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The PhD programme in Veterinary Sciences
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Investigations on *Cryptosporidium* and *Giardia* in Tigray, Ethiopia from a One Health Perspective

Undersøkelser om *Cryptosporidium* og *Giardia* i Tigray, Etiopia fra et Èn Helse perspektiv

Tsegabirhan K/yohannes Tesama

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To Tewodros (my husband), Bereketab and Yeabtsega (our children), Kifleyohannes (my father) and Lemlem (my mother)

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List of Abbreviations

ALE	Animal loss equivalent
BG	Beta-giardin
DALYs	Disability-adjusted life-years
DNA	Deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
FITC	Fluorescein isothiocyanate
GDH	Glutamate dehydrogenase
GP60	60-kDa glycoprotein
HCMP	High cysteine membrane proteins
IFAT	Immunofluorescent Antibody Test
IMS	Immunomagnetic separation
ISO	International Organization for Standardization
MASL	Meters above sea level
MLST	Multi-locus sequence typing
mZN	Modified Ziehl-Neelsen acid-fast
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pdg	6-phosphogluconate dehydrogenase
Phkg2	Phosphorylase B kinase gamma catalytic chain
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
RHP26	DNA repair and recombination
SSUrRNA	Small subunit ribosomal ribonucleic acid
TPI	Triosephosphate isomerase
USD	United States dollar
US EPA	US Environmental Protection Agency
WHO	World Health Organization

List of papers

Paper 1.

Preliminary insights regarding water as a transmission vehicle for *Cryptosporidium* and *Giardia* in Tigray, Ethiopia

Authors: **Tsegabirhan Kifleyohannes**, Lucy J. Robertson
Published: Food and Waterborne Parasitology 2020; 19, e00073.

Paper 2.

Is fresh produce in Tigray, Ethiopia a potential transmission vehicle for *Cryptosporidium* and *Giardia*?

Authors: **Tsegabirhan Kifleyohannes**, John J. Debenham, Lucy J. Robertson
Published: Foods 2021; 10(9), 1979.

Paper 3.

***Cryptosporidium* and *Giardia* in livestock in Tigray, Northern Ethiopia and associated risk factors for infection: a cross-sectional study**

Authors: **Tsegabirhan Kifleyohannes**, Ane Nødtvedt, John J. Debenham, Getachew Terefe, Lucy J. Robertson
Published: Frontiers in Veterinary Science 2021; 8, 825940.

Paper 4.

***Cryptosporidium* and *Giardia* infections in humans in Tigray, Northern Ethiopia: an unexpectedly low occurrence of anthropozoonotic transmission**

Authors: **Tsegabirhan Kifleyohannes**, Ane Nødtvedt, John J. Debenham, Kristoffer R. Tysnes, Getachew Terefe, Lucy J. Robertson
Published: Acta Tropica 2022; 231, 106450.

Additional papers not included in the thesis:

Paper 1.

Cryptosporidium infections in Africa - how important is zoonotic transmission? A review of the evidence

Authors: Lucy J. Robertson, Øystein Haarklau Johansen, **Tsegabirhan Kifleyohannes**, Akinwale Michael Efunshile, Getachew Terefe
Published: Frontiers in Veterinary Science 2020; 7, 575881.

Paper 2.

Global Goat! Is the expanding goat population an important reservoir of *Cryptosporidium*?

Authors: Kjersti S. Utaaker, Suman Chaudhary, **Tsegabirhan Kifleyohannes**, Lucy J. Robertson
Published: Frontiers in Veterinary Science 2021; 8, 183.

Abstract

Enteric protozoans *Cryptosporidium* spp. and *Giardia duodenalis* are among the leading causes of diarrhoea in children. These parasites have particular impact in low- and middle-income countries. In these countries, people often live in close contact with their animals, highlighting the potential role for zoonotic routes of transmission in disease spread. Because of the existence of resistant (oo)cysts of these parasites, which are capable of extended periods of survival in the environment, as well as their high excretion rates and low infective doses, they can be transmitted directly via the faecal oral route or indirectly, through contaminated drinking water and fresh produce.

Despite their high prevalence and considerable health effects on young animals and people, few studies have been conducted in developing countries, regarding the role of livestock in the transmission of these parasites and in the contamination of water and fresh produce.

The present PhD project was aimed at investigating *Cryptosporidium* and *Giardia duodenalis* from a One-Health perspective in Tigray, Ethiopia. The study focused on investigating the occurrence of *Cryptosporidium* and *Giardia duodenalis* in humans and livestock and determine the extent of zoonotic transmission in the region, as well as the role of different transmission routes. The occurrence of *Cryptosporidium* and *Giardia* infection in people was 6% and 26%, respectively. Molecular characterization of *Cryptosporidium* isolates revealed *C. ubiquitum*, subtype XIIa and *C. hominis* subtypes IdA17 and IbA9G3. For *Giardia*, assemblage B predominated, but we also identified a few samples with assemblage A (AII). The occurrence of *Cryptosporidium* infection was 10, 9, and 4% in calves, lambs, and goat kids, respectively; equivalent figures for *Giardia* infection were 38, 32, and 21%. Periparturient care was shown to be a particularly relevant risk factor for infection, in livestock and infections were less likely to occur under extensive management systems. Molecular characterisation of *Cryptosporidium* isolates revealed *C. ubiquitum*, subtype XIIa in all three host species; *C. ryanae*, *C. andersoni, C. bovis*, and *C. xiaoi* were also identified. For *Giardia*,

assemblage E predominated in all host species, but among calf isolates we also identified a few potentially zoonotic genotypes (assemblages A (AI) and assemblage B). Thus, there was a widespread circulation of *Cryptosporidium* and *Giardia duodenalis* in dairy calves, lambs, and goat kids, but an apparent lack of the common zoonotic species. This indicates that despite the relatively high occurrence of infection with these parasites in both people and livestock and their close contact in our rural study sites, the transmission of *Cryptosporidium* and *Giardia* between humans and their animals seems to be surprisingly uncommon.

Analysis of water and fresh produce for *Cryptosporidium* and *Giardia* to investigate their potential transmission routes for these parasite infections, found contamination in water in two of the four districts. Contamination levels in positive samples were generally low, but some samples were relatively heavily contaminated with *Giardia* cysts (highest contamination was 22 cysts per 10 L sample and 71 cysts per 30g of cabbage). Due to the limitations associated with relatively few numbers of intact (nucleated) parasites in these samples, molecular characterisation was restricted, but *Giardia* assemblage A was identified in one sample of lettuce.

In conclusion, using a One-Health approach, my study was able to identify that despite the close interaction between people and livestock in the study area, anthroponotic transmission appears to be the most likely route of infection for both *Cryptosporidium* and *Giardia.* Given the public health and veterinary burden associated with these parasites, identifying those factors that are crucial in reducing parasite circulation in communities and environments necessitates further research in Tigray, and may also be applied elsewhere.

Norsk sammendrag

De enteriske protozoene *Cryptosporidium* spp. og *Giardia duodenalis* er blant de viktigste årsakene til diaré hos barn. Disse parasittene har en spesielt viktig rolle i lav- og mellominntektsland. I disse landende lever ofte personer i nærkontakt med dyr, noe som fremhever den potensielle rollen for zoonotiske overføringsveier i sykdomsspredning. På grunn av tilstedeværelsen av resistente (oo)cyster av disse parasittene, som kan overleve lenge i miljøet, i tillegg til deres høye ekskresjonsrater og lave infeksjonsdoser, kan de bli overført direkte via den fekal-orale ruten eller indirekte via kontaminert drikkevann og ferske råvarer. Til tross for deres høye utbredelse og betraktelige helseeffekter på unge dyr og mennesker, få studier har blitt utført i utviklingsland angående husdyrenes rolle i overføring av disse parasittene og i kontamineringen av vann og ferske råvarer.

Dette PhD-prosjektet var rettet mot å undersøke *Cryptosporidium* og *Giardia duodenalis* fra et Èn Helse perspektiv i Tigray, Etiopia. Studien fokuserte på å undersøke forekomsten av *Cryptosporidium* og *Giardia duodenalis* i mennesker og fastslå omfanget av zoonotisk overføring i området, i tillegg til rollen av ulike overføringsveier. Forekomsten av infeksjon med *Cryptosporidium* og *Giardia* hos mennesker var henholdsvis 6% og 26%. Molekylær karakterisering av isolater fra *Cryptosporidium* avslørte *C. ubiquitum*, subtype XIIa og *C. hominis* subtype IdA17 og IbA9G3. For *Giardia* dominerte assemblage B, men vi fant også noen få prøver med assemblage A (AII). Forekomsten av infeksjon med *Cryptosporidium* var henholdsvis 10, 9 og 4% i kalver, lam og kje; tilsvarende tall for infeksjon med *Giardia* var 38, 32, and 21%. Omsorg etter fødsel ble funnet til å være en spesielt relevant risikofaktor for infeksjon, infeksjoner hadde lavere sannsynlighet til å oppstå hos husdyr ved ekstensiv jordbruksdrift. Molekylær karakterisering av *Cryptosporidium*-isolater avslørte *C. ubiquitum*, subtype XIIa hos alle tre vertsartene; *C. ryanae*, *C. andersoni, C. bovis* og *C. xiaoi* også ble identifisert. For *Giardia*, assemblage E dominerte i alle vertsarter, men blant isolater fra kalv ble det også identifisert noen få potensielle zoonotiske genotyper (assemblages A (AI) og assemblage B). Dermed var det utbredt

sirkulering av *Cryptosporidium* og *Giardia duodenalis* i kalver, lam og kje, men med en tilsynelatende mangel av de mest vanlige zoonotiske artene. Dette indikerer til tross for den relativt høye forekomsten av infeksjon med disse parasittene hos både mennesker og husdyr og deres nære kontakt på våre landlige studiesteder, transmisjonen av *Cryptosporidium* og *Giardia* imellom mennesker og deres dyr ser ut til å være overraskende uvanlig.

Analyse av vann og ferske råvarer for *Cryptosporidium* og *Giardia* for undersøkelse av deres potensielle overføringsveier for disse parasittinfeksjonene, ble det funnet kontaminering i vann i to av de fire distriktene. Kontamineringsnivået i de positive prøvene var generelt lavt, men noen prøver hadde relativt høy kontaminering med *Giardia* cyster (den høyeste kontamineringen var 22 cyster per 10 L prøve og 71 cyster per 30g kål). På grunn av begrensingene knyttet til veldig få antall av intakte ((oo)cyster med cellekjerne) parasitter i disse prøvene, var molekylær karakterisering begrenset, men *Giardia* assemblage A ble identifisert i en av salatprøvene.

Som konklusjon, ved bruk av Èn Helse tilnærming klarte min studie å identifisere, til tross for det nære samspillet mellom mennesker og husdyr i dette studieområdet, at antroponotisk overføring ser ut til å være den mest sannsynlige smitteveien for både *Cryptosporidium* og *Giardia*. Gitt folkehelsen og veterinærbelastningen forbundet med disse parasittene, identifisering av disse faktorene er avgjørende for å redusere sirkulering av parasittene i lokalsamfunn og miljøer nødvendiggjør videre forskning i Tigray, som også kan benyttes andre steder.

1. Introduction

1.1 Background

In developing countries, livestock contribute significantly to rural livelihoods and economies. They provide vital nutrition for billions of rural and urban households. In addition, livestock are a crucial asset and safety net for the poor (Herrero et al., 2013). However, this important agricultural sub-sector is under constant challenge from various constraints, amongst which are diseases of economic and public health significance. In this regards, parasitic diseases, including cryptosporidiosis and giardiasis, have the potential to have a significant negative impact on rural communities, both in terms of the effects on livestock and also due to the potential for transmission to humans.

1.1.1 History of *Cryptosporidium* and *Giardia*

Cryptosporidium was first recognized in the gastric glands of mice by Ernest Edward Tyzzer and called it *C. muris* (Tyzzer, 1907). He continued his research and, in 1912, described another species in the small intestine of mice that he named *C. parvum*. For many years, *Cryptosporidium* was not recognized as being of economic, medical, or veterinary importance. It was only realised as being a potential cause of disease in 1955, when *C. meleagridis* was found to be associated with diarrhoea in turkeys (Slavin, 1955). In the early 1970s, *Cryptosporidium* was reported to be associated with diarrhoea in cattle (Panciera et al., 1971; Meutin et al., 1974). In 1976, the first two human cases of cryptosporidiosis were reported (Nime et al., 1976; Meisel et al., 1976). In the 1970s, various cases were reported in cattle, sheep, pigs, horses, turkeys, rabbits, monkeys, reptiles, and guinea pigs (Fayer and Xiao, 2008). *Cryptosporidium* became widely recognized as a human pathogen related with the onset of AIDS epidemic in the early 1980s (Ahmadpour et al., 2020). Cryptosporidiosis was considered one of the original AIDS-defining illnesses and was associated with an increased risk of death compared with other AIDS-defining illnesses (O'Connor et al., 2011). The use of highly active antiretroviral therapy has enhanced survival and quality of life, and has reduced the risk of this disease in AIDS patients (Mohebali et al., 2020; O'Connor et al., 2011). However, in persons living with HIV/AIDS in developing countries, without treatment and lacking access to care,

cryptosporidiosis continues to be a major opportunistic infection and an important cause of morbidity and mortality (O'Connor et al., 2011). A dramatic increase in *Cryptosporidium* research interest was shown in 1993 following a massive waterborne outbreak in Milwaukee, Wisconsin, involving an estimated 403,000 persons (MacKenzie et al., 1994).

Giardia was first described by Anthony van Leeuwenhoek (1632-1723) in 1681 when he discovered trophozoites in his watery diarrhoea. In 1859, Vilem Lambl (1824-1895) rediscovered *Giardia* and described some of the morphological details of the trophozoite. *Cercomonas intestinalis* was the first name of the organism, *lamblia* was introduced following the name of Lambl, and *Hexamita duodenalis* from rabbits introduced the species name *duodenalis* (Smith and Paget, 2007). The genus name *Giardia* was first used by Kunstler in 1882 for a protozoan from tadpoles, *Giardia agilis* (Kunstler, 1882). In 1952, *Giardia* morphometric findings were published by Filice and he described three major type species, based primarily on the morphology of trophozoites (Filice, 1952). The species were named *Giardia agilis*, *G. muris*, and *G. duodenalis*. Filice (1952) recognised the inherent variability within *Giardia* affecting mammals, but without the tools available to distinguish reliably between variants, he opted to place many described species under the *Giardia duodenalis* 'umbrella'. In the past ten years, however, it has been possible not only to demonstrate extensive genetic heterogeneity among *Giardia* isolates from mammals but also to confirm levels of host specificity that were recognized by early taxonomists when they proposed a series of host-related species. Some researchers have suggested that *G. duodenalis* should be sub-divided into the different species and designations on the basis of the original taxonomic descriptions (Monis et al., 2009).

1.1.2 Taxonomy of *Cryptosporidium* and *Giardia*

Cryptosporidium belongs to the phylum Apicomplexa, which are unicellular eukaryotic protozoa in the class Conoidasida, order Eucoccidiorida and family Cryptosporidiidae (Taylor et al., 2016). *Cryptosporidium* differs from other apicomplexans in that it lacks various cell organelles such as the apicoplast and mitochondria, according to genomic and biochemical evidence (Ryan and Hijjawi, 2015).

Molecular studies indicate that *Cryptosporidium* is more closely related to the primitive apicomplexan gregarine parasites, rather than to coccidians (Ryan et al.,

2016). Despite similarities, *Cryptosporidium* demonstrates several peculiarities that separate it from any other coccidian. These include: (1) the location of *Cryptosporidium* within a parasitophorous vacuole of the host cell, where the developmental stages are confined to the apical surfaces of the host cell, (2) the attachment of the parasite to the host cell, where the feeder organelle is formed at the base of the parasitophorous vacuole to facilitate the uptake of nutrients from the host cell; (3) the presence of two types of oocysts, thick-walled and thin-walled; (4) the small size of the oocyst, which lacks morphological structures such as sporocyst, micropyle, and polar granules; (5) the resistance to all anticoccidial agents tested so far; (6) the presence of a gamont-like extracellular stage similar to those found in gregarine life cycles. Based on this, *Cryptosporidium* is evolutionarily different from other coccidia, but related to gregarines (Ryan and Hijjawi, 2015).

To date, more than 40 species of *Cryptosporidium* (Table 1) and over 120 genotypes of *Cryptosporidium* have been described (Ryan et al., 2021. From the measurements in the table, it is clear that attempts to differentiate between species based on oocyst size may not always be possible because of the size similarity. Several species and genotypes of *Cryptosporidium* have been reported in humans based mainly on molecular tools (Ryan et al., 2021). The most commonly used marker used for species differentiation is the small subunit ribosomal RNA (*SSUrRNA*) gene or portions thereof (Xiao, 2010). The most commonly used marker for subtyping is the 60-kDa glycoprotein gene (*gp60*) (Robinson et al., 2022). Other sub-typing markers with the potential for higher discriminatory capability than *gp60* have recently reported (Robinson et al., 2022). Markers include: *cgd1_470_1429*, *cgd4_2350_796*, *cgd5_10_310*, *cgd5_4490_2941*; *cgd6_4290*, *cgd8_4440_nc_506* and *cgd8_4840_6355* genes, but these are not yet as widely implemented as the *gp60* gene (Robinson et al., 2022).

The following subtype groups have been identified in five *Cryptosporidium* species: *C. hominis* (Ia, Ib, Id-Ij) (Ryan et al., 2014), and recently two sub-species have been reported: *C. hominis hominis* and *C. hominis aquapotentis* (Tichkule et al., 2022), *C. parvum* (IIa, IId) which are zoonotic and nonzoonotic (IIb, IIc, IIe-IIt) (Lebbad et al., 2021; Ryan et al., 2014), *C. ubiquitum* (XIIa-XIIf) (Li et al., 2014), *C. meleagridis* (XIIIa-XIIIg) (Stensvold et al., 2014), and *C. viatorum* (XVa-XVd) (Chen et al., 2019).

Table 1. *Cryptosporidium* species in different mammalian hosts

Species	**Host**	**Infection site**	**Oocyst size (µm) L/W**	**References**
C. alticolis	Voles	Small intestine	5.4 x 4.9	Horčičková et al. (2019)
C. andersoni	Cattle	Abomasum	7.4 x 5.5	Lindsay et al. (2000)
C. apodemi	Yellow-necked mice	Intestine	4.7 x 4.2	Čondlová et al. (2018)
C. bovis	Cattle	Small intestine	4.9 x 4.6	Fayer et al. (2005)
C. canis	Dogs	Small intestine	4.7 x 4.9	Fayer et al. (2001)
C. cuniculus	Rabbits	Small intestine	5.98 x 5.38	Robinson et al. (2010)
C. ditrichi	Mouse, humans	Small intestine	4.7 x 4.2	Čondlová et al. (2018)
C. erinacei	Hedgehogs	NR	4.9 x 4.4	Kváč et al. (2014)
C. fayeri	Marsupials	Small intestine	4.9 x 5.4	Ryan et al. (2008)
C. felis	Cats	Small intestine	4.5 x 5.0	Iseki (1979)
C. homai	Guinea pigs	NR	NR	Zahedi et al. (2017)
C. hominis	Humans	Small intestine	4.8 x 5.2	Ryan et al. (2002)
C. macropodum	Marsupials	Small intestine	4.9 x 5.4	Power & Ryan (2008)
C. macroti	Voles	Large intestine	4.3 x 4.1	Horčičková et al. (2019)
C. meleagridis	Turkeys, humans	Small intestine	4.5-6 x 4.2-5.3	Slavin (1955)
C. muris	Rodents	Stomach	6.1 x 8.4	Tyzzer (1907)
C. occultus	Rodents	Colon	5.20 x 4.94	Kváč et al. (2018)
C. parvum	Cattle, sheep, humans etc.	Small intestine	4.9 x 4.4	Tyzzer (1912)
C. pestis	Cattle	NR	NR	Slapeta (2006)
C. proliferans	Rodents	Stomach	7.7 x 5.3	Kváč et al. (2016)
C. rubeyi	Rodents	Small intestine	4.67 x 4.34	Li et al. (2015)
C. ryanae	Cattle	Small intestine	3.16 x 3.73	Fayer et al. (2008)
C. scrofarum	Pigs	Small intestine	5.16 x 4.83	Kváč et al. (2013)
C. suis	Pigs	Small & large intestine	4.6 x 4.2	Ryan et al. (2004)
C. tyzzeri	Rodents	Small intestine	4.64 × 4.19	Tyzzer (1912)
C. ubiquitum	Ruminants, rodents, etc.	Small intestine	5.04 x 4.66	Fayer et al. (2010)
C. viatorum	Humans	NR	5.35 × 4.72	Elwin et al. (2012)
C. wrairi	Guinea pigs	Small intestine	5.4 x 4.6	Vetterling et al. (1971)
C. xiaoi	Sheep, goats	NR	3.94 x 3.44	Fayer & Santín (2009)

NR: Not reported

Giardia are unicellular flagellated protozoa that belong to the phylum Fornicata, class Metamonadea, order Giardiida, and family Giardiidae (Taylor et al., 2016). *Giardia duodenalis* consists of eight genetically distinct (Table 2) but morphologically identical assemblages (A-H) (Thompson and Monis, 2011; Lasek-Nesselquist et al. 2010), with assemblages A and B infecting both people and animals, while C-H are generally host-adapted (Heyworth, 2016). Moreover, within assemblage A there are four sub-assemblages (AI, AII, AIII, and AIV) of varying zoonotic potential; human isolates mainly belong to AII and a lesser extent to AI, while animal isolates mainly belong to sub-assemblage AI, AIII, and AIV, and a lesser extent AII. Sub-assemblage AIII is mostly found in wild animals (Sprong et al., 2009). Assemblage B has also been divided into four sub-assemblages BI, BII, BIII, and BIV. According to a reports by Heyworth (2016) and Sprong et al. (2009) BIII and BIV occur predominantly in humans, while BI and BII appear to be more specific for animals.

Table 2. Assemblages of *Giardia* in different hosts
Source: Thompson and Monis, (2011) and Lasek-Nesselquist et al. (2010), Monis et al. (2009)

Species (=assemblage)	**Host**
G. duodenalis (= assemblage A)	Humans and other primates, livestock, dogs, cats, rodents, and other wild mammals
G. enterica (= assemblage B)	Humans and other primates, livestock, dogs, cat, rodents, Procyonidae, and some species of wild mammals
G. canis (= assemblages C/D)	Dogs and other canids
G. cati (= assemblage F)	Cats
G. bovis (= assemblage E)	Cattle and other hoofed livestock
G. simondi (= assemblage G)	Rats
Not given a species name (=assemblage H)	Seals and gull

1.1.3 Morphology of *Cryptosporidium* and *Giardia*

Cryptosporidium oocysts are rounded and measure between 4-6 μm in diameter. Oocysts contain four elongated sporozoites, which are characterized by their comma-shape and a residual body. Oocysts of many *Cryptosporidium* spp. are morphologically similar (Xiao et al., 2004), as shown in Table 1.

Giardia cysts are oval in shape, measuring 8-12 μm in length and 7-10 μm in width, with four nuclei, in contrast to two in trophozoite stage. The trophozoite has pear shape, the anterior end is broadly rounded with a ventral disc as an organ for attachment and a tapering posterior end. The trophozoites of *G. duodenalis* measures 12-15 μm in length and 5-9 μm in width. It is bilaterally symmetric, and all organs of the body are paired. They have two median bodies, two axostyles, two nuclei, and four pairs of flagella (Ankarklev et al., 2010).

1.1.4 Lifecycles of *Cryptosporidium* and *Giardia*

The lifecycle of *Cryptosporidium* begins with the ingestion of the sporulated oocyst, the oocyst releases sporozoites which glide, invade and locate in a unique location known as the parasitophorous vacuole where they transform into a uninucleate trophozoite. Trophozoites undergo asexual replication (merogony) to produce a mature type I meront with eight merozoites. Merozoites released from the type I meront invade adjacent intestinal epithelial cells to yield additional type I meronts or transform in to type II meronts that contain four merozoites. Merozoites from type II meronts are thought to transform into the sexual stages, the macrogamont (female) and microgamont (male). Female macrogamonts and male microgamonts develop and fertilization occurs. The resulting zygote develops into two different types of oocysts (thick-walled and thin-walled). Thick-walled oocysts are shed from the host into the environment, whereas thin-walled oocysts are involved in the internal autoinfective cycle and are not recovered from faeces. Oocysts are infectious upon excretion, thus enabling direct and immediate faecal-oral transmission (Figure 1. Pinto and Vinayak, 2021).

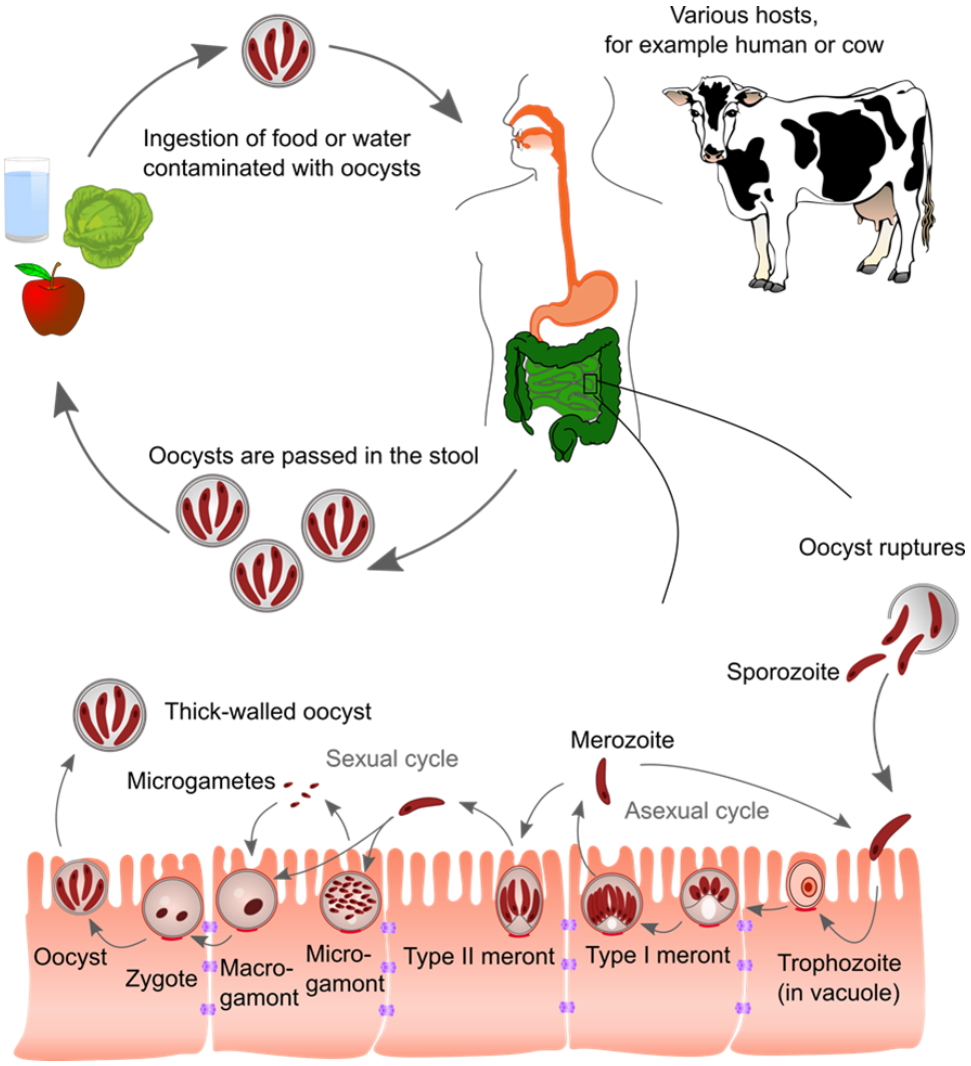


Figure 1. Lifecycle of *Cryptosporidium*
Source: Hønsvall, (2017)

Giardia duodenalis has two major stages in its lifecycle. After ingestion, they pass through the mouth, oesophagus, and stomach into the small intestine where each cyst releases two trophozoites through excystation. The trophozoites multiply by splitting into two in a process called longitudinal binary fission, remaining in the small intestine where they can be free or adhere to the luminal lining of the small intestine by their ventral sucking disc (Figure 2). After multiplication, the trophozoites pass to the terminal region of the intestine and form new cysts. Both *Giardia* cysts and trophozoites can be found in faeces. However, trophozoites are not a transmission

stage, but can be 'washed out' prior to encystation if there is profuse watery diarrhoea (Efunshile et al., 2019). The *Giardia* cyst is the stage found most commonly in faeces and are infectious immediately or shortly thereafter. *Giardia* cysts are less robust than *Cryptosporidium* oocysts and less able to withstand various environmental conditions as compared with *Cryptosporidium* oocysts (Utaaker et al., 2017).

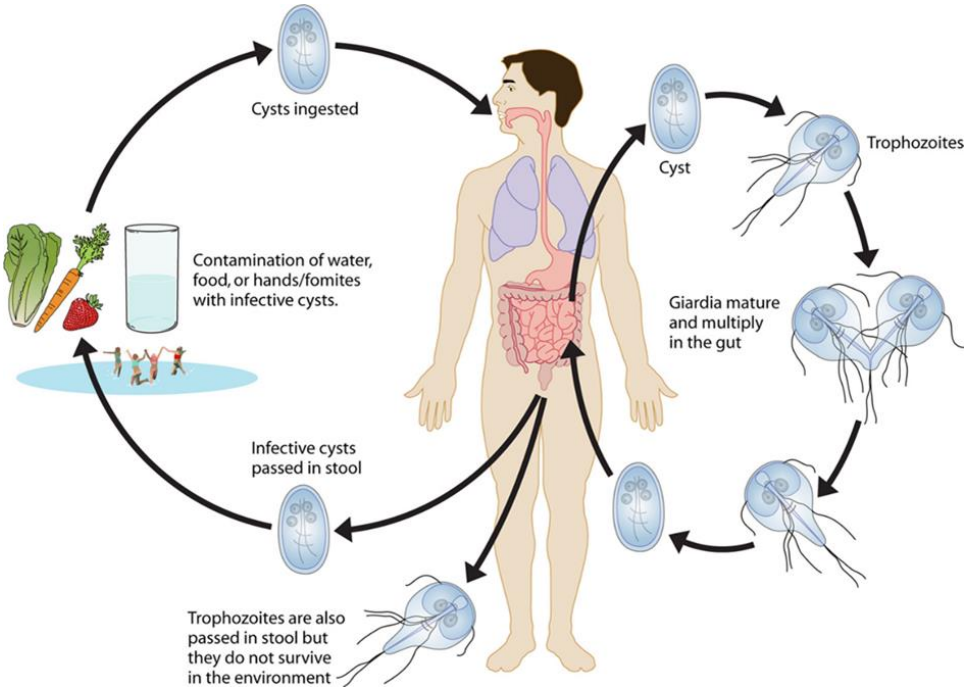


Figure 2. Lifecycle of *Giardia*
Source: Esch and Petersen, (2013)

1.1.5 Pathogenesis of *Cryptosporidium* and *Giardia*

Cryptosporidium and *Giardia* are capable of causing pathogenic changes within the host. In the intestine, *Cryptosporidium* invades the superficial surface of epithelia, causing villus atrophy, villus fusion, crypt hyperplasia, microvillus shortening and destruction, and infiltration of inflammatory cells (O' Handley et al., 2006; Certad et al., 2017). These changes can result in malabsorption through the loss of intestinal surface area, loss of membrane-bound intestinal enzymes, and impaired nutrient and electrolyte transport (O' Handley et al., 2006). *Cryptosporidium* can be viewed as 'minimally invasive' because it does not penetrate into deeper mucosal layers (Miyamoto and Eckmann, 2015). It invades the host epithelial cells of the terminal

jejunum and ileum, but can also affect the entire gastrointestinal tract. Disruption of tight junctions can lead to host cell actin polymerization and cytoskeleton remodelling, loss of barrier function, release of lactate dehydrogenase, and increased rates of cell death (Certad et al., 2017). Factors involved in excystation, gliding motility, attachment, invasion, parasitophorous vacuole formation, intracellular maintenance, and host cell damage are considered relevant virulence factors, and appear to be conserved among *Cryptosporidium* spp. The pathogenesis is similar in both humans and livestock (Tzipori and Ward, 2002; Certad et al., 2017).

Infections with *Cryptosporidium* are a common cause of diarrhoea in neonatal ruminants. Infected animals have a wide range of clinical symptoms, ranging from asymptomatic to death (Santin, 2020). Most cases of illness in cattle are caused *by C. parvum* and are characterized by an acute onset of profuse watery diarrhoea, which is frequently accompanied by loss of appetite, depression, and weakness (Santin, 2020). Cryptosporidiosis is characterized by diarrhoea and weight loss in small ruminants (Santin, 2020).

In humans, clinical manifestations following *Cryptosporidium* infection occur after an incubation of 2-14 days, and include watery, often profuse, diarrhoea, as well as abdominal cramps, nausea, vomiting, weight loss, and a low-grade fever (Certad et al., 2017). In young children, particularly if malnourished, diarrhoea can be severe and associated with significant mortality (Kotloff et al., 2013). In immunocompromised individuals, malabsorption can be present due to a decreased absorptive surface in severe infections and can contribute to the wasting syndrome seen in AIDS patients (Certad et al., 2017). Immunocompromised patients with bile-duct infection may also present with jaundice, secondary to biliary tract obstruction, or with symptoms of pancreatitis. According to Carter et al. (2020), the most commonly reported long-term sequelae of human cryptosporidiosis are diarrhoea, abdominal pain, loss of appetite, nausea, fatigue, vomiting, joint pain, eye pain, and headache. In immunocompetent individuals, disease is usually self-limited and lasts 1-3 weeks. Although asymptomatic shedding of *C. parvum* oocysts has been described (Davies et al., 2009; Eibach et al., 2015; Osman et al., 2016), information on the asymptomatic carriage of *Cryptosporidium* in the human population remains scarce.

The primary events in pathogenesis of giardiasis are the initial host-parasite interactions of attachment (Certad et al., 2017). *Giardia* usually colonises the duodenum and jejunum in the small intestine. It is extracellular with no cellular

invasion, systemic infection, or penetration of deep tissues. It causes functional lesions of brush borders that result in apical and F-actin cytoskeleton disorganization (Di Genova and Tonelli, 2016). Induction of apoptosis, blunting of villi, disaccharides deficiency, and mucosal leakage have been described. *Giardia* causes tight-junction disruption, but without penetrating the epithelium (Halliez and Buret et al., 2013). Factors involved in attachment to host cells, flagellar motility and antigenic variation, are considered relevant virulence factors (Emery et al., 2016; Ankarklev et al., 2010).

Giardiasis is a common disease in ruminants that causes diarrhoea, weight loss and malabsorption, but asymptomatic infections are also common (Santin, 2020).

Giardiasis is more frequently associated with persistent, chronic diarrhoea and illness in humans than cryptosporidiosis, even in immunocompetent persons. Watery diarrhoea, epigastric discomfort, nausea, and vomiting are some of the clinical signs of infection after a 1-2 weeks incubation period. The acute phase usually lasts 1-3 weeks, though symptoms might last months in some patients. The majority of infections are self-limiting, but in endemic areas, recurrences are common. In children from developing countries, chronic infections can cause weight loss and malabsorption (Thomas et al., 2014), as well as stunting, wasting, and cognitive impairment (Berkman et al., 2002; Nematian et al., 2008; Al-Mekhlafi et al., 2013). Asymptomatic infections are common (David et al., 2015; Tellevik et al., 2015; Osman et al., 2016), but it's unclear if this is due to low-virulence strain exposure, the host's ability to suppress infection, particularly after repeated exposure, or other factors.
In developing countries, some studies have reported *Giardia* infection in young children may mediate a protective effect (Muhsen and Levine, 2012). In a study of Israeli-Arab young children, infection with *Giardia* seems to reduce the risk of acute diarrhoea in this age group (Muhsen et al., 2014).

These varied manifestations may result from differences in the host, parasite strains, or microbiota differences (Adam, 2021). Long-term consequences may be present for several years, in the form of failure to thrive, stunting, irritable bowel syndrome, and chronic fatigue syndrome (Halliez and Buret, 2013).

1.1.6 Occurrence and impact of *Cryptosporidium* and *Giardia* infection in humans and livestock

***Cryptosporidium* and *Giardia* in humans**

Worldwide, diarrhoea is a major problem. Estimation of disease burden often uses the metric "disability-adjusted life-years" (DALYs). DALYs are the sum of years of life lost and years lived with disability in a particular population. In 2017, DALYs due to diarrhoeal diseases globally were estimated at 81 million (Karambizi et al., 2021). From a One Health perspective, it is clear that assessing parasitic losses, even when the direct costs of controlling the disease are included, does not reveal the true impact (Blake and Betson, 2017).

In 2016, *Cryptosporidium* spp. was the fifth leading cause of diarrhoeal mortality in children under 5 years of age, causing 48300 deaths, globally (Khalil et al., 2018). Close to 50% of *Cryptosporidium*-related deaths were from Nigeria and the Democratic Republic of the Congo. In addition, *Cryptosporidium* was second only to rotavirus as the major cause of moderate to severe diarrhoea in children aged 0 to 59 months in Africa and Asia (Kotloff et al., 2013; Nasrin et al., 2013). According to Kotloff et al. (2013) *Cryptosporidium* was one of the pathogens most associated with an increased risk of death in toddlers aged 12-23 months. Another study reported that *Cryptosporidium* spp. is one of the five highest attributable burdens of diarrhoea in the first year of life in Africa, Asia, and South America (Platts-Mills et al., 2015).

Cryptosporidiosis is a highly prevalent and widespread disease documented in humans in over 90 countries on all continents except Antarctica (Ryan et al., 2014). The burden of cryptosporidiosis varies considerably between and within countries. The occurrence of *Cryptosporidium* in humans was reported to be 2.6-21.3% in African countries, 3.2-31.5% in Central and South American countries, 1.3-13.1% in Asia countries, 0.1-14.1% in Europe, and 0.3-4.3% in North America (Fayer, 2004). As these data are from over 15-years ago their relevance today must be treated with caution. A more recent systematic review and meta-analysis (Dong et al, 2020) provides updated information, but it is not grouped by continent; in this article Mexico (69.6%), Bangladesh (42.5%) and Nigeria (34.0%), are reported to have the highest prevalences of infection.

In 2016, acute diarrhoeal morbidity and mortality from *Cryptosporidium* infection were associated with 4,224,000 DALYs in children younger than 5 years worldwide. The burden of *Cryptosporidium* was highest in sub-Saharan Africa. In Chad, the highest total rate of DALYs occurred (218 per 1000 child-years), the Central African Republic (164 per 1000 child-years) and Burkina Faso (128 per 1000 child-years) (Khalil et al., 2018).

Globally, giardiasis is a common parasite infection. It is especially common in areas with poor sanitation and limited water treatment facilities (Leung et al., 2019). The prevalence of giardiasis has been reported to be as high as 20 to 40% in resource-limited settings, with the highest incidence among children under five years of age (Rogawski et al., 2018). The prevalence rates in developed countries are generally reported to be 2 to 7%, with *Giardia* infecting about 2% of adults and approximately 6-8% of children (Roxstrom-Lindquist et al., 2006; Caccio and Ryan, 2008; Fink and Singer, 2017; Halliez and Buret, 2013; Tandukar et al., 2018).

According to the World Health Organization (WHO), foodborne disease burden epidemiology reference group, giardiasis resulted in 171,100 DALYs worldwide in 2010 (Torgerson et al., 2015). Globally, diarrhoeal disease is estimated to cause 89.5 million DALYs in symptomatic patients (Conlan and Lal, 2015; Murray et al., 2012).

To date, there are no vaccines available against cryptosporidiosis, drug treatment has been the primary means of controlling the disease. The only US Food and Drug Administration approved drug for treating human cryptosporidiosis is nitazoxanide Nevertheless, its efficacy in immunosuppressed individuals such as those with AIDS, and malnourished children is limited (Nguyen-Ho-Bao et al., 2022).

Various compounds are currently available for the treatment of giardiasis. Drugs that are used are metronidazole, tinidazole, nitazoxanide, albendazole and mebendazole, particularly metronidazole, are commonly used for treatment (Riches et al., 2020).

In order to reduce the risk of cryptosporidiosis and giardiasis, prevention measures are the most practical approach. Since water contamination is a major source of human infections, measures to reduce the spread of (oo)cysts in the environment by using good farming and agricultural practices to minimise contamination of water by animal faeces, water catchment protection and to improve the water treatment process are essential (Carmena, 2010).

Cryptosporidium and *Giardia* in ruminant livestock

Cryptosporidium infection has been frequently reported in livestock (Santin et al., 2013). Cryptosporidiosis causes low mortality rates, although severe cases can be fatal in young animals (Hatam-Nahavandi et al., 2019). Many reports have documented *Cryptosporidium* infection in cattle in different countries and settings, showing that *Cryptosporidium* spp. infections are common worldwide.

Dairy and beef cattle are both infected, with prevalence estimates varying greatly between studies. The prevalence at herd levels has been reported to range from 0 to 100% (compiled by Robertson et al., 2014). Infected animals have been documented in all age categories, but preweaned calves are the most commonly infected. When calves under the age of two months are examined in point-prevalence surveys, 5-93% of them shed oocysts (compiled by Robertson et al., 2014). *Cryptosporidium* infection can be seen in sheep and goats all over the world. The prevalence varies significantly between studies, ranging from 0 to 77% in sheep and 0 to 100% in goats (compiled by Robertson et al., 2014). All age groups are susceptible, however, lambs and kids are more likely to become infected than older animals (compiled by Robertson et al., 2014).

The prevalence of *Giardia* has been reported in both dairy and beef cattle from different countries. The reported prevalence varies, and infection can be as high as 100% (Siwila, 2017). *Giardia* has been found in both sheep and goats throughout the world. However, there is considerably more data available on *Giardia* infection in sheep populations than in goat populations (Siwila, 2017). In sheep, the prevalence of *Giardia* varies greatly, with adult sheep may be having as high as 38% and lambs 68% (Siwila, 2017). In goats, data tend to indicate a prevalence ranging from <10% to >40%, with similar data for cattle. In general, all ruminants are likely to be exposed to *Giardia* cyst soon after birth however, infections are most common near the end of neonatal period (O'Handley and Olson, 2006).

Using the local gross national income per capita deflator, economic losses due to zoonotic diseases can be converted to an equivalent measure for quantifying burden. This effectively converts animal production losses into years of human life. It can be converted to a DALY equivalent, called animal loss equivalents (ALE), and added to the DALY associated with human ill health to give a modified DALY. This is referred to as the “zDALY”. The zDALY is a measure of a zoonotic disease’s impact on animal

and human health (Torgerson et al., 2018). To my knowledge there have been no zDALY estimates associated with either cryptosporidiosis or giardiasis.

There are no drugs or vaccines effective against cryptosporidiosis and giardiasis in livestock, thus prevention and control rely mainly on hygienic measures and good management. This will include feeding high-quality colostrum immediately after birth, keeping the animals in a clean, dry place, avoiding contamination of feeding and drinking troughs, and regular cleaning and disinfection of surroundings using effective disinfectants (Ghazy et al., 2016; Innes et al., 2020; Santin, 2020).

1.1.7 Risk factors for *Cryptosporidium* and *Giardia* infection in humans and livestock

A higher prevalence of *Cryptosporidium* has been linked to a variety of factors, including contact with animals and manure, (Wanyiri et al., 2014) in Kenya; living in villages, drinking potentially contaminated water, and contact with animals were risk factors in a study from Egypt (Helmy et al., 2015).

In another study from Egypt, contact with livestock was not linked to giardiasis, however, it was linked to drinking tap water (Helmy et al., 2014). Malnutrition has been postulated to be an important risk factor for cryptosporidiosis and giardiasis in children (Squire and Ryan, 2017).

Studies from Australia, UK, and USA have indicated among the risk factors for sporadic cryptosporidiosis in humans, age (children under the age of five years, and to a lesser extent, young adults, who may have a greater chance of interacting with these patients), travel abroad, contact with diarrhoeic individuals, and contact with farm animals are all recognized (Robertson et al., 2002; Roy et al., 2004; Hunter et al., 2004). A finding from Australia and US indicated swimming in freshwater or public swimming pools has been positively associated with cryptosporidiosis (Caccio et al., 2005). A study from UK showed travel abroad, interacting with a diarrhoeic individual, and contact with cattle were all found to be risk factors for cryptosporidiosis. According to Caccio et al. (2005), although ingestion of contaminated fruit and vegetables with small numbers of parasites might cause subclinical infection and in turn, enhance immunity, a study by Hunter and Thompson (2005) reported strong negative associations between eating tomatoes and raw vegetables and acquiring *C. parvum* infection.

A study from Southwestern England by Stuart et al. (2003), reported swallowing water while swimming, contact (exposure) with recreational fresh water, drinking tap water, and eating lettuce as risk factors of giardiasis. However, a study from Germany reported that contact with animals and exposure to surface water (swimming/water sports) were not associated with giardiasis. In the same study, males, immunocompromised individuals and daily consumers of green salad were associated with giardiasis infection (Espelage et al., 2010).

A study from Ethiopia indicated various risk factors associated cryptosporidiosis in calves, including age, hygiene, feed source, water source and close contact with other domestic animals (Ayele et al., 2018). In Egypt, age of the animal (high in young calves), and drinking water from (possibly contaminated underground or canal rather than tap water were associated with cryptosporidiosis (Helmy et al., 2015). Overcrowded calves have been demonstrated to be at increased risk of *C. parvum* infection compared to calves in herds that are not overcrowded (Szonyi et al., 2012). Furthermore, a study from New York City dairy farms indicated other factors that significantly increased the risk of *C. parvum* infection in calves. These included weather conditions that cause the animals to be housed inside, and the use of hay bedding. Thus, factors promoting close contact between animals (herd size and housing) and oocyst survival are associated with *C. parvum* infection and this is well recognized in some non-African countries (Szonyi et al., 2012; Robertson et al., 2020). In many African countries, environmental conditions may not be optimal for prolonged oocyst survival in the environment, with desiccation and UV exposure playing a part (Robertson et al., 2020).

A study from Zambia indicated calves under three months of age and intensively managed were more likely to be positive for *Giardia* than older calves and or calves reared in a free-range system (Kakandelwa, 2015).

1.1.8 A One-Health Perspective of *Cryptosporidium* and *Giardia*

The need for a One-Health approach in tackling infection transmission has been demonstrated numerous times by experiences in the fight against zoonotic diseases. One Health is defined as a multidisciplinary endeavour at local, national, and global levels to attain optimal health for people, animals, and the environment; see figure 3 (OHCEA, 2019).

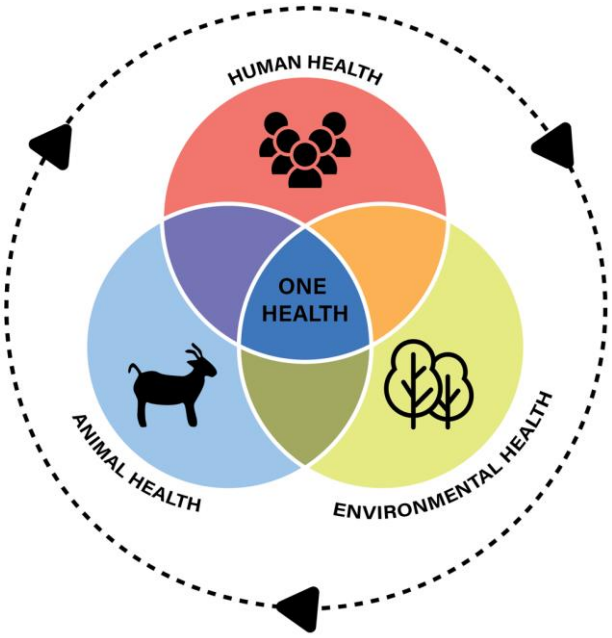


Figure 3. One Health approach to address zoonotic cryptosporidiosis and giardiasis Credit: Unni Lise, (2022)

A collaborative One-Health approach, which recognizes that humans and animals live in a shared environment, is an appropriate approach to tackling and mitigating diseases with zoonotic potential, such as cryptosporidiosis and giardiasis. By considering all sections of the One-Health triad, human health, animal health, and environmental health, different advantages and new options towards disease control may be identified (Innes et al., 2020; Archer et al., 2020).

According to Koster et al. (2022), zoonotic transmission has been suggested to be a significant route for *Cryptosporidium* spp. and *Giardia duodenalis* spreading in several settings, either in developing or developed nations. Poor hygiene is a crucial factor in enhancing the transmission of *Cryptosporidium* and *Giardia* and human infections could be derived from infected animals by direct contact, or via contaminated food, water, and environment (Koster et al., 2022). The *Cryptosporidium* oocyst load is high in livestock manure, with cattle being the predominant source of environmental contamination of zoonotic *Cryptosporidium* spp. (Vermeulen et al., 2017); use of fertilizer produced from livestock waste, surface run-off from feedlots and untreated water used for irrigation can thus lead to the contamination of food with *Cryptosporidium* oocysts. Food may also be contaminated during harvesting, packing,

transportation, selling and or preparation under unhygienic conditions (Abeywardena et al., 2015). *Cryptosporidium* and *Giardia* (oo)cysts are resistant to a variety of environmental stresses and can survive well in the aquatic environment (Carmena, 2010). A study in Canada found that water was the most commonly reported probable sources of *Cryptosporidium* infection (48%), followed by contact with livestock (21%), person-to-person contact (15%), and food-borne transmission (8%) (EFSA, 2018).

Cryptosporidium and *Giardia* are a public health concern, partly due to being widespread in ruminants and humans. This means that environmental contamination may expose humans to potentially infectious transmission stages (Santin, 2020). The excretion of infectious oocysts by infected individuals are 10^5–10^7 oocysts/g of faeces and up to 10^9 cysts in a single day (Carmena, 2010). Infected calves shed even higher numbers of oocysts, 3.89×10^{10} oocysts/g of faeces reported from 6–12 day-old calf and 3.8×10^7 cysts/g of faeces in a 50–56 day-old calf (Nydam, 2001). There is a low infectious dose (<10) (oo) cysts.

Transmission varies seasonally; many African countries have documented a higher prevalence of *Cryptosporidium* infection in human during high rainfall seasons, at the onset and higher prevalence of *Giardia* in cool seasons in Tanzania (Squire and Ryan, 2017). However, few studies have reported a higher prevalence at the end of rainy season. This variability could be due to a difference in route of transmission, host and/or *Cryptosporidium* and *Giardia* species in different places. In most European countries cryptosporidiosis cases are mainly reported in late summer and autumn (August-October) (Caccio and Chalmers, 2016) with some countries also reporting a smaller peak in spring. There is good evidence from outbreaks and molecular epidemiology that the spring peak in UK is mainly attributable to *C. parvum* and likely to be related to lambing and calving, while the summer/autumn peak is mainly *C. hominis* and associated with swimming pools and foreign travel (Chalmers et al., 2009).

Not all *Cryptosporidium* spp. in livestock are of public health significance, *C. parvum* is the species with the greatest zoonotic potential (Innes et al., 2020). Geographical analysis indicates that human-adapted species (*C. hominis*, *C. parvum* subtypes) are relatively more prevalent in resource-poor countries, while zoonotic *C. parvum* dominates in North America, Europe, Australia and parts of the Middle East (King et al., 2019).

Giardia is considered to be potentially zoonotic and has been responsible for the majority (40.6%) of recorded parasitic waterborne outbreaks globally (Lee et al., 2017). The distribution of the assemblages varies between different hosts; in people assemblages A (43%) and B (56%) are most frequently reported and, to a much lesser extent, assembalges C (0.1%), D (0.2%) E (0.2%), and F (0.2%) (Gelanew et al., 2007; Foronda et al., 2008). All these assemblages have also found in animals; thus, at this very low level of resolution, assemblages A to F could be considered zoonotic (Sprong et al., 2009). However, how often zoonotic transmission occurs is much less obvious. In African countries, the relationship between people and animals is generally much closer than in industrialized countries (Robertson et al., 2020). Thus, it seems likely that direct zoonotic transmission is more likely to occur, and, furthermore, surface water used both for drinking water and for watering animals is more likely to become contaminated by livestock. In addition, fruits and vegetables that are consumed raw are often closely associated with animals, as they are involved in harvesting and transporting. Furthermore, animal manure is used as fertilizer (Squire and Ryan, 2017).

Cryptosporidium and *Giardia* in Ethiopia

Ethiopia is located on the horn of East Africa and is the second most populous nation on the continent, with approximately 115 million inhabitants (World Bank, 2022). The country is considered the fastest-growing economy in the region, with 6.3% annual growth (financial year 2020/21, World Bank, 2022). However, it is also one of the poorest countries, with a per capita gross national income of 890 USD. Around 80% of Ethiopians are dependent on agriculture. They have direct and close contact with livestock on a regular basis. As a result, a large portion of the Ethiopian population is vulnerable to the impact of zoonotic diseases (Pieracci et al., 2016).

Tigray region in northern Ethiopia has been wracked by civil war since November 2020 when fighting broke out between the Tigrayan regional government and the Ethiopian federal army. The official death toll of civilians has exceeded 9,500, although it is fear there are many more unrecorded casualties. Furthermore, almost two million people have been displaced, with an estimated 4.6 million people facing a food shortage crisis (WFP, 2022; Nyssen et al., 2021). In such circumstances, efforts to control transmission of zoonotic parasites is going to be considered of relatively minor importance, despite the insidious effect of such diseases on the population. In addition, with breakdown of civil infrastructure and food insecurity rising towards

starvation levels, the likelihood of waterborne and foodborne transmission of pathogens is rising. It should be noted that the samples and information in the current project were gathered prior to the outbreak of civil war in Tigray, and the final sample collection was in March 2020, around 8 months before the conflict started.

In Tigray, farmers generally own small land plots (less than a hectare in total). Subsistence crops are mostly grown on rain-fed lands and cash crops on irrigated lands in the bottom of narrow river valleys. Farming practices are typically traditional and low cost but are nevertheless successful. In order to till the land, ox-drawn ploughs (or mahrasha) are commonly employed, seeds are mostly sown by hand, and most crops rely solely on rainfall without additional irrigation. Since the outbreak of the war, ploughing in Tigray has become extremely difficult due to looting and killing of oxen required to plough farmlands (Nyssen et al., 2021).

In Ethiopia, limited access to safe water and inadequate sanitation and hygiene services are responsible for 60 to 80% of communicable infections (UNICEF, 2018). According to the WHO, infections caused by environmental factors such as poor hygiene and a lack of access to clean water and sanitation are associated with 50% of undernutrition (WHO, 2008). Again, in Tigray, this is currently exacerbated by the civil war and associated breakdown of civilian infrastructure. Open defecation, which is common in many areas, results in environmental contamination with intestinal pathogens, and transmission of parasitic infections, such as cryptosporidiosis and giardiasis; such diarrhoeal diseases can cause and worsen malnutrition (Johansen et al., 2022). It has been estimated that 65% of Ethiopian households have access to improved water sources whereas only 6.3% of households have access to improved sanitation (UNICEF, 2018).

Ethiopian farmers use manure as fertilizer and dried dung as fuel in their farming practices. The risk of infection with zoonotic diseases such as *Giardia duodenalis* and *Cryptosporidium* spp. is thought to be greater in areas where people have close contact with animals and their manure (Wegayehu et al., 2013). In the same study, children who had close contact with livestock and their dung had a higher risk of *Cryptosporidium* and *Giardia* infection than children who did not.

There are very few published articles that we were able to identify regarding analysis of drinking water for these parasites in Ethiopia. The first article reports investigation of 115 samples of drinking water collected from various sources around Addis Ababa

(Atnafu et al., 2012). Among 72 tap water samples analysed, 15 (21%) were considered to contain *Cryptosporidium* oocysts and 12 (17%) were considered to contain *Giardia* cysts; among 17 storage tank samples, 6 (35%) were considered to contain *Cryptosporidium* oocysts and 5 (29%) were considered to contain *Giardia* cysts; two raw surface water samples were positive for both parasites; and the single well water sample was positive for *Cryptosporidium* oocysts, but negative for *Giardia* cysts (Atnafu et al., 2012). The second article reported investigation of 22 samples from Legedadi (Addis Ababa) municipal drinking water plant. All the samples were considered to contain *Cryptosporidium* oocysts 22 (100%), whereas *Giardia* cysts were found in 16 (73%) of the samples (Fikrie et al., 2008).

Although other studies from Ethiopia have attempted to associate infection risk of these parasites with a water source (e.g., Tigabu et al., 2010; Ayalew et al., 2008) with contrasting results, to the best of our knowledge further surveys for these parasites in drinking water in Ethiopia have not been conducted. In many African households, including in Ethiopia, untreated water is used for various purposes such as bathing, cooking, drinking, washing utensils and swimming, often exposing people to waterborne *Cryptosporidium* and *Giardia* (Tigabu et al., 2010; Ayalew et al., 2008).

Cryptosporidium and *Giardia* have also been detected in fresh fruits and raw vegetables in Ethiopia, with prevalences of *Cryptosporidium* spp. (13%) and *Giardia duodenalis* (8%) in Jimma (southwestern Ethiopia; Tefera et al., 2014), *Cryptosporidium* spp. (5%) and *Giardia duodenalis* (10%) in Arba Minch (southern Ethiopia; Bekele et al., 2017), *Cryptosporidium* spp. (8%) and *Giardia duodenalis* (9%) in Dire Dawa (eastern Ethiopia; Endale et al., 2018), *Cryptosporidium* spp. (6%) and *Giardia duodenalis* (7%) in Arba Minch (southern Ethiopia; Alemu et al., 2019), *Cryptosporidium* spp. (7%) and *Giardia duodenalis* (19%) in Aksum (northern Ethiopia; Kiros and Girmay, 2020), *Cryptosporidium* spp. (5%) and *Giardia duodenalis* (10.2%) in Bahir Dar (northwest Ethiopia; Alemu et al., 2020).

1.1.9 Knowledge gaps

Although there have been various investigations on *Cryptosporidium* and *Giardia* conducted in different parts of Ethiopia, most studies were performed using relatively non-specific microscopic identification techniques and very few studies used molecular characterisation to explore transmission routes. This hinders our understanding of which species of *Cryptosporidium* and assemblages of *Giardia* are circulating between humans and animals, and the extent of zoonotic transmission. Thus, our knowledge on the sources and transmission routes of these parasites remains poor due to the lack of targeted studies, and the contribution of zoonotic infection to the burden of human disease is also poorly understood. Lack of awareness, sharing the same house or frequent contact with animals, unsafe water supplies, inadequate sanitation, and poor hygiene are all factors that may increase transmission, but the relative importance of these factors remains unknown.
In addition, the contributions of fresh produce and water as transmission vehicles for *Cryptosporidium* and *Giardia* are unknown in Ethiopia and, more specifically, Tigray. Although the prevalence of infection of people and livestock with these parasites has been reported as high in some studies (de Lucio et al., 2016; Regassa et al., 2013; Alemu et al., 2011), variations in agroecological landscape, livestock management practices, and overall sanitary conditions in different parts of Ethiopia, means that results of studies done in different regions of Ethiopia, cannot necessarily be extrapolated to Tigray Region. Moreover, most of the studies conducted so far did not attempt to use a One-Health approach to investigate transmission and sources of infection with these two parasites. Although, as noted in the introduction, there are some published articles on contamination of drinking water with these parasites, there are various methodological issues with these papers that indicate that further investigation would be of value.

These various knowledge gaps regarding *Cryptosporidium* and *Giardia* in Ethiopia generally, and Tigray specifically, led to my study being developed, as part of the MU-HU-NMBU institutional collaboration project.

1.1.10 Aims of the study

General objective: To use a One-Health approach to investigate *Cryptosporidium* and *Giardia duodenalis* in Tigray, Ethiopia.

Specific objectives:

1. To investigate the occurrence of *Cryptosporidium* and *Giardia duodenalis* in people and livestock (dairy calves, lambs, and goat kids) in predetermined study sites in Tigray and determine the risk factors for infection.
2. To investigate the species and genotypes of *Cryptosporidium* and *Giardia duodenalis* circulating in livestock (dairy calves, lambs, and goat kids) and people in the study area as indicators for zoonotic transmission.
3. To assess the likely role of water sources and fresh produce as potential transmission routes for *Cryptosporidium* and *Giardia* infections in the study districts in Tigray.

2 Material and Methods

In this section, methodological considerations are presented rather than detailed materials and methods as these are already provided in the articles.
An overview of the materials and methods used in the different articles is provided in table 3.

Table 3. An overview of the materials and methods used in the different article

Samples collected for analysis	**Procedures used in the analysis**	**Articles**
Drinking water samples (n=37) from taps (n=3), handpumps (n=15), wells (n=1) rivers/streams (n=14), and ponds (n=4)	• Filtration • Elution • Concentration • Separation and purification by immunomagnetic separation (IMS) • Detection and enumeration using immunofluorescent antibody testing (IFAT) with DAPI staining.	Paper 1: Preliminary insights regarding water as a transmission vehicle for *Cryptosporidium* and *Giardia* in Tigray, Ethiopia. Food and Waterborne Parasitology 2020; 19, e00073.
Fresh produce commonly consumed raw (n=55) available for consumption from market-places, backyard gardens and farmlands	• Elution • Concentration • Separation and purification by IMS • Detection and enumeration using IFAT with DAPI staining. • Molecular characterization by PCR and sequencing	Paper 2. Is fresh produce in Tigray, Ethiopia a potential transmission vehicle for *Cryptosporidium* and *Giardia*? Foods 2021; 10(9), 1979.

Faecal samples from calves (n=239), lambs (n=268), and goat kids (n=250).	• Detection and enumeration of parasites using IFAT with DAPI staining • Molecular characterization by PCR and sequencing • Questionnaire survey	Paper 3: *Cryptosporidium* and *Giardia* in livestock in Tigray, Northern Ethiopia and associated risk factors for infection: a cross-sectional study. Frontiers in Veterinary Science 2022; 8, 825940.
Human faecal samples from rural study sites (n=307) and paediatric patients attending clinics in Mekelle (n=58)	• Detection and enumeration of parasites using IFAT with DAPI staining • Molecular characterization by PCR and sequencing • Questionnaire survey	Paper 4: *Cryptosporidium* and *Giardia* infections in humans in Tigray, Northern Ethiopia: an unexpectedly low occurrence of anthropozoonotic transmission Acta Tropica 2022; 231, 106450.

2.1 Study Areas

Ethiopia is located in the Eastern part of Africa (Horn of Africa) between 3° and 14.8° latitude and 33° and 48° longitude, bordering Somalia, Sudan, Djibouti, Kenya and Eritrea with a total border length of 5,311km. There is a large variation in geographical features, ranging from the highest peak at Ras Dashen (4550 meters above sea level) (m.a.s.l), to the Afar Depression (110m below sea level). Over half of the country lies above 1500 m.a.s.l. The climatic zones are broadly categorized into three; the 'Qola' or hot lands (below approximately 1500 m.a.s.l with a mean annual temperature range of 29-33°C), 'Weyna Dega' (1500-2500 m.a.s.l with 16-29°C), and Dega (above 2600 m.a.s.l and a mean annual temperatures range of 10-16°C). A characteristic feature of the highlands is that they receive more rain than the lowlands, along with erratic rainfall (World Bank, 2021).

Ethiopia is made up of eleven regional states: namely Afar, Amhara, Benishangul-Gumuz, Gambella, Harari, Sidama, Southern Nations Region of Nationalities and People, Southwest Ethiopia Peoples Region and Tigray. There are two chartered administrative cities (Addis Ababa City Administration and Dire Dawa city council) (CSA, 2022).

My study was carried out in Tigray, a regional state located in the northern part of Ethiopia. The total human population of Tigray is more than 5 million people (CSA, 2007) and the majority of these inhabitants depend on agriculture for their livelihoods (mainly subsistence mixed crop and livestock farming). Data from 2021 indicate that the livestock populations of Tigray are: approximately 4.9 million cattle, 2 million sheep, and 4.9 million goats (CSA, 2021). Most part of the region's climate is characterized as semi-arid (Haftom et al., 2019). Tigray is divided into seven administrative zones, and sub-divided into 52 woredas (districts), of which 34 are rural and 18 are urban. Among these, four districts, under the thematic area of the project were chosen including: Enderta, Hintalo Wejirat, Kilte Awulaelo, and Raya-Azebo, were included in this study (Figure 4). According to the 2015 population and housing census data, the total population of the Enderta district is approximately 114,300, Hintalo Wejirat 153,500, Kilte Awulaelo 99,700, Raya-Azebo 135,870; further details on the four rural study districts can be found in Paper II.

In Tigray, drinking water sources vary between locations and among households. Some homes are connected to a public supply with tap water, but others buy tap

water from their neighbours or from communal water-distribution posts. However, most rural communities use water obtained from controlled communal handpumps (these are locked and then opened twice/three times per day at set hours), protected or unprotected wells, or springs, rivers, or ponds (CSA, 2007). In the districts included in our study, all these water sources were used, but the majority used handpumps and streams/rivers.

The majority of farmers take their fresh produce to market to sell to individual consumers (Farm Africa, 2017). Common vegetables and fruits in Tigray include lettuce, cabbage, tomatoes, green peppers, carrot, guava, avocado (CSA, 2021).

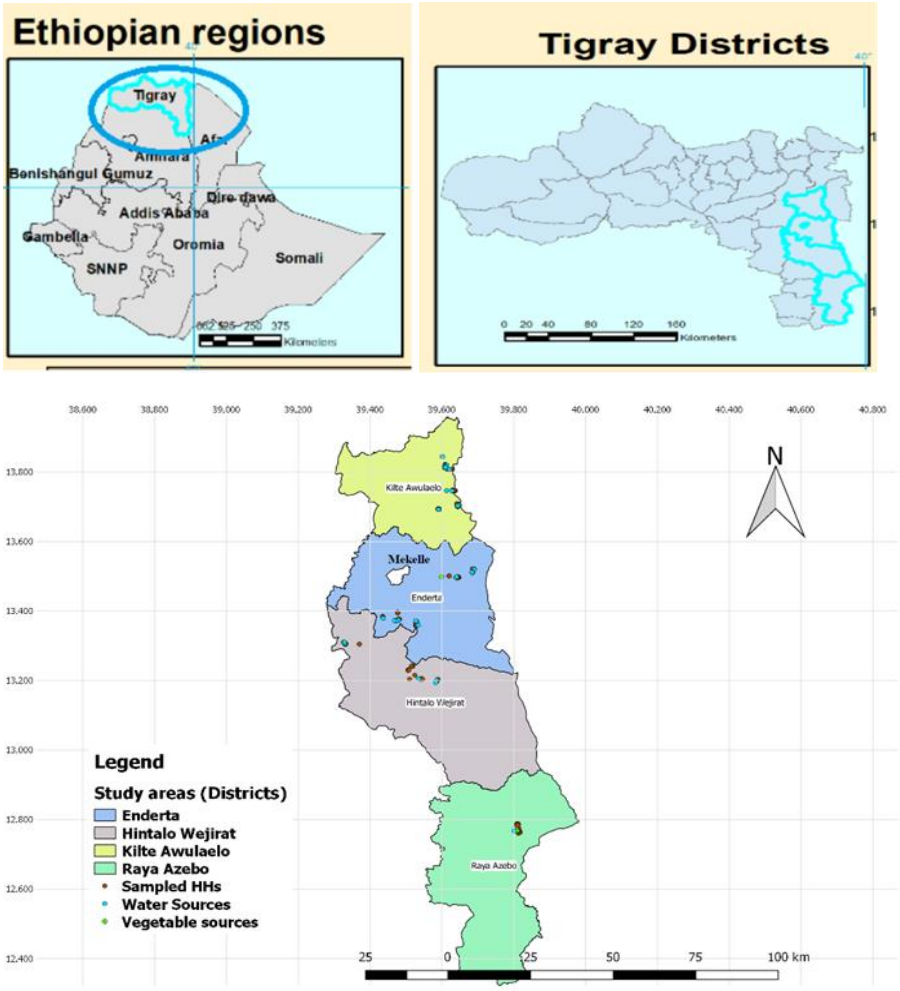


Figure 4. Location of sampling sites in the study
Credit: Habtamu Taddele

2.2. Study design and sample size

A cross-sectional study design was used to investigate contamination of water and fresh produce with *Cryptosporidium* and *Giardia* as potential transmission vehicles, as well as infections with these parasites in young ruminant livestock and people. For pragmatic reasons, convenience, rather than randomised, sampling was utilized.

Appropriate sample sizes for faecal sample investigation were determined using the formula by Thrusfield (2005), using a precision level of 5% and confidence interval of 95%. Based on previous reports in the country prevalence of 14.8 % for *Cryptosporidium* and 2.3% for *Giardia* infections in calves (Wegayehu et al., 2013; Manyazewal et al., 2017), 16.9% for *Cryptosporidium* and 2.6% for *Giardia* infections in lambs (Wegayehu et al., 2017; Ayana et al., 2015) and 11.5% for *Cryptosporidium* in goat kids; in humans 8% for *Cryptosporidium* and 11% for *Giardia* infections (Adamu et al., 2010; Flecha et al., 2015) were used in the formulae to calculate an appropriate sample size.

Samples were collected from drinking water sources according to convenience sampling (sites available, and accessible for sampling and animal owners). In addition, fresh produce samples were collected based on convenience sampling (availability of market day and market sites used by animal owners).

2.3. Sample Collection

2.3.1. Water

In rural districts of Tigray, most drinking water sources were streams and handpumps, and it was noticed that some of these were prone to contamination by human and animal faeces (personal observation). In my study areas, women are responsible for collecting water and transporting it back to their homes on their backs, occasionally with the assistance of animals. As well as the water being used for drinking (both by household members and their animals), the water collected was also used for other purposes such as washing clothes, and utensils. It was noted that although many of the rural study districts had handpumps, they were frequently inadequately maintained and often broken. In such cases, villagers used alternative water sources, such as streams or ponds (see Figure 5 for examples of water sources in the study).

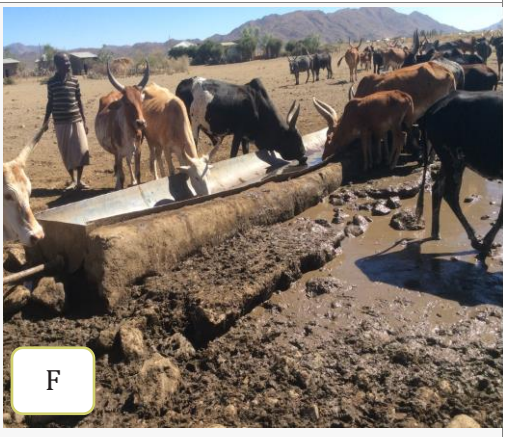
A: Stream water source at Enderta

B: Well at Kilte Awulaelo

C: Collecting pond water at Hintalo Wejirat

D: Handpump at Enderta

E: Broken handpump at Enderta



F: Animal water drinking trough at Raya Azebo

Figure 5: Water sources at the sampling locations

Photo credit: Tsegabirhan K/Yohannes and Lucy J. Robertson

2.3.2. Fresh produce

In the study districts, vegetables and fruits were available at markets (Figure 6), backyard gardens, or from farmlands. Use of animal manure as a fertiliser was common. Markets were typically held once per week, and farmers transport their produce to these markets on the backs of animals or in vehicles.
The number of fresh produce samples collected for my study was limited by the frequency of the markets. Further details on fresh produce samples are available in Paper II.

Figure 6. Vegetable market at Enderta. Note that the produce is lying on the ground, and readily accessible for contamination.
Photo credit: Lucy J. Robertson

2.3.3. Collection of faecal samples from young ruminant livestock

Animals for sampling were targeted through a door-to-door survey in all selected villages (tabia) at the study sites and all homesteads within each village were contacted. Animal owners were requested in advance not to send their animals to the field on the day of sample collection, but in some cases this was nevertheless done and the animals were not available for sampling. Within Enderta district, five sample collection sites were targeted (Dergajen, Merebmity, Chelekot, Arato, Debremarenet); for Kilte Awulaelo there were four sample collection sites (Tahtayadiksandud, Mesanu, Agula, Genfel), for Hintalo Wejirat there were two sample collection sites (Arasegeda and Dengolat), and for Raya Azebo there was a single sample collection

site (Mechare). In addition, faecal samples were obtained from calves at urban farms in Mekelle city under the advice of the local veterinary officer.

For all animals (Figure, 7), approximately 5 g of faeces were collected directly from the rectum and placed into sampling bottles. Various storage approaches and media are available for preserving faecal samples (e.g., freezing at −20 °C, ethyl alcohol, formalin, potassium dichromate), but some (such as formalin) have been associated with poor PCR amplification. Abdelsalam et al. (2017) recommend identification of *Cryptosporidium* is best performed after freezing at −20 °C as this seems to be the most suitable condition for preservation followed by potassium dichromate at 4 °C. Wilke and Robertson, (2009) recommend refrigeration in potassium dichromate or ethanol as preservation procedure of choice for molecular characterisation of *Giardia*. Thus, for this study, we chose potassium dichromate as the preservative for faecal samples. Further details are available in Paper III.

Figure 7. Goats that were sampled at Kilte Awulaelo; note the dung cakes used for fuel that are drying on the back wall.
Photo credit: Tsegabirhan K/yohannes

2.3.4. Faecal sample collection from people at rural study sites and from young children attending clinics in Mekelle

Faecal sample collection from people at the rural study sites was challenging as participants sometimes did not provide sufficient sample and sometimes none at all. As very few of the faecal samples from people at the rural study sites contained *Cryptosporidium* oocysts, and we were interested in investigating the circulating species in the human population, we conducted an additional targeted sampling from young children (target age of below 2 years) presenting at urban and peri-urban hospitals and health centres situated in and around Mekelle with diarrhoea, as this age group is more likely to suffer from *Cryptosporidium* infection. Further details are provided in Paper IV.

2.4. Laboratory analysis

2.4.1. Detection of *Cryptosporidium* and *Giardia* in faecal samples

Several tools are available for identifying *Cryptosporidium* and *Giardia* infection using a faecal sample. These include detection of the transmission stage in faeces by microscopy-based methods, detection of parasite DNA in faeces using molecular tools such as PCR, or by detecting antigens by an antibody binding method such as enzyme-linked immunosorbent assay (ELISA). In my study, a sensitive microscopy-based, antigen-binding method was used (immunofluorescent antibody test; IFAT), with characterization of the species/genotype level using molecular methods.

Faecal sample analysis

The faecal samples in my study were stored refrigerated in potassium dichromate before transport to Norway for laboratory analysis. The preservative was removed from the faecal samples by multiple wash steps before and analysis by IFAT, as described in Papers III and IV.

There are various techniques available for detecting *Cryptosporidium* oocysts and *Giardia* cysts in faecal samples, and these are often used in diagnostic laboratories. Although molecular methods (qPCR and pathogen panel kits) are beginning to predominate in medical diagnostic laboratories in many industrialised countries (Campbell et al., 2022), in Ethiopia diagnosis by microscopy remains a cornerstone,

using particular stains. Modified Ziehl-Neelsen acid-fast (mZN) staining method is simple, fast, and cost-effective for the diagnosis of *Cryptosporidium* oocysts. Oocysts stain bright red and can be confused with other structures which too stain red such as faecal debris and yeast cells, but are relatively smaller. Safranin methylene blue stains the oocysts bright orange, with sufficient colour contrast. Fluorogenic stain auramine-phenol used as an alternative to the mZN stain, it is a simple, sensitive and rapid screening procedure. The advantage of using mZN or auramine-phenol is the stained oocysts can be scraped off the slide for further DNA extraction for species identification. When a faecal concentration step is used prior to microscopy, the detection limit is improved (Khurana and Chaudhary, 2018, Ahmed and Karanis, 2018).

For identifying *Giardia* trophozoites or cysts in faecal samples, temporary stains, such as methylene blue and iodine or lugol solution are primarily used after preparation of a wet mount or concentrated smear. Some permanent stains have also been used; Giemsa stain is a readily available permanent stain for routine clinical laboratory use in which the flagella and nuclei stain reddish pink, and the cytoplasm stains grey-blue. Iron haematoxylin is also a useful staining procedure for demonstrating trophozoites and cysts of *Giardia* (Hooshyar et al., 2019).

However, all these staining methods are relatively non-specific, and we therefore used the gold standard IFAT procedure as detailed in articles III and IV.

A semi-quantitative approach was used for enumeration of parasites as an indicator of infection intensity. Infection intensity was determined based on the number of cysts/oocysts per field of view at the objective 20x according to Utaaker, (2018): +1 1–9 (oo)cysts, +2 10–50 (oo)cysts, +3 51–100 (oo)cysts, +4 >100 (oo)cysts.

2.4.2. Detection of *Cryptosporidium* and *Giardia* in water and fresh produce

Due to the low numbers of oocysts and cysts likely to occur in water samples and fresh produce, the various *Cryptosporidium* and *Giardia* detection methods described above for faecal samples are generally insufficiently sensitive or specific for water or food samples, and detection by IFAT is the standard procedure. In addition, specific concentration and purification methods need to be used to maximise the likelihood of detection.

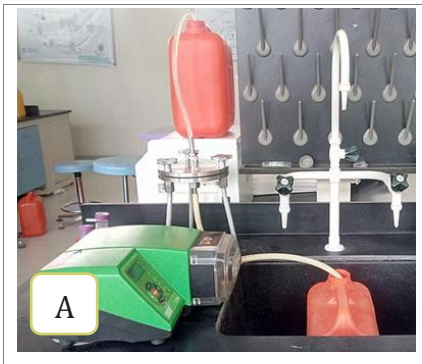
Water analysis

Standard methods have been developed to detect *Cryptosporidium* and *Giardia* in drinking water by USA government's environmental protection agency (US EPA) and the International Organization for Standardization (ISO), namely US EPA Method 1623.1 and ISO 15553 (ISO, 2006). These methods are very similar and are based on a sequence of three important steps: (1) concentration, (2) separation and isolation, and (3) detection by IFAT.

Step 1 – Concentration

Because of the low concentration of *Cryptosporidium* oocysts and *Giardia* cysts in water a concentration step is essential prior to further procedures. These include sedimentation (flocculation), flotation, or filtration (membrane filtration, cartridge filtration), often accompanied by centrifugation.

In my project, water samples were filtered through Millipore Isopore membrane filters with a pore size of 2 µm at Mekelle University, using a Watson-Marlow 520 Bp Profibus pump (Figure 8). Following filtration, the filters were placed in 50 ml tubes with the source water and stored at 4 °C until transport to Norway for further analysis.



A: The filtration apparatus in place

B: The membrane filter after filtration of a 10 L drinking water sample

Figure 8. Filtration of water samples at Mekelle University, Ethiopia. Note the extreme density of the contaminant particles in B.
Photo credit: Tsegabirhan K/Yohannes

Step 2 - Separation/isolation

As the concentration procedure will also concentrate particles that may inhibit further detection, a subsequent isolation or separation procedure is important. Although flotation has been used, immunomagnetic separation (IMS) is much more specific. IMS uses magnetic beads coated with antibodies against specific surface antigens (probably glycoproteins) occurring on the surfaces of *Cryptosporidium* and *Giardia* (oo)cysts. *Cryptosporidium* or *Giardia* (oo)cysts in a sample bind to these magnetic beads and can thus be separated from extraneous material by using a magnet. The magnetic bead complex is then detached from the (oo)cysts) by physical agitation (vortexing). Rebinding is prevented by using hydrochloric acid, which is then neutralized with NaOH after transfer to a microscope slide, and before fixing and staining.

Step 3 - Detection

The dried samples were fixed with methanol and stained with FITC-conjugated monloclonal antibodies (mABs) against *Cryptosporidium* and *Giardia* (oo)cyst walls Aqua-Glo, Waterborne Inc, NO,USA) and nuclei were stained with DAPI. A fluorescence microscope (Leica DMLB) with appropriate filters (FITC and DAPI) and Nomarski optics was used to screen the stained samples immediately after staining.

Fresh produce analysis

The standard ISO Method for analysing fresh produce (ISO 18744, 2016) is very similar to the ISO Method for water (ISO 15553, 2006), except the first steps are elution of *Cryptosporidium* and *Giardia* (oo)cysts from the surface of fresh produce by washing in a detergent solution, followed by concentration of the elute by centrifugation. The next steps, isolation by IMS and identification of the (oo)cysts by IFAT and DAPI staining using fluorescence microscopy are identical to those of ISO 15553. Due to the expense of this method, we used a modified method that has been validated by an interlaboratory trial (Utaaker et al., 2015) and found to provide equivalent results. However, it is considerably cheaper than the ISO 18744 method as it uses a smaller volume of magnetic beads in the IMS step (20 µl of each bead type and buffers that are complementary to those provided in the IMS kit. In my project, 16 samples of fresh produce from one district were processed according to the ISO

18744 (ISO, 2016) protocol, but all other samples of fresh produce were processed according to the reduced-cost protocol of Utaaker et al., (2015).

2.5. Molecular characterization

In my project, it was not possible to try to undertake molecular characterisation in all samples in which *Cryptosporidium* and/or *Giardia* were detected. Thus, a pragmatic choice was made based on the number of parasite transmission stages detected and the presence of nuclei as visualised by staining with DAPI.
In this project, water analysis showed low numbers of parasites and absence of nuclei, as indicated by the absence of DAPI staining, thus DNA isolation and molecular characterisation were not performed. For fresh produce samples, DNA was isolated from the samples with the highest number of *Giardia* cysts (71 cysts, 28 cysts, and 11 cysts) as well as the two *Cryptosporidium*-positive samples.

2.5.1. DNA isolation

There are various commercially available DNA isolation methods. For this study we used DNeasy PowerSoil kit, which uses a bead beater. This is particularly important for extracting DNA from inside a robust, protective shell such as a cyst or oocyst wall. Samples are mixed with grinding beads and buffer in a tube that is rapidly agitated for some time (Gibbons et al., 2014). One potential problem with this method is that there may be DNA degradation and the released DNA may require further purification prior to use in PCR (Liu, 2009; Vaidya et al., 2018). However, this did not seem to be a particular problem in our samples. Detailed procedures on DNA extraction from the samples are available in Papers II, III and IV.

2.5.2. Polymerase chain reaction (PCR)

Molecular characterisation for species/sub-type identification involves amplification of specific gene fragments that show variation between species or sub-types, and then comparing the sequences. There are various methods of comparison, such as by fragment size or by comparing fragment length after digestion with selected enzymes. In my project, Sanger sequencing was used. For *Cryptosporidium*, primers targeting sections of the *SSU rRNA* gene and *gp60* gene for *C. hominis* and *C. ubiquitum* were used. Four commonly targeted genes for *Giardia* genetic identification of assemblages were utilised for this study: beta-giardin (*BG*) gene, glutamate dehydrogenase (*GDH*)

gene, small subunit ribosomal ribonucleic acid (*SSU rRNA*) gene, and triosephosphate isomerase (*TPI*) gene (Read et al., 2002; Caccio et al., 2008; Lalle et al., 2005; Sulaiman et al., 2003). Multi-locus sequence typing (MLST) of *Giardia* assemblages A and B was performed: three genes were used to subtype assemblage A samples: DNA repair and recombination gene (*RHP26*), high cysteine membrane protein (*HCMP*), and NEK kinase gene (Ankarklev et al., 2018) and assemblage B isolates was performed using the suggested genetic loci, 6-phosphogluconate dehydrogenase (*pdg*), Hypothetical protein, and phosphorylase B kinase gamma catalytic chain (*phkg2*) (Seabolt et al., 2021). The PCR conditions and primers are detailed in Papers II, III, and IV. The PCR products were separated in 2% agarose gels, using SYBERsafe DNA gel stain and visualized under UV illumination. A 100 bp ready-to-use DNA ladder (Thermo Scientific) was used for fragment size determination.

2.5.3. Sanger sequencing

PCR amplicons were purified using either ExoSAP-IT PCR product clean-up reagent (Thermofisher Scientific) or PureLink Quick Gel extraction and PCR purification Combo kit (Thermo Fisher Scientific) and sent to a commercial company (Eurofins Genomics, Germany) for sequencing in both directions. For some samples for which the sequences were of poor quality, gel purification was performed but this did not improve the result. Geneious Prime software was used to check sequences, and contigs were formed before comparing them with those in GenBank using the NCBI BLAST tool, as well as a recently developed online tool, Cryptogenotyper, for *Cryptosporidium* using *SSUrRNA* and *gp60* (Yanta et al., 2021).

2.6. Questionnaire Survey

A pre-tested questionnaire was used to collect data on the study animals, their owners, and management practices. The questionnaires were completed during individual interviews with animal owners. The questionnaires are appended to Papers III and IV.

2.7. Statistics

Descriptive statistics were used to investigate and visualise occurrence data. To assess the relationship between risk factors for *Cryptosporidium* and *Giardia* infection, a database was created, and Chi-square tests and Fisher's exact tests were

used. Candidate factors for a multivariate model were variables that were significant at $P < 0.05$ in the above tests. After that, a backward stepwise logistic regression was employed to find possible *Cryptosporidium* and *Giardia* predictors.

2.8. Ethical considerations

This study was approved by Ethical Committees in both Ethiopia and Norway: the Ethiopian National Research Ethics Review Committee (Ref. No: MoSHE/RD/144/1095/19) and the Norwegian Regional Committee for Medical and Health Research Ethics, South East (REK, SE, case number 2018/1279 C) approved the study. All subjects provided written informed consent, and a parent or guardian of any child participant provided their informed consent on their behalf. Informed consent from patients was not required for samples collected from health centres, as the stool samples had been provided for routine clinical diagnostic procedures; questionnaire information associated with these patients was not collected. Information from all participants was anonymized prior to analysis of the data.

3 Results

The details of the Results are provided in the articles, and here the main points from the articles, as well as some additional information not provided in the published articles, are presented.

3.1 Occurrence of *Cryptosporidium* and *Giardia* in water (based on Paper I)

In the rural districts of Tigray region, there had previously been no monitoring of contamination of drinking water with protozoans. From the 37 water samples collected and analysed, contamination with either parasite was found in 19% of sample (Paper I), with respective pathogen occurrence being 5% for *Cryptosporidium* and 16% for *Giardia* (Table 4). The highest number of *Giardia* cysts (22) per 10 L of water was found in Enderta district. *Cryptosporidium* oocysts were detected in only two samples: one from Enderta with one oocyst and one sample from Kilte Awulaelo with three oocysts. Contamination of water by *Cryptosporidium* and *Giardia* in Enderta was significantly higher than from the other locations ($P = 0.0113$). The absence of intact parasites in the samples precluded attempts at molecular characterization.

3.2 Occurrence of *Cryptosporidium* and *Giardia* in fresh produce (based on Paper II)

Contamination of fresh produce with *Cryptosporidium* or *Giardia* was found in only two districts, Enderta and Kilte Awulaelo. In Enderta, *Cryptosporidium* and *Giardia* were found in 4% and 13% of samples, respectively. In Kilte Awulaelo, the equivalent data were 10% and 14%. Among all fresh produce samples, a cabbage sample from Enderta was found to be most highly contaminated with 71 *Giardia* cysts per 30 g of cabbage, followed by a lettuce sample from Kilte Awulaelo with 28 cysts per 30 g and a guava sample from Kilte Awulaelo with 11 *Giardia* cysts and three *Cryptosporidium* oocysts per guava (weight of approximately 50 g) (Paper II). Neither carrots nor tomatoes were found to be contaminated with these parasites. Molecular identification was attempted on the three samples with the highest number of *Giardia* cysts (71, 28, and 11 cysts) and from the two *Cryptosporidium*-positive samples.

Useful sequences was obtained only from the lettuce sample in which 28 *Giardia* cysts were detected, and revealed the contamination was with assemblage A.

Table 4. Occurrence of *Cryptosporidium* and *Giardia* in different sample types by sample site

Sampling site		Water	Fresh produce	Calf faecal samples	Lamb faecal samples	Goat kid faecal samples	Human faecal samples
Enderta	N	15	24	53	73	67	69
	% *Cryptosporidium* positive	6	4	11	16	6	9
	% *Giardia* positive	27	13	47	44	19	35
Hintalo Wejirat	N	7	5	49	73	56	43
	% *Cryptosporidium* positive	0	0	4	4	0	7
	% *Giardia* positive	0	0	27	23	30	21
Kilte Awulaelo	N	13	21	48	63	60	77
	% *Cryptosporidium* positive	8	10	21	5	5	4
	% *Giardia* positive	0	14	44	32	20	30
Raya Azebo	N	2	5	58	59	67	60
	% *Cryptosporidium* positive	0	0	5	12	5	5
	% *Giardia* positive	0	0	38	29	16	25
Mekelle	N	Not included in water and fresh produce analysis		31	No sampling from urban lambs or goat kids		58
	% *Cryptosporidium* positive			13			5.2
	% *Giardia* positive			35			13.8
TOTAL	N	37	55	239	268	250	307
	% *Cryptosporidium* positive	5	5	10	9	4	6
	% *Giardia* positive	16	11	38	32	21	26

3.3 Occurrence of *Cryptosporidium* and *Giardia* in livestock (based on Paper III)

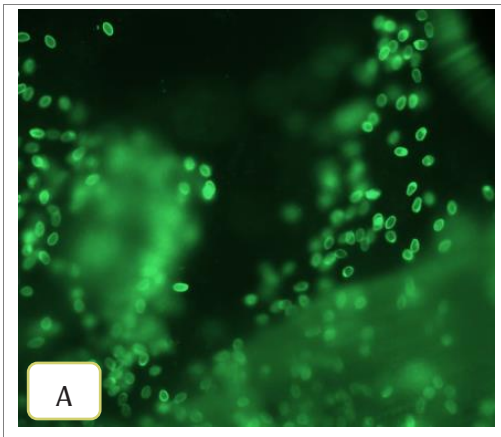
Examination of livestock faecal samples revealed the occurrence of *Cryptosporidium* and *Giardia* (oo)cysts in samples from all three animal species from all four districts, with *Giardia* infection occurring considerably more often than *Cryptosporidium*

infection (Table 4). As with water and fresh produce samples, the parasites occurred more frequently in livestock samples collected from Enderta than in the other sampling sites; whereas a statistically significant difference was found among the districts (P = 0.015) regarding the occurrence of *Cryptosporidium* among livestock samples, for *Giardia* the difference between the four districts was not significant.

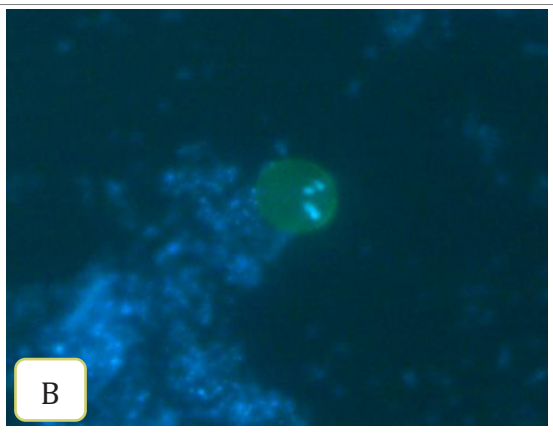
In terms of intensity of shedding, which we considered as a reflection of intensity of infection, most animals had low parasite shedding (+1) (Table 5).

Table 5. Intensity of *Cryptosporidium* and *Giardia* infection in calves, lambs, and goat kids as reflected by categories of parasite numbers detected in faecal samples

Animals	Proportion of samples with different (oo)cyst shedding categories					
	Cryptosporidium oocysts			*Giardia* cysts		
	+1	+2	+3	+1	+2	+3
Calves	95% (20)	0% (0)	5% (1)	79% (64)	14% (11)	7% (6)
Lambs	88% (22)	8% (2)	4% (1)	91% (78)	9% (8)	0% (0)
Goat kids	90% (9)	10% (1)	0% (0)	96% (51)	4% (2)	0% (0)



A: High intensity of *Giardia* shedding in a faecal sample from a child from Mekelle (magnification X 200)



B: *Giardia* cyst with DAPI-stained nuclei from a calf (magnification X 400), with zoom following photo acquisition

Figure 9: *Giardia* cysts in faecal samples collected in this study
Photo credit: Tsegabirhan K/yohannes and Lucy J. Robertson

3.4 Occurrence of *Cryptosporidium* and *Giardia* in people (based on Paper IV)

Analysis of 307 stool samples collected from people in the rural districts revealed the presence of *Cryptosporidium* oocysts in 6% (18/307) and *Giardia* cysts in 26% (79/307) of the samples (Table 4).
Again, Enderta was found to be the location where infections with these parasites were most frequently detected (Table 4).

Fifty-eight stool samples were collected from children with diarrhoea (57 of the children were ≤ 2 years and one child was 33 months old), attending seven different health centres in and around Mekelle. Among these samples, *Cryptosporidium* oocysts were detected in three of the children, giving an overall proportion of positive samples for *Cryptosporidium* 5.2% (3/58) and *Giardia* was detected in eight samples (13.8%).

The intensity of infection with *Cryptosporidium* and *Giardia* infection was low (+1) on the majority of stool samples except for few samples with above (+2) (oo)cysts (Figure 9).

3.5 Molecular characterization of *Cryptosporidium* and *Giardia* in livestock and human faecal samples (based on Paper III and IV)

Of the 291 *Cryptosporidium* and/or *Giardia* positive samples from the rural as well as urban calf samples, species determination was attempted on 98, and of these 61 were successfully sequenced such that species could be identified (Table 6). All sequences were deposited in GenBank, and their Accession Numbers are provided in Papers III and IV. The *C. ubiquitum* samples from calves, lambs, and goat kids were subtyped at the *gp60* gene and found to belong to the XIIa family. Subtyping of assemblage A *Giardia* from calves revealed sub-assemblage AI.

Among samples from people from the rural districts, of the fifteen *Cryptosporidium*-positive samples, only one was suitable for molecular characterization and revealed *C. ubiquitum*, subtype XIIa (Table 6). In addition, all three *Cryptosporidium*-positive stool samples from children attending clinics with diarrhoea were *C. hominis,* with subtypes IdA17 (one sample) and two samples with IbA9G3 (Table 6).

We selected 33 *Giardia*-positive samples from people for molecular characterization based on the intensity of infection from (+1 to +4) and visualisation of nuclei by DAPI staining. Sequence results were obtained from twenty-five of these samples at one or more genes. Three *Giardia* isolates were assemblage A (AII), and the others were all assemblage B, with subtyping revealing both BIII and BIV (Table 6).

Molecular characterization was attempted for eight *Giardia* isolates from samples collected from children with diarrhoeic stools at clinics and was successful for five of these, all of which were found to be assemblage B (Table 6).

Table 6. Species of *Cryptosporidium* and *Giardia* assemblages detected in ruminant livestock and human faecal samples

Host	**Number of isolates from which good quality sequences were obtained**	***Cryptosporidium* spp.**	***Giardia***
Calves	*Cryptosporidium* (12) *Giardia* (24)	*C. ubiquitum* (1) *C. bovis* (3) *C. ryanae* (7) *C. andersoni* (1)	Assemblage A (3) Assemblage B (1) Assemblage E (20)
Lambs	*Cryptosporidium* (5) *Giardia* (12)	*C. ubiquitum* (3) *C. xiaoi* (2)	Assemblage E (12)
Goat kids	*Cryptosporidium* (2) *Giardia* (6)	*C. ubiquitum* (1) *C. ryanae* (1)	Assemblage E (6)
People from rural districts	*Cryptosporidium* (1) *Giardia* (20)	*C. ubiquitum* (1)	Assemblage A (3) Assemblage B (17)
Children attending clinics with diarrhoea	*Cryptosporidium* (3) *Giardia* (5)	*C. hominis* (3)	Assemblage B (5)

3.6 Risk factors for *Cryptosporidium* and *Giardia* in livestock (based on Paper III)

Risk factors for *Cryptosporidium* infection were assessed in calves, lambs, and goat kids. During periparturient care, calves staying with cows were significantly more likely to be infected with *Cryptosporidium* than calves staying in their pen alone or

with other calves (P = 0.01). A significantly higher occurrence of *Cryptosporidium* infection was found in lambs housed with adults and other animals (16%) than lambs kept with ewes in their pen (5%) P = 0.002. The only significant difference was that the occurrence of *Cryptosporidium* in goat kids housed with adults and other animals (9%) was higher than in goat kids kept with does in their pen (1.3%) P = 0.01. Calves weaned at 6 months or less were more likely to have *Cryptosporidium* infection than calves weaned at older than 6 months (P = 0.001), and calves fed with concentrates were more likely to have *Cryptosporidium* infection than calves on pasture and hay (P = 0.02). *Cryptosporidium* infection was significantly more likely to be observed in calves reared under intensive management systems than extensive and semi-intensive systems, and crossbreeds were more likely to be infected with *Cryptosporidium* than local breeds.

Risk factors for *Giardia* infection were assessed in calves, lambs, and goat kids. No significant difference was observed in calves with respect to the periparturient care, weaning age, type of feed supplement, type of management system, and breeds. However, lambs, but not goat kids, kept under semi-intensive management had a significantly higher occurrence of *Giardia* infection (44%) than lambs reared in extensive management (29%). In addition, lambs housed with ewes and other animals were more likely to be infected with *Giardia* than lambs kept with ewes in their pen (P = 0.002).

3.7 Risk factors for *Cryptosporidium* and *Giardia* in humans (based on Paper IV)

The occurrence of *Cryptosporidium* and *Giardia* was significantly higher in the younger age groups and for *Cryptosporidium,* infection was more likely to be detected in females than males (Paper IV).

Risk factors of *Giardia* infection were obtaining drinking water from streams rather than from hand pumps and ponds. Furthermore, *Giardia* infections were significantly more common (P = 0.02) in individuals drinking untreated water than in those who drinking treated water.

In the samples from children attending clinics with diarrhoea, a higher rate of *Giardia* infection was found in children over 1-year old than in children ≤1 year old (P = 0.02).

4 Discussion

In my PhD study, a One-Health approach was used to investigate the occurrence and transmission routes of *Cryptosporidium* and *Giardia* in Tigray region, Ethiopia, with emphasis on exploring the role of domestic ruminant infections and contaminated water and fresh produce as potential transmission vehicles of these parasites to humans. The major findings were widespread occurrence of *Cryptosporidium* and *Giardia* in both people and livestock, and an apparent lack of the common zoonotic species or subtypes, and thus zoonotic transmission, despite the close contact between people and livestock in the rural study sites. Potential roles for drinking water and fresh produce as vehicles of infection were identified.

4.1 Water and fresh produce as sources of *Cryptosporidium* and *Giardia*

Although lower-income countries are less likely to have sophisticated infrastructure regarding sewage disposal and water treatment, and thus may be expected to report more waterborne outbreaks of disease, the majority of waterborne outbreaks have been reported from developed countries. In fact, according to Aldeyarbi et al., (2016) no outbreaks of cryptosporidiosis have been reported in Africa (by any transmission route). Furthermore, according to Ahmed et al., (2018) no outbreaks of protozoan disease transmitted by water have been reported in Africa. Despite the difficulty in determining the extent of waterborne cryptosporidiosis transmission in Africa, a water, sanitation, and hygiene (WASH) perspective is a key component in limiting the spread of diarrheal pathogens.

Diarrhoeal disease outbreaks are usually identified by medical practitioners who identify a rise in cases of diarrhoeal disease above background levels, when diarrhoeal disease, of whatever aetiology, occurs frequently, then recognising an outbreak can be challenging. However, as noted by Ahmed et al. (2018), many risk factors associated with waterborne transmission occur in African countries, and the few studies that have been conducted regarding the occurrence of parasites in water sources in Africa indicate that they do occur; an overview of some of these studies and their results is provided in Siwila et al., (2020). However, it should be noted that sampling and detection methodologies vary widely between studies, from basic

microscopy studies to molecular methods, and it seems likely that not all data are equally trustworthy. For example, a photograph of a *Cryptosporidium* oocyst from one article describing research from Cameroon (in which *Cryptosporidium* oocyst concentrations of up to 2720 oocysts per L are reported), does not appear to show a *Cryptosporidium* oocyst (Ajeagah et al., 2007).

In my study, contamination of water with *Cryptosporidium* spp. and *G. duodenalis* was 5% and 16%, respectively. There is a scarcity of data regarding water contamination with these parasites in Ethiopia. Among the few articles published, Atnafu et al. (2012) investigated treated water storage tanks and tap water using (US EPA, 2005) method in which 10 litres samples were analysed and also investigated viability using the inclusion and/or exclusion of the fluorogenic dyes, DAPI and propidium iodide (PI). Viable oocysts were DAPI + and PI-, oocysts were considered nonviable with PI+. *Giardia* cysts were considered viable with the exclusion of PI, (PI-), while inclusion of PI (PI+) indicated the cyst was non-viable. However, as the researchers dried the sample to the slide, then the results obtained must be interpreted with caution (Petersen and Enemark, 2018). They reported that 35% of samples from water storage tanks were contaminated with *Cryptosporidium* oocysts (none were considered viable), and 29% were contaminated with *Giardia* cysts (only one cyst deemed viable in one case). In the same study, *Cryptosporidium* oocysts were detected in 15 tap-water samples (21%), five of which were claimed to include viable oocysts, while *Giardia* cysts were reportedly detected in twelve tap water samples (17%). Concentrations of oocysts and cysts in the positive samples were not reported.
In addition, Fikrie et al. (2008) reported 100% contamination of all the 22 analysed samples with *Cryptosporidium*, with concentrations ranging from 1 to 7 and 33 to 53 oocysts/L in the treated and untreated water samples, respectively. *Giardia* was identified in 73% of the samples, with concentrations ranging from 0 to 3 and 13-20 cysts/L in both treated and untreated samples. These are much higher occurrences than in my study, despite being from water sources that may be considered cleaner, and the reason for this difference could be contamination from the sewage sources (Fikrie et al., 2008; Atnafu et al., 2012). However, given the number of inaccuracies in the reporting of this research, it seems likely that there was some methodological confusion in these studies.

In contrast, a study by Wolde et al. (2020) detected no parasites in 2976 water samples of 11 L of water from public tap water, service reservoirs, springs and wells of municipal drinking water in Addis Ababa city, Ethiopia. The analyses in the study

of Wolde et al (2020) were done by filtration, sedimentation and direct microscopy of the sediment and the lack of findings in their study is probably due to the use of less sensitive techniques and inappropriate detection methods for water samples.

The extent of contamination of fresh produce with *Cryptosporidium* and *Giardia* at our study sites was similar to previous reports from other parts of Ethiopia (Alemu et al., 2020; Bekele et al., 2017; Endale et al., 2018). Existing literature shows only one study from Tigray (Kiros and Girmay, 2020) which, in fact, reported a higher occurrence of the parasites (7.3% and 18.5% respectively for *Cryptosporidium* and *Giardia*) on fresh produce from markets in the town of Aksum compared to the current finding. Although other factors could not be excluded for the difference, the study by Kiros and Girmay (2021) used traditional method of microscopy for detection, which is less specific and hence may yield more false positive findings compared with the IFAT technique employed in the current report.

That contamination of fresh produce and water were found only in samples obtained from Enderta and Kilte Awulaelo in my study is interesting. Although this could be due to sampling bias or that fewer samples were obtained in other districts, it could also indicate that some communities had greater circulation of these parasites than others. In many rural parts of the country, open defecation near to rivers that are used for irrigation purposes is common, and this can result in contamination of vegetable farmlands with enteric parasites. In addition, natural fertilizer (animal excreta) is commonly used by farmers to improve crop growth (Alemu et al., 2020). It would have been of interest to explore further whether this apparent pattern was a methodological artefact or did in fact reflect more opportunities for contamination at particular locations. However, this was not possible within the scope of the project resources. It may be worth noting, however, that we noticed at Enderta, where water contamination was highest, that public handpumps were inadequately maintained such that the communities were obliged to use other water sources (see Figure 5 in Methods section of this thesis). This could mean that water sources in use were not well protected in this location.

4.2 *Cryptosporidium* and *Giardia* infection in livestock and people

In the study areas, both parasites were common among livestock, with calves having a higher occurrence of the parasites than lambs and goat kids. This is consistent with previous data, including a study by Vermeulen et al. (2017) that indicated that intensively managed calves are the most common source of oocysts compared with other livestock species. In our study, lambs were more likely to be infected with both parasites than goat kids. This difference could reflect that goats are browsers, and thus the likelihood of ingesting the parasite transmission stages from the ground is lower than for grazing species (Utaaker et al., 2021).

In the rural districts, we found that *Cryptosporidium* occurred less commonly than *Giardia* in livestock, and a similar pattern was observed in Mekelle's urban farms. Although similar findings have been reported in other studies from Europe and elsewhere (e.g., Geurden et al., 2008; Paz e Silva et al., 2014), previous reports from Ethiopia have tended to document higher prevalences of *Cryptosporidium* infection than *Giardia* among livestock (Ayana and Alemu, 2015; Abebe et al., 2008; Wegayehu et al., 2013; Wegayehu et al., 2013; Manyazewal et al., 2017; Regassa et al., 2013). Although we cannot exclude this difference being related to differences in management practices, transmission possibilities, host susceptibility, and other factors that will influence the occurrence of both parasites between studies, it seems likely that detection methods may be of importance. Most of the previous studies from Ethiopia used mZN for detection of *Cryptosporidium*; although this method can be valuable, it has been documented to result in false-positive results (Chang'a et al., 2011), and these authors commented that results obtained from studies of *Cryptosporidium* in cattle, for which mZN was the sole analytical method, should be treated with caution. In contrast, the methods most commonly used in the Ethiopian studies for detecting *Giardia* in livestock faeces used microscopy of direct wet mounts with iodine staining; this method can be useful, but often has limited sensitivity and, in particular, fails to detect empty *Giardia* cysts. Thus, as my study used IFAT for

detection of both *Cryptosporidium* and *Giardia*, it seems likely that the methodological advantage of this method is likely to be of importance.

The molecular characterisation of *Cryptosporidium* and *Giardia* isolates found in my study revealed a low occurrence of the zoonotic species despite the close contact between human and animals. In both livestock and human samples, the zoonotic species of *Cryptosporidium*, *C. parvum,* was not identified in any of the *Cryptosporidium*-positive samples investigated. This result is consistent with reports by Robertson et al. (2020) and Yang et al. (2021), who concluded that *Cryptosporidium* transmission is mainly human to human in Africa and in low- and middle-income countries, respectively. In addition, in human *C. parvum* infections in various sub-Saharan African countries, the *gp60*-subtype family (*C. parvum anthroponosum*) often predominates. This is a human-adapted subtype occurring almost exclusively in human infections. A recent systematic review and meta-analysis investigating geographical distribution of this subtype in human infections found its occurrence was associated with countries with low gross domestic product (GDP) per capita and poor sanitation (King et al., 2019).

The *C. hominis* detected from children with diarrhoea from Mekelle in this study also supports the comments by Robertson et al. (2020) and Aldeyarbi et al. (2016) that the predominant *Cryptosporidium* species infecting people in many studies from Africa is *C. hominis*, and also supports the finding of Johansen et al., (2022), that *C. hominis* is the main species of *Cryptosporidium* infecting people in Ethiopia, with the subtype families identified as Ia, Ib, Id, and Ie, as the sub-types of *C. hominis* found in my study were Ib and Id. In addition, Johansen et al. (2022) reported the anthroponotic subspecies *C. parvum anthroponosum* IIc.

My finding of *C. ubiquitum*, subtype XIIa, in calves, lambs, goat kids, and humans supports the hypothesis that this species of *Cryptosporidium* should be of public health concern, due to its wide geographic distribution and broad host range (Li et al., 2014). To the best of our knowledge, this is the first report of this subtype in humans

from Ethiopia although it has been previously reported from Nigeria, Ghana, and Tanzania (Molloy et al., 2011; Krumkamp et al., 2021).

Other species of *Cryptosporidium* reported from livestock in Ethiopia include *C. bovis, C. parvum, C. andersoni,* and *C. ryanae* in cattle (Tarekegn et al., 2021) and *C. ubiquitum* in sheep (Wegayehu et al., 2017). It is interesting that in my study, all these species were detected, except for, as previously mentioned, *C. parvum*. However, we did find other species that had not been reported in the studies of Wegayehu et al., (2016, 2017) or Manyazewal et al., (2017). These include *C. ubiquitum* in calves, *C. xiaoi* in lambs, and both *C. ubiquitum* and *C. ryanae* in goat kids. That these species have not previously reported from livestock in Ethiopia probably reflects that there have been few molecular studies characterizing this parasite in livestock infections in Ethiopia.

The majority of the *Giardia* assemblages detected in livestock in my study were assemblage E, which supports the findings of Sprong et al. (2009), as well as many other studies from different regions of the world, who have found assemblage E predominating in livestock. Although this assemblage is generally considered not to occur in human infections, a few reports have been published that purport to demonstrate the contrary. For example, Garcia-R et al. (2021) reported assemblage E in humans from New Zealand for the first time, and Zahedi et al. (2017) and Fantinatti et al. (2016) have also reported assemblage E in human samples from Australia and Brazil, respectively.

In addition, children who resided in rural villages in Egypt with high densities of cattle were reported to be shedding assemblage E *Giardia* cysts (Abdel-Moein and Saeed, 2016). Despite the suggestion by these authors that their findings demonstrate zoonotic transmission from domesticated farm animals to humans, the virulence and host-infection potential of this assemblage has yet to be conclusively demonstrated, and many studies, including ours, indicates that zoonotic transmission seems not to occur. For example, in my study, despite there being widespread infection of livestock (calves, lambs, goat kids) with *Giardia*, and molecular characterisation indicating exclusively assemblage E, no shedding of assemblage E cysts was detected among farmers, despite their frequent close contact with their livestock. It is also of interest

that although the three assemblage A isolates from calves in my study were subtyped as AI, the three assemblage A *Giardia* isolates identified in human samples were subtyped as AII, again supporting lack of zoonotic spread and indicating separate transmission cycles. Previous *Giardia* genotyping studies conducted among human isolates from different parts of Ethiopia have identified assemblages A (AII), BIII, and BIV (Flecha et al., 2015; de Lucio et al., 2016), and we also identified all three of these subtypes, with BIV predominating. Although we did find shedding of assemblage B *Giardia* cysts from both a calf and its owner, I am cautious in suggesting that this demonstrates zoonotic transmission given that it is only one potential example, and we cannot prove infection rather than carriage in the calf.

Among my study areas, Enderta not only had the highest occurrence of *Cryptosporidium* and *Giardia* contamination in water and fresh produce, but also the highest occurrence of *Cryptosporidium* and *Giardia* infection among both livestock and people compared with the other districts. This suggests a higher potential for the spread of the parasites among animals, humans, and the environment in this district, and suggest that Enderta could be an 'epicentre' for both parasites in Tigray. If investigations through a One-Health approach could identify the features or factors in Enderta that indicate the reason for this apparent high circulation of these parasites, then appropriate control measures could be implemented. Such measures could have a significant impact on limiting transmission of the parasites in this area.

In my study various factors seemed to be associated with an increased likelihood of infection. That calves staying with cows had a significantly higher *Cryptosporidium* infection than calves in their pen alone or with other calves is similar to results of a study by Manyazewal et al. (2017). This could be due to transmission from cows to calves, either directly or via contamination of pens or water troughs. That lambs and goat kids living with other animals were more likely to be infected with *Cryptosporidium* than lambs and goat kids in their pen alone could be due to overcrowding resulting in a greater infection pressure. Similarly increased infection of lambs with *Cryptosporidium* and *Giardia* under semi-intensive management compared with those in extensive farming, probably reflects greater infection

pressure due to higher animal density. For calves, the high *Cryptosporidium* infection rate in calves reared under intensive management systems compared with calves in extensive and semi-intensive systems also probably reflects confinement favouring high contact between animals and a more contaminated environment than in farms using extensive and semi-intensive management. However, a study by Abdullah et al. (2019) considered the potential effect of cattle breed on *Cryptosporidium* infection, with Mafriwal cattle (Sahiwal×Friesian crosses) and Jersey×Friesian crosses having higher rates of infection than the other breeds listed in the study, suggesting that particular breeds may be more susceptible to *Cryptosporidium* infection. This could also be contributory in my study where crossbreeds were more likely to be infected than local breeds, but this could also be a confounder due to the association of crossbreeds with more intensive farming.

In my study, calves weaned at 6 months or less were more likely to have *Cryptosporidium* infection than calves weaned when older than 6 months; this finding is in contrast to results reported by Manyazewal et al. (2017), who found that calves weaned at younger than 6 months were less likely to have *Cryptosporidium* infection than calves weaned at 6 months and above. Also, in our study, calves with additional feeding with concentrates were more likely to have *Cryptosporidium* infection than calves feeding on pasture and hay. We were unable to identify a reason why calves weaned at 6 months or younger, or fed with concentrates, were more likely to be infected with *Cryptosporidium,* but a greater potential for contamination of the feeding trough with faeces during concentrate feeding is one possible explanation. Furthermore, as (oo)cysts survive better when kept moist (Utaaker et al., 2017), they may stay infective for longer in concentrate feed than in dry hay that is exposed to UV and desiccation.

Among human participants in my study, the occurrence of *Cryptosporidium* and *Giardia* varied by age group, with the highest proportion of positive samples for both parasites observed in the age group ≤ 15 years. These findings are comparable to previous reports (Wielinga et al., 2008; Kumar et al., 2014; Painter et al., 2015), who reported the majority of cryptosporidiosis and giardiasis cases occur in younger age

groups. This could be related to the development of immunity after repeated infections, but older people tend to maintain better personal hygiene than children. The clear sex divide for infection with *Cryptosporidium* (women were more likely to be infected than men), but not *Giardia*, in my study is intriguing. In my opinion, this could indicate that the two parasites have rather different transmission paths in this situation, despite it being common for us to consider transmission of *Cryptosporidium* and *Giardia* to follow similar paths. For example, females are more likely than males to care for younger infants and babies, including changing nappies and assisting with toileting, and this could lead to increased exposure to *Cryptosporidium* oocysts (Hutter et al., 2020). It should be noted, however, that other studies have found that males are more likely to be infected with *Cryptosporidium* (e.g., in Zambia; Sinynagwe et al., 2020), but this could be due to zoonotic transmission and animal contact, which does not appear to be the case in our study.

As people who drank water from a stream had considerably higher likelihood of being infected with *Giardia* (but not *Cryptosporidium*) than people who drank water from a hand pump or pond, then waterborne transmission may be a main infection route for *Giardia* in this setting. Damitie et al. (2018) also reported a greater rate of *Giardia* infection in children in southern Ethiopia who drank water from open springs. In all the sampling sites in our study, open spring water sources were frequently unprotected. Drinking, cleaning (clothes, cars), and bathing and watering domestic animals were the most common uses of the sources (personal observation). These operations may put the sources at risk of contamination, although why this should be more relevant to *Giardia* transmission than *Cryptosporidium* is unclear.

In conclusion, using a One-Health approach my study was able to identify that in the study area, despite the close interaction between people and livestock, anthroponotic transmission appears to be the most probable route of infection with both *Cryptosporidium* and *Giardia*. The *Cryptosporidium* spp. detected in people were mainly human-specific species, except for one sample with the newly emerging, zoonotic *C. ubiquitum*. Similarly, the *Cryptosporidium* spp. identified in livestock were mainly host-specific for ruminants, with the exception of *C. ubiquitum.* Moreover, the

majority of *Giardia* infections in humans were Assemblage B, with a very few assemblages A (AII), whereas *Giardia* infections in livestock were predominantly caused by assemblage E, with some assemblage A (AI), and one anomalous identification of assemblage B. This latter finding could reflect a case of zoonotic transmission, but this does not indicate this being a common occurrence. The absence of *C. parvum* in the study areas suggest that livestock is unlikely to be the source of human infection in this area. Furthermore, both parasites were found in environmental samples (water and fresh produce). However, molecular characterisation of the parasites found on these samples was challenging due to low numbers and that many of the parasites lacked nuclei; only one fresh produce sample was found to be contaminated with *Giardia* of assemblage A, which is known to infect people and animals.

5 Future perspectives

Although the present study has provided information on contamination of water sources and fresh produce with *Cryptosporidium* and *Giardia* in rural areas of Tigray, the lack of molecular data prevented us from being able to identify the most likely sources of contamination (people or animals). Different approaches, such as using microbial source tracking tools (e.g., faecal source PCR markers, steroid markers, fluorescent whitening agents) in positive water samples may shed light on the most likely sources of contamination (e.g., Devane et al., 2019) and thus assist in developing appropriate interventions.

Although my results indicated generally low levels of contamination in positive water and fresh produce samples, the extent of contamination reveals real potential for transmission of *Cryptosporidium* and/or *Giardia* to either animals or people. Given the public health and veterinary burden associated with these parasites, trying to identify those factors important in reducing parasite circulation in the communities and environments deserves further investigation throughout Tigray, and may be applicable elsewhere also.

In my study, we sampled all animals in the predetermined age groups, regardless of any clinical signs as the emphasis was on investigation of zoonotic transmission. As we found that the *Cryptosporidium* spp. and *Giardia* assemblages circulating in livestock are generally of little zoonotic importance, a study with emphasis on diarrhoeic animals could be useful for identifying which species are associated with clinical disease in livestock and provide a basis for implementing appropriate control and prevention measures. This could also be of interest for making preliminary calculations of zDALY that would take into account not only the impact of these parasites on human health, but also any impacts on animal health.

My study was conducted in a particular region of Ethiopia (Tigray), but data from similar projects from different regions of Ethiopia have not necessarily concurred

with our findings, and whether our results could be extrapolated to other areas of Ethiopia is unclear. Further studies, preferably in rural children with diarrhoea, from different areas could provide pertinent information about the transmission routes of both parasites and the potential for zoonotic transmission in those areas. It is crucial to obtain such information for developing effective interventions against transmission that will be useful not only in Ethiopia, but throughout Africa as well. The relative importance of *C. ubiquitum* as a zoonotic species in Ethiopia is of interest and could be explored further by targeted sampling. In addition, it should not be overlooked that although zoonotic species and subtypes, particularly *C. parvum*, were not widely detected in our study, there is a real danger for such species expanding into these areas. The need for relevant interventions if or when this occurs should not be overlooked.

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7 Papers

Paper I

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Preliminary insights regarding water as a transmission vehicle for *Cryptosporidium* and *Giardia* in Tigray, Ethiopia

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ABSTRACT

This study was part of a larger One Health project with the aim of investigating the epidemiology of *Cryptosporidium* and *Giardia* infections among humans and animals in rural areas of Tigray, Ethiopia. Here we report on the contamination of different drinking water sources in four locations of this region with these *Cryptosporidium* oocysts and *Giardia* cysts; 19 samples were from unprotected surface water sources and 18 from protected water sources. A modified version of the standard ISO 15553 technique was used for analysis, and *Giardia* cysts were detected in 6 of the samples (16%) and *Cryptosporidium* in two (5%), with one of these samples containing both parasites. The number of *Giardia* cysts in positive samples ranged from 3 to 22 cysts per 10 L sample, and the number of *Cryptosporidium* oocysts in positive samples ranged from 1 to 3 oocysts per 10 L sample. Low numbers of parasites and absence of nuclei, as indicated by the absence of DAPI staining, precluded further molecular analyses. We found no association with contamination and whether the water source was protected or not, but there was an association with location, with one location more likely to have a contaminated sample than the others. These preliminary data suggest that this location should be in focus for further parts of this study.

1. Introduction

Deaths due to diarrhoeal disease among children in Ethiopia below the age of 5 years is calculated to have decreased by over 60% between 2005 and 2015. However, as the mortality rate in this age group due to diarrhoea is still at around 100.1 per 100,000 children, it remains considerably over the equivalent global mortality rate, which is estimated at 74.3 per 100,000 (GBD, 2017). Indeed, diarrhoea is considered to be the leading cause of mortality in Ethiopian children younger than 5-years of age, accounting for 23% of all deaths in this age group – more than 70,000 children a year (https://www.unicef.org/ethiopia/water-sanitation-and-hygiene-wash). Among the aetiologies associated with mortality due to diarrhoea in children under 5 years in Ethiopia, cryptosporidiosis was found to be responsible for 12% of cases, only surpassed by shigellosis and rotaviral enteritis, responsible for approximately 20% and 18% of cases, respectively (GBD, 2017). Studies on the occurrence of *Cryptosporidium* infections in Ethiopia

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have reported prevalence ranging from as low as 1% to as high as over 26% (Squire and Ryan, 2017); these estimates are presumably affected by patient characteristics (age group, clinical symptoms, immune status) as well as diagnostic technique used.

Another intestinal protozoan parasite that is often considered together with *Cryptosporidium*, is *Giardia duodenalis*. Although infection with *Giardia* is known as a cause of diarrhoea, the Global Enteric Multicentre Study (GEMS) that investigated aetiologies of moderate-to-severe paediatric diarrhoea (Kotloff et al., 2013), found no association between *Giardia* infection and these symptoms in young children; *Giardia* infection in children aged between 1-5 years was more commonly identified in children without diarrhoea. However, *Giardia* infection has been associated with persistent diarrhoea in school-age children in developing countries (Muhsen and Levine, 2012), and studies in Ethiopia have reported a prevalence of *Giardia* infection ranging between below 5% to over 50% (Squire and Ryan, 2017).

Cryptosporidium and *Giardia* are both transmitted to susceptible individuals via ingestion of infectious stages that have been excreted in faeces of infected people (or animals – some species or genotypes may be zoonotic). The infection route may be direct, hand to mouth, but ingestion via a contaminated transmission vehicle (food or water) also occurs commonly, and may result in large outbreaks (e.g., Widerström et al., 2014), particularly if the drinking water supply is contaminated. Although lower-income countries are more likely to have less sophisticated infrastructure regarding sewage disposal and water treatment, and thus may be expected to report more waterborne outbreaks of disease (e.g., Widerström et al., 2014), most waterborne outbreaks have been reported from developed countries (Omarova et al., 2018). Indeed, according to Aldeyarbi et al. (2016), no outbreaks of cryptosporidiosis have been reported from Africa (by any transmission route), leading these authors to question whether such outbreaks occur; furthermore, according to Ahmed et al. (2018), no outbreaks of protozoan disease transmitted by water has been reported in Africa either. As outbreaks of diarrhoeal disease are usually identified by medical practitioners identifying a rise of cases in diarrhoeal disease above background levels, when diarrhoeal disease occurs frequently, from different aetiologies, recognising an outbreak may be challenging. However, as noted by Ahmed et al. (2018), many risk factors associated with waterborne transmission occur in African countries and the few studies that have been conducted regarding occurrence of parasites in water sources in Africa indicate that they do occur; an overview of some of these studies and their results is provided in Squire and Ryan (2017). However, it should be noted that sampling and detection methodologies varies widely between studies, from basic microscopy studies to molecular methods, and it seems likely that not all data are equally reliable.

From Ethiopia, the only article that we were able to identify regarding analysing water for these parasites reports investigation of 115 samples of drinking water collected from various sources around Addis Ababa (Atnafu et al., 2012) using the standard US EPA methodology, with detection by immunofluorescent antibody testing (IFAT). Among 72 tap water samples analysed, 15 (21%) were considered to contain *Cryptosporidium* oocysts and 12 (17%) were considered to contain *Giardia* cysts; among 17 storage tank samples, 6 (35%) were considered to contain *Cryptosporidium* oocysts and 5 (29%) were considered to contain *Giardia* cysts; two raw surface water samples were positive for both parasites; and the single well water sample was positive for *Cryptosporidium* oocysts but negative for *Giardia* cysts (Atnafu et al., 2012). However, no attempt was made at molecular characterisation of the parasites.

Although other studies from Ethiopia have attempted to associate infection risk of these parasites with water source (e.g., Tigabu et al., 2010; Ayalew et al., 2008) with discrepant results, to the best of our knowledge further surveys for these parasites in drinking water in Ethiopia have not been conducted.

As part of a larger One Health study on the epidemiology of cryptosporidiosis and giardiasis among humans and animals in rural areas of Tigray, Ethiopia, we investigated the contamination of different drinking water sources in four locations.

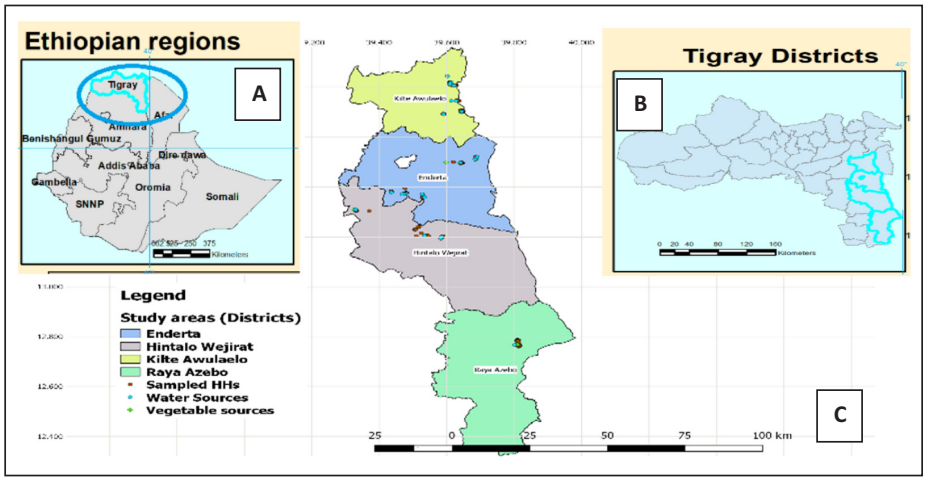

Fig. 1. Maps showing location of sampling areas. A: Map indicating location of Tigray in Ethiopia; B: Map indicating districts in Tigray; C (main map) indicating the four sampling areas.

2. Materials and methods

2.1. Sampling locations and sample collection

This project was based on four main sampling regions, Enderta, Hintalo Wejirat, Kilte Awulaelo, and Raya Azebo (see Fig. 1).

The rural area of Enderta district has around 24,600 households and an approximate total population of 114,300 (CSA, 2007). The average annual temperature in Mekelle, the main city in Enderta, is 19.1 °C and the average rainfall is 581 mm. The rural part of Hintalo Wejirat has approximately 31,000 households and the total population is approximately 141,000. The average annual temperature in Adi Gudom, the main town of Hintalo Wejirat, is 19.1 °C, and the average annual rainfall is 520 mm. The rural area of Kilte Awulaelo district has 20,222 households with a total population of 94,900. The average annual temperature in Agula (a village in Kilte Awulaelo) is 19.2 °C, and the average annual rainfall is 595 mm (https://en.climate-data.org/africa/Ethiopia/tigray). The rural area of Raya Azebo district has 27,582 households with a total population of 119,814. The area receives rainfall ranging from 300 to 750 mm, and the mean annual maximum and minimum temperatures are 25 °C and 18 °C, respectively (http://www.eiar.gov.et/mehoni).

The majority of people living in this area obtain their drinking water from unprotected water sources (streams/rivers/ponds etc.) or via communal hand pumps. In one of the districts, Raya Azebo, more water sources were unprotected (47%) than in the other three regions (21–25%) (CSA, 2007). Regarding sanitation in this area, most people do not have any access to toilet facilities and are defecating in open spaces (Table 1; CSA, 2007).

All samples were collected during the period from October 2018 until January 2019. A total of 37 samples were collected for analysis, 15 from Enderta, 7 from Hintalo Wejirat, 13 from Kilte Awulaelo, and 2 from Raya Azebo. Samples were collected into clean 10 L plastic containers and transported immediately to Mekelle University for the initial steps in the analysis. Weather during collection of all samples was consistently dry. Samples were collected from drinking water sources according to convenience sampling (sites available and accessible for sampling) and categorised as either tap water, well water, handpump, pond, or river/stream. Water sources were grouped as either untreated surface water (rivers/streams, well water, or ponds) or as protected water sources (tap water or handpump water). It should be noted that water from handpumps and tap water could have originated from unprotected surface water sources. Among the 37 samples, 14 were from rivers or streams, 4 were from ponds, and 1 was from a well (i.e., total of 19 samples grouped as untreated surface water), 15 were from handpumps, 3 were tap water, (i.e., 18 grouped as protected water sources). See Table 2 for overview of sample distribution.

2.2. Sample analysis

Standard methods for the analysis of water for contamination with *Cryptosporidium* and/or *Giardia* cysts have been developed, validated, and adopted globally. In Europe, the ISO 15553 standardized method for analyses of potable water for contamination with *Cryptosporidium* and *Giardia* is probably the most commonly used approach (ISO, 2006), and is very similar to the US EPA 1623.1 methodology (US EPA, 2012). Both these standard methods include the following steps: filtration, elution, concentration by centrifugation, separation and purification of target parasites by immunomagnetic separation (IMS), and finally detection and enumeration using (IFAT), using the fluorogenic DNA intercalator 4′,6-diamidino-2-phenylindole (DAPI) as an adjunct stage for detection of nuclei. As IMS is extremely expensive, this method is not possible for many research projects and certainly prohibitively expensive for routine monitoring in developing countries. However, a reduced cost approach has been developed and evaluated by intra-laboratory spiking studies, as well as being used with success in a project in which water samples from Northern India were analysed (Utaaker et al., 2019). The same approach was used here with the 5–7 ml sample concentrate obtained after filtration and centrifugation being further processed with IMS using a reduced volume 20 μl of each bead type (Dynabeads®: Cryptosporidium/Giardia Combo Kit, Idexx Laboratories), and the kit buffers are modified by augmenting with buffers as described in Utaaker et al. (2015). Prior to analysis of the samples, spiking experiments using this procedure were conducted with flow-sorted, commercially available *Cryptosporidium* oocysts and *Giardia* cysts (EasySeed™, TCS Biosciences, UK). Recovery efficiencies were between 30 to 40% for *Cryptosporidium* and 47 to 69% for *Giardia* (7 replicates). This is within the range considered acceptable using the ISO or US EPA methods.

For the samples in this project, the samples were filtered through Millipore Isopore membrane filters with a pore size of 2 μm at Mekelle University, using a Watson-Marlow 520 Bp Profibus pump. Following filtration, the filters were placed in 50 ml

Table 1
Sanitation facilities in sampling area (derived from: CSA, 2007).

District	Percentage of households without any toilet facility	Percentage of households with shared toilet facility (flush or latrine)	Percentage of households with private toilet facility
Kilete Awulaelo	70	6	24
Enderta	82	2	16
Hintalo Wejirat	73	3	24
Raya Azebo	78	8	14

Table 2
Sample distribution (number of samples) by location and water source.

	Enderta	Hintalo Wejirat	Kilte Awulaelo	Raya Azebo	Total
Unprotected surface water					
Ponds	0	2	2	0	4
Rivers streams	10	1	2	1	14
Well	0	0	1	0	1
Group total	10	3	5	1	19
Protected water source					
Handpump	5	3	6	1	15
Tap water	0	1	2	0	3
Group total	5	4	8	1	18
Overall total	15	7	13	2	37

centrifuge tubes that were then filled with sample water and stored at 4 °C. These sample tubes containing filters were then transported to the NMBU parasitology laboratory in Norway for the final stages of the analyses (IMS and IFAT).

In Norway, the filters were washed as according to the 15,553 protocol (ISO, 2006). The eluate was collected into 50 ml conical-base centrifuge tubes that were then centrifuged at 1690 rcf (relative centrifugal force) for 10 min. The supernatant was aspirated and the pellets concentrated into a single tube. IMS was performed as described by Utaaker et al. (2019), and the beads and any captured parasites subsequently dissociated by vigorous vortexing under acidic (0.1 M HCl) conditions. The final suspension of 50 μl was pipetted onto a single-well slide (Novakemi ab, Sweden), neutralised with sodium hydroxide, and air-dried at room temperature. The dried samples were methanol fixed before staining with FITC-conjugated monoclonal antibodies (mAbs) against *Cryptosporidium* oocyst walls and *Giardia* cyst walls (Aqua-Glo™, Waterborne™, Inc., USA). DAPI-staining was used for highlighting nuclei, both to assist in identification and for identifying samples for proposed molecular analysis for species and/or genotype identification. Samples were mounted with 1,4 diazabicyclo-octane (DABCO) antifade Mounting Medium, then each slide was covered by a glass coverslip and viewed immediately by fluorescence microscopy using a Leica DCMB microscope (20×, 40×, and 100× objectives), equipped with Nomarski differential interference contrast (DIC) optics. A blue filter block (480 nm excitation, 520 nm emission) was used for screening for *Giardia* cysts and *Cryptosporidium* oocysts labelled with the mAbs, and a UV filter block (350 nm excitation, 450 nm emission) was used for investigating DAPI staining. Morphological traits of objects reacting with the mAb were investigated further by light microscopy using DIC optics.

Each slide was scanned systematically at 20× objective, and suspect objects examined more closely at higher magnification. *Cryptosporidium* oocysts and *Giardia* cysts were enumerated according to staining characteristics and morphology.

According to laboratory protocols, samples were considered "confirmed" positive if (oo)cyst(s) exhibited typical fluorescence, with correct shape and size, and internal contents such as characteristic nuclear staining, were apparent. If the morphometry was correct and the structure had typical fluorescence, but DAPI-staining was absent and there were no discernible contents, the (oo) cysts were described as 'putative'.

2.3. Data handling

A database was created in excel and results included continuously during analysis. Contingency table analysis was used to investigate associations between occurrence of parasites in water samples and regional location and type of water source.

3. Results

3.1. Overall occurrence of Cryptosporidium and Giardia in the water samples

Among the 37 samples, parasites were detected in 7 (overall prevalence of 19%), with 5 samples being positive for only *Giardia*, 1 sample being positive only for *Cryptosporidium*, and 1 sample being positive for both parasites (16% prevalence for *Giardia*, 5% prevalence for *Cryptosporidium*). Among the samples in which *Giardia* cysts were detected, the number of cysts detected ranged from 3 cysts per sample up to 22 cysts per sample (median of 9 *Giardia* cysts per 10 L), and all of the parasites were considered putative; that is, although their morphology was correct in terms of size and shape, and their walls demonstrated characteristic apple-green fluorescence indication reaction with the mAb, none of them had visible internal contents or nuclei with DAPI staining. For the two samples positive for *Cryptosporidium* oocysts, one sample (from a river/stream in Enderta) contained a single oocyst and the other, from well water in Kilte Awulaelo, contained 3 oocysts.

The possibility of further analysis for species or genotype using molecular methods was precluded by the absence of intact *Giardia* cysts and the low numbers of nucleated *Cryptosporidium* oocysts.

3.2. Association of parasite occurrence with water source

Among the positive samples, 6 were from Enderta and 1 (*Cryptosporidium* alone in a well water sample) was from Kilte Awulaelo. Parasites were not detected among the 9 samples analysed from the other two locations (Hintalo Wejirat and Raya

Azebo). Comparison of positive samples from Enderta with those from all other locations indicates a significant association ($p = 0.0113$) between this location and the occurrence of parasites in the water supply.

As more samples from Enderta were classified as unprotected surface water (rivers/streams or ponds) than protected water sources (10 of 15 samples from Enderta; see Table 1), and the majority of the unprotected surface water samples were from Enderta (10 of 19 unprotected surface water samples; see Table 1), we investigated whether water source could be associated with whether a sample was positive or not, but no association was detected either in just Enderta or combining all locations.

4. Discussion

These data provide a preliminary snapshot of the extent of water contamination with *Cryptosporidium* oocysts and *Giardia* cysts in rural Tigray in northern Ethiopia. Although the proportion of samples positive for *Cryptosporidium* was considerably lower than previously reported from a similar study in Addis Ababa, being over 20% in Addis Ababa (Atnafu et al., 2012) compared with just 5% in our study, for *Giardia* the prevalence was similar in both studies, at around 16%. The number of parasites detected in each positive sample was not provided in the study of Atnafu et al. (2012), but as it appears that viability testing was conducted (based on exclusion and inclusion of vital dyes), it seems probable that more parasites were detected in the Addis Ababa samples than in ours. With so few data and some differences in analytical technique (for example, sucrose flotation rather than IMS was used for purification in the Addis Ababa survey), it is difficult to reach any conclusions about these differences.

Although we were unable to detect any apparent difference between the likelihood of water from different sources being contaminated, previous studies have indicated that children drinking from "protected" water supplies (handpumps) were less likely to be infected with *Giardia* than children drinking from "unprotected" water supplies (hand-dug well, spring or dam) in the Benishangul-Gumuz Region of Northwestern Ethiopia (Tigabu et al., 2010). However, unexpectedly, the same study indicated that more cases of cryptosporidiosis were detected in children using protected water sources than in children non-protected water sources (Tigabu et al., 2010). This may indicate that in this region, the transmission route for *Cryptosporidium* tends not to involve water, but may be more associated with direct transmission. Another or additional reason for this could be that the "protected" water sources are nevertheless contaminated by infectious *Cryptosporidium* oocysts. Indeed, in the Benishangul-Gumuz study, however, the water supplies themselves were not tested for contamination.

Another study from Ethiopia, this time in the Dire Dawa district of Eastern Ethiopia, found no association between the occurrence of *Cryptosporidium* or *Giardia* infections in children and whether their drinking water source was protected (springs, boreholes, protected wells) or unprotected (surface water, rivers, seepage and unprotected wells) (Ayalew et al., 2008). However, as all water sources in the study area (including that of protected sources) had previously been found to be contaminated with faecal matter (Ayalew et al., 2008), perhaps this is not surprising. Indeed, in our study, both protected and unprotected water sources seemed to have a similar likelihood of being contaminated with these parasites. Again, in the study from Dire Dawa, the water itself was not analysed.

In our study, one district seemed to be particularly associated with contaminated water, and as a further arm of our study, this area seems to have a higher prevalence of these infections among livestock (Kifleyohannes, unpublished work). This may have resulted in the greater prevalence of parasites, due to contamination, but, conversely the greater contamination could contribute to greater levels of infection. Further analyses, including molecular analyses of the parasites in faecal samples from people and their animals, should be able to provide more information on the epidemiology of these parasites in this region. It is worth noting that in this area, Enderta, fewer households have access to toilet facilities, and this may lead to greater contamination of the environment (see Table 1; CSA, 2007). This could be a contributory factor.

The relatively few samples taken in our study over a limited period are clear limitations of the study, as the results provide only an overview of the situation at a specific time point. It is possible that the contamination would be higher (samples more likely to be positive with higher numbers of parasites) immediately after precipitation. Heavy precipitation occurs more frequently in the summer months, and sampling during this time period may have provided a different picture. However, other studies that have tried to investigate whether water sources are more or less likely to be contaminated with *Giardia* and *Cryptosporidium* during rainy seasons tend to find relatively weak correlations, or correlations with only one of the parasites (e.g., Carmena et al., 2007; Mons et al., 2009; Utaaker et al., 2019). A further limitation was the period of storage and shipping between filtration of the water samples and completion of the analysis (between 2-6 months); this may have affected the oocyst integrity and hence the possibility of genetic characterisation for which the presence of sporozoite nuclei is essential. A further limitation was that sampling in the various regions was restricted by pragmatic considerations; unfortunately, the area where data from the CSA (2007) indicates that unprotected water sources are most often used as drinking water supply was actually the sampling area from which fewest samples were collected.

In conclusion, although *Cryptosporidium* contamination of water was not found to be extensive in this study, contamination of drinking water with *Giardia* appeared to occur relatively frequently but was associated with particular districts. Our results indicate the importance of the One Health approach when considering transmission routes and epidemiology of such infections, with environmental sampling providing further information to results obtained for analysis of faecal samples from both humans and animals.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Paper II

foods

MDPI

Article

Is Fresh Produce in Tigray, Ethiopia a Potential Transmission Vehicle for *Cryptosporidium* and *Giardia*?

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Abstract: In rural Ethiopia, where people often share their homes with their livestock, infections of humans and animals with *Cryptosporidium* and *Giardia* are relatively common. One possible transmission route is consumption of contaminated fresh produce; this study investigated the occurrence of *Cryptosporidium* and *Giardia* in fresh produce in four districts of rural Tigray in Ethiopia. Fresh produce samples (n = 55) were analysed using standard laboratory procedures. Overall, 15% (8/55) of samples were found to be contaminated. Although contamination levels were mostly low, a few samples had high numbers of *Giardia* cysts (up to around 70 cysts per 30 g sample). Molecular analyses were largely unsuccessful, but *Giardia* Assemblage A was identified in one sample. Contamination with these parasites was identified in two of the four districts, but, although a similar pattern has already been described for water contamination, this may be at least partially explained by sampling bias. Nevertheless, we speculate that access to clean water sources may be an important factor for reducing the occurrence of these pathogens. Given the public health and veterinary burden associated with both parasites, the factors which are of importance for their circulation in the communities and environments deserve further investigation.

Keywords: contamination; fruit; protozoa; vegetables; foodborne

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1. Introduction

Foodborne parasitic diseases are an important public health concern globally [1] resulting in significant morbidity and mortality among susceptible populations [2]; the disease burden is particularly high in low and middle-income countries (LMIC) [2]. In the developed world, such as European countries, although the disease burden is lower, foodborne parasites are nevertheless also considered important, because of the challenges in monitoring, prevention, and control [3]; issues which are also relevant in LMIC.

According to WHO estimates of disease burden, foodborne parasitic diseases, excluding enteric protozoa, caused an estimated 23.2 million cases and 45,927 deaths in 2010, resulting in an estimated 6.64 million Disability Adjusted Life Years (DALYs) [2]. The same study showed that an additional 67.2 million cases, 5560 deaths, and 492,000 DALYs were due to foodborne enteric protozoa [2].

Fresh produce can be contaminated by enteric protozoan parasites, including *Cryptosporidium* and *Giardia* [4]. Contamination of fresh produce by parasites can occur pre-harvesting [5] during cultivation, via irrigation with contaminated water or sewage or the use of animal or human faeces as fertilizer. Contamination could also occur post-harvesting, by being washed with contaminated water or from handling by infected food-handlers [4,6], or during transportation and storage [5].

The survival capabilities of *Cryptosporidium* oocysts and *Giardia* cysts in moist and refrigerated environments means that fresh produce is a suitable transmission vehicle [4]. *Cryptosporidium* oocysts are more robust than *Giardia* cysts, and oocysts can survive under less favourable storage conditions for more extended periods [7]. *Cryptosporidium* oocysts

that have contaminated fresh produce during harvesting may be still infective at the marketplace where it is sold for human consumption. Although, *Giardia* cysts are more fragile and may not survive less-favourable environmental conditions [4], contamination of fresh produce, particularly post-harvesting, may still lead to foodborne transmission. The risk of infection by *Cryptosporidium* and *Giardia* from contaminated fruits like berries can be significantly decreased by simple washing before consumption [8]. However, due to the adherent nature of some parasites, washing does not completely remove all of them [7–9], and on some fresh produce rough surfaces, crevices or hairs may entrap parasites [10].

In developing countries, like Ethiopia, enteric protozoan infections, including *Cryptosporidium* and *Giardia,* are common [11]. Both parasites are well-known causes of gastro-intestinal illness worldwide [12]. Diarrhoea is the leading cause of mortality in children younger than 5 years in Ethiopia, accounting for more than 70,000 death annually, which 23% of all under-five deaths [13]. In addition, cryptosporidiosis is of particular concern in the immunocompromised [14], or those suffering from other health insults such as malnutrition [15]; given that both malnutrition and AIDS occur at higher rates in countries of Sub-Saharan Africa than in many other global regions this is of particular concern [16]. The proportion of this diarrhoea caused by zoonotic protozoan parasites is not well understood.

Investigations of parasitic contamination of vegetables and fruits collected from local markets have been conducted in various towns in Ethiopia [5,17–22]. All these studies used a washing procedure followed by microscopy (direct wet mount, iodine wet mount, and modified Ziehl-Neelsen staining) to identify parasitic contamination. In these studies, the proportion of fresh produce contaminated by *Cryptosporidium* oocysts has been reported to range from 4.7–12.8% and with *Giardia* cysts from 1.3–18.5% indicating relatively high occurrence of contamination [5,17–22]. One limitation of these studies was quantitative data on the level of contamination of the produce samples was not reported, nor was the recovery efficiencies of the methods used provided. Additionally, no attempt was made to speciate the identified parasites by molecular methods, which is necessary to determine their threat to public health. Thus, although several studies have indicated that contamination of fresh produce with *Cryptosporidium* and *Giardia* is relatively common in Ethiopia, the importance of this contamination to human health remains unknown.

In most of Ethiopia, fruits and vegetables are sold in open-air marketplaces and streets, and often this produce is eaten directly after purchase without washing (personal observation during sample collection). Fresh produce displayed for sale without washing has been reported to be more likely to be contaminated with parasites than washed fresh produce [5]. Fresh produce is usually carried to Ethiopian marketplaces by people or animals, or in animal-drawn carts; public transport or private vehicles are sometimes used. According to Alemu et al. 2020 [5], the fruits and vegetables transported to the marketplace by animals were more likely to be contaminated with parasites than fresh produce transported in motor vehicles [5].

As part of a larger One Health study on the epidemiology of cryptosporidiosis and giardiasis among humans and animals in rural areas of Tigray, Ethiopia, we investigated contamination of vegetables and fruits collected from farmers' backyards, irrigated farmlands, and open-air local markets at four locations. In the study areas, family labour was used for all the tasks, from land preparation to harvesting and transporting of the products to the market, organic manure (faeces of the animals) used to improve production.

Our aim was to determine the occurrence of *Cryptosporidium* and *Giardia* in fresh produce at four locations in Tigray, Ethiopia, to assess the species and genotypes of these parasites in order to provide information on transmission pathways and epidemiology, and to compare these data with the occurrence of the parasites in water samples previously reported from the four sampling districts.

2. Materials and Methods

2.1. Study Areas

Between October 2018 and January 2019, samples of fresh produce (vegetables and fruits commonly eaten raw) were collected from local open-air markets, backyards of farmers, and irrigated farmlands from four selected districts of Tigray Region, Ethiopia, namely: Enderta (south-eastern zone of the region), Kilte Awulaelo (eastern zone), Hintalo Wejirat (south-eastern zone), and Raya Azebo (south zone).

Enderta (Location 1) is located at 13°14′ N and 39°40′ E with an altitude ranging from 1500 to 2300 metres above sea level (masl). The district covers a total area of 89,812 km^2 of which 30,062 ha is cultivable land [23]. The total population of the district both in rural and urban areas is approximately 114,300 according to the 2015 population and housing census data [24]. Location 1 comprises of two agro-climatic zones. In the main zone, the mean annual maximum and minimum temperature is 24 °C and 11 °C, respectively with an average annual rainfall of 601 mm [25]. A minor portion in the eastern and western parts has an elevation between 500 to 1500 masl, with an average temperature above 20 °C. The area is characterized by erratic rainfall and frequent droughts. The long rainy season is between June and September and the short rainy season is from March–May [25].

Hintalo Wejirat (Location 2) is at latitudes between 12°55′ N and 13°20′ N and longitudes 39°20′ E and 39°55′ E with an elevation area ranging from 1400 to 2850 masl. The district covers an area of 193,309 ha with an approximate total population of 153,500 [24,26]. The annual mean temperature in the area is 18 °C. There are two rainy seasons in the district, the long rainy season from June-August and the short rainy season from March-April. Rainfall patterns can be erratic and in the north-west of the region average annual rainfall is up to 850 mm, decreasing to 300–400 mm in the east [26].

Kilte Awulaelo (Location 3) is located between 13°45′ N–14°00′ N and 39°30′ E–39°45′ E. The elevation ranges from 1980 to 2500 masl. The average daily air temperature ranges between 15 °C and 30 °C. The mean annual rainfall is 601 mm. The district covers an area of 101,758 hectares, of which 21,620 hectares are farmlands [27,28]. The total population is approximately 99,700 [24].

Raya Azebo (Location 4) is located at 12°39′ N latitude and 39°44′ E longitude. The altitude ranges from 930 to 2300 masl. The area has two rainy seasons, with light rains between February to April period and heavy rains between July–September. The mean annual rainfall is 724 mm, with mean daily maximum and minimum temperatures of 18 °C and 14 °C, respectively for the western highlands and 23 °C and 20 °C, respectively in the valleys [29]. The district covers an area of about 176,210 ha with a population of 135,870 [24].

The majority of households do not cultivate vegetables, rather relying on local markets to purchase fresh produce. In Tigray, for example, production of vegetables and fruits is reported in only 14% and 8% of households, respectively [30]. Historically, most cultivated land is used to grow various types of cereal crops, with only a small portion dedicated to vegetable production. This trend is changing, however, with an increase in vegetable production since 2014 [31]. According to Tigray agricultural marketing promotion agency, an estimated 90% of the overall vegetables grown in the region are sold in the local market and the remaining 10% are used at home for family consumption [31].

2.2. Sample Collection

Of the vegetables and fruits available for raw consumption in the regions studied, the most common ones are cabbage, lettuce, carrot, pepper, tomato, and guava. These are the fresh produce types included in our study. The samples were collected from the four districts which are subdivided into 'tabias' (villages). Twenty-four samples were analysed from Location 1 (4 tabias), five samples from Location 2 (1 tabia), twenty-one samples from Location 3 (4 tabias), and five samples from Location 4 (1 tabia) (Table 1). The number of samples collected from each district varied, being affected by the schedule of the local markets and relatively low vegetable production, such that fresh produce was not always available for sampling when we were visiting each location. The total numbers of samples

analysed for each type of fresh produce were: cabbage (12), carrot (6), lettuce (13), pepper (9), tomato (9), and guava (6) (Table 1).

Study participants were interviewed regarding their sources of vegetables and fruits for consumption. Based on responses, samples of fresh produce were collected from the relevant sources. The participants reporting that they ate vegetables produced in their own backyards provided vegetable samples from this location. Some participants reported that their vegetables and fruits sources came from their own irrigated fields, and these samples were obtained from their fields. In all cases, the collected samples were put in a plastic bag, coded with date, district, and site of collection, and transported to the parasitology laboratory, College of Veterinary Medicine, Mekelle, Ethiopia.

Table 1. Sources of vegetables and fruits from the four locations.

	Backyard	Fields	Market	Total
Enderta (Location 1)				
Cabbage	0	5	2	7
Carrot	0	0	2	2
Guava	1	0	2	3
Lettuce	0	2	3	5
Pepper	1	0	3	4
Tomato	0	0	3	3
Total	2	7	15	24
Hintalo Wejirat (Location 2)				
Cabbage	0	0	1	1
Carrot	0	0	1	1
Guava	0	0	0	0
Lettuce	0	0	1	1
Pepper	0	0	1	1
Tomato	0	0	1	1
Total	0	0	5	5
Kilte Awulaelo (Location 3)				
Cabbage	0	1	2	3
Carrot	0	0	2	2
Guava	0	2	1	3
Lettuce	2	2	2	6
Pepper	1	0	2	3
Tomato	0	2	2	4
Total	3	7	11	21
Raya Azebo (Location 4)				
Cabbage	0	0	1	1
Carrot	0	0	1	1
Guava	0	0	0	0
Lettuce	0	0	1	1
Pepper	0	0	1	1
Tomato	0	0	1	1
Total	0	0	5	5
Grand total	**5**	**14**	**36**	**55**

2.3. Preparation of the Sample for Analysis

The samples were refrigerated at 4 °C for a maximum of 24 h at the parasitology laboratory and processed on the following day. For leafy vegetables (lettuce and cabbage) and peppers, 30 g were weighed into stomacher bags (Seward BA6041/STRfilter bag), 200 mL of 1 M glycine buffer was added to immerse the sample, and the samples were processed by stomacher/paddle blender for one minute. For carrots, tomatoes, and guava one or two were selected on the basis of their size and put into filtered stomacher bags and washed for 5 min by hand in 200 mL of 1 M glycine buffer [4].

The eluate was transferred into five 50 mL centrifuge tubes, the bag washed with distilled water, and the wash water transferred to the tubes. The tubes were transported

to Ayder Referral Hospital, Department of Microbiology, Mekelle on the same day, where they were centrifuged for 10 min at 1550 relative centrifugal force. Following aspiration of the supernatant, the pellets were vortexed and combined in a single tube per sample and refrigerated at 4 °C before being transported to Norway for further analysis.

2.4. Immunomagnetic Separation (IMS)

IMS was performed using Dynabeads GC-combo kit for isolation of *Cryptosporidium* oocysts and *Giardia* cysts. Although 16 samples from Location 1 district were processed according to the ISO 18 744 [32] protocol, all other samples were processed following a reduced-cost protocol that is based on this standard, but uses fewer beads (20 µL of each bead type), and modified buffers (as well as the buffers provided with the kit, PBS-Tween and SurModics StabliZyme AP buffer); the efficiency of the modified method is comparable to that of the ISO Method (30–50%) but is considerably cheaper due to the reduction in use of reagents [33].

2.5. Detection of Cryptosporidium Oocysts and Giardia Cysts Using Immunofluorescent Antibody Staining (IFAT)

Single-well slides of air-dried sample concentrates were fixed with methanol and stained with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mABs) against *Cryptosporidium* oocyst walls and *Giardia* cyst walls (Aqua-glo™, Waterborne™ Inc., New Orleans, LA, USA) and 4′,6′ diamidino-2-phenylindole (DAPI) was used to stain the DNA in the nuclei of these organisms. Samples were mounted with M101 No-Fade Mounting Medium then each slide was covered by a glass coverslip and viewed immediately.

A Leica DCMB fluorescence microscope equipped with Nomarski differential interference contrast (DIC) optics was used for examination of the slides. A blue filter block (480 nm-excitation, 520 nm-emission) was used to visualize FITC conjugated mABS labelled cysts and oocysts and a UV filter block (350 nm excitation, 450 nm emission) was used to visualize the presence or absence of DAPI-stained sporozoite nuclei. All observations were at 200× or 400× magnification.

The entire well of the stained slides was examined for the presence or absence of *Cryptosporidium* oocysts and *Giardia* cysts; preliminary identification was based on reactivity with the monoclonal antibody and appropriate shape and size. Ovoid or spherical objects with brilliant apple-green fluorescence of the appropriate size and shape were examined under the UV filter to determine the presence of nuclei, both to support identification (if present) and for assessing the sample suitability for further investigation by molecular methods. The number of *Cryptosporidium* oocysts and *Giardia* cysts observed per sample was recorded.

2.6. Recovery Efficiency of the Method

The recovery efficiencies of both the methods used were estimated using spiked samples in which known numbers of flow cytometry-sorted oocysts and cysts (AccuSpike™-IR; Waterborne Inc., New Orleans, LA, USA and EasySeed™, TCS Biosciences Ltd., Botolph Claydon, UK) were spiked onto 30 g lettuce and dried at room temperature for 2 h before analysis as described. Recovery efficiencies were estimated to be approximately 30% for *Cryptosporidium* and 55% for *Giardia*, regardless of whether the ISO 18744 [32] protocol or reduced-cost method was used [33]. This is within the range considered acceptable using the ISO or US EPA methods for analysis of water samples.

2.7. DNA Extraction

DNA extraction was conducted on positive samples using DNeasy PowerSoil Kit (Qiagen, Oslo, Norway) protocol, with some modifications. The fresh produce sample post IMS (250 µL) and 60 µL of the lysis solution (solution C1) were added to the PowerBead Tubes and vortexed together to mix. This was then subjected to bead beating to release the DNA by breaking the oo(cyst) walls using a FastPrep-24 5G (MP Biomedicals) in two cycles of 4 metre/s for 60 s with a 45 s pause between the cycles. In the end, the DNA

was eluted in 40 µL of the elution solution (solution C6) and stored at −20 °C. A recent study by Temesgen et al., 2021 [8] reported that the bead-beating approach is effective for obtaining the DNA of *Cryptosporidium* and *Giardia* from artificially contaminated berries.

2.8. Polymerase Chain Reaction and Sequencing

For *Giardia* investigation, PCR targeting the glutamate dehydrogenase gene, beta-giardin gene, and SSU gene were conducted according to published protocols [34,35]. For *Cryptosporidium*, PCR targeting the SSU-rRNA gene was conducted [35]. Details of primers and cycling conditions are listed in the Supplementary File S1. Negative and positive controls were included in each PCR run.

Reactions were carried out in a total volume of 25 µL that included 2 µL of template DNA, 0.4 µM of each primer and 12.5 µL of DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific, Oslo, Norway). Bovine serum albumin (BSA) 0.2 µL of (20 mg/mL) was used in the reaction targeting the beta giardin and GDH genes.

The PCR products were examined following separation on a 2% agarose gel, stained with SYBR Safe DNA gel stain and visualized under UV illumination. A ready-to-use DNA ladder (Thermo Scientific, Oslo, Norway) of 100 bp was used for fragment size determination.

Purification of the positive products was carried out using ExoSAP-IT PCR product clean-up reagent (Thermofisher Scientific, Oslo, Norway) and sent to a commercial company (EUROFINS GENOMICS, Ebersberg bei München, Germany) for sequencing in both directions. Sequences were checked using Geneious prime software and compared with sequences in GenBank using NCBI BLAST.

2.9. Statistics and Data Handling

Results were collected in a database in Excel. Data analysis was made using simple descriptive statistics and frequency using STATA version 15. Fisher's exact test was used to determine associations with sample location and detection of parasites and also between sources of samples and detection of parasites.

3. Results

3.1. Occurrence of Cryptosporidium and Giardia on Vegetables and Fruits

Out of 55 fresh produce samples processed, 8 (15%) were found to be contaminated with *Cryptosporidium* oocysts and/or *Giardia* cysts. DAPI staining was not observed in any of the oocysts or cysts, indicating that the oocysts or cysts had ruptured and there was no longer nuclear material within the oocysts or cysts. Of these 8 samples, 2 were contaminated with *Cryptosporidium* only, 5 were contaminated with *Giardia* only, and 1 was contaminated with both *Giardia* and *Cryptosporidium*. All 8 positive samples were from Location 1 and Location 3 districts, which were also the districts from which most samples were collected.

The contamination rate of different types of fresh produce is depicted in Figure 1. The contamination rate for cabbage was 3/12 (25%), lettuce 2/13 (15%), guava 2/6 (33%) and pepper 1/9 (11%). Contamination with these parasites was not detected on either carrot or tomato samples.

The number of contaminating oo(cysts) per sample of vegetable and fruits are described in Table 2. The degree of contamination was low for both parasites, with the exception of one cabbage sample where 71 *Giardia* cysts were counted and one lettuce sample where 28 *Giardia* cysts were counted; all other positive samples had fewer than 20 oo(cysts).

Of the positive samples, 1 (*Giardia* positive) was obtained from a backyard (of 5 total backyard samples), 3 from farmers' fields (1 *Cryptosporidium* positive, 1 *Giardia* positive, and 1 with both parasites of 14 field samples), and 4 from markets (2 *Giardia* positive and 2 *Cryptosporidium* positive of 36 market samples). No source was more or less likely to be contaminated with these parasites than another.

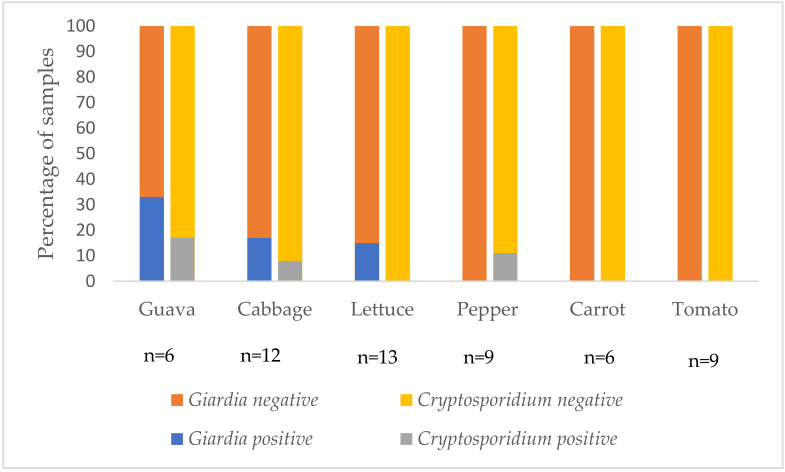

Figure 1. Proportion of *Giardia* and *Cryptosporidium* contaminated fresh produce samples.

Table 2. Intensity of contamination of fresh produce with *Cryptosporidium* oocysts and *Giardia* cysts.

Sampling Area	Type of Fresh Produce	Number of *Cryptosporidium* Oocysts Counted	Number of *Giardia* Cysts Counted
Location 1	Pepper	2	0
	Lettuce	0	3
	Cabbage	0	71
	Cabbage	0	3
Location 3	Lettuce	0	28
	Guava	0	1
	Guava	3	11
	Cabbage	2	0
Total positive samples		3	6

3.2. Genotyping of Cryptosporidium and Giardia

DNA was isolated from the three samples with the highest number of *Giardia* cysts (71, 28, and 11 cysts), in the latter of which *Cryptosporidium* oocysts were also detected, and also from the two other *Cryptosporidium* positive samples.

PCR resulted in amplification at the *Giardia* SSU gene from the sample containing 28 cysts (lettuce sample from Location 3, Kilte Awulaelo). DNA sequencing of this PCR product was successful, and revealed it to be Assemblage A, with 99% similarity to sequences at GenBank Accession numbers MH047247.1 and MH047246.1. All other PCR attempts for the other *Giardia* and *Cryptosporidium* positive samples were unsuccessful despite multiple attempts at several genes.

3.3. Comparison between Occurrence of Positive Samples in Fresh Produce and Water in the Four Districts

In our previous article [36], contamination of drinking water with *Cryptosporidium* and *Giardia* appeared to occur frequently in Locations 1 (Enderta) and 3 (Kilte Awulaelo). These are also the two locations where contamination was found in fresh produce (Table 3). In addition, the fresh produce sample with 71 *Giardia* cysts came from Location 1, and a high count of *Giardia* cysts (22 cysts in 10 L) was also observed in one water sample from this location [36].

Table 3. Occurrence of contamination of fresh produce with *Cryptosporidium* and *Giardia* oo(cysts) compared with water in the same locations.

	Proportion (%) of Samples Positive			
	Fresh Produce Samples		Water Samples	
	Cryptosporidium	*Giardia*	*Cryptosporidium*	*Giardia*
Location 1	4% (1/24)	13% (3/24)	7% (1/15)	40% (6/15)
Location 2	ND [1] (0/5)	ND (0/5)	ND (0/7)	ND (0/7)
Location 3	10% (2/21)	14% (3/21)	8% (1/13)	ND (0/13)
Location 4	ND (0/5)	ND (0/5)	ND (0/2)	ND (0/2)

[1] ND-Not detected.

4. Discussion

This study investigated the extent of contamination of fresh produce with the protozoan parasites *Cryptosporidium* and *Giardia* in four districts of rural Tigray, northern Ethiopia. The most important finding from this study is that contamination of fresh produce with these parasites occurs relatively frequently in this area, with 8 out of 55 samples (15%) positive for *Cryptosporidium* and/or *Giardia*. Contamination was only found in two districts, Location 1 and Location 3, with a district-specific occurrence of 4/24 (17%) and 4/21 (19%), respectively. However, it should be noted that considerably more samples were analysed from these districts (45 samples in total from Locations 1 and 3, and 10 samples in total from Locations 2 and 4). This sampling bias means that we cannot reach a conclusion on whether the contamination risk was higher in Locations 1 and 3 than in Locations 2 and 4.

Other reports from Ethiopia on contamination of fresh produce have produced similar results regarding the proportion of contaminated fresh produce. For example, Alemu et al., (2020) [5] reported 4.9% and 10.2% occurrence of *Cryptosporidium* spp. and *Giardia* contamination, respectively, from vegetable and fruit samples collected from local markets in Bahirdar city, northwest Ethiopia. Similarly, Bekele et al., 2017 [20] reported a prevalence of *Cryptosporidium* spp. and *Giardia* of 4.7% and 10%, respectively, from vegetables and fruits collected from local markets in Arba Minch town, southern Ethiopia. Moreover, Endale et al., 2018 [37] reported 7.7% and 9.3% prevalence of *Cryptosporidium* spp. and *Giardia*, respectively, from vegetables and fruits collected from local markets in Dire Dawa, Eastern Ethiopia. Furthermore, Tefera et al., 2014 [22], reported contamination with *Cryptosporidium* spp. (12.8%) and *Giardia* (7.5%) from fruits and vegetables collected from selected local markets of Jimma town, Southwest Ethiopia. Nevertheless, the only similar study that we could find from Tigray reported a higher occurrence than we found with 7.3% and 18.5% occurrence of *Cryptosporidium* and *Giardia* on fresh produce from markets in the town of Aksum [19]. Discrepancies between our data and others from Ethiopia could be due to real differences in the occurrence of these parasites in different geographical locations but could also reflect differences in the laboratory analysis method. As IFAT is both more sensitive and more specific than traditional microscopy methods used in these other studies, both false-negative and false-positive results should be lower.

As previously noted, none of these other studies from Ethiopia quantified the extent of contamination. Although in our study the number of parasites detected per sample was usually low (mostly fewer than 5 (oo)cysts per 30 g sample), a few of the samples were quite heavily contaminated, with one sample harbouring over 70 *Giardia* cysts. Reports from other parts of the world also generally indicate low numbers of parasites among contaminated fresh produce. For example, a study from Norway reported 1–8 *Cryptosporidium* oocysts or *Giardia* cysts in 100 g samples of various types of fruits and vegetables [38]. More relevant to this current investigation is a study of vegetables from retail in India, where among positive samples of fresh produce obtained from street vendors, parasite (*Cryptosporidium* and *Giardia*) counts were usually below 10 per 30 g sample [4]. However, as in our study, some samples had very high counts [4], although, unlike in our study, this tended to be for *Cryptosporidium*

oocysts rather than *Giardia* cysts. Another variable of interest is the type of fresh produce examined. In this study we analysed samples of vegetables commonly eaten raw (cabbage, carrot, lettuce, pepper, tomato) and guava. Although only a few guava samples were analysed, it was found to be the fresh produce most likely to be contaminated, with *Cryptosporidium* in 33% (2/6) and *Giardia* in 17% (1/6) of guava samples, followed by cabbage, lettuce, and pepper. Neither *Cryptosporidium* nor *Giardia* were detected on tomatoes and carrots. The reason for this may reflect factors associated with cultivation (carrots are below the surface of the soil and therefore less likely to be directly contaminated) or other factors, such as transport. As tomatoes are more likely to be damaged than the other fresh produces, they may be transported in containers and are therefore less likely to be contaminated during transport. Cabbage and lettuce may be more likely to be contaminated due to their high surface area, and their rough surfaces [5,7,20]. The higher contamination of guava is interesting, as they are on trees and thus unlikely to be contaminated in the field; thus, contamination during or post-harvest seems most likely.

None of the other studies from Ethiopia have investigated the species (*Cryptosporidium*) or genotype (both *Cryptosporidium* and *Giardia*) contaminating fresh produce. This information may provide clues on the source of contamination, and indicate the potential public health significance. Although we were only able to obtain data from one sample of *Giardia*, the finding of Assemblage A, which is known to infect humans and animals, opens for the possibility of multiple different transmission routes leading to contaminated produce, including zoonotic transmission.

Water analysed from all four study districts for these parasites also indicated water contamination at Locations 1 and 3 (Enderta and Kilte Awulaelo), but not at the other two locations [36]. However, as for the fresh produce samples, relatively few water samples were analysed from Locations 2 and 4. At Locations 1 and 3, there were more water sources available for sampling, whereas at Locations 2 and 4 several of the water sources were out of order (e.g., hand pumps) and at Location 4 there was a main common water source which limited sample collection. Although this sampling bias for both water and fresh produce could have influenced our results, it is also possible that these parasites are circulating more in the environment in some locations than others, and it would be of interest to examine this in greater detail. One possible explanation for the relative absence of environmental contamination at location 4 (Raya Azebo—Mechare tabia) is that the main water source was in-house tap water. Such a water supply is less likely to be contaminated by people and animals, and less likely to spread contamination to fresh produce or act as a transmission vehicle to those drinking it. Water supply has previously been shown to be a risk factor for diarrhoeal disease [39].

As with many studies, there are some important limitations in this study. The total number of fresh produce samples that we analysed was relatively low (55) compared to other studies, including those from Ethiopia where reported sample sizes were between 108 to 384 [5,17–22,37]. As our fresh produce samples were collected along with other samples, we were limited by resources. In addition, most of our study participants bought fresh produce from the market, which occurred only once per week, so opportunities for sample collection were restricted. In addition, we had challenges in our molecular analyses, and this reflects the relatively few positive samples, low concentrations of parasites, and a lack of nucleated parasites (as demonstrated by inclusion of DAPI) in those samples that were positive. Although DAPI staining was absent in the parasites detected on all positive vegetables, we speculated that the DNA may be in the suspension following elution of the samples and therefore conducted the molecular analyses. The nuclei could have been lost during transport, storage, and processing, particularly from *Giardia* cysts that are not as robust as *Cryptosporidium* oocysts [7]. Our molecular result from one of *Giardia* positive sample corroborates this suggestion, as DNA amplification was possible. It is not possible to determine whether the parasites contaminating the fresh produce were viable or infectious at the time of sample collection, and therefore presenting a public health risk to consumers. The lack of oo(cyst) nuclei at detection suggests that the parasites were

non-infective. However, it is possible that damage to the oo(cyst)s occurred after sample collection, such as during transport, storage, or processing, and were indeed infective at the time of sampling.

In conclusion, our study found that contamination of fresh produce with *Cryptosporidium* oocysts and *Giardia* cysts was relatively common in Tigray region. Despite the fact that contamination was only detected in two of the four districts in the study, this could reflect sampling bias rather than a lack of contamination at Locations 2 and 4. Although contamination levels in positive samples were generally low, a few samples were more heavily contaminated with *Giardia* cysts. Similar results have already been reported regarding contamination of water supply, with the same two districts apparently having cleaner water. Although our data may suggest certain factors in specific locations may contribute to the circulation of these parasites within the environment, sampling bias also has a role. Given the public health and veterinary burden associated with both parasites, trying to pinpoint the factors of importance in reducing the circulation of these parasites in the communities and environments deserves further investigation.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods10091979/s1, Table S1: PCR conditions and primers. Supplementary File S1.

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Paper III

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Cryptosporidium and *Giardia* in Livestock in Tigray, Northern Ethiopia and Associated Risk Factors for Infection: A Cross-Sectional Study

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The occurrence and species/genotypes of *Cryptosporidium* and *Giardia duodenalis* infecting young livestock in selected districts of Tigray, Ethiopia were investigated, along with risks associated with infection. A total of 757 faecal samples were collected from calves, lambs, and goat kids from four rural districts in Tigray, and also from calves in periurban Mekelle, Tigray's main city, and analysed for *Cryptosporidium* oocysts and *Giardia* cysts. Farmers answered questionnaires regarding potential risk factors at sample collection. Immunofluorescent antibody staining was used for parasite detection, and PCR at selected genes and sequencing of positive samples was used for molecular characterisation. The occurrence of *Cryptosporidium* infection was 10, 9, and 4% in calves, lambs, and goat kids, respectively; equivalent figures for *Giardia* infection were 39, 32, and 21%. Molecular characterisation of *Cryptosporidium* isolates revealed *C. ubiquitum,* subtype XIIa in all three host species; *C. ryanae* in calves and goat kids; *C. andersoni* and *C. bovis* were identified only in calves, and *C. xiaoi* was identified in lambs. For *Giardia*, Assemblage E predominated in all host species, but among calf isolates we also identified a few potentially zoonotic genotypes (assemblages A (AI) and Assemblage B). Periparturient care was shown to be a particularly relevant risk factor for infection, and infections were less likely to occur under extensive management systems. Our major findings were widespread occurrence of both parasites in livestock, and the apparent lack of the most common zoonotic species. Our results are discussed in relation to other relevant studies. As our study was conducted in Tigray, further investigation in different settings in Ethiopia could provide relevant information on transmission and zoonotic potential. In addition, given the dependency on healthy animals for the livelihoods of the population of Tigray, investigation of the effect of these common parasites on livestock productivity is important.

Keywords: ***Cryptosporidium*, Ethiopia, *Giardia*, livestock, risk factor**

INTRODUCTION

In developing societies, the livelihoods of many people are dependent on livestock. In most parts of Africa, people and animals live in close proximity, and children have a substantial role in livestock-rearing activities (1). In low and middle-income countries, livestock, primarily cattle and small ruminants (sheep and goats), represent much more than food and nutrition (2) and are also used for income and employment, as well as a means of storing wealth. Livestock is also particularly valuable to women in Africa because it is among the few assets in many societies that women can own and manage themselves (3). Furthermore, livestock manure is used for fertiliser, as fuel for cooking, and for building materials, which are typically prepared by hand by women and children (4). However, this close association between people and their animals also provides the interface for the transmission of zoonotic pathogens (5).

Ethiopia has the largest livestock population in Africa. In Tigray, one of the regional states in northern Ethiopia, the livestock populations are estimated to be approximately 5 x 10^6 cattle, 2 x 10^6 sheep, and 5 x 10^6 goats (6).

There are three main livestock production systems in Ethiopia: extensive, intensive, and semi-intensive (7). In Tigray, the livestock system is usually extensive, with animals mostly kept in small groups in simple pens close to the farmer's home (personal observation). These animals are often under the care of younger family members, and communal grazing is common (7). Drinking water for ruminant livestock in grazing areas is mainly provided from untreated sources (personal observation). Feeding strategies use naturally available forage (pasture and browse), with farmers using crop residues and hay as supplements during the dry season (7).

The protozoan parasites *Cryptosporidium* (Phylum Apicomplexa) and *Giardia* (Phylum Fornicata) are extremely common in ruminants, with worldwide distribution (8, 9). Both parasites can cause gastrointestinal disease in infected hosts, sometimes leading to considerable economic losses, and both also have zoonotic potential (8, 10).

Cryptosporidium infection is an important cause of clinical morbidity, mortality, and associated loss of production in ruminant livestock, particularly in young, especially neonatal, animals (8). Clinical signs of cryptosporidiosis in calves include pasty to watery diarrhoea, sometimes accompanied by lethargy, inappetence, fever, dehydration, and poor body condition. Similarly, neonatal lambs and goat kids with cryptosporidiosis have pasty to watery, yellow foul-smelling diarrhoea, anorexia, depression and abdominal pain (11). Infection in older animals is usually subclinical, but can still have a negative effect on production, with a lower body condition score, slower growth rate, and lower carcass weight and dressing percentage at slaughter (11).

Giardiasis in livestock is also characterised by diarrhoea, weight loss, and malabsorption. Infection may also be subclinical, but still affect growth and productivity (9). Chronic *Giardia* infections tend to occur toward the end of the neonatal period in ruminants, but their importance as a cause of diarrhoea is still ambiguous (8).

Over 30 species and over 70 genotypes of *Cryptosporidium* have been identified, some of which are host specific (12, 13). The four major *Cryptosporidium* spp. that infect cattle throughout the world are *C. parvum, C. bovis, C. ryanae,* and *C. andersoni,* of which *C. parvum* and *C. andersoni* are the two species that have been most associated with clinical disease in cattle (14). In sheep and goats, the most common species identified are *C. parvum, C. ubiquitum*, and *C. xiaoi,* and all three can be responsible for mild to severe diarrhoea and mortality (9, 11).

The zoonotic potential of *Cryptosporidium* depends on the species / genotype (11). *C. parvum* (subtypes IIa and IId) is the dominant zoonotic species in cattle and humans. Although information is scarce, some evidence suggests that sheep and goats can also be important as reservoirs for the zoonotic transmission of *Cryptosporidium*, particularly *C. parvum* (15, 16).

Giardia duodenalis is a species complex with eight distinct assemblages or genotypes, with some degree of host specificity (17–19). Globally, sub-assemblage AI is predominantly found in livestock and to a lesser extent in humans, whereas in most parts of the world, AII is mainly found in humans and a minor proportion in animals. In contrast, AIII has exclusively been found in animals, mainly in wildlife (17). Sub-assemblages AI, AII, BIII, and BIV are considered potentially zoonotic (17).

Among *Giardia* infections in domestic ruminants (cattle, goats, sheep) globally, including in Africa, Assemblage E is the predominant species reported and is not usually considered zoonotic (20, 21). However, Assemblages A (AI and AII) and B (BIV) have been reported in goats, cattle, and other animal species, both domesticated and wild, from various parts of Africa (18).

Although livestock infections with *Cryptosporidium* and *Giardia* have been reported from many parts of Ethiopia (**Table 1**), all published studies are from Addis Ababa and Oromia region (120 km distance from Addis Ababa) and very few studies included molecular characterisation (species and subtypes of *Cryptosporidium* or Assemblages of *Giardia*). The only published article on the molecular study of *Cryptosporidium* and *Giardia* in lambs from Addis Ababa and its surroundings reported *C. ubiquitum* and Assemblage E *Giardia* (21).

The epidemiology, impact, and zoonotic potential of these parasites in livestock in Ethiopia are therefore poorly understood. This is partly because most previous studies in livestock were based solely on light microscopy, using modified Ziehl-Neelsen (mZN) staining (23, 24). This method has poor sensitivity and specificity and cannot be used to identify species (and subtypes) or genotypes. Thus, knowledge on the epidemiology of *Cryptosporidium* and *Giardia* in young livestock in Ethiopia would be highly valuable, particularly from regions beyond the area in the vicinity of the capital, Addis Ababa, and also regarding zoonotic (public health) aspects.

This need underpinned the objectives of this study, which were specifically: i) to determine the occurrence of *Cryptosporidium* and *Giardia* infection in dairy calves, lambs, and goat kids in Tigray, Northern Ethiopia; ii) to identify the species (and subtypes) of *Cryptosporidium* and *Giardia duodenalis* genotypes in infected dairy calves, lambs, and goat kids, and thereby determine the zoonotic potential; and iii) to

TABLE 1 | Studies from Ethiopia on *Cryptosporidium* and *Giardia* infections in ruminant livestock, with detection methods and prevalence data.

Region	Host animal	Age group	Number sampled	Detection method	Molecular characterisation	Prevalence (%)	References
***Cryptosporidium* infections**							
Bishoftu (Debre Zeit) Oromia	Calves	<1 year	364	mZN	Not done	13.6	(22)
	Lambs	<1 year				16.9	
	Goat kids	<1 year				11.5	
Addis Ababa and Debre Zeit	Calves	<1 year	580	Sheather's flotation, mZN	Not done	17.6	(23)
North Shewa Zone, Oromia	Cattle	age not reported	384	mZN	Not done	7.8	(24)
Oromia special zone	Calves	<5 months	449	mZN PCR	*C. bovis, C. ryanae, C. andersoni*	15.8	(25)
Addis Ababa surrounding (Oromia special Zone)	Lambs	<5 months	389	mZN, PCR	*C. ubiquitum*	2.1	(21)
Addis Ababa and its surroundings	Calves	<2 months	392	mZN	Not done	17.3	(26)
		2–6 months				18.2	
		>6 months				21.5	
Holeta, Oromia	Dairy calves	<3 months	270	mZN, PCR	*C. parvum, C. andersoni*	14.8	(27)
Eastern Hararghe	Calves	<12 months	237	Sheather's flotation, mZN	Not done	27.8	(28)
	Lambs	<6 months				22.2	
	Goat kids	<6 months				12.2	
North West	Calves	<12 months	360	Sheather's flotation, mZN	Not done	18.6	(29)
Southern zone	Calves	<12 months	330	IFAT	Not done	13	(30)
***Giardia* infections**							
North Shewa Zone, Oromia	Cattle	age not reported	384	Direct wet mount with saline and iodine stain	Not done	2.3	(24)
Addis Ababa surrounding (Oromia special Zone)	Lambs	<5 months	389	Lugol's iodine PCR	Assemblage E	2.6	(21)
Southern zone	Calves	<12 months	330	IFAT	Not done	10	(30)

identify risk factors associated with the infection of livestock with these parasites.

MATERIALS AND METHODS

Study Areas

The study was carried out between October 2018 and January 2019 in four districts of Tigray, Ethiopia; these are: Enderta (south east zone of the region), Kilte Awulaelo (east), Hintalo Wejirat (south east zone of the region), and Raya Azebo (south zone). Detailed information about these districts can be found in Kifleyohannes et al. (31). In addition, in February 2020, calf samples from urban farms were collected from Mekelle, the capital city of Tigray.

Study Design and Sample Size Determination

A cross-sectional study design, based on a convenience sample rather than randomised sampling, was used to determine the occurrence in livestock from these four districts of Tigray by analysing faecal samples collected from calves, lambs, and goat kids. Sample size was determined by using expected prevalence of 14.8 % for *Cryptosporidium* and 2.3 % for *Giardia* infections in calves (24, 27), 16.9 % for *Cryptosporidium* and 2.6 % for *Giardia* infections in lambs (21, 22), and 11.5 % for *Cryptosporidium* in goat kids (22) using the formula given by Thrusfield (32). To calculate the sample size, 5 % absolute precision and 95 % confidence intervals were used for each species.

The calf samples from Mekelle were collected on an availability basis, with input from the city's animal production and health experts regarding where young calves were available for sampling within Mekelle.

Study Animals

Animals were targeted through a door-to-door survey in all selected villages (tabia) at the specified study sites, and all homesteads within each village were contacted. Across the four

districts, faecal samples were collected from 208 calves, 270 lambs, and 248 goat kids, all of which were ≤ 6 months of age and kept under intensive, semi-intensive, or extensive management systems. Of the calves, 65 (31%) were ≤ 8 weeks of age, and of these 21 (32%) were under 3 weeks of age.

For calves in Mekelle city, a total of 31 faecal samples were collected from 12 urban farms (two cooperative farms and 10 private farms). All calves were aged ≤ 3 months.

Information was recorded on animal age, sex, management system, faecal characteristics (consistency, colour, presence or absence of mucus), cleanliness of the pen and perianal area. For calves, the breed (local or crossbreed) was also recorded.

Sample Collection and Analysis for *Cryptosporidium* Oocysts and *Giardia* Cysts

Approximately 5 g of faeces were collected directly from the rectum of each animal and placed into sampling bottles. The samples were preserved by mixing in 2.5 % potassium dichromate and then refrigerated at 4°C at the Parasitology Laboratory, at the College of Veterinary Medicine, Mekelle University (Mekelle, Ethiopia) before shipping to Norway. Potassium dichromate was removed from the faeces by repeated washings at the Parasitology Laboratory, Faculty of Veterinary Medicine, at the Norwegian University of Life Sciences (Oslo, Norway).

The subsamples were then homogenised, concentrated by centrifugation, and approximately 20 µl of the resulting pellet was placed on a microscope slide using a plastic loop. The samples were air-dried and then fixed with methanol before being analysed by using immunofluorescent antibody test (IFAT), with staining with 15 µl of fluorescein isothiocyanate (FITC)-labelled monoclonal antibody (Aqua-Glo, Waterborne Inc, NO, USA) for the detection of *Cryptosporidium* or *Giardia* (oo)cysts. After incubation of the samples and antibody at 37°C for 20 min, 10 µl of 4', 6' diamidino-2-phenylindole (DAPI) was added. After rinsing the samples, a drop of 1,4 diazabicyclo (2.2.2) octane (DABCO) anti-fade mounting medium was added and a coverslip placed over the sample. The stained samples were screened immediately after staining using a fluorescence microscope equipped with appropriate filters (FITC and DAPI) and Nomarski optics.

The entire slide was scanned under the FITC filter and, for presumptive (oo)cysts based on morphometry and staining, the morphology and intactness were checked by light microscopy with Nomarksi optics, as well as visualisation of DAPI staining of nuclei under the DAPI filter. A semi-quantitative measure of infection intensity was determined based on the number of cysts/oocysts per field of view at the objective 20x according to Utaaker, (33): +1 1–9 (oo)cysts, +2 10–50 (oo)cysts, +3 51–100 (oo)cysts, +4 >100 (oo)cysts.

DNA Isolation

The samples containing higher numbers of (oo)cysts or where (oo)cysts were positive for DAPI (and therefore containing nuclei), were preferentially selected for DNA extraction. DNA was isolated using the DNeasy PowerSoil Kit protocol (Qiagen, Oslo, Norway), with slight modifications. The faecal pellet (250 µl) and 60 µl of the lysis solution (solution C1) were added to the PowerBead Tubes and vortexed together to mix. This was then subjected to bead beating to release the DNA by breaking the (oo)cysts walls using a FastPrep-24 5G (MP Biomedicals) in two cycles of 4 m/s for 60 s with a 45 s pause between the cycles. Finally, the DNA was eluted in 50 µl of the elution solution (solution C6) and stored at −20°C. Studies by Elwin et al. (34, 35) reported that the bead-beating approach is effective for obtaining the DNA of *Cryptosporidium* and *Giardia* from human stools.

Polymerase Chain Reaction and Sequencing

For samples positive for *Cryptosporidium*, primers targeting sections of the SSU-rRNA gene and gp60 gene for *C. ubiquitum* were used for sequence investigation by conventional PCR. Four genes were targeted for genotyping investigations of the *Giardia*-positive samples: glutamate dehydrogenase (GDH) gene, beta-giardin (BG) gene, triosephosphate isomerase (TPI) gene, and SSU (rRNA) gene. In addition, three genes were used to subtyping Assemblage A samples: DNA repair and recombination gene (RHP26), high cysteine membrane gene (HCMP), and NEK kinase gene. Assemblage B subtyping were performed using the following three genes, 6-phosphogluconate dehydrogenase (pdg), Hypothetical protein, and Phosphorylase B kinase gamma catalytic chain (phkg2). Primers and reaction cycles, as well as other details, for all PCR used in this study are provided in **Supplementary File 1**.

The PCR products were examined after separation on a 2% agarose gel, stained with SYBERsafe DNA gel stain and visualised under UV illumination. A 100 bp ready-to-use DNA ladder (Thermo Scientific) was used for fragment size determination. Purification of the positive products was carried out using either ExoSAP-IT PCR product clean-up reagent (Thermofisher Scientific) or PureLink Quick Gel extraction and PCR purification Combo kit (Thermo Fisher Scientific) and sent to a commercial company (Eurofins Genomics, Germany) for sequencing in both directions. Sequences were checked using Geneious Prime software, contigs made, and compared with sequences already deposited in GenBank using the Basic Local Alignment Search Tool (NCBI BLAST), along with a recently developed online tool, SSU and gp60 Cryptogenotyper for *Cryptosporidium* sequences (36).

Questionnaire Survey

A pre-tested questionnaire was used to collect data on the study animals, their owners, and management practises. The questionnaires were filled out during individual interviews with the animal owners by the first author, supported by colleagues from the College of Veterinary Medicine of Mekelle University, along with veterinarians and technicians at the study districts. Data on risk factors of *Cryptosporidium* and *Giardia* infections such as age, feeding method, source of drinking water, the occurrence of diarrhoea, pen cleanliness, etc. were collected (see **Supplementary File 2** for questionnaire).

The questionnaires were designed to contain mainly closed-ended questions.

Statistics

A database was created in Excel and analysed using STATA 15 software (STATA SE Corp, TX, USA) software. The Chi-square test and Fisher's exact test were used to evaluate the association between risk factors for *Cryptosporidium* and *Giardia* infection. Variables significant at $P < 0.05$ in the above tests were considered as candidates for a multivariable model. Subsequently, a backward stepwise logistic regression was used to identify possible predictors of the *Cryptosporidium* and *Giardia* infection out of the following variables: districts, periparturient care, weaning age, management system and breed. At each step, the variable with the highest *p*-value in the multivariable logistic regression model was removed before re-running the multivariable logistic regression model and these iterations of elimination were continued as long as the values of the Akaike information criterion (AIC) of the new models were decreasing. The confidence level was maintained at 95 % and $P < 0.05$ was considered for the significance level.

RESULTS

Overview of *Cryptosporidium* and *Giardia* Infections in Calves, Lambs, and Goat Kids From the Four Districts

The overall combined occurrence of *Cryptosporidium* spp. and *Giardia* spp. in the four districts in all livestock (calves, lambs, and goat kids) were 8 % (56/726) and 30 % (220/726), respectively. Investigation of associations between the occurrence of infection and livestock species showed statistically significant differences for both *Cryptosporidium* ($P = 0.024$) and *Giardia* ($P = 0.001$). The highest occurrence of *Cryptosporidium* was observed in calves and the lowest in goat kids, and the same pattern was found with *Giardia*.

Statistically significant differences ($P = 0.015$) were found between the occurrences of *Cryptosporidium* in the four districts: the highest occurrence was in Enderta (11 %) and the lowest occurrence was found in Hintalo Wejirat (3 %). No statistically significant difference was observed between the presence of *Giardia* in the four districts.

The intensity of *Cryptosporidium* infection between livestock species was as shown in **Figure 1**, with most infected animals shedding relatively low numbers of oocysts (+1). The intensity of infection in *Giardia*-infected animals varied between livestock species. Although most infected animals shed only low quantities of cysts (+1), relatively high *Giardia*-cyst counts were recorded in several calves.

The occurrence of *Giardia* infection among the three species of the study animals was compared in different age groups of animals using the chi-square test. Calves and lambs ≤8-weeks were more likely to have *Giardia* infection than calves and lambs of >8 weeks, whereas the opposite was seen for goat kids; goat kids over 8 weeks were more likely to be infected with *Giardia* than goat kids of 8 weeks and younger (**Table 2**). Although *Cryptosporidium* infections tended to occur more often within the younger age group for all livestock species, no clear age-related associations were identified.

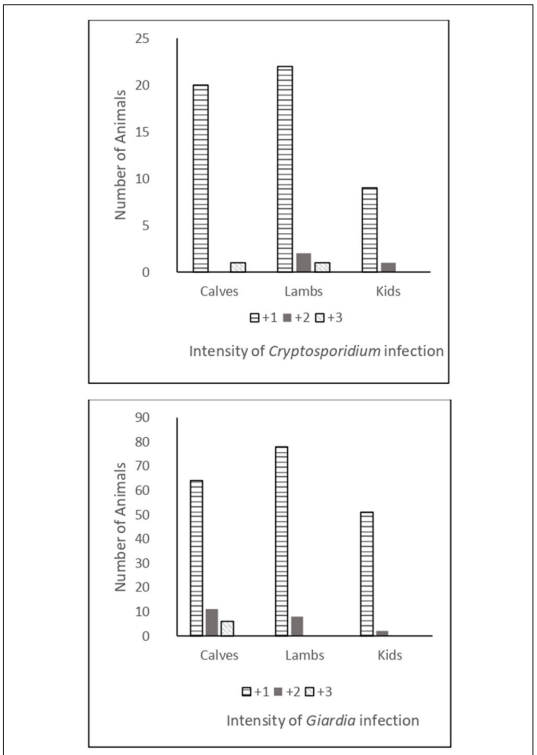

FIGURE 1 | Intensity of *Cryptosporidium* and *Giardia* infections in calves, lambs, and goat kids.

Cryptosporidium and *Giardia* Infections in Calves by District and Different Management Systems

The Chi-square test showed a significant difference in *Cryptosporidium* infection in calves in all four districts. The occurrence of infection in the calves of Kilte Awulaelo district (21%) was significantly higher than that in the other districts; see Supplementary File 3 (**Supplementary Table 1**). The occurrence of infection was significantly higher in crossbreed calves (33 %) than in local breed calves (7%); see Supplementary File 3 (**Supplementary Table 1**).

However, when multivariable logistic regression analysis was used, only management system and periparturient care were found to be significant. Calves in an intensive management system were more likely to be infected with *Cryptosporidium* than

TABLE 2 | Comparison of the occurrences of *Cryptosporidium* and *Giardia* infections between different age groups of calves, lambs, and goat kids.

Animals	Age	Total	*Cryptosporidium*			*Giardia*		
			Positives	Proportion (%)	*P*-value	Positives	Proportion (%)	*P*-value
Calves	≤ 8 weeks	65	9	14	0.226	32	49	0.040
	> 8 weeks	143	12	8		49	34	
Lambs	≤ 8 weeks	78	8	10	0.738	33	42	0.022
	> 8 weeks	190	17	9		53	28	
Goat Kids	≤ 8 weeks	65	4	6	0.303	8	12	0.041
	> 8 weeks	185	6	3		45	24	

calves in an extensive management system (OR = 7.8, 95% CI = 2.46–24.49: $P = 0.001$). Calves that stayed in the same barn with cows after birth were at higher risk of *Cryptosporidium* infection than calves that stayed in their pen alone or with other calves (OR = 3.2, 95% CI = 1.02–10.08; $P = 0.04$).

The occurrence of *Giardia* infection in calves was similar among the four districts and with respect to their periparturient care, weaning age, type of feed supplement, type of management system, and breeds see Supplementary File 3 (**Supplementary Table 2**).

In calves in urban farms in Mekelle city, out of 31 calf faecal samples collected from 12 urban farms, 13% contained *Cryptosporidium* spp. oocysts and 35% contained *Giardia* cysts.

Molecular Characterisation of *Cryptosporidium* and *Giardia* From Calf Infections

Of the 21 *Cryptosporidium*-positive samples from the four districts, species determination was attempted on 15, but only nine were successfully amplified and sequenced. Additionally, of the four *Cryptosporidium*-positive calf samples from Mekelle city, molecular characterisation was successful for three. Accordingly, *C. andersoni* ($n = 1$), *C. bovis* ($n = 3$), *C. ryanae* ($n = 7$), and *C. ubiquitum* ($n = 1$) were identified from the four districts, whereas only *C. andersoni* and *C. ryanae* were identified in the urban farms. The *C. ubiquitum* sample was subtyped and found to belong to the XIIa family.

Of the 81 *Giardia*-positive calf samples from the four districts, 31 were selected for molecular characterisation, of which 18 provided good sequence results; 14 were Assemblage E, three were Assemblage A, and one was Assemblage B. Subtyping the Assemblage A isolates revealed that two were AI and one could not be subtyped. Subtyping of the Assemblage B using multilocus sequence typing showed a close relationship with Algerian samples (37). Out of 11 *Giardia*-positive calf samples from urban farms, molecular characterisation was successful for six and all were Assemblage E.

Representative sequences have been deposited in GenBank and can be found under Accession numbers OK336067, OK336072-OK336079, OK336082-83 and OK358701-OK358702 (*Cryptosporidium*) and OK523961-OK523964, OK523969-OK523975, OK626272-OK626274, OK626276-OK626277 (*Giardia*).

Cryptosporidium Infection in Lambs and Goat Kids by District and Different Management Systems

Based on the Chi-square test, the occurrence of *Cryptosporidium* infection in lambs showed significant differences among the four districts. The occurrence of infection in lambs in the Enderta district (16%) was significantly higher than in the other districts; see Supplementary File 3 (**Supplementary Table 3**). However, such differences were not observed in goat kids among different districts. The only significant difference was that the occurrence of *Cryptosporidium* in goat kids housed with adults and other animals (9%) was higher than in goat kids kept with does in their pen (1.3%) see Supplementary File 3 (**Supplementary Table 4**). Lambs kept under a semi-intensive management system had a significantly higher occurrence of *Cryptosporidium* infection (19%) than lambs reared under an extensive management system (7%); see Supplementary File 3 (**Supplementary Table 3**).

However, analysis using multivariable logistic regression only showed a significantly higher occurrence of *Cryptosporidium* infection in lambs housed with adults and other animals than lambs kept with ewes in their pen (OR = 3.7, 95% CI = 1.12–11.19; $P = 0.020$). Lambs from Enderta were more likely to be infected with *Cryptosporidium* infection than lambs from Kilte Awulaelo and Hintalo Wejirat (OR = 5.1, 95% CI =1.07–23.88; $P = 0.040$). Lambs from Raya Azebo were more likely to be infected with *Cryptosporidium* infection than lambs from Kilte Awulaelo and Hintalo Wejirat (OR = 6.1, 95% CI = 1.14–32.4; $P = 0.035$).

Giardia Infection in Lambs and Goat Kids by District and Different Management Systems

Analysis using Chi-square test showed that the occurrence of *Giardia* infection in lambs was similar among the four districts, and not affected by age at colostrum feeding and type of feed supplement. However, lambs, but not goat kids, kept under semi-intensive management had a significantly higher occurrence of *Giardia* infection (44%) than lambs reared in extensive management (29%); see Supplementary File 3 (**Supplementary Tables 5**, **6**). Multivariable logistic regression

revealed that lambs housed with ewes and other animals were more likely to be infected with *Giardia* than lambs kept with ewes in their pen (OR = 2.7, 95% C = 1.42–5.21; $P = 0.002$).

Molecular Characterisation of *Cryptosporidium* and *Giardia* From Lamb Infections

Out of 93 lamb faecal samples positive for *Cryptosporidium* oocysts and/or *Giardia* cysts, DNA was extracted from 20. Of the 25 *Cryptosporidium*-positive lamb samples, only seven were selected for molecular characterisation. For five of them, amplification was successful and resulted in good sequences; three were identified as *C. ubiquitum* and two as *C. xiaoi*. All three *C. ubiquitum* isolates were subtyped as belonging to the XIIa family.

Of the 86 *Giardia*-positive lamb samples, most were very light infections containing few cysts; however, 17 were selected for molecular characterisation, and amplification and sequencing were successful for 12 of these. All the samples were found to be Assemblage E.

Representative sequences have been deposited in GenBank and can be found under Accession numbers OK336068-OK336070, OK336084-OK336087, OK358703-OK358704 (*Cryptosporidium*) and OK523965-OK523966, OK523976, OK626275 (*Giardia*).

Molecular Characterisation of *Cryptosporidium* and *Giardia* From Goat Kid Infections

Out of 61 goat kid faecal samples positive for *Cryptosporidium* oocysts and/or *Giardia* cysts, DNA was extracted from 11. Of the three *Cryptosporidium*-positive goat-kid samples selected for molecular characterisation, DNA from two of them was amplified and sequenced successfully and revealed *C. ryanae* and *C. ubiquitum*. The latter was subsequently identified as the XIIa subtype.

Of 10 *Giardia*-positive goat kids samples selected for molecular characterisation, only six samples provided good sequence results at one or more genes; all were identified as Assemblage E.

Representative sequences have been deposited in GenBank and can be found under Accession numbers OK336071, OK336080-81 and OK358705-06 (*Cryptosporidium*) and OK523967-OK523968, OK523977-OK523978 (*Giardia*).

DISCUSSION

Cryptosporidium and *Giardia* Infections in Calves, Lambs, and Goat Kids

The main finding of this study is that both parasites occur widely and frequently within livestock in rural Tigray, with a significantly higher occurrence in calves than small ruminants, particularly goat kids. The low infection in goat kids may reflect that they are browsers, and thus the likelihood of ingesting the transmission stages of these parasites from the ground is lower than for the other grazing species (38). Another important finding was that in the four study districts, *Cryptosporidium* infection of livestock occurred significantly less frequently than *Giardia* infection and a similar pattern was seen among calves from the urban farms in Mekelle. However, for calves, more variables had significant association with *Cryptosporidium* infection than with *Giardia* infection. This might be attributed to the robustness of *Cryptosporidium* and survival capability to stay in the environment and contaminate the barn. In addition, in the study areas, although intensive management and cross breeds were reported by some farmers concerning calves, for lambs and goat kids only local breeds were reported, with extensive management almost exclusively predominating, and intensive management not reported at all. This is in line with a report by Vermeulen et al. (39), who found that intensively managed calves are the dominant source of oocysts compared with other livestock species. Although *Giardia* cysts are robust and can survive well in aquatic environments, they are relatively fragile compared with *Cryptosporidium* oocysts and are unlikely to survive for prolonged periods in the animal houses (40).

Unlike in our study, other studies of livestock in Ethiopia for both parasites have tended to find higher prevalences of *Cryptosporidium* infection than *Giardia* (see **Table 1**). However, most other studies focused only on *Cryptosporidium*, and generally report higher prevalences than found in our investigation. Differences in occurrence for both parasites should be expected between studies, due to variations in management practises, transmission possibilities, host susceptibilities, and other parameters. Of particular relevance may be that most of the samples in our study were from extensively managed animals, but in some of the other studies from Ethiopia the animals were intensively managed [e.g., 22, 28]. Intensive management may promote transmission, due to closer contact between infected animals and build-up of oocyst contamination within a restricted environment.

However, it also seems likely that methodological specificities and sensitivities may be of relevance. Most other studies from Ethiopia used mZN for detection of *Cryptosporidium* (21, 24, 27), which is less sensitive and less specific than the IFAT technique used in our study. A study from Tanzania on *Cryptosporidium* in calves (41) found that misidentification occurred when mZN was used, and commented that false-positive results may occur when mZN is the sole detection method used.

There are only three published reports from Ethiopia on the prevalence of *Giardia* in livestock (**Table 1**), and our results describe a considerably higher prevalence than all the previous studies. Methodological differences may also play a role here, as a less sensitive method (light microscopy with Lugol's iodine staining) was used in one study for identifying *Giardia* infection in lambs (21). However, another study that investigated *Giardia* shedding in calves in southern Ethiopia (30) used IFAT, but also found a much lower prevalence (10%; see **Table 1**).

Although both infections were found in all species of livestock in all districts investigated, and occurrences were similar for *Giardia* among districts, for *Cryptosporidium* the occurrences varied significantly according to district. That we found the highest occurrence of *Cryptosporidium* infection in Enderta followed by Kilte Awulaelo, is interesting as surveys of fresh

produce and water in all four districts found contamination with these parasites in these two districts only (31, 42). This might suggest that this parasite is circulating more successfully among animals and in the environment in these two districts.

Most animals included in our study had relatively low intensities of infection. Most studies from different parts of the world do not report the intensity of infection. The low levels of intensity of infection found in our study may reflect that the majority of samples were collected from extensive management systems where trickle infections, with concomitant build-up of immunity, may be more likely to occur than abrupt exposure to a massive infectious dose.

The prevalence of *Cryptosporidium* infection was similar in the different age groups of animals investigated in our study, although there was a trend that the occurrence was higher within the younger age group for all livestock species. In a report from Nigeria, young calves (< 3 months old) had higher infection rates of *Cryptosporidium* than calves > 3 months old (43).

The occurrence of *Giardia* infection was higher in calves and lambs ≤ 8weeks old than older calves and lambs suggesting that the infection rates decline as the age of the animals increases. Similar results have been reported in other countries. In Canada and China the prevalence of *Giardia* infection was greater in calves and lambs than adults (44, 45) and this is mostly consistent with our findings. However, we found that *Giardia* infection in goat kids was significantly higher in the age group > 8 weeks than ≤ 8 weeks old.

Compared with *Giardia* cyst excretion from calves and lambs, relatively few cysts were found in most *Giardia*-positive goat kids. This low shedding of *Giardia* cysts from infected goat kids might be related to goats being selective browsers, and therefore less exposed to infectious cysts.

Risk Factors for *Cryptosporidium* and *Giardia* Infections in Calves, Lambs, and Goat Kids

We found that *Cryptosporidium* infection occurred more often in crossbreed calves than local breeds. The potential effect of cattle breed on *Cryptosporidium* infection has been previously discussed (13). In a study from Malaysia (46), it was found that Mafriwal cattle (Sahiwal × Friesian crosses) and Jersey × Friesian crosses had higher rates of infection. This could also be due to the different management system, as crossbreed calves tended to be intensively managed compared with local breeds. Higher rates of *Cryptosporidium* infection in lambs under semi-intensive systems probably reflects the relatively high confinement in semi-intensive management, which results in greater crowding and a more contaminated environment than in farms using an extensive system. No significant difference was observed in goat kids under both management systems in our study.

Calves housed with cows had a significantly higher *Cryptosporidium* infection than calves housed in their pen alone or with other calves in our study; this finding is similar to those reported by Manyazewal et al. (27). This strong association between periparturient care and *Cryptosporidium* infection in calves is probably associated with the level of hygiene when calves are kept alone or with other animals. This suggestion is supported by Abebe et al. (23), who reported a substantial connexion between *Cryptosporidium* infection and the hygienic status of dairy animals and their farms. Similarly, Ayele et al. (29) stated that poor hygiene increases the infection rate and spread of *Cryptosporidium* among animals. This is probably because dirty and muddy farms may create favourable conditions for the presence or survival of *Cryptosporidium* oocysts. This increases the likelihood of feed and water contamination, which, in turn, might favour exposure of calves to *Cryptosporidium* oocysts. We found similar results for ewes and lambs and for goat kids and does housed together in a single pen, and, again, a likely explanation is close contact between the animals and contamination, along with less exposure to inactivation pressures such as desiccation and UV-irradiation (13).

Molecular Characterisation of *Cryptosporidium* and *Giardia* in Calves, Lambs, and Goat Kids

Perhaps the most important finding of this cross-sectional study is that, despite the close contact between animals and humans, the main zoonotic species and subtypes of *Cryptosporidium* and *Giardia* were not detected in any of the animal samples. However, species such as *C. andersoni* and *C. ubiquitum*, which are usually considered to have limited zoonotic potential (47, 48), were detected. Similarly, the *Giardia* assemblages identified were mostly non-zoonotic Assemblage E, but a few *Giardia* isolates detected in calves were of the potentially zoonotic Assemblages A and B.

Globally, four species of *Cryptosporidium* occur commonly in cattle: *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* (14). However, the majority of studies from Africa have indicated that *C. parvum* occurs relatively infrequently in cattle here (13). Nevertheless, one study from Egypt reported all the common *Cryptosporidium* spp., except for *C. andersoni* (49), and, in Ethiopia, all four common species have been reported in calves from the Oromia region by Wegayehu et al. (25) and Manyazewal et al. (27). Our findings seem more similar to those from a study from Nigeria that reported all species of *Cryptosporidium* from calves, except *C. parvum* (43). It could be argued that *C. parvum* was not detected in calves in our study, as it is more common in very young calves. Of the 65 calves ≤8 weeks old included in our study, the age range was from 4 days, with 21 ≤ 3 weeks of age. Interestingly, our findings also differ from other reports from Africa, as we also detected *C. ubiquitum,* subtype XIIa in cattle. *C. ubiquitum* is considered an emerging zoonotic pathogen, with a wide geographic distribution and broad host range (47). However, to the best of our knowledge, there is only a single report of human infection with *C. ubiquitum* in Africa, with just one child from Nigeria reported to be infected with *C. ubiquitum* (then described as cervine genotype) among a survey of 692 children, of whom 134 were considered to be infected with *Cryptosporidium* (50). Thus, it appears that zoonotic transmission of this species of *Cryptosporidium* is not well established in Africa; reasons for this are discussed in the review article of Robertson et al. (13).

Globally, the most common species of *Cryptosporidium* identified in sheep are *C. ubiquitum* and *C. xiaoi*, although in

some European countries *C. parvum* is also prevalent in sheep (51). The report by Robertson et al. (13), that *C. parvum* occurs relatively infrequently in African small ruminants, with *C. xiaoi* and *C. ubiquitum* predominating, is in line with our findings.

In goats, the most common *Cryptosporidium* spp. identified from different parts of the world are *C. ubiquitum*, *C. xiaoi,* and *C. parvum* (9, 52, 53). However, in our study only *C. ryanae* and *C. ubiquitum* were detected in goat kids. A recent review of *Cryptosporidium* in goats (38), reported that in studies from different parts of the world (Europe, Asia, Africa, South America, and Oceania) where *C. ubiquitum* has been reported, only subtype-XIIa has been detected (where subtyping has been conducted) and we also found this subtype.

The *Giardia* assemblages identified in this study indicated that calves mainly carried Assemblage E infections and all *Giardia* isolates from lambs and goat kids belonged to Assemblage E. This Assemblage, which is generally not considered zoonotic, is usually found in cattle, sheep, goats, and pigs (9, 17). However, we did identify a few potentially zoonotic Assemblages in calves, with three Assemblage A isolates and one Assemblage B isolate, and consistent results among the different genetic loci amplified by PCR. Our finding of Assemblage B in calves is rather unusual, but Sprong et al. (17) also noted a very low percentage of Assemblage B in cattle. Our case could, potentially, indicate carriage rather than infection. Subtyping of two Assemblage A isolates revealed AI, which is mainly found in animals (17).

CONCLUSION

Our study found a widespread occurrence of *Cryptosporidium* and *Giardia* in livestock in Tigray, Ethiopia. Five *Cryptosporidium* species, namely *C. bovis*, *C. ryanae*, *C. andersoni*, *C. xiaoi*, and *C. ubiquitum*, were identified in calves, lambs, and goat kids, with *C. ryanae* and *C, ubiquitum* being the predominant species. The zoonotic species, *C. parvum,* was not detected in any of the animal samples. Similarly, the majority of the *Giardia* assemblages detected in the samples were Assemblage E.

Periparturient care seemed to be particularly important regarding these infections in livestock, with crowding among calves, lambs, and goat kids, particularly mixing with adults and/or other animals, increasing the vulnerability to infection with both these parasites. Similarly, if the management system was more intensive, then the likelihood of infection was greater.

Conducting further research from different locations and settings in Ethiopia could provide relevant information regarding transmission dynamics for both parasites and the potential for zoonotic transmission. It would also be important to ascertain the effect that these parasites have on the productivity of the livestock, given that the farmers are dependent on healthy animals for their livelihoods.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, OK336067, OK336072-OK336079, OK336082-83, OK358701-OK358702; OK523961-OK523964, OK523969-OK523975, OK626272-OK626274, OK626276-OK626277, OK336068-OK336070, OK336084-OK336087, OK358703-OK358704, OK523965-OK523966, OK523976, OK626275, OK336071, OK336080-81, OK358705-06, OK523967-OK523968 and OK523977-OK523978.

ETHICS STATEMENT

The animal study was reviewed and approved by the Regional Committee for Medical and Health Research Ethics, South East; REK, SE, case number 2018/1279 C and the National Research Ethics Review Committee in Ethiopia (Ref. No: MoSHE/RD/144/1095/19). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

TK, AN, JD, GT, and LR: conceptualization, methodology, and writing-review and editing. LR: validation. TK, AN, JD, and LR: formal analysis. TK: investigation, data curation, and writing original draft preparation. TK and LR: visualization and funding acquisition. LR, AN, JD, and GT: supervision. LR: project administration. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.825940/full#supplementary-material

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Cryptosporidium and *Giardia* infections in humans in Tigray, Northern Ethiopia: an unexpectedly low occurrence of anthropozoonotic transmission

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ABSTRACT

Enteric protozoans *Cryptosporidium* spp. and *Giardia duodenalis* are among the leading causes of diarrhoea in children. These parasites have particular impact in low- and middle-income countries. In these countries, people often live in close contact with their animals, highlighting the potential role of zoonotic routes of transmission in disease spread. The occurrence and species/genotypes of *Cryptosporidium* and *Giardia duodenalis* infecting humans in Tigray, Ethiopia were investigated, along with the risk associated with infection. Stool samples from 249 asymptomatic people (4-80 years of age) in four rural districts in Tigray and 58 from symptomatic young children (1-33 months) attending health centres in Mekelle, Tigray's main city, were analysed for *Cryptosporidium* oocysts and *Giardia* cysts. Participants in the rural areas completed questionnaires regarding potential risk factors, with emphasis on livestock contact and sources of water. The occurrence of *Cryptosporidium* infection was 6% and 5% in people in the rural districts and young children from Mekelle, respectively; equivalent figures for *Giardia* infection were 29% and 14%. Molecular characterization of *Cryptosporidium* isolates revealed *C. ubiquitum,* subtype XIIa in a sample from rural districts, and *C. hominis* subtype IdA17 (1 sample) and IbA9G3 (2 samples) in infants from Mekelle with diarrhoea. For *Giardia*, Assemblage B predominated (22/25; 88%), but we also identified three samples with Assemblage A (AII).

Our major finding was that, despite the close contact between people and livestock in our rural study sites, transmission of *Cryptosporidium* and *Giardia* between humans and their animals seems to be surprisingly uncommon. Our results are discussed in relation to other relevant studies, and also draws attention to the possibility that introduction of zoonotic species and/or subtypes, such as *C. parvum*, could have serious consequences for both human and animal health. As our study was conducted in Tigray, further investigation in different settings in Ethiopia could provide relevant information on transmission and zoonotic potential, and the potential for spread of zoonotic transmission. In addition, given the importance of these two parasites in causing diarrhoea in children, this information is vital for developing effective appropriate interventions against transmission that can be applied not only in Tigray or Ethiopia, but throughout Africa and beyond.

1. Introduction

Cryptosporidiosis and giardiasis are enteric protozoan diseases caused, respectively, by *Cryptosporidium* spp. and *Giardia duodenalis* with a worldwide distribution in humans and animals. Both pathogens are important causes of diarrhoeal diseases (Santin, 2020), although *Giardia* infection is often asymptomatic, particularly in endemic areas (Bartelt and Platts-Mills, 2016). Cryptosporidiosis ranks second to rotavirus infection as a cause of death due to diarrhoea in children under the age of five years, killing around 525,000 children every year (World Health Organization, 2017; Kotloff et al., 2013).

In sub-Saharan Africa, a 3-year case-control study showed that

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approximately 2.9 million episodes of diarrhoea annually in children aged < 24 months each year were attributed to *Cryptosporidium* (Sow et al., 2016). In 2010, 179 million cases of giardiasis were reported globally (Pires et al., 2015). In developing countries, *Giardia* infection is often acquired during early childhood and the prevalence often reaches 15-20% in children under 10 years of age (Nkrumah and Nguah, 2011). Transmission of *Cryptosporidium* and *Giardia* to people mostly occurs directly from person to person, via zoonotic transmission directly from animals, or via contaminated drinking water or contaminated food (Cacciò et al., 2005).

Most cases of human cryptosporidiosis are due to five species: *C. hominis, C. parvum, C. meleagridis, C. felis,* and *C. canis*, with the vast majority of cases due to *C. hominis* and *C. parvum* (Ryan et al., 2021). Although *C. parvum* is generally considered zoonotic, and this is true for sub-types IIa and IId, other subtypes, notably IIc (*C. parvum anthroponosum)*, IIe, and IIm, have only been reported in humans and are not considered zoonotic (Nader et al., 2019). Although *C. hominis* is considered to be primarily anthroponotic, it has been suggested that its host range may be wider than previously assumed (Widmer et al., 2020).

There are geographical differences in the distribution of *C. hominis* and *C. parvum.* In some parts of the world, infected animals have been implicated as the source of cryptosporidiosis outbreaks in humans (Chalmers et al., 2019; Kiang et al., 2006; Smith et al., 2004), but in low- and middle-income countries (LMIC), including the majority of African countries, infection with zoonotic *Cryptosporidium* species, particularly *C. parvum,* is infrequently reported (Robertson et al., 2020; Yang et al., 2021). In most African countries, it appears that *Cryptosporidium* transmission to people is largely anthroponotic (human-to-human), and various theories have been suggested for this including, but not limited to, immunity development, genetic differences in the host populations, differences in livestock husbandry, and environmental differences (Robertson et al., 2020). Routes of transmission are probably either direct or via a transmission vehicle, such as food or water; however, direct evidence of foodborne or waterborne transmission is lacking in most African countries.

A large number of different mammals serve as hosts for *G. duodenalis*. Assemblages A and B are responsible for human infection, and these assemblages have also been detected in a variety of mammals (Sprong et al., 2009). In Africa, sub-assemblage AII appears to be more common (88%) than sub-assemblage AI (12%) in humans; however, the reverse trend is reported in African animals (Sprong et al., 2009). In Africa, the data suggest that sub-assemblage BIII is more prevalent (81%) in human infections than infection with sub-assemblage BIV (19%) (Sprong et al., 2009).

In Ethiopia, *Cryptosporidium* spp. and *G. duodenalis* infections are endemic in humans and animals. Systematic reviews by Tarekegn et al. (2021) and Tegen et al. (2020) reported 11% and 10% prevalences of *Cryptosporidium* and *Giardia,* respectively, in humans. Seven *Cryptosporidium* spp., namely *C. hominis, C. parvum, C. meleagridis, C. viatorum, C. felis, C. canis,* and *C. xiaoi,* have been reported in people from Ethiopia (Tarekegn et al., 2021). For *G. duodenalis* infections from the southern part of Ethiopia, both Assemblages A and B and mixed infections were reported from humans, with most Assemblage A isolates typed as sub-assemblage AII (Damitie et al., 2018). In stool samples from school children in Bahir Dar, Ethiopia, sub-assemblages AII, BIII, and BIV were reported (de Lucio et al., 2016).

In Ethiopia, farmers live in close proximity with their livestock, providing a strong potential for anthropozoonotic transmission of pathogens, including *Cryptosporidium* and *Giardia*. In addition, children, who are often most susceptible to infection with these parasites, play a major role in caring for these animals, and children and women prepare the manure as cakes that are used as fuel and building materials (Tegegne, 2004). Nevertheless, there is a paucity of data regarding the extent to which zoonotic transmission of *Cryptosporidium* spp. and *Giardia duodenalis* occurs in Tigray, northern Ethiopia.

Our overarching aim was to investigate the extent to which zoonotic transmission of these parasites occurs in this region, with the intention of developing relevant interventions.

2. Materials and Methods

2.1. Study areas, study design, and sample collection

A cross-sectional study design was used to investigate the occurrence of *Cryptosporidium* and *Giardia* in humans from four districts of Tigray (from mid-October 2018 to mid-January 2019); detailed information about these districts can be found in Kifleyohannes et al. (2021). Accordingly, a sample size of 265 was determined by using expected prevalences of 8% and 11% for *Cryptosporidium* and *Giardia,* respectively and 5 % absolute precision and 95 % confidence intervals (Adamu et al., 2010; Flecha et al., 2015), using Epitools (https://epitools.ausvet.com.au/oneproportion). However, 307 humans were sampled to increase the precision. We went from door-to-door in the various study sites, visiting all homesteads to obtain participants. All those who fulfilled the inclusion criteria and were willing to participate were included, with emphasis on those who owned or had daily contact with livestock (cattle, sheep, goats) less than 6-months old. Those who only owned livestock over 6-months of age were excluded.

Those who agreed to participate in our study were provided with sample bottles. These were collected when a stool sample had been provided and were transported to the Parasitology Laboratory, at the College of Veterinary Medicine, Mekelle University (Mekelle, Ethiopia).

It should be noted that at the time of sample collection, samples of animal faeces, fresh produce, and water from local sources were also collected. The results from analysis of these samples have already been published (Kifleyohannes and Robertson, 2020; Kiflyohannes et al., 2021, 2022).

In addition, during February and March 2020, stool samples were collected from children ≤ 2 years of age with diarrhoea. These samples were obtained from children attending 7 urban and peri-urban hospitals and health centres situated in and around Mekelle, the capital city of Tigray Province: Mekelle Hospital, Quiha, North (Semein), Beza, Kassech, Adishumdihun, and Rimna Hospital. Sample bottles were provided to the health centres. Samples were transported to the Parasitology Laboratory, at the College of Veterinary Medicine, Mekelle University where they were preserved by mixing in 2.5% potassium dichromate and then refrigerated at 4°C. Following shipment to the Parasitology Laboratory, at the Norwegian University of Life Sciences, Norway, potassium dichromate was removed from the stool samples by repeated washings before further processing and investigation.

2.2. Immunofluorescent antibody testing

Approximately 20 μL of stool pellet was placed on a microscope slide using a plastic loop, air dried, and then fixed with methanol. The fixed samples were stained by incubation at 37°C for 20-30 minutes with 15 μL of fluorescein isothiocyanate (FITC)-labelled monoclonal antibody for the detection of *Cryptosporidium* or *Giardia* (oo)cysts (Aqua-Glo, Waterborne Inc., NO, USA). Following antibody staining, 10 μL of 4`, 6` diamidino-2-phenylindole (DAPI; 0.5 μg/ml) was added for staining of nuclei for one minute. After removal of excess DAPI, a drop of 1,4 diazabicyclo (2.2.2) octane (DABCO) anti-fade mounting medium was added, and a coverslip placed over the sample. The stained samples were screened immediately after staining using a fluorescence microscope (Leica DMLB) equipped with appropriate filters (FITC and DAPI) and Nomarski optics. Intensity of infection was determined based on the number of cysts/oocysts per field of view at the objective 20x: +1 (1-9) (oo)cysts, +2 (10-50) (oo)cysts, +3 (51-100) (oo)cysts, +4 >100 (oo) cysts.

2.3. DNA extraction, polymerase chain reaction, and sequencing

DNA was extracted from approximately 250 μl of selected positive stool samples using a DNeasy PowerSoil Kit protocol (Qiagen, Oslo, Norway), with slight modifications of the elution volume from 100 μl to 50 μl. Finally, DNA was eluted in 50 μl of the elution solution and stored at -20°C until analysis by molecular methods.

Cryptosporidium DNA present in the stool samples was detected by nested PCR amplification of the small subunit (*SSU) rRNA* gene, as previously described by Jiang et al. (2005). In addition, primers targeting sections of the *gp60* gene for *C. hominis* and *C. ubiquitum* were used for sequence investigation by conventional PCR (Alves et al., 2003; Li et al., 2014). Genetic identification of *Giardia* Assemblages present was performed by PCR and sequencing targeting four genes: glutamate dehydrogenase (*GDH*) gene, beta-giardin (*BG*) gene, triosephosphate isomerase (*TPI*) gene, and *SSU* gene (*rRNA*) (Read et al., 2002; Cacciò et al., 2008; Lalle et al., 2005; Sulaiman| et al., 2003). In addition, three genes were used to subtype Assemblage A samples: DNA repair and recombination gene (*RHP26*), high cysteine membrane gene (*HCMP*), and *NEK* kinase gene (Ankarklev et al., 2018). Multi-locus sequence typing (MLST) of Assemblage B isolates was performed using the suggested genetic loci, 6-phosphogluconate dehydrogenase (*pdg*), Hypothetical protein, and phosphorylase B kinase gamma catalytic chain (*phkg2*) (Seabolt et al., 2021).

Primers and reaction cycles, as well as other details, for all PCR used in this study are provided in Supplementary File 1.

Each amplification run included a negative control (nuclease-free water) and two positive controls. The PCR products were visualized in 2% agarose gels, using SYBERsafe DNA gel stain and visualized under UV illumination. A 100 bp ready-to-use DNA ladder (Thermo Scientific) was used for fragment size determination. PCR amplicons were purified using either ExoSAP-IT PCR product clean-up reagent (Thermofisher Scientific) or PureLink Quick Gel extraction and PCR purification Combo kit (Thermo Fisher Scientific) and sent to a commercial company (Eurofins Genomics, Germany) for sequencing in both directions. Geneious Prime software was used to check sequences, and contigs were formed before comparing them with those in GenBank using the NCBI BLAST tool, as well as a recently developed online Cryptogenotyper for *Cryptosporidium* using *SSU* and *gp60* (Yanta et al., 2021). All the sequences have been deposited in GenBank, and their respective Accession Numbers are provided in the Results section.

2.4. Questionnaire survey

Pre-tested questionnaires were administered to collect data on potential risk factors for *Cryptosporidium* and *Giardia* infections in people in the districts. This encompassed gathering general personal information, including information on illness in themselves and children, information on livestock ownership and management, and information on sources and consumption of fresh produce and water.

The questionnaires (see Supplementary File 2) were designed to contain mainly closed-ended questions. Individual interviews were made by the first author, supported by colleagues from the College of Veterinary Medicine of Mekelle University, along with veterinarians and technicians in the study districts, and the information was obtained from the interviewees in a language with which they felt comfortable (Tigrigna).

2.5. Ethical statement

This study was approved by ethical committees in both Ethiopia and Norway: the Ethiopian National Research Ethics Review Committee (Ref. No: MoSHE/RD/144/1095/19) and the Norwegian Regional Committee for Medical and Health Research Ethics, South East (REK, SE, case number 2018/1279 C). All subjects provided written informed consent, and a parent or guardian of any child participant provided their informed consent on their behalf. Informed consent from patients was not required for samples collected from health centres, as the stool samples had been provided for routine clinical diagnostic procedures; questionnaire information associated with these patients was not collected. Information from all participants was anonymized prior to analysis of the data.

2.6. Statistics

A database was created in Excel and analyzed using STATA 15 software (STATA SE Corp, TX, USA) software. To evaluate associations between risk factors for *Cryptosporidium* and *Giardia* infection, the Chi-square and Fisher's exact tests were used. The confidence level was maintained at 95% and $P < 0.05$ was established for the significance level.

3. Results

3.1. Participant characteristics and occurrence of Cryptosporidium and Giardia in samples from the four districts

3.1.1. Participant characteristics and overall occurrence of the parasites

Samples were collected from 249 participants among the four rural sampling districts. The age of the participants ranged from 4-80 years (median = 19, mean = 25), with 77 female and 172 male. All the stool samples were of normal consistency; none of the participants exhibited diarrhoea at sample collection.

Cryptosporidium oocysts were detected in 6% (15/249) of stool samples (Table 1). All 15 positive samples contained 9 oocysts or fewer per microscope field (+1), and only two samples contained DAPI-positive oocysts.

Giardia cysts were detected in 29% (71/249) of the stool samples examined (Table 1), occurring significantly more frequently than *Cryptosporidium* oocysts ($P<0.0001$). The number of cysts ranged from below 9 cysts (+1) to more than 100 cysts (+4) per microscope field of view. Of the 15 *Cryptosporidium*-positive samples, 13 also contained *Giardia* cysts.

The proportion of positive samples for *Cryptosporidium* was very similar in each of the four districts, ranging from 4% to 9%. For *Giardia,* the proportion of positive samples was highest in Enderta (35%) and lowest in Hintalo-Wejirat (21%) (Table 1), but there was no significant difference in occurrence between districts.

Only a few participants among all the districts did not own any livestock (n=38) and were from Enderta (13), Kilte Awulaelo (17), and Raya Azebo (8). The occurrence of these parasites in those are included in the data in Table 1; there was no difference in occurrence between those people owning livestock and those not owning livestock (data not shown).

3.1.2. Occurrence of Cryptosporidium and Giardia by age group

The proportion of positive samples for *Cryptosporidium* and *Giardia* was related to age ($P= 0.032$ and $P=0.005$, respectively). For both *Cryptosporidium* and *Giardia,* a higher proportion was found in the younger age group (≤ 15 years). Whereas 11% and 40% of participants in the younger age group were shedding *Cryptosporidium* or *Giardia*, respectively, these proportions in participants over 15 years were 4% and 23%).

3.1.3. Occurrence of Cryptosporidium and Giardia by sex

Females were significantly more likely to be infected with *Cryptosporidium* than males ($P=0.012$), with 12% (9/77) of females infected with *Cryptosporidium* compared with 3.5% (6/172) of males (Fig. 1). No significant association was observed between sex and *Giardia* infection.

Table 1
Cryptosporidium and *Giardia* detected in people in the four districts

		Cryptosporidium			*Giardia*		
Districts	**No.**	**No. Positive**	**Proportion (%)**	**P-value**	**No. Positive**	**Proportion (%)**	**P-value**
Enderta	69	6	9	0.628	24	35	0.395
Kilte-Awulaelo	77	3	4		23	30	
Hintalo-Wejirat	43	3	7		9	21	
Raya-Azebo	60	3	5		15	25	
Total	**249**	**15**	**6**		**71**	**29**	

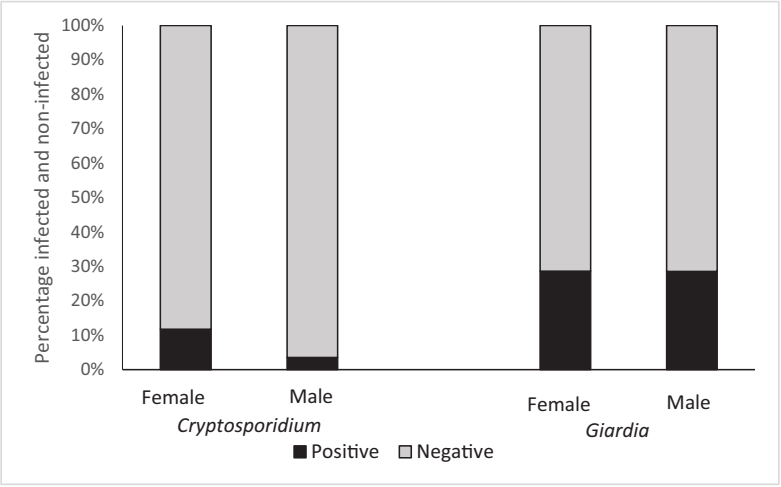

Fig. 1. Occurrence of *Cryptosporidium* and *Giardia* by sex

3.2. Occurrence of Cryptosporidium and Giardia in paediatric patient samples from urban and peri-urban health centres

In seven health centres in urban and peri-urban areas, 58 stool samples were collected from children two years and under, except for one child 33 months old. All had diarrhoea.

Among these, *Cryptosporidium* oocysts were detected in three of the children, giving an overall proportion of positive samples for *Cryptosporidium* 5.2% (3/58). *Giardia* was detected in eight (13.8%) of the samples from these children, with a higher rate of infection noted in children over 1-year old (7 in 27; 26%) than in children ≤1 year old (1 in 31; 3%) (P = 0.02). The intensity of infection in the different age groups is shown in Table 2.

Table 2
Intensity of shedding of *Cryptosporidium* and *Giardia* (oo)cysts in paediatric patient samples from urban and peri-urban health centres

Age (months)	Total	Intensity per field of view
Cryptosporidium		
6	1	+1*
17	1	+2*
20	1	+1
Giardia		
9	1	+1
17	2	+1
20	2	+2
22	1	+2
22	1	+3*
33	1	+3

*+1, 1–9 (oo)cysts, *+2, 10–50 (oo)cysts, *+3, 51–100 (oo)cysts

3.3. Cryptosporidium genotyping

Of the *Cryptosporidium*-positive samples collected from the four rural districts, only one was appropriate for molecular characterization. The single good quality sequence, which was obtained from *Cryptosporidium* oocysts from a 12-year old girl, revealed *C. ubiquitum*, subtype XIIa. Of the three *Cryptosporidium*-positive samples from young children (all baby boys) attending urban health centres, molecular characterization revealed *C. hominis;* subtyping indicated one subtype IdA17 and the other two subtype IbA9G3. The two children with *Cryptosporidium* of the same subtype did not attend the same health centre.

Sequences have been deposited in GenBank and can be found under Accession numbers OL889916, OM241502- OM241503, OL952819, OM273872-OM273873.

3.4. Giardia genotyping

From the *Giardia*-positive samples obtained from the four rural districts, 25 were selected for molecular characterization, based on the intensity of infection from (+4 to +1) and presence of nuclei as shown by DAPI staining. Sequence results were obtained from 20 of these samples at one or more genes. Three samples belonged to Assemblage A (AII) and the other 17 were Assemblage B. Multi-locus typing of 17 Assemblage B samples using three genetic loci suggested by Seabolt et al. (2021), resulted in quality sequences at one or more genes. However, sequences from the same samples using the three genes were not concordant with the genetic lineages described by Seabolt et al. (2021) (see Supplementary file 4). The majority of samples, particularly at *pdg* and *phkg2* genes, were in the same group as those described as being of Algerian origin (Seabolt et al., 2021). However, at the *hyp* gene,

the majority of samples were most similar to those classified as being of Peruvian origin by Seabolt et al. (2021). For sixteen samples, sequencing at *GDH* and *TPI* loci identified 37.5% (6/16) BIII and 62.5% (10/16) BIV.

Of the eight *Giardia*-positive samples from young children attending urban health centres, molecular characterization was attempted on all; successful amplification at two genes (*GDH* and *SSU rRNA*) in five of these samples revealed Assemblage B.

Representative sequences have been deposited in GenBank and can be found under Accession numbers OL952820 - OL952856, OM273874 - OM273888, OM732417- OM732429.

3.5. Risk factors associated with Cryptosporidium and Giardia infection

Potential risk factors for *Cryptosporidium* spp. or *G. duodenalis* infection were categorized into three groups: (i) general information on the participants, (ii) their living conditions and livestock contact, and (iii) their sources of water and fresh produce. Apart from age and sex (higher occurrence in children and females), none of the risk factors investigated were significantly associated with *Cryptosporidium* infection (see supplementary file 3, Table 1).

Among factors related to the general information, district and level of education were not significantly associated with *Giardia* infection. Higher occurrence of *Giardia* infection was found in children than adults. Among the other factors, the occurrence of *Giardia* was significantly higher in people who used stream water 42.9% (27/63) as a source of drinking water than in people obtaining water from hand pumps 26.6% (53/199) and ponds 25% (4/16) ($P = 0.004$) (see supplementary file 3, Table 2). In addition, *Giardia* infections occurred significantly more often in those who drank untreated water 50% (11/22) than treated water 26.6% (60/226) ($P = 0.02$) (see supplementary file 3, Table 2).

4. Discussion

The main finding from our study was that anthropozoontic cycling of these parasites between people and their livestock appears to occur only rarely in our study area. This was unexpected as there is close contact between humans and young livestock at the district-level sampling sites, and both *Cryptosporidium* spp. and *G. duodenalis* infections occur relatively commonly in people (6% and 29% occurrence, respectively, from the district sampling) and livestock (8% and 30% occurrence, respectively; Kifleyohannes et al., 2022) in these locations. However, in our previous description of livestock infections in these districts (Kifleyohannes et al., 2022), we found the following five *Cryptosporidium* species, namely *C. bovis, C. ryanae, C. andersoni, C. xiaoi*, and *C. ubiquitum*; the most zoonotic species, *C. parvum,* was not detected in any of the animals (calves, lambs, goat kids) investigated. Our results support the findings of Robertson et al. (2020) who reported that anthroponotic transmission predominates in Africa at present. In addition, a review article by Yang et al. (2021) also notes that in LMIC, anthroponotic transmission of *Cryptosporidium* plays a major role.

Although *C. hominis* was identified among the young children with cryptosporidiosis in Mekelle, as has been indicated in other studies of young Ethiopian children with diarrhoea (e.g., Johansen et al., 2022), the one *Cryptosporidium* sample from the rural districts that we were able to characterise molecularly was the newly emerging *C. ubiquitum* subtype XIIa, which does have zoonotic potential. Although we believe this to be the first report of human infection with *C. ubiquitum* in Ethiopia, it has been reported from other countries, including in Africa (Nigeria; (Molloy et al., 2011), Ghana, Tanzania (Krumkamp et al., 2021)). Our sample with *C. ubiquitum* was detected in a 12-year-old girl who had been in contact with adult cattle and goat kids daily. Although none of the goat kids (with which she had contact and that were investigated), were positive for *Cryptosporidium* at the time of sampling, investigations in the same study area (Kifleyohannes et al., 2022) found *C. ubiquitum* in calves, lambs, and goat kids. A recent review of *Cryptosporidium* in goats also showed that *C. ubiquitum* has been detected in goats as well as other animals globally. This species has been suggested to represent a greater threat to human and animal health than most other *Cryptosporidium* species, except *C. parvum*, given its ability to infect both humans and livestock (Utaaker et al., 2021).

Similarly, most of the *Giardia* Assemblages detected in the livestock samples in our study were Assemblage E, with just a few Assemblage A and B *Giardia* isolates detected in samples from calves (Kifleyohannes et al., 2022). All 3 Assemblage A isolates from people belonged to AII, which is mainly found in humans, whereas all 3 Assemblage A isolates from calves in these districts were subtyped as AI (Kifleyohannes et al., 2022), which occurs mainly in animals (Sprong et al., 2009). The various owners of these three animals shedding AI *Giardia* were negative for *Giardia*. However, one of the 17 people found to be infected with *Giardia* Assemblage B owned a calf that was also shedding Assemblage B *Giardia* (Kifleyohannes et al., 2022). Alignment of the sequences obtained from PCR amplification of DNA from these two isolates at two different genes (*phkg2* and *BG*) showed that they were identical apart from one single nucleotide polymorphism. Given that infection with Assemblage B *Giardia* was only found in one calf in this study (Kifleyohannes et al., 2022), the results suggest that transmission between these hosts may have occurred, but the direction of transmission cannot be determined.

In our study, we found a slightly higher proportion of Assemblage BIV *Giardia* than BIII; previous reports have indicated that among human infections, BIII occurs more commonly in Africa than BIV (Sprong et al., 2009), but it is not stated which countries are included in these data. The multi-locus subtyping using the genetic loci suggested by Seabolt et al. (2021), showed a lack of concordance at the sample level with the lineages indicated. Although Assemblage E has been suggested to have zoonotic potential (Fantinatti et al., 2016), infection in humans with Assemblage E *Giardia* appears to occur rarely. It was not detected at all in the current study of human infections, despite a high prevalence of Assemblage E *Giardia* infections amongst the livestock owned by these farmers (Kifleyohannes et al., 2022).

The lack of molecular evidence for extensive zoonotic transmission of these parasites is supported by the risk factor investigations that demonstrated that the likelihood of infection was not increased by contact with calves, lambs, or goat kids. This result differs from another study from Ethiopia (Hailu et al., 2021), who stated the presence of animals (specifically cows and calves) were a positive predictor for *Cryptosporidium* infection; however, that investigation was from two other regions of Ethiopia, Wurgissa in Amhara region, around 300 km south of Tigray, and Hawassa, over 1000 km south of Tigray, and zoonotic *C. parvum* was the main species reported. As suggested in the review by Robertson et al. (2020), it is possible that *C. parvum* may spread further into Africa, especially with globalisation, travel, and more intensive farming practices. In Tigray, as in other areas of Africa, the introduction and spread of this highly zoonotic parasite could be disastrous for both human and animal health. Screening of new livestock obtained from different districts or regions for this parasite may be a relevant intervention.

The occurrence of *Cryptosporidium* spp. infection in our study areas was lower than from most other reports in Ethiopia. However, these previous studies have often focused on specific population groups who may be more likely to be infected with *Cryptosporidium,* such as patients with diarrhoea (Adamu et al., 2010; Andualem et al., 2007) and HIV-infected patients (Alemu et al., 2011; Adamu et al., 2014). Thus, the low occurrence of *Cryptosporidium* in the present study might be related to the target population, apparently healthy individuals in rural Tigray, but does not explain the low occurrence also found in diarrhoeic paediatric patients in Mekelle.

We reported a higher occurrence of *Giardia* infection than indicated by a systematic review of intestinal protozoal infections in humans in Ethiopia by Tegen et al. (2020) who reported a pooled prevalence of 10%. Two other reports on *Giardia* from Ethiopia have also reported a higher occurrence, one focusing on school children in the rural Amhara

region (55%) (de Lucio et al., 2016) and one focusing on Lege Dini children (35%) by Ayalew et al. (2008). The overall lower prevalence of *Giardia* infection in people indicated by the systematic review might reflect that the laboratory method used in most of the research works included in the systematic review was light microscopy (see supplementary file 5), which is less sensitive than IFAT as used in our study.

The highest proportions of positive samples for *Cryptosporidium* and *Giardia* in our study were from people living in Enderta district, 9% and 35%, respectively. It is of interest that the occurrence of these parasites in livestock species, fresh produce, and water samples was always greatest in Enderta compared with the other districts participating in the study (Kifleyohannes et al., 2022; Kifleyohannes et al., 2021; Kifleyohannes and Robertson, 2020). This might indicate that these parasites seem to have a greater rate of spread among animals, humans, and the environment in this particular district. As suggested in an article by Vermeulen et al. (2017), new methods to treat faecal waste may reduce contamination of the environment and help protect water catchments. As per our findings, Enderta seems the epicentre of both parasites and appropriate interventions in this area could have a substantial effect in controlling transmission of these parasites here.

The occurrence of *Cryptosporidium* varied among the different age groups, with the highest proportion of positive samples for *Cryptosporidium* (10.5%) observed in the age group $\leq$ 15 years. This finding is similar to a report by Wielinga et al. (2008), who reported the majority of cryptosporidiosis cases occurred in the children's age group (0-9 years).

Among the samples from people living in the rural districts, the occurrence of *Giardia* was higher in the younger age group. There was a statistically significant decline in the occurrence of *Giardia* with increasing age. The lower occurrence in the adult age group might be attributed to the development of immunity after repeated infections, as suggested by Kumar et al. (2014). However, it may also reflect the fact that older people tend to maintain better personal hygiene than children. Similar findings were reported in Uganda (Johnston et al., 2010), who found a higher prevalence of *Giardia* in individuals 15 years or younger than in individuals between 16 and 75 years.

In the urban (Mekelle) city samples, there was a significantly higher occurrence of *Giardia* among children with diarrhoea aged 13-33 months than children aged 1-12 months. This age-associated increase in *Giardia* infection after the first year of life was similar to a study carried out in Brazil that reported higher risk of *Giardia* infection after the first year of life (Pereira et al., 2007). Moreover, a report by Painter et al. (2015) showed giardiasis cases were most frequently reported in children aged 1-4 years. In contrast to our findings and the above reports, Efunshile et al. (2019) found an apparent absence of *Giardia* infections among children under 5 years of age with acute watery diarrhoea in Nigeria. This discrepancy could be due to the difference in sampling. In the Nigerian study, the samples were collected from children admitted to hospital with acute diarrhoeal cases. In such cases, *Giardia* trophozoites may not get a chance to encyst before being washed away by profuse watery diarrhoea due to infections with other pathogens, such as rotavirus, bacteria, or *Cryptosporidium*.

In the present study, *Cryptosporidium,* but not *Giardia,* infection had a pronounced sex distribution, with women more likely to be infected than men. This is interesting and possibly indicates different transmission routes of the parasites in this setting. For example, consistent with other reports, females are more likely than males to care for younger children and babies, particularly changing nappies and assisting with toileting, and this may result in greater exposure to *Cryptosporidium* oocysts (Hutter et al., 2020). However, other reports have suggested that males are more likely to be infected with *Cryptosporidium* (e.g., in Zambia; Sinynagwe et al., 2020), but this may be more associated with zoonotic transmission and animal contact, which seems not to be the case in our study. Other studies have reported no difference between sexes regarding *Cryptosporidium* infection, as we found for *Giardia*, perhaps indicting a common exposure, such as drinking water.

Our study showed a significantly higher *Giardia* infection in people who used stream water as a source of drinking than people who used drinking water from hand pump and pond. This finding is consistent with a report by Damitie et al. (2018) who found higher rate of *Giardia* infection in children who used open spring water. Open spring water sources were often unprotected in all the districts. The sources were mainly used for drinking, washing (clothes, vehicles, and bathing) and watering domestic animals (personal observation). These activities might predispose the sources to contamination.

There was also a significantly higher occurrence of *Giardia* infection in people who used untreated drinking water than those used treated drinking water. This result was also similar to a finding from Malaysia where significantly lower *Giardia* infection was reported in people who drank boiled water (Choy et al., 2014).

Our main conclusion from this study is that, despite the close contact between people and livestock in our study sites, anthroponotic transmission seems the most probable route of infection with both *Cryptosporidium* and *Giardia.* Although we identified the newly emerging zoonotic *C. ubiquitum* from a single human sample from the districts, only *C. hominis* was identified from Mekelle city (urban) children with diarrhoea. Moreover, the majority of *Giardia* infections were Assemblage B, with a very few Assemblage A (AII). Although *C. ubiquitum* and *G. duodenalis* Assemblages A and B have zoonotic potential, *C. parvum* was not detected in either humans or animals in the study areas and Assemblage A (AI) was not detected in human samples, suggesting that livestock may not be the source of human infection in this area.

Our unexpected findings, given the closeness of people and their livestock in rural Tigray, may reflect that extensive farming practices and environmental factors may mitigate against zoonotic transmission at this time. However, the likelihood for the introduction of *C. parvum*, along, with the potential impacts of this highly zoonotic parasite on human and animal health, should not be overlooked.

Water source seemed to be particularly important regarding *Giardia* infection, with drinking from a stream increasing the likelihood of infection. Conducting further research from several regions of Ethiopia, preferably in rural children with diarrhoea, could provide relevant information on the transmission of both parasites and the potential for zoonotic transmission. Such information is vital for developing effective interventions against transmission that will be of relevance not only for Ethiopia, but throughout Africa. In addition, emphasis should be placed on the potential for zoonotic species and subtypes, particularly *C. parvum*, expanding into these areas, and the need for relevant interventions if or when this occurs.

CRediT authorship contribution statement

Tsegabirhan Kifleyohannes: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Ane Nødtvedt:** Methodology, Formal analysis, Data curation, Writing – review & editing, Visualization, Supervision. **John James Debenham:** Conceptualization, Formal analysis, Writing – review & editing, Supervision. **Kristoffer R. Tysnes:** Writing – review & editing, Supervision. **Getachew Terefe:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Lucy J. Robertson:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2022.106450.

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8 Appendices

Appendix 1. The supplementary materials for the PCR conditions and primers that were used for *Cryptosporidium* and *Giardia* identification in calves, lambs and goat kids (Data sheet 1); questionnaires for calves, lambs and goat kids owners (Data sheet 2); *Cryptosporidium* and *Giardia* infection in calves, lambs, goat kids and various risk factors (Data sheet 3) can be accessed through this link,
click here to download
https://www.frontiersin.org/articles/10.3389/fvets.2021.825940/full#supplementary-material

Appendix 2. The supplementary materials that were used for *Cryptosporidium* and *Giardia* identification in human can be accessed through the following links,
click here to download

1. PCR conditions and primers
   https://ars.els-cdn.com/content/image/1-s2.0-S0001706X22001425-mc1.docx
2. Questionnaire for livestock and non-livestock owners
   https://ars.els-cdn.com/content/image/1-s2.0-S0001706X22001425-mmc2.docx
3. Occurrence of *Cryptosporidium* and *Giardia* infection in humans and associated Risk Factors
   https://ars.els-cdn.com/content/image/1-s2.0-S0001706X22001425-mmc3.docx
4. Subtyping of *Giardia duodenalis* assemblage B using five genes
   https://ars.els-cdn.com/content/image/1-s2.0-S0001706X22001425-mmc4.docx
5. Published studies from Ethiopia on *Cryptosporidium* spp. and *Giardia duodenalis* infections in humans
   https://ars.els-cdn.com/content/image/1-s2.0-S0001706X22001425-mmc5.docx

Introduction

This information sheet and consent form is designed for different age groups, adults 18 years and older, children/adolescents aged 12 to 17 (under the age of 18) and children under the age of 12. The information sheet for the different age groups are nearly identical, but explanations for children were supported by images. To avoid repeating some ideas, the information sheet we used for adults and the consent form for all ages are included in this supplementary file.

INVITATION TO PARTICIPATE IN ICGOH

Project title: Investigations on *Cryptosporidium* and *Giardia* in Tigray, Ethiopia from a One Health Perspective

Introduction

Greetings

My name is Tsegabirhan K/yohannes, a PhD student at NMBU, Norway. I would like to invite you to participate in this research project. This study is concerned with two parasitic diseases, cryptosporidiosis and giardiasis, which cause diarrhoea in both humans and animals.

The purpose of the study is to understand the prevalence and importance of cryptosporidiosis and giardiasis in your community and to identify whether animals are sources of these infections in your community and whether water, fruits and vegetables act as routes of transmission.

You are invited to participate in this research project because your participation will help in obtaining the information we need and that could be used to improve prevention and control of these diseases, and provide better health care in your area.

Further details about the project are described in this form.

I hope you have time to read this information, and that it will help you to decide to participate in the study project. The responsible research participants will give more explanations to you, and you are welcome to ask questions at any time.

Your participation is voluntary, and we will ensure that you will remain anonymous. If you decide to participate, you will be asked to sign the informed consent form at the end of this document.

WHAT IS ICGOH ABOUT?

Although we know that cryptosporidiosis and giardiasis occur in Ethiopia, we do not know how important infections in cattle, sheep, and goats are for human infections. This is because most previous studies on cryptosporidiosis and giardiasis in these animals have not used tools that can investigate this. In this study, we will use more advanced techniques to analyse samples, and this will provide clues about the extent to which people are infected by animals.

In addition, as *Cryptosporidium* and *Giardia* are in the environment, water and vegetables may be contaminated, and people can become infected from consuming these. We will therefore, investigate whether this is how these infections spread in your community.

Procedures

Research professionals will ask you various questions and help you to complete the questionnaire form. The right answers are those that are correct for you.

You will be given a sampling bottle for the collection of a stool sample. We will collect your sample container and analyse the sample in the laboratory. If you would like to know what we find, we will communicate the results to you.

POSSIBLE BENEFITS AND EXPECTED DISADVANTAGES OF TAKING PART IN ICGOH

There is no risk associated with being a participant of this study.

Although you may not see a direct benefit from participating in this study, you will have a free examination for these diseases, and the information obtained may have a great impact in providing a better understanding of cryptosporidiosis and giardiasis in your area. This may assist in reducing the spread of these diseases and may help to improve control and prevent the diseases in the country.

Risks

WHAT WILL HAPPEN TO YOUR HEALTH INFORMATION?

The records of this study will be kept securely and will be confidential. All faecal specimens will be coded, and information that can identify the person who provided the sample will be removed. Only study staff will be able to link the code with his/her name. To ensure total anonymity, any identifier such as name or address will never be used in connection with any of the information or specimen provided.
All data will be kept completely confidential, and all test results will be treated confidentially. However, if samples are found positive for active parasitic infections, local health professionals will be informed so that you obtain the necessary treatment and advice.

VOLUNTARY PARTICIPATION AND THE POSSIBILITY TO WITHDRAW CONSENT

Participation in this project is voluntary. You are free to leave the project at any time you want without providing any reason for doing so. If you decide to leave the project, any clinical care and management will not be affected.

Contact address: If you have any questions about the study, you can ask the nearby health professionals involved in the study.

CONSENT FOR PARTICIPATING IN ICGOH

I AM WILLING TO PARTICIPATE IN ICGOH

I confirm that the document describing the purpose, benefits, risks, and confidentiality of the research project entitled 'Investigations on *Cryptosporidium* and *Giardia* in Tigray, Ethiopia from a One Health Perspective (ICGOH)" 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 laboratory results, as it is coded by numbers rather than names of people.

I realized that there are no personal benefits apart from the clinical services related to stool examination. In addition to the above-mentioned, I have no objection if part of the stool specimen is shipped to another country for further examination. Therefore, with a full understanding of the importance of the study, I hereby agreed to sign this consent form.

Signature_____Date _____ Kebele _____ Village _____

Name of the professional taking consent_____Signature_____ Date_____

CONSENT FORM FOR PARTICIPATING IN ICGOH FOR CHILDREN/ADOLESCENTS BETWEEN THE AGES OF 12 TO 17 YEARS (UNDER THE AGE OF 18)

I AM WILLING TO PARTICIPATE IN ICGOH

I confirm that the document describing the purpose, benefits, risks, and confidentiality of the research project entitled 'Investigations on *Cryptosporidium* and *Giardia* in Tigray, Ethiopia from a One Health Perspective (ICGOH)" 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 child/relative results, as samples will be coded by numbers rather than names of people.

I realized that there are no personal benefits apart from the clinical services related to stool examination. In addition to the above mentioned, I have no objection if part of the stool specimen is shipped to another country for further examination. Therefore, with a full understanding of the importance of the study, I hereby agreed to sign this consent form on behalf of my child/relative.

Name of the professional taking consent: _____

Signature:_____ Date:_____

Participant's Name:_____ Participant's Signature_____ district_____

As parents/guardians of:_____ (Full name), we consent for him/her to participate in the Research Project

Parent's/Guardian's Signature_____

CONSENT FORM FOR PARTICIPATING IN ICGOH FOR PARENTS/GUARDIANS OF CHILDREN UNDER THE AGE OF 12

I AM WILLING TO PARTICIPATE IN ICGOH

I confirm that the document describing the purpose, benefits, risks, and confidentiality of the research project entitled 'Investigations on *Cryptosporidium* and *Giardia* in Tigray, Ethiopia from a One Health Perspective (ICGOH)" 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 child/relative results, as samples will be coded by numbers rather than names of people.

I realized that there are no personal benefits apart from the clinical services related to stool examination. In addition to the above mentioned, I have no objection if part of the stool specimen is shipped to another country for further examination. Therefore, with a full understanding of the importance of the study, I hereby agreed to sign this consent form on behalf of my child/relative.

Name of the professional taking consent: _____

Signature:_____ Date:_____

Participant's Name:_____ Participant's Signature_____ district_____

As parents/guardians of:_____ (Full name), we consent for him/her to participate in the Research Project

Parent's/Guardian's Signature_____

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