Prevalence of *Cryptosporidium* infection and associated risk factors in ruminants in Gauteng Province, South Africa

By

Tebogo Atlivia Seanego

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DECLARATION

As declared by Tebogo Atlivia Seanego, this dissertation is my original work that has never been submitted for consideration for a degree to any other academic university institution. It is being presented here for the Master of Science degree at the Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences, University of Pretoria, South Africa.

Dr Tebogo A Seanego  Date: 19/03/2024

Approved by:

Prof Munyaradzi Christopher Marufu  Date: 19/03/2024

Prof Lise Korsten  Date: 26/05/2023

Dr Charles Byaruhanga  Date: 26/05/24

DEDICATION

I dedicate this work to my wonderful parents, Caroline and Rosbert Seanego, who always believed in me even when I did not believe in myself, to my son, Tshepiso, and to my siblings Jerry, Rethabile and Serema Seanego for being there for me when I needed emotional support. To more wisdom and interest in research work for the future

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LIST OF ABBREVIATIONS

AgriSETA	Agricultural Sector Education and Training Authority
DNA	deoxyribonucleic acid
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
EITB	enzyme linked immunoelectron transfer blots
IFA	immunofluorescent antibody
MZN	modified Ziehl-Neelsen Staining
PCR	polymerase chain reaction
RT-	real-time
RFLP-	restricted fragment length polymorphism
RAPD-	random amplified polymorphic DNA
AP-	arbitrarily primed
PMA-	propidium monoazido
sp or spp.	species
WHO	World Health Organization
%	percentage
°C	degrees celsius

ABSTRACT

Prevalence of *Cryptosporidium* infection and associated risk factors in ruminants in Gauteng Province, South Africa

Cryptosporidiosis, an emerging enteropathogenic disease with negative implications for public and livestock health, remains poorly investigated in Africa, necessitating focused epidemiological studies. To determine the prevalence and risk factors associated with *Cryptosporidium* infections in domesticated ruminants, a longitudinal study was conducted on eight farms in Rust de Winter, Gauteng Province, South Africa. During winter (2022) and summer (2023), 370 faecal samples were collected from cattle (n=146), sheep (n=105), and goats (n=119) kept in extensive and semi-intensive production systems. Microscopic analysis using the gold standard Modified Ziehl Neelsen (MZN) test was employed, followed by screening of the positive samples for *Cryptosporidium parvum* using quantitative real-time PCR (qPCR). Semi-structured interviews were conducted with the farmers to establish demographic, behavioural and management practices that could pose a risk for *Cryptosporidium* infection between animals and humans. The microscopic analysis identified *Cryptosporidium* oocysts in 57 (15.4%) of the 370 faecal samples. However, the qPCR detected *Cryptosporidium* DNA in only one (1.8%) of the 57 MZN-positive samples, possibly because of low oocyst concentration, below the detection limit of the qPCR, or the presence of *Cryptosporidium* species other than *C. parvum*. The *Cryptosporidium* data from the MZN test revealed that infection was significantly higher ($p=0.0133$) in summer (19.4%) than in winter (11.6%). No statistically significant differences ($p>0.05$) in infection were observed between male (16.2%) and female

(14.0%) animals and across ruminant species, although cattle (11.6%) tended to have lower infection rates than sheep (20.0%. OR=1.9; p=0.075) and similar infection rates to goats (16.0%; OR=1.16; p=0.695). All interviewed farmers (n=13) were unaware of cryptosporidiosis, highlighting the importance of awareness and training to reduce the potential risk for disease transmission to humans and other animals. Despite the limitations associated with the qPCR method, this research provides valuable insights into the epidemiology of *Cryptosporidium* infections in ruminants in Gauteng Province. The observed seasonal variation and the need for farmer education underscore the significance of proactive measures to mitigate livestock mortality and prevent the potential spread of infection to humans. Further investigation involving more animal farms and assessment of the occurrence of infections in humans will contribute to better understanding of *Cryptosporidium* infections. There is also a need to evaluate clinical vs sub-clinical cases on farms with regards to the detection limit of nucleic acid-based methods.

Keywords: one health**,** cryptosporidiosis, epidemiology, farmer education, zoonosis

CHAPTER 1: Introduction

1.1 Background

Cryptosporidium species are predominant enteric, obligatory protozoan parasites with a proclivity for infecting the gastrointestinal epithelium across a diverse array of vertebrate hosts including humans (Díaz *et al.*, 2018; Gharieb *et al.*, 2019). They cause gastroenteritis that manifests as diarrhoea of varying severity in both humans and animals. The degree of diarrhoea depends on the host's immunity. Immunocompetent hosts, usually have diarrhoea, which is mild and usually resolves on its own, whereas in immunocompromised hosts, it can be severe, chronic, and life-threatening (Samra *et al.*, 2016). The disease in animals has debilitating consequences such as poor growth rates, anorexia, increased morbidity, mortality, and decreased milk production, exerting a negative impact on animal production. Neonates face a heightened risk of infection resulting in considerable economic losses to farmers due to poor feed conversion and growth rates (Amer *et al.*, 2013; Chang'a *et al.*, 2011). Threats to public health arise from the possibility that infected animals have a significant influence in the emergence of outbreaks of cryptosporidiosis in people (Diaz *et al*., 2018).

The World Health Organization (WHO) ranks *Cryptosporidium* fifth amongst important foodborne parasites in the world (Odeniran and Ademola, 2019). Apart from being a foodborne parasite, it is also waterborne. Transmission of the parasite is anthroponotic (human to human or animal) and zoonotic (animal to human) (Benhouda *et al.*, 2017; Xiao, 2010). Infection occurs through fully sporulated oocysts, primarily via ingestion

of faecal-contaminated food and/or water or by direct contact with infected hosts (Xiao, 2010). *Cryptosporidium* resistance to common disinfectants and water-cleaning agents permits its persistence in recreational water sources and potable water posing a substantial public health risk (Hatam-Nahavandi *et al.*, 2019).

Over the years, researchers identified numerous *Cryptosporidium* species with Tyzzer being the first to identify *Cryptosporidium* spp. In mice in 1910 (Tyzzer *et al*., 1910). The first human case was documented in 1976, garnering increased attention following the discovery of cryptosporidiosis as a causative agent in the death of an AIDS patient (Meisel *et al*., 1976). The early 1980s witnessed the emergence of *Cryptosporidium* in veterinary medicine due to neonatal calf diarrhoea (Meuten *et al*., 1974) strengthening its status as a main enteric pathogen (Heine *et al*., 1984). Some *Cryptosporidium* species are host-specific, and others infect multiple hosts. *Cryptosporidium parvum*, a zoonotic strain, and *Cryptosporidium hominis*, a non-zoonotic strain, mostly infect people (Kange'the *et al.*, 2012). Cattle on the other hand are susceptible to three additional species: *Cryptosporidium andersoni*, *Cryptosporidium bovis,* and *Cryptosporidium ryanae* (Amer *et al*., 2013). Zoonotic *Cryptosporidium parvum* finds a major reservoir in young dairy calves globally (Mahfouz, 2014). Sheep and goats serve as significant sources for the transmission of *Cryptosporidium ubiquitum* to humans (Mahfouz, 2014; Soltane *et al.*, 2007). Small stock to a lesser extent are affected by the host-adapted *Cryptosporidium andersoni*, *Cryptosporidium parvum*, *Cryptosporidium bovis* and *Cryptosporidium xiaoi* (Wegayehu *et al.*, 2016).

In South Africa, researchers have utilised diverse diagnostic techniques for detecting *Cryptosporidium* species (Leav *et al*., 2002; Samie *et al*., 2017; Samra *et al*., 2013). These diagnostic techniques can be divided into three groups i.e microscopic and serological which have been used for detection on faecal samples, as well as molecular techniques used for detection on extracted DNA of the faecal samples (Helmy and Hafez, 2022). All three techniques were employed in a study by Samra *et al.* (2011). The widely employed microscopic technique, Modified Ziehl Neelsen (MZN) conventional acid-fast staining, was used for *Cryptosporidium* oocysts screening. The second technique used was the immunofluorescent antibody test (IFA), a serological

method which targeted *Cryptosporidium parvum* antibodies. The molecular techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) of the 18S rRNA gene, and sequencing have been instrumental in species identification and genotyping of *Cryptosporidium* (Samra *et al.*, 2013). Another study incorporated quantitative real-time PCR (qPCR) and 18S rRNA gene sequencing on selected samples (Samie *et al.*, 2017).

Data on the prevalence of cryptosporidiosis based on the number of articles published on domesticated ruminants in South Africa is scarce. The reported prevalence of cryptosporidiosis has been mainly in the Mpumalanga Province at the interface area between wildlife, livestock and humans. In this area, low prevalence was reported in cattle and wildlife (Samra *et al.*, 2013). Wildlife prevalence of 25.8%, 5.5%, and 4.2% were noted in elephants, buffalo, and impala, respectively, using MZN (Samra *et al*., 2013). Calves in the same area exhibited a prevalence of 0.6% (Samra *et al.*, 2016) while childhood prevalence was reported at 12.2% (Samra *et al.*, 2013) and 5.5% in Mpumalanga Province (Samra *et al*., 2016). Samie *et al.* (2017) reported a prevalence of 47.7% (goats), 26.8% (cattle), 7.4% (chicken), and two positive sheep out of four that were tested in Limpopo Province, South Africa. Ovine and caprine cryptosporidiosis has not been thoroughly investigated in South Africa, even though epidemiological studies indicate that the disease is prevalent in sheep and goats.

The key risk factors for cryptosporidiosis in humans include contact with infected livestock particularly in contaminated environments, and heightened susceptibility in immunocompromised individuals (Adamu *et al.*, 2014; Samra *et al.*, 2011). Faecal-contaminated recreational waters and day-care centres pose a high risk (Samie *et al*., 2017). Fetching water from unprotected sources is an implicated probable risk factor for cryptosporidiosis in people (Salyer *et al.*, 2012). Though Samra *et al.* (2013) did not investigate risk factors, their study showed that the shared use of grazing and water resources by cattle and wildlife poses a transmission threat at the interface between wildlife, livestock and people.

1.2 Problem Statement

While cryptosporidiosis has been studied in certain provinces of South Africa, such as Mpumalanga and Limpopo (Samra *et al.*, 2013; 2016; Samie *et al.*, 2017), a critical gap exists in understanding its prevalence and associated risk factors within the densely populated Gauteng Province. Unlike other regions, Gauteng's unique combination of high human and ruminant populations, intensified resource sharing, and increased human-animal interactions creates an environment with distinct dynamics for *Cryptosporidium* transmission. Despite the potential consequences for both livestock production and public health, there is a notable absence of studies exploring the prevalence of cryptosporidiosis in domesticated ruminants in Gauteng. There is a need to fill this crucial knowledge gap by providing valuable insights into the epidemiology of *Cryptosporidium* in a region that plays a pivotal role in South Africa's economy and food security.

While Samra (2011) initially conducted wildlife-focused research, subsequent studies extended to indigenous cattle, young children, and diverse wildlife species (Samra *et al.*, 2013; Samra *et al.*, 2016). Despite indications of widespread ovine and caprine cryptosporidiosis with documented fatalities in lambs and goat kids, comprehensive prevalence data for small ruminant species are lacking (Castro-Hermida *et al.*, 2002; Samie *et al.*, 2017). Existing prevalence studies by Samie and colleagues (2017) in Limpopo Province, lack comprehensive cover for ruminant species, indicating a critical dearth in knowledge regarding the prevalence of cryptosporidiosis in ruminant livestock in South Africa.

The molecular characterization of *Cryptosporidium* species and genotypes, while contributing to understanding the biological diversity of this parasite, remains limited in the context of ruminant infections (Silverlås *et al.*, 2010; Samra *et al.*, 2013). Current molecular tools, such as polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) analysis of the 18S rRNA gene, offer valuable insights into species, genotypes, and subtype identities of *Cryptosporidium* (Odeniran and Ademola, 2019). The 60-kilodalton glycoprotein (gp60) gene serves as a discriminative marker for identifying subtype families in DNA sequencing analysis (Gharieb et al., 2019). Molecular diagnostics for cryptosporidiosis have been scarcely applied to free-ranging African wildlife in South Africa (Samra et al., 2013). Despite the

demonstrated utility of molecular diagnostics in wildlife studies, their application to ruminant infections in South Africa remains limited. There is, thus, a need for studies determining the species identity of *Cryptosporidium* infecting ruminants in South Africa using molecular methods.

Contact with cattle was a notable risk factor for zoonotic transmission of *Cryptosporidium parvum* in children and HIV/AIDS patients in Egypt and Ethiopia (Adamu *et al.*, 2014). Additionally, keeping animals in closed shade on dirty floors predisposes them to a higher prevalence of cryptosporidiosis than animals kept in open shade (Samie *et al.*, 2017). The factors associated with increased risk of *Cryptosporidium* infections in neonatal ruminant species in South Africa particularly in Gauteng Province remain unexplored and need to be investigated.

1.3 Justification

Cryptosporidium stands as a major entero-pathogen negatively impacting ruminant production globally. Ruminant farming is one of the largest contributors to food security and South Africa's economy through local and international trade of animals and animal products. Gauteng Province is densely populated, with a large ruminant population, and increased sharing of resources, e.g., water and manure, between people and animals, giving rise to an increased risk of transmission of cryptosporidiosis. Despite the potential severe economic impact on livestock production and human health, the occurrence of cryptosporidiosis in domesticated ruminants in Gauteng Province has not undergone comprehensive investigation.

To this extent, understanding the prevalence and predisposing factors of *Cryptosporidium* infection in ruminants in Gauteng Province is crucial for mitigating the spread and risk of infection. The resulting prevalence data will enable effective control measures for infections with severe public health consequences, contributing to both animal welfare and human health. The use of molecular tools for species characterization is pivotal for unravelling the species diversity of the pathogen and discerning host specificity within ruminant populations. This knowledge is fundamental for instituting targeted control measures to combat the impact of cryptosporidiosis in both animals and humans in Gauteng Province.

1.4 Aim

The current study aimed to investigate the prevalence of *Cryptosporidium* spp. and explore associated risk factors in ruminants on farms within Gauteng Province, South Africa.

1.5 Objective

a) To estimate the prevalence of cryptosporidiosis using MZN and qPCR tests, in ruminants on farms in Rust de Winter, Gauteng Province, South Africa.
b) To establish, through a questionnaire survey, the risk factors associated with *Cryptosporidium* infection in ruminants on farms in Rust de Winter, Gauteng Province, South Africa.

1.6 Hypotheses

H_{0i}: The prevalence of *Cryptosporidium* infection in domesticated ruminants in Gauteng Province, South Africa, is high.

H_{0ii}: Infected animals pose a risk of infection to healthy animals and humans.

1.7 Structure of the dissertation

This dissertation comprises four chapters. Chapter 1 provides a comprehensive introduction detailing the background, problem statement, justification, aims, and objectives, and the overall structure of the study. To achieve the aim and objectives of this study, Chapter 2 presents a review of relevant published literature. Chapter 3 outlines the methodology of the study, incorporating materials and methods for investigating the prevalence of *Cryptosporidium* using microscopic and molecular techniques and associated risk factors through a questionnaire survey. Chapter 4 details the results of the microscopic and molecular techniques used for the diagnosis of cryptosporidiosis. Chapter 5 is the general discussion, and the final chapter (Chapter 6) encompasses the conclusions and recommendations based on the findings of the study.

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CHAPTER 2: Literature review

The prevalence and risk factors of *Cryptosporidium* infection with a focus on South Africa and selected African countries

2.1 Introduction

Cryptosporidium species have been reported in human and animal populations, each exhibiting distinct prevalence, geographic distribution, and health implications. Vulnerability to illness is heightened in young children and individuals with compromised immune systems. Newborn calves and lambs are also susceptible to infection, releasing substantial quantities of oocysts, that contaminate the environment, posing infection risks for humans and animals (Berhanu *et al*., 2022). Asymptomatic weaned and adult cattle, acting as reservoir hosts, also contribute to environmental oocyst release (Noorden *et al*., 2000). A single adult bovine excretes approximately 36 million oocysts daily (Scott *et al*., 1994).

Sub-clinically infected ewes are identified as a potential infection source for lambs, particularly in the period around parturition (Ye *et al*., 2013). The substantial oocyst release, of up to 10^7 oocysts per gram of faeces, from infected animals underscores the public health risks associated with inadequate control measures (Lasprilla-Mantilla *et al*., 2019). Apart from direct faecal deposition, additional pathways involve surface transfer from manure-applied land or leaching through soil to groundwater. Runoff from contaminated fields is crucial for oocysts to reach water sources, highlighting cattle farms as significant sources of infection for people and animals (Ogendo *et al.,* 2015). The persistence of *Cryptosporidium* oocysts in infected animal manure further establishes it as an enduring reservoir, leading to cryptosporidiosis outbreaks and ensuring severe economic and health ramifications associated with water contamination.

The prevalence of *Cryptosporidium parvum* and zoonotic transmission significance in disease epidemiology in low and middle-income countries, remains poorly researched (Adamu *et al.*, 2014). In Ethiopia, however, the prevalence of

cryptosporidiosis caused by *Cryptosporidium parvum* in HIV-positive patients together with other non-zoonotic *Cryptosporidium* species that occur in domesticated ruminants is well recognized (Wegayehu *et al.*, 2016). Although research is limited in South Africa on the prevalence of cryptosporidiosis in small ruminants, the disease is said to be common (47.4-50.0%) in small ruminant farms in Spain, though the incidence is low (Díaz *et al.*, 2018). *Cryptosporidium* infection, therefore, holds particular relevance in domestic ruminants due to its frequent association with diarrhoeal illness and substantial economic implications (Castro-Hermida *et al.,* 2002).

This chapter comprehensively explores the aetiology, transmission, life cycle and clinical signs of cryptosporidiosis. It also reviews the prevalence of the disease, with a focus on South Africa and select African countries. Diagnostics tools used in the detection of the disease and associated risk factors are also reviewed in this chapter.

2.2 Aetiological agent

Cryptosporidiosis is a global parasitosis known to cause diarrhoea in newborn lambs, goat kids, foals, piglets, and humans. These intracellular protozoan parasites within the phylum Apicomplexa, order Eucoccidiorida, family Cryptosporiidae, and genus *Cryptosporidium* have been extensively investigated to reveal their diversity and detection through molecular epidemiology (Xiao *et al.*, 2004; Soltane *et al.*, 2007). To date, 26 species and approximately 50 genotypes have been described in humans and animals using molecular techniques, highlighting the extensive genetic variability of the organism (Ayinmode *et al.*, 2010; Elwin *et al.*, 2012). Geographic variations exist in the distribution of major species causing human infections. *Cryptosporidium parvum* predominates in the United Kingdom (UK), Europe, and New Zealand, whereas *C. hominis* is prevalent in low and middle-income countries (Xiao, 2010). The remaining species affecting humans are *C. meleagridis, C. felis, C. canis, and C. ubiquitum* (Figure 1) (Ryan *et al*., 2021).

In cattle, studies on *Cryptosporidium* species distribution have primarily focused on dairy breeds in industrialized nations rather than low and middle-income countries (Amer *et al.*, 2013). Notably in Nigeria, Ayinmode *et al*. (2010) reported 27.7% and 7.7% of calves, had *C. bovis* and *C. ryanae* infections respectively, with an additional

16.9% experiencing mixed infections. The absence of *C. parvum* suggests limited zoonotic infection risk for humans (Ayinmode *et al*., 2010). South African studies detected, *C. parvum* and *C. hominis* in hospitalized diarrhoeic children (Leav *et al.*, 2002; Samra *et al.*, 2013; Samie *et al*., 2017) while *C. parvum* and *C. andersoni* were detected in cattle and goats. *Cryptosporidium ubiquitum*, *C. bovis* and *C. andersoni* were detected by Samra *et al*. (2013) in impala and African buffalo, domestic calves and impala and domestic calves respectively. Notably, the zoonotic species *C. meleagridis* was first detected in South Africa in 2016 (Samra *et al.*, 2016).

Among *Cryptosporidium* species, *C. parvum* and *C. hominis* are paramount species due to their widespread distribution (Plutzer and Karanis, 2009; Fayer, 2010). These species are major contributors to clinical diarrhoeal cases in young children globally (Benhouda *et al.*, 2017). The prevalence of cryptosporidiosis in cattle has been reported to be age-related, with *C. parvum* predominantly found in pre-weaned calves causing watery diarrhoea and significant economic losses (Fayer, 2010; Xiao, 2010; Ogendo *et al.*, 2017). Additionally, lambs, goat kids, and foals are potential sources of *C*. *parvum* for people (Hatam-Nahavandi *et al.*, 2019). *Cryptosporidium bovis* and *C*. *ryanae* were observed mostly in weaned calves, with *C. andersoni* occurring in yearlings and adult cattle (Wegayehu *et al.*, 2016). The epidemiological significance of young native breed calves in human cryptosporidiosis requires further investigation (Maikai *et al* 2011).

Cryptosporidium hominis is typically maintained in human-human cycles, suggesting that it is anthroponotic (Kange'the *et al.*, 2012). In Egypt *C*. *parvum* was detected in cattle and buffalo while C. *hominis* and C. *parvum* were found in humans (Ibrahim *et al.*, 2016). An uncommon species, C. *viatorum* was described in two Swedish nationals who had travelled to Africa and Latin America (Adamu *et al.*, 2014). Although *C. xiaoi* was detected in HIV/AIDS patients in Ethiopia, little research has been conducted on species detected in Kenya and Tanzania (Chang'a *et al.*, 2011).

Studies in various African nations identified an early development of *C. bovis* and *C*. *ryanae* in native breeds and traditionally-reared cattle (Maikai *et al.*, 2011; Wegayehu *et al.*, 2016; Hatam-Nahavandi *et al.*, 2019). This pattern contrasts the prevailing

pattern of *C*. *parvum* dominance in young calves (Wang *et al.*, 2008), suggesting that young native calves may not be important in the epidemiology of human cryptosporidiosis (Maikai *et al.*, 2011). However, more epidemiological studies are required to support this observation. Adult livestock, though often asymptomatic, act as cryptic carriers contributing to herd-level re-infections (Hatam-Nahavandi *et al.*, 2019).

Several *Cryptosporidium* species have been documented in domesticated ruminants within the African context, with limited exploration in South Africa. The primary species affecting neonatal ruminants appears to be *C. parvum*, although the significance of other species in disease aetiology cannot be dismissed. There is a paucity of studies investigating the prevalence of *C. parvum* across various age groups in domestic ruminants in South Africa, underscoring the need for additional research in this area.

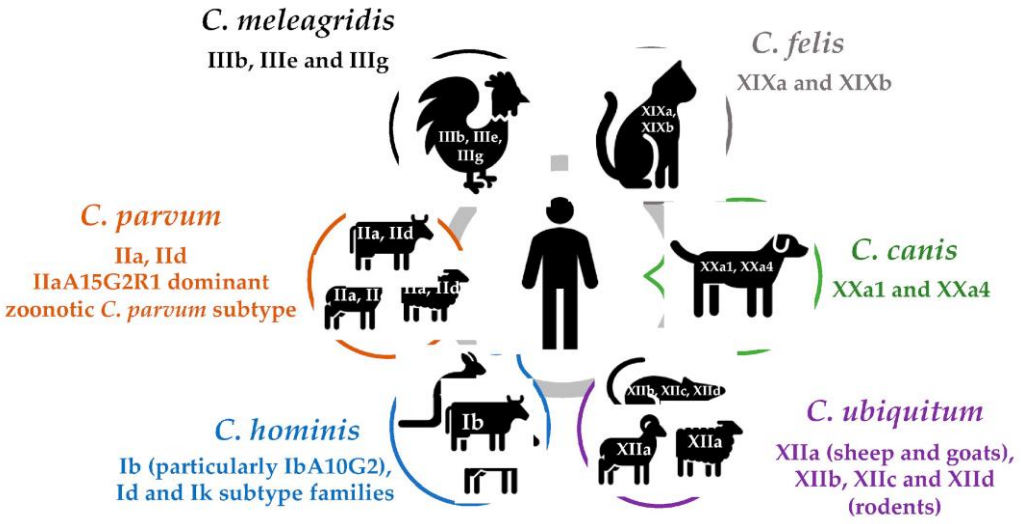

Figure 1: Zoonotic transmission of the six most common *Cryptosporidium* species and genotypes. Adapted from (Ryan et al., 2021). https://doi.org/10.3390/ani11113307 Accessed 28 September 2023.

Table 1: Most predominant *Cryptosporidium* species: major hosts, oocyst sizes and locations – Adapted from Helmy and Hafez (2022).

***Cryptosporidium* species**	**Host**	**Size (µm)**	**Parasite location in the host**
C. hominis	Humans	4.5 × 5.5	Small intestine
C. parvum	Ruminants, humans, deer	4.5 × 5.5	Small intestine
C. bovis	Ruminants	4.2–4.8 × 4.8–5.4	Small intestine
C. andersoni	Ruminants	5.5 × 7.4	Abomasum
C. rynae	Ruminants, camel	3.2 × 3.7	Small intestine
C. xiaoi	Sheep	3.9 × 3.4	Small intestine
C. ubiquitum	Sheep/Wildlife	5.2 × 4.9	Small intestine

2.3 Transmission

Cryptosporidium oocysts are resilient, exhibiting prolonged survival for up to months, particularly in faecal matter and its surroundings (Mahfouz *et al*., 2014). Environmental dispersal occurs when oocysts are released by humans or animals spreading through soiled water or food (Samra, 2013). Farmyard manure, known to harbour a substantial oocyst load, poses a significant risk as it may contaminate water sources and crops, leading to human exposure (Ogendo *et al*., 2017). Inhalation of oocysts via aerosolized droplets or fomite is an alternative zoonotic transmission route (Helmy and Hafez, 2022). Additionally, *Cryptosporidium* transmission can occur through direct contact with an infected person, and the consumption of contaminated food or water (Xiao, 2010; Khosravani *et al.*, 2017). Furthermore, synanthropic flies within the suborder Cyclorapha, play a crucial role in the mechanical transmission and dissemination of cryptosporidiosis (Helmy and Hafez, 2022). Cryptosporidiosis is a classic example of a One Health disease complex, involving intricate transmission cycles among animals, humans and the environment as depicted in Figure 2.

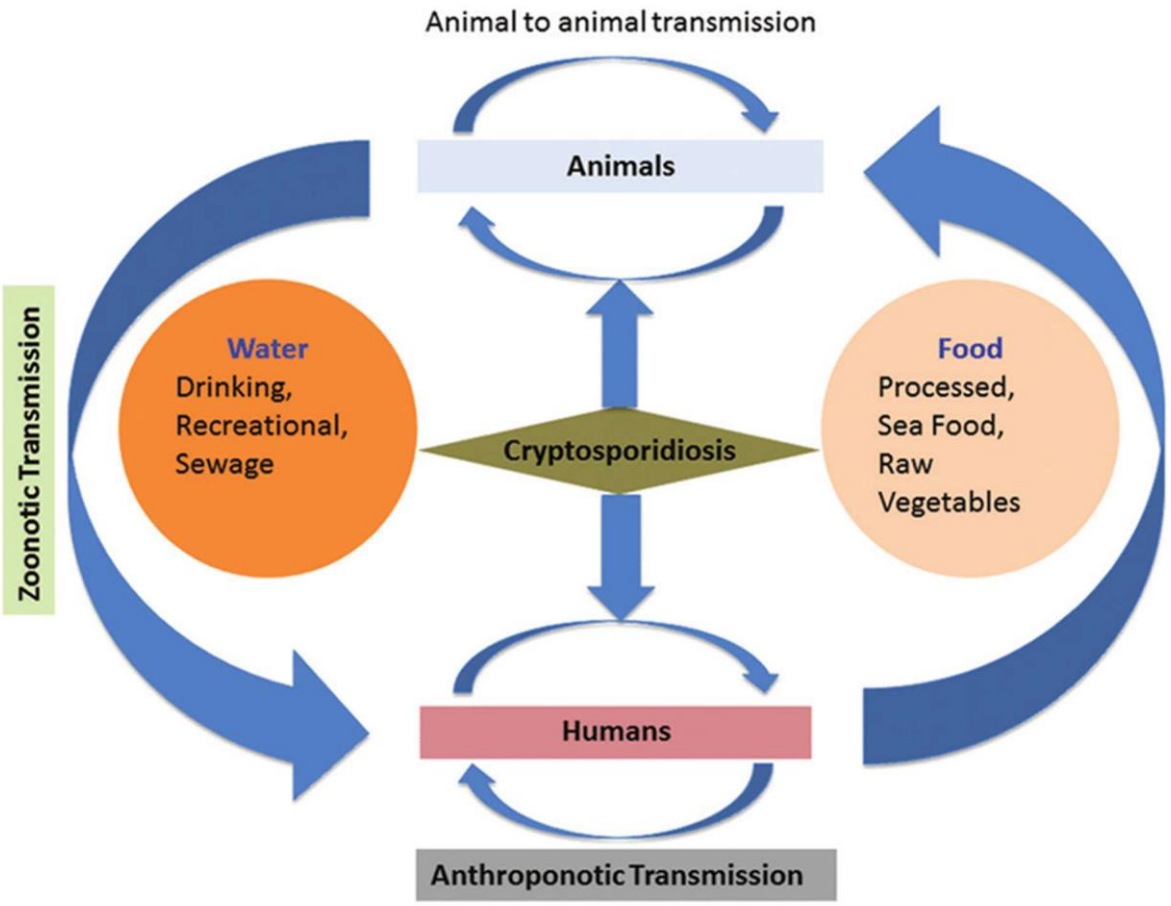

Figure 2: A depiction of the transmission cycles of cryptosporidiosis between animals, humans and the environment. The figure highlights the anthroponotic, zoonotic and animal to animal transmissions. Adapted from (Shrivastava *et al*., 2017). https://www.researchgate.net/figure/Transmission-cycle-of-*Cryptosporidium*-*Cryptosporidium*-transmission-via-zoonotic-and_fig2_316148931

The infectivity of *Cryptosporidium* oocysts is remarkable, as very few oocysts can lead to infection in vulnerable hosts upon ingestion (Ogendo *et al.*, 2017). While the contribution of animals, particularly young ones, to the spread of human *Cryptosporidium* remains unclear (Paul *et al*., 2009), zoonotic transmission particularly from calves to humans, may occur easily, seasonally, and frequently, posing an occupational hazard (Hatam-Nahavandi *et al.*, 2019). The typical *C*. *parvum* infection dose for humans ranges from less than 10 to more than 1000 oocysts. Historical waterborne outbreaks, such as the Milwaukee outbreak in the United States

of America (USA), reported infections in individuals exposed to as few as 1 to 10 *Cryptosporidium* oocysts. Environmental factors, including a warm and humid climate that promotes *Cryptosporidium* persistence and dissemination, coupled with poor hygiene standards elevate the risk of infection in calves. Due to their underdeveloped immune systems and high susceptibility to infections, neonates are highly susceptible to infection and can contract the parasite by ingesting small amounts of oocysts (Ogendo *et al.*, 2017).

Cryptosporidium oocysts exhibit prolonged viability in humid environments, being more prevalent in the rainy season than in dry periods (Samra *et al.*, 2011; Samra, 2013). The oocysts can survive for more than 140 days in water (Ramirez *et al.,* 2004) and remain infectious outside the host's body for 6-8 months (Weir, 2001).

2.4 Life Cycle

The life cycle of *Cryptosporidium*, a complicated monoxenous process has an asexual phase encompassing sporogony and schizogony/merogony, and a sexual phase involving gamogony (O'Donoghue, 1995; Helmy *et al*., 2022) as illustrated in Figure 3. *Cryptosporidium* localizes intracellularly but extracytoplasmically. The initial step involves the parasite affixing itself to the host's cell surface before entering. This interaction triggers the production of an actin-rich disk, serving as both a feeder organelle and a conduit into the host cell's cytoplasm (Sulaiman *et al*., 2002). Following internalization, sporozoites, exhibiting a rough surface and a pointed apical portion measuring 5 x 0.5 µm, undergo division within the parasitophorous vacuole to produce spherical trophozoites with a smooth surface and hoodlike shape measuring 1–2.5 um in length (Leitch and He, 2011; Helmy and Hafez, 2022).

A large type I meront with eight merozoites forms from a trophozoite after asexual division (Thompson, 2008). After emerging from the parasitophorous vacuole, merozoites initiate asexual development in the epithelial cells, leading to the formation of type I meronts before trophozoite formation. Simultaneously, merozoites initiate sexual development by differentiating into type-II meronts (English *et al.*, 2022; Helmy and Hafez, 2022). Four merozoites in the meront, undergo asexual division to yield micro- and macrogametes post-infecting additional enterocytes. Upon maturation,

microgametes leave the host cell and fertilize the macrogametes. The resultant zygote undergoes syngamy, develops and proceeds through sporogony (Pinto and Vinayak, 2021). Thin and thick-walled oocysts develop inside the parasitophorous vacuole (Rossele and Latif 2013). Thin-walled oocysts excyst in the host's alimentary canal, instigating endogenous autoinfection, while thick-walled counterparts are excreted with the faeces into the environment (Leitch and He, 2011), contributing to external transmission. A representation of the *Cryptosporidium* life cycle is depicted in Figure 3 (CDC, 2019).

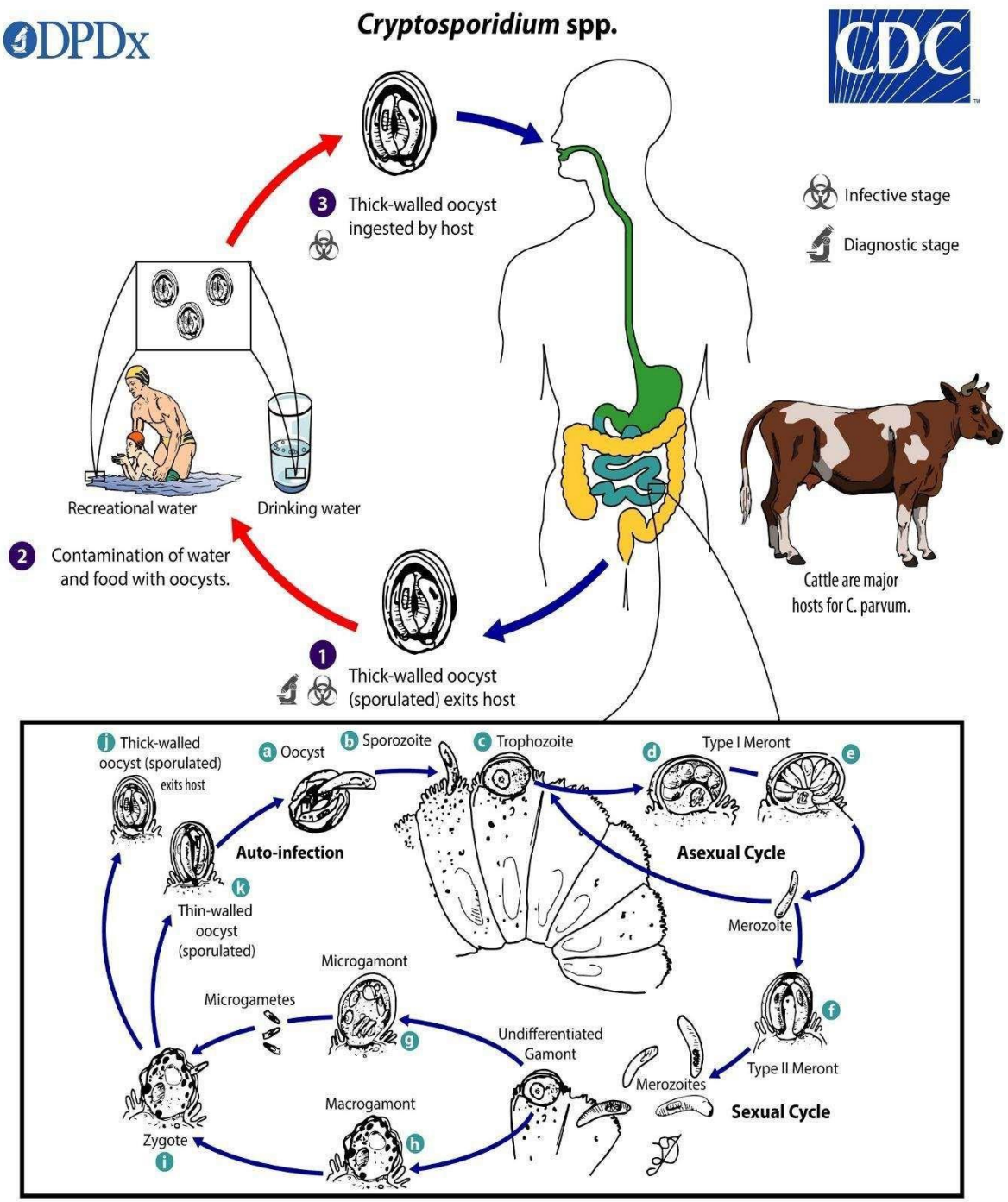

Figure 3. Life Cycle of *Cryptosporidium* Cryptosporidiosis, Global Health, Division of Parasitic Diseases and Malaria, May 20, 2019. https://www.cdc.gov/dpdx/*cryptosporidiosis*/modules/*Cryptosporidium*_LifeCycle_lg.jpg. Accessed 14 December 2021.

2.5 Pathogenesis

The pathogenesis of cryptosporidiosis is subject to variations depending on the host's immune system. Early host-parasite interactions encompassing attachment, invasion, and parasitophorous vacuole formation, are identified as pivotal events (Tzipori and Ward, 2002). Pinto and Vinayak (2021) accentuate the influential role of *Cryptosporidium*-host interactions in determining the development or protection from the disease. Notably, the pathogenesis of *Cryptosporidium* remains less explored compared to other apicomplexans like *Toxoplasma*, *Eimeria*, and *Plasmodium* (Pinto and Vinayak, 2021). Mucin-like glycoproteins which are also targets of protective cellular immune responses, facilitate *Cryptosporidium* adherence and penetration of intestinal epithelial cells, setting it apart from other apicomplexans (Bhalchandra *et al*., 2013). The intricate host-parasite interaction of *Cryptosporidium parvum* is depicted in Figure 4. Understanding these interactions becomes imperative for identifying key biological processes warranting future research efforts directed toward efficacious vaccines and treatments for cryptosporidiosis.

In the area of *Cryptosporidium*, recent significant technological advancements in genetics, cell culture platforms, and novel animal infection models have greatly accelerated researchers' foundational comprehension of the parasite's lifecycle and illness aetiology (Pinto and Vinayak, 2021). Using these technologies, researchers hope to gain a better understanding of the disease's pathophysiology and biology, leading to the development of vaccines and treatments to prevent cryptosporidiosis (Pinto and Vinayak, 2021). Calves exhibit acute catarrhal enteritis characterized by hyperemic and edematous mucosa, somewhat swollen and edematous mesenteric lymph nodes, and modest to moderate villus atrophy with sporadic villus fusion (Tzipori and Ward, 2002; Fayer *et al*., 1990). Histologically, modest to moderate villus atrophy with sporadic villus fusion is seen. These pathogenic changes result in reduced intestinal absorption surface and subsequent malabsorption (Helmy and Hafez, 2022).

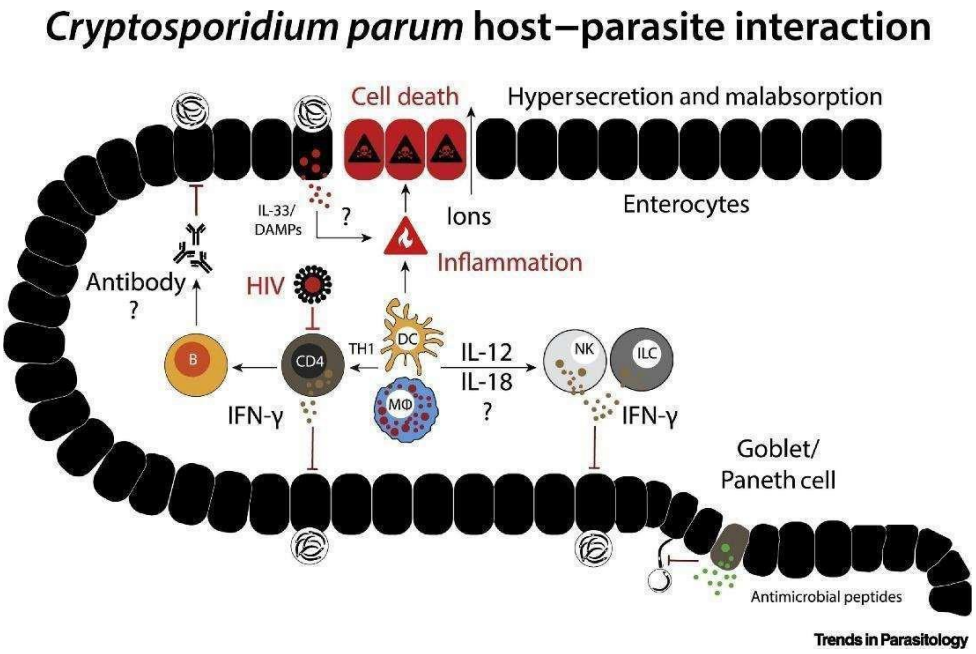

Figure 4: The host-parasite interaction of *Cryptosporidium parvum*. Adapted from Dumaine *et al.,* (2019). https://doi.org/10.1016/j.pt.2019.11.003. Accessed 08 September 2023.

2.6 Clinical signs

2.6.1 Cattle

The prevalence of cryptosporidiosis is higher in young calves, and the disease's severity is influenced by several variables, including the host's immunity, age, infectious dosage, season, location, and combined infections with other pathogens (Hatam-Nahavandi *et al*., 2019). Clinical symptoms might range from being asymptomatic to having pasty or watery copious diarrhoea known as scours, dehydration, and even death. Co-infection of C. *parvum* with enterotoxin *Escherichia coli*, Coronaviruses, and Rotaviruses, contribute to calf mortality, particularly within the first three weeks of life (Helmy *et al.,* 2017). Factors such as oocyst excretion peak, immunological response development, and virulence, play roles in the absence of clinical signs in positive hosts (Causapé *et al.,* 2002; Olson *et al.,* 2004 and Huber *et al.,* 2005).

2.6.2 Humans

Clinical indications in humans include abdominal discomfort, nausea, vomiting, flatulence, lethargy, and anorexia, often coexisting with diarrhoea in humans, which ranges from moderate to profuse watery to catarrhal. Inhalation of oocysts from polluted air can cause respiratory signs (Sponseller *et al.,* 2014).

2.6.3 Small ruminants

Sheep and goats infected by *C. parvum* exhibit acute diarrhoea typically appearing between five and 20 days of age. The severity of the diarrhoea correlates with the quantity of excreted oocysts (Paraud *et al.,* 2010). While many infections in lambs are asymptomatic (Pritchard *et al*., 2008; Paraud *et al*., 2009), some studies suggest an association between oocyst excretion and diarrhoea (Paraud et al., 2010; Pritchard et al., 2008; Causape et al., 2002; Ryan et al., 2005).

2.7 Epidemiology

Cryptosporidium infections pervasive among ruminants and other vertebrate hosts globally, were initially isolated in a case of calf diarrhoea in 1971 (Sulaiman *et al*., 1999). Subsequently, cryptosporidiosis has been established as the cause of calf diarrhoea outbreaks across multiple countries. Prevalence is notably higher in North America and Europe (Hatam-Nahavandi *et al*., 2019), due to the intensive farming practices (Atkinson *et al.*, 2020). While often affecting younger animals, healthy asymptomatic carriers, including adults, contribute to disease epidemiology by shedding oocysts into the environment (Glaberman *et al.* 2002). Cryptosporidiosis displays a diverse host range, affecting humans, domesticated ruminants, rodents, pigs, birds, and reptiles (Maikai *et al*., 2011; Sulaiman *et al*., 1999). Neonates are particularly vulnerable with a reported mortality exceeding 35%.

Certain studies indicate that cryptosporidiosis presents as self-resolving diarrhoea in immunocompetent hosts (Helmy *et al.,* 2013; Mahfouz *et al*., 2014). On the contrary, it can be severe, fatal, and prolonged in immunocompromised individuals such as HIV/AIDS patients, young children or cancer patients (Chalmers and Davies 2010; Mahfouz *et al*., 2014). These findings support the conclusion by Laurent and Lacroix-

Lamandé, (2017) emphasizing the control of cryptosporidiosis through immunological mechanisms. In immunocompetent individuals, there is spontaneous termination of oocysts, production of antibodies in infected calves, resistance to infection in adult animals, while disease is severe in incompetent individuals.

Mahfouz *et al*. (2014) highlight the global distribution of infection, especially in developing countries, where prevalence varies based on socio-economic factors and animal farming intensity. In South Africa, a study conducted by Samra *et al.* (2016) reported a prevalence of 0.6 % in calves. This low prevalence was consistent with studies conducted in Egypt (7.09 %) (Mahfouz *et al.,* 2014), Ethiopia (9.4 %) (Wegayehu *et al.*, 2016), Tanzania (1.8 %) (Chang'a *et al.*, 2011), Kenya (8.3 %) (Ogendo *et al.*, 2017) and Mozambique (3.7 %) (Miambo *et al.*, 2019). Factors influencing prevalence rate include seasonality, targeted sampling of diarrheic calves, and the presence of coinfections (Ibrahim *et al.*, 2016; Samra *et al.*, 2016; Ogendo *et al.*, 2017). Elsewhere in Africa, the reported prevalence of cattle was contradictory and higher than in the above-mentioned countries. In Algeria, Egypt, Ethiopia, Kenya, Nigeria and Tunisia, the highest reported prevalence in these areas was 84% (Benhouda *et al.*, 2017), 30.2% (Amer *et al.*, 2010), 18.7% (Ayele *et al*., 2018), 16% (Kange'the *et al.*, 2012), 53.2% (Ayinmode *et al*., 2010) and 21.4% (Rahmouni *et al.*, 2014) respectively. Poor hygiene was associated with an increased rate and spread of infection in these areas (Helmy *et al.*, 2013; Wegayehu *et al.*, 2016). Dirty and muddy farms provide favourable microclimatic conditions for the survival and persistence of the oocysts (Ayele *et al*., 2018), leading to heightened feed and water contamination, and increased exposure to the pathogen.

In Africa, *Cryptosporidium* prevalence in sheep was reported in four countries, Ethiopia (2.1%) (Wegayehu *et al.*, 2016), Egypt (2.5%) (Mahfouz *et al.*, 2014), Tunisia (11.2%) (Soltane *et al.,* 2007) and Nigeria (16.6%) (Odeniran and Ademola, 2019). Prevalence in goats varied, with reported rates of 0% in Tunisia (Soltan *et al*., 2007) and Mozambique (Miambo *et al.,* 2019), while Nigeria reported a prevalence of 26%, (Odeniran and Ademola, 2019). The observed differences in prevalence among these species may be attributed to herd size, environmental exposure, and management practices, with sheep generally kept in larger groups in commercial farming systems,

increasing their exposure to environmental contamination compared to goats (Odeniran and Ademola, 2019). Additionally, the rearing of goats in smaller extensively managed groups may contribute to the lower cryptosporidiosis prevalence than in sheep (Soltan *et al*., 2007).

Based on the foregoing, it can be inferred that the available data on the prevalence of cryptosporidiosis in ruminants in South Africa is insufficient. The existing studies exhibit inadequacies in comprehensively addressing all ruminant species, with a notable gap in coverage for sheep and goats. Considering the potential of these species to serve as reservoirs for zoonotic infection, it is imperative to undertake more extensive prevalence studies encompassing all ruminant species and geographical localities throughout South Africa.

2.8 Diagnosis

In previous studies, the reported prevalence was detected using microscopic techniques which cannot distinguish between species or genotypes (Ayinmode *et al*., 2010; Maikai *et al.,* 2011). The primary method of detection in most studies was the Modified Ziehl-Neelsen (MZN) technique (Rahmouni *et al.*, 2014; Samra *et al.,* 2016; Miambo *et al.*, 2019). Molecular tools, particularly PCR-RFLP, have facilitated species-level identification (Maikai *et al.,* 2011; Helmy *et al.,* 2013; Manyazewal *et al.,* 2018) offering improved sensitivity and specificity. These advancements allow for a comprehensive understanding of *Cryptosporidium* at both the species/genotype and subtype family level (Ayinmode *et al*., (2010); Fereig *et al.*, 2016). Table 2 highlights three different methods of detection, encompassing microscopy, serological and molecular techniques, with advantages and disadvantages of each method.

Table 2: Diagnostic techniques for cryptosporidiosis detection.

Technique	Diagnostic targets	Advantages	Disadvantages	References
Microscopy ● No stain ● Modified Ziehl-Neelsen Staining ● Heine technique ● Kinyoun's Carbol Fuschin staining technique. ● IFA-Immunoflourescent antibody-based staining technique	External Morphology or internal structures	Inexpensive and available worldwide. Highly sensitive and cheaper.	Cannot differentiate viable and non-viable oocysts. Low sensitivity	(Delafosse *et al.,* 2006, Reshmi *et al*., 2006)
Serological ● EIA-Enzyme Immunoassay ● ELISA-Enzyme Linked Immunosorbent assays. ● EITB-Enzyme Linked immunoelectron transfer blots. ● Strip-Immunochromatography	*Cryptosporidium* surface antigens	Best for screening a large number of samples. More sensitive than microscopy Faster and easy to perform. Normally used for epidemiological surveys	Limited to trained lab and expensive	(Leitch and He., 2011, Helmy and Hafez., 2022)
* Molecular (Nucleic Acid detection) ● RT-PCR ● RFLP-PCR ● RAPD-PCR ● AP-PCR ● Crypto PMA-PCR ● DNA Sequencing	DNA	Identifies viable and non-viable oocyts Identifies species, genotypes and subtypes More sensitive; Gold standard	Expensive	(Nichols et al., 2002, Brescia et al., 2009)

* RT-PCR; Reverse transcription polymerase chain reaction, RFLP-PCR; Restriction fragment length polymorphisim polymerase chain reaction, RAPD-PCR; Random amplified polymorhic DNA polymerase chain reaction, AP-PCR; Arbitrarily primed polymerase chain reaction, Crypto PMA-PCR; Cryptosporidium Propidium monoazide polymerase chain reaction.

Adapted from: Shrivastava *et al*., (2017).

2.8.1 Microscopy

The Modified Ziehl-Neelsen (MZN) technique, widely adopted by researchers, is employed for *Cryptosporidium* detection through microscopic examination of oocysts. Positive samples exhibit specific morphological characteristics including size (4-6 micromole), colour (refractive pink), shape (spherical round to oval), and presence of a residuum and sporozoites (Figure 4) (Manyazewal *et al*., 2018). Evaluations of the MZN's specificity and sensitivity vary across studies, contingent on matrix variables such as oocyst concentration and the presence of acid-fast oocyst-sized particles within faecal samples (Chang'a et al., 2011; Miambo et al., 2019). While MZN is lauded for its speed, cost-effectiveness, and reliability, it is not without drawbacks, including the potential for false positives due to staining artifacts and its lower sensitivity in cases of sparse oocyst shedding (Samra et al., 2016; Chang'a et al., 2011).

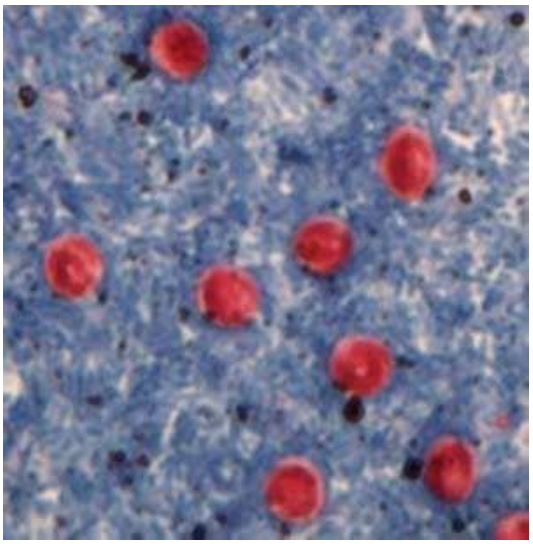

Figure 5: *Cryptosporidium* species oocysts stained with Ziehl-Neelson modified acid-fast. https://www.cdc.gov/dpdx/cryptosporidiosis/index.html. Accessed 08 September 2023.

2.8.2 Nucleic acid-based tests

Researchers have increasingly turned to nucleic acid-based tests such as polymerase chain reaction (PCR), quantitative real-time polymerase chain reaction (qPCR) and polymerase chain reaction restricted fragment length polymorphism (PCR-RFLP) for precise identification and characterization of *Cryptosporidium* genotypes and sub-genotypes (Coupe *et al*., 2005). These molecular methods offer advantages over traditional microscopic approaches, enabling accurate, sensitive, and robust identification of parasitic protozoans in the environment (Adeyemo *et al*., 2018). In epidemiological surveillance studies, nucleic acid-based methods can determine the source and severity of diseases. They can determine whether cysts and oocyst species are exclusive to certain hosts. Adeyemo *et al*. (2018) further explain that these procedures can identify species genotyping, which is then used to decide epidemic prevention measures.

Nucleic acid-based methods have enhanced precision, high throughput capabilities, and reliability, constituting notable advantages (Adeyemo *et al.*, 2018). The PCR is a genotyping technique that amplifies specific gene sequences with heightened sensitivity compared to traditional approaches (Hatam-Nahavandi *et al.*, 2019). While PCR offers superior sensitivity, it requires expensive equipment and is prone to false positives. To augment the sensitivity of primary PCR, nested PCR is employed, utilizing dual primer sets to boost specificity. Tzipori and Ward (2002), applied nested PCR to amplify a 590 bp fragment of a small sub-unit rRNA (SSU rRNA) gene for species identification. Subsequent sub-genotyping of the Glycoprotein 60 (GP60) gene involved amplifying a 300-bp gene segment. Although different fragments have been used by different researchers, the GP60 and SSU rRNA genes are frequently used for species identification, genotyping, and sub-genotyping (Benhouda *et al*., 2017; Ibrahim *et al*., 2016; Rahmouni *et al*., 2014).

For the selective amplification of various genetic loci, PCR-RFLP selectively amplifies diverse genetic loci using primer pairs (Samra *et al*., 2013) followed by enzymatic cleavage or sequencing for *Cryptosporidium* species or genotype categorisation (Adeyemo *al*., 2018). Quantitative real-time PCR has been widely used in the detection and quantification of pathogens in both environmental and clinical samples. This

approach enables real-time DNA amplification monitoring, offering practical advantages over traditional PCR (Adeyemo *et al*., 2018). The advantages of qPCR are a measurement of genetic targets across a broad range, increased sensitivity and specificity, quicker time and less labour (Adeyemo *et al.*, 2018). Previous studies have described qPCR techniques targeting the SSU rRNA gene to detect the whole *Cryptosporidium* genus, with assays targeting the 18s rRNA gene demonstrating efficacy in detecting all *Cryptosporidium* species (Maikai *et al*., 2011; Wegayehu *et al*., 2016).

A paucity of investigations is evident in the detection of cryptosporidiosis in ruminant species utilising molecular approaches within the South African context. The existing studies predominantly employ conventional microscopy methods, while the utilization of PCR, known for its heightened sensitivity and specificity, is limited. Consequently, to rectify the knowledge deficit regarding the prevalence of *Cryptosporidium* species impacting ruminants in South Africa, further research initiatives employing both molecular and microscopic detection methodologies are warranted.

2.9 Risk Factors

The risk factors associated with cryptosporidiosis encompass three different categories i.e. management, environmental and animal factors, which may exhibit interconnections.

2.9.1 Animal factors

The age of the animal; plays a pivotal role with goat kids, lambs and calves below three months showing heightened susceptibility (Mahfouz *et al*., 2014). Although juveniles, yearlings and adults may succumb to infection, the incidence is lower than in younger animals (Manyazewal *et al.*, 2018).

2.9.2 Environmental factors

Contaminated water sources, seasonal patterns, particularly during the rainy season, contaminated manure, and poor waste management. Ogendo *et al.* (2017) established a strong association between exposure to contaminated manure and increased prevalence of cryptosporidiosis in humans and animals. Most of the people

interviewed during the study of Ogendo *et al.* (2017) who used manure for fertilization and building purposes. Some of the interviewees were not observing the protocol of frequently washing their hands after handling manure and were not regularly cleaning manure from their farms.

2.9.3 Managemental factors

Poor hygiene, intensive farming practices, failure to separate calves from adult cows, delayed colostrum feeding, overcrowded living conditions and occupational exposure (veterinary students, children in agricultural camps, research technicians) amplify the risk of infection (Helmy *et al.*, 2013).

While the risk factors associated with cryptosporidiosis have been extensively investigated in various regions of Africa, the research in South Africa is notably deficient. A discernible knowledge gap exists regarding the risk factors for cryptosporidiosis on farms in South Africa, necessitating focused attention and comprehensive investigation to bridge this gap.

2.10 Control and Prevention

There are currently few safe and efficient therapeutic alternatives for treating cryptosporidiosis in humans or livestock, as well as no viable vaccines to prevent the disease. Only one FDA-approved drug, nitazoxanide, is effective in immunocompetent individuals, but ineffective in highly immunocompromised people (Sparks *et al*., 2015). In calves, halofuginone lactate (Trotz-Williams *et al*., 2011), the only registered product in South Africa, is used as a prophylactic treatment and is administered within 24 hours of the development of clinical indications and continued for seven days. Although it can be lethal in dehydrated animals, the medicine works to limit the parasite's shedding and to lessen the severity and duration of diarrhoea. Recently, paromomycin (Grinberg *et al*., 2002) has become accessible in the UK under veterinary prescription and can be used for seven days after a diagnosis of *Cryptosporidium* infection. It has been found to minimize oocyst shedding and diarrhoea, however there is a risk of toxicity with this medication.

Environmental public health strategies involving hand washing and education on disease transmission routes are vital in lowering disease occurrence. Due to the absence of vaccines for cryptosporidiosis in livestock, disease prevention and control currently entails enhancing calf resistance through colostrum feeding in the first hours of life (Meganck *et al*., 2014), maintaining hygienic living conditions (Kay 2012) and effective manure management to reduce exposure to *Cryptosporidium* oocysts (Vermeulen et al., 2017). A reduction in the viability of *Cryptosporidium* oocysts due to appropriate management of manure and slurry on farms will minimize the danger of disease for the surrounding environment, especially for water catchments (Kay 2012). These on-farm treatments include composting manure properly (heat of 60 °C will inactivate the oocysts), storing slurry (ammonia and low pH will aid to inactivate the oocysts), and treating oocysts using mesophilic and thermophilic anaerobic digestion to drastically reduce oocyst viability (Vermeulen *et al*., 2017).

2.11 Conclusion

Cryptosporidiosis significantly impacts domesticated ruminants in Africa, particularly in South Africa. The detection of the disease in Africa is constrained, predominantly relying on traditional microscopic methodologies rather than molecular techniques. There exists a research gap concerning the specific *Cryptosporidium* species affecting ruminants, particularly small stock, in South Africa. While risk factors of cryptosporidiosis have been elucidated in other African regions, in South Africa these remain inadequately explored. There is a need for more studies on the prevalence and associated risk factors of cryptosporidiosis in ruminants in South Africa. Priority should be given to studies focusing on diagnostic methodologies to enhance understanding of the disease.

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CHAPTER 3: Materials and Methods

3.1 Ethical approvals

This study received ethical clearance (see Appendix 1 – 3) from the University of Pretoria Faculty of Veterinary Sciences Research Ethics Committee, the Animal Ethics Committee (reference number REC112-21), and the Faculty of Humanities Ethics Committee (reference number HUM011/0522). Approval of the study in accordance with Section 20 (reference number 12/11/1/1/6 (2099MVA)) of the Animal Diseases Act of 1984 and was granted by the Department of Agriculture, Land Reform and Rural Development (Pretoria, South Africa), authorizing the collection and movement of samples from the farms to the laboratory. Prior to participating, farmers involved in the study provided written informed consent for collection of faecal samples from their animals and for the questionnaire interviews.

3.2 Study area

This research was conducted in Rust de Winter area, situated at the border between Gauteng and Limpopo Provinces of South Africa (Qekwana *et al*., 2010). The location comprises a small residential area of 0.38 km² coupled with an expansive region featuring a game reserve and a 16.5 km² dam. In the Rust de Winter area, there are both livestock farmers and wildlife farmers. According to Botha et al. (2005), the farms in this area occupy approximately 380 km², of which 120 km² fall under the Limpopo Province and 260 km² fall under the Gauteng Province. The type of management systems in the area ranges from extensive to semi-intensive. They further mention that animals kept in these farms range from cattle, sheep, goats, chickens, ducks, to pigs, with sheep and goats often grazing together with cattle. According to their observation, there is close interaction between animals and humans. The area has both hot and dry climatic conditions, with predominantly summer rainfalls of approximately 600 mm (Botha *et al.,* 2005), which may favour the survival of oocysts. The average minimum temperatures between 2022 and 2023 ranged from 2.2 to 6.0 °C from May to August and 9.0 to 16.7 °C from September to August. The average maximum temperatures range from 20.2 to 23.04 °C from May to August and 26.7 to 29.6 °C from September

to April. The area often experiences a dry year once every 10 years (Botha *et al*., 2005). The majority of the farmers practice livestock farming, and the rest practice mixed or solely crop farming (Botha *et al.*, 2005). The most reliable water source in the area is a borehole.

3.3 Study design and sample size

In this study, we analysed faecal samples collected from ruminants and interviewed farm workers/owners about their practices and knowledge related to cryptosporidiosis.

The faecal-sample study adopted a repeated cross-sectional, two-stage stratified design, where faecal samples were collected from individual domestic ruminants (cattle, sheep and goats) from farms, during both winter (July 2022) and summer (February 2023), using a systematic random sampling method. The sample size was determined following a formula by Dohoo *et al*. (2003), considering a 95% confidence level (Z = 1.96), an estimated prevalence of 8% (p), and a margin of error (d) of 0.06.

$$n = \frac{Z^2 p(1\text{-}p)}{d^2}$$

A sample size of 79 was calculated using an estimated prevalence of 8% from a previous study with calves in a communal farming area of Mnisi in Mpumalanga Province, South Africa (Samra et al., 2013). Farm- then individual-level sampling was applied in this study, therefore, the sample size was adjusted by multiplying it with the design effect (DE), to take into consideration the expected dependence of *Cryptosporidium* exposure in each farm (cluster). We used the design effect (2.53) and intracluster correlation (0.18) for Eimeria species (Orjuela et al., 1991 cited in Otte and Gumm, 1997), whose transmission and maintenance in the environment is similar to that of *Cryptosporidium* species. The calculated effective sample size obtained for each of the two seasons was 199 domestic ruminants (total 398 for the two seasons). However, a total of 370 faecal samples were collected from cattle (n=146), sheep (n=119), and goats (n=105) in both seasons (Table 3) due to less animals on some farms and accessibility challenges.

The farms were selected through simple random sampling from a list provided by the state veterinarian. Only farms with at least 30 animals were included in the sampling list. The number of animals sampled (n1) from each farm in each season was calculated following the formula described by Elbers *et al*. (1995):

$$n1 = \sqrt{\frac{[\frac{T1}{T2}]\,(1-\rho)}{\rho}}$$

taking in to account the ratio of the cost of sampling one animals (T1) to the cost of sampling all animals on the farm (T2). Considering an estimated distance between farms (T1/T2=300), and the intra-cluster correlation coefficient (ρ=0.18), a total of 36 animals were to be sampled per farm. Initially five farms (n/n1), each contributing 36 animals (12 of each species) were ear marked for sampling in each season, however, eventually six farms were sampled in each season due to limitations in the availability of sheep and goats on some farms. Individuals of each species were selected by systematic random sampling on the farm. A sampling interval K will be determined beforehand and the starting point was determined randomly from the first to last animal. The questionnaire study was of a purposive design, in which farm owners and workers on or around the farms that had been sampled for faecal samples were interviewed.

Table 3: An illustration of the number of sampled animals per species per season.

Species	**Dry season**	**Wet season**	**Total**
Cattle	73	73	146
Sheep	57	48	105
Goats	60	59	119
Total	190	180	370

3.4 Faecal sample collection

Faecal samples were collected from the same farms during wet and dry seasons, except for two farms that were only sampled in the dry season with two new farms introduced in the wet season to replace the unrepeated farms. Fresh faeces were directly obtained from the rectum of each animal using a hand/finger dressed in a

lubricated latex glove. Individual animal signalment data were recorded. Samples were stored in airtight faecal pots, labelled accordingly, and promptly transported to the laboratory in a cooler box until laboratory processing. In the laboratory, a portion of the fresh faeces was stored in 2 ml centrifuge tubes and frozen at -20 ºC for DNA extraction. The second portion of approximately 10 g was stored in 3-4 ml of formalin in 15 ml tubes at 4° C for microscopic examination of the oocysts.

3.5 Laboratory analysis for the occurrence of *Cryptosporidium* in domesticated ruminants

Laboratory analysis was done in the Helminthology Diagnostic Laboratory and the Research and Training Laboratory in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa.

3.5.1 Concentration of the oocysts

Prior to undertaking the MZN and molecular procedures, each of two faecal sample aliquots (one in 10% formalin and the other as fresh sample without formalin) underwent a sedimentation procedure to concentrate the oocysts (Casemore, 1991). This widely adopted method, integral to comprehensive ova and parasite testing, ensures optimal recovery and facilitates the detection of low organism counts overlooked by direct smears. Ethyl acetate was used to separate debris and fat from the faecal material, with a resultant sediment containing parasites. About 1 g of solid faecal sample or 2ml of liquid faecal sample was added to a 15-ml tube. This was followed by addition of 10 ml of saline solution (1.5 g of sodium acetate, 2 ml of acetic acid, 4 ml of 37-40% formaldehyde and 92.5 ml of distilled water) to the tube. The tube was closed, and the content mixed well by tilting the tube. The sample mixture was filtered into a new 15 ml tube through a gauze (size: 100 mm X 100 mm) placed in a filter funnel. The gauze was squeezed to get all the filtrate into the new tube. The filtrate was centrifuged using a bench centrifuge (Compact Centrifuge Z206A, Hermle, Germany) for two minutes at 500 relative centrifugal force (rcf). The supernatant was decanted, and 8 ml of normal saline was added to resuspend the sediment, followed by addition of 3ml of ethyl acetate, and the content was mixed well by shaking and inverting the tube. The mixture was centrifuged for five minutes at 500 rcf. The supernatant was poured off, followed by addition of 1 ml of saline to the sediment. The

content was mixed well by shaking, ultimately providing material for subsequent Ziehl-Neelsen staining and DNA extraction for PCR.

3.5.2 Modified Ziehl-Neelsen (MZN) staining and microscopy

All 370 faecal samples were screened using the Modified Ziehl-Neelsen (MZN) staining technique for the detection and identification *Cryptosporidium* oocysts (Manyazewal *et al.*, 2018). One drop of the mix from the concentration technique was transferred onto a slide to make thin smears. The slides were heat-fixed, covered with carbolfuchsin stain, and heat-steamed for 5 minutes, then rinsed with tap water. This was followed by decolorization by rinsing with acid alcohol for two minutes and rinsing with tap water, then counterstaining with malachite green for 0.5–1 minute, rinsing with tap water, and drying. A cover slip was then mounted on the slide. The staining procedure was performed in a fume hood as standard operating procedure for safety in the laboratory. Stained slides were observed under a compound microscope under the 50X objective lens. *Cryptosporidium* oocysts appeared as pink-to-red spherical structures.

3.5.3 DNA extraction

The concentrated fresh faecal samples from section 3.5.1 were immediately subjected to DNA extraction using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hildenn, Germany), following the manufacturer's instructions, but with some modifications. The sample was transferred from the 15-ml tube into a 2-ml Eppendorf tube and centrifuged at 21913 g for 2 minutes to further sediment the sample. About 0.2 g of sediment was transferred using a tongue depressor into 2-ml MagNA Lyser Green Beads tube (Roche Diagnostics, Rotkreuz, Switzerland) to which 600 µl of lysis buffer, AL, from the DNA extraction kit had been added. The mixture of beads, sample and lysis buffer was homogenised using a Precellys 24 homogenizer (Bertin Technologies, Montigny, France) at 5500 units per second for 18 x 4 seconds, followed by incubation on a heating block (Labnet International Inc, Woodbridge, USA) for five minutes at 85 °C and then centrifuged in a table microcentrifuge using the Eppendorf benchtopcentrifuge 5810 R (Eppendorf South Africa (PTY) Ltd, Johannesburg, South Africa) for 1 minute. About 300 µl of the supernatant was transferred to 2-ml tube containing 15 ml of proteinase K from the DNA extraction kit. InhibEX reagent from the

extraction kit was added to the mixture, followed by incubation overnight at 70°C. The following day, about 400 µl of absolute ethanol was added to the mixture. The rest of the protocol, from the addition of ethanol was as described for the QIAamp Fast DNA Stool Mini Kit.

3.5.4 Quantitative real-time PCR for *Cryptosporidium parvum*

The PrimerDesign™ Ltd genesig® Standard Kit for *Cryptosporidium parvum* (Eastleigh, England) was used to detect the presence of *C. parvum* in the DNA samples, following the manufacturer's instructions. The kit targets the *C. parvum* Zinc protease telomerase (ZPT) gene. Each PCR reaction mixture of total volume 20 µl comprised 10µl of the PrecisionPlus 2X qPCR Master Mix, 1µl of *C. parvum* primer/probe mix (FAM labelled), 4µl Rnase/Dnase-free water and 5µl of genomic DNA template. The thermocycling conditions involved 50 cycles for each of the following steps: activation cycle for at 95 °C for 2 minutes, denaturation at 95 °C for 10 seconds, and with a fluorogenic data collection step at 60 °C for 60 seconds. In each PCR run, we included two 10-fold serial dilutions (2×10^4 and 2×10^3 copy number per µl) of the original positive *C*. *parvum* DNA template (2×10^5 copy number per µl), which comes with the kit. The negative control was Rnase/Dnase-free water.

3.6 Questionnaire Survey

Before conducting the questionnaire survey, informed consent was obtained from participating farmers and this was facilitated by the Rust de Winter state veterinary animal health technician. Farmers were provided detailed explanations regarding the study's objectives, animal sampling procedures and the questionnaire methodologies. Emphasis was placed on the voluntary nature of their participation, and each farm owner signed an informed consent form (Appendix IV), ensuring confidentiality. The interview-based questionnaire was translated from English to the indigenous languages (Sepedi and isiZulu) and administered by the principal researcher and two University of Pretoria interns. Each questionnaire interview lasted approximately 20 minutes.

The semi-structured questionnaire (Appendix IV) aimed to determine the risk factors associated with *Cryptosporidium* infection and the participants were farm owners or workers. Topics covered during the interviews included the animal demographics, animal-human interaction frequency, and the overall farming environmental conditions. Questions about animals included signalment (age, sex, breed, species), illness history, presence of diarrhoea, and herd size. Farm related inquiries included water source type (borehole or municipal water), housing conditions (muddy or concrete), farm type (intensive or extensive), feeding practices, proximity of animal housing to other on-farm activities, presence of vegetables near animal housing, manure management procedures, and waste disposal methods. The questionnaire also included questions on water sources for human and animal use, basic farm or household hygiene practices after animal handing and the frequency of children's exposure to animals.

3.7 Data analysis

Data from the questionnaire were managed in Microsoft Excel sheet (Microsoft version 2401) and given the small number of respondents (n=13), these data were analysed by descriptive tools (number and percentage of responses). For laboratory data, descriptive statistics were used to establish the frequency (percentage) of *Cryptosporidium* positive animals as determined by the Ziehl Neelsen stain method, across different predictor variables, including animal species, sex, age category, and season. This was followed by univariate analysis, in which the chi-squared test was used to determine the association between each of the four factors and faecal smear positivity. All variables, irrespective of p-value from univariate analyses, were entered into multivariable mixed-effects Generalised Linear model with a stepwise backward elimination procedure and Akaike Information Criteria, to determine the risk factors for *Cryptosporidium* infection. The random effect was farm of origin, while fixed effects were animal species, sex, age category and season. Analyses were conducted using R statistical software version 4.3.1 (RCore Team, 2023) at 5% significance level.

3.8 References

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CHAPTER 4: Results

4.1 Microscopy – Modified Ziehl Neelsen (MZN) staining

4.1.2 Descriptive and univariate analyses for occurrence of *Cryptosporidium* species

Microscopic analysis identified *Cryptosporidium* oocysts in 57 (15.4%) of the 370 faecal samples. Four factors (animal species, sex, age category, season) were analysed with respect to occurrence of *Cryptosporidium*. Univariate analysis showed that only season had a significant effect (p=0.0362) on the occurrence of Cryptosporidium species (Table 4). All the four factors regardless of p value were entered into a multivariable logistic regression model.

Table 4: Descriptive and univariate analyses for the association between animal or environmental factors and the occurrence of *Cryptosporidium* species. Occurrence of infection was established using the Modified Ziehl-Neelsen method.

Factor	**Level**	**Number and percentage (in brackets) of animals detected positive for *Cryptosporidium* spp.**	***p*-value**
Animal species			
	Cattle (n=146)	17(11.6)	0.1906
	Goats (n=119)	19(16.0)	
	Sheep (n=105)	21(20.0)	
Sex			
	Female (n=285)	40(14.0)	0.6348
	Male (n=74)	12(16.2)	
Age (months)			
	>36 (n=74)	9(12.2)	0.657
	4 to 11 (n=136)	23(16.9)	
	12 to 36 (n=160)	25(15.6)	
Season	Winter (n=190)	22(11.6)	0.0362
	Summer (n=180)	35(19.4)	

4.1.3 Multivariable analysis

The final multivariable model comprised only three variables (sex, animal species, season) (Table 5). Sheep (20.0%; OR=1.9, p=0.075) showed a higher likelihood, and goats (16.0%; OR=1.16, p=0.695) showed a similar likelihood, for *Cryptosporidium* infection compared to cattle (11.6%), but with no statistical significance (Table 5). On the other hand, season significantly influenced *Cryptosporidium* infection, with higher odds of infection in summer (19.4%; OR=2.2; p=0.0133) compared to winter (11.6%). With regards to sex, there was a similar likelihood of *Cryptosporidium* infection between male (16.2%) and female animals (14.0%), with an odds ratio of 1.1 (Table 5).

Table 5: Final multivariable model for the association between three factors on one hand and the occurrence of *Cryptosporidium* as established using the Modified Ziehl-Neelsen method.

Variable	Category	Odds ratio (CI)	*p*-value
Animal species			
	Cattle (ref)		
	Sheep	1.91(0.94, 3.89)	0.0754
	Goat	1.16(0.55, 2.46)	0.6950
Sex			
	Female (ref)		
	Male	1.09(0.53, 2.22)	0.8208
Season			
	Winter (ref)		
	Summer	2.19(1.18, 4.08)0	* 0.0133

*indicates significant difference at P<0.05

4.2 Quantitative real-time PCR detection of *Cryptosporidium* spp.

Samples that were positive from the microscopic method were tested using qPCR. Of the 57 samples tested, only one (1.8%, 1/57) was positive for *Cryptosporidium*, which although showed a high Cq value (around the cut-off point), this was a positive quantitative result. This qPCR positive sample was from the summer batch 1/34 (2,9%), while none of the samples from the winter batch was positive (0/23, 0%).

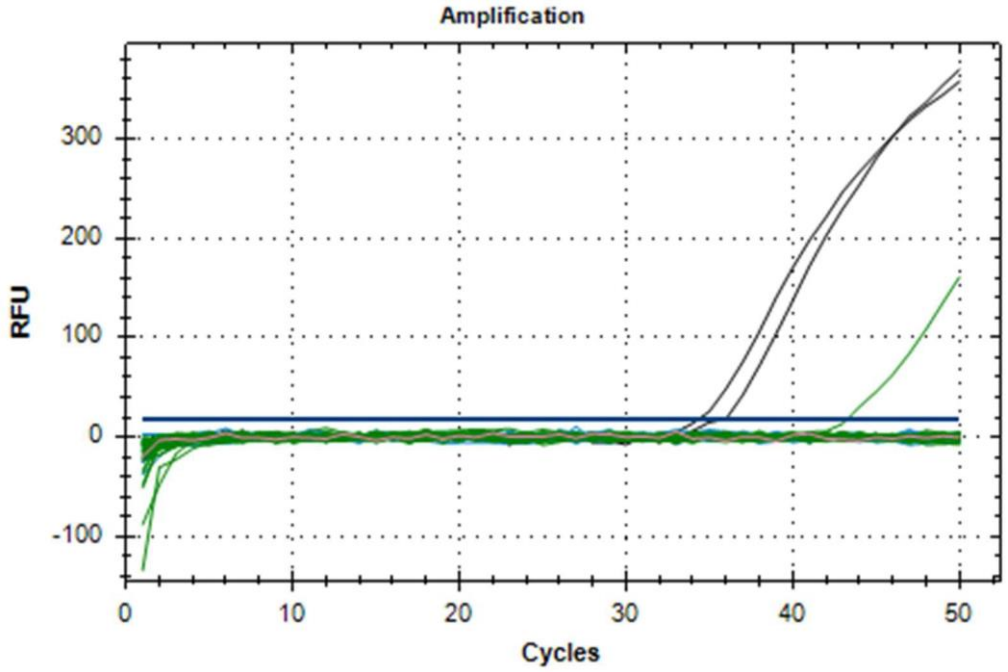

Figure 6: Quantitative real-time PCR detection of *Cryptosporidium parvum* oocysts from ruminant faecal samples collected during the summer months of 2022. Of the 57 samples tested, one was positive (green colour amplification). The solid line represents the baseline (threshold), while the two black lines peaking represent amplification of the positive control DNA (10-foldserial dilutions of *C. parvum* DNA). The orange line represents the negative control, and the green lines represent the samples tested. Any line below the thresholds is negative, and the lines spiking over the baseline represent positive samples.

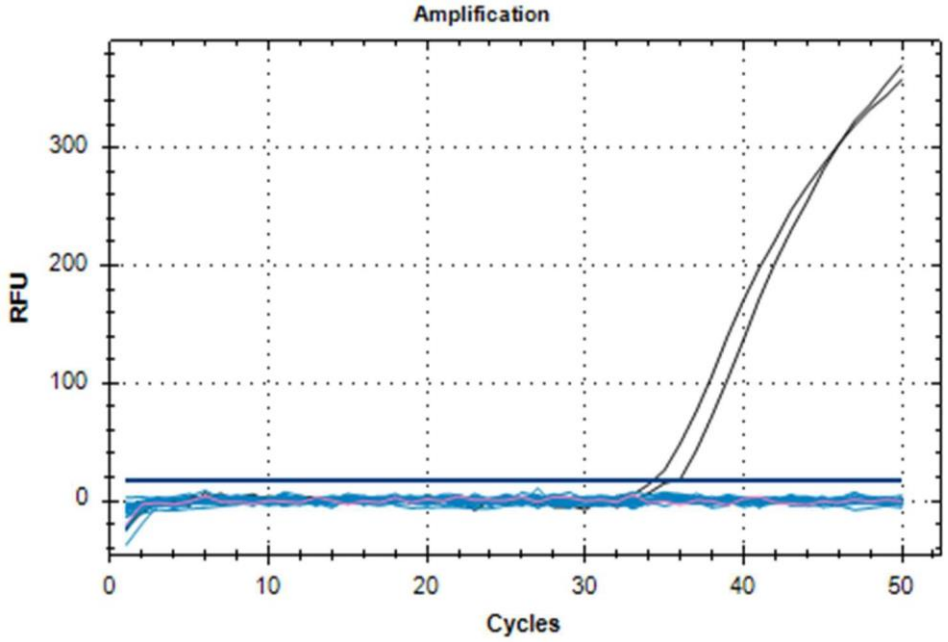

Figure 7: Detected *Cryptosporidium parvum* oocysts by quantitative real-time PCR from ruminant faecal samples collected during the winter months of 2022. The solid line represents the baseline (threshold), while the two black lines peaking represent the 10-fold serial dilutions of the positive control DNA template from *C. parvum* oocysts. The pink line represents the negative control, and the blue lines represent the samples tested. All samples were below the baseline; therefore, no oocysts were detected by real-time PCR during winter.

4.3 Questionnaire interviews

4.3.1 Demographics of the respondents

The questionnaire survey targeted participants that attended regular farmers' meetings, garnering responses from only 13 individuals from the studied farms. Most of the respondents were male (n=11/13), while 2 were female (Table 6). The 11 interviewed males were farm owners, while the female respondents were farm workers. Most participants (n=12) were above 30 years of age (6 participants of each of the 31-50 and 51-70 age categories) (Table 6). Most participants (n=11) had at least secondary education (8 with secondary and 3 with tertiary), while the rest had primary (n=1/13) or lacked formal education (n=1/13) (Table 6). In terms of experience with

raising animals, most of the farmers (n=8/13) had been raising animals for more than 20 years (Table 6).

Table 6: Socio-demographic characteristics of the participants for the *Cryptosporidium* risk factors survey in the Rust de Winter community, Gauteng Province in South Africa.

Variable (n=13)	**Level**	**Frequency of the respondents**	
		No.	**%**
Farm role	Owner	11	84.6
	Farm manager	1	7.6
	Animal caretaker	1	7.6
Gender	Male	11	84.6
	Female	2	15.4
Age category (years)	18-30	1	7.6
	31-50	6	46.2
	51-70	6	46.2
Marital Status	Married	5	38.5
	Never Married	8	61.5
Household size	<5	2	15.4
	5-10	7	53.8
	>10	4	30.8
Highest formal education level	None	1	7.6
	Primary	1	7.6
	Secondary	8	61.5
	Tertiary	3	23.1
Experience with raising animals (years)	<5	3	23.1
	>5-20	2	15.4
	21-40	8	61.5

4.3.2 Farm management characteristics

Most of the farmers kept three (n=5/13) or four (n=5/13) animal species, while others kept one species (n=2/13) and only one farmer kept five species (n=1/13). Regarding domestic ruminants, most farmers reared cattle (n=12/13), followed by goats (n=11/13) and some kept sheep (n=9/13). The other species kept include chicken, pigs, and ducks. Bonsmara was the most common cattle breed kept (9/13 farmers), followed by Brahman (7/13 farmers), while other farmers kept Nguni (n=3) or other breeds (n=5) (Table 7). Since 12/13 farmers owned cattle, the section on cattle herd information had 12 respondents. A higher proportion (n=10/12) of participant farmers reported that their cattle had a good body condition score of at least 3 (on a scale of 1 to 5). Equal number of farmers practiced intensive or extensive management systems (each 5 farmers), while the rest engaged in backyard (cattle enclosure erected behind the house) system. Most of farmers (n=7/12) used boreholes as a water source for their livestock, followed by streams (n-6) and wells (n=6). All farmers (n=12) applied grazing as the primary source of feeding (n=12/12), and 8/12 applied mixed feeding (grazing and supplements), while 6/12 provided concentrates as supplements for their cattle.

Table 7: Farm management characteristics from participant farms during a survey on *Cryptosporidium* risk factors in the Rust de Winter community, Gauteng Province in South Africa.

Variable (n=12)	Level	Frequency of farms	
		No.	%
Cattle breed	Brahman	7	58.3
	Bonsmara	9	75.0
	Nguni	3	25.0
	Other	5	41.7
Average herd body condition	Good	10	83.3
	Average	1	8.3
	Poor	1	8.3
Management system	Intensive	5	41.7
	Extensive	5	41.7
	Backyard	2	16.7
Water source for livestock	Well	6	50.0
	Borehole	7	58.3
	Stream	6	50.0
	Dam	2	16.7
	Tapped public water (Municipality)	5	41.7
	Water tank	1	8.3
Feed source	Grazing	12	100
	Concentrates	6	50.0
	Mixed feeding (Grazing and supplements)	8	66.7
Provision of colostrum (first 24h of birth)	Yes	12	100
	No	0	0
Stocking density	Average	5	41.7
	High	4	33.3
	Very high	3	25.0

4.3.3 Hygiene characteristics of the farms

In terms of farm hygiene level, 5 of the 13 farmers did not remove animal manure from their farms, while only 2 farmers removed animal manure daily. The manure was either re-used at the farm (n=3/13), dumped in a ditch hole at farm (n=1/13), surrounding area (n=1/13), or dumped near a water source (n=1/13). One farmer sold the manure to crop farmers, and another burnt it. When it came to handling animals at the farm for routine procedures such as ear tagging, vaccinations, deworming and random health checks, most respondents (n=7/13) handled the sick ones first. All but two farmers (n=11/13) were not aware of cryptosporidiosis and had never received any form of awareness about the disease.

Table 8: Hygiene characteristics of participant farms during a study on risk factors for *Cryptosporidium* in Gauteng Province, South Africa.

Variable (N=13)	Level	Frequency	
		No.	**%**
Frequency of manure removal	Daily – weekly	2	15.4
	Fortnightly-monthly	3	23.7
	Three – six months	3	23.7
	Never	5	38.5
Manure dumping	Farm reuse	3	23.7
	Around area	1	7.6
	Near water source	1	7.6
	Ditch hole at farm	1	7.6
	Burn	1	7.6
	Crop Farmers	1	7.6
Animal care order	Young 1st	3	23.7
	Sick 1st	7	53.8
	Healthy 1st	3	23.7
***Cryptosporidium* awareness**	Yes	1	7.6
	No	11	84.6
	Maybe	1	7.6

4.3.4 Household practices

Four of the 13 farmers shared their water source with the animals, while 9/13 refrained from this practice. The communal water source served various purposes including drinking, cleaning, food preparation and washing. Animals were mostly (5/13) handled on a weekly basis, and only 5/13 farmers reporting that they washed their hands once after handling the animals. Most farms (10/13) indicated minimal contact between children and the animals.

Table 9: Zoonotic risk factors according to owner's household practices and level of hygiene.

Variable	Level	Frequency	
		No.	**%**
Humans share water with animals	Yes	4	30.8
	No	9	69.2
Water used for	Drinking	1	7.7
	Cleaning	3	23.1
	Food prepping	2	15.4
	Washing	2	15.4
	N/A	9	69.2
Animal handling	Daily	4	30.8
	Weekly	5	38.5
	Fortnightly	3	23.1
	When sick	1	7.7
Hand-washing before/after handling animals	Once	5	38.5
	Before and after	2	15.4
	In between animals	4	30.8
	All the time	1	7.7
Exposure of children to animals	Minimal	10	77
	Moderate	2	15.4
	Frequent	1	7.7

4.3.5 Association between risk factors mentioned by the farm participants and diarrhoea or *Cryptosporidium* occurrence

Based on the sampled farms, 4/8 did not remove manure from the premises, 7/8 farmers handled sick animals first when conducting animal care procedures, and 7/8 farmers were not aware of cryptosporidiosis (Table 10). Of the eight farms, equal number practiced extensive and intensive management. Each farm had a unique

feeding source, but e grazing was a common all the farms. Similarly, different water sources were used, but 2/8 farmers used public water for their animals. Comparison of *Cryptosporidium* occurrence amongst farms, as determined by the microscopic method, showed a significant difference in presence of oocysts (Table 10). Two farms showed no infection, while infection in the other six farms ranged from 4.3 to 31.9% (Table 10). There was no clear trend in infection in relation to management practices; for example, some farms that removed manure daily, cared for healthy animals first and practiced intensive management had higher infection than those that never removed manure, cared for sick animals first and practiced extensive management (Table 10).

Table 10: Association between risk factors mentioned by the farm participants and diarrhoea or *Cryptosporidium* occurrence *Cryptosporidium*

Farm and number of animals sampled	**Farm factors (**Frequency of manure removal, manure dumping, animal care order, *Cryptosporidium* awareness, feed source, water source, management system)	**Number and % of positive animals**	**p-value**
Bezuidenhout (n=71)	Frequency of manure removal: 3-4months Manure dumping: Burn. Animal Care order: Sick first *Cryptosporidium* awareness: Yes Feed Source: Grazing, mixed feed. Water source: Well, Borehole Management system: Intensive	3(4.23)	0.00049
Matshene (n=36)	Frequency of manure removal: Never Manure dumping: N/A Animal Care order: Sick first *Cryptosporidium* awareness: No Feed Source: Grazing, mixed feeding.	8(22.22)	

	Water source: Public water Management system: Extensive		
MF5 (n=62)	Frequency of manure removal: Fortnightly Manure dumping: Farm reuse. Animal Care order: Sick first *Cryptosporidium* awareness: No Feed Source: Grazing, Mixed feed Water source: Well, Borehole Management system: Intensive	4(6.45)	
MF9 (n=72)	Frequency of manure removal: Never Manure dumping: N/A Animal Care order: Sick first *Cryptosporidium* awareness: No Feed Source: Concentrates, Grazing, Mixed feed Water source: Stream, Borehole, Public, Well Management system: Rural	19(26.39)	
Mohuni (n=34)	Frequency of manure removal: Daily Manure dumping: Farm reuse. Animal Care order: Healthy 1st *Cryptosporidium* awareness: No Feed Source: Grazing, concentrate Water source: Well, Borehole, Stream Management system: Intensive	0(0.0)	
Rooikop (n=16)	Frequency of manure removal: Never Manure dumping: N/A Animal Care order: Sick first *Cryptosporidium* awareness: No	1(6.25)	

	Feed Source: Grazing, winter supplement. Water source: Stream Management system: Extensive		
Sizalo (n=69)	Frequency of manure removal: Never Manure dumping: N/A Animal Care order: Sick first *Cryptosporidium* awareness: No Feed Source: Grazing, mixed feed, concentrate. Water source: Stream, Borehole, well Management system: Intensive	22(31.88)	
Zak (n=10)	Frequency of manure removal: Monthly Manure dumping: Farm reuse. Animal Care order: Sick first *Cryptosporidium* awareness: No Feed Source: Grazing, mixed feed. Water source: Stream, well, Borehole Management system: Extensive	0(0.00)	

CHAPTER 5: Discussion

5.1 Discussion

The current study highlights the occurrence and risk factors for *Cryptosporidium* infection in cattle, sheep, and goats in the Rust de Winter region of Gauteng Province, South Africa, using a combination of microscopic and nucleic acid-based analyses.

About 15.4% of the sampled cattle, sheep and goats were detected as positive for *Cryptosporidium* using the MZN method. Our observed occurrence is higher than that reported by Samra et al. (2016) in calves (0.6%) in Mpumalanga Province of South Africa, but lower than the overall 31.2% for goats, cattle, chicken, and sheep in Limpopo Province (Samie et al., 2017). Variations in climate and husbandry systems may contribute to observed differences in occurrence of the pathogen in different studies. For instance, in Zambia, oocysts are widely distributed in the majority of sheep and goats that are intensively managed in large outdoor paddocks or on pasture (Goma *et al*., 2006). Also, minimal stocking rates contribute to minimal infection pressure. In addition to the type of animal management, other factors that may influence the observed variation in cryptosporidiosis prevalence include study design, timing of sampling, animal age, and housing conditions (Hatam-Nahavadi *et al* 2019). Also, different ages of animals in the different studies may contribute to a possibility of sporadic oocyst excretion patterns in some categories such as post-weaned and adult animals, and therefore an apparently less severe infection (Santin, 2013).

In the current study, prevalence was higher in sheep (20.0%), followed by goats (16.0%) and lowest in cattle (11.6%). This aligns with previous studies in Ghana (sheep: 34,1%, goats: 33.3% and cattle: 26,5% using qPCR) and in Limpopo Province of South Africa (sheep: 50.0%, goats: 47.7% and cattle: 26.8% using MZN), demonstrating a consistent pattern of higher prevalence in small ruminants compared to cattle (Samie *et al*., 2017; Squire *et al.,* 2017; Causape *et al*., 2002). Higher occurrence of the pathogen in sheep than goats can be explained by the way the two species feed: sheep are mainly grazers, and therefore more likely to ingest parasites

released in faeces (Robertson, 2009), while goats are mainly browsers, with less contact for contaminated feeds. Infection was higher in small ruminants than cattle, perhaps attributed to better immunity developed in cattle with age, compared to the young age of ruminants.

Comparing the different laboratory methods used, qPCR revealed a lower detection (1.8%) than MZN. This was surprising as PCR-based methods are of higher sensitivity and specificity for identification of *Cryptosporidium* infection (Samra, 2013; Paul *et al*., 2009; Xiao, 2010). The present study employed a *C. parvum-specific* qPCR kit and therefore lower detection can be explained by the possibility that some samples contained species or genotypes other than *C. parvum*. A study by Jothikumar *et al*. (2008) employed a generic 18S RNA TaqMan assay, and also developed a dual TaqMan assay targeting the 18S RNA gene specific for *C. hominis* and *C. parvum* in human and cattle faecal samples from USA and Botswana. Their *C. parvum* TaqMan assay was only able to amplify *C. parvum* and *C. wrairi,* while the *C. hominis* TaqMan probe did not cross-react with any other species on their panel. A study in France by Morio *et al*. (2019) employed a multiplex PCR assay (ParaGENIE Crypto-Micro Real-Time PCR kit) in humans for the identification of different *Cryptosporidium* species, which was able to detect *C. parvum, C. hominis, C. meleagridis, C. felis, and C. cuniculus* which were all included on their panel. Therefore, this shows that species specific kits will most likely detect *Cryptosporidium* species specific to them. In the present study, qPCR detected a positive sample only after concentration of oocysts in faecal samples using the methyl-ether sedimentation technique prior to DNA extraction. Therefore, to increase the sensitivity of the PCR-based methods, it may be useful for future research to incorporate this oocyst concentration step.

Other studies have also shown lower detection from the MZN method than PCR, for example Samra *et al*. (2013) reported a prevalence of 54/442 (12.2%) in children and in the wildlife population *Cryptosporidium* spp. was highest in elephants (25.8%) followed by buffalo (5.5%) and impala (4.3%). A total of 28 MZN-positive samples were retested using PCR and 14 (50%) were positive with rtPCR. It is possible for reports of false positive results from the MZN method, accounting to reports of higher

prevalences in some studies, as observed in East African countries such as Tanzania where 13/710 (1.4%) were suspected to have *Cryptosporidium* oocysts, however none of the samples had oocysts on re-examination by IFAT and by PCR (Chang'a *et al*., 2011). False positives can be due to several matrix factors, such as the presence of acid-fast, oocyst-sized organisms or objects in the faecal samples, leading to variations in the sensitivity and specificity of the MZN method in identifying *Cryptosporidium* oocysts (Changa'a *et al*., 2011). False positives can also be caused by differences in expertise of the investigators, with a possibility of human error in the MZN assay (Kange'the *et al*., 2012). Despite human error being a possibility for false positive MZN results, this is reduced in our case as we used expertise of highly trained laboratory technicians. On the other hand, good sensitivity (79.3%) and specificity (100%) of the MZN method have been reported (Muriuki *et al.,* 1997), while Rickard *et al*. (1999) found a specificity of only 40.6% and sensitivity of 52.0%.

In the current study *Cryptosporidium* infection was reported more in females (40/285) than in males (12/74). Females are affected by lower immunity and prone to physiological stressors especially during pregnancy and lactation. Management of pregnant/lactating females together with neonates which are known to have high rates of shedding may increase the risk of females getting infected. Sex-related variations have previously been reported in males (12.4 % in cattle and 14.5 % in buffaloes) than in females (9.1 % in cattle and 10.7 % in buffaloes) in Egypt (Ibrahim *et al*., 2016), sheep in Pakistan males were reported to be less affected 17.03% than females 18.80% (Khan *et al*., 2022), cattle in Nigeria males also less affected 14/84 than females 17/110 (Maikai *et al.,* 2011), these reported findings were obtained using microscopic technique. The present findings are, however, consistent with studies in India (Hingole *et al.*, 2017; Maurya *et al.,* 2013) and Nigeria (Maikai *et al*., 2011), where statistically insignificant differences in infection between males and females were reported. The inconclusive insignificant sex impact on the prevalence of *Cryptosporidium* infection was also observed in studies on sheep and goats in Kuwait (Majeed *et al*., 2018), Ethiopian lambs and goat kids (Wegayehu *et al*., 2016), and goat kids in India (Dixit *et al*., 2019).

Seasonal dynamics in the current study showed a higher occurrence during wet-summer season than the dry-winter period, consistent with other studies in Sub-Saharan Africa, in which seasonal peaks of cryptosporidiosis were documented during the rainy season (Ojuromi and Ashafa, 2018; Morse *et al*., 2007; Tumwine *et al*., 2003; Nchito *et al*., 1998). The increased risk of *Cryptosporidium* infection during summer may be attributed to factors such high humidity and rainfall, and therefore increased survival of oocysts in wet environments (Berhanu *et al*., 2022; Bouzid *et al*., 2018; Diaz *et al*., 2018; Maurya *et al*., 2013). Becher *et al*. (2004), in contrast, demonstrated that the infection was not affected by the time of year. Other studies reported similar seasonality like the present study: Samra et al. (2013) reported a lower prevalence in winter (dry season) and Moodley *et al*. (1991) reported high frequency of infection during the rainy season (summer). The current findings thus emphasize the potential impact of climatic conditions, especially summer rainfall, on *Cryptosporidium* prevalence in ruminants.

The questionnaire survey elucidated that most respondents, predominantly male farm proprietors, typify a specific demographic profile within the local farming community in Gauteng Province, which is in concordance with that of Jiyana et al. (2021). The present finding strengthens the assertions of Olaogun et al. (2023) that males are more suited to the physically demanding work of livestock farming than females. The prevalence of respondents in the age bracket of 31-50 years signifies a workforce in the prime years of agricultural activity, atypical of rural farming communities which are normally ravaged by rural-urban migration of the working age population (Idamokoro et al., 2019; Tada et al., 2012). The various demographic factors of the respondent's influence the farmers decision making in animal health and management practices. The results reporting these factors thus provide insights into the potential risk factors and transmission dynamics of cryptosporidiosis in livestock populations. Varied educational backgrounds were observed, with a preponderance possessing secondary education, followed by tertiary education, underscoring the necessity for multifaceted awareness campaigns tailored to diverse educational strata. Most farmers had more than 21-40 years livestock rearing experience, signifying a wealth of experience and deep-rooted connection to the farming lifestyle.

Insight into farm-related practices revealed the dominance of livestock farming activities, encompassing three or more animal species, and an extensive duration of farming engagement. The current findings support the assertion by Jiyana et al. (2021) that farming communities in Gauteng Province consider livestock farming as a full-time job that needs complete dedication. Bonsmara and Brahman breeds dominated, suggesting an alignment with local environmental adaptability (Webb et al., 2018). Most farmers reported good body condition scores and average stocking density, implying prudent livestock health management practices within the community, most likely attributed to the numerous years of experience and full-time nature of farming engagement.

Many respondents (7/12) used boreholes and wells as sources of drinking water for livestock which may increase the risk of infection. Notably, *Cryptosporidium* is not only confined to surface water sources but frequently occurs in groundwater sources, making use of potable groundwater a latent animal and public health concern (Chique et al., 2020). Farm hygiene practices revealed a range of approaches, with some farmers acknowledging average hygiene levels and variations in the frequency of manure removal. The diversity in manure disposal practices reported in the current study, such as reuse, dumping in surrounding areas, or near water sources are high risk practices (Vermeulen et al., 2017), underscoring the need for promoting standardized hygiene practices to mitigate the risk of *Cryptosporidium* transmission. The questionnaire survey highlighted concerning gaps in knowledge among farmers regarding cryptosporidiosis. Lack of awareness poses significant risks to both humans and animals, especially children in the area as they are frequently exposed to the animals (Khan et al., 2019). Factors such as farmers not frequently cleaning their hands, not cleaning the manure frequently and sharing a water source with the animals were highlighted as significant risk factors for possible zoonotic transmission of the disease. This remains a plausible concern, emphasizing the need for targeted educational interventions to enhance farmer awareness and implement preventive measures.

There was no clear trend between frequency of *Cryptosporidium* infection on farms and the management practices. Absence of infection in some farms could just be due

to absence of oocysts circulating in the area, while higher infections in some farms can be attributed to already existing latent infections or from introduced animals, that slowly spreads to other animals in the housing units. However, the sampled farms are still few to observe a statistical trend with good power. Perhaps, further investigations with more farms and more animals will provide a clearer insight.

The study had limitations, including a relatively low number of farmers surveyed. Future research should consider more farms and higher number of animals and over all the four seasons for a number of years so as obtain more comprehensive data on seasonal variations. The present study hardly obtained qPCR-positive samples, probably due to low concentration of oocysts in the faecal samples, below the detection limit of the assay. Additional studies evaluating more locations over an extended period may discover infections and these need to be assessed using additional molecular markers and genotyping to understand *Cryptosporidium* diversity. Notwithstanding the limitations, this study provided valuable insights into the prevalence and factors influencing *Cryptosporidium* infection in the Rust de Winter region. The combination of microscopic and molecular approaches, along with questionnaire data, contributed to a holistic understanding of the epidemiology of cryptosporidiosis in the studied population. The seasonal variations observed underscore the complexity of *Cryptosporidium* epidemiology, necessitating delicate approaches to future research and public health interventions.

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CHAPTER 6: Conclusions and Recommendations

The prevalence of *Cryptosporidium* recorded in this study, although low, is probably underestimated because of the irregular oocyst-excretion of post-weaned and adult animals. Given that they might play a major role in the emergence of outbreaks of cryptosporidiosis in both human and domestic ruminant neonates, it is acceptable to see animals as possible risks to both animal productivity and human health. The choice of MZN staining for *Cryptosporidium* detection is a common approach, but caution is warranted due to its reported variations in sensitivity and specificity. To comprehend the dynamics of the infection's spread and to create efficient management strategies, it is critical to investigate the prevalence of *Cryptosporidium* in various geographic areas. To prevent and control the disease in both humans and cattle, it is also crucial to increase public awareness of risk factors, infection sources, and mechanisms of transmission. To ascertain prevalence and species allocation, more molecular epidemiology studies in different parts of the country are recommended.

APPENDIX I

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Humanities
Fakulteit Geesteswetenskappe
Lefapha la Bomotho

Humanities 100. Since 1919

8 August 2022

Dear Dr TA Seanego

Project Title:	Prevalence of Cryptosporidium infection and associated risk factors in ruminants in Gauteng Province, South Africa
Researcher:	Dr TA Seanego
Supervisor(s):	Dr MC Marufu
Department:	Veterinary Tropical Diseases
Reference number:	14169101 (HUM011/0522)
Degree:	Masters

Thank you for the application that was submitted for ethical consideration.

The application was ***conditionally*** **approved** by the **Research Ethics Committee** on 28 July 2022 due to the following:

1. Please clarify the inclusion and exclusion criteria, for example will only Gauteng farms be included?

2. Revise section 4 of the ethics application form to mention the questionnaire.

3. With regard to informed consent:
a. The participants should be informed about what the research entails, what is required of them, how much time would be needed to complete the questionnaire etc. before signing consent. Please provide an information form for the participants in addition to the informed consent form.
b. If the consent form is completed as "I" then the language of sentences such as the following should also be written in the first person: "the owner of the livestock's personal information will not be disclosed".

4. The letterhead on the consent form is the "Animal ethics committee". Is this correct, should the document not be presented on a UP departmental letterhead?

Please note that data collection may not commence. Once the outstanding documentation and sufficient clarification is submitted, full ethical clearance will be granted. To facilitate the administrative process, please log onto the Peoplesoft ethics platform and select the 'Docs due Conditional approval' tab to upload a cover letter, addressing each of the above issues, together with any supporting/ outstanding documents.

Sincerely,

Prof Karen Harris
Chair: Research Ethics Committee
Faculty of Humanities
UNIVERSITY OF PRETORIA
e-mail: tracey.andrew@up.ac.za

Room 7-27, Humanities Building, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa
Tel +27 (0)12 420 4853| Fax +27 (0)12 420 4501|Email pghumanities@up.ac.za | www.up.ac.za/faculty-of-humanities

APPENDIX II

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

10 May 2022

CONDITIONALLY APPROVAL

Ethics Reference No	**REC112-21**
Protocol Title	**Prevalence of Cryptosporidium infection and associated risk factors in ruminants in Gauteng Province, South Africa**
Principal Investigator	**Dr TA Seanego**
Supervisors	**Dr MC Marufu**

Dear Dr TA Seanego,

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC112-21) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Conditionally approved (pending obtaining ALL other relevant approvals).

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

Room 6-6, Arnold Theiler Building
University of Pretoria, Faculty of Veterinary Science
Private Bag X04, Onderstepoort, 0110, South Africa
Tel +27 (0)12 529 8390
Email marie.watson-kriek@up.ac.za
www.up.ac.za

APPENDIX III

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

22 June 2022

Approval Certificate
New Application

AEC Reference No.:	REC112-21
Title:	Prevalence of Cryptosporidium infection and associated risk factors in ruminants in Gauteng Province, South Africa
Researcher:	Dr TA Seanego
Student's Supervisor:	Dr MC Marufu

Dear Dr TA Seanego,

The **New Application** as supported by documents received between 2021-10-08 and 2022-05-30 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-05-30.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Number
Cattle - Various breeds	120
Goats - Various breeds	120
Sheep - Various breeds	120
Samples	Number
Bovine - Faeces (Samples from live animals)	120
Caprine - Faeces (Samples from live animals)	120
Ovine - Faeces (Samples from live animals)	120

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-06-06.
3. Please remember to use your protocol number (REC112-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

APPENDIX IV

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

03 July 2023

Approval Certificate
Annual Renewal
(EXT1)

AEC Reference No.: REC112-21
Line 1
Title: Prevalence of Cryptosporidium infection and associated risk factors in ruminants in Gauteng Province, South Africa
Researcher: Dr TA Seanego
Student's Supervisor: Dr MC Marufu

Dear Dr TA Seanego,

The **Annual Renewal** as supported by documents received between 2023-05-26 and 2023-06-26 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2023-06-26.

Please note the following about your ethics approval:

1. The use of species is approved:

Species	Approved
Cattle - Various breeds	120
Goats - Various breeds	120
Sheep - Various breeds	120
Samples	Approved
Bovine - Faeces – (Samples from live animals)	120
Caprine - Faeces - (Samples from live animals)	120
Ovine - Faeces (Samples from live animals)	120

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2024-07-03.
3. Please remember to use your protocol number (REC112-21) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. All incidents must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

APPENDIX V

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development Private Bag X138, Pretoria 0001
Enquiries: Ms Marna Laing • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: MarnaL@Dalrrd.gov.za
Reference: 12/11/1/1/6 (2099MVA)

Dr Tebogo Atlivia Seanego
1st street, Old Industrial area
Potgietersrus
Zebediela Road
Mokopane
0601
Email: chris.marufu@uo.ac.za
Tel: 0737350932

Dear Dr Seanego/ Dr Marufu

PERMIT TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your application dated 2021/08/19 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. The study is approved as per the application form dated 2021/08/19 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to MarnaL@Dalrrd.gov.za;
2. No part of the study may begin until the valid ethical approval has been obtained from the relevant South African authority;
3. Samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);
4. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to MarnaL@Dalrrd.gov.za;
5. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;

6. All bio-safety and biosecurity protocols as outlined in the application must be followed.
7. It is the responsibility of the researcher and laboratory/facility manager to ensure that the human health and safety aspects of this study are adequately addressed;
8. Only a waste disposal company registered for the disposal of biohazardous waste may be used for the removal of all potentially infectious waste from the research project;
9. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes. A dispensation application may be considered by the Director Animal Health in the event that any of the above is to be stored or distributed;
10. Only cattle, sheep and goats from the areas as specified in the application and with valid State Vet letters may be used for sampling. It is further the responsibility of the researcher to consult with the local state veterinarian responsible for the state veterinary area to ensure the area of origin is not under veterinary restriction and to determine if veterinary movement permits are required for transportation prior to the movement of each batch of samples;

Title of research/study: Prevalence of Cryptosporidium infection and associated risk factors in ruminants in Gauteng Province, South Africa.
Researcher: Dr Tebogo Atlivia Seanego
Institution: University of Pretoria, Department of Veterinary Tropical Diseases, Soutpan Road (M35), Onderstepoort, 0110
Permit Expiry date: 16 February 2025
Our ref Number: 12/11/1/1/6 (2099MVA)
Your ref:

Kind regards,

DR MPHO MAJA

DIRECTOR: ANIMAL HEALTH

Date: 2022 -02- 17

APPENDIX VI

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA
Faculty of Veterinary Science

Consent form for farmers to participate in the study
Prevalence of Cryptosporidium infection and associated risk factors in ruminants in Gauteng Province, South Africa

Introduction
This research study is being conducted by Dr. Tebogo Seanego registered as a Masters student at the University of Pretoria, South Africa, to determine the prevalence and associated risk factors of cryptosporidiosis in ruminants in Gauteng Province, South Africa.

Procedure
The participants will be asked to respond to closed and open-ended questions prepared on a questionnaire. The interview will take at most 15 minutes per individual farmer. The questions will include details about farmer demographics, ruminant herd/flock demographics, knowledge about the occurrence of cryptosporidiosis and risk factors.

Risks
There are no risks for participation in this study.

Benefits
There will be no monetary benefits from participation in this study. The information that will be obtained during the study on
Prevalence of *Cryptosporidium* infection and associated risk factors in ruminants in Gauteng Province, South Africa will equip the farmer with knowledge about cryptosporidiosis, transmission, and prevention and enable the formulation of disease intervention strategies.

Participation
Participation of respondents in this study is voluntary. The participants may refuse to participate or withdraw at any time during the study. However, non-participation will disadvantage the farming community in that the results obtained from the study will not be a true representation of the data in the study area. A misrepresentation of the results will affect the true representation of associated risk factors of cryptosporidiosis and affect possible future intervention from possible assistance from relevant stakeholders.

Confidentiality
All the information will remain confidential. The data will be recorded as group data with no identifying information and data will be destroyed should participants withdraw from the study. The data obtained from the participants will be secured in the Institutional Repository for a period of 15 years. Only the Supervisors, the researcher and other persons directly involved in the research will have access to the information.

Access to the researcher
If participants have enquiries or wish to withdraw from the study, they may contact Dr. Tebogo Seanego at 0790808978

Location ..... Date .....

Signature of Participant..... Signature of Researcher.....

APPENDIX VII

Class	Cattle	Sheep	Goats	Llama	Other Specify ……
Number					
Rank					

10. What are your sources of income? (Rank 1 as most important source)

Source	Rank
Livestock	
Crop	
Old age pension	
Salary/wages	
Other (specify)…….	
Not applicable	

SECTION B (CATTLE HERD INFORMATION)

11. What breed of cattle do you keep? (Rank 1-5 based on number).

Type	**Nguni**	**Bonsmara**	**Brahman**	**Nondescript**	**Other Specify**
Rank					

12. What are the sexes of your cattle: (Rank 1-3 based on number)

Sex	**Male**	**Castrated male**	**Female**
Number			
Rank			

13. What is the body condition score of your herd (on a 5 point scale)?**:**

☐ **Poor** ☐ **Average** ☐ **Good**

14. Do you have calving pens (space for giving birth): **Yes** ☐ **No** ☐

15. Do you have restraining equipment: **Yes** ☐ **No** ☐

16. Do you have weight estimation equipment: **Yes** ☐ **No** ☐

17. Which system of management do you adopt in your farm:

Rural Household/Backyard ☐ **Extensive** ☐ **Semi-Intensive** ☐ **Intensive** ☐

18. What is your cattle's major source of water? (Rank 1-5 based on importance)

Water sources	**Stream/Flowing River**	**Public Water**	**Well**	**Borehole**	**Other Specify** .....
Rank					

19. What is your major source of cattle feed? (Rank 1-4 based on importance).

Feed sources	**Concentrate**	**Grazing grass**	**Mixed Feeding**	**Other Specify** .....
Rank				

SECTION C (RISK FACTORS AND AWARENESS)

1. Do you ensure that your newborn animals get colostrum within the first 24hrs of life?

Yes ☐ **No** ☐

2. How would you rate your stocking density? **Very low** ☐ **Low** ☐ **Average** ☐ **High** ☐ **Very high** ☐

3. What is the level of hygiene at your farm? **Very low** ☐ **Low** ☐ **Average** ☐ **High** ☐ **Very high** ☐

4. How frequently do you clean/remove manure?

Daily ☐ **Weekly** ☐ **Fortnight** ☐ **Never** ☐ **Others specify……**

5. Where do you dump your waste?

Near the water source ☐ **Municipality Dumping site** ☐ **Other** ☐ **Specify……..**

What system do you use to handle your animals if there are sick ones or young ones involved? **Sick 1st** ☐ **Healthy 1st** ☐ **/ Adults 1st** ☐ **Young 1st** ☐

7. Are you aware of Cryptosporidiosis? **Yes** ☐ **No** ☐ **Maybe** ☐
8. What was the source of training? **……**
9. If you are aware of it, what is the local name for Cryptosporidiosis?**……**
10. What clinical signs do animals exhibit when infected?

Diarrhea: Yes ☐ **No** ☐ **Maybe** ☐

Vomiting: Yes ☐ No ☐ **Maybe** ☐

Anorexia: Yes ☐ **No** ☐ **Maybe** ☐

Poor body condition: Yes ☐ **No** ☐ **Maybe** ☐

Blisters: Yes ☐ **No** ☐ **Maybe** ☐

Bloat: Yes ☐ **No** ☐ **Maybe** ☐

11. What is the most likely mode of transmission?

Contact ☐ **Aerosol** ☐ **Fecal-oral route** ☐

SECTION D (ZOONOTIC IMPLICATIONS)

1. Can cryptosporidium affect people? **Yes** ☐ **No** ☐
2. Can infected people spread cryptosporidiosis to animals? **Yes** ☐ **No** ☐
3. What may increase the spread of cryptosporidiosis to animals?
4. If yes, what do you think is the most correct mode of transmission?

Contact ☐ **Aerosol** ☐ **Fecal-oral route** ☐

5. Do you share your water sources with your animals? **Yes** ☐ **No** ☐
6. If yes, please state what you use the water for i.e food preparation, cleaning, washing etc.:

……..

7. How frequently do you handle your animals?

Daily ☐ **Weekly** ☐ **Fortnightly** ☐ **Other** ☐

8. How frequently do you wash your hands before and after handling your animals?

Once ☐ **In Between animals** ☐ **Never** ☐ **Other Specify** ☐ **……**

What is the frequency of children's exposure to animals?

Minimal contact ☐ **Moderate** ☐ **Frequent** ☐

THANK YOU FOR YOUR PARTICIPATION