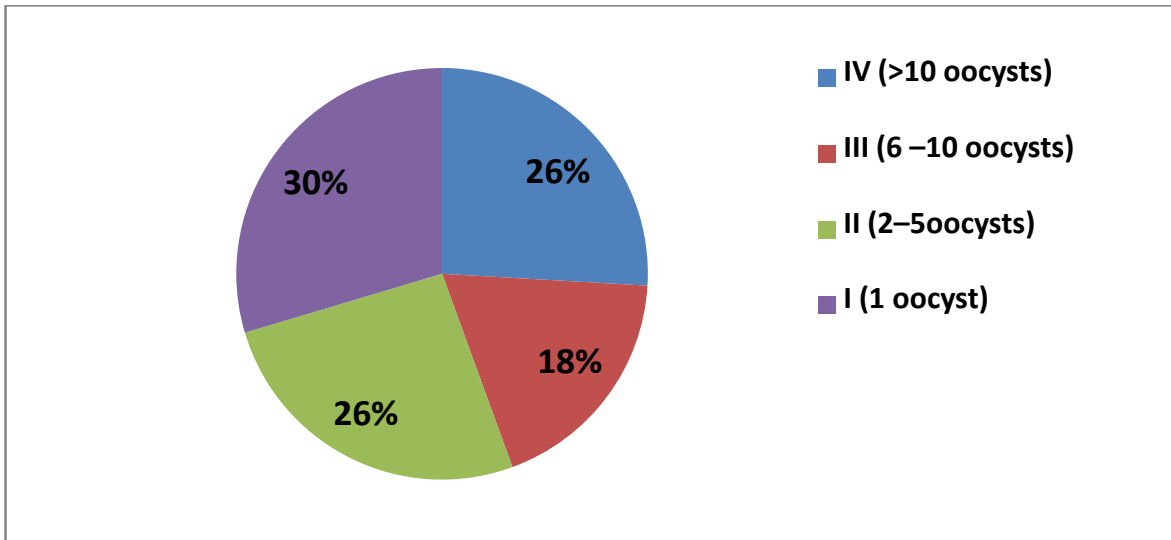


Figure 12: Intensity of *Cryptosporidium* infection in humans in Addis Ababa and its vicinity, June/2014-June/2015.

#### 4.2.3. Risk factors of human *Cryptosporidium* infection

Risk factors considered to be associated with human *Cryptosporidium* and assessed in this study includes: educational level, presence of animals at home, type of animal in contact, level of contact with animals and their faeces, duration of animal contact, source of drinking water, treatment of water at home and contact with diarrheic persons especially children. Among these factors, statistically significant associations were encountered with educational level ( $p = 0.037$ ), presence of animals at home ( $p = 0.024$ ), type of animal in contact ( $p = 0.015$ ), level of contact with animals and their faeces ( $p = 0.008$ ), duration of contact with animals ( $p = 0.014$ ) and source of drinking water ( $p = 0.004$ ) (Table 9). Whereas, no associations ( $p > 0.05$ ) were encountered with the rest of the assessed factors. People having high school or college level education were 3.5 times less likely infected compared to people without formal education or people having elementary level of education (OR=3.68, 95% CI: 1.08–12.55). Individuals having animals at home were about 3 times more likely to be infected as compared to those lacking animals at home (OR =2.96, 95% CI: 1.16–7.55). People possessing cattle, having high contact with animals and their faeces and people having animal contact for more than a year were about 3 times more likely

infected compared to people without animals and people having no contact with animals and their faeces (Table 9). The prevalence of *Cryptosporidium* infections in study participants using well or stream water for drinking, 16%, was statistically significantly higher ( $p = 0.037$ ) than the prevalence in people using pipe water as drinking water source, 5%. The infection had occurred about three times more likely in persons using well or stream water compared to people using pipe water (OR=3.2, 95% CI: 1.5–7.4). Furthermore, associations of *Cryptosporidium* infection with frequency and duration of diarrhoea, consistency and colour of faeces, and presence of mucus or blood in faeces were assessed (Table 10). It was found that *Cryptosporidium* were more prevalent in persons with history of diarrhoea sometimes in the past compared to those without history and current episode of diarrhoea (OR=4.43, 95% CI: 1.86–10.54). The infection rate was higher in people with diarrhoea, up to 5 times per day, compared to those without diarrhoea incident ( $p = 0.004$ , OR =3.28, 95% CI: 1.46–7.35). People with diarrhoea for a week period showed infection rate of 17.9% which was significantly higher ( $p = 0.001$ ,  $\chi^2 = 10.76$ ) than persons without diarrhoea, 5.3%. *Cryptosporidium* was more common in persons with mucoid faeces, 14.7%, compared to persons without mucoid faeces, 6.3% (OR=2.55, 95% CI: 1.15–5.67). Magnitude of *Cryptosporidium* was not significantly different in relation to presence of blood as well as colour and consistency of faeces ( $p > 0.05$ ) (Table 9). Among the HIV positive patients, individuals on ART (Anti Retroviral Treatment) medication showed prevalence of 0.83%, this group was about 32 times less likely to acquire the infection as compared to pre ART group (OR=32.92, 95% CI: 4.19–58.71). Persons with CD4+ count of less than 200 cell/ $\mu$ l showed 25 times more infection compared to those with CD4+ count of 200 - 500 cell / $\mu$ l. *Cryptosporidium* was not detected in patients with CD4+ cell count  $> 500/\mu$ l (Table 10).

Table 10: Risk factors of *Cryptosporidium* infection in humans in Addis Ababa and its vicinity, June/2014-June/2015.

Risk factors	group	<sup>1</sup> No exam	<sup>2</sup> Prev %	Adjusted OR		Pvalue
				<sup>3</sup> OR	95% CI <sup>4</sup>	
Education level	highschool/colleg	84	3.4			
	no/elementary	216	11.4	3.68	1.1– 12.6	0.037
Presence of animals at home	No	131	4.58			
	Yes	169	12.43	2.96	1.2 - 7.6	0.024
Type of animal	no	131	4.58			
	cattle	74	14.87	3.64	1.3- 10.3	0.015
	cattle & pets	59	13.56	3.27	1.1 - 9.9	0.036
Level contact c anim & faeces	No	131	4.58			
	high	121	14.88	3.64	1.4 - 9.5	0.008
Duration of contact animals & faeces	no animal	131	4.58			
	≥1yr	98	14.29	3.47	1.3 - 9.4	0.014
Source of drinking water	pipe	200	5.50			
	stream/well	100	16.00	3.27	1.5 - 7.4	0.004

<sup>1</sup> = Number examined,

<sup>2</sup>: = prevalence (%),

<sup>3</sup> = Odds Ratio

<sup>4</sup>: = Confidence interval

Table 11: Features associated with *cryptosporidium* infection in humans in Addis Ababa and its vicinity, June/2014-June/2015.

Features	Label	<sup>2</sup> Prev %	Adjsted OR		
			OR	95% CI <sup>3</sup>	P value
Presence of diarrhoea	no	6.1			
	in the past	22.2	4.43	1.0- 10.5	0.001
Frequency of diarrhoea	no	5.5			
	≤5 x/day	16.0	3.27	1.5- 7.4	0.004
Duration of diarrhoea	no	5.3			
	≤ a week	17.9	3.91	1.7- 8.9	0.001
Presence of mucus in faeces	no	6.3			
	yes	14.7	2.55	1.2 - 5. 7	0.021
ART status	ART	0.8			
	pre ART	21.7	32.92	4.3 - 58.7	0.001
CD4+ cell count	200-500	1.7			
	< 200	30.8	25.33	5.4-119.9	0.000

<sup>1</sup>= number examined, <sup>2</sup>: prevalence (%), <sup>3</sup>: Confidence interval

#### 4.2.4. Molecular study results

All of the 27 microscopy positive human samples examined by the nested PCR targeting the SSU rRNA gene of *Cryptosporidium* generated the expected PCR product of approximately 830-bp. RFLP restriction of the purified secondary PCR products by *SspI* enzyme showed bands of *C. parvum* at the level of 449, 254 and 108 base pairs as described previously (Feng *et al.*, 2007a). Further RFLP restriction by *AseI* (*VspI*) enzyme showed bands of *C. parvum* ‘bovine genotype A’ at the level of 628,104 and 102 base pairs (Xiao *et al.*, 1999a) (Figure 14). The ‘bovine genotype A’ was detected in samples from the farm community as well as HIV+ patients.

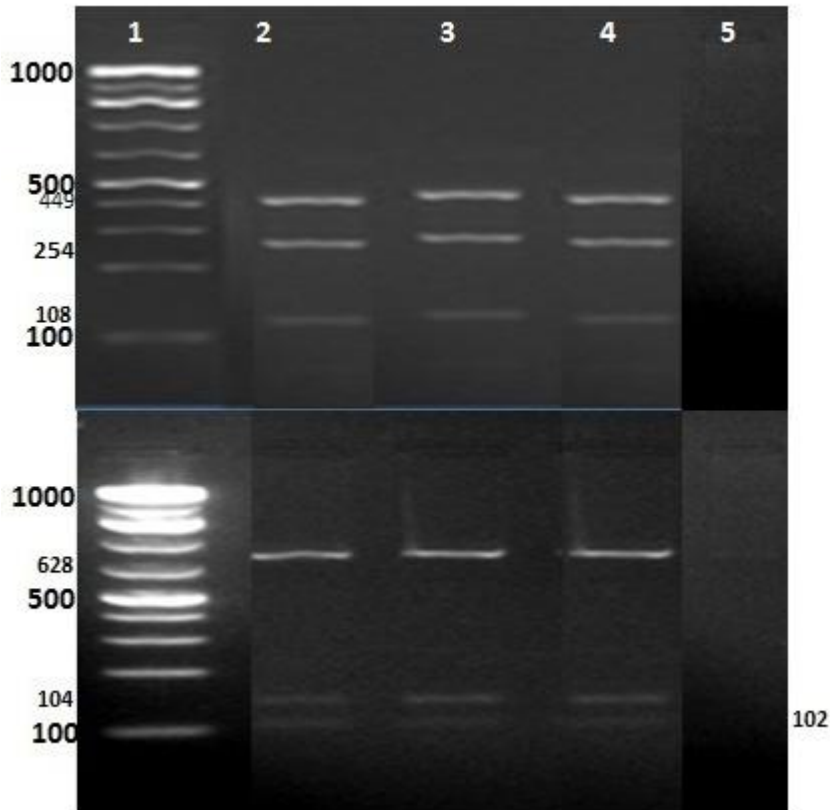


Figure 13: RFLP digestion of the 18S SSU rRNA gene nested product by *SspI* (upper panel) and *AseI* (lower panel) enzymes: Lane 1:100 bp ladder; Lanes 2, 3, 4: *C. parvum*, lane 5: negative control

#### 4.2.5. Sequence analysis

Clear sequences of the SSU rRNA gene obtained from the 27 microscopy and PCR positive samples were successfully sequenced and analysed. Homology search in the GenBank indicated that 22 of the 27 isolates (81.5%) were *C. parvum* and 5 of the isolates (18.5%) were *C. hominis*. These results confirmed the *C. parvum* species previously identified by the RFLP analysis whereas, *C. hominis*, which was not identified by RFLP was confirmed by sequence analysis. BLAST (Basic Local Alignment Search Tool) searches of the partial SSU rRNA gene sequences of 18 of the 22 *C. parvum* positive samples showed 100% similarity with the GenBank reference sequences for *C. parvum* AH006572.2, KX082687.1, KT151548.1, KP994662.1, L16996.1, KM012044.1, AB746195.1, JX298604.1, AF108865.1, EU331237.1, DQ656355.1, AY204237.1,

and L16996; three of the *C. parvum* positive samples showed 100% similarity with the GenBank reference sequences for *C. parvum* AF161856.1, AB089290.1, AF108863.1 ; and one *C. parvum* positive sample showed 100% similarity with the GenBank reference sequence for *C. parvum* AF159112.1. Of the 5 *C. hominis* positive samples 3 had 100% similarity with *C. hominis* nucleotide sequences EU186156.1, AB434890.1, KM085019.1, KP098564.1, KC734570.1, JQ313989.1, JN833575.1 and GQ183510.1; one had 100% similarity with *C. hominis* nucleotide sequences KM215744.1 and GQ983348.1 and 1 *C. hominis* positive sample showed 100% similarity with *C. hominis* nucleotide sequence GU319779.1.

(<http://www.ncbi.nlm.nih.gov/blast/>).

### 4.3. Longitudinal study of calves

#### 4.3.1. Prevalence of *Cryptosporidium* infection

Two hundred seventy faecal specimens were collected from the study calves and examined, out of which, 40 samples (14.8%) were found *Cryptosporidium* positive. Twelve of the thirty calves (40%) shedded *Cryptosporidium* oocyst at least once during the three month study period. with the exception of day one calves, oocysts were detected throughout the sampling dates. the initial infection was noticed in seven day old calves, of which, 10% shedded oocysts. the highest infection rate was at two weeks of age when 12 of the 30 calves (40%) excreted oocysts. The lowest prevalence of infection, 6.7%, was observed in 2 and 3 month old calves (Figure 15). The overall prevalence in neonates, 19.3%, was significantly higher ( $p = 0.022$ ) than the prevalence in post-neonatal calves, 9.2% ( $\chi^2 = 5.684$ ). The chance of infection in pre-weaned calves was 2.7 times more likely as compared to post-weaned calves (OR= 2.66, 95% CI= 1.1– 6.3).

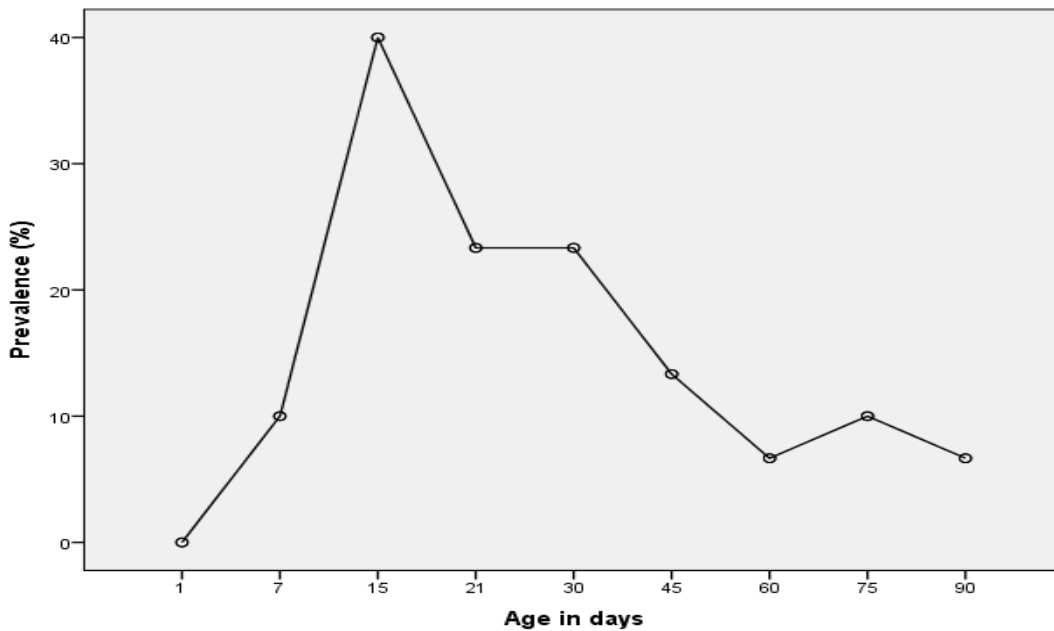


Figure 14: Prevalence of *Cryptosporidium* infection across age of calves (July/2014-June/ 2015)

### 4.3.2. Intensity of infection

Out of 30 semi-quantitatively examined samples, the average number of oocysts in 13 samples was > 10 oocysts, 6 samples showed 6-10 oocysts, 7 showed 2-5 oocysts and 4 showed only 1 oocyst (Figure 16).

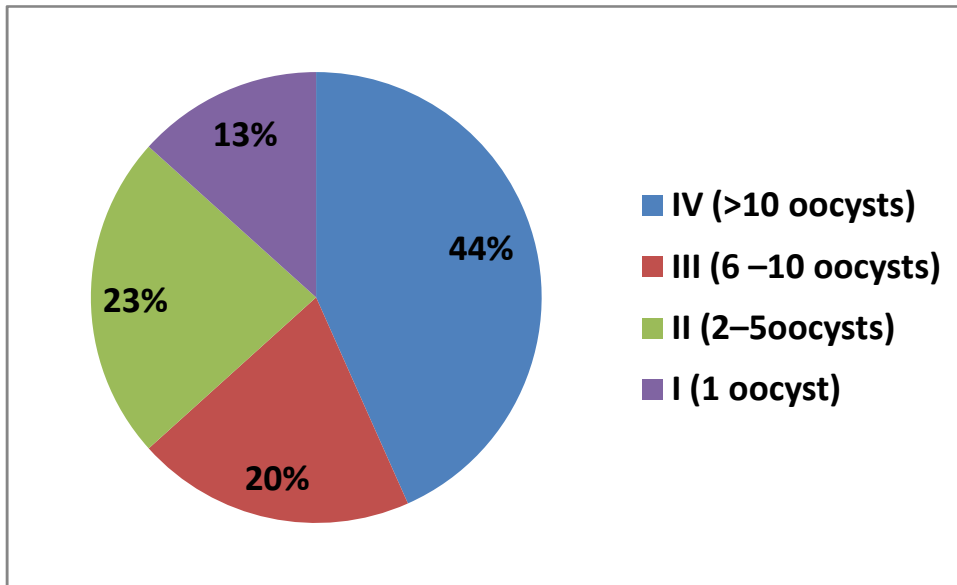


Figure 15: Intensity of *Cryptosporidium* infection in longitudinal study calves (July/2014-June/2015)

### 4.3.3. Risk factors of *Cryptosporidium* infection

Risk factors of *Cryptosporidium*:- including farm management, sex, genetic blood level (50-75%, and >75% Holstein Friesian x local zebu crossbreed), time of birth (day, night) and parity of the dam (first, second, > second parity) were investigated, consistency and colour of faeces as well as presence of mucus or blood in faeces were assessed for associations with *Cryptosporidium* infection. The prevalence in the two study farms HARC (13.3%) and HCGI (17.8%), as well as, the prevalence in male (15.2%) and female calves (14.6%), were statistically not different (Table 12). Calves with > 75% Holstein Friesian x local zebu crossbreed showed prevalence of 18.1% which was higher and statistically significant (OR=2.21, 95% CI: 1.0-4.9) than the prevalence in

the 50-75% Holstein Friesian crossbreed, 9.1%. Calves born during night-time and calves born to cows above second parity were 2.6 and 3.6 times more likely to acquire *Cryptosporidium* infection as compared to calves born during daytime and calves born to cows in their first or second parity (Table 12). Pre-weaned calves acquired *Cryptosporidium* infections 2.66 times more likely as compare to post weaned calves (OR=2.66, 95% CI: 1.1–6.3). *Cryptosporidium* infections showed significant associations with diarrheic faeces (OR=11.29, 95% CI: 4.0–31.9), creamy orange coloured faeces (OR=3.73, 95% CI: 1.6–8.5) and faeces containing mucus (OR=3.61, 95% CI: 1.8–7.4) (Table 13).

Table 12: Risk factors of *Cryptosporidium* infection in calves from birth to 3 months of age (July/2014-June/ 2015)

Risk factors	category	No of samples	<sup>1</sup> Prev (%)	Adjusted OR		P value	<sup>1</sup> =
				<sup>2</sup> OR	95% CI <sup>3</sup>		
Dairy farm	HARC	180	13.33				
	HCGIC	90	17.78	1.41	0.7– 2.8	0.334	
Sex	female	171	14.6				
	male	99	15.2	0.96	0.5– 1.9	0.906	
Genetic blood level	50-75	99	9.1				
	>75	171	18.1	2.21	1.0 - 4.9	0.048	
Age group	Post wean	90	7.78				
	Pre wean	180	18.33	2.66	1.1– 6.3	0.025	
Age group 2	Post-neonate	120	9.17				
	neonate	150	19.33	2.38	1.1– 5.0	0.022	
Time of birth	Day	207	11.1				
	night	63	27.0	2.60	1.5–6.0	0.003	
Parity of dam	2 <sup>nd</sup> parity	99	10.10				
	>2 <sup>nd</sup> parity	63	28.57	3.56	1.5–8.4	0.003	

prevalence

<sup>2</sup>= Odds Ratio

<sup>3</sup> = Confidence Interval

Table 13: Macroscopic characters of faeces related to *Cryptosporidium* infection in calves (July/2014-June/ 2015)

Macroscopic character	Label	No of samples	<sup>1</sup> Prev (%)	Adjusted OR		P value
				<sup>2</sup> OR	95% CI <sup>3</sup>	
Consistency of faeces	Soft	92	5.43			
	Liquid	61	39.34	11.29	4.0 - 31.9	0.000
Colour of faeces	dark brown	120	7.50			
	creamy	99	23.23	3.73	1.6 - 8.5	0.002
	creamy brown	51	15.69	2.30	0.8 - 6.3	0.109
Presence of mucus	No	159	8.18			
	Yes	111	24.32	3.61	1.8 - 7.4	0.000
Presence of blood	No	225	13.33			
	Yes	45	22.22	1.86	0.8 - 4.1	0.130

<sup>1</sup>= prevalence      <sup>2</sup>= Odds Ratio      <sup>3</sup> = Confidence Interval

#### 4.3.4. Molecular study and Sequence analysis

All of the 40 microscopy positive samples from the study calves were also positive by the nested PCR and sequenced successfully. Sequence analysis of the specimens identified *C. parvum* in thirty-five and *C. andersoni* in five specimens with proportions of 87.5% and 12.5 %, respectively (Figure 17). *C. parvum* constituted 100% and 54.5% of the infections in neonates and in calves >1 month, respectively, in contrast, *C. andersoni* was detected only in post weaned calves (> 2 months old) and not detected in preweaned calves (Figure 18).

BLAST (Basic Local Alignment Search Tool) searches of the partial SSU rRNA gene sequences of the 23 *C. parvum* positive samples showed 100% similarity with *C. parvum* nucleotide sequences AB513881.1, AB513858.1, L16997.1, AF108865.1 and EU660038.1, while 12 of the *C. parvum* positive samples showed 100% similarity with *C. parvum* nucleotide sequences AH006572.2, AB513880.1, KT151548.1, KP994662.1, L16996.1, AB089290.1, AY204238.1 and

AF040725.1. All of the five *C. andersoni* positive samples showed 100% similarity with the gene bank *C. andersoni* nucleotide sequences KJ917578, AB777193, AB513856, AB089285, HM002493, FJ608606, KM199850 and KF826311.

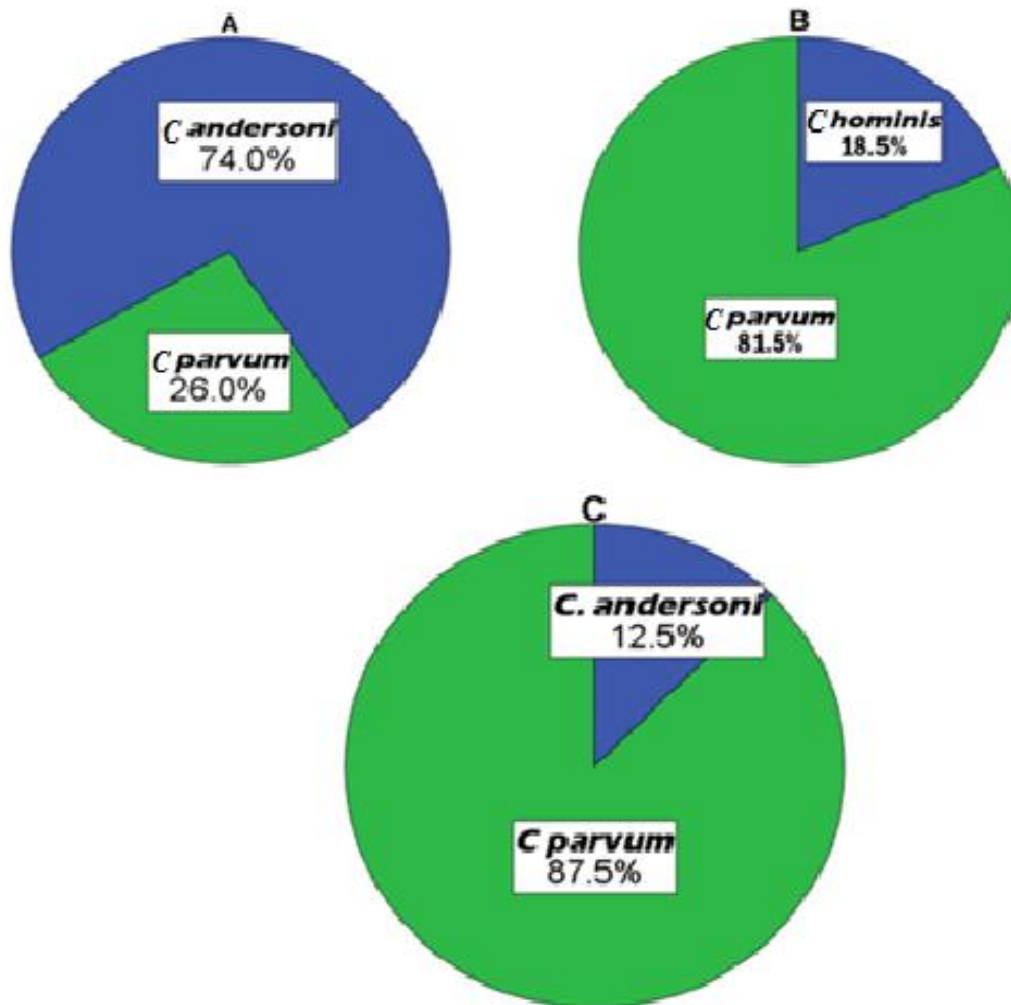


Figure 16: Proportion of *Cryptosporidium* species identified by sequence analysis and PCR-RFLP. (June/2014-June/ 2015) a) cattle b) humans and c) calves (Longitudinal study)

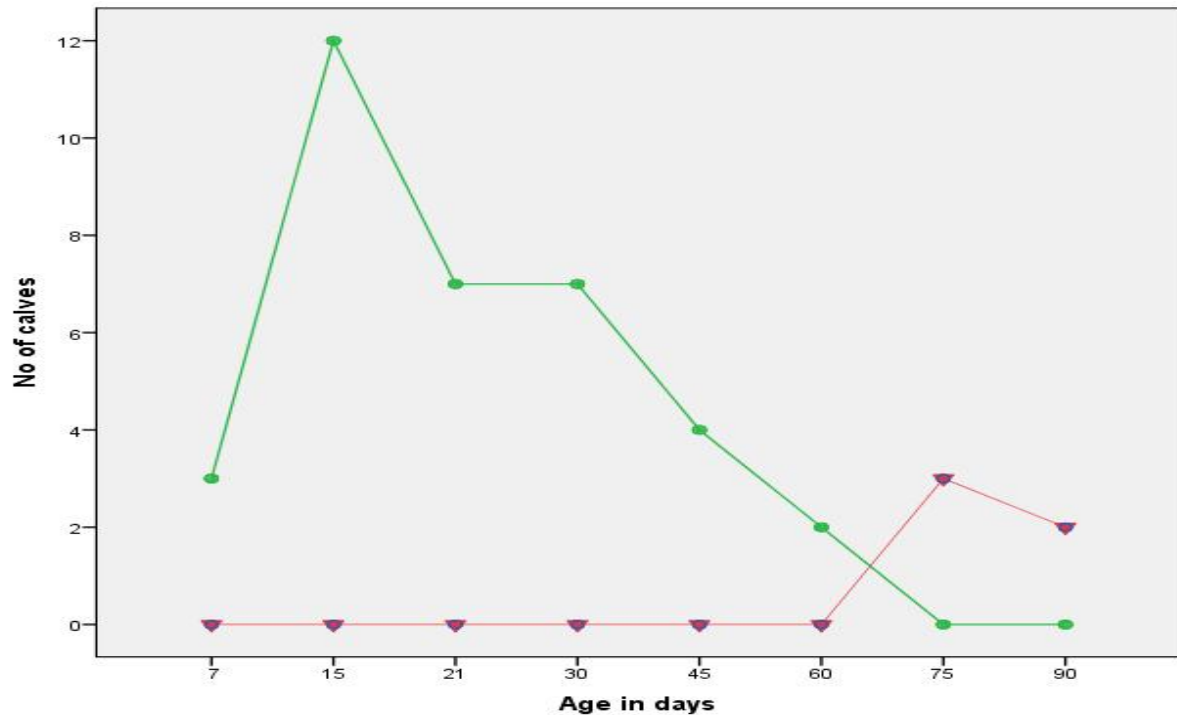


Figure 17: Distribution of *Cryptosporidium* species infection along age of calves  
● *C. parvum* , ▼ *C. andersoni* (July/2014-June/ 2015)

#### 4.4. Phylogeny

All isolates from this study grouped with their homologous GenBank retrieved sequences of *Cryptosporidium* species. The three *Cryptosporidium* species obtained in this study formed three clades with full statistical reliability. The first clade contained *C. andersoni* showing bootstrap values of 100%, the second clade contained *C. hominis* and *C. parvum* showing bootstrap values of 84% and the third clade contained *C. parvum* showing bootstrap values of 97%. The *C. parvum* isolates from this study clustered under three different clades: *C. parvum* isolate KX856003 grouped in the first clade with GenBank retrieved isolates AB513881 and KM870599 showing 84% similarity. The *C. parvum* isolates KX264363, KX264364 and KX264365 clustered under the second clade with GeneBank isolates S40330 and X64341 showing bootstrap values of 99%. The remaining four *C. parvum* isolates (KX855997, KX855998, KX855999 and KX856004) from this

study grouped under the third clade with five GenBank retrieved isolates (AB089290, AF040725, AY204238, AB513880, EU660038) (Figure 19). Genetic distance analysis using Kimura-2 Parameter confirmed the above results (Table 14) showing small genetic distances (0.011 - 0.017) between GenBank isolates S40330 and X64341 and isolates KX264363, KX264364 and KX264365 from this study. GenBank isolate AB513881 showed identical genetic distance (0.000) with isolate KX856003. the remaining four *C. parvum* isolates KX855997, KX855998, KX855999 and KX856004 from this study showed genetic distances varying from 0.000 - 0.002 with the five GenBank retrieved sequences (AB089290, AF040725, AY204238, AB513880, EU660038). The genetic distance between *C. andersoni* isolates from this study and their corresponding GenBank isolates showed genetic distance of 0.000 to 0.001 and the *C. hominis* isolates of this study showed genetic distance ranging from 0.000 to 0.007, with their analogous GenBank isolates (Table 15, 16).

Table 14: Genetic distances using Kimura-2 Parameter, comparing *C. parvum* sequences from this study (bolded 1-8) and *C. parvum* sequences retrieved from GenBank (9-17).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 KX264361 <i>C parvum</i> cattle																	
2 KX264360 <i>C parvum</i> cattle	0.000																
3 KX856003 <i>C parvum</i> cattle	0.095	0.097															
4 KX856004 <i>C parvum</i> cattle	0.969	0.962	0.956														
5 KX264362 <i>C parvum</i> cattle	0.089	0.091	0.060	1.042													
6 KX855999 <i>C parvum</i> human	1.028	1.023	1.002	0.000	1.085												
7 KX855997 <i>C parvum</i> human	1.026	1.021	1.011	0.000	1.083	0.002											
8 KX855998 <i>C parvum</i> human	1.204	1.194	0.914	0.000	1.228	0.000	0.000										
9 KM870599.1 <i>C parvum</i>	0.106	0.106	<b>0.020</b>	0.894	<b>0.065</b>	0.962	0.958	1.132									
10 S40330.1 <i>C parvum</i>	<b>0.011</b>	<b>0.011</b>	<b>0.099</b>	1.030	<b>0.091</b>	1.058	1.074	1.321	0.126								
11 X64341.1 <i>C parvum</i>	<b>0.016</b>	<b>0.017</b>	0.102	1.040	<b>0.096</b>	1.070	1.087	1.335	0.130	0.004							
12 AB513881.1 <i>C parvum</i>	<b>0.090</b>	<b>0.092</b>	<b>0.000</b>	0.956	<b>0.052</b>	0.997	1.011	1.288	0.025	0.340	0.340						
13 AF040725.1 <i>C parvum</i>	1.247	1.238	1.030	<b>0.000</b>	1.292	<b>0.000</b>	<b>0.002</b>	<b>0.001</b>	1.188	1.684	1.675	1.619					
14 EU660038.1 <i>C parvum</i>	1.247	1.238	1.038	<b>0.000</b>	1.292	<b>0.002</b>	<b>0.000</b>	<b>0.001</b>	1.188	1.407	1.422	1.394	0.026				
15 AB513880.1 <i>C parvum</i>	1.247	1.238	1.038	<b>0.000</b>	1.292	<b>0.002</b>	<b>0.000</b>	<b>0.001</b>	1.188	1.407	1.422	1.394	0.023	0.003			
16 AY204238.1 <i>C parvum</i>	1.247	1.238	1.039	<b>0.000</b>	1.292	<b>0.000</b>	<b>0.000</b>	<b>0.001</b>	1.188	1.429	1.444	1.415	0.023	0.003	0.000		
17 AB089290.1 <i>C parvum</i>	1.247	1.238	1.030	<b>0.000</b>	1.292	<b>0.000</b>	<b>0.002</b>	<b>0.001</b>	1.188	1.399	1.413	1.386	0.022	0.003	0.001	0.000	
18 AF026388.1 <i>Eimeria tenella</i>	1.495	1.496	1.331	0.215	1.566	0.178	0.185	0.156	1.419	2.070	2.070	1.833	1.608	0.587	0.589	0.595	0.581

Table 15: Genetic distances using Kimura-2 Parameter, comparing *C. andersoni* sequences from this study (bolded 1-3) and sequences retrieved from GenBank (4-6) and *E. tenella*

		1	2	3	4	5	6	7	8	9	10	11	12
1	AB089285 <i>C. andersoni</i>												
2	AB513856 <i>C. andersoni</i>	0.000											
3	AB777193 <i>C. andersoni</i>	0.000	0.000										
4	FJ608606 <i>C. andersoni</i>	0.000	0.000	0.000									
5	HM002493 <i>C. andersoni</i>	0.000	0.000	0.000	0.000								
6	KF826311 <i>C. andersoni</i>	0.000	0.000	0.000	0.000	0.000							
7	KJ917578 <i>C. andersoni</i>	0.000	0.000	0.000	0.000	0.000	0.000						
8	KM199850 <i>C. andersoni</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000					
9	<b>KX264360 <i>C. andersoni</i></b>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000				
10	<b>KX264361 <i>C. andersoni</i></b>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000			
11	<b>KX264362 <i>C. andersoni</i></b>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		
12	AF026388 <i>E. tenella</i>	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.112	

Table 16: Genetic distances using Kimura-2 Parameter, comparing *C. hominis* sequences from this study (bolded 1-3) and sequences retrieved from GenBank (unbolded 4-6) and *E. tenella*

		1	2	3	4	5	6	7
1	<b>KX856000 <i>C. hominis</i></b>							
2	<b>KX856001 <i>C. hominis</i></b>	0.000						
3	<b>KX856002 <i>C. hominis</i></b>	0.000	0.000					
4	JQ313989 <i>C. hominis</i>	0.000	0.007	0.004				
5	EU186156 <i>C. hominis</i>	0.000	0.000	0.003	0.000			
6	KM085019 <i>C. hominis</i>	0.000	0.000	0.003	0.000	0.000		
7	AF026381 <i>E. tenella</i>	1.312	1.291	1.361	1.347	1.357	1.394	

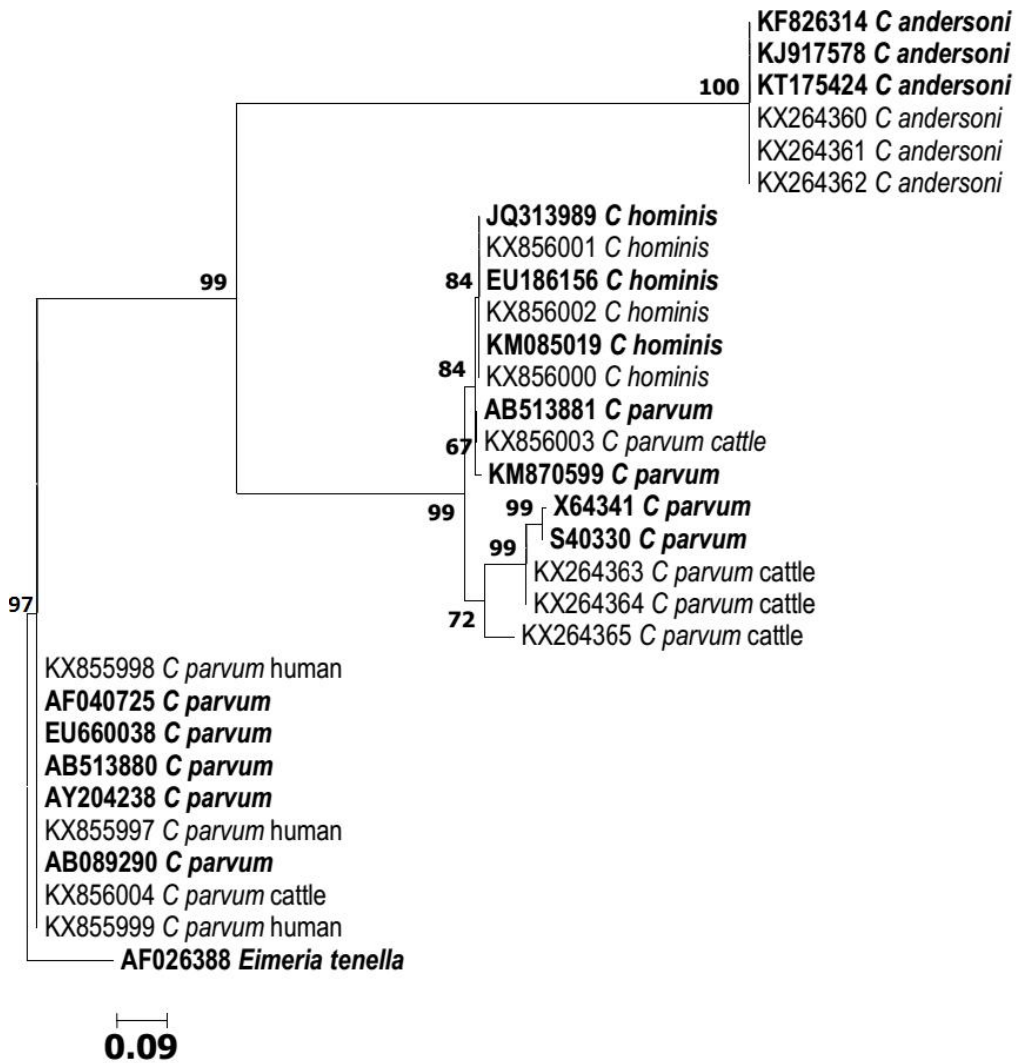


Figure 18: Phylogenetic relationship among *Cryptosporidium* sequences retrieved from the GenBank (bolded) and sequences from this study inferred by the Maximum Likelihood method based on the Kimura 2-parameter model.

The bootstrap tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach. The scale bar indicates an evolutionary distance of 0.09 nucleotides per position in the sequence.

## 5. DISCUSSION

### 5.1. *Cryptosporidium* infection in cattle

The overall prevalence of *Cryptosporidium* infection in cattle obtained in this study, 18.6%, was comparable to the prevalence of 17.6% reported in central Ethiopia (Abebe *et al.*, 2008), lower than the prevalence report of 27.8% by Alemayehu *et al.* (2013) and higher than the 7.8%, 13.6% and 15.8% prevalence reports by Wegayehu *et al.* (2013), Dinka and Berhanu (2015) and Wegayehu *et al.* (2016), respectively. Studies conducted in other parts of the world also stated varied prevalence rates: comparable values of 18.8%, and 17% were reported by Budu-Amoako *et al.* (2012) and Keshavarz *et al.* (2009), respectively. Higher prevalence rates ranging from 27% to 86.7% had been reported (Santin *et al.*, 2004; Nguyen *et al.*, 2007 and Venu *et al.*, 2012) and lower values of 12% and 11.7% were reported by Hamnes *et al.* (2006) and Khan *et al.* (2010), respectively.

The difference in the overall prevalence of *Cryptosporidium* among different studies could be due to variations in ecology, study design, season, management system, age, herd size and laboratory techniques employed. Animals reared under intensive management system were more affected by *Cryptosporidium* (21.4%) than those under the extensive system (11.2%) which could be due differences in breeds of animals as well as confinement, higher stocking rate and crowding in the intensive dairy farms favouring more contamination of barns, high contact of animals and rapid dissemination of oocysts compared to extensive farms. In the semi-intensive or intensive management system of rearing animals are confined to a restricted area, thus continuously contaminated the surroundings (Maikai *et al.*, 2011). This result is in agreement with the findings of Geurden *et al.* (2006) that reported prevalence of 42.8% for animals reared under intensive system and 6.3% for animals under extensive system. Comparable lower prevalence had been reported in extensive farms compared to intensive farms (Ralston *et al.*, 2003; Santin *et al.*, 2004). Disposal of farm waste water to ground wells was associated with increased risk of *Cryptosporidium* compared to disposal to distant fields. Contamination of farm water or feed store by the nearby waste-water wells might be the reason for the observed difference as this site favours survival of oocysts; conversely, disposal of farm waste-water over wider field area may

exposé oocysts to high environmental temperature and desiccation for which Oocysts are susceptible (Li *et al.*, 2010). Reduction in viability and infection pressure has been reported due to dispersal of oocysts over larger surface that exposes them to direct sunlight (Li *et al.*, 2010). Although *Cryptosporidium parvum* is considered a cause of diarrhoea in neonates and many previous studies reported significant association between diarrhoea and shedding (Geurden *et al.*, 2006; Karanis *et al.*, 2010), no association was detected in this study which might be due to differences in pathogenicity of the strains or co-infections by other diarrhoea causing enteropathogens that could mask the effect of *Cryptosporidium*. Similar results showing absence of association between diarrhoea and shedding had been reported (Abebe *et al.*, 2008; Rieux *et al.*, 2013). Animals from farms without provision calf bedding acquired more *Cryptosporidium* compared to animals from farms with provision of calf bedding. Since daily appliance and disposal of calf bedding has major effect in lessening oocyst persistence in pens, this could explain the reduced contamination of farms and lower infection prevalence in animals for which calf bedding is provided. Maddox-Hyttel *et al.* (2006) stated that addition of clean bedding and its daily disposal had significantly declined the risk of *Cryptosporidium* infection. Other studies also showed that bedding and hygiene related factors had significant effect on the odds of infection (Castro-Hermida *et al.*, 2006; Brook *et al.*, 2008b). Significant association was observed between group penning of calves and weaning age  $\geq 6$  months with increased infection rate. Both of these risk factors could lead to sound contact of mature and young calves and transmission of infection to neonates. Calf-to-calf contact is the most likely route of transmission, and averting of this tends to decrease the infection (Kvác *et al.*, 2006).

*Cryptosporidium* was significantly associated with absence of calving facilities and practice of dam suckling; higher chance of infection might have resulted due to exposure of neonates to their dams or other group of the herd in farms where calving facilities are absent, or if newborns stayed with their dams in maternity pens in case of farms with calving facilities. Our result is in agreement with findings of Del Coco *et al.* (2008) that reported higher exposure and prevalence of the disease in newborns that stayed with their dams in maternity pens. Higher infection pressure of *Cryptosporidium* occurs in calves less than 3 weeks of age due to their closeness with their dams (Santin *et al.*, 2008; Bjorkman *et al.*, 2015). *Cryptosporidium parvum*-like oocysts were detected at two days of age and *C. parvum* was confirmed by molecular analysis at an age four days

indicating transmission of oocysts either from the dam or from contamination of calving pens (Silverlås, 2010). The present study illustrates that infections were significantly higher in farms with previous record of Foot and Mouth Disease (FMD) or Pasteurellosis compared to farms without these diseases. It is likely to get higher prevalence in such farms given that *Cryptosporidium* is an opportunistic parasite mostly affecting immunocompromized animals (Fayer and Xiao, 2008; Sonia, 2011) and these diseases are highly infectious and known to cause severe illness with immune suppression effect.

Increased risk of *Cryptosporidium* was seen in farms using river/stream water sources; this could be due to exposure of these water sources to faeces of human, domestic and wild animals which have been contaminated with oocysts of *Cryptosporidium*. River water is heavily contaminated with oocyst of *Cryptosporidium* in proportion to the number of cattle in the adjacent area and livestock waste were more pollutant of river water compared to sewages (Yang *et al.*, 2008). Results of this study showed that animals having unclean hindquarters and/or housed in unclean pens showed higher infection rates than animals with clean hindquarters and/or housed in clean pens. This could be due to the fact that wet and soiled pen floors create favourable environment for the persistence of oocysts and spread of the infection among the herd. Our results are in accord with the findings of Abebe and colleagues (2008) that reported a 5.2 times odds of infection in calves housed in poorly cleaned farms compared to calves in well-cleaned farms. Zhang *et al.* (2013) and Maddox-Hyttel *et al.* (2006) illustrated significant association between daily cleaning of pens and reduction in the risk of *Cryptosporidium* infection. Contradictory to earlier studies that reported higher risk of infection in larger herds (Silverlås, 2010; Inpankaew *et al.*, 2014) findings of this study demonstrated more infection rate in medium and small size dairy farms. The reason for higher infection rate in medium and small size dairy farms might be due to implementation of better management practices in larger farms, mainly supervised by animal health and production professionals, compared to medium and small sized farms run by owners or non professional personnel.

*Cryptosporidium parvum* and *C. andersoni* are the two species identified in the current study which is in agreement with reports within the country (Adamu *et al.*, 2010; Wegayehu *et al.*, 2016) and reports from outside that reported infection of cattle as usually being associated with

four main species, *C. parvum*, *C. andersoni*, *C. ryanae*, and *C. bovis* (Geurden *et al.*, 2006; Nguyen *et al.*, 2007; Santin *et al.*, 2008; Keshavarz *et al.*, 2009; Silverlas *et al.*, 2010; Xiao, 2010; Rieux *et al.*, 2013). The occurrence of *C. parvum* and *C. andersoni* in this study showed an age related distribution pattern in that *C. parvum* was encountered only in calves less than two months of age, while *C. andersoni* was observed in calves older than three months, in heifers and adult animals. Comparable age related distribution pattern results had been reported by different researchers (Santin *et al.*, 2004; Feng *et al.*, 2007a; Plutzer and Karanis, 2007; Thompson *et al.*, 2007; Keshavarz *et al.*, 2009; Liu *et al.*, 2009; Silverla *et al.*, 2010). In support of this finding, studies on calves of 12–24 weeks and 2–6 month old from Nigeria and Vietnam reported the absence of the zoonotic *C. parvum* species and suggested that these age groups of calves were unlikely to contribute to human cryptosporidiosis (Ayinmode *et al.*, 2010; Nguyen *et al.*, 2012). *C. parvum* infection rates of 0.4 % and 0.7%, was reported in milking cows and heifers, respectively, and it was explained that yearling and mature dairy cattle are relatively low risk sources of infection to humans (Fayer *et al.*, 2006, 2007).

Majority of the *C. parvum* infections in this study were encountered in neonates below one month of age which is in concord with findings of Castro-Hermida *et al.*(2002a) and Santin *et al.*(2004) that reported occurrence of most *C. parvum* infections between the first and fourth week of life. In addition, Díaz-Lee *et al.* (2011) and Del Coco *et al.* (2008) reported the highest *C. parvum* infection rates in calves 7-14 days and 8-21 days of age, respectively. Contrary to the present finding Castro-Hermida *et al.* (2007) reported only *C. parvum* in cows, and Wells *et al.* (2015) reported that 96% of the infections in adult cattle were due to *C. parvum*. Although not frequent, infection of dairy calves <2 months of age by *C. andersoni* and *C. ryanae* (Liu *et al.*, 2009), calves < month of age by *C bovis* and *C. ryanae* (Santin *et al.*, 2004; Silverlas *et al.*, 2010; Wegayehu *et al.*, 2016) and pre-weaned dairy calves by *C. andersoni* (Kvac *et al.*, 2011; Venu *et al.*, 2012) had been reported. The difference in the occurrence and distribution of major species infecting dairy calves suggest that the transmission of *Cryptosporidium* may be different among different herds of cattle (Ma *et al.*, 2015).

## 5.2. *Cryptosporidium* infection in humans

The overall prevalence of *Cryptosporidium* infection in humans obtained in this study, 9.0%, was comparable to earlier reports of 8.1% - 9% from Ethiopia (Tigabu *et al.*, 2010) and the prevalence of 9% reported in children in Pakistan (Mumtaz *et al.*, 2010). The prevalence obtained in the farm community, 10.8%, was within the range of 7.3-12.2% prevalence reports in apparently normal children (Ayalew *et al.*, 2008, Tigabu *et al.*, 2010; Wegayehu *et al.* 2013) but lower than the prevalence report of 14.8% in diarrheic children (Teshome *et al.*, 2014). The prevalence of *Cryptosporidium* obtained in the HIV seropositive study group, 7.8%, was lower than earlier reports of 20.1% (Assefa *et al.*, 2009) and 17.7% (Dawit *et al.*, 2014) in HIV seropositive persons at different parts of the country. It is also lower than the prevalence of 12% in Indonesia (Kurniawan *et al.*, 2009) and 52.7% (Adesiji *et al.*, 2007) in HIV patients with chronic diarrhoea. In developed nations, the use highly active antiretroviral therapy (HAART) from the mid-1990s proved highly successful in HIV-infected persons in suppression of HIV viral load and restoration of immunity, improvements in qualitative and quantitative CD4+ T-cells counts, significant decreases in opportunist infections such as those caused by protozoa and decline in AIDS morbidity and mortality rates (WHO, 2006; Delpierre *et al.*, 2008). The difference in prevalence among various studies could be due to differences in geographical location, strain of the parasite, sensitivity of employed laboratory methods, and existing control measures against intestinal parasitosis or CD4+ count. The high prevalence of cryptosporidiosis in HIV positive individuals in developing countries could be due to drinking contaminated water and lack of personal hygiene. Furthermore, appropriate diagnostic laboratory investigations are not routinely done for this parasite as is for the other enteric protozoan parasites.

Majority of the human infections in this study were due to *C. parvum* while few were caused by *C. hominis*, this result is in agreement with findings of Adamu (2010) that reported 95% and 2.5% of the human infections in Ethiopia being due to *C. parvum* and *C. hominis*, respectively. Studies on human cryptosporidiosis in the Middle East and the UK reported *C. parvum* as the major species infecting humans (Sulaiman *et al.*, 2005; Leoni *et al.*, 2006; Meamar *et al.*, 2007; Tamer *et al.*, 2007). A study in Thailand showed that all stool samples from HIV patients were characterize as *C. parvum* (Nuchjangreed *et al.*, 2008). However, other studies in the UK and the Netherlands

reported higher *C. hominis* infections compared to *C. parvum* (Nichols *et al.*, 2006; Wielinga *et al.*, 2008). The variation in the distribution and proportion of *C. parvum* and *C. hominis* infection depends on sources of infective oocysts (feces of domestic/wild animals, human stool, sewage contaminated water, contaminated vegetables and other food types) and means of transmission either as zoonotic or anthroponotic. Infections were more prevalent in persons with history of diarrhoea up to 5 times per day for about a week, indicative for the chronic nature of diarrhoea in this group. Hijjawi *et al.* (2010) stated that the most common clinical symptoms in all *Cryptosporidium* positive cases were diarrhoea and abdominal pain; which were experienced by majority of patients. *Cryptosporidium* oocyst discharge was associated with prolonged duration of diarrhoea vomiting and abdominal cramps (Mumtaz *et al.*, 2010). Chronic diarrhoea was the major finding of *Cryptosporidium* infection in HIV+ patients in Indonesia (Kurniawan *et al.*, 2009) and 100% of the HIV+ seropositive patients in Nigeria showed chronic diarrhoea (Adesiji *et al.*, 2007).

Significant association of *Cryptosporidium* infection was seen with owning animals and having high level of contact with their faeces, and this association was particularly evident in persons having contact with cattle but not with pet animals. Moreover, among farm community participants of the study, high prevalence (85%) of *Cryptosporidium* was obtained in persons working in dairy farms with high prevalence of the infection. Our finding is in agreement with earlier studies that reported close contact with cattle and their faeces as the major risk factor of *Cryptosporidium* infections in humans (Nuchjangreed *et al.*, 2008; Ng *et al.*, 2012; Wegayehu *et al.*, 2013; Adamu *et al.*, 2014; Ehsan *et al.*, 2015). In a study in Pakistan majority of the infected children had history of contact with animals and the authors suggested that animals could be reservoirs of human infection (Mumtaz *et al.*, 2010). *Cryptosporidium* parasites are not host specific and infections can spread from infected animals to humans (Laubach *et al.*, 2004). A study on *Cryptosporidium* species in Thailand showed that all HIV and cattle stool samples were characterized as *C. parvum*, suggesting possible zoonotic transmission of the parasite between cattle and humans (Nuchjangreed *et al.*, 2008). In this study, no association was detected between infections of humans and having contact with pet animals and their faeces, however some studies have reported the occurrence of *C. canis* and *C. felis* oocysts in pets and humans in the same house (Traub *et al.*, 2003, 2009). While a study in Thailand reported that 30%-40% of the infections in

dogs and cats were attributed to *C. parvum*, and suggested the potential role of zoonotic transmission.

The prevalence of *Cryptosporidium* was higher in people using well or stream water than people using pipe water, which could be due to more exposure and contamination of well and stream water with faeces of animals and humans compared to pipe water. This result is in agreement with findings of Mumtaz *et al.* (2010) that reported 77.8% of the total *Cryptosporidium* infections in children using well water. A similar finding, showing significant association of *Cryptosporidium* infection with consumption of contaminated water, was reported from Kuwait (Sulaiman *et al.*, 2005).

This study showed that the prevalence of *Cryptosporidium* was much higher in HIV patients not receiving ART compared to patients on-ART medication. The infection rate was higher in HIV positive persons with CD4+ count <200 cell/ $\mu$ l compared to those with CD4+ count 200-500 cell/ $\mu$ l, and no infection was detected in those with CD4+ count greater than 500 cells/ $\mu$ l. Similar results signifying the outcome of ART medication in reducing *Cryptosporidium* infection rate had been reported from Ethiopia (Dufera *et al.*, 2008; Adamu and Petros, 2009; Adamu *et al.*, 2013; Girma *et al.*, 2014; Shimelis *et al.*, 2016). Clinical recovery of cryptosporidiosis and resolution of diarrhoea is due to increased CD4+ (Morpeth and Thielman, 2006), and HAART is considered as the only modality of treatment to prolong and improve quality of life in people living with HIV+ (Karunamoorthi *et al.*, 2009). *Cryptosporidium* infection is usually of short duration and self-limiting in immunocompetent individuals (Moghaddam, 2007). This might be due to the significant protection role of cellular immunity in *Cryptosporidium* infection. Patients with defect of innate and cellular immunity can experience severe or prolonged illness and association of lower numbers of CD4+ T cells (<200 cells/ $\mu$ l) with risk of acquiring cryptosporidiosis had been reported (Morpeth and Thielman, 2006). Kurniawan *et al.* (2009) stated that risk of faecal carriage, severity of illness and development of unusual complications of cryptosporidiosis are directly related to the CD4+ cell count. *Cryptosporidiosis* is unlikely to be more serious than normal except in individuals with impaired T-cell function equivalent to a CD4+ count of < 50 cell/ $\mu$ l. Intestinal polyparasitism occurred in all CD4+ count groups, with the exception of those with CD4+ counts >400 cells/mm<sup>3</sup> (Kurniawan *et al.*, 2009). Whatever the cause of the impairment;

whether inherited or due to HIV infection, other disease, or therapy. Such patients should be advised about the need to avoid the risk of cryptosporidiosis as far as possible (John and Petri, 2006; CDC, 2015). Our results illustrate that people having high school or college level education showed less infection rate compared to people without formal education or having elementary level education. This could be due to better personal hygiene and better knowledge on disease prevention and control measures by more educated people in contrast to people without formal education or low grade education.

### **5.3. *Cryptosporidium* infection in dairy cattle calves**

Thirty dairy calves were followed-up from birth to three months of age to determine the age related occurrence of *Cryptosporidium* infection. Twelve of the thirty calves (40%) shed oocysts of *Cryptosporidium* sometimes during the study period and *Cryptosporidium* was detected in calves from week 1 to 3 months of age. The first appearance of oocysts was on day 7 when 7 of the 30 calves (23.3%) showed the infection, the highest prevalence was on day 15 followed by day 21 and 30. It is hard to realize the source of infection at such early age since calves were segregated from their dams within hours of birth, housed individually till weaning age and had no contact with other calves. Silverlås (2010) stated that detection of *Cryptosporidium parvum*-like oocysts at two days of age and molecular confirmation of *C. parvum* at an age four days indicate transmission of oocysts either from the dam or contaminated calving pens. In addition, study reports point out a range of possible sources of calf hood infection such as contaminated pens, water supplies, buildings, tools and contact surfaces, shoes and clothing of animal handlers as well as flies and birds serving as mechanical vectors (Conn *et al.*, 2007). Since the reported prepatent period for *C. parvum* infection is 3-14 days (Fayer *et al.*, 2008; Silverlås *et al.*, 2010), it is likely to detect this species at 7 days of age and even expected to detect *C. parvum* infections at an earlier date than the present one if our sampling schedule had been on daily basis. Our results are in accord with findings of a longitudinal study that reported the highest prevalence of infection at 2 weeks of age when 29 of the 30 calves excreted oocysts (Santin *et al.*, 2008). Another study on dairy calves less than one month of age in Argentina reported that all infections (100%) occurred in calves less than 14 days old (Del Coco *et al.*, 2008). *Cryptosporidium* infection is realized

during the first 2 weeks of life in dairy cattle calves (Castro-Hermida *et al.*, 2002b; Trotz-Williams *et al.*, 2007).

Of the total 30 calves used in this study 12 were infected by *C. parvum* with a prevalence rate of 40% and 3 were infected by *C. andersoni* showing a prevalence of 10% (three calves infected by *C. andersoni* had been previously infected by *C. parvum*). The distribution of *C. parvum* and *C. andersoni* infections showed a strictly age-related pattern across the ages of calves, in that, *C. parvum* infections were observed from day 7 up to 60 days of age, while *C. andersoni* infections were observed from day 75 up to day 90 of age. This finding is in agreement with a similar longitudinal study in Maryland in which *C. parvum* was detected in 80% of one week old calves and in 96.6% of two week old calves, while *C. andersoni* was detected only in heifers, 12–24 months of age (Santín *et al.*, 2008). In this study, *C. parvum* constituted 100% of the infections in preweaned calves which is in accord with findings of Santín *et al.* (2008) that reported 97% of the total infections in pre-weaned calves were attributed to *C. parvum* infections. A study on newborn calves with diarrhoea in dairy farms in the Metropolitan (Santiago), Chile, confirmed *C. parvum* as the major parasitic disease agent of neonatal calves in the region. We haven't detected *C. bovis* and *C. ryanae* infections in this study; even though these two species may occur in this age group of calves but an overwhelming *C. parvum* infection may mask the concurrent infection of pre-weaned calves by *C. bovis* or *C. ryanae* (Feng *et al.*, 2007b).

Calves infected with *Cryptosporidium* showed more signs of diarrhoea and mucoid faeces without blood as compared to non-infected calves, this could be due to the invasion and colonisation of the epithelial surface by the parasite which results in loss of epithelial cells and microvillus brush border, increased epithelial permeability, impaired nutrient and electrolyte transport, altered solute transportation and osmotic diarrhoea (Chen *et al.*, 2002; Robinson *et al.*, 2003). Similar findings indicating association of *Cryptosporidium* with diarrhoea and mucoid faeces had been reported by other researchers (Del Coco *et al.*, 2008; Diaz-Lee *et al.*, 2011). Enemark *et al.* (2002) stated that clinical diarrhoea was restricted to calves younger than two months in which the highest number of oocyst was detected. The findings of yellowish colour faeces in most infected neonatal calves compared to non-infected ones in the current study was in agreement with results of (Lendner and

Dauguschies, 2014) that reported calves at the age of 7–12 days are the main hosts of *C. parvum* where it causes watery, yellowish and foul-smelling diarrhoea.

Crossbred calves possessing more Holstein Friesian blood level showed higher prevalence of *Cryptosporidium* compared to crossbreds having lower Holstein Friesian blood level. This difference might be due to more susceptibility of the high producer, Holstein Friesian breed, to infections compared to local zebu breed, which is low producer but having some sort of resistance to infections. A study in china reported that different cattle breeds had shown significant differences in the prevalence rate and species of *Cryptosporidium* (Gong *et al.*, 2017). Calves born during night time showed higher prevalence of *Cryptosporidium* compared to calves born during daytime. This could be due to extended period of contact of night born calves with their dams, on occasions where there are no attendants to isolate them, favouring exposure of calves to more infections. In support of the above finding Del Coco *et al.* (2008) reported higher exposure of newborns that stayed in maternity pen with their dams. Results of the current study showed that Calves born to cows above second parity were more infected by *Cryptosporidium* as compared to calves born to cows at first or second parity. Assuming that calves might be infected by their dams at birth, this result could be explained by higher chance of infection of older cows, above second parity, compared to cows in their first or second parity, especially in less contaminated farms.

#### **5.4. Phylogenetic analysis**

Phylogeny analysis of the isolates from this study showed that all isolates had statistically significant similarity with the already recognized GeneBank isolates. All of the *C. andersoni* isolates from this study clustered with their respective GeneBank isolates in one group. Likewise all *C. hominis* isolates of this study clustered with similar GeneBank isolates in another group demonstrating that isolates of the two species circulating in the study area have very close intraspecies similarity. Conversely, the *C. parvum* isolates from this study clustered under three different groups suggesting that multiple subtypes of *C. parvum* bovine genotype might be circulating concurrently in the study area. Although this study has not employed subtyping of the *C. parvum* isolates using the GP60 gene analysis and couldn't confirm the reality of the subtypes, earlier studies had shown the possibility of concurrent circulation of multiple subtypes of *C.*

*parvum* in an area. Xiao *et al.* (2001b) reported concurrent circulation of four subgenotypes of *C. parvum* in calves in central Ohio and four subtypes of *C. parvum* human genotype in AIDS patients in New Orleans. a study in Ethiopia reported the simultaneous circulation of the zoonotic *C. parvum* subtype IIa and IIc in HIV+ Patients (Adamu *et al.*, 2014). Results obtained from the PCR-RFLP and sequence analysis of the 18S rRNA genes demonstrate that *C. parvum* bovine genotype 'A' and *C. hominis* are responsible for human infections in the study area; hence, zoonotic transmission plays major role in the epidemiology of the disease in humans.

## 6. CONCLUSION AND RECOMMENDATION

### 6.1. Conclusion

Results of the current study demonstrated that *Cryptosporidium* infection is widely distributed in dairy cattle and human population of the study area with prevalence of 18.6% and 9%, respectively. The species of *Cryptosporidium* identified in Addis Ababa and its surrounding area, in order of their prevalence, are *C. andersoni* and *C. parvum* in cattle, and *C. parvum* and *C. hominis* in humans. Detection of the zoonotic *C. parvum* bovine genotype in humans and cattle, as well as, evidence of a strong association between human infection and ownership of cattle suggests significance of the zoonotic transmission and complexity in the epidemiology of the disease. The study highlighted risk factors of *Cryptosporidium* infection in dairy farms and human population. Knowledge on risk factors of the disease and its application in the control and prevention of *Cryptosporidium* in dairy farms and humans is considered to reduce incidence of the disease and dissemination of oocysts. Moreover, familiarity with the risk factors is crucial to lessen the risk that humans and cattle pose to other humans, domestic animals, wildlife and the environment and to develop cost-effective management strategies. The present study revealed that *C. parvum* is the major species infecting pre-weaned calves during the first four weeks of life; therefore, individuals closely working with young calves are at greater risk of the infection. Dairy farm attendants, veterinarians, Animal production scientists and farm visitors in close contact with this age group and their faeces should take the necessary precautions. River and stream water as well as contaminated pipe water are the major routes of transmission for *Cryptosporidium* in both cattle and humans in the study area, hence, it is recommended to avoid faecal contamination of nearby water and soil through proper management of pre-weaned calves and farm waste-water disposal. The magnitude and distribution of *Cryptosporidium* infection was higher in cattle compared to humans and it was higher in pre-weaned calves than heifers or adult cattle. The study demonstrated that *C. parvum* infection is more prevalent in young calves compared to adult cattle which were mostly infected with *C. andersoni*. This fact testifies the importance of pre-weaned calves as sources of human infection rather than heifers or adult cattle.

## 6.2. Recommendations

Based on the findings of this study the following recommendation points are forwarded

- Policy makers should be aware of the importance of this zoonotic disease from dairy development and public health perspective and incorporate cryptosporidiosis in future disease control plan of the country.
- Awareness on cryptosporidiosis its risk factors and control methods should be created in the community by the concerned Ministries (Ministry of Health and Ministry of Livestock and Fisheries Resource).
- Drinking water contaminated by sewage is the major vehicle for *Cryptosporidium* oocyst. Hence, public health and municipal water authorities should regularly check safety of the water supply from *Cryptosporidium* oocyst and provide the community with sufficient information for control opportunities
- Drinking water could be freed from *Cryptosporidium* oocyst by boiling at 100 °C for 1 minute/rolling boil or filtering with a filter of  $\leq 1 \mu$  (micron) pore size (CDC, 2015).
- In humans, the only control option is avoiding ingestion of oocysts via water (drinking/swimming pool) or contaminated food, however, regular hand washing and escaping from contact with faeces of animals or humans is an important hygienic measure.
- Intensive dairy farms are of paramount importance in boosting milk production for the ever increasing demand of human population. To attain this goal farms should pursue standard hygienic management practice that could enable them to prevent/control zoonotic infections. Among these practices the major ones are:-
  - Farms should have adequate number and dimension of barns and pens in proportion to their herd size.
  - Farms should have sufficient number of calving pens
  - Calves should be provided with adequate bedding which has to be changed at daily or two days interval.
  - Calves should be separated from their dams immediately after birth and provided with colostrum, calf suckling should be avoided
  - Weaning age of calves should not be too long; calves should be kept separately in individual pens during the weaning period.

- Farms should have a clean water supply, preferably private wells with piping system, use of river/stream water should be avoided.
- Animals should have free access to drinking water all over the day.
- Farm waste water should be disposed in such a way that it won't contaminate pasture land and drinking water of the farm.
- Preventive and control measures against endemic infectious diseases should be in place to avoid occurrence of opportunistic diseases like cryptosporidiosis.
- Dairy farm barns, pens and indoor animals should always be clean and hygienic, farm gates should be provided with disinfectant solution to decontaminate humans, animals and vehicles entering in to the farm.

### Limitations of the research

- Following the SSU rRNA gene analysis, attempts made to subgenotype *C. parvum* and *C. hominis* isolates using the GP60 gene analysis failed due to unpredicted degradation of reserved genomic DNA of samples.
- This study was not capable to determine the magnitude of oocyst contamination of drinking water sources due to shortage of materials to investigate water samples.

Table 17: Major findings, implication and future research plan

Major findings	<ul style="list-style-type: none"> <li>➤ <i>Cryptosporidium</i> infection was detected in 37.7% of investigated farms with an overall prevalence of 18.6, of which 21.4% were in the intensive and 11.2% were in the extensive farms.</li> <li>➤ Intensive urban farming, medium herd size, absence of calving pen, absence of calf bedding, weaning age <math>\geq</math> 6 months, river/stream water sources, disposal of farm waste water to nearby wells, unhygienic pens, group penning of calves and occurrence of endemic infectious diseases were identified as risk factors of the infection in dairy farms</li> <li>➤ <i>Cryptosporidium</i> infection was detected in 88.9% of the health centers and 61.5% of the dairy farms investigated with an overall prevalence of 9%. 10.8% was</li> </ul>
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	<p>in dairy farm community and 7.8% was in HIV+ patients.</p> <ul style="list-style-type: none"> <li>➤ The prevalence in HIV positive non-ART group was 21.7% and in those on ART was 0.83%</li> <li>➤ Low education level, possession of cattle/cattle &amp; pets, high contact with animals and their faeces and drinking well/stream water were identified as risk factors for human infections.</li> <li>➤ Three <i>Cryptosporidium</i> species were identified:- <i>C.andersoni</i> (73.3%) and <i>C. parvum</i> (26.7%) in cattle and <i>C. parvum</i> (81.5%) and <i>C. hominis</i> (18.5%) in humans.</li> <li>➤ The age related distribution of <i>Cryptosporidium</i> infection was determined. Oocyst shedding started at the age of 7 days and continued till day 90 of age (end of the study period), the highest prevalence, 40%, was in two week calves.</li> <li>➤ The prevalence in neonates, 19.3%, and pre-weaned calves, 18.3%, was higher compared to 9.2% and 7. 8% in post- neonate and post-weaned calves, respectively.</li> <li>➤ <i>C. parvum</i> accounted for 87.5% of the total infections and 100% of the infections in pre-weaned calves, while <i>C. andersoni</i> was identified in post-weaned calves only and constituted 12.5 % of the total infections.</li> </ul>
Implication	<ul style="list-style-type: none"> <li>➤ Possibility of zoonotic transmission was verified due the following epidemiological and molecular study evidences <ul style="list-style-type: none"> <li>❖ higher prevalence of human <i>Cryptosporidium</i> were detected in dairy farms with high infection rate than low/nill infections</li> <li>❖ Possession of cattle or cattle and pets was highly associated with <i>Cryptosporidium</i> infection in humans.</li> <li>❖ <i>C. parvum</i> isolates of human and cattle grouped under same subgenotype group</li> </ul> </li> <li>➤ The restricted distribution of <i>C. parvum</i> infections in pre-weaned calves indicated the importance of pre-weaned calves for human infections.</li> <li>➤ River and stream water sources were identified as the common risk factors for both human and cattle infections</li> </ul>
future	<ul style="list-style-type: none"> <li>➤ Molecular studies aimed at subgenotyping of cattle isolates are required to</li> </ul>

research plan	<p>complete the molecular epidemiology of the parasite in the country.</p> <ul style="list-style-type: none"><li>➤ Molecular studies should expand to contain drinking water samples, and other farm animals and pets living in close proximity with humans.</li><li>➤ The research on <i>Cryptosporidium</i> should extend to pastoral area and to areas where intensive dairy farms are on the rise</li></ul>
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## 8. APPENDICES

### Appendix I: Forms and questionnaires used

#### Appendix Table 1: Questionnaire: general information about dairy farms

##### 1. Farm description

1.1. Name of the Farm ..... Owners' name: .....

1.2. Address: Kebele..... House no ..... Tel. no .....

1.3. Farm location:            a) urban            b) periurban            c) rural

2. Production system        a) intensive        b) semi- intensive        c) Extensive

2.1. Herd size:    cows..... Male calves.....Bulls..... female calves.....Heifers.....

2.2. Breed of animals .....

2.3 Age of animals (range): .....

2.4. Age of the farm: .....

##### 3. Management data

3.1. Calf caretaker (attendant):    a) owner (family member)            b) hired

3.2. Sex            a) male            b) female

3.3. Experience    a)  $\leq 5$  years            b)  $>5$  years

3.4. Manager's educational status

a) people without formal education b) Read and write c) Elementary school d) High school graduate e) Professional

3.5. If professional    a) Related to animal production    b) Unrelated to animal production

##### 4. Periparturient care

4.1. Calving facilities            a) calving pen            b) the same barn

4.2. Navel treatment            a) practiced            b) not practiced

4.3. Awareness of importance of colostrum to neonates            a) yes            b) no

Method of colostrum feeding            a) suckling            b) hand feeding

Time of first colostrum feeding            a) 6 hours            b) 6-24 hours            c)  $> 24$  hours

Duration of colostrum feeding            a) for 24 hour            b) 24 hour-4 days            c)  $> 4$  days

If hand feeding source of feeding            a) dam            b) another cow

4.4. Weaning age .....

**5. Feeding**

5.1. Type of feed            a) milk            b) milk replacer

5.2. Amount of milk/milk replacer given daily per unit of body weight.....

5.3. Frequency    a) once/day    b) twice/day    c) thrice/day

5.4. Time of introducing feed other than milk or milk replacer .....

5.5. Type of supplementary feed and quantity given per unit of body weight

          a) Grazing (hours of grazing)    b) concentrates    c) hay

**6. Access to water**                    a) free access        b) limited access

6.1. Source of drinking water    a) well                b) river        c) pipe

6.2. Disposal of waste water    a) to a well        b) to a river    c) to a grazing field

**7. Housing**

7.1. Housing    a) separate pen        b) together with cows in the cow barn    c) other

7.2. If separate pen            a) individual pen    b) group pen

7.3. Type of the floor    a) concrete    b) soil/stone    c) wooden

7.4. Bedding            a) present            b) absent

7.5. If present what is the bedding material and how frequently is it changed

          a) more than once per week        b) once per week        c) less than once per week

**8. Experience on calf health problems, prevention, and control of problems**

8.1. Major health problem of the farm.....

8.2 Number of calves lost during the last one year.....

8.3. Disease or disease syndrome responsible for sickness and death of calves in order of

Importance: 1. ....

                  2. ....

                  3.....

8.4. Measures taken to treat sick calves.....

8.5. Measures taken to prevent disease problems.....

Appendix Table 2: Sample calf/cow/heifer/ bull information

1.1. Name of the Farm \_\_\_\_\_ Owners' name: \_\_\_\_\_ date \_\_\_\_\_

1.2. Identity of the animal \_\_\_\_\_ Code No. \_\_\_\_\_

1.3. Date of birth      Date \_\_\_ Month \_\_\_ year \_\_\_ age \_\_\_ Site name \_\_\_\_\_

Sex of the animal                      a) male      b) female

Exotic blood level                      a)  $\leq 50\%$       b) 50-75%      c)  $\geq 75\%$

Consistency of faeces                      a) liquid      b) soft      c) solid

Color of the faeces                      a) cream/orange b) creamy brown      c) brown

Presence of mucus                      a) yes      b) no

Presence of blood                      a) yes      b) no

History of diarrhoea in the past      a) yes      b) no

Cleanness of the Pen                      a) clean      b) medium c) unclean

Cleanliness of the tail, hindquarters and flank      a) clean      b) medium      c) unclean

**Appendix Table 3:** Questionnaire format for human participants (English)

Code No. \_\_\_\_\_ Name: \_\_\_\_\_ date \_\_\_\_\_

1. Age: \_\_\_\_\_ Sex: \_\_\_\_\_ district: \_\_\_\_\_ kebele: \_\_\_\_\_
2. Educational background: a) without formal education      b) elementary school level  
c) High school level      d) college/university level
3. Presence of Abdominal pain: a) Yes b) No c) sometimes in the past \_\_\_\_\_
4. Presence of Diarrhoea:      a) present      b) absent      c) sometimes in the past
5. Frequency of diarrhoea:      a)  $\leq 5$  X/day      b) 5-10 X /day      c)  $>10$  X/day
6. Duration of diarrhoea:      a)  $\leq$  a week      b) for weeks      c) for months
7. Have you been treated? :-      a) Yes      b) No
8. Type of drug (if known): \_\_\_\_\_
9. Treatment response: a) good \_\_\_\_\_ b) excellent \_\_\_\_\_ c) No response \_\_\_\_\_
10. Do you have animals at home: a) Yes \_\_\_ b) No \_\_\_ c) some times in the past \_\_\_\_\_
11. which animal type: a) Cattle \_\_\_ b) Dogs \_\_\_ c) Cat \_\_\_ d) all e) others specify \_\_\_\_\_
12. Where do they live: a) at home with the family \_\_\_\_\_ b) in separate room \_\_\_\_\_
13. Contact with animals/their manure: a) high \_\_\_ b) medium \_\_\_ c) low \_\_\_ d) no \_\_\_
14. Source of drinking water: a) well \_\_\_ b) pipe \_\_\_ c) stream \_\_\_\_\_
15. Treatment of water at Home: a) filtration \_\_\_ b) boiling \_\_\_\_\_ c) chemicals d) no
16. Use of swimming pools: a) yes \_\_\_ b) No \_\_\_ c) some times in the past \_\_\_\_\_

17. Contact with a person with diarrhoea, especially a child: a) yes\_b) No c) some times in the past

18. Changing nappies or toileting young children: a) yes \_\_ b) No \_\_ c) some times in the past \_\_\_\_

19. Attendance at childcare settings: a) yes \_\_\_\_ b) No \_\_\_\_ c) some times in the past \_\_\_\_

**For dairy farm workers only**

Farm name: \_\_\_\_\_ district: \_\_\_\_\_ kebele: \_\_\_\_\_

20. Service year as farm attendant: a) <=5 yrs \_\_\_\_ b) 5-10 yrs \_\_\_\_ c) >10 yrs \_\_\_\_

21. which animal type you attend: a) cows \_\_\_\_ b) heifers \_\_\_\_ c) Calves \_\_\_\_ d) all \_\_\_\_

22. Do you use hand gloves while working: a) yes \_\_\_\_ b) no \_\_\_\_ c) sometimes \_\_\_\_

23. Availability of water and soap for hand washing: a) yes \_\_\_\_ b) no \_\_\_\_ c) sometimes

24. Do you wash your hands after contact with animals: a) yes \_\_\_\_ b) no \_\_\_\_ c) sometimes

25. Source of drinking water: a) well \_\_\_\_ b) pipe \_\_\_\_ c) stream \_\_\_\_

**Appendix Table 4: Questionnaire format for human participants (Amharic)**

የክሊኒክ/ የእርባታ ጣቢያው ስም \_\_\_\_\_

የኮድ ቁጥር. \_\_\_\_\_ ስም: \_\_\_\_\_ ቀን \_\_\_\_\_

1. ዕድሜ: \_\_\_\_\_ ጾታ: \_\_\_\_\_ ወረዳ: \_\_\_\_\_ ቀበሌ: \_\_\_\_\_

2. የትምህርት ደረጃ: a) ያልተማረ b) አንደኛ ደረጃ c) ሁለተኛ ደረጃ d)

ኮሌጅ/ዩኒቨርሲቲ

3. የሆድ ህመም ሁኔታ: a) ጤነኛ b) ህመም አለ c) ቀደም ብሎ ነበረ

4. የሆድ ማስቀመጥ ሁኔታ: a) አለ b) የለም c) ቀደም ብሎ ነበረ

5. በቀን ውስጥ የማስቀመጥ ብዛት: a) እስከ 5 ጊዜ b) ከ5-10 ጊዜ c) ከ10 ጊዜ በላይ

6. የማስቀመጥ የጊዜ ርዝመት: a) ለአንድ ሳምንት b) ለሳምንታት c) ለወራት

7. ለሆድ ማስቀመጥ ህክምና ተካሂዷል? :- a) አዎን b) አልተካሄደም

8. ህክምና ተካሂዶ ከሆነ የመድሀኒቱ አይነት (ከታወቀ): \_\_\_\_\_

9. በመድሀኒቱ የተገኘ ፈውሰ: a) ጥሩ b) በጣምጥሩ c) ፈውሰ የለውም

10. በመኖርያዎ የቤት እንስሳት አሎት? a) አዎን b) የሉም c) ቀደም ብሎ ነበሩ

11. የትኛው የቤት እንስሳት a) ውሻ b) ድመት c) የቀንድ ከብት d) ሌሎች

12. እንስሳት የሚኖሩት የት ነው: a) መኖርያ ቤት ውስጥ b) በተለየ ክፍል

13. ከቤት እንስሳትና ከእዳሪያቸው ጋር ያሉት ንክኪ: a) ከፍተኛ b) መካከለኛ c) ዝቅተኛ d) የለም

14. የመጠጥ ውሀ ምንጭ a) ጉድጓድ b) ቧንቧ c) ምንጭ
15. በቤት ውስጥ ውሀን የማጽዳት ስራ : a) በማጣሪያ b) በማፍላት c) በኬሚካል d) የለም
16. የመዋኛ ገንዳ ይጠቀማሉ ? a) እጠቀማለሁ b) አልጠቀምም c) ቀደም ብሎ
17. ሆዱን ከሚያስቀምጠው ሰው ጋር በተለይም ከህጻናት ጋር ንክኪ አሎት? a) አዎን b) የለኝም c) ቀደም ብሎ
18. የህጻናትን ጨርቅ የመቀየር ሀላፊነት አሎት ? a) አዎን b) የለም c) ቀደም ብሎ
19. በህጻናት ማሳደጊያ ይሰራሉን ? a) አዎን b) የለም c) ቀደም ብሎ

**በእንስሳት እርባታ ጣቢያዎች ለሚሰሩ የጥናቱ ተሳታፊዎች ብቻ**

የእንስሳት እርባታው ስም \_\_\_\_\_ ወረዳ: \_\_\_\_\_ ቀበሌ: \_\_\_\_\_

20. በእርባታ ጣቢያው የሰሩባቸው አመታት ብዛት: a) ≤5 b) ከ5-10 አመት c) >10 አመት
21. የየትኞቹ እንስሳት ተንከባካቢ ነዎት a) የላሞች b) የጊደሮች c) የጥጆች d) የሁሉም
22. በስራ ወቅት የእጅ ጓንት ይጠቀማሉን a) አዎን b) የለም c) አንዳንድ ጊዜ
23. በእርባታ ጣቢያው የእጅ ውሀና ሳሙና አቅርቦት: a) አለ b) የለም c) አንዳንድ ጊዜ
24. ከእንስሳት ጋር ከሰሩ በሀላ እጅዎን ይታጠባሉን: a) አዎን b) የለም c) አንዳንድ ጊዜ
25. የእርባታ ጣቢያው የውሀ ምንጭ : a) ጉድጓድ b) ቧንቧ c) ምንጭ

consistency of the faeces	liquid	Soft	solid
color of the faeces	cream/orange	creamy brown	brown
presence of mucus	Yes	No	
presence of blood	Yes	No	
ART status	Yes	No	
how long on ART			
CD4 counts			

**Appendix Table 5:** Cryptosporidiosis research, participant information sheet (English)

Title of the project: “Molecular Epidemiology of *Cryptosporidium* infections in Cattle and Humans in Addis Ababa and Its environs, Ethiopia”

Principal investigator: Dr. Manyazewal Anberber, PhD student, Addis Ababa University, College of Veterinary Medicine and Agriculture.

Introduction: Cryptosporidiosis is one of the important zoonotic diseases affecting animals and humans. Cattle, primarily dairy calves, are considered sources of the infection to humans and the

disease is very severe in children and immune-compromised people with diseases such as HIV/AIDS. This disease is caused by a protozoan parasite and its transmission to humans occurs through contaminated water. Therefore you are invited to participate in a study about the “public health significance of cryptosporidiosis in Addis Ababa and Oromia special zone” The study is conducted by a PhD student of Addis Ababa University, College of Veterinary Medicine and Agriculture in collaboration with the health bureaus of Addis Ababa and Oromia special zone. This form provides you the information about the study, thus, reading the document here under is very crucial. More explanations will be given to you by the research participant professionals, you have the right to raise questions and get answers before you decide to participate / not on the study.

**Objective of the study:** The purpose of the study is to know the situation of cryptosporidiosis in the communities of the study areas. Therefore, your participation will help to achieve concrete information that could be used to improve the cryptosporidiosis prevention and control program and to provide better health care in the area.

**Duration of the study:** The duration of this study is about three months, as part of the study you will be requested to answer some questions focusing on your contact with cattle, dogs and cats and on your water consumption habits. In addition, you will be asked to give about 2 gm of stool. The estimated time taken to participate in this study is approximately half an hour.

**Benefits of being in the study:** you will not receive any personal benefit attributable to participating on this study, except free clinical service for the diagnosis of the disease.

**Potential risks of being in the study:** There is no risk resulting from being a participant of this study.

**Right of the participant:** Your participation is entirely based on voluntary basis; you can refuse to participate without penalty or loss of benefits to which you are otherwise entitled. You can stop your participation at any time and your refusal will not have any impact on your current or future relationships with the people or institutions carrying out this study.

**Confidentiality and privacy protections:** The records of this study will be kept securely and confidential. All publications arising from the study will exclude information about your identity. However, if you are found positive for active parasitic infections, the local health professionals will be informed so as to provide you the necessary treatment and advices.

**Contact address:** If you have any question about the study, you can ask the nearby health professionals involved in the study, furthermore, you can also use the following addresses to communicate with the principal investigator of the study.

Dr. Manyazewal Anberber  
Addis Ababa University  
College of Veterinary Medicine and Agriculture  
Tel Office: +251-114338533, Mobile -----+251-913-01-35-35  
Addis Ababa Health Bureau Review Board : Tel: +251-115-538193

**Appendix Table 6:** Cryptosporidiosis research, participant information sheet (Amharic)

**የምርምር ፕሮጀክቱ ርዕስ:** የክሪፕቶስፖሪዲዮሲስ በሽታ በአዲስ አበባና አካባቢ ህብረተሰብና የቀንድ ከብቶች ውስጥ ያለው ስርጭት

**ዋና ተመራማሪ:** ዶ/ር ማንያዘዋል አንበርብር, በአዲስ አበባ ዩኒቨርሲቲ የእንስሳት ህክምናና ግብርና ኮሌጅ የፒኤችዲ (PhD) ተማሪ

**መግቢያ:** ክሪፕቶስፖሪዲዮሲስ ከእንስሳት ወደ ሰው ከሚተላለፉ በሽታዎች አንዱ ሲሆን ሰውንና የተለያዩ እንስሳትን ያጠቃል. የዳልጋ ከብቶች በተለይም ትናንሽ ጥጆች ወደ ሰው ለሚተላለፈው በሽታ ዋና ምንጮች ሲሆኑ ህመሙ በተለይም በህጻናትና በሽታን የመቋቋም አቅም ባጠራቸው እንደ በኤች ኤይቪ ኤድስ (HIV/AIDS) በሽታ ቢታመሙ ሰዎች ላይ የጠነከረ ይሆናል። ይህ በሽታ ፕሮቶዞዎች (protozoa) በተባለ ተህዋስ የሚመጣ ሲሆን ወደ ሰው የመተላለፊያው ዋና መንገድ በተህዋሱ የተበከለን ውሀ በመጠጣት ነው።. ስለዚህም እርስዎን በዚህ የክሪፕቶስፖሪዲዮሲስ በሽታ በአዲስ አበባና በአሮሚያ ልዩ ዞን ወረዳዎች በሚኖረው ህብረተሰብ ውስጥ ያለውን ስርጭትና አንደምታ ለማወቅ በሚደረገው ጥናት ላይ እንዲሳተፉ ጋብዘንዎታል። ጥናቱ የሚካሄደው በአዲስ አበባ ዩኒቨርሲቲ የእንስሳት ህክምናና ግብርና ኮሌጅ ከአዲስ አበባና ከአሮሚያ ልዩ ዞን ወረዳዎች የጤና ቢሮዎችና ተቋማት ጋር በመተባበር ነው። ይህ ፎርም ለእርስዎ ለጥናቱ ተሳታፊ የሚሆኑ ዝርዝር መረጃዎችን የያዘ በመሆኑ በደንብ ማንበብና መረዳት ያስፈልጋል። ተጨማሪ መግለጫዎች በጥናቱ ባለሙያዎች የሚሰጥ ሲሆን በጥናቱ ላይ ለመሳተፍ / ላለመሳተፍ ከመወሰንዎ በፊት ጥያቄዎችን የመጠየቅና መልሶችን የማግኘት መብትዎ የተጠበቀ ነው።. የጥናቱ ተሳታፊ ከሆኑ በሽታውን በሚመለከት ለተዘጋጁ ጥያቄዎች መልስ እንዲሰጡ ይጠየቃሉ። በተጨማሪም 5 ግራም የሰገራ ናሙና እንዲሰጡ ይጠየቃሉ።

**የጥናቱ አላማ:** የዚህ ጥናት አላማ ጥናቱ በሚካሄድበት አካባቢ ባለው ህብረተሰብ ውስጥ የክሪፕቶስፖሪዲዮሲስ በሽታ ያለውን ስርጭት ለማወቅ ነው። ስለዚህ እርስዎ የሚሰጡት መረጃ የክሪፕቶስፖሪዲዮሲስን በሽታ በአካባቢውም ሆነ በመላው አገሪቱ በተሻለ መንገድ ለመቆጣጠርና የተሻለ የጤና ሽፋን እንዲኖር የሚረዳ ይሆናል።

**ጥናቱ የሚቆይበት ጊዜ:** ጥናቱ የሚካሄደው ለስድስት ወራት ገደማ ሲሆን እርስዎ በጥናቱ የሚቆዩበት ጊዜ ለሰላሳ ደቂቃ ያህል ብቻ ነው።

**የጥናቱ ተሳታፊ መሆን የሚያስገኘው ጥቅም:** በጥናቱ ላይ የሚደረገው ተሳትፎ በፍቃደኝነት ላይ የተመሰረተ ስለሆነ የተለየ ጥቅም አያስገኝም።

**በጥናቱ በመሳተፍ የሚያጋጥሙ ችግሮች:** በጥናቱ ላይ በመሳተፉ በተሳታፊው ላይ የሚያስከትለው ምንም ዓይነት የጤና ችግር አይኖርም።

**የጥናቱ ተሳታፊ መሆኑ:** የጥናቱ ተሳትፎ በፍቃደኝነት ላይ የተመሰረተ በመሆኑ ተሳታፊው በፈለገበት ጊዜ የለምንም ተጠያቂነት ጥናቱን ማቀረጥ ይችላል ይህም ሁኔታ & ተሳታፊው ከተቃማቱ ጋር ያለውን የወደፊት ግኑኝነት አያበላሽም።

**የጥናቱ ተሳታፊ የግል መረጃዎች ሚስጥራዊነትና ጥበቃ:** የጥናቱ የላብራቶር ውጤቶችና ሌሎችም መረጃዎች ሁሉ በመመዘገቢያ ቅጽ ላይ ተመዘግበው በሚስጥር የሚሰጡ ሲሆን የጥናቱን ውጤት የሚገልፁ ህትመቶችም የጥናቱን ውጤቶች እንጂ የተሳታፊውን ማንነት አይጠቅሱም። ነገር ግን በተሳትፎው ሂደት በሚደረገው የምርመራ ውጤት መሰረት ተሳታፊው የአንጀት ውስጥ ጥገኞች በሽታ ከተገኘበት ውጤቱ ለአካባቢው የጤና ባለሙያ ይገለጻል ይህም የሚሆነው ተሳታፊው አስፈላጊውን የህክምና እርዳታና ምክር በጤና ማእከሉ ማግኘት እንዲችል ታስቦ ነው።

**አድራሻ:** ተሳታፊው በሚረዳው ቋንቋ ጥያቄዎችን መጠየቅና አጥጋቢ ማብራሪያዎችን በጥናቱ ተሳታፊ ከሆነ የአካባቢው ባለሙያ ማግኘት እንዲችል ይመቻቻል። ይህም ሆኖ ተሳታፊው ተጨማሪ ጥያቄዎችን መጠየቅና መረጃ ማግኘት ከፈለገ የሚከተለውን አድራሻ መጠቀም ይችላል።

ዋና ተመራማሪ: ዶ/ር ማንያዘዋል አንበርብር  
በአዲስ አበባ ዩኒቨርሲቲ የእንስሳት ህክምናና ግብርና ኮሌጅ  
ስልክ ቁጥር: +251-114-338533 ፣ +251-913-013535  
የአዲስ አበባ ጤና ቢሮ ስልክ ቁጥር +251-115-538193

Appendix Table 7: Written consent form (English)

S/No.: \_\_\_\_\_

Card No.: \_\_\_\_\_

Full Name: \_\_\_\_\_

I the above mentioned confirm that the document describing the purpose, benefits, risks, and confidentiality of the research study on ‘ the public health significance of cryptosporidiosis’ that is being conducted at/in ----- district, has been read and explained to me. During the process I was encouraged to ask questions, understood that I have the right to withdraw from the study at any time and have been informed that other people will not know my results as it is coded by numbers rather than names of people. I realized that there are no personal benefits to me apart from the clinical services related to my stool examination. In addition to the above mentioned I have no objection if part of the stool specimen is shipped to other country for further examination and agreed to use this sample for the same experiment in the future. Therefore, with full understanding of the importance of the study, I hereby confirm that I have voluntarily participated on the research.

Signature \_\_\_\_\_ Date \_\_\_\_\_

Urban \_\_\_\_\_ Kebele \_\_\_\_\_ Rural \_\_\_\_\_ Village \_\_\_\_\_

Name of the professional taking consent \_\_\_\_\_ Signature \_\_\_\_\_ Date \_\_\_\_\_

Appendix Table 8: Written consent form (Amharic)

ተራ ቁጥር: \_\_\_\_\_

የካርድ ቁጥር: \_\_\_\_\_

ሙሉ ስም \_\_\_\_\_

እኔ ከዚህ በላይ በስም ተጠቃሹ የክሪፕቶስፖሪዲዮሲስ በሽታ በሰዎች ላይ ስለሚያደርሰው ጉዳት በሚል ርዕስ የቀረበውንና በ ----- ወረዳ የሚካሄደውን ጥናት አላማ ጥቅም ችግርና አስተማማኝነት የተገለፀበትን መረጃ አንብቤ/ተነባልኝ እና ተብራርቻለሁ። ተረድቼአለሁ። የጥናቱን አላማ በተመለከተ ማንኛውንም ጥያቄ እንድጠይቅ ተበረታትቼ ጥያቄዎችን የጠየኩ ሲሆን በቂ የሆነ ማብራሪያና መልስ ተመልሶልኛል። በጥናቱ ውስጥ መሳተፍ በፍቃድኝነት ላይ የተመሰረተ መሆኑንና በማንኛውም ጊዜ ተሳትፎን ማቋረጥ እንደሚችል፤ ይህም ከተቋሙ ጋር ያለኝን ግንኙነት እንደማያበላሽው ተገንዝቤአለሁ። የምርመራው ውጤት ሌሎች ሰዎች በማያውቁበት ሁኔታ በኮድ ተመዝግቦ እንደሚያዝና የጥናቱም ውጤት

ለጥናቱ አላማ ብቻ ለሚመለከታቸው ክፍሎች የሚገለጹ መሆኑን ተረድቻለሁ። ከዚህ በላይ ከተጠቀሰው በተጨማሪ የዚህ ናሙና ክፍል ለበለጠ ምርመራ ወደ ውጪ አገር ቢላክ የማልቃወም መሆኔንና ለወደፊትም ቢሆን ናሙናው ለዚህ ጥናት ስራ እንዲውል የተስማማሁ መሆኔን አስታውቃለሁ። ስለሆነም የጥናቱን ጠቃሚነት ሙሉ በሙሉ በመረዳቴ በፍቃደኝነት የጥናቱ ተሳታፊ መሆኔን ከዚህ በታች በሰፈረው ፊርማዬ አረጋግጣለሁ።

የተሳታፊው ፊርማ \_\_\_\_\_ ቀን \_\_\_\_\_  
ከተማ \_\_\_\_\_ ቀበሌ \_\_\_\_\_ ገጠር \_\_\_\_\_ ቀበሌ \_\_\_\_\_  
ስምምነቱን የተቀበለው ባለሙያ \_\_\_\_\_ ፊርማ \_\_\_\_\_ ቀን \_\_\_\_\_  
የእማኝ ስም \_\_\_\_\_ ፊርማ \_\_\_\_\_ ቀን \_\_\_\_\_

**Appendix Table 9:** Cryptosporidiosis research, participant information sheet (Oromiffa)

Ragaalee hirmatootaa dhukuba kriptosporidiosisii

**Mataduree qoranno pirojekti:** Dhukubni cryptosporidiosis godina oromiyaa liyu anaalee filataman sadii keessa tamsaaina beeyladaa manaa keessa qabu fi sababotaa tamsaaina isaa ciimsan akasumaas umatta irrati midhaa inii fidu qorachuf ta'aa.

**Qorataa dura anaa:** Dr Maanaazawaal Anbarbiir, yunivarsitti Adis Ababaa kolleeji fayyaa beeyladaa fi Qonnaa Bushoofutti Barataa digrii PhD

**SENSAA:** Dhukubni cryptosporidiosis ilbisoota xixiqaa protozu'aa jadamaniin kan dhufu yemu ta'uu beyladotaa manaa, bileensotta bosonaafi ilma namaa kan midhudha. Dhukubni Kun ilmoo namaa irati ciiminna kan argatu keysaahu umata dhukubotta humna namaa dadhabsisan kaneen aka Edsii /AIDS/ fakaataniin kan qabaman irratidha. Dhukubni kan namatti dabru bishaan protozu'aa kanaan walitimakamen yemu ta'uu guyyaa toko keesa yero 6-12 tti garaa kaasisuu danda'aa, sababa kanaan bishaan hanga litrii qudha torbaa sagaraa waliin makamee qaama dhukubsataa kesaa aka bahu ni godhaa. kanaafu midhaa dhukubni cryptosporidiosis ilma namaa irati fidu beekudhaaf qorano gageefamu kanaa irrati aka hirmatan yamamteniru. Qoranoon kun kan gageefamu Qajelcha fayaa godina oromiyaa liyu fi hojjatota wajjiraa fayaa Anotaa wajjin tahudhaani.

Unkaan kun waa'ee qoranichaa gadi-fageenyaan kan ibsu waan ta'eef siritii dubisun barbaachisadha. Hirmaataan qoranicha hirmaataa ta'uu fi dhabu isaa murtesu duratti waa'ee qoranicha irratti hubanoon ga'aa ta'ee ogeesaan ni-keenamaaf. Gafiin yojiratees debiin nikenamaaf.

**Kayoon qoranichaa:** Qoranoon kun kan gageefamu wa'ee tamsaiina dhukubni cryptosporidiosis haawasa naanichaa keesa qabufii sababota tamsaiina issaa gargaran bekuf yamu ta'uu hirmaanaan qorano kana iratii gotan raggaale qabatamaa ta'aan kan argamsisu fi foyainan dhukubicha to'ano jala olchuuf kan gargaru ta'aa.

**Yerro qoranaan itti turu:** Qoranoon yeroon itti gageefamu jia jehaaf tahulle hirmaatan qorano keysati hirmaachuf hanga daqiqaa sodomaadhaa. Hirmaata qoranichaa yoo taatan waa'ee dhukuba kanaa ilalchise gaafille aka walqunamti horii maalaa, Adureefi saree wajjin qabdan nigaafatamtu idaamaanis faki sagraa aka keenitan nigafatamtu.

**Hiramaata qoranichaa tahun faydaa inni argamsisu:** Hirmataa qoranichaa tahun kan fedhinaa irraa kae male faydaa birraa hinqaabu.

**Hiramaata qoranichaa tahu iratii rakkon qunamu danda'uu:** fakin qoranoo kanaaf fudhatamu fakii sagraa waan ta'eef rakkon qunamu danda'uu hinjiruu.

**Mirga hirmaataa:** Hirmanaan fedhi irati kan hundaawe waan ta'eef hirmaataan yeroo fedheeti hirmachumaa issaa dhaabuu nidanda'aa kun amoo walqunamti dhabaa keenyaa wajjin qabnu kan baleesu mitti.

**Waaee egumsaaa fi iccitti raggaalle hirmaata qoranichaa:** buaan qoranno laaboratori fi raggaaleen biraa unkaadhaan galmaawee iccittin kan qabamu fi unkaan kan dhunfaa waan ta'eef Barullen buaa qoranicha ibsan bu'aa qoranichaa male enyumaa hirmaatichaa kan hinibsinedhaa. Garu yeroo hirmataa keesatti dhukubni cryptosporidiosis hirmaatichaa iratii yoo argame raggaan hojatota fayyaa naano sanif ni ibsama kunis hirmaatichi gorsaa fi degarsa ogumaa aka argatu tasisuf yaadatameeti.

**Iddo:** Hirmaataan afaan beekun gaafi gaafachu fi debii argachu yo barbaadee ogeessa hirmataa qoranicha nanno san jiru irraa aka argatu ni godhamaa. Debiidaannoo argachu yo berbadess iddo teesoo kanaan gaafachu ni-danda'aamaa.

Dr maanaazawaal Anbarbiir

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Birro unkaa mirkanese: Birro Egumssa Fayyaa Bulchinssa magaalaa Addis Ababaa

Lakofsa bilbia : +251-115-538193

Appendix Table 10: Written consent form (oromiffa) Unkkaa waligalte hirmaatichaa

Anni ----- dokumentiin armaan ollitti caqafamee kan kaayo, faaydaa, rako fi icitii qorano dhukuba Cryptosporidiosis umata (hawaasa) irrati aannaa -----kessa gageefamaa jiru naaf dubifamee ibsaaniis nakeenamu isaa nin mirkanesaa. Akasumatis ragaan buu'aa qoranichaa umani birraa aka hinbeeyneeti kodin galmaawee aka qabamu mirkaneefadheerra. Qoronnoo bodattis bu'aan qoranichaa kaayoo qoranichaa qofaaf aka olufii qaamma illaalu qofaaf aka keenamu (dabru) beekeerra. Hirmanaan qoranichaa feedhii irrati hundaawuu isaafi yerroo barbaadameeti dhabun aka danda'amuus naaf ibsamerra. Kanaafu,

faaydaa qoranichaa guttu guttdhaan waanin ittii amaneef, feedhii kiyaan qoranichaa irratti hirmaachuf walligalu kiyaan malattoo kiyaan armaan gaditti mirkaneserra.

Kodii qoranichaa \_\_\_\_\_

Maqaa hirmaatichaa \_\_\_\_\_ Mallattoo \_\_\_\_\_ Guyya \_\_\_\_\_

Magaalaa \_\_\_\_\_ Aradda \_\_\_\_\_ Badiya \_\_\_\_\_

Maqaa ogesa \_\_\_\_\_ Mallattoo \_\_\_\_\_ Guyya \_\_\_\_\_

Maqaa ragaa \_\_\_\_\_ Mallattoo \_\_\_\_\_ Guyya \_\_\_\_\_

## Appendix II: Supplementary tables and figures

**Appendix Table 11:** *Cryptosporidium parvum* isolate ET28 710 bp small subunit ribosomal RNA gene, partial sequence. Gene bank accession number KX856003

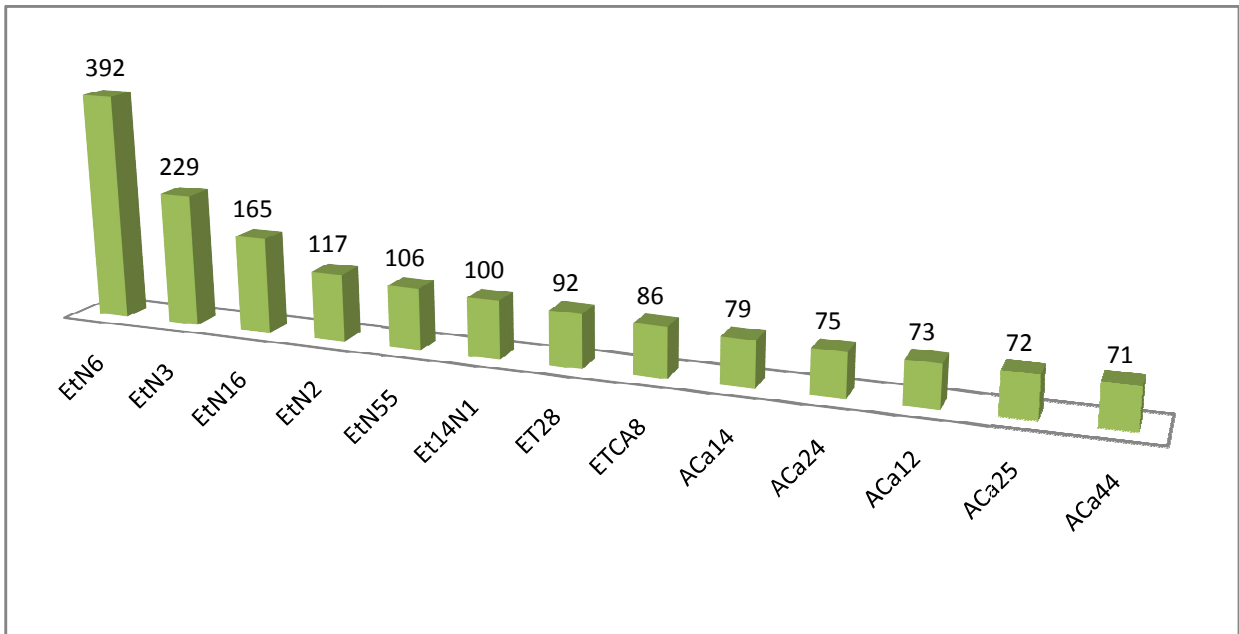
```
1 GCCTGAGAAA CGGCTACCAC ATCTAAGGAA GGCAGCAGGC GCGCAAATTA CCCAATCCTA
61 ATACAGGGAG GTAGTGACAA GAAATAACAA TACAGGACTT TTTGGTTTTG TAATTGGAAT
121 GAGTTAAGTA TAAACCCCTT TACAAGTATC AATTGGAGGG CAAGTCTGGT GCCAGCAGCC
181 GCGGTAATTC CAGCTCCAAT AGCGTATATT AAAGTTGTTG CAGTTAAAAA GCTCGTAGTT
241 GGATTTCTGT TAATAATTTA TATAAAATAT TTTGATGAAT ATTTATATAA TATTAACATA
301 ATTCATATTA CTATATATTT TAGTATATGA AATTTTACTT TGAGAAAATT AGAGTGCTTA
361 AAGCAGGCAT ATGCCTTGAA TACTCCAGCA TGAATAATA TTAAAGATTT TTATCTTCT
421 TATTGGTTCT AAGATAAGAA TAATGATTAA TAGGGACAGT TGGGGGCATT TGTATTTAAC
481 AGTCAGAGGT GAAATTCTTA GATTTGTTAA AGACAAACTA ATGCGAAAAGC ATTTGCCAAG
541 GATGTTTTCA TTAATCAAGA ACGAAAGTTA GGGGATCGAA GACGATCAGA TACCGTCGTA
601 GTCTTAACCA TAAACTATGC CAACTAGAGA TTGGAGGTTG TTCCTTACTC CTTCAGCACC
661 TTATGAGAAA TCAAAGTCTT TGGGTTCTGG GGGGAGTATG GTCGCAAGGC.
```

**Appendix Table12:** *Cryptosporidium hominis* 723 BP, Isolate ETN2 small subunit Ribosomal RNA gene, partial sequence. Gene bank accession number KX856000

1 TTACGGATCA CAATTAATGT GACATATCAT TCAAGTTTCT GACCTATCAG CTTTAGACGG  
61 TAGGGTATTG GCCTACCGTG GCAATGACGG GTAACGGGGA ATTAGGGTTC GATTCCGGAG  
121 AGGGAGCCTG AGAAACGGCT ACCACATCTA AGGAAGGCAG CAGGCGCGCA AATTACCCAA  
181 TCCTAATACA GGGAGGTAGT GACAAGAAAT AACAATACAG GACTTTTTGG TTTTGTAATT  
241 GGAATGAGTT AAGTATAAAC CCCTTACAA GTATCAATTG GAGGGCAAGT CTGGTGCCAG  
301 CAGCCGCGGT AATTCCAGCT CCAATAGCGT ATATTAAGT TGTTGCAGTT AAAAAGCTCG  
361 TAGTTGGATT TCTGTTAATA ATTTATATAA AATATTTTGA TGAATATTTA TATAATATTA  
421 ACATAATTCA TATTACTATT TTTTTTTTAA GTATATGAAA TTTTACTTTG AGAAAATTAG  
481 AGTGCTTAAA GCAGGCATAT GCCTTGAATA CTCCAGCATG GAATAATATT AAAGATTTTT  
541 ATCTTTTTTA TTGGTCTAA GATAAGAATA ATGATTAATA GGGACAGTTG GGGGCATTG  
601 TATTTAACAG TCAGAGGTGA AATTCTTAGA TTTGTAAAG ACAAACTAAT GCGAAAGCAT  
661 TTGCCAAGGA TGTTTTCAAT AATCAAGAAC GAAAGTTAGG GGATCGAAGA CGATCAGATA  
721 CCG

**Appendix Table 13:** *Cryptosporidium andersoni* 738 bp isolate ACA12 small subunit ribosomal RNA gene, partial sequence. Gene bank accession number KX264363

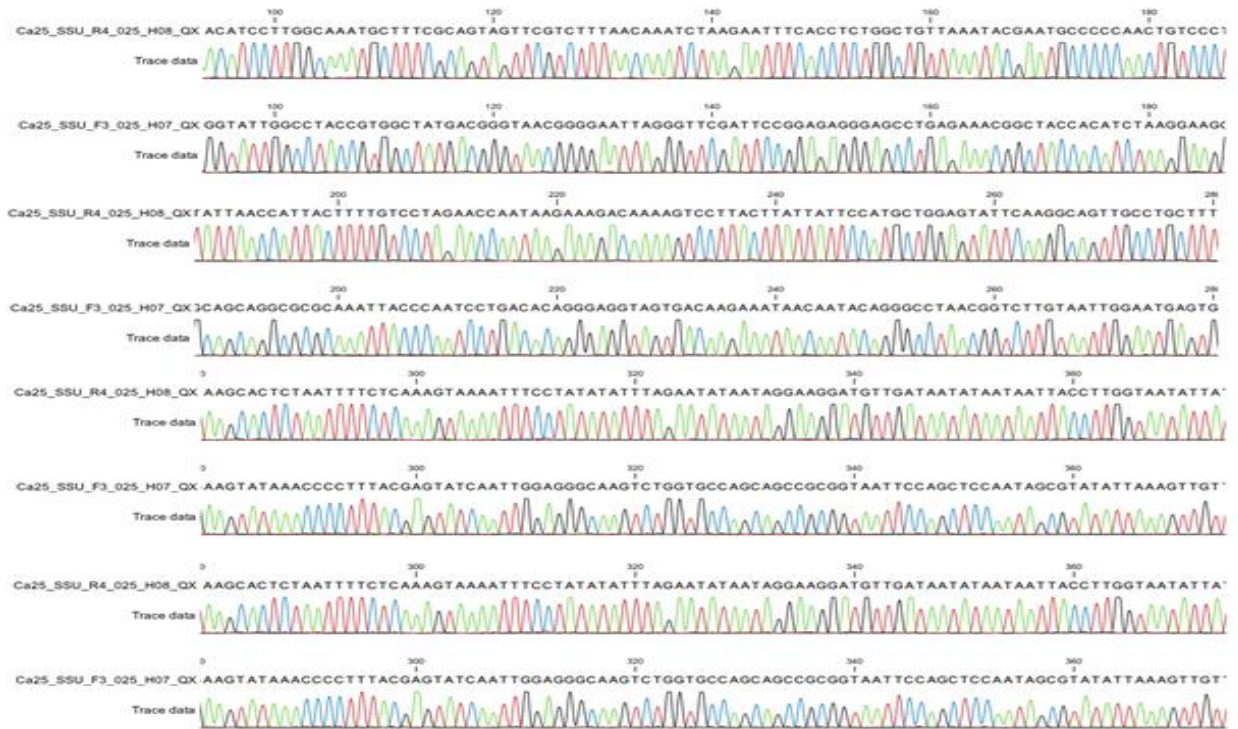
1 TAAGACTACG ACGGTATCTG ATCGTCTTCG ATCCCCTAAC TTTCGTTCTT GATTAATGAA  
61 AACATCCTTG GCAAATGCTT TCGCAGTAGT TCGTCTTTAA CAAATCTAAG AATTTACCT  
121 CTGGCTGTTA AATACGAATG CCCCCAAGT TCCCTATTAA CCATTACTTT TGCCTAGAA  
181 CCAATAAGAA AGACAAAAGT CCTTACTTAT TATTCATGC TGGAGTATTC AAGGCAGTTG  
241 CCTGCTTTAA GCACTCTAAT TTTCTCAAAG TAAAATTCC TATATATTTA GAATATAATA  
301 GGAAGGATGT TGATAATATA ATAATTACCT TGGTAATATT ATAAAATTAT ACAACAGAAA  
361 TCCAACACTAG AGCTTTTTTAA CTGCAACAAC TTTAATATAC GCTATTGGAG CTGGAATTAC  
421 CGCGGCTGCT GGCACCAGAC TTGCCCTCCA ATTGATACTC GTAAAGGGGT TTATACTTCA  
481 CTCATTCCAA TTACAAGACC GTTAGGCCCT GTATTGTTAT TTCTTGTCAC TACCTCCCTG  
541 TGTCAGGATT GGGTAATTTG CGCGCCTGCT GCCTTCCTTA GATGTGGTAG CCGTTTCTCA  
601 GGCTCCCTCT CCGGAATCGA ACCCTAATC CCCGTTACCC GTCATAGCCA CGGTAGGCCA  
661 ATACCCTACC GTCTAAAGCT GATAGGTCAG AAACCTGAAT GATATGTCGC ATCAGAGATG  
721 CGATCCGTAA AGTTATTA



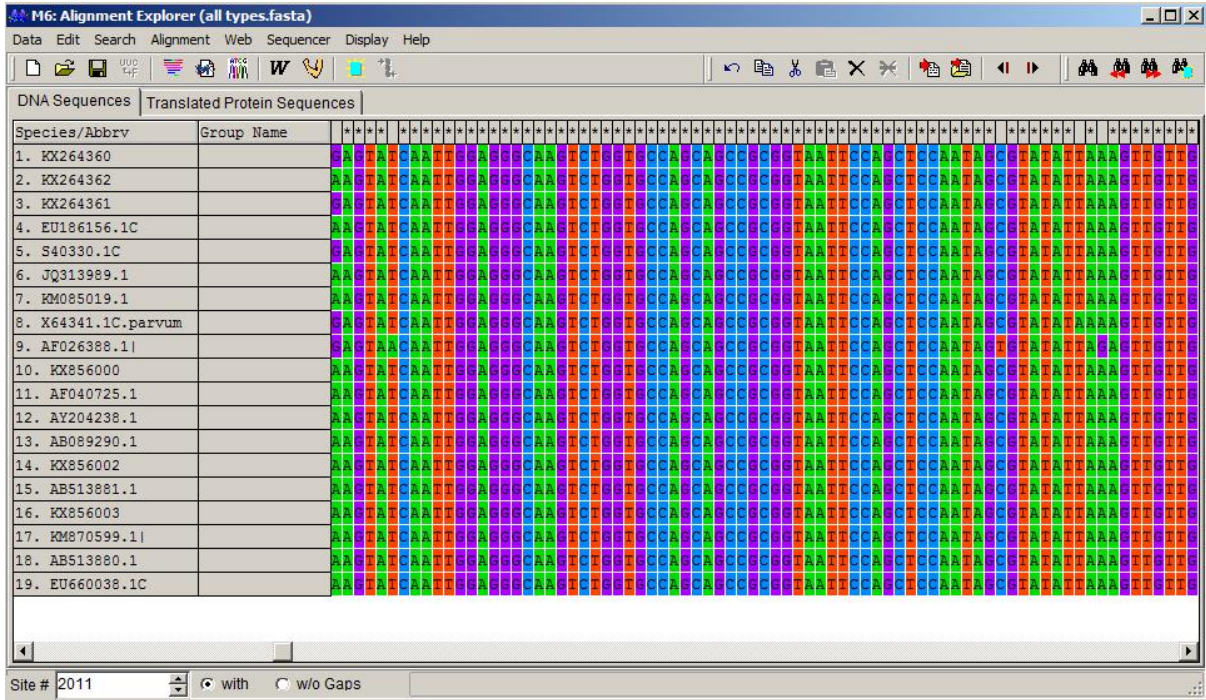
**Appendix Figure 1:** Genomic DNA concentration (ng/μl) of *Cryptosporidium* isolates in 2 μl sample from cattle and human (June 2014–Dec 2015)

### Appendix III: published papers

- 1) Manyazewal A., Francesca S., Gezahegne M. and Getachew T.(2017): *Cryptosporidium* Infection in dairy cattle calves and its Public health significance in Central Ethiopia. *J. Adv. Vet. Res.*, **7**: 59–65. ISSN: 2090-6277/2090-6269/



**Appendix Figure 2:** DNA sequence contigs trace data of *C. parvum* Nucleotides  
 A= green, C= blue, G= black, T= red



**Appendix Figure 3:** Multiple alignment of sequences retrieved from the genbank and sequences from this study

Appendix Table 14: Curriculum Vitae

**Personal information**

Name: Manyazewal Anberber Zeleke

Nationality: Ethiopian

Sex: Male

Place of birth: Gursum, East. Hararghe, Ethiopia

Date of birth: 10/6/1963

Marital status: Married

Academic qualification: - MSc in Tropical veterinary Epidemiology (MSc TVE)

Doctor of Veterinary Medicine (DVM)

Language proficiency: English, Amharic, Oromiffa, Somali

Scientific Membership: Member of the Ethiopian Veterinary Association (EVA)

## **Educational Background**

2011-2012- PhD student Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit, Ethiopia

2005-2007: Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit, Ethiopia  
Award: MSc in Tropical Veterinary Epidemiology

13/1/-17/5/2003: University of London, The Royal Veterinary College, London, UK  
Award: Certificate in Wild Animal Health, MSc course

13/3/-19/6/2000: Freie University of Berlin, Faculty of Veterinary Medicine, Berlin, Germany

Award: Certificate in Veterinary Epidemiology, MSc course

1981-1987: Addis Ababa University, Faculty of Veterinary Medicine, Debre Zeit, Ethiopia.  
Award: DVM (Doctor of Veterinary Medicine) degree

1978-1981: Grade 9-12. Harar Junior Secondary School, Hararghe, Ethiopia

1970-1977: Grade 1- 8, Gursum Elementary and junior secondary school, Hararghe, Ethiopia

## **Other trainings**

Nov.6-10/1995:-Seminar on Parasitology Research in Africa, International Foundation for Science (IFS) held in Bobo-Dioulasso, Burkina Faso

Award: Certificate of participation

August 21-26/1996: Training Course on Integrated Tsetse and Trypanosomoses control, held in southern Ethiopia, ICIPE, Nairobi, Kenya

Award: Training Certificate

Oct.2-6/2001: Training workshop on Project Planning, Monitoring and Evaluation

Held in Jimma town, Ethiopia, Ethiopian Agricultural and Research Organization

Award: Training Certificate

1/1/-30/4/2002: Training in computer courses, Tropical Business Service, Jimma, Ethiopia

Award: Diploma in Computer End user courses

Additional Skills: Driving License Grade 2

## Work Experience

Sep/2008-to present: Assistant professor, head, Department of veterinary laboratory  
Technology, Ambo University

2007-2008: Associate Researcher, Ethiopian Agricultural Research Institute

9/5/2004-2/7/2005: Head of Parasitology department, Bedelle Regional Veterinary Laboratory

17/5/95-8/5/2004: Technical coordinator and Research Officer, Bedelle Regional Veterinary  
Laboratory

1/2/94-16/5/95: Head of Protozoology department, Bedelle Regional Veterinary Laboratory

1995-2005: Coordinator of the Serosurveillance team of the PACE project, Bedelle Regional  
Veterinary Laboratory

1992-1994: Veterinary team leader of Jimma zone.

1987-1991: Veterinary Officer at various districts of Jimma zone.

## Publications

1. Manyazewal A.Z. (1987): Diseases of Camel in Keren and Key-Bahir Awraja, Eritrea province, Northern Ethiopia. DVM thesis, Addis Ababa University, Faculty of Veterinary Medicine, June 1987.
2. Manyazewal, A.Z. (2007): Assessement of the tsetse and trypanosomosis control program in chewaka settelement station, Illubabour zone, Southwest Ethiopia. MSc thesis, Addis Ababa University, Faculty of Veterinary Medicine, June 2007.
3. Anberber M., Asfaw Y., Basu A.K. (2012): Success in tsetse and trypanosomosis control in the newly established Chewaka settlement station, in southwest Ethiopia. *Journal of Natural History*, **8**: 50–64.
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6. Pal M. and Manyazewal A. (2014): Monograph on microbiology of drinking water. LAP Lambert Academic Publishing, Saarbrücken, Deutschland/Germany. ISBN-13 978-3-659-53825-4. EAN: 9783659538254. ISBN-10 3659538256
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8. Pal M., Yirgalem M., Anberber M., Giro B., Dasgupta R. (2015): Impact of environmental pollution on Animal health. *Journal of Natural history* **11**:4-21
9. Pal M., Ketema A., Anberbe M.r, Mulu S.and Dutta Y. (2016): Microbial quality of Fish and Fish products. *Beverage & Food World*, **43**: 46–49.
10. Pal M., Tafese W., Tilahun G., Anberber M. (2016): Cryptosporidiosis: An emerging food and waterborne protozoan disease of global significance. *Beverage & Food World* **43**: 43–48.
11. Olani A., Habtamu Y., Wegayehu T. and Anberber M. (2016): Prevalence of camel trypanosomosis (surra) and associated risk factors in Borena zone, southern Ethiopia. *Parasitol Res.*, **115**:1141–1147, DOI 10.1007/s00436-015-4845-9
12. Wegayehu T., Karim R., Anberber M., Adamu H., Erko B., Zhang L., Tilahun G. (2016) Prevalence and Genetic Characterization of *Cryptosporidium* Species in Dairy Calves in Central thiopia. *PLoS ONE 11(5)*: e0154647. doi:10.1371/journal.pone.0154647.
13. Pal M., Mulugeta B., Anberber M., Mulu S.,Shukla PK.(2016) Public Health significance of Pesticides in Foods of Animal origin. *Beverage & Food World* **43**: 20–23

## References

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