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MOLECULAR DETECTION AND IDENTIFICATION OF *Cryptosporidium* Species ISOLATED FROM HUMAN AND ANIMAL SOURCES IN LIMPOPO AND GAUTENG PROVINCES

A Dissertation Submitted in Fulfilment of the Requirements for the award of a Master of Science (M.Sc.) Degree in Microbiology

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Declaration

I, Hasani Alone Hlungwani, hereby declare that the research contained in this dissertation is my original work and has not been submitted for any degree at any other University or institution. The dissertation does not contain other person's writing unless specifically acknowledged and referenced accordingly.

Signature (Student): ___________________________________ Date: ___________________

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Dedication

This study is dedicated to my late father, Risimati Joel Hlungwani and my mother Mthabini Annah Chauke. Thank you so much for the love and support you gave me.

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Research output

Publications

Hlungwani H.A., Mbati P.A. and Samie A. Prevalence and risk factors of *Cryptosporidium* species among domestic animals in rural communities in Northern South Africa. It has been reviewed (MS No: 299/161) and is to be accepted for publication in Tropical Biomedicine.

Hlungwani H.A., Mbati P.A. and Samie A. Molecular detection and identification of *Cryptosporidium* species isolated from human and animal sources in Limpopo and Gauteng provinces. To be submitted to Veterinary Parasitology Journal.

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Table of contents

Contents **Page**

Declaration i

Dedication ii

Research output iii

Publications .... *iii*

Acknowledgement iv

Table of contents v

List of Tables viii

List of Figures ix

Abbreviations x

Abstract xii

CHAPTER 1: INTRODUCTION, RATIONALE AND OBJECTIVES 1

1.1 INTRODUCTION 1

1.2 STUDY RATIONALE 3

1.3 HYPOTHESIS 4

1.4 OBJECTIVES OF THE STUDY 4

1.4.1 Main objective *4*

1.4.2 Specific objectives *4*

1.5 RESEARCH QUESTION 4

CHAPTER 2: LITERATURE REVIEW 5

2.1 Classification of *Cryptosporidium* spp 5

2.2 Life Cycle of *Cryptosporidium* Spp. 7

2.3 Pathogenesis of *Cryptosporidium* Spp. 9

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2.4 Epidemiology of *Cryptosporidium* Spp. **11**

2.5 Transmission of *Cryptosporidium* Spp. **12**

2.6 Symptoms of Cryptosporidiosis **13**

2.7 Diagnosis of Cryptosporidiosis **14**

2.8 Treatment of Cryptosporidiosis **15**

CHAPTER 3: MATERIALS AND METHODS **18**

3.1 ETHICAL CLEARANCE **18**

3.2 STUDY SITE AND SAMPLE COLLECTION **18**

3.3 MICROSCOPY **19**

3.4 ELISA FOR THE DETECTION OF *CRYPTOSPORIDIUM* FROM STOOLS **20**

3.5 MOLECULAR CHARACTERIZATION OF *CRYPTOSPORIDIUM* SPP. **20**

3.5.1 Extraction of genomic DNA from stool samples *20*

3.5.2 Detection of Cryptosporidium by conventional PCR *21*

3.5.3 Detection of Cryptosporidium spp. by Real-Time PCR using SYBR green *22*

3.5.4 Detection of Cryptosporidium by Real-Time PCR using probes *22*

3.5.5 DNA sequences analysis *25*

3.5.6 Statistical analysis *26*

CHAPTER 4: RESULTS **27**

4.1 MICROSCOPIC DETECTION OF *CRYPTOSPORIDIUM* **27**

4.2 IMMUNOLOGICAL DETECTION OF *CRYPTOSPORIDIUM* **28**

4.3 GENETIC DIVERSITY OF *CRYPTOSPORIDIUM* IN HUMANS **29**

4.3.1 Demographic characteristics of the study population *29*

4.3.2 Comparison of selected diagnostic methods for identification of Cryptosporidium in human stool samples *30*

4.3.3 Distribution of human Cryptosporidium in rural and urban areas *31*

4.3.4 Occurrence of Cryptosporidium in humans according to their geographical region *32*

4.3.5 Cryptosporidium detection by real-time PCR. *33*

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4.3.6 Phylogenetic and evolutionary distances analysis from human samples 36

4.4 GENETIC DIVERSITY OF *CRYPTOSPORIDIUM* IN ANIMALS **38**

4.4.1 Demographic characteristics of animal study population 38

4.4.2 The possible household risk factors that predispose animals to infection with Cryptosporidium spp. 39

4.4.3 Demographic characteristics of the participants who owned animals in the households 40

4.4.4 Demographic characteristics of the participants and the infection risk factors.........42

4.4.5 Occurrence of Cryptosporidium spp. in goats and cattle 45

4.4.6 Occurrence of Cryptosporidium isolates based on the consistency of animals stool .46

4.4.7 Amplification of 18S rRNA gene by conventional PCR 47

4.4.8 Phylogenetic analysis from animal samples 48

4.4.9 Evolutionary distances analysis of the sequences from animal samples 49

CHAPTER 5: DISCUSSION, CONCLUSION, RECOMMENDATIONS AND LIMITATIONS **50**

5.1 DISCUSSION **50**

5.2 CONCLUSION **58**

5.3 RECOMMENDATIONS **58**

5.4 LIMITATIONS OF THE STUDY **59**

REFERENCES **60**

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List of Tables

Tables **Page**

Table 2.1: *Cryptosporidium* species commonly detected in humans and animals as reported in the literature 6

Table 3.1: Primers used for the detection of *Cryptosporidium* with the specific gene target 24

Table 4.1: Demographic characteristics of the study population 29

Table 4.2: Comparison of selected diagnostic methods for identification of *Cryptosporidium* in human stool samples 30

Table 4.3: Occurrence of human *Cryptosporidium* spp. in the study sites based on stool type and gender of the participant 32

Table 4.4: Demographic characteristics of animal sample population used in the study 38

Table 4.5: The possible household risk factors that predispose animals to infection with *Cryptosporidium* 39

Table 4.6: Demographic characteristics of the participants who owned animals in the households 41

Table 4.7: Demographic characteristics of the participants and the infection risk factors 43

Table 4.8: Occurrence of *Cryptosporidium* in animals according to diagnostic technique used 44

Table 4.9: Occurrence of *Cryptosporidium* spp. in goats and cattle 45

Table 4.10: Occurrence of *Cryptosporidium* isolates based on the consistency of animal stool 46

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List of Figures

Figures **Page**

Figure 2.1: Schematic representation of Cryptosporidium parvum life cycle (Bouzid et al., 2013). 8

Figure 2.2: Schematic representation of the pathogenesis of C. parvum (Bouzid et al., 2013). 10

Figure 3.1: Map of South Africa showing the study sites (www.mapsofworld.com). 19

Figure 4.1: Cryptosporidium oocysts representation taken at 100X magnification on a light microscope from one of the samples tested. 27

Figure 4.2: Microassay plate showing some positive samples for Cryptosporidium oocyst by ELISA. 28

Figure 4.3: Prevalence of Cryptosporidium infections in rural and urban areas. 31

Figure 4.4: A 2% agarose gel picture showing the bands of the PCR product using 18S rRNA gene 33

Figure 4.5: A 2% agarose gel picture showing the bands of 18S SSU rRNA gene at 300bp 34

Figure 4.6: Sequence alignment of the 18SrRNA gene of Cryptosporidium from human stool samples using Bioedit package (Hall, 1999) 35

Figure 4.7: Molecular Phylogenetic analysis of Cryptosporidium spp. by Maximum Likelihood method 36

Figure 4.8: Estimates of Evolutionary Divergence between Sequences of the different isolates 37

Figure 4.9: An agarose gel showing the target bands of the 18S rRNA gene detection from conventional PCR. The product size was 553bp. 47

Figure 4.10: Molecular Phylogenetic analysis by Maximum Likelihood method of COWP gene 48

Figure 4.11: Estimates of Evolutionary Divergence between the COWP gene sequences of Cryptosporidium isolates from animals 49

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Abbreviations

Abbreviation	Meaning
>	= Greater than
<	= Less than
%	= Percentage
≥	= Greater or equal
°C	= Degrees Celsius
µl	= Micro liter
18S rRNA	= 18S ribosomal ribonucleic acid
AIDS	= Acquired Immunodeficiency Syndrome
bp	= Base pairs
C.	= *Cryptosporidium*
CDC	= Centres for disease control
COWP	= *Cryptosporidium* oocyst Wall Protein
DFA	= Direct fluorescent antibody
DNA	= Deoxyribonucleic Acid
dNTP	= deoxynucleotide triphosphate
EDTA	= Ethylene diamine tetraacetate
ELISA	= Enzyme-Linked Immunosorbent Assay
et al	= *Et al*ia (and others)
g	= Grams
HIV	= Human Immunodeficiency Virus
HPLC	= High Performance Liquid Chromatography
i.e.	= That is
IC	= Internal control
ICZN	= International Code for Zoological Nomenclature

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kb	= Kilobase
kDa	= Kilodalton
M	= Molarity
mM	= Mili-molar
NCBI	= National Center for Biotechnology Information
NTZ	= Nitazoxanide
O_2	= Oxygen
OD	= Optical density
PCR	= Polymerase Chain Reaction
qPCR	= Quantitative PCR
RFLP	= Restriction Fragment Length Polymorphism
Spp	= Species
SPSS	= Statistical Package for the Social Sciences
SSU rRNA	= Small subunit ribosomal ribonucleic acid
Taq	= an enzyme obtained from – *Thermus aquaticus*
USA	= United State of America
UV	= Utraviolet
V	= Volts
v/v	=Volume per Volume
w/v	= Weight per Volume
WHO	=World Health Organization
χ^2	= Chi-square

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Abstract

Background: Diarrheal diseases constitute an important problem among children but also among HIV positive patients particularly in developing countries such as South Africa. *Cryptosporidium* infect humans and has been shown to be an important cause of infection among different types of animals. Because of its small size, *Cryptosporidium* can easily go through the water purification system and can easily become a cause of an epidemic. Previous studies have shown that *Cryptosporidium* is an important cause of diarrhea in Limpopo Province. However, very few studies have been conducted on the genetic diversity of these organisms in the region. Therefore, the aim of this study was to detect and identify the genetic diversity of *Cryptosporidium* species from humans and animals in Giyani situated in the northern part of South Africa and Pretoria situated in the central part of the country.

Methodology: A total of 560 samples were collected from human and animals and were all screened by microscopy using modified Ziehl-Neelsen staining technique. All the samples were tested by Enzyme-Linked Immunosorbent Assay (ELISA) using the *Cryptosporidium* II kits from Techlab, Virginia, USA. Positive samples from microscopy and ELISA were examined by different PCR protocols including conventional PCR for amplification of *Cryptosporidium* oocyst wall protein (COWP) region; Real-time PCR employing SYBR Green detection format for amplification of 18S rRNA region; Real-time PCR employing Hydrolysis probes detection format for amplification of SSU rRNA region; Real-time PCR specific for amplification of *C. hominis* region and *C. parvum* region. Positive samples from real-time PCR that gave clear bands on gel electrophoresis were sent for sequencing. The sequences were analysed using Staden package software to edit the nucleotides, Bioedit and MEGA6 software were used to align sequences and draw phylogenetic trees. The SPSS software was used for statistical analysis.

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Results: The overall prevalence of *Cryptosporidium* as detected by ELISA method from the samples collected from humans was 41.2% (239/580). The prevalence was higher from the rural area 73.0% (159/218) compared to the urban area 22.1% (80/362) and the difference was statistically significant ($\chi^2 = 145.1$; p = 0.0001). Due to the limited amount of samples, only 134 ELISA-positive samples were tested using real-time PCR. Of these samples, 35.8% (48/134) tested positive. Of 48 real-time positive samples 25 were successfully sequenced and two different species (*C. hominis* and *C. muris*) were identified. Of all the sequences obtained, one (4.0%) was *C. muris* and 20 (80%) were *C. hominis* isolated from rural area, whereas 16.0% (4/25) were also *C. hominis* isolated from samples obtained from urban area. *Cryptosporidium* was not associated with diarrhea in the present study.

A total of 85 samples were collected from animals (52 from cattle and 33 from goats) and of these 4 (4.7%) were positive by microscopy and ELISA. All these samples were non diarrheal. Conventional PCR also detected a similar number. Of these 4 positive samples, 1 was from a male goat, while the 3 others were obtained from female adult goats.

Real-time PCR detected 56.5% (48/85) positive samples. Only 12 of the 85 animal samples were diarrheal and of these 4 were positive for *Cryptosporidium*. The prevalence of *Cryptosporidium* infection was higher 68.4% (13/19) in male animals compared to female animals 53.0% (35/66). The prevalence rates in cattle and goats were 55.8% (29/52) and 60.6% (20/33) respectively.

Of 48 real-time positive samples from animals, 12 (25.0%) were successfully sequenced and two species (*C. parvum* and *C. andersoni*) were identified. Of these 6 were from cattle and the other 6 were from goats. Out of the 12 samples 10 (83%) were *C. parvum* while 2 (17%) were *C. andersoni*. Of the two *C. andersoni*, one was from a goat and one was from a cow. Of the 10 *C. parvum*, 5 were from goats and 5 were from cattle.

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In conclusion, microscopy remains the low sensitive tool for the detection of *Cryptosporidium* while real time PCR appeared to be far much more sensitive by detecting more samples than all the three other methods combined. Closer to the real time PCR was ELISA that detected also more samples compared to conventional PCR and microscopy.

The present study identified *C. muris* from humans' samples in our area for the first time. However, *C. hominis* remains the dominant species that infects humans in our area. *Cryptosporidium* species was mostly found in samples from asymptomatic individuals. In animals, *C. parvum* was the most commonly isolated organism while *C. andersoni* was identified in our region for the first time as well and occurred in both goats and cattle.

Populations in the affected areas need to be made aware of the infections so that care should be taken to avoid the spread of infection in water sources or in immunocompromised individuals.

Key words: *Cryptosporidium*; detection; identification; isolates; ELISA; PCR

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CHAPTER 1: INTRODUCTION, RATIONALE AND OBJECTIVES

1.1 INTRODUCTION

Cryptosporidium is an enteric protozoan parasite, which infects a wide range of vertebrate hosts including humans and animals (Ericsson *et al.,* 2001), causing disease called cryptosporidiosis (Carey, 2004). *Cryptosporidium* is generally recognised by its hardiness-tiny resistant form called oocyst, which is responsible for causing infections once ingested by a susceptible host. The oocyst is a stage which plays a role in the dispersal, survival and infectivity of *Cryptosporidium*, which also facilitates the detection and identification of this parasite. This infectious form generally survives for a very long time under a range of environmental pressures including freezing, desiccation, and water treatment processes and in physical environments such as faeces and various water types commonly associated with oocysts (Robertson *et al.,* 1992; Alum *et al.,* 2014). It appears nearly spherical, measuring about 4 to 6 µm in diameter and has unclear internal structures. Furthermore, these oocysts are shed in millions in stool of infected humans or animals. Using a routine diagnostic tool such as microscopy in laboratories, one can easily identify *Cryptosporidium* oocysts in an infected specimen (Chalmers and Chalmers, 2012; Ghaffari, and Kalantari, 2014).

It has been recorded that *Cryptosporidium* was first described by Ernest Edward Tyzzer in 1907, and it was not recognized as a pathogen until an outbreak of diarrhea in a turkey flock in 1955. Carey, (2004) further described that infection was mainly observed in calves and

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lambs until 1976, when two cases of human cryptosporidiosis were reported. However, the parasite was not recognized as an agent causing a waterborne disease in immunocompetent humans until 1987 (Rose, 1988). To date, only one moderately effective drug (nitazoxanide) is available for the treatment of cryptosporidiosis (Miyamoto and Eckmann, 2014). Nitazoxanide has partial efficacy in immunocompromised individuals. Resolution of cryptosporidiosis can be maintained with effective Highly Active Antiretroviral Therapy (HAART) (Abd-Ella, 2014).

Like many important health pathogens, *Cryptosporidium* consists of several gene loci commonly used as targets for its scientific study, and they include: 18S rRNA gene which is highly polymorphic within the genus and is useful as a target for the identification and differentiation of *Cryptosporidium* species and genotypes (Xiao *et al.,* 1999); *Cryptosporidium* oocyst Wall Protein (COWP) gene which is a single copy gene encoding a major constituent of the inner layer of the *Cryptosporidium* oocysts wall protein (Spano, *et al.,* 1997); and heat shock protein 70 kDa (HSP70) gene which is a good target for sub-typing and multi-locus study of *Cryptosporidium* isolates, because of its high level of heterogeneity spread over the entire sequence of a variety of *Cryptosporidium* isolates from human and animal hosts (Sulaiman *et al.,* 2001). These genes are best identified by Polymerase Chain Reaction (PCR), the mostly used molecular technique in the diagnostic settings.

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1.2 STUDY RATIONALE

Cryptosporidium species causes infections in humans and animals, and the infection is life-threatening in children and immunocompromised individuals worldwide. It was estimated that about 8 – 19% of diarrheal diseases were attributed to *Cryptosporidium* in developing countries (Gatei *et al.,* 2006). Even though the number of valid species remains constant, some new isolates of *Cryptosporidium* are continuously discovered as studies are being conducted in other continents (Ghaffari, 2014). Infections with the human specific *C. hominis* or the zoonotic *C. parvum* were suggested to be the cause of human cryptosporidiosis (Ehsan et al., 2015), with some other species being detected in humans less frequently (Elwin et al., 2012).

Large genetic diversity of *Cryptosporidium* was observed in 18S rRNA gene and it depends on infection pressure and transmission intensity in the susceptible hosts (Ruecker *et al*., 2012). Infections depends on socioeconomic and educational status in the community (Region *et al*., 2016). Therefore, identification of the diversity of *Cryptosporidium* in the community would improves our understanding of parasite and adequate diagnosis and treatment of the disease it causes (Ghazy *et al*., 2015). However, very few molecular studies have been conducted to determine the current frequency and diversity of *Cryptosporidium* genotypes of human and animal populations in South Africa. The available molecular data was restricted to primary school children and hospitalized patients' survey in the Venda region, South Africa (Samie *et al*., 2006). Therefore, this study focused on the detection and identification of *Cryptosporidium* species in the rural area of Limpopo Province and urban area in Gauteng Province in order to determine the genetic diversity of this parasite and verify its distribution in humans and animals.

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1.3 HYPOTHESIS

There are unknown *Cryptosporidium* species infecting humans and animals in South Africa.

1.4 OBJECTIVES OF THE STUDY

1.4.1 Main objective

The aim of this study was to isolate and identify genotypes of *Cryptosporidium* from humans and animals in the Limpopo and Gauteng Provinces.

1.4.2 Specific objectives

a) To identify *Cryptosporidium* spp. and determine their occurrence in humans and domestic animals from Limpopo and Gauteng Provinces.

b) To determine the distribution and host specificity of *Cryptosporidium* spp. between humans and animals.

c) To determine the sequence similarities of *Cryptosporidium* isolates detected using the similar gene.

1.5 RESEARCH QUESTION

a) Which common *Cryptosporidium* species are dominating in humans and animals?

b) Could a new *Cryptosporidium* species be discovered in South Africa?

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CHAPTER 2: LITERATURE REVIEW

2.1 Classification of *Cryptosporidium* spp.

For the isolates to receive taxonomic status, sufficient morphological, biological, and molecular data are required and names have to comply with the rules of the International Code for Zoological Nomenclature (ICZN) (Bouzid *et al.,* 2013). Historically, the taxonomy of *Cryptosporidium* has been based on morphologic characteristics, infectious niche, and strict host-specificity for species designation. This approach resulted in a variety of species being described (Xiao *et al.,* 2004), and although *Cryptosporidium* is still considered to be relatively host specific, cross-transmission studies coupled with molecular epidemiological investigations have revealed that transmission of some species and genotypes between different hosts can occur.

Morphology and cross-transmission studies remain the fundamental basis for *Cryptosporidium* taxonomy (Xiao *et al.,* 2004) even though they do not allow the clear differentiation of the full range of *Cryptosporidium* spp. (Fall *et al.,* 2003). Among 26 valid *Cryptosporidium* species currently described, at least 13 have been found in humans (with the most important for public health appearing to be *C. hominis, C. parvum, C. cuniculus, C. canis, C. felis,* and *C. meleagridis*). There is an increasing number of isolates being identified in humans and other animals in addition to more than 60 *Cryptosporidium* spp that were already described, although it is uncertain if they are pathogenic or not (Bouzid *et al.,* 2013).

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There are some *Cryptosporidium* species that were often detected in a particular susceptible host. Table 2.1 below summarises some of the common species isolated from humans and animals in other studies, with some of the references.

Table 2.1: *Cryptosporidium* species commonly detected in humans and animals as reported in the literature

Susceptible Host	***Cryptosporidium* species in animals**	**Reference**
Humans	*C. hominis;*	(Hadfield *et al.,* 2011; Samie *et al.,* 2006)
	C. parvum	Alves *et al.,* 2003
	C. ubiquitum	Hadfield *et al.,* 2011
Cattle	*C. parvum*	Hunter and Thompson, 2005
	C. andersoni	Hadfield *et al.,* 2011
	C. bovis	Chalmers and Chalmers, 2012
Goats	*C. xiaomi*	(Hadfield *et al.,* 2011; Koinari *et al.,* 2014)
	C. hominis	Koinari *et al.,* 2014
Sheep	*C. xiaomi*	Hadfield *et al.,* 2011
	C. parvum	Koinari *et al.,* 2014
Chickens	*C. baileyi*	(Fayer, 2004; Current *et al.,* 1986)
	C. galli	(Chalmers and Chalmers, 2012)

Cryptosporidium parvum represents the species of most concern to human health, and could be identified from other species within the *Cryptosporidium* genus based on morphological characteristics and host niche (Bonnin *et al.,* 1996).

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The human genotype of *C. parvum* was elevated to its own species status in 2002 and renamed *C. hominis* (Morgan-Ryan *et al.,* 2002), while many other parasites previously described under the *C. parvum* have been elevated to species status.

Those isolates that lacked taxonomic status have not been elevated to species status, are now simply referred to as *Cryptosporidium* genotypes, and are typically identified by their host origin. Removing these genotypes from the designated *C. parvum* species has greatly simplified the taxonomy (Ruecker, 2009; Fayer, 2010). All species of *Cryptosporidium* are classified taxonomically within the family Cryptosporidiidae, suborder Eimeriorina, order Eucoccidiorida, subclass Coccidiasina and class Coccidia (Carey, 2004), phylum Apicomplexa, super-phylum Alveolata, kingdom Chromalveolata and domain Eukaryota. Studies are being conducted in order to characterise and group this genus, *Cryptosporidium* (Egyed, *et al.,* 2003; Carey, *et al.,* 2004).

2.2 Life Cycle of *Cryptosporidium* Spp.

Cryptosporidium species have a monoxenous life cycle completed within the gastrointestinal tract. During the whole cycle, the different forms of the oocysts are confined to the apical surfaces of the host cells. During the infective stage, an oocyst attaches to the apical surfaces by a poorly understood process (Barta, *et al.,* 2006), which is thought to be initiated by changes in temperature, pH, presence of bile salts and pancreatic enzymes (Reduker, *et al.,* 1985). The suture dissolves and four sporozoites excyst from each oocyst. The anterior end of the sporozoite adheres to the luminal surface of the epithelial cell of the infected tract. The sporozoites are subsequently surrounded by the microvilli, rendering them intracellular but extra cytoplasmic forming a protective membrane called parasitophorous vacuole where the endogenous life cycle of the parasite occurs (Tzipori, *et al.,* 2000).

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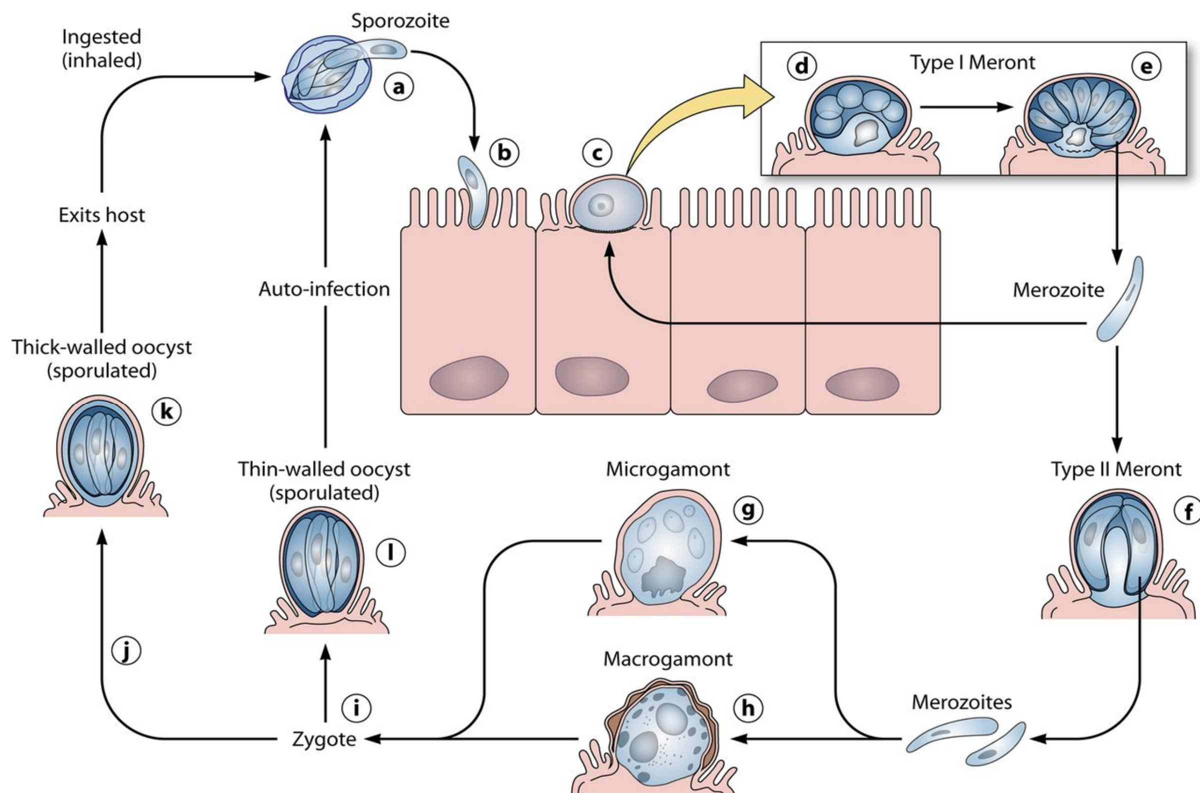

Figure 2.1: Schematic representation of *Cryptosporidium parvum* life cycle (Bouzid et al., 2013).

Within parasitophorous vacuole, sporozoites mature into trophozoites which then progress through asexual replication in 24 hours to form type I meronts that release merozoites. These merozoites infect new epithelial cells to either repeat asexual replication generating more type I meronts, or through an unknown process, progress through sexual development resulting in type II meronts. These merozoites, when released, attach again to the epithelial cell, but instead of developing into further meronts, they initiate gametogony. Individual merozoites produce either microgamonts or macrogamonts (Smith *et al.,* 1998). Each microgamont undertakes nuclear division and differentiates to form microgametes which, when released from the parasitophorous vacuole, locate and fertilize a unicellular macrogametocyte that has developed from a macrogamont. The product of fertilization, zygote, undergoes two asexual cycles of sporogony to produce an oocyst with either a thick wall or a thin wall, containing four sporozoites (Current, *et al.,* 1986).

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The thick-walled oocysts are released into the lumen of the intestine, are excreted from the host in the feces, and are immediately infective, allowing the spread of infection to other susceptible hosts (Smith *et al.,* 1998). In addition, *Cryptosporidium* is able to auto infect the same host. Autoinfection occurs through the thin-walled oocysts, which excyst once they are separated from the epithelium, and the cycle starts again (Current, *et al.,* 1986).

2.3 Pathogenesis of *Cryptosporidium* Spp.

Infection with *Cryptosporidium* begins when the ingested oocysts release sporozoites, which subsequently attach to and invade the intestinal epithelial cell. Attachment to and invasion of host cells are crucial primary events in pathogenesis. Once the parasite has gained entry, virulence proteins including CSL, GP900, p23/27, TRAP C1, gp40/45, cp47, and gp15/Cp17 (Brett *et al.,* 2003) can affect the host at any time during the life cycle from the time when the parasite enters the body until it is killed or completes the cycle and exits the host (Fayer *et al.,* 2009). This could result in direct or indirect injury to the host epithelial cells through the effect of inflammatory cells and cytokines recruited to the site of infection (Okhuysen, *et al.,* 2002).

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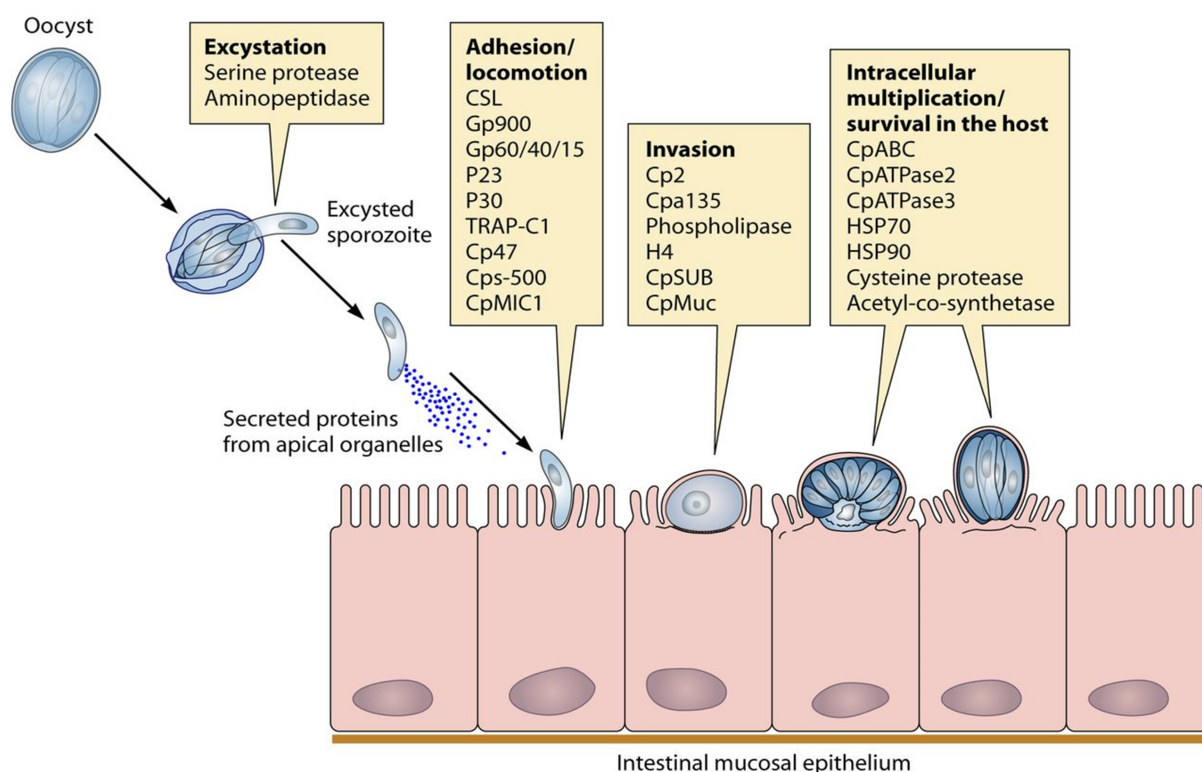

Figure 2.2: Schematic representation of the pathogenesis of *C. parvum* (Bouzid et al., 2013).

Cryptosporidium does not normally cause a systemic infection or penetrate deep tissue; rather, the parasite establishes itself in a membrane-bound compartment on the apical surface of the intestinal epithelium (Okhuysen *et al.,* 2002). However, it causes significant abnormalities in the absorptive and secretory functions of the gut, and both of these processes are regulated by the intestinal epithelial cells which are infected by *Cryptosporidium*. The mechanism by which *Cryptosporidium* infection causes diarrhea remains elusive, but it has been reported that transport defects and abnormalities in the barrier properties of the intestinal epithelium, contribute to *Cryptosporidium* diarrhea (Douglas, 1999). The diarrhea is typically non-inflammatory and is often profuse (Gargala, 2008).

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2.4 Epidemiology of *Cryptosporidium* Spp.

In the Venda region of South Africa, Samie, *et al.,* (2006) reported the high prevalence of *Cryptosporidium* infections and its implications in causing diarrhea. Real time PCR (qPCR) was used for initial screening to detect positive samples, while an 18S rRNA nested PCR followed by restriction fragment length polymorphism was used to determine the species genotype. From a total of 244 stool samples tested, 44 (18%) had *Cryptosporidium* with no significant difference between samples collected from patients attending hospitals 36/197 (18%) and the samples from primary schools 8/47 (17%). *C. hominis* (82%) was more common than *C. parvum* (18%). In an extended outbreak of cryptosporidiosis in China, four *Cryptosporidium* spp. and 6 *C. hominis* subtypes were isolated from 102 of 6,284 patients in three paediatric hospitals in the People's Republic of China. It was reported that cryptosporidiosis outbreak was identified retrospectively and that the outbreak lasted more than a year and affected 51.4% of patients in one hospital ward, where two *C. hominis* subtypes with different virulence were found (Feng *et al.,* 2012).

In a study in Malawi, DNA from 69 *Cryptosporidium*-positive human faecal samples were examined by multi-locus genetic analyses. From 43, 27 and 28 of the samples, the SSU rRNA, 70 kDa heat shock protein (HSP70) and 60 kDa glycoprotein (GP60) genes, respectively, were successfully PCR amplified (Peng *et al.,* 2003). Restriction analysis of the SSU PCR products showed that 41 of the 43 PCR positive samples had *C. hominis* and two had *C. parvum*. Sequence analysis of the HSP70 and GP60 gene confirmed the species identification by SSU rRNA PCR-RFLP analysis, but also revealed high intra-specific variations (Peng *et al.,* 2003). In another Malawian study, the incidence of cryptosporidiosis in children aged more than five years with diarrhea in an urban and rural hospital-based setting was examined (Morse *et al.,* 2007). A range of microscopic methods were used to

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determine the presence of *Cryptosporidium* spp. oocysts. Species determination was carried out by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of oocyst-extracted DNA using 18S rRNA and COWP gene loci. *Cryptosporidium* spp. oocysts were seen in 5.9% (50/848) of samples, of which 43 amplified by PCR-RFLP indicated the following species: *C. hominis, C. parvum, C. meleagridis, and C. andersoni* (Morse *et al.,* 2007).

2.5 Transmission of *Cryptosporidium* Spp.

The most common mode of transmission of *Cryptosporidium* species is faecal-oral route. It involves passing faecal particles carrying the oocyst from one host and introduced into the oral cavity of another host. The process of transmission may be simple or involve multiple steps. For instance, water that comes in contact with faeces and is then inadequately treated before drinking; food that has been prepared in the presence of faecal matter; disease vectors like houseflies, spreading contamination from inadequate faecal disposal; poor or absent cleaning after handling faeces or anything that has been in contact with it; and sexual practices that may involve oral contact with faeces. The faecal-oral route of transmission, from either humans or animals, depends on the *Cryptosporidium* species; for example, *C. hominis* has a human infection cycle while *C. parvum* also has susceptible animal hosts causing mainly gastrointestinal disease in young ruminants.

It is the parasite's ability to survive in the environment and its resistance to chlorine disinfection that support transmission via drinking and recreational waters, and other vehicles such as food (Chalmers, 2012). The other mode of transmission includes faecally contaminated material; through environmental contamination such as release of faeces, sewage or wastewater, or slurry in an open environment (often as overflow following heavy-

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rain events) (Aguirre *et al.,* 2016). *Cryptosporidium* can also be transmitted via inhalation of oocysts (Sponseller *et al.,* 2014). The symptoms associated with this route are respiratory and could be accompanied by mild diarrhea. This mode of transmission was reported for immunocompromised patients and children.

2.6 Symptoms of Cryptosporidiosis

Cryptosporidium has been recognized as a cause of gastrointestinal illness in both immunocompromised and immunocompetent people (Rossle and Latif 2013). Symptoms of cryptosporidiosis, which may resolve after 2 and 3 days include: self-limiting diarrhea (Rossle *et al*., 2013), although it may also be asymptomatic in immunocompetent individuals, whereas in immunocompromised people, children and young animals it manifests as mild to severe diarrhea that can be fatal (Omoruyi *et al*., 2014; Ravaszova *et al*., 2012); abdominal pain which has been reported in some humans; nausea, vomiting which is rare; low-grade fever and malaise (Bouzid *et al*., 2013; Moon *et al*., 2013; Widerström *et al*., 2014).

These symptoms may last for up to three weeks during which time apparent recovery may be followed by temporary recurrence. Occasionally, nonspecific symptoms such as myalgia, weakness, headache, and anorexia occur. Studies suggested an association with long-term health sequelae, such as reactive arthritis and post-infection irritable bowel syndrome (Hunter *et al.,* 2004). However, these classifications are not concrete and sometimes mixed infections are detected in patients, particularly in endemic areas with poor hygiene and sanitation practices (Rossle and Latif, 2013). In developing countries, where children are more susceptible to infection, cryptosporidiosis was suspected to be associated with successive impaired physical and cognitive development, even in the absence of diarrhea in early childhood (Rossle and Latif, 2013).

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2.7 Diagnosis of Cryptosporidiosis

The morphological features of *Cryptosporidium* oocysts, which are usually 4–6 µm in size (Omoruyi *et al*., 2014), are recognized through observation using microscopy after Ziehl Neelson staining method, and it is the convention in the diagnosis of cryptosporidiosis. However, this technique is laborious and less sensitive and thus prone to error (Bialek *et al.,* 2002; Cimino *et al*., 2015; Omoruyi *et al*., 2014). *Cryptosporidium* oocysts are quite tiny, and consequently can easily be mistaken in stool debris as an artifact. Also, they may be easily confused with other oocysts, such as those of *Cyclospora* species and yeast cells (Connelly *et al.,* 2008). This method would not differentiate between *Cryptosporidium* species oocysts because they similarly take up a red to pink colour as do other faecal components, which is the shortfall of this technique compared to others. However, it is cheap and affordable; hence resource-poor countries still rely on the technique as has been variously reported (Omoruyi *et al.,* 2014). Microscopy is often considered the diagnostic reference standard when samples are tested by a skilled microscopist (Elsafi *et al*., 2013).

A combination of Ziehl-Neelsen technique and either ELISA or PCR techniques was reported to be a "gold standard" as specificity and sensitivity could be very high, thus ensuring *Cryptosporidium* infections do not go undiagnosed (Omoruyi *et al*., 2014). ELISA is a simple method, easy to perform in a short time, applicable for large number of samples, very easy to read and interpret the results, it can detect antigen of different species of *Cryptosporidium* (Ghaffari and Kalantari, 2014). However, there could be false negative results obtained by the ELISA test due to condition of the samples. For example, the test is less sensitive for formed specimens; antigenic variability within clinical isolates of *Cryptosporidium*; very high or low parasite density in the sample, and low parasite densities due to the late stage of infection or

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asymptomatic persons; or it could be the result of difficulty in homogenizing of semi-solid or solid samples (Elsafi *et al*., 2014).

Polymerase Chain Reaction (PCR) is still a leading molecular genetics techniques currently used for identification, characterization and genotyping of this parasite in research and health laboratory settings (Hasajová et al., 2014; Wegayehu et al., 2016). PCR technique was developed more than two decades ago to detect and differentiate *Cryptosporidium* species at species or genotype and subtype level. It was recommended for epidemiological applications and also tracking the sources of infection for identification of *Cryptosporidium* spp. in the event of asymptomatic cryptosporidiosis (Mirhashemi et al., 2016). This assay has shown that some parasites are very host-specific while others have a wide host range (Li et al., 2014; Widmer et al., 2012). PCR is sensitive and specific as it can detect oocysts occurring in small numbers in most environmental samples (Smith and Grimason, 2003). The disadvantage of using PCR is that empty oocysts cannot be detected by as they do not contain sporozoite DNA, whereas in microscopy, the intact and empty oocysts can be observed. Therefore, microscopic examination adds significant value to PCR negative results since empty oocysts are an indicator of contamination (Smith *et al.,* 2010). However, stool sample that test positive with microscopy are assumed to contain high parasite loads, whereas samples that are negative by microscopy but diagnosed as positive by PCR test are likely to have low to moderate parasite loads (Hawash, 2014).

2.8 Treatment of Cryptosporidiosis

The report of the past indicated that effective drugs were not formulated because the eukaryotic parasite had a condensed genome lacking many of the traditional drug targets, and that most of the remaining genes remained functionally uncharacterized, thereby limiting

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pharmacological targets (Mauzy et al., 2012). Recent studies referred cryptosporidiosis as Neglected Disease with low funding priority and limited commercial interest which is the most challenge for anti-cryptosporidial drug development (Alum et al., 2014; Lal et al., 2013; Miyamoto and Eckmann, 2015). To date, only one moderately effective drug (nitazoxanide) is available for the treatment of cryptosporidiosis (Miyamoto, and Eckmann, 2014). Nitazoxanide has partial efficacy in immunocompromised individuals. Resolution of Cryptosporidiosis can be maintained with effective Highly Active Antiretroviral Therapy (HAART) (Abd-Ella, 2014).

The other commonly used treatments against cryptosporidiosis are paromomycin, and azithromycin, which are partially effective and not FDA-approved for treating cryptosporidiosis and their efficacy is generally lower than nitazoxanide (Vandenberg *et al*., 2012; Hussien *et al*., 2013). Nitazoxanide (NTZ)'s effectiveness was demonstrated in vitro, and in vivo using several animal models and finally in clinical trials. It significantly shortened the duration of diarrhea and decreased mortality in adults and in malnourished children. Nitazoxanide remains the most effective current therapeutic agent available against cryptosporidiosis in immunocompetent individuals and the drug is not consistently effective against infection under conditions of immunodeficiency (Miyamoto, and Eckmann, 2014), it only gives a partial efficacy in immunocompromised individuals (Abd-Ella, 2014).

The requirement for extensive studies regarding the parasitological and clinical effectiveness of nitazoxanide has been indicated. In AIDS patients, combination therapy restoring immunity along with antimicrobial treatment of *Cryptosporidium* infection is necessary (Gargala, 2008). Thus, resolution of cryptosporidiosis can be maintained with effective Highly Active Antiretroviral Therapy (HAART) (Abd-Ella, 2014).

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Most people who have healthy immune systems recover without treatment. Treatment is by supportive therapy, which is preferred in both humans and animals (Rossle and Latif, 2013). Diarrhea can be managed by replacement of fluid and electrolyte, for example drinking a plenty of fluids to prevent dehydration and nutritional support as well as antidiarrheal drugs (Efunshile et al., 2015).

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CHAPTER 3: MATERIALS AND METHODS

3.1 ETHICAL CLEARANCE

Ethical clearance was obtained from the Research and Ethics Committee of the University of Venda, and the Department of Health and Welfare in Giyani and Pretoria. A signed consent forms was given to the study participants before sample collection. The information of the participants was kept confidential.

3.2 STUDY SITE AND SAMPLE COLLECTION

A total of 714 samples (629 human stools and 85 animal stools) were collected randomly from rural area and urban areas in South Africa in 2011 and 2014. Human samples were obtained from people of all age groups who were visiting to the clinics in Giyani region in the Limpopo Province (n=248) and Pretoria areas in Gauteng Province (n=381). In the animal population, all 85 samples were collected from rural areas, with most of the samples obtained from cattle (n=52) and some samples (n=33) obtained from goats. The samples were transported to the laboratory at the University of Venda and were stored in the freezer at -20ºC until the time of analysis. Figure 3.1 shows the study sites.

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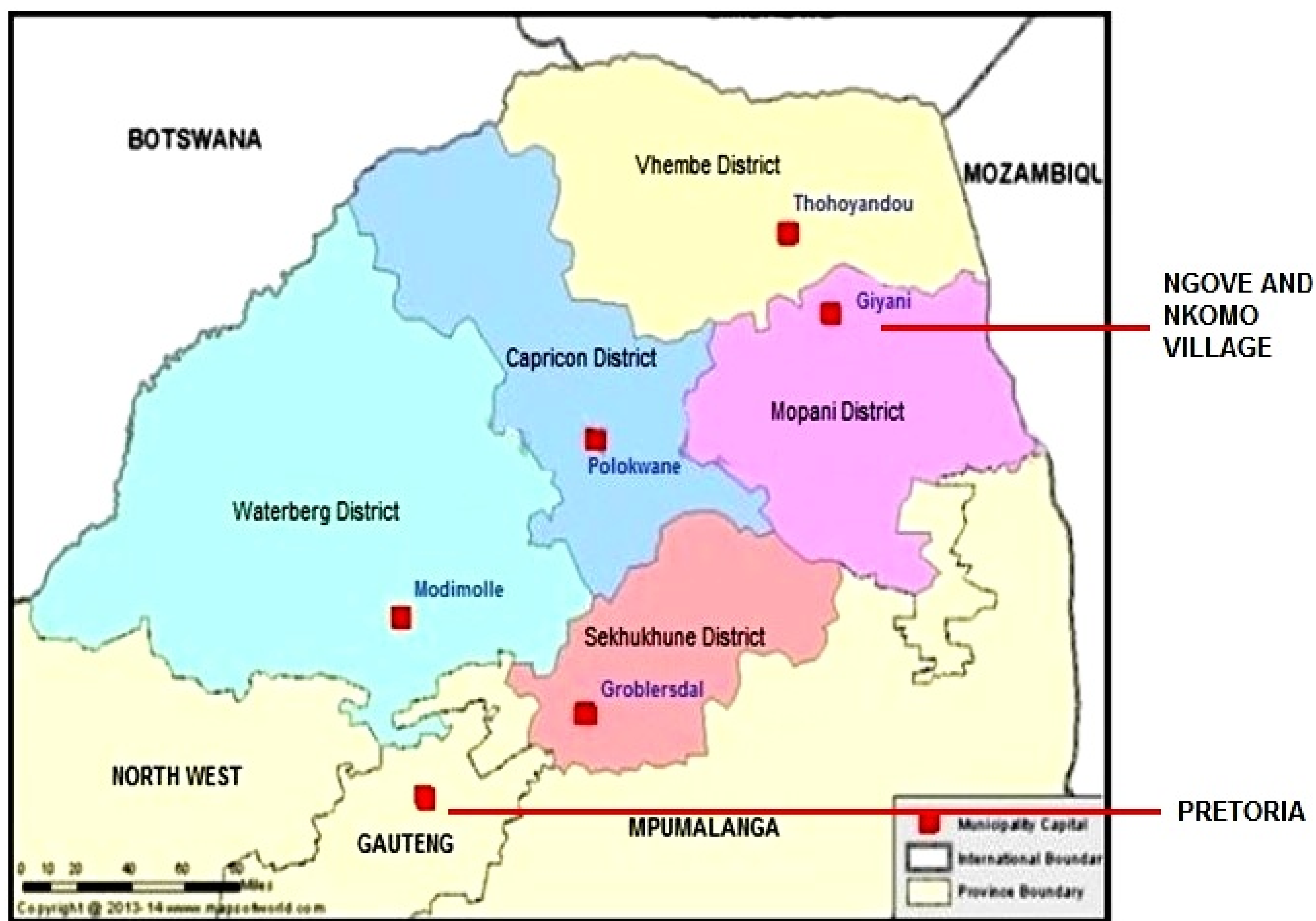

Figure 3.1: Map of South Africa showing the study sites (www.mapsofworld.com).

3.3 MICROSCOPY

Once in the laboratory, the samples were analysed for *Cryptosporidium* using the modified Ziehl-Neelsen technique as previously described by Fayer *et al.,* (2000) and Casemore *et al*., (1985). Briefly, 10 µl of sample was smeared on a slide and left to air dry. The smear was fixed using 100% and flooded with Carbol-fuchsin and rinsed with tap water after for one minute. The slide was decolorized with 5% sulphuric acid and then counter stained with methylene blue for 1-2 minutes. Finally the slides were rinsed with water and allowed to air dry. The slides were then observed under the microscope at 100 x magnification using immersion oil for the presence or absence of the oocysts (Fayer *et al.,* 2000; Casemore *et al*., 1985).

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3.4 ELISA FOR THE DETECTION OF *CRYPTOSPORIDIUM* FROM STOOLS

Cryptosporidium spp was detected using Enzyme-linked Immunosorbant assay (ELISA) kit, manufactured by TECHLAB® (Blacksburg, VA 24060 U.S.), following the manufacturer's instructions. Briefly, 100 µl of diluents was transferred to each well of the microassay plate. One drop of diluted sample was added using a Pasteur pipette to each well already containing diluents, and was gently tapped to mix. The plate was sealed with adhesive sheet and incubated for 1 hour at room temperature. The contents were shaken out of the well after incubation and the wells were washed 5 times with 1 x washing solution by directing it to the bottom of the wells. The plate was tapped hard onto a dry paper towel to remove residual liquid. One drop of conjugate was added to each well, mixed, sealed, and incubated for 30 minutes at room temperature. The washing procedure was repeated. Two drops of substrates were added to each well, mixed and incubated at room temperature for 10 minutes. One drop of stop solution was added to each well, mixed and waited for 2 minutes before reading. The absorbance was read at 450/620 nm. According to the manufacturer, results are considered positive at absorbance value of ≥ 0.090 OD and considered negative at < 0.090 OD.

3.5 MOLECULAR CHARACTERIZATION OF *CRYPTOSPORIDIUM* SPP.

3.5.1 Extraction of genomic DNA from stool samples

Genomic DNA was extracted from the stool samples according to manufacturer's instruction. Briefly, 0.3g of faecal sample was added to a ZR Bashing Beads ™ Lysis Tube following which 750 µl Lysis solution was added to the tube. A bead beater fitted with a 2 ml tube holder assembly was secured (e.g., Disruptor Genie ™) and processed at maximum speed for 5 min. ZR BashingBeads ™ Lysis Tube was then centrifuged in a microcentrifuge at 13,000

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rpm for 1 min. After centrifugation 400 µl supernatant were transferred to a Zymo-Spin ™ IV Spin Filter in a collection tube and centrifuged at 7,000 rpm for 1 min. After centrifugation 1,200 µl of Fecal DNA Binding Buffer was added to the filtrate in each of the collection tube from Step 4. Then 800 µl of the mixture was transferred to a Zymo-Spin ™ IIC Column in a collection tube and centrifuged at 10,000 rpm for 1 minute. The flow through was discarded from the collection tube. Up to 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin ™ IIC Column in a new Collection Tube and centrifuged at 10,000 rpm for 1 min. Then 500 µl Fecal DNA Wash Buffer was added to the Zymo-Spin ™ IIC Column and centrifuged at 10,000 rpm for 1 min. The Zymo-Spin ™ IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl DNA Elution Buffer was added directly to the column matrix. They were centrifuged at 10,000 rpm for 30 seconds to elude the DNA. The eluted DNA was transferred to prepared Zymo-Spin ™ IV-HRC Spin Filters (green tops) in a clean 1, 5 ml microcentrifuge tube and centrifuged at exactly 8,000 rpm for 1 minute. The filtered DNA was then suitable for PCR.

3.5.2 Detection of *Cryptosporidium* by conventional PCR

Conventional PCR was performed following the conditions described by Spano *et al.,* (1997), with minor changes. Briefly, the genomic DNA of *Cryptosporidium* was used in standard PCR reaction mixtures of 25 µl containing 30 pmoles of each COWP-specific primer and 1 unit of Taq DNA polymerase. The primer sequences and the fragment size were shown in Table 3.1 below with primer names: “Cry-9” and “Cry-15”. The templates were subjected to 30 amplification cycles (94 °C for 50 s, 55°C for 30 s, 72°C for 50 s) followed by one cycle of 10 minutes at 72°C (Spano *et al.,* 1997)

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3.5.3 Detection of *Cryptosporidium* spp. by Real-Time PCR using SYBR green

Genomic DNA was extracted from both positive and some selected negative samples by Microscopy and ELISA. A real-time PCR protocol previously described by Samie *et al.,* was used for the detection of *Cryptosporidium* spp targeting 18S rRNA gene. The primers used were shown in Table 3.1 below with primer names: "Crypt F" and "Crypt R". With minor changes, the reaction was performed in a total volume of 25 µl containing 12.5 µl of 2X MaximarM SYBR@ Green Supermix (Fermentas, Glen Burnie, MD), 0.4 µl of each primer (20 pmol/ µl), 6.7 µl of DNase-, RNase- and proteinase-free water (Fisher Biotech, Bridgewater, Nl) and 5 µl of genomic DNA. Thermocycling (conducted in a LightCycler® 480 96 System II, Roche) conditions were as follows: 13.5 minutes at 95^0C, followed by 50 cycles of 15 seconds at 95^0C, 15 seconds at 60^0C and 20 seconds at 72^0C (with data collection at the end of each cycle) (Samie *et al.,* 2006).

3.5.4 Detection of *Cryptosporidium* by Real-Time PCR using probes

For the specific detection of *C. hominis* and *C. parvum*, real-time PCR was carried out in two duplex reactions: (i) a genus-specific PCR amplifying ~300 bp of the *Cryptosporidium* SSU rRNA gene, duplexed with a *C. parvum*-specific PCR amplifying 166 bp of the LIB13 locus, and (ii) a *C. hominis*-specific PCR amplifying 169 bp as previously described by (Hadfield *et al.,* 2011). Each 25µl reaction mixture contained 12.5µl of TaqMan environmental mastermix 2.0 (Applied Biosystems). All primers (Integrated DNA Technologies, Glasgow, United Kingdom) were included at 900 nM except CRULib13RCh, which was at 300 nM. The minor groove binding (MGB) TaqMan probes (Applied Biosystems) CRU18STM (6-carboxyfluorescein [FAM] labelled) and CRULIB13Cp and CRULIB13Ch (both VIC labelled) were at 100 nM, 150 nM, and 100 nM, respectively. The *C. hominis* LIB13-IC tube

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contained 1µl of primer/probe (FAM labelled) mix and 5µl of a 1:20 dilution of the IC DNA (PrimerDesign). To each tube, 2µl of DNA was added. *C. hominis*, *C. parvum* and no-template PCR controls were included in each run. Thermocycling conditions were as follows: 95°C for 10 min, followed by 55 cycles of 95°C for 15 s and 60°C for 60 s. Data were collected from the green (FAM), yellow (VIC), and orange (ROX normalization dye) channels during each 60°C annealing/extension phase (Hadfield *et al.,* 2011).

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Table 3.1: Primers used for the detection of *Cryptosporidium* with the specific gene target

Locus	Primer name	Sequence (5'-3')	Fragment size	Reference
SSU rRNA gene	CRU18SF	GAGGTAGTGACAAGAAATAACAATACAGG	~300	Hadfield *et al.*, 2011
	CRU18SR	CTGCTTTAAGCACTCTAATTTTCTCAAAG		
	CRU18STM	FAM-TACGAGCTTTTTAACTGCAACAA		
***C. hominis* specific gene**	CRULib13F*	TCCTTGAAATGAATATTTGTGACTCG	~169	
	CRULib13RCh	AAATGTGGTAGTTGCGGTTGAAA		
	CRULib13TMCh	VIC-CTTACTTCGTGGCGGCGT MGB-NFQ		
***C. parvum* specific gene**	CRULib13F*	As above	~167	
	CRULib13RCp	TTAATGTGGTAGTTGCGGTTGAAC		
	CRULib13TMCp	VIC-TATCTCTTCGTAGCGGCGTA MGB-NFQ		
18S rRNA gene (Used in the SYBR Green qPCR)	Crypt F	CTGCGAATGGCTCATTAIACCA	~267	Samie *et al.*, 2006
	Crypt R	AGGCCAATACCCTACCGTCT		
COWP gene	Cry-15	GTAGATAATGGAAGAGATTGTG	~553	Spano *et al.*, 1997
	Cry-9	GGACTGAAATACAGGCATTATCTTG		

*Similar primers

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3.5.5 DNA sequences analysis

Briefly, 20µl of the PCR products of some amplicons were sequenced at Inqaba Biotechnologies (Inqaba, Pretoria, South Africa). Sequences were edited using the Staden package (Staden *et al.,* 1999). This package allows room for changes of nucleotides from the sequence during editing. During the editing process, unidentified nucleotide present in the sequence were removed and replaced by appropriate nucleotides in line with the reference sequence that came with the package. Thereafter, Bioedit (Hall, 1999) was used to edit the nucleotide sequences which were then aligned using the Clustal W multiple aligner. With this package the nucleotides for all genes were aligned alongside a referral strain from Genbank to determine the similarity and differences between the various gene products from all strains. After this, a similarity plot was then performed to determine points of mutation that must have taken place in each strain in line with the reference strain.

The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.12564987 is shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.,* 2011).

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3.5.6 Statistical analysis

The results were entered into an excel spread sheet, edited appropriately (Microsoft office package) and analyzed using Statistical Package for the Social Sciences (SPSS for WINDOWS version 21.0). Data was summarized using frequency tables and bar graphs. Contingency tables were used and the strength of association was measured using the chi-square and its associated p-value at asymptotic significance. The results were considered to be statistically significant when the p-value obtained is ≤ 0.05.

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CHAPTER 4: RESULTS

4.1 MICROSCOPIC DETECTION OF *CRYPTOSPORIDIUM*

Sample was observed for the presence of oocyst using modified Ziehl-Neelsen staining technique. The oocysts are normally coloured pink to red while the background is blue.

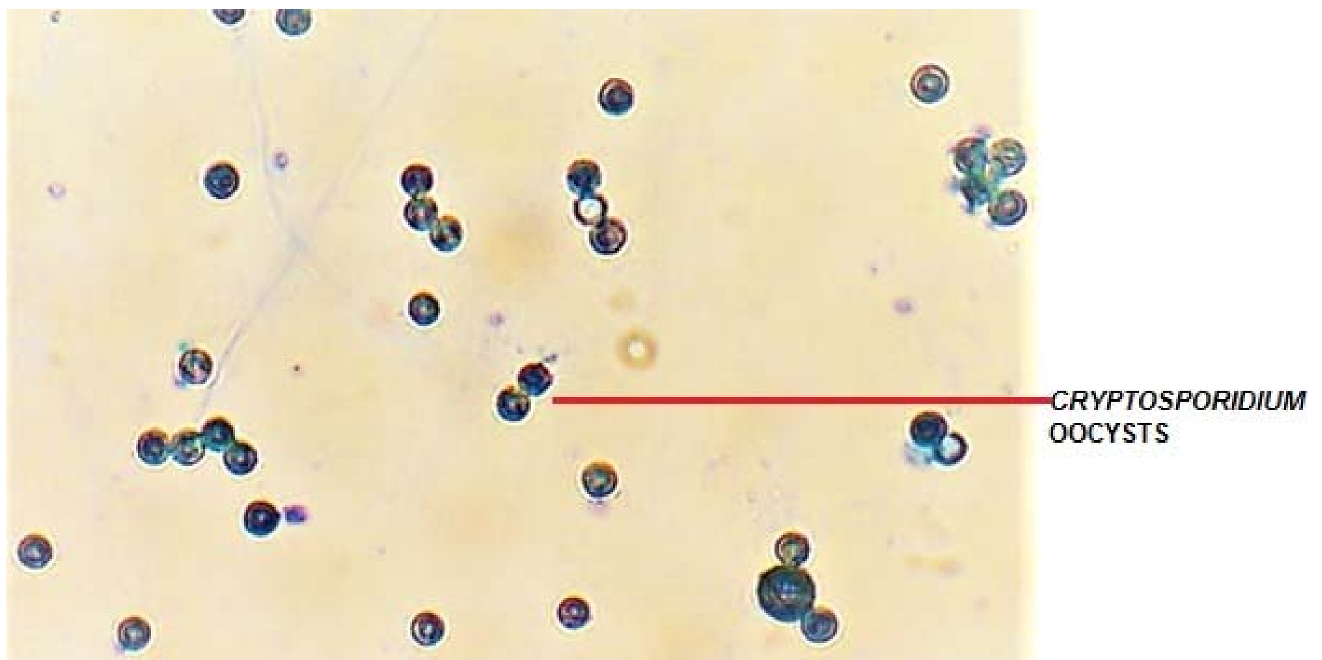

Figure 4.1: Cryptosporidium oocysts representation taken at 100X magnification on a light microscope from one of the samples tested.

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4.2 IMMUNOLOGICAL DETECTION OF *CRYPTOSPORIDIUM*

A qualitative detection of *Cryptosporidium* oocyst antigen in stool samples was performed using the *Cryptosporidium* II test from Tachlab (Virginia, USA). The overall prevalence of *Cryptosporidium* was 41.2% (239/580) (figure 4.2).

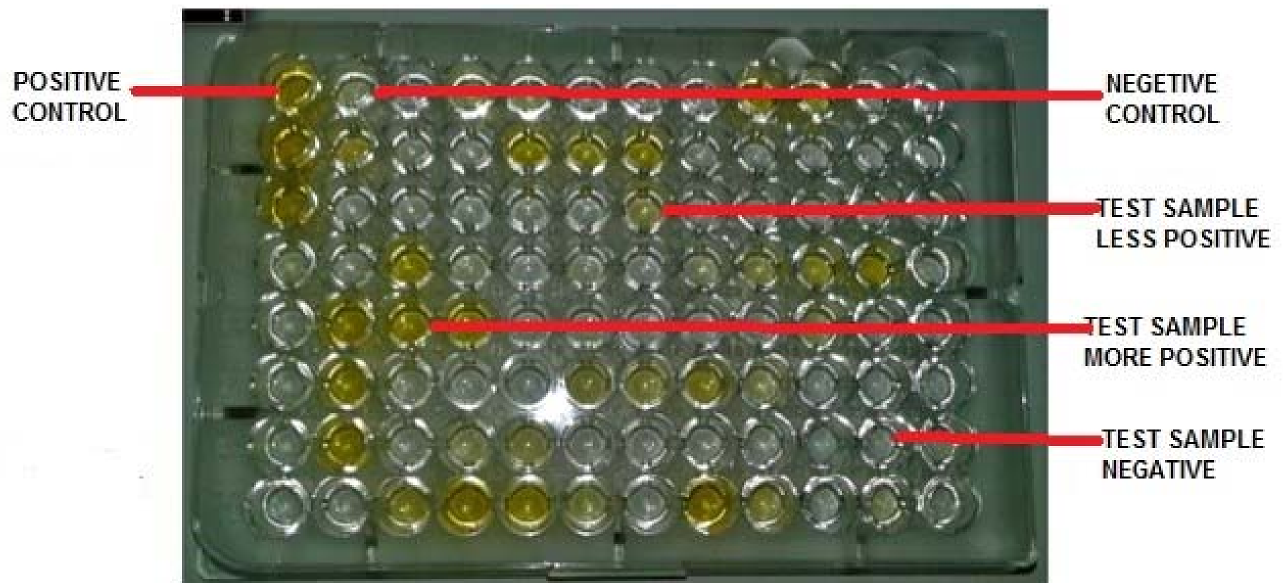

Figure 4.2: Microassay plate showing some positive samples for *Cryptosporidium* oocyst by ELISA.

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4.3 GENETIC DIVERSITY OF *CRYPTOSPORIDIUM* IN HUMANS

4.3.1 Demographic characteristics of the study population

.A total of 629 human stool samples were obtained from Giyani (n=248) and Pretoria (n=381). Female participants were more 48.8% (307) than males 43.4% (273) in both study areas. Watery stools were recorded as diarrheal and the formed ones as non-diarrheal. Of all the samples collected 325 (51.7%) were diarrheal. (Table 4.1)

Table 4.1: Demographic characteristics of the study population

Characteristics		**Frequency**	**Percent (%)**
Origin	Rural	248	39.4
	Urban	381	60.6
Gender	Female	307	48.8
	Male	273	43.4
	Missing information*	49	7.8
Stool Consistency	Diarrheal	325	51.7
	Non-diarrheal	255	40.5
	Missing information*	49	7.8
Total		**629**	**100**

*The gender and stool consistency of some stool samples were not recorded, because only the DNA samples were available for use in the present study.

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4.3.2 Comparison of selected diagnostic methods for identification of *Cryptosporidium* in human stool samples

This study involved the comparison of different methods for routine diagnostics of Cryptosporidium spp., Microscopy (modified Ziehl-Neelsen staining), ELISA, Real-time PCR and conventional PCR. Our results shows that ELISA is the best method for detection of Cryptosporidium, a total of 239 (41.2%) samples were positive by ELISA.

Table 4.2: Comparison of selected diagnostic methods for identification of *Cryptosporidium* in human stool samples

Technique used	Frequency	Total number of samples tested	Samples not tested	Valid Percent (%)
Microscopy	1	580	49*	0.2
ELISA	239	580	49*	41.2
Real-time PCR of Hydrolysis probe	48	134	495**	35.8
Real-time PCR of SYBR Green	0	134	495**	0.0
Conventional PCR	0	25	604***	0.0

*Some of the stool samples (49) were no longer available for test with microscopy and ELISA; however, their DNA was kept frozen and was used in this study.

**Only ELISA positive samples (239) were considered for PCR tests due to cost effects. However, not all the ELISA positive samples were found during DNA extraction and therefore, the available frozen DNA was used, which reduced the tested number of samples to 134 instead of 239.

***Conventional PCR was terminated after a several trials using 25 ELISA positive samples and getting negative results, as a result, many samples were left un-tested to avoid cost effects

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4.3.3 Distribution of human *Cryptosporidium* in rural and urban areas

Comparison of the prevalence of *Cryptosporidium* infections between rural and urban areas was done using the ELISA test results (Figure 4.3). The sample size from rural area was lower than that of urban area; however, high prevalence was observed in the rural (73.0%) 159/218 than in urban area 22.1% (80/362). The difference between the infection rates in the village and town was statistically significant ($\chi^2 = 145.1$; $p < 0.0001$).

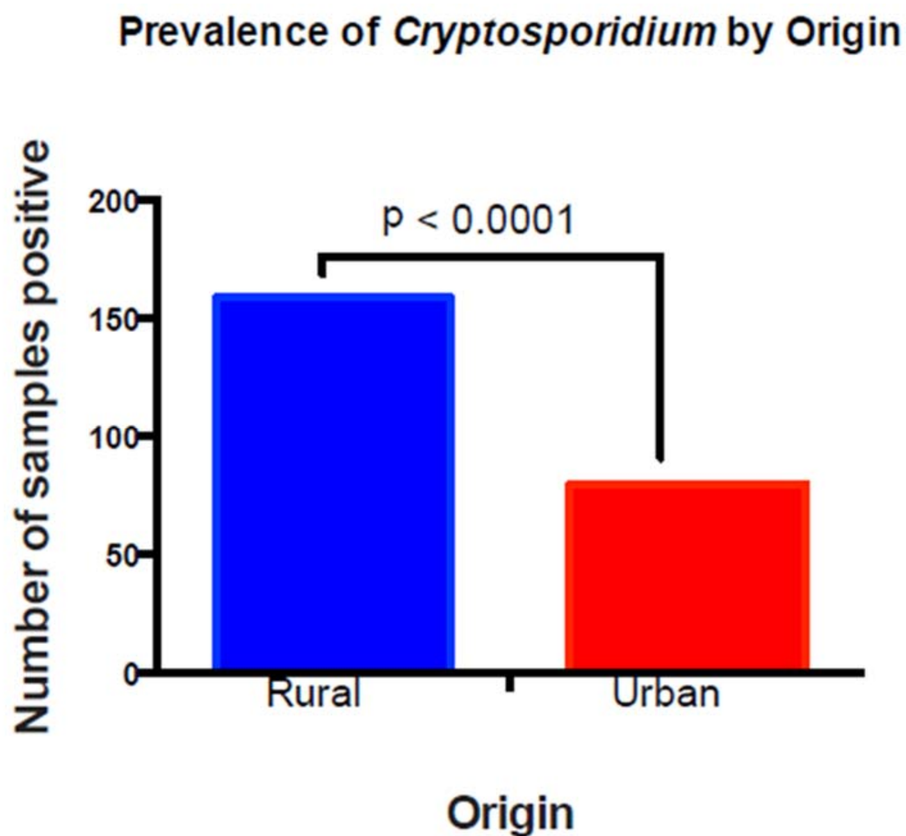

Figure 4.3: Prevalence of *Cryptosporidium* infections in rural and urban areas.

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4.3.4 Occurrence of *Cryptosporidium* in humans according to their geographical region

Twenty five human samples were successfully sequenced and two *Cryptosporidium* species were identified. *C. hominis* was the most prevalent species detected 96.0%, (24/25) followed by *C. muris* in 4.0% (1/25). The *C. muris* came from the rural area, and there was no demographic information* about its stool sample. Twenty (80.0%) *C. hominis* isolates were identified in samples from rural area and diarrheal and non-diarrheal samples had 36.0% (9/25) each. *C. hominis* was highly prevalent in female 40.0% (10/25) than in males 32.0% (8/25). Of 4 isolates obtained from urban area, 12.0% (3/25) *C. hominis* isolates were identified in males as shown in Table 4.3.

Table 4.3: Occurrence of human *Cryptosporidium* spp. in the study sites based on stool type and gender of the participant

Species	Origin	Stool	Gender
C. hominis **24/25 (96.0%)**	Rural **20/25 (80.0%)**	Diarrheal **9/25 (36.0%)**	Female **10/25 (40.0%)**
		Non-diarrheal **9/25 (36.0%)**	Male **8/25 (32.0%)**
		*Missing **2/25 (8.0%)**	*Missing **2/25 (8.0%)**
	Urban **4/25 (16.0%)**	Diarrheal **3/25 (12.0%)**	Female **1/25 (4.0%)**
		Non-diarrheal **1/25 (4.0%)**	Male **3/25 (12.0%)**
C. muris **1/25 (4.0%)**	Rural **1/25 (4.0%)**	*Missing **12/25 (4.0%)**	*Missing **1/25 (4.0%)**

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*The stool samples were no longer available. However, the DNA samples that were previously extracted and kept in the freezer were used in this study. Therefore "gender of the participants" and "stool consistency" of those samples were not kept on record.

4.3.5 *Cryptosporidium* detection by real-time PCR.

Real-time PCR diagnostic method was employed using Hydrolysis probes to detect *Cryptosporidium* genus from human fecal DNA. Out of 134 samples tested, 48 (35.8%) were positive with *Cryptosporidium.* In order to confirm the results and have some aplicon sequenced, gel electrophoresis was used to separate the DNA fragments. Figure 4.4 and 4.5 show PCR products of the 18S rRNA gene and SSU rRNA gene for *Cryptosporidium* respective. A 2% agarose gel was run in 100V for 45 minutes with a 100bp Marker.

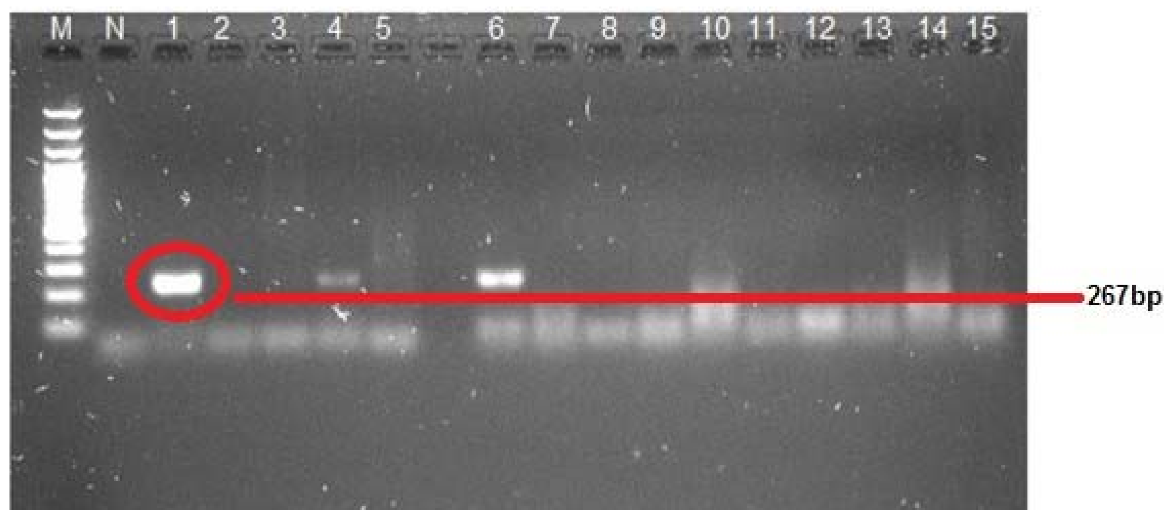

Figure 4.4: A 2% agarose gel picture showing the bands of the PCR product using 18S rRNA gene

Keys: **M** = Marker; **N** = Negative control; **Lane** (1 – 15) = Test samples

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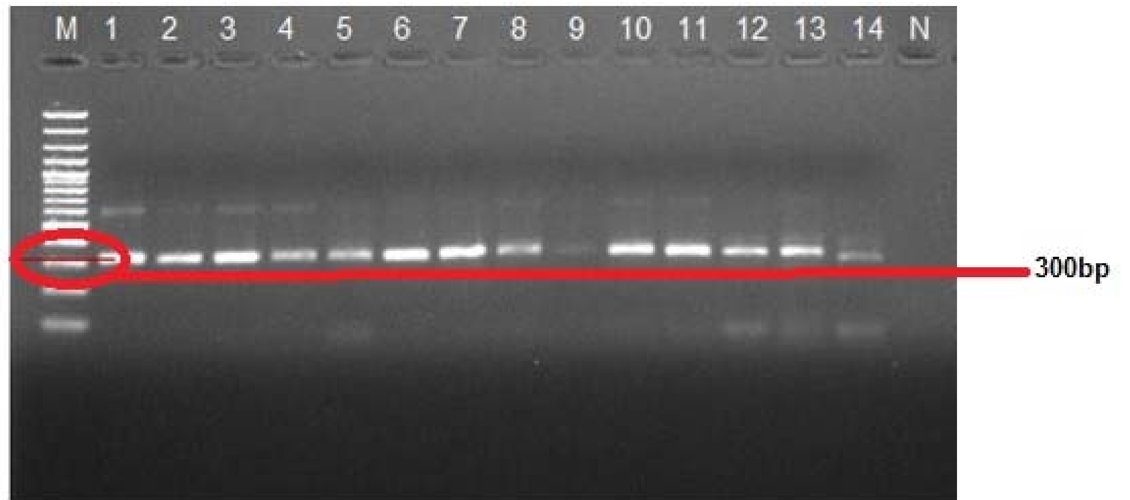

Figure 4.5: A 2% agarose gel picture showing the bands of 18S SSU rRNA gene at 300bp

M = Marker; **N** = Negative control; **Lane** (1 – 14) = Test samples

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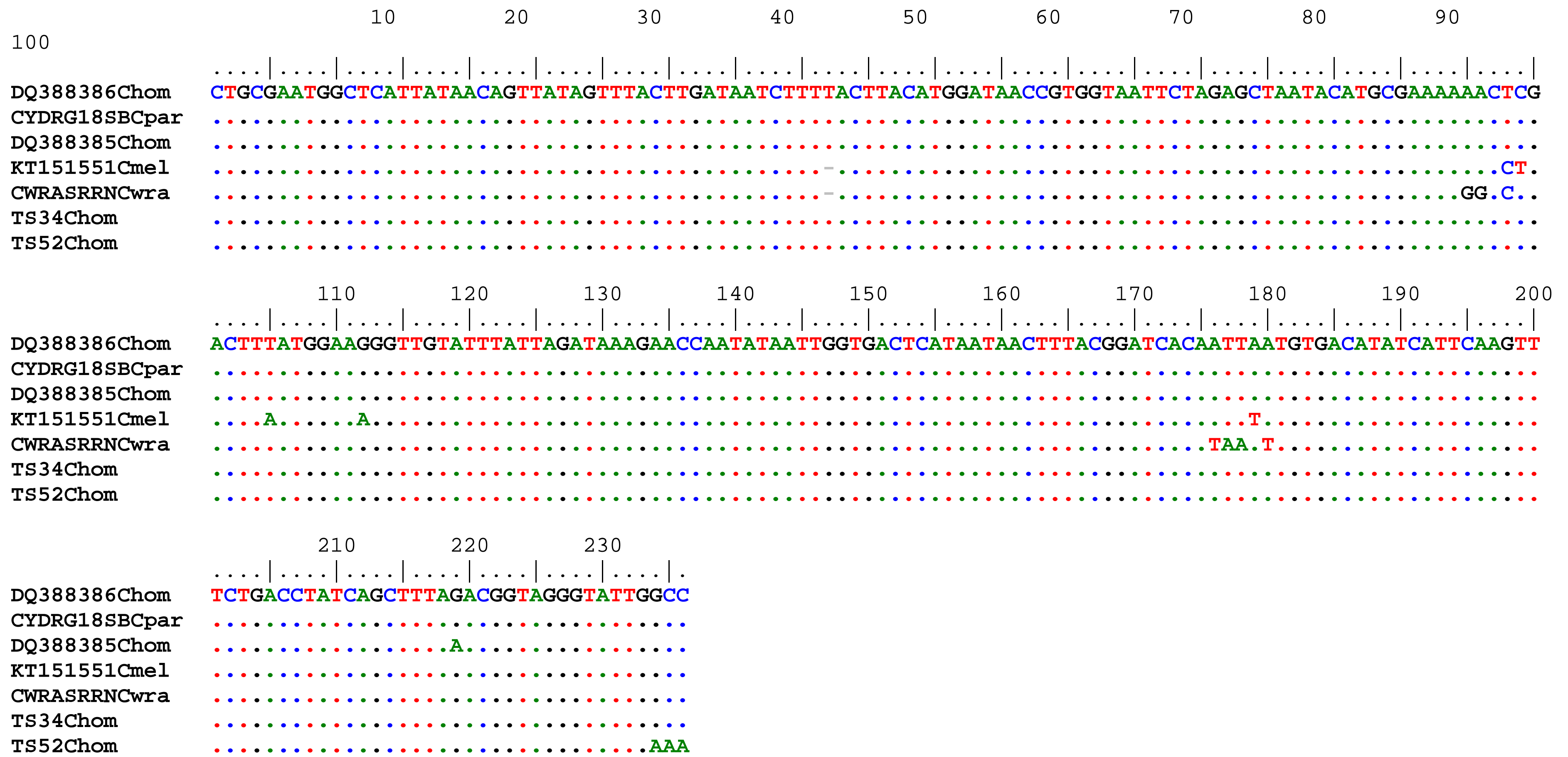

Figure 4.6: Sequence alignment of the 18SrRNA gene of *Cryptosporidium* from human stool samples using Bioedit package (Hall, 1999)

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4.3.6 Phylogenetic and evolutionary distances analysis from human samples

A phylogenetic tree was constructed using neighbour-joining method (Saitou and Nei, 1987) in order to determine the potential interrelation of *C. hominis* isolates in the study. Each of the sequences from the present study was found to be closely related to two referral sequences from GenBank (TS34; DQ388386; CYDRG18SB) and (TS52; KT151551; CWRASRRN), while the other reference sequence (DQ388385) was not related but originated from the same root or shared the same ancestor in Cluster 1. The percentage of trees in which the associated taxa clustered together is shown next to the branches. There were a total of 235 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

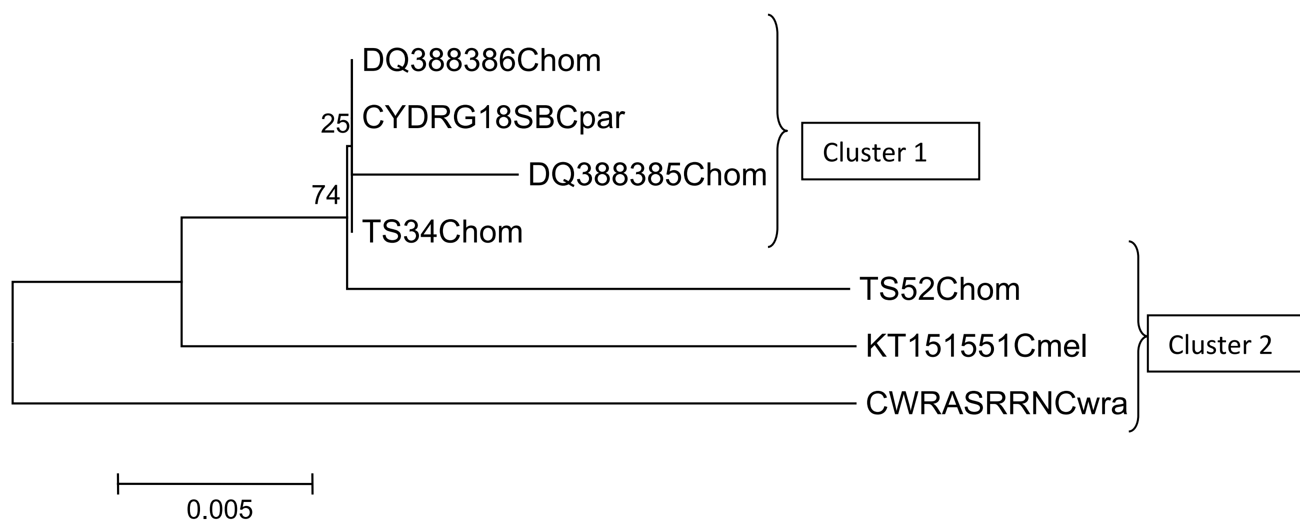

Figure 4.7: Molecular Phylogenetic analysis of *Cryptosporidium spp.* by Maximum Likelihood method

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The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2013) and represented as the number of base substitutions per site. The similarities are indicated by 0.000 units in the table, and all the values that are greater than 0.000 indicates the genetic differences of the 18S rRNA gene.

	1	2	3	4	5	6	7
1. DQ388386Chom							
2. CYDRG18SBCpar	0.000						
3. DQ388385Chom	0.002	0.002					
4. KT151551Cmel	0.009	0.009	0.011				
5. CWRASRRNCwra	0.013	0.013	0.015	0.019			
6. TS34Chom	0.000	0.000	0.002	0.009	0.013		
7. TS52Chom	0.005	0.005	0.007	0.015	0.019	0.005	

Figure 4.8: Estimates of Evolutionary Divergence between Sequences of the different isolates

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4.4 GENETIC DIVERSITY OF *CRYPTOSPORIDIUM* IN ANIMALS

4.4.1 Demographic characteristics of animal study population

A total number of 85 animal samples were examined in the present study, all collected from 2 types of domestic animals in rural areas. Fifty-six samples were collected from Ngove village and thirty-three were collected from Nkomo village, and both villages are situated near the town of Giyani in Vhembe district in the Limpopo Province. The stool consistency, like in human samples, was arranged according to diarrheal (loose and watery stools) and non-diarrheal (formed stools). The non-diarrheal stools were more (85.9%) than the diarrheal samples. Many of the samples (61.2%) came from cattle, followed by goats' samples which were (38.8%). The female animals were found to be high in number (77.6%) than males, and when they were assessed according to their age groups, adult animals were high in number (80.0%) than young animals.

Table 4.4: Demographic characteristics of animal sample population used in the study

Characteristics		**Frequency**	**Percent (%)**
Animal type	Cow	52	61.2
	Goat	33	38.8
Gender	Female	66	77.6
	Male	19	22.4
Age group	Young animal	17	20.0
	Adult animal	68	80.0
Origin	Ngove	53	62.4
	Nkomo	32	37.6
Stool Consistency	Non-diarrheal	73	85.9
	Diarrheal	12	14.1
Total		**85**	**100.0**

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4.4.2 The possible household risk factors that predispose animals to infection with *Cryptosporidium* spp.

The findings of this study shows that most households 27 (84.4%) were keeping their animals in the open sheds whereas only 5 (15.6%) households kept their animals in the closed sheds. Twenty-five (78.1%) households mentioned that they let their animals scavenge whereas 7 (21.9%) households were feeding them in containers or on the floor. Thirty-one (96.9%) households confirmed their animals were healthy, but 1 (3.1%) were concerned about the health of one or two animals in the shed, reporting that they were sick (a cow having diarrhea and weight loss)

Table 4.5: The possible household risk factors that predispose animals to infection with *Cryptosporidium*

Characteristics		**Frequency**	**Percent (%)**
Animal shed	Close shed	5	15.6
	Open shed	27	84.4
Animal feed	They scavenge	25	78.1
	Fed on the container/floor	7	21.9
Animal Health	Good	31	96.9
	Sick	1	3.1
Total		**32**	**100.0**

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4.4.3 Demographic characteristics of the participants who owned animals in the households

Animals were owned mostly by males 20 (62.5%) than females 12 (37.5%), and especially married people makes about 59.4% in 35 participated households. Those who are less than 35 years old but owning animals in the households were only 3 (9.4%), and 23 of them were older whereas the age of 6 people was not provided during the survey. Most of them 20 (62.5%) went to school even if some of them did not reach the higher education level, but the number of people who did not go to school at all was low 12 (37.5%). It was discovered that 19 people who owned animals were unemployed, most of them 25 (78.1%) fall under the category of income between R1 – R3000 per month.

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Table 4.6: Demographic characteristics of the participants who owned animals in the households

Characteristics		**Frequency**	**Percent (%)**
Gender	Male	20	62.5
	Female	12	37.5
Marital status	Married	19	59.4
	Unmarried	13	40.6
Age	Less than 35 years	3	9.4
	More than 35 years	23	71.9
	Unknown age*	6	18.8
Education	Went to school	20	62.5
	Did not go to school	12	37.5
Occupation	Employed	13	40.6
	Unemployed	19	59.4
Income per month	R1-R3000	25	78.1
	R3001-R12000	5	15.6
	R12001+	2	6.3
Total		**32**	**100**

*Age of the participants was not provided during the survey for personal reasons

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4.4.4 Demographic characteristics of the participants and the infection risk factors

It was found that 19 (59.4%) of the participant households had less than 6 people. About 40% of the households had children less than 5 years, but the diarrhea was only observed in 3 households out of the 32. A huge number (90.6%) of households were depending on public clinics and hospitals for treatment.

Municipal water is the primary water source for many 27 (84.4%) households whereas few (15.6%) depend on borehole water. A small number of households (21.9%) mentioned that they treat their drinking water, either by boiling or chlorination (adding JIK). Almost all households 30 (93.7%) store water for days, even weeks. The same number pours directly or use cup to draw water from the storing containers while two households use spigot. Again, the 93.7% use open pit toilets whereas 2 (6.3%) said that they use flush toilets. When asked about cleaning water storing containers, 31 (96.9%) said they clean storing containers after some days or weeks, whereas 1 (3.1%) said it is not cleaned at all.

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Table 4.7: Demographic characteristics of the participants and the infection risk factors

Characteristics		**Frequency**	**Percent (%)**
Number of people in the household	Less than 5 people	31	96.9
	10 people and above	1	3.1
Children in the household	Less than 5 years	13	40.6
	5 and above years	19	59.4
Diarrhea observed	Yes	3	9.4
	No	29	90.6
Treatment centre	Public clinic/hospital	29	90.6
	Private clinic/hospital	3	9.4
Primary water use	Municipal water	27	84.4
	Borehole water	5	15.6
Water treatment	Boiling and chlorination (JIK)	7	21.9
	No water treatment	25	78.1
Water storage	Store water for days or weeks	30	93.7
	Do not store water	2	6.3
Cleaning storing container	Cleaned after days or weeks	31	96.9
	Do not clean containers	1	3.1
Drawing water	Use spigot	2	6.3
	Pour directly or use cup	30	93.7
Type of toilet used	Flush toilets	2	6.3
	Open pit and the bush	30	93.7
Total		**32**	**100**

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4.4.5 Comparison of selected diagnostic methods for identification of *Cryptosporidium* in animal stool samples

Microscopy detected about 1.2% (1/85) samples positive for *Cryptosporidium*. ELISA assay and conventional PCR detected 3 (3.5%) and 4 (4.7%) positive samples respectively. The sensitive real-time assay detected a high number 56.5%, (48/85) of positive samples. Twelve out of 48 (25.0%) samples that were positive with real-time and conventional PCR were sent for sequencing and the results of the sequences showed that 10 (83.3%) animals were infected with *C. parvum* while the other 2 (16.7%) were *C. andersoni*.

Table 4.8: Occurrence of *Cryptosporidium* in animals according to diagnostic technique used

Characteristics		**Frequency**	**Percent (%)**
Microscopy	Positive	1	1.2
	Negative	84	98.8
ELISA	Positive	3	3.5
	Negative	82	96.5
Conventional PCR	Positive	4	4.7
	Negative	81	95.3
Real-time PCR	Positive	48	56.5
	Negative	37	43.5
Total		**85**	**100**

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4.4.5 Occurrence of *Cryptosporidium* spp. in goats and cattle

Cryptosporidium species have been detected in almost all domestic animals for many years, and cattle are the leading *Cryptosporidium* infected animals. Twelve samples from cattle and goats were sequenced and two *Cryptosporidium* species were identified. These species were isolated from 11.5% (6/52) of samples obtained from cattle and 18.2% (6/33) of samples obtained from goats.

Table 4.9: Occurrence of *Cryptosporidium* spp. in goats and cattle

Animals	*Cryptosporidium* positive	*Cryptosporidium* negative	Total	χ^2	*p*-Value
Goats	6 (18.2%)	27 (81.8%)	33	0.213	0.644
Cattle	6 (11.5%)	46 (88.5%)	52		

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4.4.6 Occurrence of *Cryptosporidium* isolates based on the consistency of animals stool

Cryptosporidium can be asymptomatic or symptomatic mostly in young animals, and diarrhea is one of the signs of *Cryptosporidium* infection even in animals. In the present study, only 1 (20.0%) isolate was identified in diarrheal samples out of 5 diarrheal samples of goats while 5 (17.9%) isolates were identified in non-diarrheal samples. In cattle, *Cryptosporidium* isolates were only identified in 6 (13.3%) non-diarrheal samples.

Table 4.10: Occurrence of *Cryptosporidium* isolates based on the consistency of animal stool

Stool consistency	Goats		Cattle	
	Sequenced samples and Isolates identified	**Non-sequenced samples**	**Sequenced samples and Isolates identified**	**Non-sequenced samples**
Number of diarrheal stool	1 (20.0%)	4 (80.0%)	0 (0.0%)	7 (100.0%)
Number of non-diarrheal stool	5 (17.9%)	23 (82.1%)	6 (13.3%)	39 (86.7%)

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4.4.7 Amplification of 18S rRNA gene by conventional PCR

The genus specific primers (18S rRNA gene) were used to detect *Cryptosporidium* from animal stool samples (figure 4.9). Two percent agarose gel was run in 100V for 45 minutes with a 100bp marker.

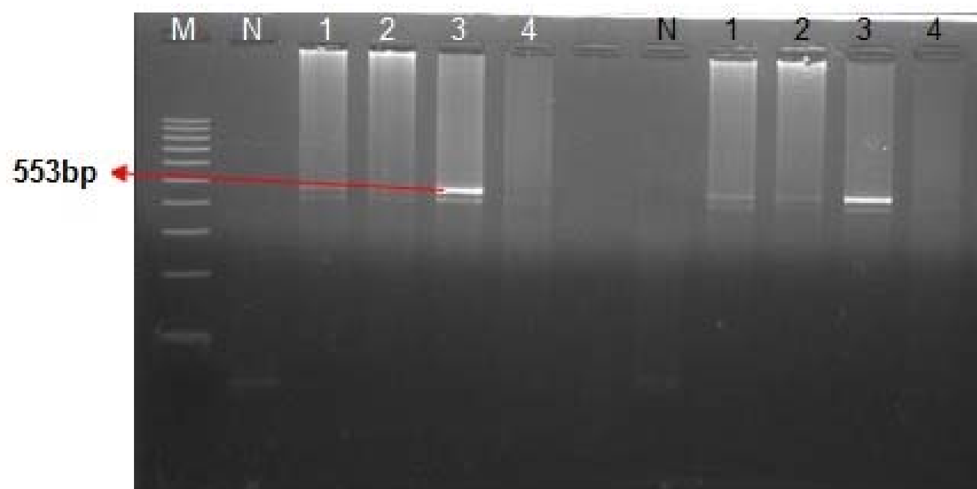

Figure 4.9: An agarose gel showing the target bands of the 18S rRNA gene detection from conventional PCR. The product size was 553bp.

M = Marker; **N** = Negative control; **Lane** (1 – 4) = Test samples

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4.4.8 Phylogenetic analysis from animal samples

To determine the sequence diversity of *C. parvum* isolates from animal population in this study, the sequences were aligned using multiple sequene alignment by Clustal-W. The alignment was edited manually in order to align all of the homologous sequences to each other. The total sequence diversity was identified in the alignment. Those sequences were also found to be diverse from the sequences used in this study as reference sequences from GenBank. Although AN220 seemed to be related with XM002141621, they both seemed to be originated from different ancestors.

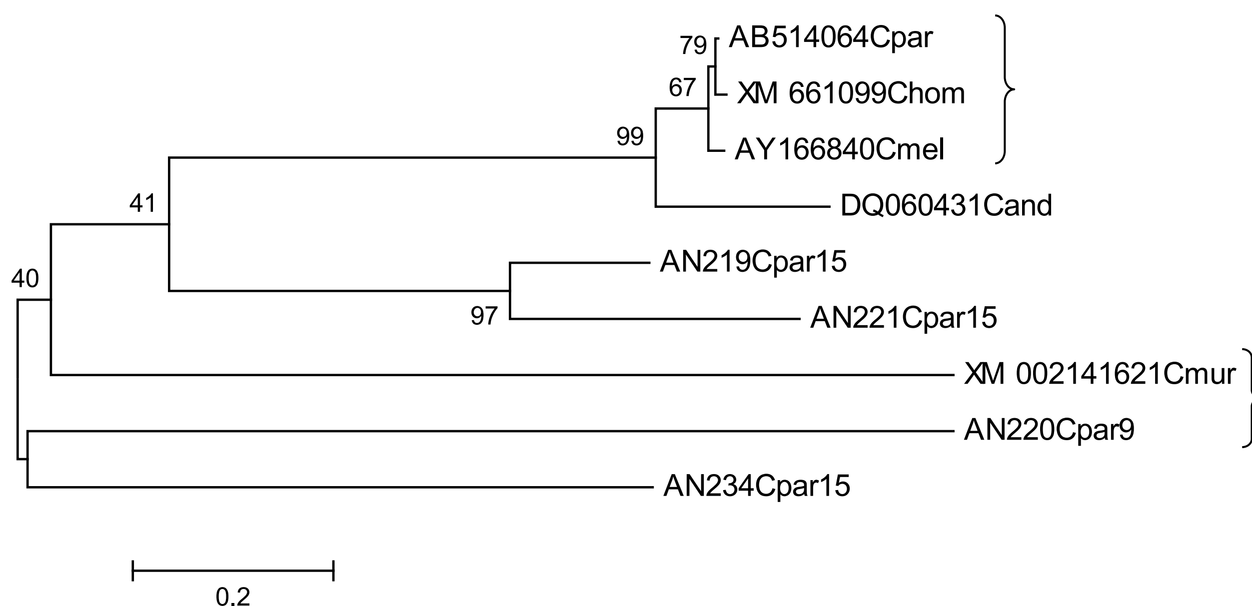

Figure 4.10: Molecular Phylogenetic analysis by Maximum Likelihood method of COWP gene

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4.4.9 Evolutionary distances analysis of the sequences from animal samples

The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2013) and represented as the number of base substitutions per site. The similarities are indicated by 0.000 units in the table and all the values greater than 0.000 indicates the genetic differences of the 18S rRNA gene.

	1	2	3	4	5	6	7	8	9
1. AB514064Cpar									
2. AY166840Cmel	0.025								
3. XM 661099Chom	0.014	0.034							
4. XM 002141621Cmur	1.577	1.551	1.548						
5. DQ060431Cand	0.246	0.252	0.250	1.786					
6. AN219Cpar15	1.218	1.210	1.210	1.709	1.389				
7. AN220Cpar9	2.186	2.229	2.176	2.084	2.086	2.016			
8. AN221Cpar15	1.319	1.314	1.323	2.221	1.446	0.469	1.831		
9. AN234Cpar15	1.873	1.870	1.853	2.087	2.202	1.559	2.239	1.754	

Figure 4.11: Estimates of Evolutionary Divergence between the COWP gene sequences of *Cryptosporidium* isolates from animals

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CHAPTER 5: DISCUSSION, CONCLUSION, RECOMMENDATIONS AND LIMITATIONS

5.1 DISCUSSION

Cryptosporidium is an important pathogen that infects both animals and humans and is responsible for economic loss due to absence from work as well as hospitalization in case of diarrhea. The present study showed high prevalence of *Cryptosporidium* in humans at 41.2% (239/580) compared to the work reported previously by Samie *et al*., (2006) who showed the prevalence of *Cryptosporidium* to be 18% (36/197) using real-time PCR in the Vhembe region, South Africa. This difference could be due to the difference in terms of the study population used but could also reflect negligence in hygiene form the time the previous study was conducted until the present study was conducted, as poor hygiene may result in *Cryptosporidium* infection in humans (Ryan et al., 2014), In this study the prevalence data were obtained by ELISA, which was the most sensitive tool than microscopy. ELISA was reported as the most useful diagnostic tool to rule out the infection in an area where non-pathogenic species are endemic (Efunshile *et al.,* 2015). This is in line with the results of a study conducted in Turkey whereby 24.0% positive samples were detected by ELISA. They described it as the sensitive and specific diagnostic method and its simple, rapid and reliable tool which could be useful for large-scale of epidemiological studies of cryptosporidiosis (Elgun *et al.,* 2011). However, ELISA showed less detection of *Cryptosporidium* in animal samples in the present study, which could be associated with low excreted oocysts leading to false negative results like what was reported in central Spain by De la Fuente *et al.,* (1998).

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The present study showed that microscopy is less sensitive in detecting *Cryptosporidium* because only a few number (0.2%, 1/158) of human samples were positive for this organism and in animals the prevalence was 1.2% (1/85). Similar result was detected in the study from Tunisia where 2.7% positive samples with *Cryptosporidium* were detected among 708 samples examined by microscopy (Essid *et al.,* 2008). Other studies reported microscopy as a low sensitive tool when compared with other tools such as ELISA in testing for *Cryptosporidium* from preserved and non-preserved stool samples (Van Gool, *et al.,* 2003). In Turkey, 5.2% positive samples were detected from patients by modified acid-fast staining method (Elgun *et al.,* 2011). This is in agreement with the study conducted by Savioli *et al.,* (2006) which described that sensitivity in the detection of microorganisms is influenced by the method(s) used and that the presence or absence of morphological stages in a test sample determines sensitivity for microscopic methods, whereas the presence or absence of target antigens or DNA determines the sensitivity of parasite antigen or parasite DNA tests, and no test is necessarily 100% sensitive and specific. However, microscopic examination of a single stool sample cannot rule out infection, therefore at least three stool specimens should be examined by a competent microscopist (Savioli *et al.,* 2006).

In most cases, the results obtained by screening methods are not the same when a different technique is used. For example, Ghaffari and Kalantari, (2012) found 26 positive samples with *Cryptosporidium* from screening method, but only 13 out of the 26 were positive for the 18S rRNA gene by PCR. This was the case in the present study where the positive samples from ELISA were further tested by real-time PCR for genetic analysis and was detected 35.8% (48/134) positive samples with *Cryptosporidium*, which was lower than the results obtained from ELISA.

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A study by Da Silva *et al.,* (2003), identified *Cryptosporidium* species using the COWP gene from all (n=4) samples which were found positive in microscopy. The study in Korea showed low sensitivity of *Cryptosporidium* 6.1% with PCR versus the results of ELISA 9.7% (Lee *et al.,* 2016). Their results correlate with the results of the present study which showed less sensitivity of Conventional PCR even though it tested only positive samples from ELISA. This could be due to the presence of inhibitors or the loss of sensitivity of the primers during the course of the tests. Real-time PCR has been reported as high sensitive tool for detecting *Cryptosporidium* in Australia (Stark *et al.,* 2010), in United Kingdom (Hadfield *et al.,* 2011) and in South Africa (Samie *et al.,* 2006). These results are in agreement with the results of the present study which reports a high prevalence of 58 (61.0%) by real-time PCR. Although these results are higher than that from of Northern Spain which reported 12.4% out of the 362 animals tested using real-time PCR, where they were investigating asymptomatic adult cattle (Cano *et al.,* 2015). This huge difference in prevalence may be due to the fact that in the present study, domestic animals from the rural areas were investigated, whereas in Spain farm animals which may be getting some treatment were investigated. In this case the infection rates could be too different between the two geographical areas.

The prevalence of *Cryptosporidium* infection in rural and urban areas were 64.1% (159/248) and 21.0% (80/381) respectively, and the difference of infections between the two study sites was statistically significant (χ^2 = 145.1; $p < 0.0001$). This may be caused by zoonotic infection, since in the rural people are more associated with domestic animals such as cattle and goats which could be disseminating *Cryptosporidium* oocysts in the community. The 1994 USEPA *Cryptosporidium* Criteria Document cited adequate evidence for the transmission of *Cryptosporidium* from animals, particularly livestock, to humans. Recent evidence indicated that ruminants are the common reservoir of zoonotic *Cryptosporidium*

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and humans get infected through direct contact with livestock, for example animal handlers (Ehsan et al., 2015). This could be the cause of high prevalence in the rural than in the urban area,

Diarrheal stool has been associated with *Cryptosporidium* infection in some studies, but in the present study the prevalence of infection was low in diarrheal samples 38.2% (124/325) compared to that of non-diarrheal samples 45.1% (115/255), however there was no difference ($p > 0.05$). This means that the diarrhea occurring in these two study sites was probably not due to *Cryptosporidium*; instead it could be some other intestinal parasites such as *Giardia duodenalis* or some other bacteria (Cardona *et al*., 2016; Ehsan *et al*., 2015). There was no difference with infection rates between males 41.4% and females 41.0% in general, meaning that *Cryptosporidium* infection is not a gender specific organism, therefore, it can infect both males and females equally.

The primary *Cryptosporidium* species detected in humans was *C. hominis*. The present study obtained 24 (96%) *C. hominis* isolates by sequencing. The other species identified from sequencing was one (4.0%) *C. muris* isolate. To the best of our knowledge, this is the first time *C. muris* is described in South Africa, this adds to the diversity of *Cryptosporidium* spp infecting humans. Similar species was identified in Slovakia in two immunocompetent patients with clinical symptoms (Petrincová *et al.,* 2015) and in children of between 0 to 14 years old, where it was reported that *C. muris* infection was associated with zoonotic potential of rodent-borne *Cryptosporidium* in areas with poor hygiene conditions and living with animals (Hasajová *et al.,* 2014). Therefore, since these species were identified in the rural areas, similar situation could apply.

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Twenty *C. hominis* isolates were identified in rural areas. The number of *C. hominis* isolates identified in diarrheal and non-diarrheal samples was equal (36.0% each), suggesting that *C. hominis* cause both symptomatic and asymptomatic infections. Females were more infected compared to males. In urban area, our findings suggested that *C. hominis* comes from symptomatic individuals. This is similar with the case of a 2 year old girl reported in Northern Iran (Ghaffari and Kkalantari, 2012). It is difficult to say that the infection was coming from being in contact with animals, since in urban areas there are no such animals like cattle and goats except few pets kept by few individuals.

Most animal samples that were tested in the present study were from adult cattle and goats. According to the previous reports adult animals are mostly asymptomatic while shedding small numbers of oocysts (Casemore *et al.,* 1997; Fayer, 1997; O'Donoghue, 1995). Therefore, low prevalence of *Cryptosporidium* infection was expected in this study. Twelve animal samples were sequenced and two *Cryptosporidium* species were identified from cattle and goats. *C. parvum* was found to be the dominant species in cattle. A study in China reported the same results where they found *C. parvum* as the dominant species in pre-weaned calves whereas *C. andersoni* was identified in post- weaned calves and adult cattle (Gong *et al.,* 2017). The present study identified three *C. andersoni* isolates in samples obtained from adult cattle, and our results correlated with the results of the study from Indonesia (Ananta *et al.,* 2014) and United Kingdom (Wells *et al.,* 2015), meaning that *C. andersoni* is one of the common species in cattle of South Africa. However, there is still a need to investigate this with a large number of sequences.

Cryptosporidiosis can be asymptomatic or symptomatic in animals, and diarrhea is one of the signs of *Cryptosporidium* infection even in animals. The present study showed that *Cryptosporidium* isolates were mostly identified in non-diarrheal samples in which 11

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(91.7%) isolates were identified out of 12 sequences than in diarrheal samples. These results indicated that there was no association between infection with *Cryptosporidium* and the symptoms in animals. These results correlate with the results of the study in Korea where *Cryptosporidium* was not considered to cause hemorrhagic diarrhea in young calves due to low prevalence of *C. parvum* in calves with hemorrhagic diarrhea (Lee et al., 2016).

Out of 32 households participated in this study, 78.1% let their animals scavenge whereas few (21.9%) fed them in a container or on the floor at their compound. Animals that scavenge are at high risk of getting infected with *Cryptosporidium*, for example by consuming the oocysts associated with the grass in the bush, or by drinking water contaminated with *Cryptosporidium* from the rivers.

The owners of the cattle or goats in the households were mostly males 23 (65.7%) and especially the married people 62.9%. About 72% were elderly people of more than 35 years who owned animals, and most owners at least went to school but they mentioned that they were unemployed.

Other studies reported that thorough cleaning and changing the location of animals before reutilization maximizes the efficiency of preventive method from *Cryptosporidium* infection (Ruest *et al*., 1998; Mohammed *et al*., 1999). The present study showed that most households (84.4%) were keeping their animals in an open sheds which were not cleaned or either animals being changed to other locations. This means that chances of *Cryptosporidium* infections may increase.

The higher population density and lack of sanitation in a particular area can pose a risk of infection with *Cryptosporidium* in some instances. High population density was found to be

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associated with *C. hominis* cases in Scotland (Pollock *et al.,* 2009) and with the diversity of infections due to fewer community services such as sanitation in Argentina (Cimino *et al*., 2015). Although there were no figures to show the specific threshold for population density, there was no more than ten people in 32 households investigated in this study. However, most of them (93.7%) were using pit toilets and the bush to show that there is a lack of sanitation in the area. Therefore, there is a potential risk of *Cryptosporidium* infection in the community.

The present study found that about 40% of households were having at least one child less than 5 years, but the diarrhea was observed in only 3 households out of 35. There is no specific drug for the treatment of *Cryptosporidium* infections, but general parasitic drugs and advices on how to control and to prevent the infection can be given for free in public health centres in the study area. The majority of households 32 (91.4%) depend on public clinics and hospitals for treatment, whereas the studies indicated that person-to-person transmission of cryptosporidiosis infection may occur where population densities are high, including hospitals (USEPA, 1994). Therefore, regardless of them going for treatment, they are exposed to the person to person infection with *Cryptosporidium*.

Un-purified drinking water was reported as one of the risk factors for *Cryptosporidium* infection in three communities lacking adequate municipal water and sewage services in the United States-Mexico Border where *Cryptosporidium* infection rate was 70% (Redlinger *et al.,* 2002). The introduction of faecal matter to stored drinking water may result from several activities which includes scooping with contaminated hands in the container (Copeland *et al.,* 2009). The present study investigated the primary water source in the participating households and it was discovered that many (85.7%) households were depending on municipal water in the community. The advantage is that the water comes well treated and

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safe to drink, but the challenge is that most households (94.3%) stored water in the collection containers for days or even weeks, and they drew water from the storing containers by pouring directly or use cup instead of using spigot. Therefore, contamination of that water may be possible and only a small number (20.0%) of households practice drinking water treatment using the boiling method.

The study reported by Sauch *et al.,* (1991) showed that boiling drinking water for one minute was the best method for eliminating viable *C. parvum* oocysts. Their results were in agreement with a study from North Thames which found that *Cryptosporidium* outbreak was associated with un-boiled tap water (Willocks *et al.,* 1998). However, a study in the United States-Mexico Border by Redlinger *et al.,* (2002) found that treating water with sodium hypochlorite decreased the presence of *Cryptosporidium.* This practice may help the communities in our rural area since they lack sewage services hence storing water for a long time in their houses.

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5.2 CONCLUSION

Cryptosporidium was detected in high number in our study areas in general, and the species identified by direct sequencing were the most commonly detected around the world, *C. hominis* being the leading infectious species of *Cryptosporidium* in humans. *C. hominis* is still the dominant species infecting humans even in our area, and it is highly threatening in humans particularly those who are immunocompromised. The most interesting finding is, the first discovery of *C. muris* in humans in the South African population

In animals, *C. parvum* is the most dominant species in cattle and goats, however, *C. andersoni* is being described in our region for the first time. Therefore, attention is needed to prevent multiple infections in animals because it is a threat of zoonotic infections to humans. The increased infection could be a result of ignorance of advices from the health centres about preventing the infections, meaning that public awareness campaign on hygiene and good hygiene practices while caring for domestic animals is still needed in the rural areas.

5.3 RECOMMENDATIONS

The recommendation would be that people should take responsibility of keeping themselves away from contact with animals or at least reduce contacts with animals.

Practicing hygiene such as washing hands on regular basis after using the latrine or after changing diapers is essential.

Most importantly, they should take precautions on what they eat or drink particularly in the crowded places.

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5.4 LIMITATIONS OF THE STUDY

There was no clinical information of the samples used in the study, which could have affected the statistical analysis. This was due to the fact that the samples were collected from the clinics and they were de-identified.

Non homogenous sample size between rural and urban areas, and also between human and animal population. Sample collection depended mostly on the clinic staff who did not want to provide patients' information to third party.

The use of direct sequencing from the PCR products has limited the chances of identifying various species, which if cultured, possibly many species would have been identified and the method used for sequencing (the Sanger method) might have limited the identification of different species, which could have yielded better results if the next generation sequencing method was used.

The ELISA method appeared to be very efficient for the screening of samples as it detected more positive samples and was easy to perform. The advantage of ELISA clearly shows that *Cryptosporidium* antigen is detected even in non-viable oocysts. In both PCR techniques a number of disadvantages could have been occurred, which include presence of inhibitors, DNA denaturation after it has been extracted, as well as difficulties in optimisation. However, the findings suggest that real-time PCR is more sensitive for the detection of *Cryptosporidium* species from frozen stool samples. Further studies are needed in order to improve the detection and genotyping of *Cryptosporidium* in these communities.

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