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COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF PARASITOLOGY AND PATHOLOGY

***CRYPTOSPORIDIUM* IN DAIRY CATTLE, SHEEP, HUMANS, AND MANURE IN
AND AROUND ADAMA AND ASELLA TOWNS, OROMIA REGIONAL STATE,
ETHIOPIA**

MSc THESIS

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BISHOFTU, ETHIOPIA

***CRYPTOSPORIDIUM* IN DAIRY CATTLE, SHEEP, HUMANS, AND MANURE IN
AND AROUND ADAMA AND ASELLA TOWNS, OROMIA REGIONAL STATE,
ETHIOPIA**

A thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa
University in partial fulfillment of the requirements for the degree of Master of Science in
Veterinary Parasitology

By

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**June, 2022
Bishoftu, Ethiopia**

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DEDICATION

This thesis manuscript is dedicated to Meseret, for her love, moral and spiritual support. To my father and mother for their dedication in the success of my life. Finally, I dedicate this thesis to the Almighty God, praising him for his direction, strength, mental power, protection, and providing me with a healthy existence.

STATEMENT OF AUTHOR

First, I declare that this thesis is my *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 an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare 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 ABBREVIATIONS

HIV	Human Immune deficiency Virus
MZN	Modified Zeihl Neelsen
PA	Peasant Association
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
µm	Micrometer
ml	Millimeter

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	I
LIST OF ABBREVIATIONS.....	II
TABLE OF CONTENTS.....	III
LIST OF TABLES.....	V
LIST OF FIGURES.....	VI
LIST OF APPENDICES.....	VII
ABSTRACT.....	VIII
1. INTRODUCTION.....	1
2. LITERATURE REVIEW.....	4
2.1 Etiology and Taxonomic classification.....	4
2.2. Epidemiology.....	4
2.2.1. <i>Geographical Distribution</i>	4
2.2.2. <i>Life cycle of Cryptosporidium</i>	5
2.2.3. <i>Host range</i>	6
2.2.4. <i>Source of infection and transmission</i>	7
2.2.5. <i>Manure as source of Cryptosporidium infection</i>	7
2.2.6. <i>Risk factors</i>	8
2.2.7. <i>Zoonotic Importance</i>	8
2.3. Economic loss.....	9
2.4. Clinical sign and pathological conditions.....	9
2.5. Diagnostic methods.....	10
2.6. Treatments.....	11
2.7. Control and prevention measures.....	11
2.8. Challenges and Future action to overcome <i>Cryptosporidium</i> infection in ruminants..	12
2.9. <i>Cryptosporidium</i> infection in Ethiopia.....	13
3. MATERIALS AND METHODS.....	15
3.1. Study areas.....	15
3.2. Study population.....	16
3.3. Study design and sample size determination.....	17
3.3.1. <i>Sample size determination for dairy cattle and sheep</i>	17
3.3.2. <i>Sample size determination for humans and manure samples</i>	17

TABLE OF CONTENTS (CONT'D)

3.4. Sampling method	17
3.5. Sample collection, handling and transportation.....	18
3.6. Laboratory examination	19
3.6.1. <i>Sheather's sugar floatation method</i>	19
3.6.1. <i>Modified Ziehl Neelsen staining method</i>	19
3.7. Data management and analysis	20
4. RESULTS.....	21
4.1. Prevalence of <i>Cryptosporidium</i> infection in dairy cattle, sheep, human, and manure storage.....	21
4.2. Risk factors for the occurrence of cryptosporidium infection in dairy cattle, sheep, humans, and manure.....	22
4.3. Intensity of <i>Cryptosporidium</i> oocyst in dairy cattle, sheep and humans.....	27
4.4. Age related intensity of <i>Cryptosporidium</i> oocyst in dairy cattle and sheep	28
4.5. Manure handling practices in dairy farm owners and farmer households	30
5. DISCUSSION	31
5.1. <i>Cryptosporidium</i> infection in Dairy cattle	31
5.2. <i>Cryptosporidium</i> infection in Sheep.....	32
5.3. <i>Cryptosporidium</i> infection in human	34
5.4. <i>Cryptosporidium</i> in manure.....	34
5.5. Intensity of <i>Cryptosporidium</i> oocyst	35
6. CONCLUSION AND RECOMMENDATIONS	37
7. REFERENCES	39
8. APPENDICES	50

LIST OF TABLES

Table 1. Molecularly identified <i>Cryptosporidium</i> species in Ethiopia.....	13
Table 2. Prevalence of <i>Cryptosporidium</i> infection in ruminants in Ethiopia.....	14
Table 3. Overall prevalence of <i>Cryptosporidium</i> in dairy cattle, sheep, human, and manure samples	21
Table 4. Simple logistic regression of host and management related risk factors for prevalence of <i>Cryptosporidium</i> infections in dairy cattle.....	23
Table 5. Simple logistic regression of host and environment related risk factors for prevalence of <i>Cryptosporidium</i> infections in sheep	24
Table 6. Simple logistic regression for occurrence of <i>Cryptosporidium</i> oocysts in manure ..	25
Table 7. Simple logistic regression of human-related risk factors for prevalence of <i>Cryptosporidium</i> infections	26
Table 8. Final multiple logistic regression model of potential risk factors for the occurrence of <i>Cryptosporidium</i> infection in dairy cattle, sheep, humans, and manure	27
Table 9. Manure handling practices in dairy farms and farmers house holds.....	30

LIST OF FIGURES

Figure 1. Life cycle and transmission of zoonotic species of <i>Cryptosporidium</i>	5
Figure 2. Species of <i>Cryptosporidium</i> in Animals	6
Figure 3. Map showing study areas	16
Figure 4. Images of oocysts of <i>Cryptosporidium</i> by different techniques	22
Figure 5. Intensity of <i>Cryptosporidium</i> oocysts per-field of 100x magnification from positive samples of dairy cattle, sheep, and humans.....	28
Figure 6. Age related intensity of <i>Cryptosporidium</i> oocyst in dairy cattle and sheep	29

LIST OF APPENDICES

Appendix 1. Sample collection format sheet.....	50
Appendix 2. Sugar floatation technique	50
Appendix 3. Modified Ziehl-Neelsen staining method	51
Appendix 4. Semi-structured questionnaire to gather information on manure handling practices	53
Appendix 5. Body condition scoring of dairy cattle and sheep.....	54
Appendix 6. Criteria used to categorize the hygiene of the dairy farms	54

ABSTRACT

In Ethiopia, several studies have reported the occurrence of *Cryptosporidium* infection in young calves, lambs, and HIV positive patients. However, studies on cattle and sheep of all age categories, and humans (farm workers and animal owners) were limited. A cross-sectional study design was thus conducted from October 2021 to April 2022 to study the occurrence of *Cryptosporidium* in dairy cattle, sheep, humans, and manure in and around Adama and Asella towns, Oromia, Ethiopia. Dairy cattle and sheep were stratified according to their age and randomly sampled. Stool samples from volunteered farm workers and household members were collected purposefully. A semi-structured questionnaire was also used to collect information about manure handling practices. All samples (dairy cattle = 234, sheep = 200, humans = 56, manure = 58) were processed with sugar floatation technique and the modified Ziehl-Neelsen staining method. A univariate regression analysis was used to test the association of individual factors with the occurrence of *Cryptosporidium*. Associated factors were subjected to multivariate regression analysis to identify potential risk factors. The overall prevalence of *Cryptosporidium* in dairy cattle, sheep, humans, and manure was 20.5%, 14%, 16%, and 13.20%, respectively. The occurrence of *Cryptosporidium* infection in dairy cattle at the ages of 1–12 months and 13–36 months was 3.48 and 3.07 times higher, respectively, in comparison to the age above 36 months. Similarly, the occurrence was 2.69 times higher in sheep aged 1–6 months than in those aged older than 6 months. And also, a higher average level of oocyst count (above 10 oocysts) per-field was examined in dairy cattle (3/48) aged 1–12 months, followed by sheep (1/28) aged 1–6 months. Furthermore, the likelihood of infection was 13 times greater in farm workers compared with the rural farmer members. In addition, the occurrence of oocysts was 22.8 times higher in manures from dairy cattle than manure from sheep. From data on manure handling practices, only 16.8% of them had manure storage holes, 98.1% used manure for crop production, and all of them used manure without treatment. The current study revealed the occurrence of *Cryptosporidium* infection in all age groups of dairy cattle and sheep, farm workers, animal owners, and manures. Therefore, more studies on adult animals and manure is required to investigate their contribution to environmental contamination as well as their role as a source of infection in young calves and lambs. In addition, molecular characterization is also required to identify the zoonotic species of *Cryptosporidium*.

Keywords: Adama, Asella, Dairy cattle, *Cryptosporidium*, Human, Manure

1. INTRODUCTION

Cryptosporidium is a unicellular protozoan parasite that causes cryptosporidiosis in a variety of animals, including humans. Tyzzer, (1910) discovered the first parasites in laboratory mice in 1907. However, the clinical significance of bovine Cryptosporidiosis was not recognized in the veterinary and medical fields for 70 years, until the parasites were discovered in an 8-month-old calf with chronic diarrhea in 1971 (Pancier *et al.*, 1971). Later, in Australia, the infection was discovered in lambs with diarrhea (Barker & Carbonell, 1974). In the early 1980s, investigations on natural and experimental infections established the protozoan's function as a key etiological agent in bovine diarrhea (Tzipori *et al.*, 1980).

According to a recent study, more than 44 *Cryptosporidium* species have been identified (Ryan *et al.*, 2021). From these, *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium andersoni*, and *Cryptosporidium ryanae* are the major species infecting cattle. Sheep are infected with three main *Cryptosporidium* species; *Cryptosporidium parvum*, *Cryptosporidium ubiquitum*, and *Cryptosporidium xiaoi*, and humans are most commonly infected with *Cryptosporidium parvum* (the most zoonotic species), and *Cryptosporidium hominis* (Ryan *et al.*, 2014). Other *Cryptosporidium* species are also reported from different animals, and each of which has its own prevalence, geographic range, and public health significance (Baroudi *et al.*, 2018).

The infection is transmitted to animals and humans orally through the ingestion of sporulated oocysts. The sexual and asexual life cycles are completed in the same host (monoxenous) and have a unique location within the host cell, situated between the cytoplasm and the cell membrane (Goebel, 1982; Tzipori & Ward, 2002). Even though *Cryptosporidium* infection causes clinical disease in neonatal calves and lambs, there is evidence that adult cattle reduce milk production during shedding of *Cryptosporidium andersoni* (Shaw *et al.*, 2021). The economic losses associated with this disease are not only due to mortality but also to production loss, retarded growth, and the cost of drugs (De Graaf *et al.*, 1999).

In particular, neonatal calves and lambs are vulnerable to *Cryptosporidium* infection and shed billions of oocysts, which results in huge environmental contamination and a risk of infection to other animals and humans (Kerie, 2019). Meanwhile, asymptomatic post-weaned and adult cattle also excrete oocysts into the environment (Pam *et al.*, 2013). According to Scott *et al.* (1994), a single adult bovine might possibly excrete more than 36 million oocysts every day. Other researchers indicated that the sub-clinically infected ewes are a source of infection for lambs, especially during the peri-parturient period (Ye *et al.*, 2013). Without adequate control, this contamination represents a human health hazard because animals infected with *Cryptosporidium* could be excreting up to 10^7 oocysts per gram of feces (Bordes *et al.*, 2020).

Oocysts are resistant to environmental conditions and survive for a months in environments and animal manures in cool and wet conditions. Infected animal manure also serves as a significant reservoir for *Cryptosporidium* infection (Lasprilla-Mantilla *et al.*, 2019). Other studies indicates that contaminated manures from dairy or beef cow operations can be substantial sources of *Cryptosporidium* oocysts for humans and animals, unless manure management or treatment measures are implemented to decrease oocyst viability or transmission to water (Kuczynska and Shelton, 1999). Surface transfer from land-applied manures or leaching through the soil to groundwater are two additional mechanisms of conveyance to potable or recreational water, in addition to direct fecal deposition. Runoff from polluted soil might act as a vehicle for *Cryptosporidium* oocysts to enter water sources. As a result, farms that maintain cattle might be a source of *Cryptosporidium* infection for animals and humans (Ogendo *et al.*, 2017).

In Ethiopia, several studies have been conducted on *Cryptosporidium* infection both in animals and humans. The prevalence ranges from 10.8 to 27.8% (Regassa *et al.*, 2013; Mullusew *et al.*, 2020) and 2.1 to 22.2% (Regassa *et al.*, 2013; Wegayehu *et al.*, 2017) in calves and lambs, respectively. On the other hand, Hailu *et al.* (2021) reported the highest prevalence (46%) of *Cryptosporidium* infection in humans having contact with animals. The majority of these reports were from young calves, lambs, and HIV positive patients, and with few reports from adult cattle and sheep (Ayana *et al.*, 2009; Wegayehu *et al.*, 2013; Mullusew *et al.*, 2020). The occurrence of *Cryptosporidium* infection in adult cattle has been not well investigated. In addition, the occurrence of infection in animal manure was not yet documented.

Investigation of *Cryptosporidium* infection in the adult age group of healthy cattle and sheep is important because they act as reservoirs of *Cryptosporidium* species and sources of infection for their calves and lambs, respectively (Díaz *et al.*, 2021). In addition, infected animal manure serves as a significant reservoir for *Cryptosporidium* oocyst. When pathogen-laden manure is sprayed as fertilizer on fields, there is a risk of environmental contamination and increase animal exposure. Intense rainfall results in surface water runoff, which can transport oocysts from farms into surrounding watersheds, resulting in high pathogen loads and more potential for human infection (Brankston *et al.*, 2018).

Therefore, data regarding the occurrence of *Cryptosporidium* infection in different age groups of dairy cattle and sheep, humans, and manure samples are important to understand the source of infection, risk factors, and transmission route in dairy cattle, sheep, and humans. It is also crucial to design and establish effective measures to prevent and control the infection in farm animals and humans. The general objective of this study was thus designed to study the occurrence of *Cryptosporidium* in Dairy cattle, Sheep, humans, and manure in and around Adama and Assela Towns, Oromia, Ethiopia.

The specific objectives to be dealt in this research are:

- ✓ To estimate the prevalence of *Cryptosporidium* in dairy cattle, sheep, humans, and manure.
- ✓ To identify the potential risk factors of *Cryptosporidium* infection in the study subjects
- ✓ To determine the intensity of *Cryptosporidium* oocysts in fecal samples, stool samples and manure samples
- ✓ To assess manure handling practices in dairy farms and farmers house holds

2. LITERATURE REVIEW

2.1. Etiology and Taxonomic classification

The *Cryptosporidium* infection is caused by the unicellular protozoan parasite called *Cryptosporidium*. For a long period of time, the parasite has been classified together with other enteric coccidian parasites in the order Eucoccidiidae, suborder Eimeriina, and family Cryptosporididae on the basis of many similarities in their morphological characteristics and life cycles (Xiao *et al.*, 2004). However, recent taxonomic studies have placed *Cryptosporidium* as separate a clade from Coccidia. They also explain why *Cryptosporidium* has several characteristics that distinguish it from other coccidians, including host infection confined to the apical region of epithelial cells, small oocyst size, the formation of both thick- and thin-walled oocysts, and resistance to anti-coccidian agents (Hijjawi *et al.*, 2002).

By recent molecular and biological studies *Cryptosporidium* is closely related to gregarines due to some similarities; the absence of a sporocyst, the presence of a feeding organelle, and its epi-cellular localization (Carreno *et al.*, 1999; Hijjawi *et al.*, 2002). This classification is not without doubt as transcriptomic analysis of apicomplexan parasite indicate *Cryptosporidium* have different lineage from gregarines and have the earliest diverging lineage from apicomplexan parasites (Ryan *et al.*, 2021). Therefore, *Cryptosporidium* may have its own separate lineage is likely to reveal new avenues of investigation into pathogenesis, epidemiology, treatment and control of these ubiquitous pathogens.

2.2. Epidemiology

2.2.1. Geographical Distribution

Different *Cryptosporidium* species infect wide ranges of animals, including humans, and have a global distribution. The prevalence of infection differs from region to region and country to country. This difference in prevalence might be due to study design, sampling nature, sample size, breed of animal, geographical location, climate, and management practices (Thomson *et al.*, 2017; Santin, 2020). Several factors have contributed to the epidemiology of *Cryptosporidium*; the ability of oocysts to survive in warm and moist conditions for a long period of time, high production of oocysts, and the ability to cause infection by a small number

of oocysts (Ulutas, 2017).

2.2.2. Life cycle of *Cryptosporidium*

Both types of life cycle (sexual and asexual) occur in *Cryptosporidium* and all stages of development take place in one host (Figure 1). The cycle starts when the infective oocyst (thick-walled) is ingested by another animal. The infective oocyst contains four sporozoites and is excysted after entering the gut by the action of temperature, pH, parasite proteases, and bile salts (Fayer and Leek, 1984). The four naked sporozoites invade the epithelial cells and start the asexual development (Merogony). The individual trophozoites undergo successive generations of merogony (type I and type II) and yield eight and four merozoites, respectively (Saul and Ward, 2002).

The sexual reproduction starts from type two meronts which develop to merozoites and transforms into male (micro) and female (macro) gamonts. Finally, a zygote is produced from the union of male and female gamonts followed by two asexual reproduction to produce thin and thick walled oocyst (Goebel, 1982). The oocyst (thick walled) passed to the environment to be ingested by another host for the continuation of the life cycle. The thin-walled oocyst separated from the epithelium and excysted to cause autoinfection (Current & Reese, 1986).

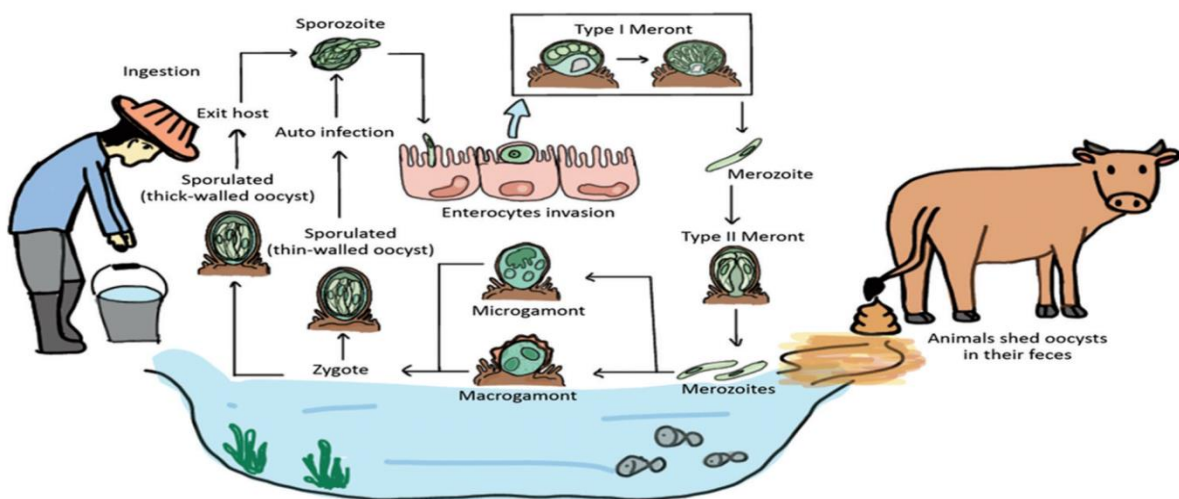


Figure 1. Life cycle and transmission of zoonotic species of *Cryptosporidium* (Source: Pumipuntu and Piratae, 2018)

2.2.3. Host range

A recent studies indicate over 44 species and several genotypes have been recognized (Pane & Putignani, 2022). From a total 25 species of *Cryptosporidium* identified in animals, *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium andersoni*, and *Cryptosporidium ryanae* are frequently infect cattle (Dankwa *et al.*, 2021). Also, *Cryptosporidium felis*, *Cryptosporidium hominis*, *Cryptosporidium scrofarum*, *Cryptosporidium serpentis*, and *Cryptosporidium suis* are reported as sporadic infection in (Robertson *et al.*, 2014). On the other hand, *Cryptosporidium parvum*, *Cryptosporidium ubiquitum*, and *Cryptosporidium xiaoi* are the main species occurred in sheep and goats. (Baroudi *et al.*, 2018). Out of 19 species reported in humans, *Cryptosporidium parvum*, *Cryptosporidium meleagridis*, *Cryptosporidium hominis*, and *Cryptosporidium felis* are the most frequent occurrences. Other *Cryptosporidium* species are also identified in wild and domestic animals, including fish, birds, amphibians, and reptiles, as indicated in Figure 2.

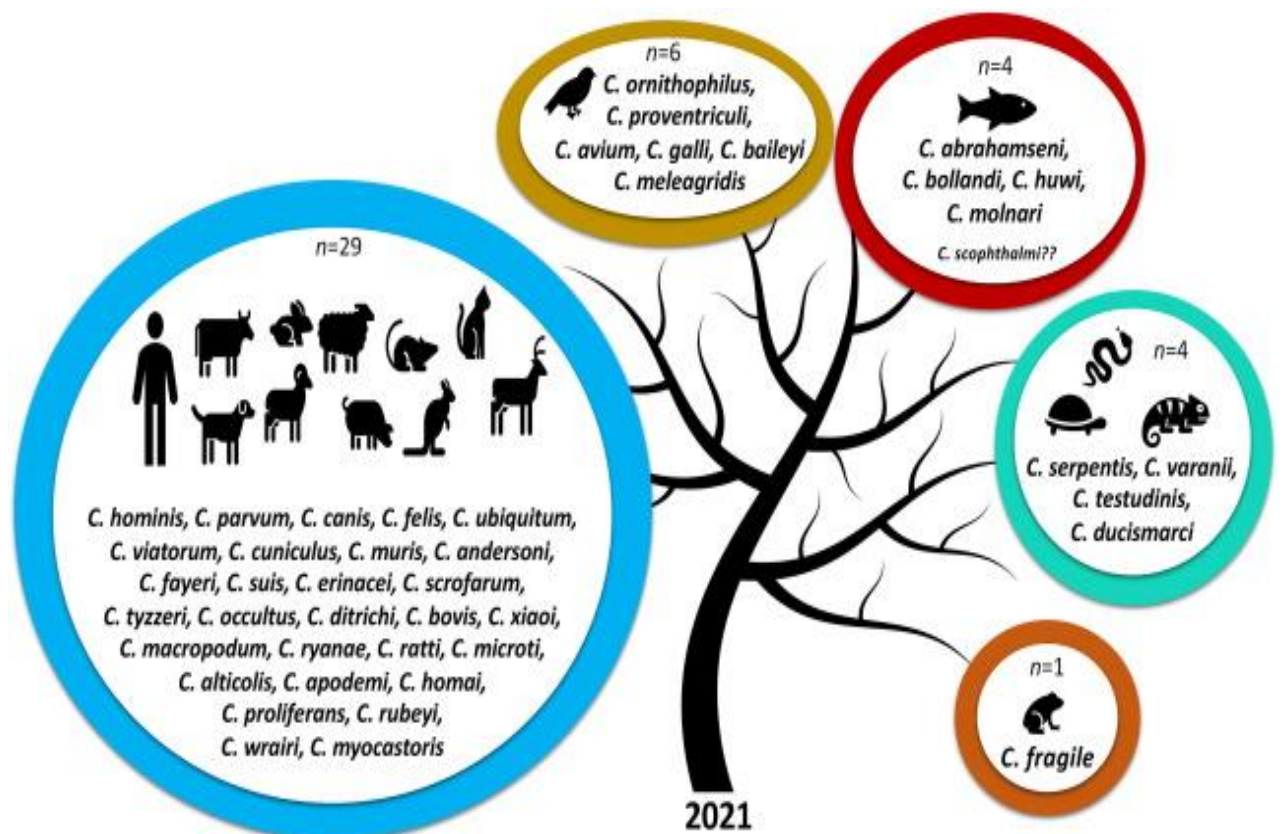


Figure 2. Species of *Cryptosporidium* in Animals (Source: Ryan *et al.*, 2021)

2.2.4. Source of infection and transmission

The sporulated oocyst released from infected adult and new born animals contaminate the environment and act as a source for *Cryptosporidium* infection. For instance, infected young calves excrete about 1×10^6 to 2×10^6 oocyst/gram of feces to the environment. This high level of oocyst shedding from young animals cause for environmental contamination, feed and water supply (De Graaf *et al.*, 1999). Although, ruminants such as cattle, sheep and goats are the main reservoir for zoonotic *Cryptosporidium* species (*Cryptosporidium parvum*) (Robertson *et al.*, 2020).

The infection caused by *Cryptosporidium* transmitted directly from ingesting sporulated oocyst and indirectly through contaminated feed and water (Paraud and Chartier, 2012). Oocysts are environmentally resistant to some disinfectants and can survive for long periods (> 6 months) in cool, moist environments, and on fomites such as farm gates, buildings and utensils (Bushkin *et al.*, 2013). This characteristic plays a role in transmission of *Cryptosporidium* infection to animals and humans.

2.2.5. Manure as source of *Cryptosporidium* infection

In an intensive dairy farm production system, a large amount of manure is produced. The *Cryptosporidium* oocyst shed from infected animals survives in manures, and cause environmental contamination (Lasprilla-Mantilla *et al.*, 2019). Furthermore, infective oocysts are viable for a long period of time in animal manure. The runoff manure to water sources increases the risk of infection for animals and humans (Bordes *et al.*, 2020).

Using spatially explicit process-based modeling, a recent study estimated worldwide *Cryptosporidium* loads in livestock manure to be in the range of 3.2×10^{23} oocysts per year, with cattle being the most common source. When manure is applied to land, oocysts can be carried into surface waterways through run-off, posing a risk of infection to both animals and people. The oocyst load from cattle dung is largest in Asia, followed by Africa, South America, and Europe (Vermeulen *et al.*, 2017).

Effective manure and slurry management on farms to minimize *Cryptosporidium* oocyst viability will have an influence on disease risk in the wider environment, particularly for water

catchments. On-farm measures include adequate manure composting (>60°C), slurry storage (ammonia and low pH assist inactivate oocysts), and treatment with mesophilic and thermophilic anaerobic digestion to greatly reduce oocyst viability. The use of vegetation and riparian buffer strips, as well as fencing of cattle away from streams and water courses, can assist to reduce the entry of *Cryptosporidium* oocysts from livestock faecal matter into water courses (Innes *et al.*, 2020).

2.2.6. Risk factors

Different factors, including hygiene status, age of animals, management systems, source of feed and water, type of bedding, colostrum feeding, diarrheic conditions, climate and geographic conditions are a risk for *Cryptosporidium* infection in animals (Abraham *et al.*, 2018). The age-related transmission, occurrence, and clinical disease of *Cryptosporidium* infection are commonly reported in ruminants (Santin, 2020). In addition, intensive management systems and overcrowding of animals in farms accelerate the contamination and transmission of infection between calves from contaminated udders and floors (De Graaf *et al.*, 1999).

Host immunity level and concurrent infection encourage the transmission of infection in young animals. Clinical disease mostly occurs in young animals (immature immune systems) and immune compromised individuals rather than adult and immune-competent individuals. During infection, specific antibodies are produced by the host, which both humoral and cell-mediated antibodies are important for protection as well as the local immune system in young animals (Anderson, 1998).

2.2.7. Zoonotic Importance

Animals (cattle, sheep, and goats) act as reservoirs for zoonotic *Cryptosporidium* species, *Cryptosporidium parvum*, although other species have been reported in humans. According to Ng *et al.* (2012), having close contact with cattle increases the risk of infection and is identified as a risk factor. In addition, Zambrano *et al.* (2014) reported about high risk of infection from infective oocyst excreted by diarrheic calves. The high level of oocyst excretion and the nature of oocysts (thick-walled) that resist environmental hazards increase the viability of oocysts in the environment and contaminated objects, which favor the transmission between animals and

humans. Sporulated oocysts are released from animals, meaning they cause infection immediately and do not require sporulation in the environment. So, humans get the sporulated oocyst directly from infected people or animals (Casemore *et al.*, 1985), or indirectly from feed and water contaminated with the oocyst (Tzipori *et al.*, 1980). The number of cases and outbreaks of *Cryptosporidium* infection in humans has occurred throughout the world, mainly caused by infected people and contaminated water (Hassan *et al.*, 2021).

The occurrence of *Cryptosporidium* in humans has a worldwide distribution, with a higher prevalence in developing countries where the health system is in poor condition. Poor sanitary conditions, concurrent infection (virus infection, cancer), and age of the individual determine the occurrence in humans. Young children and elderly people are more likely to be infected than adult people (Chalmers *et al.*, 2011).

2.3. Economic loss

Even though *Cryptosporidium* infection causes diarrhea, weight loss, and mortality in young animals, its growth effect on farm animals was not clearly identified. It also increases drug costs, production losses, and labor costs in the livestock production (De Graaf *et al.*, 1999). According to Shaw *et al.* (2020), the infection has a long-term effect on young calves by lowering their weight gain and decreasing their production performance. As a result, farmers lose their income in the farm animals due to high costs for treatment, lower carcass weight and additional costs for feed and labor (Zhu *et al.*, 2021).

2.4. Clinical sign and pathological conditions

Diarrhea is a typical clinical sign observed in infected animals. The severity of diarrhea and related clinical signs varies according to species, immunity level, age of animals, and concurrent infection. For example, in lambs and kids, it is mild to moderate and persists for several days regardless of treatment; it also causes depression, dehydration, anorexia, and tenesmus (Tzipori *et al.*, 1981). However, the disease is self-limiting in adult animals and young with competent immunity. The recovered infected host becomes a reservoir for infection and acts as a source of infection for other animals (Ulutas, 2017). In the adults, the infection becomes chronic and is characterized by progressive loss in body weight, but most of the infected animals remain asymptomatic (Paul *et al.*, 2014).

Inflammatory changes due to interaction of *Cryptosporidium* sporozoites and merozoites with host cells result in signaling cascades with molecules (such as proteases and hemolysins) that damage cells and cause pathological conditions. In addition, local inflammatory responses such as prostaglandins and several cytokines, particularly interferon- γ , become activated (Gookin *et al.*, 2002). The reaction between the immune system and parasite brings an adverse effect on cellular activity (increases permeability, loss of fluid and electrolytes) (Gerace *et al.*, 2019). Some pathological changes can occur in the affected animal's intestine, such as villus atrophy, villus degeneration, crypt hyperplasia, inflammatory infiltrations, and microvillus degeneration (Gookin *et al.*, 2002).

2.5. Diagnostic methods

The most widely used diagnostic method of *Cryptosporidium* infection in ruminants is the fecal floatation technique with Sheather's sucrose solution and modified Ziehl–Neelsen (MZN) staining method. In the staining method, the oocyst stained appears red to pink in color, round to oval in shape, with distinct internal structures measuring 3-6 μm (Casemore *et al.*, 1984). The method is time consuming and requires well-trained and experienced people to interpret the results. However, it is cheaper than advanced serological and molecular methods (Shanmathi *et al.*, 2020).

Clinical diagnostic laboratories use other advanced methods, including direct or indirect immunofluorescence microscopy and enzyme linked immunosorbent assay (Marks *et al.*, 2004). However, it is not as simple to use them in developing countries because of the expensive nature of the kits and a lack of experienced personnel (Khurana and Chaudhary, 2018). The most advanced molecular method (PCR) is used for species identification. PCR is rapid and accurate, and the results are also easy to interpret (Thomson *et al.*, 2016). However, it cannot be used for routine diagnosis of *Cryptosporidium* oocysts due to its technical complexity and interference of results by inhibitors (like bilirubin, bile salt, and complex polysaccharides) that reduce its sensitivity. However, these inhibitors can be eliminated by *Cryptosporidium* oocyst purification from fecal samples, which involves density gradient concentration of fecal samples (Shanmathi *et al.*, 2020).

2.6. Treatments

Despite the fact that *Cryptosporidium* infection is important for both veterinary and public health, treatment options for both animals and humans are limited. Only one drug (Halofuginone lactate) is approved to be used in calves and lambs in some country. The drug does not cure disease in animals, but it does reduce oocyst excretion and shortens diarrhea duration (Giadinis *et al.*, 2007). Other drugs are in experimental stages reported by different authors with a finding of a reduction in clinical symptoms and shedding of oocysts in experimental animals. For example, antibiotics (Paromomycin), coccidiostats (Decoquinate and Nita-zoxanide), and novel bumped kinase inhibitors are among drugs tested for treating *Cryptosporidium* infection in animals, showing positive results on decreasing clinical symptoms and shedding of oocysts (Thomson *et al.*, 2017).

Alternative therapies such as passive immune therapy using hyper immune, serum, and bovine colostrum containing antibodies against *Cryptosporidium parvum* have been tested with promising but inconclusive results (Naciri *et al.*, 1994). Several colostrum preparations have been used to treat infections, with the best results being obtained with hyper-immune bovine colostrum harvested from dairy cows vaccinated during gestation with *Cryptosporidium parvum* oocyst/sporozoite antigens. Treatment with hyper-immune colostrum containing high levels of antibodies against *Cryptosporidium* has been associated with the symptomatic improvement or resolution of infection (Ghazy *et al.*, 2016).

2.7. Control and prevention measures

Understanding the risk factors for *Cryptosporidium* infection in livestock will allow for the logical development of preventive hygiene measures. Prevention of *Cryptosporidium* infection transmissions is important because the infective dose of oocyst is low, making it difficult to control. Sanitation and manure management can help to reduce the level of exposure to oocysts and transmission among animals and from the environment to the host (Shanmathi *et al.*, 2020).

In the livestock industry, appropriate management systems (cleaning of floors, removing of infected oocysts by chemicals and heat, reducing stock density, separation of diseased animals from healthy animals) are important to minimize the occurrence of infection in farm animals (Kerie., 2019). Proper application of chemical disinfectants (aldehyde, ammonia alcohol,

chlorine or alkaline compounds) is used to remove resistant *Cryptosporidium* oocysts from animal houses and utensils. In addition, steam heat sterilization and fumigation with formaldehyde or ammonia gas have also been recommended as appropriate forms of decontamination (Ghazy *et al.*, 2016).

2.8. Challenges and Future action to overcome *Cryptosporidium* infection in ruminants

Different factors make it difficult to control *Cryptosporidium* infection in animals and humans. The parasite has unique characteristics; it is located intracellularly but extra-cytoplasmic, has a low infective dose, a resistant oocyst, and a high level of oocyst production, making it difficult to control the infection in animals and humans (Thomson *et al.*, 2017). In addition, a lack of simple in vitro cultivation and small animal models limits the development of effective chemotherapeutic agents (Siddiki *et al.*, 2009).

Several chemotherapeutic agents have been tested for the treatment of *Cryptosporidium* infection in livestock, but they resulted only in a reduction of shedding oocysts and clinical symptoms. However, the recent animal-based in vitro culture method of *Cryptosporidium parvum* for replication of the life cycle and the Clustered Regularly Interspaced Short Palindromic Repeats associated protein based gene editing tool facilitate vaccine development (Hijjawi *et al.*, 2002). According to a recent study, the parasite develops and completes its life cycle in stem-cell-derived epithelial organoids (Florin-Christensen *et al.*, 2021). Thus, genome editing and the established animal model will allow functional studies of parasite proteins and antigens to assess their suitability for vaccine candidates. The availability of such in vitro cultivation systems will allow testing parasite neutralization by antisera raised against vaccine candidates before their use in vaccination trials (Regina *et al.*, 2021).

Systemic exposure of drugs cannot treat *Cryptosporidium* infection because of its location in the epithelium of the gastrointestinal brush border. For further research into the parasites, advanced technologies such as genetic engineering, cell culture modeling, and bio-computational methods are required. It will be critical to model this for both humans and cattle in order to develop vaccines and effective treatments (Putignani, 2021). In addition to therapeutic and vaccine development, the application of one health approach is crucial as the infection circulates between humans and animals. Collaboration of all stakeholders is required to control this global disease with important mitigation strategies (Innes *et al.*, 2020).

2.9. *Cryptosporidium* infection in Ethiopia

Cryptosporidium infection is common and has been identified as a major cause of calf, lamb, and kid enteritis in a number of places across the world, including Ethiopia. From the findings of several authors, the prevalence of *Cryptosporidium* infection in both animals and humans shows variation. The variation might be due to climatic conditions (determining oocyst viability), management systems (intensive, semi-intensive, extensive), hygiene conditions, contact with animals (human), co-infection, age (determining immunity status) (Tarekegn *et al.*, 2021).

In Ethiopia, the prevalence of *Cryptosporidium* infection ranges from 10.8 to 27.8% in calves, 2.1 to 22.2% in lambs, and 4 to 22.2 in goat kids (Table 2). In addition, the highest (46%) and lowest (1.1%) prevalence of infection reported from humans by Hailu *et al.* (2021) and Flecha *et al.* (2015), respectively. Molecularly, five, three and seven species of *Cryptosporidium* species have been identified from cattle, sheep/goat and humans, respectively (Table 1).

Table 1. Molecularly identified *Cryptosporidium* species in Ethiopia. (Source: Wegayehu *et al.*, 2017; Tarekegn *et al.*, 2021; Kifleyohanes *et al.*, 2022)

Cattle	Sheep and goat	Human
<i>Cryptosporidium andersoni</i>	<i>Cryptosporidium ubiquitum</i>	<i>Cryptosporidium parvum</i>
<i>Cryptosporidium parvum</i>	<i>Cryptosporidium ryanae</i>	<i>Cryptosporidium hominis</i>
<i>Cryptosporidium bovis</i>	<i>Cryptosporidium xioai</i>	<i>Cryptosporidium canis</i>
<i>Cryptosporidium ryanae</i>		<i>Cryptosporidium xioai</i>
<i>Cryptosporidium. ubiquitum</i>		<i>Cryptosporidium viatorum</i>
		<i>Cryptosporidium felis</i>
		<i>Cryptosporidium melagridis</i>

Table 2. Prevalence of *Cryptosporidium* infection in ruminants and humans in Ethiopia

Study Area	Study animals	Sample size	Prevalence (%)	Reference
Central Ethiopia	Calves	580	17.6	(Abebe <i>et al.</i> , 2008)
North West Ethiopia	Calves	360	18.6	(Ayele <i>et al.</i> , 2018)
South Eastern Ethiopia	Calves	384	24.0	(Birhanu <i>et al.</i> , 2017)
Bishoftu	Calves, lamb, goat kids	214,89,61	13.6,16.9, 11.5	(Ayana and Alemu, 2015)
Central Ethiopia	Calves	270	14.8	(Zelege <i>et al.</i> , 2017b)
Addis Ababa and its Environs	Calves	392	18.6	(Manyazewal <i>et al.</i> , 2018)
North Showa	Cattle	384	7.8	(Wegayehu <i>et al.</i> , 2013)
Haramaya	Calves, lamb, goat kids	133, 63, 41	27.8, 22.2, 12.2	(Regassa <i>et al.</i> , 2013)
Central Ethiopia	Calves	449	15.8	(Wegayehu <i>et al.</i> , 2016)
West Shoa	Calves	378	10.8	(Mullusew <i>et al.</i> , 2020)
Southern Ethiopia	Calves	330	13.0	(Hailu <i>et al.</i> , 2020)
East Arsi (Ziway)	Lamb and kids	384	15.4	(Ali <i>et al.</i> , 2019)
Northern Ethiopia	Calves, lambs, goat kids	208, 270, 248	10, 9, 4	(Kifleyohanes <i>et al.</i> , 2022)
East Wollega	Calves	384	13.8	(Ebiyo and Haile, 2022)
North West Ethiopia	Calves	193	15.5	(Tekle, 2021)
Gonder	Calves	384	21.4	(Birhan <i>et al.</i> , 2019)
Central Ethiopia	Lambs	389	2.1	(Wegayehu <i>et al.</i> , 2017)
Southern Ethiopia	Human	92	1.1	(Flecha <i>et al.</i> , 2015)
North Showa	Children	384	7.3	(Wegayehu <i>et al.</i> , 2013)
Across Ethiopia	Human	1038	7.6	(Adamu <i>et al.</i> , 2010)
North west Ethiopia	children	384	8.1	(Tigabu <i>et al.</i> , 2010)
Eastern Ethiopia	children	1259	11.5	(Ayelaw, 2006)
Across Ethiopia	Human	187	46	(Hailu <i>et al.</i> , 2021)

3. MATERIALS AND METHODS

3.1. Study areas

The study was conducted in and around Adama and Asella towns. Adama Town, capital of East shawa zone, is found in rift valley between 8° 33' to 8°36'N latitude and 39° 11' 57" to 39° 21' 15"E longitude. The town is located 99 Km south-east of Addis Ababa and with average altitude of 1712 meters above sea level. Adama receives an annual range of rainfall approximately from 600-1150 mm and temperatures range between 12 and 33°C. The livestock populations of the area are estimated to be 103,440 cattle, 45,554 sheep, 54,112 goats, and 87,341 poultry (Gebregziabhear, 2018).

The second study area, Asella town, is the capital of the Arsi zone, located 175 km to the south-east of Addis Ababa. The town is located between altitudes of 1780 and 3100 m above sea level, with a longitude and latitude of 6° 59 to 8° 49 N and 38° 41 to 40° 44 E, respectively. The average annual rainfall in the area is 1200 mm, with minimum and maximum temperatures of 5 °C and 28 °C, respectively. According to Alemu (2019), the population of livestock in the area was estimated at 82,190 cattle, 52,292 sheep, 11,479 goats and 162,015 poultry.

A mixed farming system of crop-livestock production is practiced around both study areas. From small to large, communal and private dairy farms are found in and around the towns and are managed in an intensive to semi-intensive type of production system. In addition, extensively reared local sheep breeds by household farmers are found in and around the towns.

Study Areas

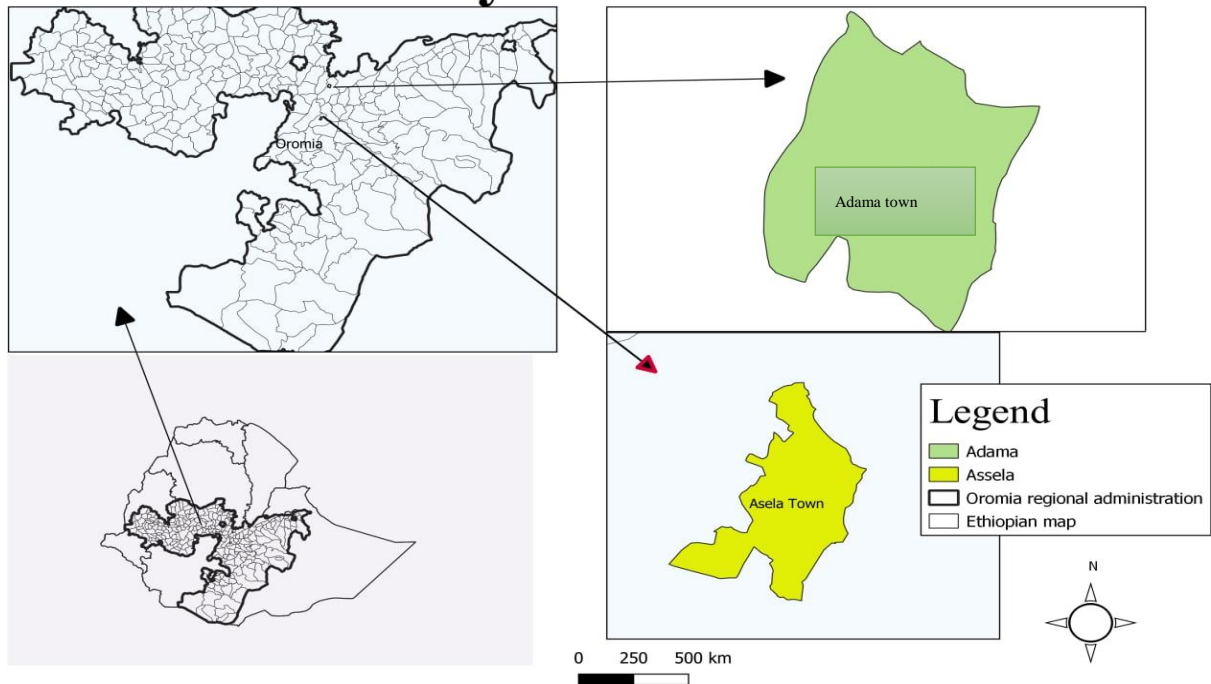


Figure 3. Map showing study areas

3.2. Study population

Dairy cattle and sheep from selected dairy farms and household farmers, respectively, were study population. Dairy cattle of all age groups and sexes were selected for sampling. The dairy cattle on the selected farm are managed under intensive to semi-intensive management systems. The Holstein breed is the most breed owned by farmers, followed by crosses and the local Borena. Similarly, local sheep breeds of all age groups and sex from farmers located in and around Adama and Asella towns were recruited for the study. Criteria were developed to categorize the hygiene of dairy farms (Appendix 6). The body condition of dairy cattle and sheep were categorized according to Klopčič *et al.* (2011) and (Meyer, 1994), respectively (Appendix 5). The age of dairy cattle can be determined by looking at their ear tags; sheep, conventionally by asking their owner.

In addition to cattle and sheep, people working on dairy farms and rural farmers were included to investigate the occurrence of *Cryptosporidium* infection. All individuals were selected based on their consent to participate in the study. Manure samples were also collected from all selected dairy farms and rural farmers' houses.

3.3. Study design and sample size determination

A cross sectional study design was conducted from October 2021 to April 2022 to study the occurrence of *Cryptosporidium* infection in dairy cattle, sheep, human, and manure in study area and to identify the potential risk factor for the infection.

3.3.1. Sample size determination for dairy cattle and sheep

The required sample size for the study was determined using the formula given by (Thrusfield, 1986). From previous study, the prevalence of *Cryptosporidium* infection in dairy calve and lamb are 18.6% (Manyazewal *et al.*, 2018) and 15.4 (Ali, 2019), respectively. The prevalence 18.6% (cattle) and 15.4% (sheep) from previous study was used as expected prevalence at 95% confidence interval and 5% absolute precision.

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

Where, N= Sample size, P_{exp}= Expected prevalence, d = Desired absolute precision at 95% confidence interval.

Accordingly, 234 dairy cattle and 200 sheep were required for the study.

3.3.2. Sample size determination for humans and manure samples

A manure sample was collected from dairy farms and rural farmer's that owned manure in their compound. A total of 68 sites were identified (six dairy farms and 62 rural farmer households) and used for sampling. Similarly, 56 people (farm workers and household members) were gave their consent to participate on the study.

3.4. Sampling method

Dairy cattle used in this study were from purposefully selected dairy farms found in the study areas, and sheep from farmers volunteer to participate in the study. From a total of 14 dairy farms from Adama and Asella Town agricultural bureau, six dairy farms (three from each study areas) were purposefully selected based on their animal population. Eight peasant association (PA) (four from each study site) in and around Adama and Asella towns were selected for

sampling sheep. From the selected PA, households willing to participate in the study were included to sample their sheep until the required sample size was attained. Before sampling, age stratification was made for both cattle and sheep. Accordingly, dairy cattle stratified into three age groups; 1-12 months, 13 – 36 months and above 36 months, and sheep into two age groups (1-6 months) and (above 6 months). Simple random sampling method was used to sampling dairy cattle and sheep from all age groups. Important information about the age of animals, body condition, sex, species, breed, agroecology, fecal consistency, and their management systems were recorded from face-to-face interviews with farm owners and animal attendants. Purposive sampling method was applied for sampling people working in dairy farm and farmer house hold members owned flock of sheep. Before sampling, the people were informed about the objective of the study and asked for their consent. Additionally, manure sample was collected from all dairy farms included in this study and manure from rural farmer's compound.

3.5. Sample collection, handling and transportation

About 10 gram of fresh fecal samples were collected from rectum of dairy cattle and sheep using disposable gloves. Similarly, about 10 gram of fresh stool sample was collected from each consented people. In addition, 20 gram of manure sample were collected from each dairy farms and household that owned manure storage in their compounds. Manure samples collected from thoroughly mixed manures storages of each study site. The fecal and manure samples were placed in a sterile, airtight plastic bottle. After labelling, the sample was transported in ice box to parasitology laboratory of Addis Ababa University, college of veterinary medicine and agriculture. The samples that were not examined on the same day were stored in refrigerator at +4 °C.

During sample collection, important information about age of animals, body condition, breed, sex, species of animals, consistency of feces (normal, soft, diarrheic), management system (Intensive and semi-intensive), and hygiene status of the farm were recorded on sample record sheet. In addition, history of contact with animal feces and manure, place of manure disposal, use of manure, frequency of manure disposal, and age of people participating in the study were also recorded on semi-structured data collection sheet.

3.6. Laboratory examination

In the laboratory, the samples were processed by floatation technique using Sheather sugar solution and Modified Ziehl–Neelsen staining methods.

3.6.1. Sheather's sugar floatation method

The collected samples were examined by Sheather's sugar solution of 1.27 specific gravity; to detect *Cryptosporidium* oocyst microscopically as described by Trotz-Williams *et al.* (2005). About 3 grams of feces was diluted in 42 ml of sugar solution and passes through sieve gauze to remove the solid particles. The concentrated solution was poured in 15ml test tube, covered with cover slip on the tube and was kept at standing for 20 minutes. Then, the cover slip was lifted carefully and placed on microscope slide for oocyst observation under 40x and 100x magnification. A similar method was used for stool and manure samples. Microscopically, *Cryptosporidium* oocysts are spherical or slightly oval in shape, colorless, thick-shelled, and have four elongated sporozoites.

3.6.1. Modified Ziehl-Neelsen staining method

Thin smear was prepared from fecal, stool, and manure samples following the procedure Casemore, (1991). For manure smear preparation, the dry sample (manure) were mixed well using a drop of water to create a uniform suspension. Once the sample was well mixed, it was distributed on a microscope slide using plastic stick. Two thin smear was prepared for each of manure samples to increase the detection of *Cryptosporidium* oocysts. The prepared smear was air dried and fixed with concentrated methanol for three minutes. After staining by carbolfuschin for twenty minutes, the smears were washed in running water for 1-2 minutes. It was then decolorized for 30 seconds in 1% hydrochloric acid in ethanol and counterstained for one minute in 3% methylene blue. Finally, the slides were examined under a microscope at 100x objective lenses. The oocysts were identified according to standard methods, which appear as pink-stained, round to oval structures of about 3 to 6 μm in diameter, containing distinct internal structures.

The intensity of *Cryptosporidium* oocysts was determined semi-quantitatively MZN stained smear by counting the average number of oocysts in 10 randomly selected fields (oocysts per field) at 100x magnification. The intensity of oocyst was graded as: low (1–5 oocyst), medium (6–10 oocyst), and high (above 10 oocysts) (Dessi *et al.*, 2020).

3.7. Data management and analysis

All data collected from study sites and laboratory result were entered and stored in a Microsoft excel spread sheet program and analyzed by R statistical software (R version 4.2.0, R Statistical Computing). The prevalence of the *Cryptosporidium* was calculated separately for dairy cattle, sheep, manure and humans. Then, the association of individual factor with the occurrence of *Cryptosporidium* infection were determined by univariate regression analysis and variable at $p \leq 0.1$ were selected for further multiple logistic regression to identify the potential risk factors (Graham, 2003). Multicollinearity test or independency of explanatory variables was checked by variance inflation factor and values less than 10 are selected for model construction. For multiple logistic regression, best predictor variables were selected using stepwise tool depending on Akaike information criterion. Hosmer-Lemeshow Goodness of Fit test and the receiver operating characteristic curve were used to test the best fitted model. Results was considered statistically significant when the P value is ≤ 0.05 at 95% confidence interval.

3.8. Ethical considerations

The study protocol was reviewed and approved by the research ethics review committee of Addis Ababa University, college of veterinary medicine and agriculture (Ref. No: VM/ERC/12/02/14/2022). The objective and procedure of the study were explained to farm owners and farmers for their voluntary use of their animals. The Oromia health bureau provided official permission regarding human-related studies (Ref. No: BFO/HBTFH/2-/6/11093). Before sample collection, informed consent was given for all individuals participating in the study.

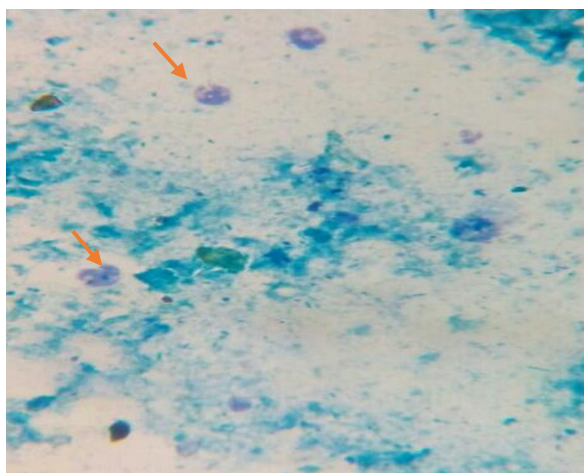
4. RESULTS

4.1. Prevalence of *Cryptosporidium* in dairy cattle, sheep, human, and manure storage

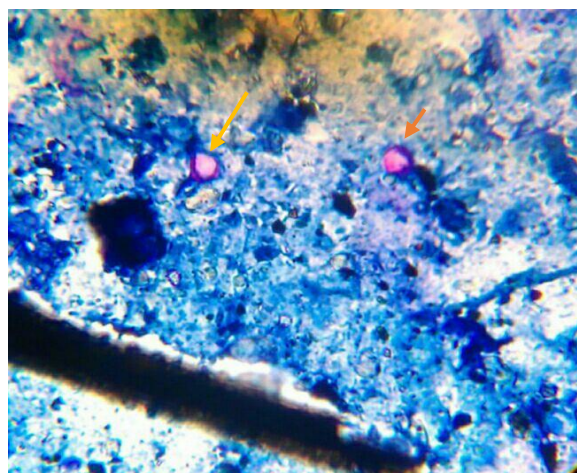
From total of 234 microscopically examined samples, the prevalence of *Cryptosporidium* infection in dairy cattle was 20.5% (48/234). The prevalence in sheep, humans and manure was 14% (28/200), 16% (9/56), and 13.2% (9/68), respectively (Table 3).

Table 3. Overall prevalence of *Cryptosporidium* infection in dairy cattle, sheep, human, and manure samples

Study subject	Study site	Animal examined	Number of positive	Percentage (%)
Dairy cattle	Adama	132	25	18.9
	Asella	102	23	22.5
	Total	234	48	20.5
Sheep	Adama	92	16	17.3
	Asella	108	12	11.1
	Total	200	28	14
Human	Adama	25	5	20
	Asella	31	4	13
	Total	56	9	16
Manure	Adama	33	3	9
	Asella	35	6	17.1
	Total	68	9	13.2



Cryptosporidium oocyst under Modified Ziehl-Neelsen staining method (100x): Cattle



Cryptosporidium oocyst under Modified Ziehl-Neelsen staining method (100x): Sheep



Cryptosporidium oocyst under sheather sugar solution (100x): Cattle

Figure 4. Images of oocysts of *Cryptosporidium* by different techniques

4.2. Risk factors for the occurrence of *Cryptosporidium* infection in dairy cattle, sheep, humans, and manure

Dairy cattle: In simple logistic regression, eight independent variables/predictors were used to determine the association with the occurrence of *Cryptosporidium* infection in dairy cattle (Table 4).

Table 4. Simple logistic regression of host and management related risk factors for prevalence of *Cryptosporidium* infections in dairy cattle

Variables	Category	Animals examined	Number of positive (%)	Odds ratio	95% CI	p-Value
Age (month)	1-12	91	26 (28.6)	3.48	1.61 – 8.05	0.002
	13-36	46	12 (26)	3.07	1.22 – 7.93	0.018
	Above 36	97	10 (10.3)	Ref		
Sex	Female	182	40 (22.0)	0.65	0.26 – 1.42	0.302
	Male	52	8 (15.4)	Ref		
Body condition	Medium	132	30 (22.7)	1.66	0.84 – 3.42	0.156
	Poor	9	4 (44.4)	4.51	1.01 – 19.2	0.039
	Good	93	14 (15)	Ref		
Breed	Pure	154	29 (18.8)	1.06	0.44 – 2.83	0.899
	Local	41	12 (29)	1.89	0.67 – 5.70	0.238
	Cross	39	7 (17.9)	Ref		
Farm hygiene	Medium	20	9 (45)	3.67	1.39 – 9.48	0.007
	Good	214	39 (18.2)	Ref		
Agro-ecology	Midland	132	25 (18.9)	0.80	0.42 – 1.52	0.498
	High land	102	23 (22.5)	Ref		
Management system	Semi-intensive	82	14 (17.1)	0.71	0.35 – 1.40	0.340
	Intensive	152	34 (22.4)	Ref		
Fecal consistency	Normal	146	30 (20.5)	2.07	0.36 – 39.14	0.501
	Soft	79	17 (21.5)	2.19	0.36 – 42.14	0.473
	Diarrhea	9	1 (1.1)	Ref		
Total observation		234	48(20.5)			

Three risk factors (age, body condition, and farm hygiene) were significantly associated ($p \leq 0.05$) with *Cryptosporidium* infection in dairy cattle. Age below 12 months, between 13 and 36 months, and poor body condition showed effect on the occurrence of *Cryptosporidium* infection in dairy cattle. The occurrence of infection was 3.48 times higher in calves of 1-12 months (OR= 3.48, 95%CI= 1.61 – 8.05, $p = 0.002$) than in age above 36 months. Also, it was

3.07 times higher in age of 13-36 months (OR = 3.07, 95%CI = 1.22–7.93, p = 0.018) than in (above 36 months). Similarly, the occurrence of *Cryptosporidium* infection in poor cattle was 4.51 times higher than in cattle with a good body condition score. Furthermore, cattle in medium hygienic conditions have 3.67 times more likely to be infected than cattle in good hygienic conditions. Other factors (sex, breed, management system, agro ecology and fecal consistency) could not affect the occurrence of cryptosporidium infection in this study.

Sheep: From five independent variables/predictors included in simple logistic regression, age has significantly associated with likelihood of *Cryptosporidium* infection (Table 5). Accordingly, sheep of age 1- 6 months (OR = 2.69, 95%CI = 1.19 – 6.38, p = 0.020) were 2.69 times more likely exposed to *Cryptosporidium* infection than sheep above 6 months. In this study, sex, body condition, agro-ecology and fecal consistency were not significantly associated with the occurrence of infection.

Table 5. Simple logistic regression of host and environment related risk factors for prevalence of *Cryptosporidium* infections in sheep

Variables	Category	Animals Examined	Number of positive (%)	Odd ratio	95% CI	p-Value
Age (months)	1- 6 months	87	18 (20.7)	2.69	1.19 – 6.38	0.020
	> 6 months	113	10 (8.8)	Ref		
Sex	Male	94	11 (11.7)	0.69	0.30 – 1.55	0.380
	Female	106	17 (16.0)	Ref		
Body condition	Medium	115	15 (13.0)	1.07	0.46 – 2.55	0.871
	Poor	2	2 (100)	2.56	0.50 – 10.47	0.211
	Good	83	11 (13.3)	Ref		
Agro-ecology	Midland	92	16 (17.4)	1.68	0.76 – 3.85	0.205
	High land	108	12 (11.1)	Ref		
Fecal consistency	Normal	181	22 (12.2)	0.14	0.01 – 3.58	0.167
	Soft	17	5 (29.4)	0.42	0.01 – 11.94	0.562
	Diarrhea	2	1 (50.0)	Ref		
Total observation		200	28(14)			

Manure: In simple logistic regression, manure type was significantly associated with the occurrence of *Cryptosporidium* oocyst (Table 6). The occurrence of *Cryptosporidium* oocyst was 22.80 times higher in manure from dairy cattle (OR = 22.80, 95%CI = 3.60 – 198.92, p = 0.001) than in manure from sheep. Agro-ecology (P = 0.35) was not included in the multiple regression model.

Table 6. Simple logistic regression for occurrence of *Cryptosporidium* oocysts in manure

Variables	Category	Manure examined	Number of positive (%)	Odd ratio	95% CI	p-Value
Manure types	Cattle manure	6	4 (66.67)	22.80	3.60 – 198.92	0.001
	Sheep manure	62	5 (8.06)	Reference		
Agro-ecology	Highland	35	6 (17.1)	2.07	0.50 – 10.54	0.335
	Midland	33	3 (9.1)	Reference		
Total observation		68	9 (13.2)			

Human: In simple logistic regression, occupation (p = 0.046) was significantly associated with the occurrence of *Cryptosporidium* infection (Table 7). The likelihood of *Cryptosporidium* infection in individual working in dairy farm (OR = 13.14, 95%CI = 1.12 – 306.22, p = 0.046) was 13.14 times higher when compared to rural farmers.

Table 7. Simple logistic regression of human-related risk factors for prevalence of *Cryptosporidium* infections

Variables	Category	Number of Examined	Number of positive (%)	Odds ratio	95% CI	p-Value
Age	Young	21	2(9)	0.42	0.06 – 1.97	0.312
	Adult	35	7(20)	Ref		
Occupation	Farm worker	3	2(67)	13.1	1.12 – 306.22	0.046
	Rural farmer	53	7(13)	Ref		
Sex	Male	46	8(17.4)	1.89	0.29 – 37.45	0.569
	Female	10	1(10)	Ref		
Total observation		56	9(16)			

Multiple logistic regression analysis was used to identify the potential risk factors for occurrence of *Cryptosporidium* infection in dairy cattle, sheep, human, and manure samples (Table 8). For dairy cattle, age, farm hygiene, and body condition of animals were entered to into multivariate regression. Variance inflation factor was calculated for independent variable and no collinearity was found between age, farm hygiene, and body condition. In sheep, only age of animal was indicated as potential risk factor for *Cryptosporidium* infection in multivariate regression. Occupation and manure types had significantly associated with occurrence of *Cryptosporidium* infection in humans and manure samples, respectively.

Table 8. Final multiple logistic regression model of potential risk factors for the occurrence of *Cryptosporidium* in dairy cattle, sheep, humans, and manure

Study subject	Variables	Category	Animal examined	Number of positive (%)	Odd Ratio	95% CI	p-Value
Dairy cattle	Farm hygiene	Medium	20	9 (45)	3.67	1.39 – 9.5	0.007
		Good	214	39 (18.2)	Ref		
	Age (month)	1-12 months	91	26 (28.6)	2.60	1.13 – 6.27	0.027
		13-36 months	46	12 (26)	2.93	1.15 – 7.61	0.024
	>36 months	97	10 (10.3)	Ref			
	Observations		234	48 (20.5)			
Sheep	Age (month)	1- 6 months	87	18 (20.7)	2.69	1.19 – 6.38	0.020
		> 6 months	113	10 (8.8)	Ref		
	Observations		200	28 (14)			
Human	Occupation	Farm worker	3	2 (67)	13.14	1.12 – 306.2	0.046
		Rural farmer	53	7 (13)	Ref		
	Observations		56	9(16)			
Manure	Manure types	Cattle manure	6	4 (66.67)	22.8	3.60 – 198.9	0.001
		Sheep manure	62	5 (8.06)	Ref		
	Observations		68	9 (13.2)			

4.3. Intensity of *Cryptosporidium* oocyst in dairy cattle, sheep and humans

For all positive results, the average of oocyst count per field was determined by selecting 10 fields of 100x magnification randomly (Figure 5). In the dairy cattle sample, 32 showed an average of (1–5 oocysts), 13 showed an average of (6–10 oocysts), and 3 showed an average of (> 10 oocysts) from 48 positive results. Similarly, 28 positive samples were examined for intensity in sheep, showing an average of (1–5 oocysts) in 18 samples, 9 (6–10 oocysts), and 1 (> 10 oocysts) per-field. Furthermore, nine positive samples were examined for intensity in human samples and the average of an oocyst recorded was 8 (1–5 oocysts), and one (6–10

oocysts). All of nine manure samples showing an average of (1–5 oocysts). The highest average oocyst count was recorded in samples from dairy cattle.

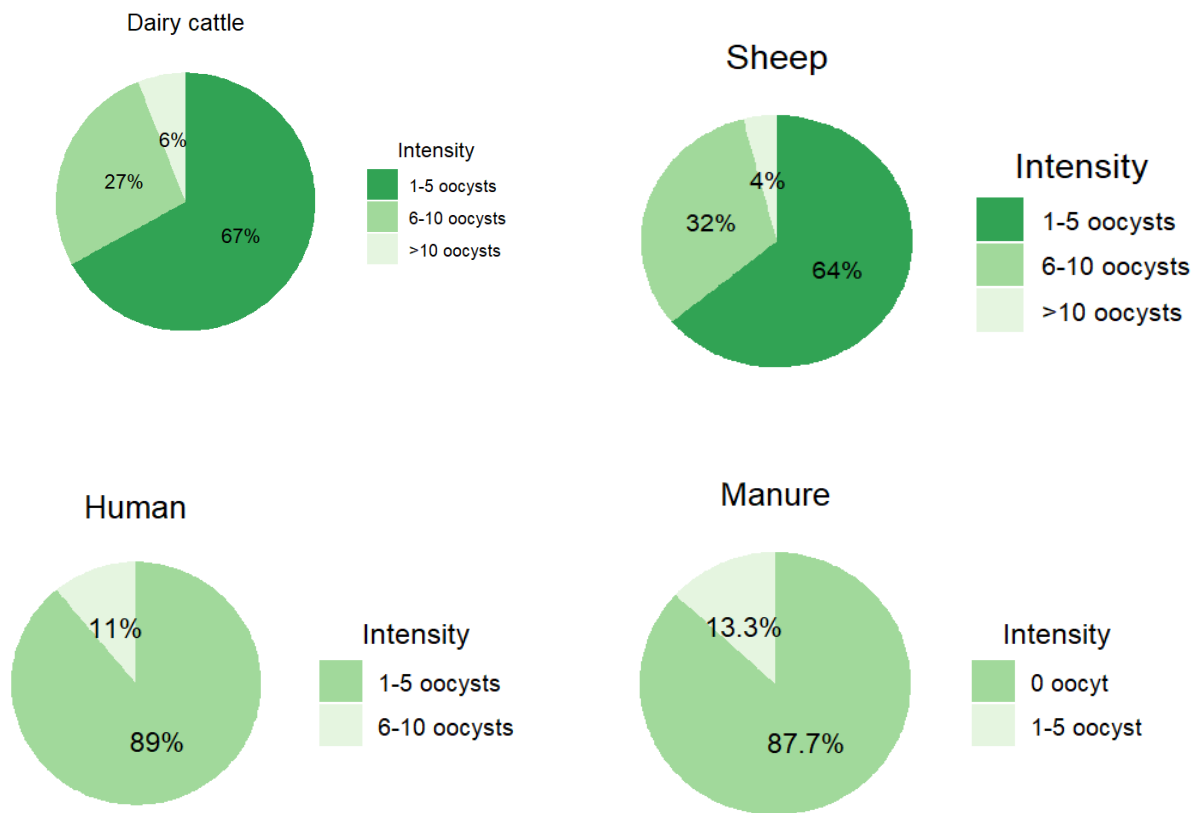
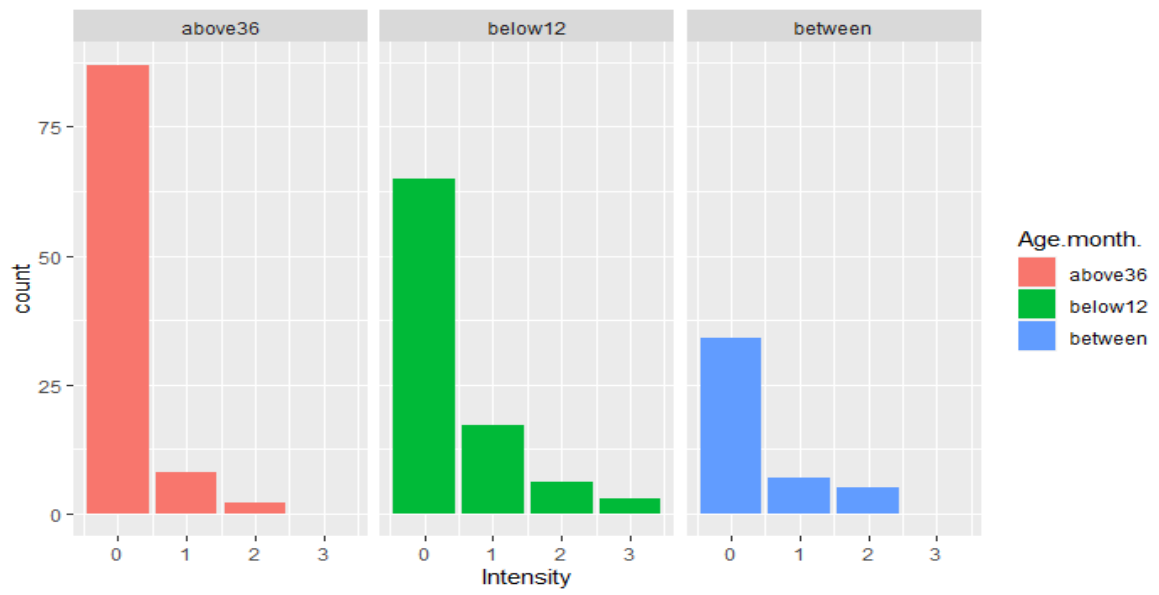


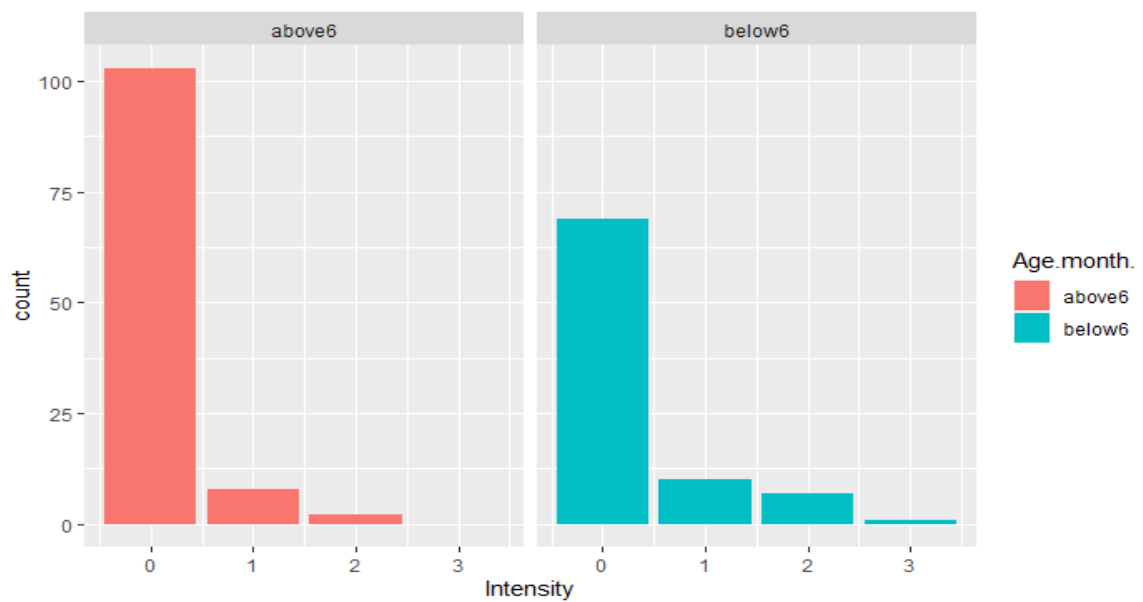
Figure 5. Intensity of *Cryptosporidium* oocysts per-field of 100x magnification from positive samples of dairy cattle, sheep, and humans.

4.4. Age related intensity of *Cryptosporidium* oocyst in dairy cattle and sheep

A higher average level of oocyst intensity (> 10 oocysts) per field was examined in dairy cattle aged 1–12 months (Figure 6). The moderate (6-10 oocysts) and lower average of oocyst counts (1-5 oocysts) per field were examined at ages of 12–36 months and above 36 months, respectively. Similarly, higher average oocyst counts (>10 oocysts) per field were examined in sheep at an age of 1-6 months, followed by moderate intensity (6-10 oocysts) at an age of over six months.



Dairy cattle



Dairy cattle

Sheep

Intensity: 1=(1-5 oocysts), 2= (5-10 oocysts), 3= (>10 oocysts)

Figure 6. Age related intensity of *Cryptosporidium* oocyst in dairy cattle and sheep

4.5. Manure handling practices in dairy farm owners and farmer households

In manure handling practice, frequency of removal, place of manure removal and uses of manure was assessed during sample collection from dairy farm and farmer house hold (Table 9). Only (16.1%) of interviewers dispose the manure daily from their animal house. And, 5% of them had storage hole, and 8% dispose the manures in grazing land. 98.5% of them used manure for crop production, and all of them did not apply any chemicals for manure treatment.

Table 9. Manure handling practices in dairy farms and farmers house holds

Variables	Category	Number of respondents	Percentage (%)
Frequency of manure disposal	Monthly	21	30.8
	Weekly	36	52.9
	Daily	11	16.1
Place of manure disposal	Grazing land	6	8
	Manure storage hole	4	5
	Crop land	58	85.2
	Biogas tank	1	1.4
Use of manure	Crop production	67	98.5
	Energy production	1	1.4
Application of manure treatment	Yes	0	0
	No	68	100

5. DISCUSSION

5.1. *Cryptosporidium* infection in Dairy cattle

The current study found the overall prevalence of *Cryptosporidium* infection in dairy cattle was 20.5%. These findings relatively agree with the results of other studies in cattle, with a prevalence of 23.7% from Ghana (Dankwa *et al.*, 2021), 22.3% from Nigeria (Adamu *et al.*, 2015), and 26.5% from Iraq (Saeed *et al.*, 2019). However, the current result (23.7%) is higher than the report of Wegayehu *et al.* (2013), who reported a 4.7 percent prevalence in dairy cattle reared under an extensive management system. Also, Geurden *et al.* (2006) reported a higher prevalence (42.8%) in intensive-managed cattle than in extensively managed cattle. The difference in prevalence might be due to the management systems in which the extensively reared cattle remain in large outdoor areas, reducing contact and oocyst contamination. In addition, cattle in extensive management systems have lower exposure to infection where oocysts are dispersed on a large surface and are exposed to direct sunlight, which reduces the oocysts' viability, resulting in a reduced infection pressure (Lefay *et al.*, 2000).

Considering the age category of cattle, a higher prevalence (28.6%) was reported in the (1-12 months) age group. This result was in line with the prevalence of 27.8% in Haramaya (Alemayew *et al.*, 2013), 24% in South Eastern Ethiopia (Birhanu *et al.*, 2017), 28.1% in Nigeria (Ayinmode and Fagbemi, 2010), and 25.7% in Brazil (Conceição *et al.*, 2021). However, it is higher than the findings from different parts of Ethiopia: 13.8% from East Wollega, 10% from Tigray, 13% from Southern Ethiopia; 13.6% from Bishoftu; and 10.8% from West Showa (see Table 3). The prevalence (26%) in the (13–36) age groups is comparable with the findings from Ghana (Dankwa *et al.*, 2021) and Iraq (Saeed *et al.*, 2019), with a prevalence of 27.3% and 21.88%, respectively.

In multiple logistic regression analysis, age was significantly associated with the occurrence of *Cryptosporidium* infection. The finding was supported by Ayana and Alemu (2015); Ebiyo and Haile (2022); Venu *et al.* (2013); and Santin *et al.* (2004), which explained the importance of age in the occurrence of *Cryptosporidium* infection. In current findings, age groups (1–12 months) were significant association ($p = 0.027$) and more likely to be exposed to infection (OR = 2.60) than those above 36 months of age. This is due to the fact that the underdeveloped immune system of calves results in their being less resistant to infection and highly susceptible

to *Cryptosporidium* infection (Díaz *et al.*, 2021). Furthermore, cattle aged 12–36 months were significant association with *Cryptosporidium* infection ($p = 0.024$) and were 2.93 times more likely to be exposed than cattle aged greater than 36 months.

The current study also confirms that adult dairy cattle (above 36 months) shed *Cryptosporidium* oocyst with a prevalence of 10.3% even though the age group was not significantly associated. The result indicates that adult cattle shed the oocyst and play a role in environmental contamination. Similarly, Díaz *et al.* (2021) explain that adult cattle have a role in the outbreak of cryptosporidiosis in calves and humans, even though healthy calves under one month are the main carriers of *Cryptosporidium* species. Other researchers also found that the level of oocyst shedding becomes increased in dams during parturition as a result of immune depression (T helper 1-type) and increases the risk of infection for their new born (Faubert and Litvinsky, 2000). Due to this, recent studies have given attention to adult cattle and their contribution to the transmission and environmental contamination (Shaw *et al.*, 2021).

Farm hygiene is another risk factor that showed an association ($p = 0.007$) with the occurrence of *Cryptosporidium* infection in dairy cattle. Locally, Abraham *et al.* (2018), Abebe *et al.* (2008), and Ebiyo and Haile (2022) reported similar findings from different parts of the country. Similarly, Castro-hermida *et al.* (2002) indicated animals are less at risk of infection when their house is frequently cleaned. Improper hygienic conditions create a dirty and muddy environment, which is favorable for the survival of oocysts and environmental contamination.

5.2. *Cryptosporidium* infection in Sheep

In this study, two hundred fecal samples from sheep were microscopically examined using sheather sugar solution and the MZN staining method, and 14% of the overall prevalence was recorded. In Ethiopia, an investigation has been conducted on sheep lambs and the prevalence of *Cryptosporidium* infection in all age categories was limited in general. From Iran and Poland, comparable findings were reported by Gharekhani *et al.* (2014) and Majewska *et al.* (2000), with a prevalence of 11.3% and 10.1%, respectively. Similarly, Dessì *et al.* (2020) from Italy report a prevalence of 10.1%.

However, the current finding is lower than the reports from Mexico (Romero-salas *et al.*, 2016), Brazil (Snak *et al.*, 2017) and Iraq (Al-zubaidi, 2017) with high prevalence; 72.5%, 59%, and 48%, respectively. These discrepancies in prevalence might be attributed to the management system in which the present study collected the sample from extensively reared sheep. The sampling animals (sheep) in this study are kept out of doors and are less exposed to *Cryptosporidium* oocyst than intensively managed sheep, whose crowding and confinement to small areas favor environmental contamination with oocyst and exposure to infection (Snak *et al.*, 2017). Furthermore, in an extensive management system, the oocyst excreted from the oocyst spreads to a large surface area, resulting in low infection pressure (Geurden *et al.*, 2006).

In multiple logistic regression analysis, only age shows a significant association ($p = 0.02$) with the occurrence of *Cryptosporidium* infection. The occurrence of infection is more likely in sheep under 6 months (OR = 2.69, 95% CI = 1.19–6.38, $p = 0.020$) than above 6 months. Similar findings were reported from other countries, including Spain (Castro-Hermida *et al.*, 2007), Nigeria (Abare *et al.*, 2019), and England (Smith *et al.*, 2010). The prevalence of infection under 6 months of age (20.7%) is comparable with the findings of Regassa *et al.* (2013) and Ayana and Alemu (2015) from Ethiopia, with a prevalence of 22.2% and 16.9%, respectively. However, it is higher than Wegayehu *et al.* (2017), who report a 2.1% prevalence. The variation might be due to the difference in methodological method; the present study used microscopical examination versus the highly sensitive molecular method used by the former authors.

The present study also found a prevalence of 8.8% in sheep of age above 6 months. The finding was in line with Kifleyohannes *et al.* (2022), who report a prevalence of 9% for sheep lambs. Other authors, including Castro-Hermida *et al.* (2007) and Abare *et al.* (2019), reported lower (5.3%) and higher (21.4%) rates, respectively. This finding indicates that adult sheep may serve as a reservoir for *Cryptosporidium* infection and increase the risk of infection for their lambs. Similarly, Firoozi *et al.* (2019) and Dessì *et al.* (2020) support the finding that there is a high risk of maternal transmission in lambs that live with their dams. Furthermore, Chikweto *et al.* (2019) explained the role of adult sheep as a source of environmental contamination by producing a large volume of feces.

5.3. *Cryptosporidium* infection in human

A total of 56 individuals were selected from selected dairy farms and farmers' households in the current study to examine the presence of *Cryptosporidium* oocyst in their stools. All have animals in their houses and a history of contact with their animals and their feces. The *Cryptosporidium* oocyst was examined in nine of them, with a prevalence of 16%, which is comparable with the reports of Tekle, (2021), whose finding is 11.5%. The prevalence of *Cryptosporidium* infection in Ethiopia ranges from 1.1 to 46% (Flecha *et al.*, 2015; Hailu *et al.*, 2021). The higher prevalence (46%) was reported from individual contact with animals (Hailu *et al.*, 2021).

The occurrence of *Cryptosporidium* infection is 22.3 times greater in dairy farm workers than in farmers' households rearing sheep in their houses. Siwila *et al.* (2007) also agree with the current findings; dairy farm workers were more likely to be infected with *Cryptosporidium* than their household members not working on the farm. This is due to the fact that dairy farm workers have frequent contact with their animals, which increases the risk of infection from zoonotic *Cryptosporidium* species. Even if the current study has not been done on species identification in both humans and animals (cattle and sheep), different studies using molecular methods have found a greater prevalence of *Cryptosporidium* infection in people who have had contact with animals (Wegayehu *et al.*, 2013; Zeleke, 2017a).

5.4. *Cryptosporidium* in manure

Sixty-eight manure samples were collected from six dairy farms and sixty-two rural farmer households to determine the presence of *Cryptosporidium* oocyst in stored animal manure. The overall prevalence (13.2%) of *Cryptosporidium* oocysts in the present study was smaller than the reports of Lasprilla-Mantilla *et al.* (2019) and Faubert and Litvinsky (2000), with a prevalence of 50% and 80%, respectively. However, it is larger than the findings of Fleming *et al.* (1997) and Ogendo *et al.* (2017), who reported 7.4% and 8.1%, respectively. The variation of results may come from improper manure handling practices; the manure samples used in this study were not treated by chemicals, which increased the survival of oocysts in manures.

In the current study, the manure type has a significant association ($p = 0.001$) with the occurrence of *Cryptosporidium* infection. The occurrence of *Cryptosporidium* oocyst in manure collected from dairy farms was 22.8 times higher than in manure collected from rural farmer households. This finding was agreed with by Pam *et al.* (2013), who reported the highest percentage of *Cryptosporidium* oocyst in cattle manure than in sheep manure. Similarly, Fleming *et al.* (1997) discovered that dairy farms had a higher prevalence of *Cryptosporidium* oocyst than swine farms. The global *Cryptosporidium* oocyst load indicates that cattle are the most abundant source of oocyst for the environment, followed by sheep, goats, swine, and other domestic animals (Vermeulen *et al.*, 2017).

The assessment on manure handling practice indicates only 16.1 percent of interviewer's dispose of the manure from animal houses daily. 85% of them disposed of the manure on the cropland and 98.5% used the manure for crop production. In addition, all of them did not use any treatment during manure storage. Improper handling of animal manure results in environmental contamination. Even though the role of livestock manure as a source of environmental contamination was not investigated in our country, it is the main problem in North America and Europe (Vermeulen *et al.*, 2017). The spread of manure on grazing and cropland promotes the propagation of infection to large areas and drinking water (Bordes *et al.*, 2020). In addition, the use of untreated animal manure on vegetable crops increases the risk of contamination by *Cryptosporidium* oocyst (Tram *et al.*, 2022). This results in indirect transmission of *Cryptosporidium* infection in humans from contaminated feed and water (Ryan *et al.*, 2014).

5.5. Intensity of *Cryptosporidium* oocyst

The intensity of *Cryptosporidium* oocysts was determined semi-quantitatively by taking the average of oocyst counts from 10 randomly selected fields as described by (Dessi *et al.*, 2020). The highest intensity of oocyst count (above 10 oocysts) was examined in a fecal sample collected from dairy cattle (6%), followed by a fecal sample from sheep (4%). The lowest oocyst (1–5 oocyst) count was recorded from manure storage. Vermeulen *et al.* (2017) support this finding by stating that cattle are the most abundant source of oocysts among domestic animals. He also added that collecting the manure in storage and application of treatment reduces the concentration of oocyst.

Age level intensity indicates that young calves (1–12 months) and lambs (1–6 months) showed a higher level of oocyst (above 10) count per field than adult cattle and sheep. The finding was supported by Brook *et al.* (2008), who found moderate to intense oocyst shedding in dairy calves. Research findings from naturally infected calves also indicate the number of oocysts shed decreases as the age of the calf increases (Nydam *et al.*, 2001; Zambriski *et al.*, 2013). This is due to the fact that young calves and lambs are more susceptible to infection and shed a large amount of oocyst compared to adult animals.

Even if the oocyst intensity was higher in young animals, a significant amount of oocyst count (2–6 oocysts) was examined in fecal samples from adult cattle and sheep. The research done by Scott *et al.* (1994) and Pam *et al.* (2013) indicates asymptomatic post-weaned and adult cattle shed millions of oocysts into the environment. Similarly, the findings suggest adult ewes shed about 1×10^6 *Cryptosporidium* oocysts into the environment, especially during the lambing season (Li *et al.*, 2018). However, the large volume and fibrinous nature of adult feces reduce the concentration of oocyst and limit the threshold level examined under the microscope (Shaw *et al.*, 2021). This underestimates the prevalence of *Cryptosporidium* in adult animals and their contribution to the transmission of infection to young animals. It is important to correctly assess the role of adult animals in the transmission of infection by using modified techniques used to detect the minimum level of oocyst concentration (Wells *et al.*, 2016).

6. CONCLUSION AND RECOMMENDATIONS

In conclusion, the current study revealed the presence of *Cryptosporidium* infection among dairy cattle, sheep, humans, and manure in and around Adama and Asella towns. The infection was prevalent in young animals, especially in dairy calves aged under 12 months (28.6%) and sheep lambs aged under 6 months (20.7%). The occurrence was not only limited to young calves and lambs, but a significant percentage of *Cryptosporidium* infection was also found in adult cattle (10.3%) and sheep (8.8%). This implies asymptomatic adult cattle and sheep shed oocysts into the environment and act as a source of infection for their calves and lambs, respectively.

Regarding risk factors, age and farm hygiene were identified as potential risk factors for the occurrence of *Cryptosporidium* in dairy cattle. Similarly, age was identified as a potential risk factor for the occurrence of infection in sheep. Young calves and lambs had a high risk of infection due to poorly developed immunity systems and thus required more attention from dairy farmers and sheep owners to prevent mortality and low growth rate. The study also found a higher prevalence of *Cryptosporidium* infection in farm workers due to frequent contact with animals and their feces than in household members.

In addition, *Cryptosporidium* oocyst was detected in manure samples (13.2%), which indicates the presence of *Cryptosporidium* oocyst in manures. Furthermore, dairy farmers and rural farmers dispose of animal manure on agricultural and grazing lands without any chemical treatment. The direct application of untreated manure to agricultural lands pollutes the environment, water sheds, and agricultural vegetables. Both humans and animals had a high risk of infection from contaminated feed and water due to improper handling of manure on dairy farms and rural farmers' households.

Based on the current findings the following recommendation were forwarded;

- ✚ To date, there has been no effective treatment or vaccination for *Cryptosporidium* infection in animals hence maintaining proper hygiene and management systems should be the first option to prevent the transmission of infection in young calves and lambs.

- ✚ Since adult cattle and sheep excrete oocysts into the environment, isolation of young calves and lambs from their dam is important to decrease the possibility of acquiring infection from their dams.
- ✚ Few animals were diarrheic and clinically look healthy. So, health and economic importance of the parasites on livestock should be studied.
- ✚ Proper wearing of protective gloves, clothes and shoes should be important during handling animals to prevent the transmission of Zoonotic *Cryptosporidium* species.
- ✚ Dairy farmers and farmer households should handle, treat, and store animal manure properly in order to reduce the risk of infection to both animals and humans.
- ✚ Further investigation is necessary on the contribution of adult animals and manure in environmental contamination.
- ✚ Further molecular studies is required to identify species of *Cryptosporidium* and their epidemiological distribution in both animals and humans.

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8. APPENDICES

Appendix 1. Sample collection format sheet

Dairy cattle											
Date	Site of study	Farm name	Species	Breed	Age	Sex	Body condition	Management system	Fecal consistency	Crypto	
										MZN	SSF
Sheep											
Date	Site of study	Spp	Breed	Age	Sex	Body condition	Fecal consistency	Crypto			
								MZN	SSF		
Human											
Date	Site of study	Occupation		Age	Contact with animal		Fecal consistency	Crypto			
		Farm worker	House hold member		Yes	No		MZN	SSF		

Appendix 2. Sugar floatation technique

Preparation and procedures of sugar floatation fluids (Specific gravity ≈ 1.27)

Table sugar (454 gram), 355 ml of tap water, and 6 ml of 37% formaldehyde were used for preparation of floatation fluid.

Materials and reagents

Microscopic slides	Tea spoon	Table	37% Formalin (6ml)
Measuring cylinder	Balance	Stove	Microscope
Pistil and mortem	Stick	A tea strainer	Cover slip
Table sugar	Test tube rack	Beaker	Tap water
Disposable plastic glove	Applicator		

Preparation: 454 gram of table sugar was dissolved in 355 ml of tap water using heat. Then 6 ml of 37% formaldehyde was added to fluid to prevent the growth of mold.

Procedures

- ✓ Three grams of feces were weighted from all samples and mixed with 42ml of sugar solution.
- ✓ Then the mixture was poured through tea strainer into a beaker and added into 15ml test tube.
- ✓ More floatation fluid was added until convex meniscus at the top of the tube and kept at standing for 20minutes.
- ✓ Finally, the cover slip was carefully lifted off from the tube vertically, together with the drop of fluid adhering to it, and placed on a microscope slide labeled with respective code number.
- ✓ The entire cover slip was examined under 40x magnification and 100x oil immersion microscope to identify cryptosporidium oocysts.

Appendix 3. Modified Ziehl-Neelsen staining method

Modified Ziehl-Neelsen staining technique was used to examine acid fat organisms such as *Cryptosporidium* parasite. These organisms are difficult to decolorize once stained with a specific dye, and even when treated with a mixture of acid and alcohol, they keep their red color (while all other structures present in the sample will decolorize). Though methylene blue is used as a counter stain, only acid-fast organisms appear red-colored and may thus be clearly identified in a fecal sample.

Material and reagents:

Carbolfuchsin base	Methanol alcohol 95%	Pipette
1% HCL	Microscopic slide	Microscope
Methylene blue	Rack	Tap water

Procedure

The preparation of smear take place by mixing the sample (fecal, stool and manure) to create a uniform suspension. Once the sample was well mixed, it was distributed on a microscope slide using plastic stick. Two thin smear was prepared for each of manure samples to increase the detection of *Cryptosporidium* oocysts. The slides were then air dried.

- ✓ Then fixed with methanol for three minutes
- ✓ A few drops of carbolfuchsin will be added to the smear for twenty minutes
- ✓ The slide was washed in running water for 1-2 minutes
- ✓ Then, it was decolorized in acid alcohol (1% HCL) for 30 second and washed in tap water
- ✓ Then the slide were counterstained by methylene blue for 30 seconds and washed in tap water
- ✓ Finally, the slide were dried and examined using 40x and 100x (under oil immersion) objective lenses

The oocysts were identified according to standard methods, which appear as pink-stained, round to oval structures of about 3 to 6 m in diameter, containing distinct internal structures.

Appendix 4. Semi-structured questionnaire to gather information on manure handling practices

Date of collection_____

1. Site of manure collection

Dairy farm

Rural Farmers

2. What do you do with animal manure?

Crop production

Energy production

3. How frequently do you remove manure from animal houses?

Daily

Weekly

Monthly

4. Where do you dispose the manure?

Crop land

Grazing land

Manure storage hole

Biogas tank

5. Do you apply any chemicals for manure treatment?

Yes

No

Appendix 5. Body condition scoring of dairy cattle and sheep

Dairy cattle

Condition	Description
------------------	--------------------

Poor	Individual spinous processes no longer apparent. Skin fairly supple but coat condition often rough. Rear ribs, hip, and pin bones evident. Slight fat cover over shoulder and fore ribs only.
------	---

Medium	Last two ribs noticeable. Small amount of fat over shoulder, fore ribs, and loin. Slight or no fat on brisket or over hip and pin bones.
--------	--

Good	Very smooth profile due to fat deposits. Considerable fat covering over shoulder, rib, loin, and hip. Fat fills out brisket, flanks, and tail head.
------	---

Sheep

Poor	Spinous processes are sharp and prominent. Loin eye muscle has little fat cover but is full. Trans-verse processes are smooth and slightly rounded. It is possible to pass fingers under the ends of the transverse processes with a little pressure.
------	---

Medium	Spinous processes are smooth and rounded and one can feel individual processes only with pressure. Transverse processes are smooth and well covered, and firm pressure is needed to feel over the ends. Loin eye muscle is full with some fat cover.
--------	--

Good	Spinous processes can be detected only with pressure as a hard line. Transverse processes cannot be felt. Loin eye muscle is full with a thick fat cover.
------	---

Appendix 6. Criteria used to categorize the hygiene of the dairy farms

Category	Criteria
-----------------	-----------------

Poor	Dirty floor and water tank, feces present all over, calves dirty
------	--

Medium	Separate calf pens which are washed occasionally, minimal feces present on the floor and on the calves
--------	--

Good	Separate calf pens, which are dry, spacious, no feces, cleaned daily, calves clean, clean floor
------	---