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MOLECULAR CHARACTERIZATION OF *CRYPTOSPORIDIUM* FROM HUMAN AND ANIMAL STOOLS IN THE VHEMBE AND MOPANI REGIONS OF LIMPOPO PROVINCE

By

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DEDICATIONS

For Constance, my sister.

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"No matter what accomplishment you have-somebody helped you"-Althea Gibson.

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DECLARATION

I declare that the contents of this dissertation entitled "**Molecular characterization of *Cryptosporidium* from human and animal stools in the Vhembe and Mopani regions of Limpopo province**" was written and completed by myself, unless otherwise acknowledged and has not been submitted in support of an application for a degree or qualification in this or any other university or institution.

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ABSTRACT

Cryptosporidium species are important causes of diarrhoea especially in developing countries. The epidemiology of *Cryptosporidium* has been dynamically changing over the past decade from that of a largely asymptomatic infection to an acute enteric disease of animals and humans. *Cryptosporidium parvum* has been reported in both animals and humans. This study sought to determine the prevalence of *Cryptosporidium* in animals and humans, to identify the prevalent genotype and to determine the zoonotic threat of *Cryptosporidium* in the Vhembe and Mopani regions.

In the present study, a total of 333 animal and 313 human stool samples were collected from the Mopani and Vhembe regions of Limpopo province. The samples were screened for *Cryptosporidium* using the modified Ziehl-Neelson acid fast staining. Caesium chloride flotation was used to purify oocysts that were further used to extract genomic DNA. Molecular detection was done by a multilocus PCR using 18SrRNA, HSP-70 and GP60 as target genes. PCR amplicons were further sequenced using the Sanger DNA sequencing method. Sequences were aligned using BioEdit and phylogenetic trees computed using MEGA5.

The present study reports a Cryptosporidium prevalence of 10.5% among the humans and 31.3% among the animals by Ziehl-Neelson microscopy. From the 10.5% found positive by microscopy, 85% of these were confirmed *C. hominis* in humans by PCR using HSP-70 as the target gene. From the 31.3% prevalence in animals stool samples, we found 7.4% of *C. parvum*, 33.3% of *C. suis*, 41% of *C. andersoni*, 4% of *C. galli* and 15% of *C. scrofarum* using 18SrRNA as the target gene. The GP60 gene failed to produce any desired amplicons probably due to lack of sensitivity in our region.

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This study can conclude that *Cryptosporidium* is common among inhabitants of the Mopani and Vhembe regions and this may account for the persistent diarrhoea commonly observed in the region although this remains to be confirmed. Moreover, cattle are in abundance in these regions and interaction with humans increases the risks of cross transmission. Further, this study reports a rare occurrence of *C. suis* in cattle and a lower prevalence of *C. parvum* in cattle while there was high prevalence of *C. andersoni*.

Keywords: *Cryptosporidium*, human, animal, 18S rRNA, HSP-70

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
BHK	Baby Hamster Kidney
BSA	Bovine Serum Albumin
CA	California
CD4	Cluster of Differentiation 4
CF	Complement Fixation
C-src	Proto-oncogene protein pp60
DFA	Direct Immunoflurescent Antiboby
DNA	Deoxyribose Nuceic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
gp60	Glycoprotein 60
gp900	Glycoprotein 900
HAART	Highly Active Antiretroviral Therapy
HFL	Human Fetal Lung

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HIV	Human Immunodeficiency Virus
HR	Pradish peroxidase
HRT	Human Rectal Tumour
HSP70	Heat Shock Protein 70
IFA	Indirect Immunofluorescent Antibody
JHB	Johannesburg
K_2Cr_2O7	Potassium Dichromate
LDH	Lactate dehydrogenase
MEGA6	Molecular Evolutionary Genetics Analysis 6
MLGs	Multilocus genotypes
NF-κB	Nuclear Factor-kappa B
PCK	Primary Chicken Kidney
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
PK-10	Porcine Kidney
qPCR	quantitative Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
rDNA	Recombinant Deoxyribonucleic Acid

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RFLP	Restriction Fragment Length Polymorphism
RPMI 1640	Roswell Park Memorial Institute medium 1640
rRNA	ribosomal Ribonucleic Acid
UK	United Kingdom
USA	United States of America
WHO	World Health Organization
ZN	Ziehl-Neelson

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CHAPTER ONE

BACKGROUND AND STUDY OBJECTIVES

1.1 Historical Background

Clarke J.J. first described *Cryptosporidium* as swarm spores lying upon the gastric epithelium of mice in 1895 (Clarke, 1895). These "spores" which were probably the motile merozoites of *Cryptosporidium muris* were later confirmed and described as *Cryptosporidium* in 1907 by the American parasitologist Ernest Edward Tyzzer from the gastric epithelium of laboratory mice (*Mus musculus*) (Tyzzer, 1907). Consequently, because unlike the previously known coccidian, the oocyst of this parasite did not have sporocysts surrounding the sporozoites, these small coccidian organisms were placed in a new genus called *Cryptosporidium* which means hidden sporocysts (Current and Garcia, 1991). Following these findings, in 1910, Tyzzer proceeded to give a plausible and detailed description of the various life cycle stages of *C. muris* (Tyzzer, 1910). In 1912, Tyzzer reported the description of a protozoan parasite smaller than *C. muris* and based on its life cycle, experimental infection and morphology, he proposed a new *Cryptosporidium* species called *C. parvum* (Tyzzer, 1912). Moreover, in 1929, Tyzzer also reported the first description of *Cryptosporidium* in birds and considered it to be *C. parvum*; this finding was however not confirmed (Tyzzer, 1929).

In spite of all these findings, *Cryptosporidium* was still not recognized as a pathogen until 1955 during an outbreak of diarrhoea in a turkey flock (Slavin, 1955). However, it was only until 1970 that *Cryptosporidium* species were considered economically or medically important with the veterinary significance highlighted by the association of *C. parvum* with bovine diarrhoea (Panciera *et al.*, 1971). Since then, a number of sporadic cases and outbreak

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reports have been published and *C. parvum* is now considered as an important cause of neonatal diarrhoea in calves and lambs associated with economic loses (Meuten *et al.*, 1974; Tzipori *et al.*, 1980; Holland, 1990; Moore and Zeman, 1991; Current and Garcia, 1991; De Graaf *et al.*, 1999; O'Handley and Olson, 2006). The first case of human cryptosporidiosis associated with watery diarrhoea was reported in 1976 (Meisel *et al.*, 1976, Nime., 1976). However, its medical significance was highlighted when in one study report *Cryptosporidium* was recognized as a common cause of acute diarrhoea in immune competent individuals when *Cryptosporidium* occurred in twelve immune competent individuals who had direct contact with the faeces of infected calves during three unrelated outbreaks of calf cryptosporidiosis. Among these twelve, nine of them had with abdominal cramps that lasted 1 to 10 days (Current *et al.*, 1983). A separate study also reported coinciding findings with a 4.1% of 884 hospital patients with gastroenteritis excreting *Cryptosporidium* oocysts in their stools. These infections were also accompanied by vomiting, anorexia and abdominal pains (Tzipori et al., 1983). Although the first waterborne cryptosporidiosis outbreak was in 1985 (D'Antonio et al., 1985), the 1993 Milwaukee massive outbreak was the highlight of the significance of *Cryptosporidium* as a waterborne pathogen affecting 403 000 persons (Mackenzie et al., 1994) and costing state over 90 million US dollars (Corso *et al.*, 2003).

To date, according to Xiao and Fayer (2008), there are 18 valid *Cryptosporidium* species with different specific host and among the 18; eight are known to also infect humans. Below is a table summarising the valid *Cryptosporidium* species, their original description and the first host in which they were first found.

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Table 1: Representation of valid *Cryptosporidium* species and their original description and host (Reproduced from Xiao and Fayer (2008)).

Species	First description	Host at first description
Cryptosporidium andersoni	Lindsay *et al.*, 2000	*Bos Taurus* (domestic cattle)
Cryptosporidium baileyi	Current *et al.*, 1986	*Gallus gallus* (chicken)
Cryptosporidium bovis	Fayer *et al.*, 2005	*Bos Taurus* (domestic cattle)
Cryptosporidium canis	Fayer *et al.*, 2001	*Canis familiaris* (domestic dog)
Cryptosporidium fayeri	Ryan *et al.*, 2008	*Macropus rufus* (red kangaroo)
Cryptosporidium felis	Iseki, 1979	*Felis catus* (domestic cat)
Cryptosporidium galli	Pavlasek, 1999	*Gallus gallus* (chicken)
Cryptosporidium hominis	Morgan-Ryan *et al.*, 2002	*Homo sapiens* (human)
Cryptosporidium macropodum	Power and Ryan, 2008	*Macropus giganteus* (grey kangaroo)
Cryptosporidium meleagridis	Slavin, 1955	*Meleagris gallopavo* (turkey)
Cryptosporidium molnari	Avarez-Pellitero and Sitja-Bobadila, 2002	*Sparus aurata* (gilthead sea bream) *Dicentrarchus labrax* (European seabus)
Cryptosporidium muris	Tyzzer, 1910	*Mus musculus* (house mouse)
Cryptosporidium parvum	Tyzzer, 1912	*Mus musculus* (house mouse)
Cryptosporidium scophthalmi	Alvarez-Pellitero *et al.* (2004)	*Scophthalmi maximus* (turbot)
	Levine (1980)	*Elaphe guttata* (corn snake)
Cryptosporidium serpentis	Brownstein *et al.*, (1977)	*Elaphe subocularis* (rat snake)
Cryptosporidium suis	Ryan *et al.*, 2004	*Sus scrofa* (domestic pig)
Cryptosporidium varanii	Pavlasek *et al.*, 1995	*Varamus prasinus* (Emerald monitor)
Cryptosporidium wrairi	Vetterling *et al.*, 1971	*Carvia porcellus* (guinea pig)

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1.2 Rationale

Cryptosporidium species are important causes of diarrhoea in developing countries (Griffiths, (1998); Dillingham *et al.*, (2002); Huang *et al.*, (2004); Shirley *et al.*, (2012)). To date, a vaccine is still lacking for the prevention of cryptosporidiosis. However, nitazoxanide, the only drug that has shown efficacy in immune-competent individuals (Smith and Corcoran, 2004) is still ineffective in immune-compromised individuals (Abubakar *et al.*, (2007); Amadi *et al.*, (2009)). The epidemiology of cryptosporidiosis, an infection caused by several genotypically diverse *Cryptosporidium* species, has been dynamically changing over the past decade from that of a largely asymptomatic infection to an acute enteric disease of animals and humans (Ramirez *et al.*, 2004). Transmission of the bovine genotype of *C. parvum* from calves to humans has been reported (Miran *et al.*, (1991); Lengerich *et al.*, (1993); Al Mawly *et al.*, (2015)) and it is estimated that 50% or more of all dairy calves will shed detectable numbers of oocysts and that the parasite is present on more than 90% of all USA dairy farms (Sischo *et al.*, 2000). Further, it has been noted that there is no significant difference between children and HIV-1 adults in the distribution of *C. hominis, C. parvum, C. meleagridis, C. felis, and C. canis* (Xiao *et al.*, (2001); Cama *et al.*, (2003)). From these we can extrapolate that in as much as there is treatment for immune-competent individuals, preventative measures are failing to contain zoonotic transmission of *Cryptosporidium.*

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1.3 Aim

The absence of parallel data on the epidemiological trends and molecular characterization of animal and human isolates in the Limpopo region has made diagnosis of *Cryptosporidium* challenging and transmission trends more complex to deal with. Culminately, this study sought to determine the genetic characteristics of *Cryptosporidium* from human and animal isolates in the Limpopo region.

1.4 Objectives

- To determine the prevalence of *Cryptosporidium* among animals and humans in the Vhembe and Mopani Districts of the Limpopo Province.
- To identify the prevalent *Cryptosporidium* genotypes in both human and animal samples.
- To determine the zoonotic threat through correlation of data received from both isolate types.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomy of *Cryptosporidium*

An experimental study documented in 1980 demonstrated that isolates of *Cryptosporidium* obtained from lambs, calves and humans were able to infect seven different animals (Tzipori *et al.*, 1980). Moreover, the acute diarrhoea in lambs caused by the isolate transmitted from humans was indistinguishable from that caused by other animal isolates. This observation indicated the potential zoonotic nature of *Cryptosporidium* (Tzipori *et al.*, 1980). It was based on these observations that the naming of *Cryptosporidium* species after their hosts became questionable (Tzipori *et al.*, 1980).

According to Lee *et al.*, 2000, the most current classification of *Cryptosporidium* as published by the Protozoologists Society is as follows:

Phyllum : **Apicomplexa** determined by Levine, 1970

Class : **Conoidasida** determined by Levine, 1988

Subclass : **Coccidiasina** determined by Leukart, 1879

Order : **Eucoccidiorida** reported by Leger and Duboscq, 1910

Suborder : **Eimeriorina** reported by Leger, 1911

Family : **Cryptosporidiidae** reported by Leger, 1911

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Genus : ***Cryptosporidium*** reported by Tyzzer, 1907.

Since the introduction of molecular evidence tools in the classification of *Cryptosporidium*, the taxonomic status of *Cryptosporidium* has been undergoing rapid changes because of new information that is supported by molecular data (Fayer, 2004). Interestingly, based on SSU rRNA gene sequences, Carreno *et al.*, (1999) showed that *Cryptosporidium* species (*C. baileyi, C. parvum, C. wrairi, C. serpentis, C. muris*) form a monophyletic clade that is a sister group to the gregarines which are parasites of invertebrates belonging to the subclass Gregarinasina. Further, this gregarine-*Cryptosporidium* clade was separated from the coccidian clade which comprised of *Sarcocystis, Toxoplasma* and *Eimeria* and supported by a 95% bootstrap value. Since the original description of *C. muris* and *C. parvum*, *Cryptosporidium* was commonly confused with members of the *Sarcocystis* as they have similar morphological characteristics such as thin walled oocysts which burst releasing free sporocysts each containing four sporozoites (Xiao *et al.*, 2004). This confusion was however resolved when the criteria of possession of a unique attachment organelle was used as a key issue to define the family Cryptosporidiidae and the genus *Cryptosporidium* (Xiao *et al.*, 2004).

2.2 Life Cycle

The *Cryptosporidium* life cycle is initiated by changes in temperature, pH changes, presence of bile salts and pancreatic enzymes (Reduker *et al.*, 1985). The life cycle begins with the ingestion of sporulated oocysts by the susceptible host; the oocystsexcysts and release four infective sporozoites through the suture (Morgan-Ryan *et al.*, 2002). The released sporozoites glide over the intestinal wall and releases materials from the apical complex (Okhuysen and Chappell, 2002) allowing the zoites to migrate across the surface of host cells and to actively invade them (Wetzel *et al.*, 2005). During this motility, the sporozoites deposits trails of

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proteins which facilitate attachment and invasion of the parasite into the host cells as they glide over the intestinal walls (Wanyiri and Ward, 2006).

The sporozoites develop into spherical trophozoites, which in turn undergoes merogony and forms type I meronts containing eight merozoites (O'Donoghue, 1995). The merozoites are released and return back and attach to the surface of an epithelial cell where they undergo merogony again and either form a type I or type II meront. Type II meronts each contain four merozoites which are released to attach to the epithelium but instead of developing into further meronts, they initiate gametogony (Robinson, 2005). Individual merozoites produce either microgamonts which undergoes nuclear division and differentiates to form up to 16 microgametes which, when released from the parasitophorous vacuole, they locate and fertilise a unicellular microgametocyte that has developed from a macrogamont. The product of fertilization, the zygote, undergoes two sexual cycles of sporogony to produce an oocyst with either a thick or a thin wall containing four sporozoites (Figure 1) (Current and Reese, 1986). The thick walled oocysts are then released into the lumen of the intestines and excreted from the host with the faeces and are immediately infective allowing spread of the infection to vulnerable hosts (Smith and Rose, 1998). However, the thin walled oocysts excyst and induce auto-infection and contribute to persistent chronic infections and the very low infective dose of *Cryptosporidium* (DuPont *et al*., 1995; Okhuysen *et al*., 1999; Jokipii and Jokipii, 1986).

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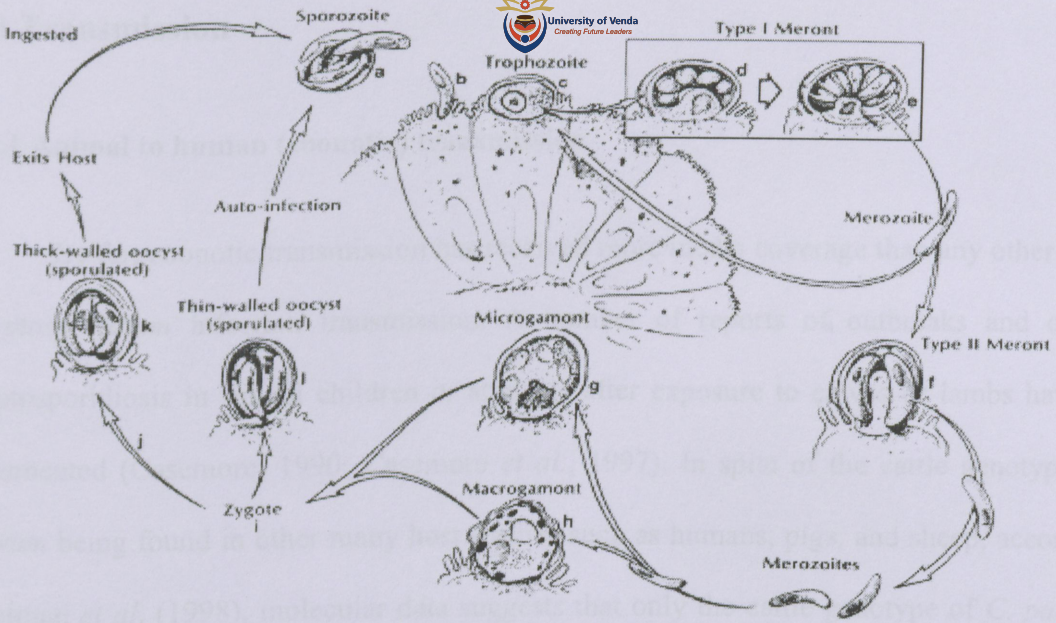


Figure 2.1: Life cycle of *Cryptosporidium* (Current and Garcia, 1991).

(a) The oocyst excysts in the lumen of the intestine, releasing the infective sporozoites. (b) The sporozoite attaches to the surface of the enterocyte. (c) The sporozoite penetrates into host cells and develops into trophozoites within a parasitophorous vacuole. (d) and (e) The trophozoites undergo merogony to form merozoites. (f) After being released from type I meront, the invasive merozoites enter adjacent host cells to form additional type I meronts (recycling of type I meronts) or to form type II meronts. (g) and (h) Type II meronts enter host cells to form the sexual stages microgamonts and macrogamonts. (i) The zygote, product of fertilization of the macrogamont by the microgametes (released from microgamont). (j) The majority (80%) of the zygotes develop into environmentally resistant, thick-walled oocysts. (k) The thick walled oocysts undergo sporogony to form sporulated oocysts containing four sporozoites, which will be released in the faeces and allow transmission of the infection. (l) A smaller proportion (20%) of the zygotes have a unit membrane surrounding the four sporozoites, these are the thin-walled oocysts, which are the auto-infective forms that can maintain the parasites in the host without repeated exposure.

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2.3 Transmission

2.3.1 Animal to human (zoonotic) transmission

By far, zoonotic transmission has received more media coverage than any other form of *Cryptosporidium* infection transmission. A number of reports of outbreaks and cases of cryptosporidiosis in school children or students after exposure to calves or lambs have been documented (Casemore, 1990; Casemore *et al.*, 1997). In spite of the cattle genotype of *C. parvum* being found in other many host species such as humans, pigs, and sheep, according to Sulaiman *et al.* (1998), molecular data suggests that only the cattle genotype of *C. parvum* is capable of zoonotic transmission. The high prevalence of *C. parvum* in cattle and sheep with the high count of oocysts shed by infected newborn animals makes cattle and sheep significant sources of environmental contamination with *Cryptosporidium* oocysts that are able to infect humans (WHO, 2009). In addition, according to Casemore (1990), there exists indirect evidence suggesting that contact with horses or horse manure is a risk factor for cryptosporidiosis. However, according to WHO (2009), only immunosuppressed horses have been shown to carry the cattle genotype of *C. parvum* whereas the immunocompetent horses carry a specific horse genotype. It has been demonstrated that a variety of wildlife animals are hosts to specific *Cryptosporidium* species that are not found in other animals including humans (Samra *et al.*, 2013). More importantly to this point, is that these animals shed oocysts into the environment thus contributing to the total *Cryptosporidium* load in drinking water sources (WHO, 2009).

2.3.2 Human to human transmission

In the views of Fayer and Ungar, (1986), Casemore, (1990) and Cordell and Addis, (1994), the human to human transmission route has been abundantly reported in day care

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centres among care givers and children. Moreover, Casemore *et al.* (1994), has reported possibilities of patient to patient or patient to health care staff transmission in hospitals. Irresponsible oro-anal sexual practices yield a high risk for exposure to *Cryptosporidium* oocysts (WHO, 2009). Foreign travel to countries with a high *Cryptosporidium* prevalence has also been implicated (WHO, 2009).

2.4 Epidemiology

Before we can deliberate on the epidemiological patterns of *Cryptosporidium*, it is imperative that we acknowledge several factors that are pivotal to the epidemiology of *Cryptosporidium* as outlined by Dillingham *et al.*, 2002 as these factors render the control and total eradication of cryptosporidial infections somewhat difficult.

i. Fayer, 2008, reported that the *Cryptosporidium* oocysts are highly resistant and can survive freezing at -10°C for a week and up to 4 days in drying stool.

ii. Further, the oocysts can withstand the effects of most disinfectants, most notably chlorination, iodophor, cresylic acid, 5% formaldehyde (Barbee *et al.*, 1999; Biswas *et al.*, 2003; Chauret *et al.*, 2001; Drescher *et al.*, 2001; Venczel *et al.*, 1997). In 1999, Okhuysen and colleagues reported that the infective dose of *Cryptosporidium* is very low and as low as nine oocysts of have been proven to be infectious for humans. Moreover, 50 oocysts have been shown to cause infection in calves (Moore *et al.*, 2003).

iii. The sporulated oocysts are readily infective at shedding and consequently, a new host can be infected immediately (Current and Garcia, 1991).

iv. Zoonotic transmission can take place through direct contact or contamination of water, food, soil or tools.

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The infection rate of cryptosporidiosis differs significantly from one region to the other. However, it has been extensively shown that *Cryptosporidium* diarrhoeal infection is much greater in the third world countries than in more developed countries whereby infection is influenced by gender, ethnic background, different locations and age group and socio-economic conditions (Xiao *et al.*, 2004). *Cryptosporidium* parasites are estimated to be around 500 million annually in developing countries with the African continent accounting for about 20-35% of annual infections and reportedly 32.5 to 40% of infections in the Sub-Saharan Africa (Kfir *et al.*, 2000). A comparative survey reported an average of 2-3 and 5% of patients hospitalized for diarrhoea in the United Kingdom and United States to be infected with chronic cryptosporidiosis whereas 40% in some third world countries was reported (Xiao *et al.*, 2004).

According to Current and Garcia (1991) however, prevalence rates of *Cryptosporidium* are 1-4% in Europe and North America and 3-20% in Africa, Asia, Australia and South and Central America. A lot of parasite distribution studies in other African countries such as Kenya have also been conducted. A survey done in Kenya over a year period reported 4% cryptosporidiosis prevalence (Gatei *et al.*, 2006). Most alarming however the prevalence is rates of *Cryptosporidium* among children and young animals. A study done in North Eastern Brazil by Lee *et al.* (2005), reported a cryptosporidiosis prevalence of 94% among children. In KwaZulu Natal, east coast of South Africa, *Cryptosporidium* was found to be endemic with a laboratory-confirmed prevalence ranging from 2.9 to 3.7% and 2.9 to 3.0% of cases submitted for analysis respectively (Jarmey-Swan *et al.*, 2001). In the Venda region of the Limpopo province, far north-eastern parts of South Africa, Samie *et al.* (2006) documented the highest prevalence of *Cryptosporidium* among people in the age range of 50-59 years with individuals in the age range of 2-5 years having the second highest prevalence.

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2.4.2. Epidemiology of *Cryptosporidium* in animals

The reporting of *Cryptosporidium* species from more than one host harbouring many species that can cross host species barriers to other hosts has led to a rather more complex epidemiological trend of the coccidian parasite (Fayer, 2008; Smith, 2008). A lot of studies have been done on the prevalence of cryptosporidiosis in both tamed or domestic and wild animals. Most notably, cryptosporidiosis prevalence have been on the rise in cattle with different species including *C. bovis*, *C. andersoni* and *C. parvum* being responsible with *C. parvum* proving the most predominant parasite in cattle, goats, and sheep (Zintl *et al*, 2006). This protozoal infection has reportedly been characterised by diarrhoea, anorexia, abdominal pains, apathy and depression in calves, lambs and goat kids which led to suggestions by Casey, (1991); de Graaf *et al*., (1999) and Lefay *et al*., (2001) that the parasite destruction of the intestinal epithelia of its host may render increased susceptibility to other enteric pathogens. In older animals, a possible explanation for the subclinical infections and lower *Cryptosporidium* prevalence may be attributed to a lot of factors such as age-related resistance due to maturation of the intestinal mucosa (Harp *et al*., 1990). The latter notion was further strengthened by the accounts of Harp (2003) and Akili *et al*., (2006) who reported that a 54-kDa protein present in the intestinal mucosa from adult mice and bovines prevented *Cryptosporidium* infection in mouse pups.

In Malawi, a comparative study on the seasonal prevalence of *Cryptosporidium* reported the parasite to be more prevalent in cattle with 37.7%, 7.46% and 7.8% in the cool, dry and rainy seasons respectively (Banda, *et al*., 2009). Interestingly and congruent to other reports as those reiterated by Casey *et al*., (1991); de Graaf, *et al*., (1999); and Lefay *et al*., (2001); Banda *et al*., (2009) also reported calves and heifers having an infection rate going up

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to 50% in one particular season as compared to adult animals. In the Iringa and Tanga regions of Tanzania *Cryptosporidium* prevalence was found to be highest at 57% for calves less than 3 years of age compared to 31% and 12% of those between 3 to <6 and >6 years respectively (Widmer and Lee, 2010). Animals such as chickens and sheep have received a lot of research attention as some of the hosts for *Cryptosporidium*. Similar to cattle, the prevalence is a lot high in younger chickens and sheep than in matured ones.

In sheep, the infection causes mild to severe diarrhoea in lambs of between 5 to 12-day-old and this is mostly characterized by symptoms such as depression, anorexia, weight loss and the shedding of a large number of oocysts in the stool (Angus *et al.,* (1982); Thompson *et al.*, (2005)). Cryptosporidiosis was first described in sheep in Australia in 3-weeks old lambs which had diarrhoea in 1974 (Barker and Carbonell, 1974) and this was later confirmed in 1982 by Angus and colleagues in an experimental and natural infections studies. Beyond this, infections were reported from various regions such as USA (77.4%) (Santin *et al.*, 2007), Canada, Iran (17.2%) as reported by Nouri and Karami (1991), Spain (59%) which was reported by Causape *et al.*, (2002) and Italy with a significant prevalence that varied from 4%-85% (de Graaf *et al.*, 1999). Moreover, further studies which have been carried throughout the world about the spread of *Cryptosporidium species* in sheep includes Zambia (12.5%) (Goma *et al.*, 2007), Poland (10.1%) (Majewska *et al.*, 2000), Tunisia (11.2%) (Soltane *et al.*, 2007) and the list is endless.

2.4.3. Zoonotic cryptosporidiosis.

Cryptosporidium-contaminated drinking water, food or recreational water and direct contact with infected persons or animals are raw examples of common infection sources for humans. The zoonotic *C. parvum* which has been widely reported in young livestock has also been reported in humans (Caccio and Putignani, 2014). The first human cryptosporidiosis

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causative species, *C. hominis,* which was widely accepted as human-host-bound (Morgan-Ryan *et al*., 2002) was recently reported in calves' population (Ghaffari *et al*., 2014). Furthermore, the report by Ghaffari *et al*., (2014) highlights the significant dangers of zoonosis. However, other reports on multilocus subtype analysis of human and cattle isolates have determined that zoonotic and anthroponotic *C. parvum* are present in human cases (Leoni *et al*., 2007; Ngouanesavanh *et al*., 2006; Mallon *et al*., 2003a; Mallon *et al*., 2003b). It was also reported by Mallon *et al*., (2003a, b) that most of the cattle and human *C. parvum* mulitlocus genotypes (MLGs) were identical and there was evidence of random mating in theses *C. parvum* populations which was an indication of frequent zoonotic transfer. In 2007, Hunter and colleagues reported a higher frequency of pre-infection animal contact in humans with certain alleles at two loci, these records were supportive of the zoonotic *C. parvum* transfer findings by Mallon *et al*., (2003a, b). Interestingly, of the anthroponotic alleles one was detected in urban areas and the other zoonotic allele was detected mostly in rural areas (Hunter *et al*., 2007).

Reports from Kiang *et al*., (2006); Roberson *et al*., (2006); Preiser *et al*., (2003) and Pohjola *et al*., (1986) imparts information about human *C. parvum* cases caused by caused by contact with infected calves. Notwithstanding, Silverlas *et al*, (2010), argued that even when zoonotic *C. parvum* gp60 subtypes or MLGs are isolated, cattle are not necessarily the source of infection as these subtypes may circulate in the human population in addition to the anthroponotic ones.

2.5 Pathogenesis of *Cryptosporidium*

Infection with *Cryptosporidium* begins when the ingested oocysts release sporozoites which attach to and invade the intestinal epithelial cells. The parasite mainly attacks the jejunum and terminal ileum but in patients with Acquired Immune Deficiency Syndrome (AIDS), other sites within the gastrointestinal tract which may include the stomach, duodenum

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and colon may be implicated (Hunter and Nichols, 2002). *Cryptosporidium* possesses quite a number of surface glycoproteins which are thought to play a role in the parasite pathogenesis. The antigens which have been shown to be important for the attachment and invasion of *Cryptosporidium* into the host cell are all mucin-like glycoproteins such as gp60 (Xiao *et al.*, 2002). The glycoproteins are located at the apical end of sporozoites and in microemes of merozoites and attach intimately to the microvillus membrane and causes loss of microvilli and effacement which results in mal-absorption. Following this, second-signal pathways, such as the nuclear factor-kappa B (NF-κB) and proto-oncogene protein pp60 (C-src) systems are activated. Activation of the (NF-κB) induces the production of cytokines and chemokines such as interleukin-8 (IL-8), which trigger an inflammatory reaction and stimulates anti-apoptotic survival signals in directly infected cells. Activation of C-src is associated with host cell cytoskeletal reorganization and perhaps dysfunction of tight junctions (Cevallos *et al.*, 2002).

2.5.1 Pathology of diarrhoea caused by *Cryptosporidium*

Presentation of clinical symptoms and their consistency is entirely dependent on the host immune response (Carey *et al.*, 2004). In immune competent individuals with a rather an immature mucosal for instance, diarrhoea is self-limiting and can take up to two weeks, whereas in immune compromised individual such as those with HIV/AIDS, *Cryptosporidium*-induced diarrhoea can take a minimum 2 days to a lifetime (Chen *et al.*, 2002). Thus far, no solid pathogenicity mechanism have been adopted (Carey *et al.*, 2004), however, a few have been suggested including the disruption of the microvillus surface area which compromises the normal functioning of internal absorption and digestion (Bird and Smith, 1980), presence of an enterotoxin activity which is possibly produced by the parasite or host (Argenzio *et al.*, 1993; Argenzio et al., 1990; Okhuysen and Chappell, 2002) and the adhesion factors affecting

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attachment of the parasite to host cells (Ceva *et al.*, 2000; Peterson *et al.*, 1992; Nesterenko *et al.*, 1999; Riggs *et al.*, 1997).

In an experimental study, Perkin and colleagues (1999) evaluated the effect of *C. parvum* infection on the epithelial barrier function by using matured polarized colonic epithelial cells. This was evaluated according to the electrical resistance across the monolayer, level of the intracellular enzyme lactate dehydrogenase (LDH), and the flux of horse radish peroxidase (HRP) across the monolayer as compared to heat-inactivated oocyst control (Adams *et al.*, 1994).

2.6 Diagnosis

Microscopy has been used as the primary detection method for many years by examining the stool samples for the presence of oocysts with the use of stains. Oocysts are recovered from stools and identified using differential staining methods such as safranin-methylene blue stain, modified Kinyoun's acid-fast method, modified Ziehl-Neelson and DMSO-carbolfuchsin stain (Cruz *et al.*, 2006). Sample preparation for direct observation is however time consuming and strenuous and so proper diagnosis depends on qualified laboratory personnel (Cruz *et al.*, 2006). The bare truth is, in areas of endemic prevalence where there are limited resources and skills, there is often misdiagnosis and thus inconvenience of health care.

All major intestinal parasite infections are still to date solely dependent on microscopy for diagnosis. Other parasite infections however, are confirmed after microscopy by serological and molecular based assays (Omoruyi *et al.*, 2010). The most widely used serology-based assay includes enzyme-linked immunosorbent assay (ELISA) which is also called enzyme

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immunoassay (EIA) (Moro and Schantz, 2009). Other serological assays less frequently used in comparison to ELISA include hemagglutination test, direct or indirect immunofluorescent antibody (IFA) or direct immunoflurescent antiboby (DFA) tests, complement fixation (CF) and immuno-blotting and rapid diagnostic tests (Moro and Schantz, 2009).

Although serology-based assays have some advantages to an extent, molecular based assays are by far the most convenient giving the most accurate results. PCR is capable of discriminating, among others, the taxonomic membership between *Cryptosporidium* strains that infect humans. Moreover, besides the conventional PCR, including the nested and multiplexed PCR, more robust and highly sensitive PCR methods such as the real-time PCR are available for the diagnosis of parasitic infections (Gasser, 2006).

2.7. Treatment, Prevention and Control

It was documented in two separate reports that nitazoxanide significantly shortened the duration of diarrhoea and decreased mortality in adults and malnourished children (Gargala, 2008; Cohen, 2005). Because this success was only limited to immune-competent patients, a combination therapy restoring immune response along with antimicrobial treatment of *Cryptosporidium* infection was introduced to control the infection (Gargala, 2008; Smith and Corcoran, 2004). Following an observation that the highly active antiretroviral therapy (HAART) increase patients' immune response, viral proliferation suppression and boosting the levels of CD4 lymphocytes (Schmidt *et al*., 2001), the HAART has been successfully used to treat cryptosporidia and microsporidia in HIV bound patients (Miao *et al*., 2000).

In 2001, following the food and mouth epidemic in the United Kingdom (UK), control measures such as limited access to the countryside, containment of animals and restriction of

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livestock for trade or to pastures were introduced. The implementation of these control measures saw a 36.5% decline in human cryptosporidiosis cases caused by *C. parvum* (Smerdon *et al.*, 2003; Hunter *et al.*, 2003). Apart from this, Ramirez *et al.* (2004) suggested ways to decrease the spread of oocysts in the environment and to prevent water supply contamination including good hygiene, good cattle husbandry, early diagnosis of cryptosporidiosis in domestic animals and household decontamination. Moreover, Hunter and colleagues (2004 b) reported that the awareness of specific risk factors and taking relevant actions such as washing hands after changing nappies should reduce *C. hominis* infection. In addition, avoiding sexual practices that can result in hand or mouth exposure to stool bearing oocysts is likely to assist the prevention and control of cryptosporidiosis (Robertson and Bruno, 1997).

2.8 Molecular Characterization of *Cryptosporidium*

In light of the failures presented by morphological discriminations between *Cryptosporidium species*, genetic characterization methods have been developed. *Cryptosporidium spp.* are morphologically similar; hence we can no longer describe them solely based on developmental studies and past morphological descriptions (Xiao *et al.*, 2004). Further, Xiao *et al.*, 2004 elaborated that most animals can be naturally infected with multiple *Cryptosporidium* species which cannot be differentiated from each other on the basis of methodology and developmental studies.

Most molecular methods currently used to discriminate between *Cryptosporidium* species and genotypes are based on polymerase chain reaction (PCR) followed by subtyping with restriction fragment length polymorphism (RFLP) and/or sequencing techniques (Jiang and Xiao, 2003). For identification of *Cryptosporidium* species, most recent studies have

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employed SSU rRNA gene based diagnostic tools it is sensitive and genus specific due to higher copy number of the target (Xiao, 2009). However, in many subtyping reports, the gp60 genetic locus is widely used. This is the case mainly because as Leave *et al.*, (2002) elaborate, "the gp40/15 alleles and proteins have an unprecedented degree of sequence diversity especially among the human genotype which thus far is greater than any other gene or protein studied in *C. parvum*". Molecular techniques for the detection of intraspecific variations have been developed. Such include isoenzyme profiles (Awad-El-Kariem *et al.*, 1995), random amplified polymorphic DNA (RAPD) (Morgan *et al.*, 1996), polythreonine motifs and oocyst wall protein (Carraway *et al.*, 1997; Spano *et al.*, 1997) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Bonnin *et al.*, 1996).

2.9 Molecular characterizing tools

2.9.1. The usage of the 18S rRNA as a genotyping tool

The first reported cloning and sequencing of the 18S rRNA was from *C. parvum* (Cai *et al.*, 1992). Some authors later determined the ribosomal RNA gene organisation in the same organism based on the hypothesis that the genes encoding the cytoplasmic ribosomal RNAs (rRNA) in most eukaryotes are organized into transcriptional units with a small subunit rRNA gene, a 1-5.8S rRNA gene in a 5'-3'orientation separated by internal transcribed spacers, which they called the rDNA unit which consists of two subunits, namely small and large subunits which are 1.7 and 3.6kb respectively (Le Blancq *et al.*, 1997). Further, Le Blancq and colleagues determined the size of the large subunit rRNA and concluded that the rDNA unit in *C. parvum* has the standard arrangement of 5' small subunit rRNA – internal transcribed spacer 1 – 5.8S rRNA internal transcribed spacer 2 – large subunit rRNA 3', the minimum size would

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be 6.5kb, there are five copies of the rDNA unit per haploid genome in *C. parvum*, there are two types of rDNA unit in *C. parvum* and rDNA units are dispersed through the genome of *C. parvum*. (Le Blancq *et al*., 1997).

2.9.2. Heat Shock Protein 70 (HSP70)

The HSP-70 gene codes for the 70-kDa heat shock protein family which is one that assist in protein folding in a variety of cellular components and that is broadly observed among prokaryotes and eukaryotes (Bakau and Horwich, 1998). The hsp70 was first described in *C. parvum* in 1955 (Khramtsov *et al*., 1995). Khramtsov and colleagues cloned the HSP 70 gene and described it as an intronless gene that codes for a protein of 674 amino acid residues with molecular mass of 73403 Da. In *C. parvum* this gene is located in chromosome 2 but it has orthologs or paralogs genes in other genomes regions as in *C hominis* and *C. muris* (CryptoDB). In a comparative study of the efficacy of the HSP-70 gene and the SSU rRNA loci, Sulaiman *et al*., (2000) reported that the HSP-70 gene locus offers several advantages over the SSU rRNA gene for phylogenetic studies of *Cryptosporidium* parasites as it is more permissive to nucleotide changes making it heterogeneous and makes it better for genotyping.

2.9.3 Intra-species molecular analysis

2.9.3.1 Gp15/45/60

Genotypic characterisation of numerous isolates of *Cryptosporidium* has led to the uncovering of new species and subtypes and a better understanding of the epidemiology, host adaptation, infection sources, taxonomy and transmission dynamics of the parasite (Widmer

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and Lee, 2010; Li *et al*., 2014; Xiao, 2010). Among others, the most commonly employed subtyping is based on the sequence analysis of the gp15/40 or gp60 gene.

The glycoprotein 60 kDa (gp60) protein was first described in *C. parvum* in the year 2000 (Strong *et al*., 2000). The gp60 was discovered by mistake by Strong and colleagues when they had actually sought to clone and sequence the sequence of an 11A5 antigen known to react with a monoclonal antibody which recognised a 15kDa surface glycoprotein left behind by motile sporozoites and recognised by several lectins that neutralised parasite infectivity in cultured epithelial cells. Astonishingly, Strong and colleagues realised that the cloned gene coded for a 330 amino acid, a mucin like glycoprotein that was predicted to contain an N-terminal signal peptide, a homopolymeric tract of serine residues, 36 sites of O-linked glycosylation, and a hydrophobic C-terminal peptide specifying attachment of a glycosyl-phosphatidylinositol anchor (Strong *et al*., 2000). Moreover, according to Strong *et al*., (2000), this gene is localised in chromosome 6 and has orthologs or paralogs in other genome regions in *C hominis* and *C. muris* and is expressed during merogony to produce a 60kDa precursor protein which is proteolytically cleaved to 15 and 45 kDa grycoprotein products which are localised to the surface of the sporozoites and merozoites. This gene, now named gp15/45/60, display a very high degree of sequence diversity among *C. parvum* isolates, with single-nucleotide and single-amino acid polymorphisms defining five to six allelic classes, each characterized by additional intra-allelic sequence variation (Strong *et al*., 2000). As a result, the greatest advantage of this gene is that it enables the discrimination of subtype families within the same genotype (Quilez *et al*., 2011) and can be used for establishing meaningful relationships between *Cryptosporidium* genotypes and phenotypes (O'Brien *et al*., 2008; Power *et al*., 2009; Strong *et al*., 2000).

Because of the prerogatives this gene holds, there has been widespread adoption of this marker, and with it, a number of alleles have been identified (Widner and Lee, 2010).

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However, because of the natural genetic recombination that occurs in *C. parvum* and *C. hominis* population structures, it has been suggested that a single locus marker such as the gp60 is not reliable (Widner, 2009).

2.10. *In vitro* propagation of *Cryptosporidium*

The cultivation of *Cryptosporidium in vitro* has not been marred with failures. In fact, a lot of researchers still struggle to reproduce published cultivation protocols. In 1983, the first successful cultivation of *Cryptosporidium* was reported using human rectal tumour (HRT) cell line maintained in RPMI 1640 which was supplemented with 10% heat inactivated fetal bovine serum (FBS) at 37°C and 5% of CO_2 (Woodmansee and Pohlenz, 1983). Following the latter, Current and Haynes (1984) reported the successful propagation of an anthroponotic *Cryptosporidium* isolate in human fetal lung (HFL), primary chicken kidney (PCK) and porcine kidney (PK-10) cells using isolates derived from an AIDS patient. Moreover, Current and Haynes (1984) further found that the HFL cells gave the greatest yield. The successful growth of a human *Cryptosporidium* isolate was described in baby hamster kidney (BHK) cells and chicken embryos (Naciri *et al*., 1984) and as with Current and Haynes (1984), Naciri and colleagues also obtained the isolates from an immunocompromised patient but propagated in goats. Bonnin and colleagues (1990) also reported the description of the asexual stages of *Cryptosporidium* species of human and animal origin in Lewis rat small intestine carcinoma cells. Cultivated oocysts storage is the same irrespective of which cell lines were used for propagation. The above reports all reported storage in 2.5% potassium dichromate (K_2Cr_2O7).In spite of these said successes, the past decade have seen less and less research output regarding successful cultivation of *Cryptosporidium in vitro*. Instead, researchers have resorted to animal infectivity models. Notwithstanding, the latter is the most direct method to

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assess the viability and infectivity of *Cryptosporidium* oocysts and this is done by inoculating an animal with oocysts followed by monitoring oocyst shedding and examining histological evidence (Carey *et al*., 2004). Moreover, animal infectivity models have been pace setters in the understanding of the pathology of *Cryptosporidium*, drug testing and immunology (Tzipori, 1998).

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CHAPTER THREE

METHODOLOGY

3.1 Ethical Considerations

Ethical clearance was obtained from the University of Venda Research and Ethics Committee. Authorization through a signed informed consent to collect samples was obtained from the patients and ethical clearance was obtained from hospital officials. Consent was also obtained from the owners of the cattle.

3.2 Human Subjects

All study participants wilfully filled up and signed a consent form which was binding but still made provision for them to terminate participation at any time should they feel uncomfortable. Confidentiality of information provided by volunteering subjects was guaranteed by keeping all records under lock and key where only the study leader has access.

3.3 Animal Subjects

Samples collected from the cattle subjects did not involve any form of physical contact whatsoever with the cows. There was no use of any instruments which may inflict pain or cause any discomfort to the animals

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3.4 Subject Selection and Data Collection

Three-hundred-and-thirty-three (333) human stool samples and 313 cattle stool samples were collected. Human stool samples were collected from Nkomo and Makhuva clinics in Giyani. Biographical information such as gender, age, and stool characteristics (consistency), was recorded. For animals, fresh stool samples were collected in Giyani at Nkomo, Ngove and Makuleke villages. Information such as location (village), age, source of drinking water and diarrhoea were also collected.

3.5 Sample Processing

Upon arrival at the laboratory, both human and animal stool samples were observed in a modified Ziehl-Neelson microscopy test for screening of *Cryptosporidium* oocysts as described by Adamu *et al*., (2010).

3.6 Cesium Chloride Flotation Oocyst Purification

Stool samples were be diluted with 0.85% saline, homogenized and passed through a #60 mesh sieve to remove large particles and the supernatant allowed to settle overnight. The stool suspension was then be applied to a sucrose gradient and centrifuged at 13,400rpm. The oocyst-enriched layer that forms was recovered between the sucrose layers. The sucrose gradient purified oocysts was then applied to a cesium chloride gradient and centrifuged at 13,400rpm. Oocyst enriched layer that forms at the top of the sample was recovered and preserved in 2.5% of potassium dichromate (Arrowood, 2002) for further analysis.

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3.7 DNA Extraction

Genomic DNA was isolated from the purified oocysts using the QIAamp Stool Mini Kit from Qiagen (Valencia, CA, USA). Acid-washed glass beads were added to 2ml eppendorf tubes and 0.25g of the purified oocyst from the faecal samples was added to the 2ml tubes. 1.4ml of ASL buffer will be added to each sample tube. Tubes were placed in the mini bead-beater (Lasec SA) for 1 minute for each sample tube to disrupt the oocyst wall. An inhibiTEX tablet was be used to remove DNA inhibitors to optimise DNA recovery. After numerous washes with buffers, DNA was eluted to new 1.5ml Eppendorf tubes with buffer AE and stored at -20°C for further analysis. For quality control purposes, eluted DNA was run in a gel to observe presence of DNA.

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3.8. Molecular detection of *Cryptosporidium*

Molecular characterization of *Cryptosporidium* from animal isolates.

Table 3.1. PCR primers and their specifications used for screening and sequencing of animal isolates.

Target gene	Primer set	Annealing Temp. °C	Fragment Size	Reference
SCREENING				
18S rRNA	18SiF (5′-AGT GAC AAG AAA TAA CAA TAC AGG-3′) 18SiR (5′-CCT GCT TTA AGC ACT CTA ATT TTC-3′).	60	298bp	Morgan *et al.*, (1997)
SEQUENCING				
18S rRNA	18SiF (5′-AGT GAC AAG AAA TAA CAA TAC AGG-3′) 18SiR (5′-CCT GCT TTA AGC ACT CTA ATT TTC-3′).	60	298	Morgan *et al.*, 1997

A 25µl reaction consisting of 12.5µl of OneTaq® mastermix, 0.5µl of 18SiF 0.5µl of 18SiR primers, 0.5µl of BSA, 6µl of PCR grade water ad 5µl of genomic DNA. This mixture

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was preheated at 94°C for 2min, amplified for 40 cycles at 94°C for 30s, 60°C for 30s, 72°C for 30s, and subjected to a final extension at 72°C for 7min (Morgan *et al.*, 1997).

Molecular characterization of *Cryptosporidium* from human isolates

Table 3.2. PCR primers and their specifications used for molecular analysis of the human isolates

Target gene	Primer set	Anneal Temp.	Size	Ref
SCREENING				
18S rRNA	18SiF (5′-AGTGACAAGAAATAACAATACAGG-3′) 18SiR (5′-CCTGCTTTAAGCACTCTAATTTTC-3′).	60	298	Morgan *et al.*, 1997
GENOTYPING				
Hsp1	5′-GTTGGTGTATGGAGAAACGATA-3′	55	Forward primer	
Hsp13	5′-CTGTGTTTTCTGGATTTCTAGCAACT-3′	55	148	*C. hominis*
Hsp5	5′-CAGGTACGGTGACGACGGCGTTCTTAATTG-3′	55	394	*C. canis*
Hsp6	5′-TGTACCACCTCCCAGATCGAAAATCAGG-3′	55	564	*C. felis*
Hsp8	5′-CGCATTGAGTTCTGAGTCTTCTTAAAG-3′	55	757	*C. parvum*
SUBTYPING				
GP60	Cpgp401 F-(5′-ATGAGATTGTCGCTCATT-3′) Cpgp401R-(5′-TTACAACACGAATAAGGC-3′	55	850	Leave *et al.*, 2002
GP60	Cpgp402F-5′ -ATGAGATTGTCGCTCATT-3′ Cpgp402F-5′ -TTACAACACGAATAAGGC-3′	55	850	Leave *et al.*, 2002

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A 25µl reaction consisting of 12.5µl of OneTaq® mastermix, 0.5µl of 18SiF 0.5µl of 18SiR primers, 0.5µl of BSA, 6µl of PCR grade water ad 5µl of genomic DNA. This mixture was preheated at 94°C for 2min, amplified for 40 cycles at 94°C for 30s, 60°C for 30s, 72°C for 30s, and subjected to a final extension at 72°C for 7min (Morgan *et al.*, 1997).

Multiplex primers for HSP-70 were run in a 25µl reaction consisting of OneTaq® mastermix, 0.5µl of the forward primer HSP1, 0.5µl of HSP5, HSP6, HSP8 and HSP13 respectively, 0.5µl of BSA, 4.5µl of PCR grade water and 5µl of genomic DNA. This mixture was preheated at 94 for 3min and amplified for 45 cycles of 94 °C for 50 sec, 55 °C for 50 sec, with final elongation at 72 °C for 1 min. Polymerase chain reactions were performed in a G-storm (Vacutec) thermocycler (Lindergard *et al.*, 2003).

A highly polymorphic region of the gp60 was amplified using primers Cp(GP401) F (5′-ATGAGATTGTCGCTCATT-3′) and Cp (GP401)R (5′-TTACAACACGAATAAGGC-3′ which produce a 850bp product. In a 25µl reaction, 12.5µl OneTaq, 0.5µl of 18SiF, 0.5µl of 18SiR, 0.25µl of BSA, 6.25µl of nuclease free water and 5µl of genomic DNA. The mixture will be subjected to an initial hot start at 95°C for 2 minutes followed by 40 cycles each consisting of 95°C for 45 seconds, 55°C for 45 seconds and 72°C for 2 minute and a final extension at 72°C for 10 minutes. For samples in which PCR products were not obtained using the first set of primers, a second PCR was performed using primers

5′-ATGAGATTGTCGCTCATT-3′ and 5′ -TTACAACACGAATAAGGC-3′ (Leave *et. al.*, 2002).

The PCR products were resolved in a 2.0 % agarose gel and visualized by ethidium bromide staining in a Gel doc XR+ (BioRad Laboratories, JHB, South Africa).

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3.9 Sequencing and Sequence Analysis

A total of 36 samples (inclusive of human and animal isolates) were sequenced. However, we only used 27 sequences in the alignments.

A 20µl aliquot of each PCR product that showed successful amplification from the 18S rRNA was prepared and sent to Inqaba Biotechnical PTY (Ltd) (Pretoria, South Africa) for sequencing. DNA sequences were aligned with previously reported sequences using BioEdit software and phylogenetic trees were constructed with MEGA 6 software to compute evolutionary relationships.

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CHAPTER FOUR

RESULTS

PART I: Prevalence and molecular characterization of *Cryptosporidium* from human isolates

4.1. Biographical details of sample population

The sample population comprised largely of females with 57.7% (192/333) compared to the males with 42.3% (141/333). The subjects age distribution was divided into three age groups with most of the subjects 60.7% (202/333) falling within the 0-25 years age group. Subjects older than 45 years had the second highest amount with 26.1% (87/333). Table 4.1

Table 4.1: Biographical details of the humans sample population

Gender	Frequency	Percent
Female	192	57.7
Male	141	42.3
Total	333	100
Age Group		
0-25	202	60.7
26-45	33	9.9
>45	87	26.1
Unknown	11	3.3
Total	333	100

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4.2. Prevalence of *Cryptosporidium* among humans population

A total of 333 human samples were collected and 35 samples were found positive with Ziehl-Neelson staining microscopy amounting to a prevalence of 10.5% of *Cryptosporidium* species among humans A significant amount 7.2% (24/333) of the participants had diarrhoea whereas 48.7% (162/333) of the subjects provided a hard stool which meant they were constipated at the time of sample collection. Table 4.2.

Table 4.2: Prevalence of *Cryptosporidium* based on microscopy and stool characteristics

ZN Microscopy	**Frequency**	**Percent**
Negative	298	89.5
Positive	35	10.5
Total	333	100
Stool Consistency		
Hard	162	48.7
Soft	147	44.1
Watery	24	7.2
Total	333	100
Diarrhoea		
No diarrhoea	309	92.8
Diarrhoea	24	7.2
Total	333	100

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4.3. Molecular characterization of *Cryptosporidium* human isolates.

4.3.1. *Cryptosporidium* screening by 18S rRNA

PCR amplification with 18SrRNA for screening of *Cryptosporidium* was successful yielding a 298bp fragment.

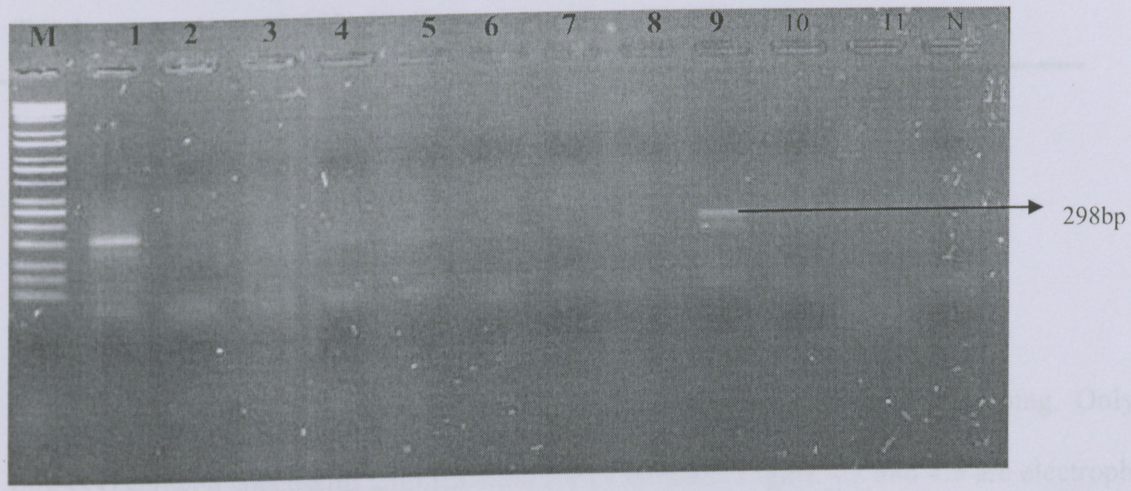


Figure 4.1: Agarose gel of one step PCR products amplified by specific *Cryptosporidium* screening primers. Products were electrophoretically resolved in a 2% agarose gel. Lane 1 is a Kappa universal ladder and lanes 2-10 are test samples, lane 11 is empty and lane 12 is the negative control. Lanes 2 and 9 were positive for *Cryptosporidium*

Table 4.3 below describes the distribution of *C. hominis* among three different age groups. The 26-45 age group cohort had the most recorded prevalence with 15.2% (5/33) as compared to the 12.5% and 11.5% of the 0-25 and >45 years age group cohorts respectively. However, the 0-25 age group had the highest number of subjects (202) as compared with 33 and 87 for the 26-45 and >45 years age groups respectively.

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Table 4.3. Prevalence of *C. hominis* by age group

	HSP-70	
Age group	Positive	Negative
0-25	(25/202) 12.4%	(177/202) 87.6%
26-45	(5/33) 15.2%	28/33 84.8%
>45	(10/87) 11.5%	(77/87) 88.5%
Total	(40/322) 12.4%	87.6%

4.3.2. Genotyping by Heat Shock Protein-70 Multiplex PCR

A total of 333 human samples were analysed for *Cryptosporidium* genotyping. Only 12% (40/333) showed successful amplification for *C. hominis*. Figure 4.2 and 4.3 are electrophoresis gels with *C. hominis* being the only amplifies fragment with 148bp.

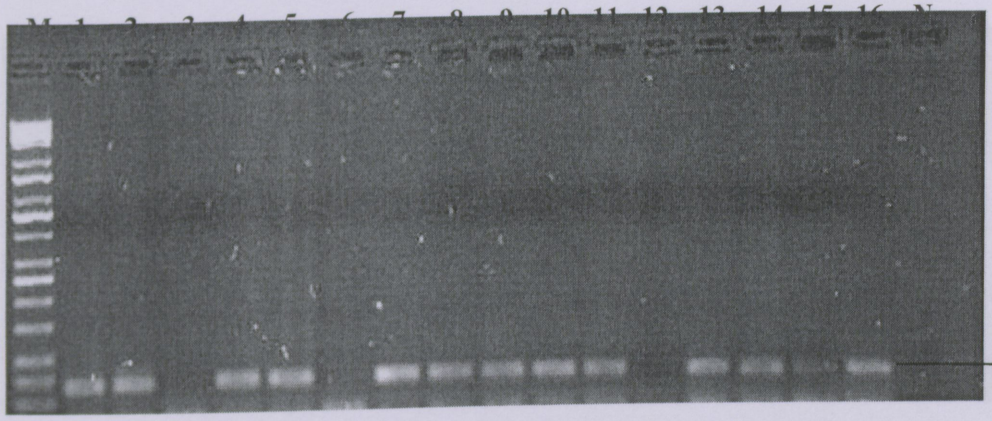


Figure 4.2. Agarose gel of PCR products amplified by multiplex HSP70 primers targeting four genotypes. The only genotype with successful amplification was a 148bp *C. hominis*. The products were resolved electrophoretically in a 2% agarose gel.

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Figure 4.3. Agarose gel of PCR products amplified by multiplex HSP70 primers targeting four genotypes. The only genotype with successful amplification was a 148bp *C. hominis*. The products were resolved electrophoretically in a 2% agarose gel.

4.3.3. Glycoprotein 60

The gp60 primers were unable to produce the desired fragment size when ran with both the primary and alternative primer sets. Nonetheless, a rather interesting gel was produced. This being the most widely used subtyping locus, the results were expected to be like this but in a more sensible manner.

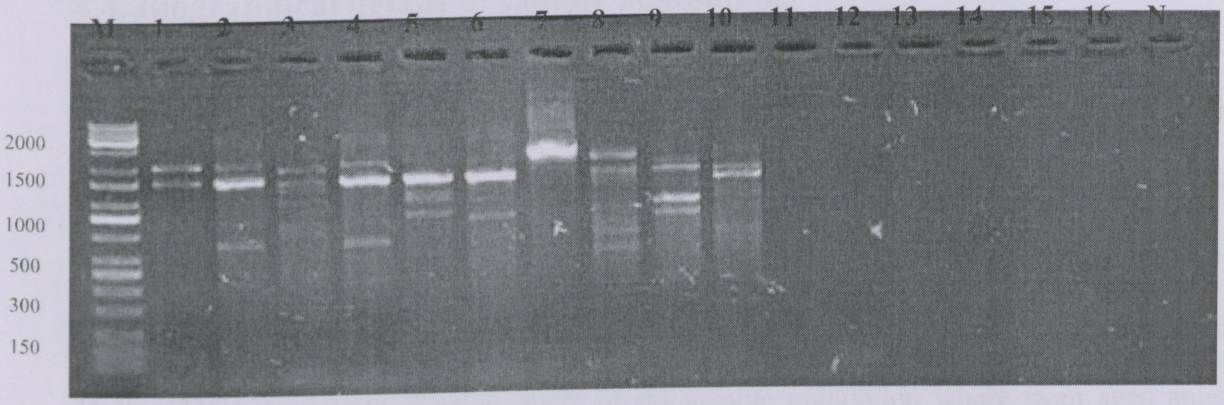


Figure 4.4. Agarose gel of PCR products amplified by primers targeting the gp60 gene for genotyping *Cryptosporidium*. The PCR products were electrophoresed for resolution with 2% agarose gel at 100V for 45min.

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We attempted to sequence the purified gel product but the results were not good for reporting in this study. We sought to explain this and decided to purify the fragments from gel. Figure 4.5 is a gel image of the agarose gel purification.

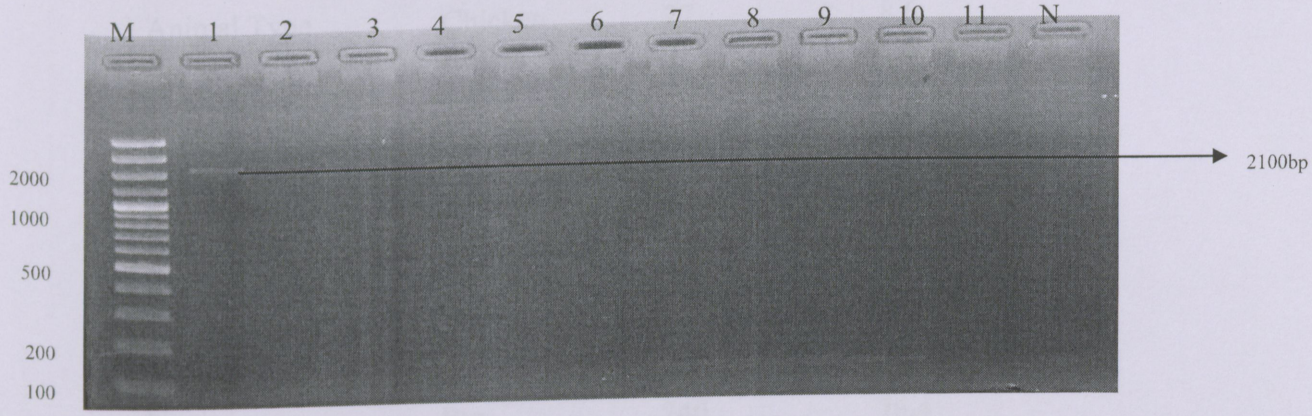


Figure 4.5. Electrophoresed agarose gel of gel purified gp60 PCR products.

PART II: Prevalence and molecular characterization of *Cryptosporidium* from animal isolates

4.4. Biographical details of sample population.

The animal sample population consisted largely of cattle 62.0% (194/313), and this is mainly the case because *Cryptosporidium* molecular studies in cattle are well documented. Goat samples were 28.1% (88/313) and chicken and sheep were 8.6% (27/313) and 1.3% (4/313) respectively. Moreover, most of the samples were female with 73.6% (231/313) compared to 23.2% (73/313) of male. Further, most of the sample population where from aged animals 76.4% (240/313) compared with 23.6% (74/313). Consistency of the stool samples were mostly solid at 88.12% (277/313) compared to 10.5% (33/313) of loose and 1.3% (4/313) were diarrheic. Table 4.4

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Table 4.4 Biographical description of animal study population.

		Frequency	Percent
Animal Type	Cattle	194	62.0
	Goat	88	28.1
	Chicken	27	8.6
	Sheep	4	1.3
	Total	313	100
Animal Sex	Female	231	73.6
	Male	73	23.2
	Unknown	10	3.2
	Total	313	100
Size	Big	240	76.4
	Small	74	23.6
	Total	313	100
Stool Consistency	Solid	277	88.2
	Loose	33	10.5
	Watery	4	1.3
	Total	313	100

4.5. Socio-economic considerations of study animals' owners

Hygiene plays a significant role in the transmission of *Cryptosporidium*; therefore, basic sanitary activities such as washing hands with sanitizers and having clean running water for drinking are important loopholes in the prevention of cryptosporidiosis. Besides financial considerations, most preventative measures in place require at least basic education to implement successfully. The sampled subjects' level of education ranged from grade 3 to a degree graduate. However, most of the subjects (47.3%) did not have a matric certificate compared to only 10.5% of the owners who had grade 12. Further, only 2.5% and 1.9% had completed a first degree and diploma respectively. Withal, there were 33.9% of subjects who had no formal education at all.

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A rather compelling observation is that 27.2% of these people earned a collective household salary below R1000 as compared to the 24.6% who earned less than R5000. However, a certain portion (8.6%) earned above R12 000. More isolates were obtained from married owners with 72.5% compared to 24.9% from single owners and 1.9% from widowed owners. Table 4.5

Table 4.5: Socio-economic background of study animals' owners

		Frequency	Percent
Educational Level	Grade 1-11	148	47.3
	Grade 12	33	10.5
	Post Matric	12	3.8
	Completed Diploma	6	1.9
	Completed degree	8	2.5
	No formal education	106	33.9
	Total	313	100
Household Income	R1-R500	85	27.2
	R501-R1000	65	20.8
	R1001-R3000	77	24.6
	R3001-R6000	17	5.4
	R6001-R12000	23	7.3
	R12000+	27	8.6
	Total	313	100
Marital status	Single	78	24.9
	Married	227	72.5
	Widowed	6	1.9
	N/A	3	0.9
	Total	313	100

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4.6. Potential reoccurrence of diarrh

Among the subjects, it was observed that the occurrence of diarrhoea was reoccurring. About 15.7% (49/313) of the subjects reported to having diarrhoea in the previous three months previous months and small but non-negligible portion responded to getting used to the diarrhoea as it happens annually. Interestingly, 19.2% responded to having diarrhoea that lasted up to 3 days whereas 4.5% responded to having diarrhoea lasting for up to 2 weeks.

4.7. Prevalence of *Cryptosporidium* from animal isolates

4.7.1. Effect of water safety on prevalence of *Cryptosporidium*

Contaminated water represents a major source of *Cryptosporidium* infections for humans (Ramirez *et al*., 2004). An overwhelming majority of 74.4% (233/313) of the animals' keepers did not treat the water they and the animals drank. A rather insignificant number of subjects (0.6%) did not respond to whether they treat their water or not. However, a 25.0% of the subjects reported to be treating their drinking water. Table 4.6

Table 4.6 Study subjects who treated water before consumption

Do you ever treat water?	Frequency	Percent
No	233	74.4
Yes	79	25.0
N/A	2	0.6
Total	313	100

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4.7.2. Microscopy

A total of 313 animal samples were collected with biographical information and 98 were found positive by Ziehl-Neelson staining microscopy amounting to a detection percentage of 31.3% of *Cryptosporidium spp.* in animals.

4.7.3 Molecular screening of *Cryptosporidium*

A 298bp fragment was successfully amplified for 28 animal stool samples. Of the 28 samples, 27 were sent for sequencing to ascertain the genotypes. Table 4.8 is a detailed description of the distribution of all determined genotypes across all study animals.

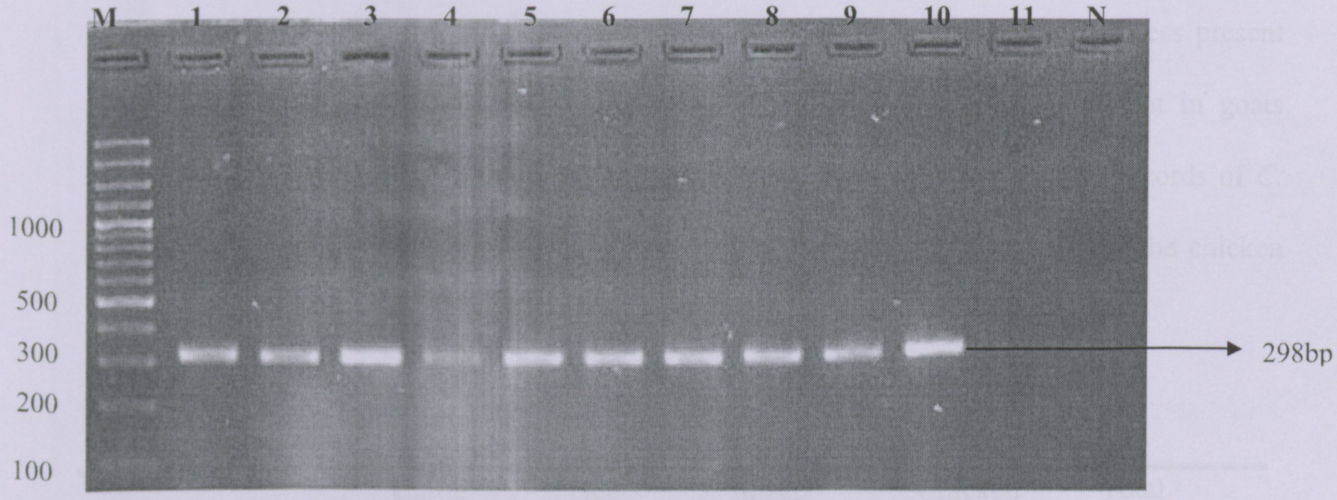


Figure 4.6. Agarose gel of PCR products amplified by 18S rRNA primers. Products were resolved in a 2% agarose gel run at 100V for 45min. The molecular marker used was a 100bp plus ladder. Lane 1 is the marker, lanes 2-11 are positive samples and lane 12 and 13 are negative controls (sterile PCR water, no template)

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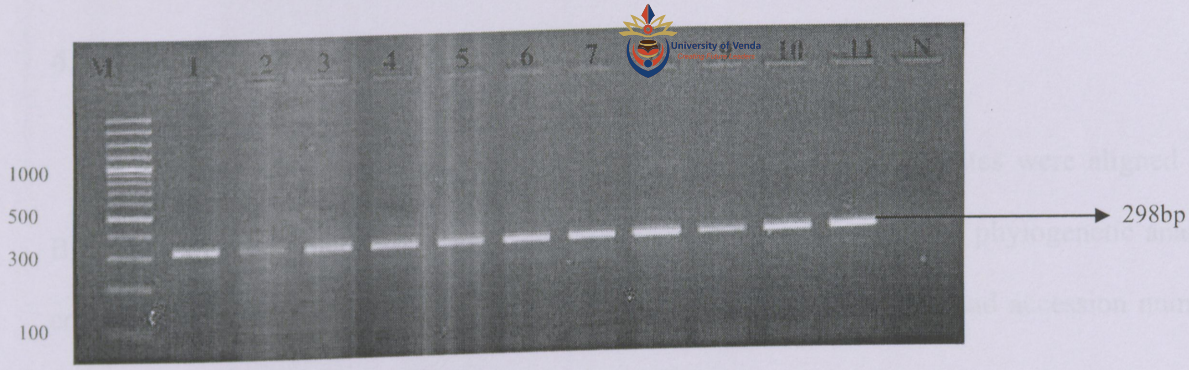


Figure 4.7. Agarose gel of PCR products amplified by 18S rRNA primers. Products were resolved in a 2% agarose gel run at 100V for 45min. The molecular marker used was a 100bp plus ladder. Lane 1 is the marker, lanes 2-12 are positive samples and lane 13 is the negative control (sterile PCR water, no template)

Among cattle, *C. suis* had the highest prevalence compared to *C. andersoni* and the others. More interesting is the low presence of *C. parvum* in cattle, *C. andersoni* was less present compared with an unlikely *C. suis*. However, *C. andersoni* was more dominant in goats compared with 1 and 2 records of *C. parvum* and *C. suis* respectively. Only 3 records of *C. parvum* was recorded in sheep and only 2 records of *C. galli* was registered for the chicken host. Table 4.7

Table 4.7 Distribution of genotypes per animal subject

	Cattle	Goat	Sheep	Chicken	Total
C. parvum	5	3	3	0	11
C. suis	25	7	0	0	32
C. andersoni	14	25	0	0	39
C. galli	1	0	0	2	3
C. scrofarum	10	3	0	0	13
Total	55	38	3	2	98

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4.8 Sequencing and phylogenetic ana

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The obtained sequences from both the human and animal isolates were aligned with BioEdit using reference sequences obtained from NCBI gene bank and phylogenetic analysis computed with MEGA6 (Tamura *et al.*, 2013). Reference sequences had accession numbers KJ020907 and KT02743 respectively.

Figure 4.8 is a phylogenetic analysis inferred by a Neighbour-Joining method (Saitou and Nei, 1987). Three distinct evolutionary relationships were formed which we named cluster 1, 2 and 3 and outgroup. Interestingly, cluster 1 which included the only three human sequences (HS025 and HS060 grouped very close to the reference sequences with bootstrap values almost 80%. Most interesting however, is having the other human isolate (HS062) clustering on the same clade with the reference sequences with a bootstrap value of 95%. Moreover, isolates, 131Cattle-*C. andersoni* and 146Sheep-*C. parvum* clustered together with a bootstrap value of 99% showing almost perfect relatedness despite these sequences originating from different animal isolates. This serves as more evidence of possibility of cross-transmission of previously described host-bound (Nemejc *et al.*, 2013) *Cryptosporidium* genotypes.

More fascinating was cluster 2 which consisted of cattle and goat isolates. All the isolates contained porcine genotypes (*C. suis* and *C. scrofarum*) which have been previously described to be host specific too (Fayer *et al.*, 2000). Moreover, cluster 2 was too different from cluster 1 with the bootstrap values confirming that at 24%. Cluster 3 was more the same with cluster 1 with most of the isolates from cattle origin and the genotypes being mostly *C. andersoni*.

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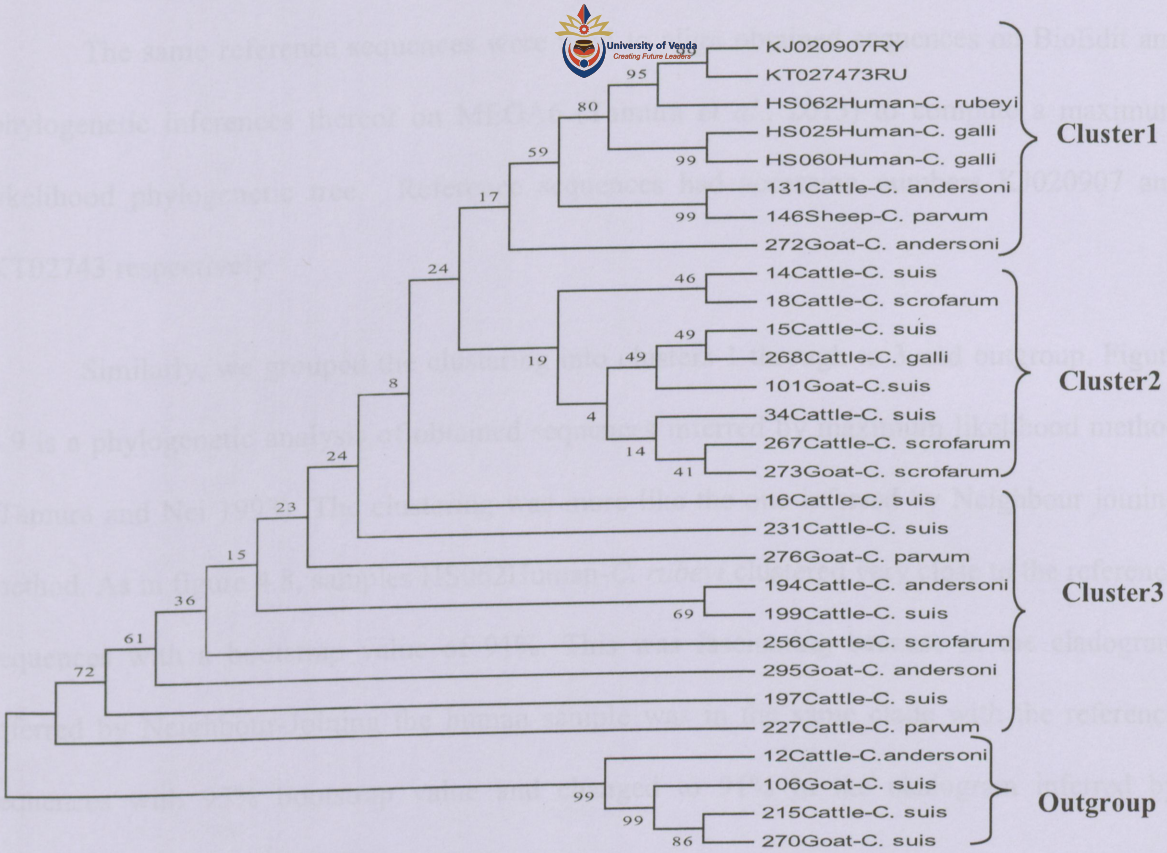


Figure 4.8. Evolutionary relationships of taxa of both animals and humans.

The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.34935103 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). These Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

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The same reference sequences were used to align obtained sequences on BioEdit and phylogenetic inferences thereof on MEGA6 (Tamura *et al.*, 2013) to compute a maximum likelihood phylogenetic tree. Reference sequences had accession numbers KJ020907 and KT02743 respectively.

Similarly, we grouped the clustering into clusters 1 through to 3 and outgroup. Figure 4.9 is a phylogenetic analysis of obtained sequences inferred by maximum likelihood method (Tamura and Nei 1993). The clustering was more like the one inferred by Neighbour joining method. As in figure 4.8, samples HS062Human-*C. rubeyi* clustered very close to the reference sequences with a bootstrap value of 91%. This was fascinating because in the cladogram inferred by Neighbour-Joining the human sample was in the same clade with the reference sequences with 95% bootstrap value and changed to 91% in the cladogram inferred by maximum likelihood method.

The other two human samples (HS025human-*C.galli* and HS060Human-*C. galli*) clustered together with a bootstrap value of 99% but there was only 66% relatedness between the latter and the reference sequences. Cluster 2 showed a clear diversion from cluster 1 with a bootstrap value of 1%. Cluster 3 showed a further diversion to cluster 2 with a bootstrap value of 4%.

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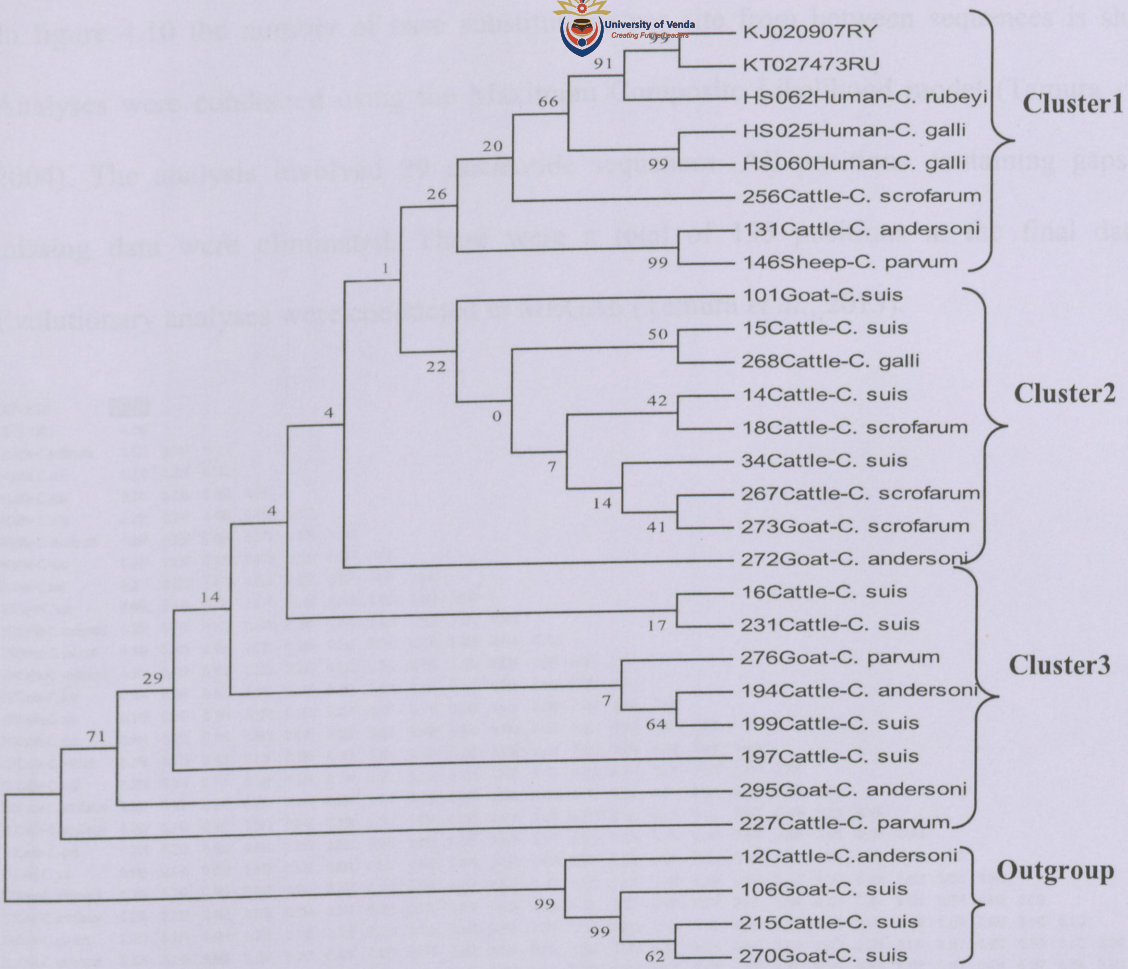


Figure. 4.9. Molecular Phylogenetic analysis of animal and human isolates.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-2453.7399) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. These Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

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In figure 4.10 the number of base substitutions per site from between sequences is shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al.*, 2004). The analysis involved 29 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 195 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

KJ020907RY																													
KT027473RU	0.059																												
12Cattle-C.andersoni	0.522	0.527																											
14Cattle-C. suis	0.220	0.226	0.511																										
15Cattle-C. suis	0.247	0.226	0.462	0.091																									
16Cattle-C. suis	0.258	0.247	0.489	0.118	0.102																								
18Cattle-C. scrofarum	0.237	0.226	0.484	0.070	0.075	0.097																							
34Cattle-C. suis	0.247	0.237	0.473	0.065	0.059	0.097	0.070																						
101Goat-C.suis	0.237	0.220	0.473	0.065	0.027	0.097	0.048	0.043																					
106Goat-C. suis	0.645	0.640	0.371	0.634	0.618	0.613	0.613	0.613	0.634																				
131Cattle-C. andersoni	0.258	0.247	0.522	0.183	0.167	0.204	0.183	0.183	0.161	0.629																			
146Sheep-C. parvum	0.306	0.301	0.489	0.220	0.204	0.253	0.247	0.204	0.204	0.634	0.102																		
194Cattle-C. andersoni	0.317	0.317	0.478	0.253	0.220	0.215	0.242	0.199	0.226	0.624	0.296	0.301																	
197Cattle-C. suis	0.306	0.296	0.403	0.145	0.134	0.151	0.145	0.118	0.129	0.559	0.237	0.253	0.242																
199Cattle-C. suis	0.242	0.247	0.484	0.172	0.151	0.124	0.172	0.134	0.145	0.618	0.258	0.258	0.156	0.183															
215Cattle-C. suis	0.694	0.683	0.392	0.683	0.640	0.661	0.683	0.688	0.661	0.349	0.651	0.651	0.613	0.624	0.629														
227Cattle-C. parvum	0.349	0.333	0.409	0.226	0.199	0.183	0.194	0.188	0.220	0.538	0.333	0.333	0.258	0.188	0.237	0.629													
231Cattle-C. suis	0.285	0.269	0.500	0.145	0.124	0.134	0.151	0.151	0.151	0.602	0.237	0.280	0.247	0.220	0.210	0.634	0.226												
256Cattle-C. scrofarum	0.263	0.258	0.441	0.226	0.194	0.204	0.220	0.210	0.199	0.565	0.237	0.242	0.231	0.247	0.183	0.602	0.269	0.215											
267Cattle-C. scrofarum	0.263	0.242	0.462	0.081	0.043	0.108	0.081	0.059	0.059	0.608	0.188	0.215	0.231	0.129	0.151	0.645	0.177	0.113	0.199										
268Cattle-C. galli	0.253	0.237	0.462	0.091	0.032	0.091	0.075	0.059	0.027	0.618	0.177	0.220	0.220	0.134	0.151	0.640	0.199	0.145	0.194	0.065									
270Goat-C. suis	0.656	0.640	0.317	0.672	0.629	0.651	0.651	0.645	0.645	0.274	0.677	0.661	0.602	0.581	0.613	0.280	0.565	0.651	0.581	0.634	0.629								
272Goat-C. andersoni	0.258	0.242	0.484	0.102	0.091	0.102	0.102	0.086	0.065	0.624	0.172	0.220	0.215	0.156	0.156	0.667	0.237	0.172	0.188	0.091	0.091	0.661							
273Goat-C. scrofarum	0.258	0.237	0.462	0.075	0.054	0.097	0.081	0.038	0.048	0.618	0.177	0.210	0.220	0.140	0.129	0.667	0.199	0.124	0.199	0.032	0.054	0.645	0.070						
276Goat-C. parvum	0.280	0.274	0.484	0.151	0.118	0.124	0.156	0.118	0.124	0.591	0.237	0.253	0.210	0.183	0.140	0.656	0.215	0.156	0.188	0.140	0.129	0.602	0.145	0.113					
295Goat-C. andersoni	0.306	0.296	0.468	0.194	0.167	0.129	0.183	0.172	0.183	0.575	0.258	0.269	0.194	0.161	0.172	0.645	0.172	0.167	0.177	0.172	0.167	0.602	0.199	0.172	0.140				
HS025Human-C. galli	0.226	0.247	0.489	0.156	0.156	0.188	0.177	0.161	0.140	0.651	0.210	0.247	0.242	0.237	0.172	0.651	0.301	0.237	0.204	0.172	0.151	0.634	0.167	0.156	0.204	0.237			
HS060Human-C. galli	0.220	0.242	0.495	0.161	0.161	0.194	0.177	0.167	0.145	0.651	0.210	0.253	0.253	0.242	0.177	0.656	0.301	0.242	0.204	0.177	0.156	0.640	0.167	0.161	0.210	0.242	0.011		
HS062Human-C. rubeyi	0.204	0.204	0.532	0.215	0.242	0.247	0.220	0.231	0.215	0.656	0.247	0.296	0.349	0.280	0.258	0.694	0.355	0.296	0.247	0.242	0.242	0.683	0.242	0.242	0.285	0.285	0.188	0.194	

Figure 4.10. Estimates of Evolutionary Divergence between Sequences

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DISCUSSION

Cryptosporidiosis is a major cause of diarrheal infections in both developing and developed countries, but accounts for a higher prevalence in developing countries (Areshi *et al.*, 2007; Dillingham, 2002). Moreover, *Cryptosporidium spp.* are important parasitic diarrheal agents and have been problematic as an opportunistic infection among HIV patients (Tzipori and Widmer, (2008); Cabada and White, (2010); Kotloff *et al.*, (2013)). Additionally, *Cryptosporidium* is a persistent challenge for livestock production and have presented itself as a common infection in calves (Fayer *et al.*, 2000). *Cryptosporidium* epidemiological studies among humans and animals in the Limpopo region have been understudied. However, isolated studies have been conducted among school children and adults who reported *C. hominis* as the most predominant *Cryptosporidium spp.* (Samie *et al.*, 2006). A prevalence of 32.0% among cats and 44% among dogs has been reported (Samie *et al.*, 2013). Samra *et al.*, (2013) reported a varying prevalence between *C. andersoni, C. bovis* and *C. ubiquitum* among free roaming wildlife animals of the Kruger National Park (KNP) in South Africa. The prime aim of this study was to determine the genetic characteristics of *Cryptosporidium* isolates from human and animal subjects in the Limpopo province and this was informed by the lack of data regarding the circulation of *Cryptosporidium spp.* in this epidemiological context. From the 10.5% found positive by microscopy, 85% were confirmed *C. hominis* in humans by PCR using HSP-70 as the target gene. From the 31.3% prevalence in animals stool samples, we found 7.4% of *C. parvum*, 33.3% of *C. suis*, 41% of *C. andersoni*, 4% of *C. galli* and 15% of *C. scrofarum* using 18SrRNA as the target gene. The GP60 gene failed to produce any desired amplicons. Furthermore, we report in the Mopani and Vhembe regions of Limpopo province, a rare

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occurrence of *C. suis* and *C. scrofarum* in cattle. This could be of particular importance especially in these rural communities among whom HIV prevalence is high and exposure to zoonotic pathogens can be life threatening.

5.1. Prevalence and molecular characterization of *Cryptosporidium* from human isolates

The first cases of human cryptosporidiosis were reported in 1976 (Nime *et al.*, 1976; Meisel *et al.*, 1976). This was found in two different patients of 3 and 39 years old respectively who had a skin disease and were treated with cyclophosphoamide and prednisolone. Interestingly, both of the patients lived near a farm. Diagnosis was based purely on microscopic examination of rectum and jejunal biopsy specimens. Unluckily, identification to the species level was not possible in these times and infections were diagnosed just as *Cryptosporidium spp.* (Nime *et al.*, 1976; Meisel *et al.*, 1976). Further, the role of *Cryptosporidium* as a serious human pathogen was emphasized in the 1980s in HIV positive individuals who experienced a persistent and life threatening infection that involved disseminating the parasite to the respiratory and hepatobiliary tracts in addition to the normal gastrointestinal tract (Ma and Sloave, 1983; Navin and Juranek, 1984); and in 1993 in the Milwaukee outbreak that involved at least 403,000 people (McKenzie et al., 1995). These landmark incidents accelerated the development of molecular methods for the detection and identification of *Cryptosporidium spp.* and their application has greatly increased our understanding of the taxonomy and epidemiology of *Cryptosporidium* (Caccio and Putignani, 2014). In the present study, we report 12.8% prevalence in humans with *C. hominis* being the only detected *Cryptosporidium spp.* by HSP-70 genotyping. This resonates well with other previous studies conducted in the same region as the present study which reported *C. hominis*

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as the most prevalent *Cryptosporidium spp.* with a prevalence rate of 14.2% compared to 4.1% for *C. parvum* (Samie *et al.*, 2006). Similar studies conducted in Pretoria and Johannesburg which looked at children younger than 5 years who attended health care facilities and had diarrhoea also reported high prevalence of *Cryptosporidium* with 18.8% and 25.6% for Johannesburg and Pretoria respectively (Berkowitz *et al.*, 1988; Geyer *et al.*, 1993). Together with this, numerous other studies in East and Southern Africa have demonstrated that the great majority of infections are caused by *C. hominis* (Mor and Tzipori, 2008). A study done in Uganda reported that approximately 75% of childhood case resulted from *C. hominis* (Tumwine *et al.*, 2003). Interestingly, this appears to be a stable phenomenon, because studies there are numerous studies which have reported congruent results (Tumwine *et al.*, 2005) and these includes among others reports from Malawi (Morse *et al.*, 2007; Peng *et al.*, 2003) and Kenya (Gatei *et al.*, 2006).

The present study reports a 7.2% portion of the study individuals who had watery diarrhoea. Referring from Samie *et al.*, (2006) equips us that watery diarrhoea is the most common symptom of cryptosporidiosis and other symptoms including, weight loss, stomach cramps, dehydration, fever, nausea, and vomiting. Moreover, Abdel-Messih *et al.*, (2005) showed that clinical findings associated with *Cryptosporidium* diarrhoea included vomiting, persistent diarrhoea and the need for hospitalization. Therefore, in cases of diarrhoea, there is probability to detect *Cryptosporidium* as observed by other studies (Pilarczyk and Balicka-Ramisz, (2002); Sobieh *et al.*, (1987); Uga *et al.*, (2000)).

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5.2 Prevalence and molecular characterization of *Cryptosporidium* from animal isolates

Cryptosporidium spp. are coccidian parasites that have a wide spectrum of farm livestock hosts including cattle, sheep, goats, pigs, horses, and chicken, and remain a veterinary problem in neonatal ruminants (Frazer *et al.*, 2011). No doubt, the effects of *Cryptosporidium* on livestock production has been emphatically demonstrated (Samra *et al.*, (2011); Wang *et al.*, (2010); Bejan *et al.*, (2009); Sari *et al.*, (2009); Castro-Hermida *et al.*, (2002); Lefay *et al.*, (2000)). In this study, we sampled isolates from cattle (62%, 194/313), goats (28.1%, 88/313), sheep (1.3%, 4/313) and chicken (8.6% 27/313) to determine the prevalence and genotype distribution among these livestock animals. The overall prevalence of *Cryptosporidium* was recorded to be 31.3% with cattle contributing the most infections.

Accordingly, of the 27 genotyped samples 55.6 % (15/27) originated from cattle with *C. suis, C. andersoni, C. galli* and *C. scrofarum* being the detected genotypes. Interestingly, *C. suis* and *C. scrofarum* were previously known to be obligate porcine genotypes (Guselle *et al.*, (2003); Maddox-Hyttel *et al.*, (2006); Vitovec *et al.*, (2006); Hamnes *et al.*, (2007), Jenikova *et al.*, (2011); Kvac *et al.*, (2009b; 2012; 2013). However, a recent study conducted by Bodager *et al.*, (2015) also reported a 27.4% incidence of *C. suis* in cattle. Together with this, they noted the absence of common cattle *Cryptosporidium species* (*C. parvum* and *C.andersoni*). Likewise, Fayer *et al.*, (2000) and Tzipori and Widmer, (2008) also noted what they termed the "uniqueness" of the broad range of *C. suis* and the absence of *C. parvum* and *C. andersoni* from cattle isolates. Interestingly, in the present study, we report *C. suis* in both cattle and goat which demonstrates emphasis to the broad range of *C. suis*. In another independent study, in Papua, New Guinea, *C. scrofarum* was reported in goats (Koinari *et al.*, 2014). Additionally, in

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line with Koinari *et al.*, (2014), the present study found a significant amount of *C. andersoni* in both cattle and goat.

5.3. Zoonotic risks of *Cryptosporidium*

Cryptosporidium parvum has reportedly been the *Cryptosporidium species* that have contributed immensely to the zoonotic transmission of the parasite (Feltus *et al.*, 2006). The past decade has however seen a lot of developments regarding the transmission and host range of *Cryptosporidium spp. C. hominis* has been for a long time been regarded as an exclusive human genotype until Ghaffari *et al.*, (2014) reported it in calve samples and concluded that there is a rare possibility of transmission of this species from calves to humans. Moreover, *C. suis* which has also been believed to be an exclusive porcine genotype, has been reported in humans (Xiao *et al.*, 2002; Leoni *et al.*, 2006; Wang *et al.*, 2013) and in cattle (Bodager *et al.*, 2015) as has also been demonstrated in the present study.

The real threat of the zoonotic nature of *Cryptosporidium* becomes even more pronounced when the basic generic preventative and control measures such as provision of dedicated water sources for animals and washing of hands after interaction with animals. However, education and economic backgrounds play pivotal roles in ensuring that these preventative measures which have been set down are actually practised on a daily basis. In the present study, over 33% of the subjects did not have formal education and only 3.8% had completed matric. Further, a mere 1.9% and 2.5% had a diploma and degree respectively. In as much as these are just basic measures to carry out, adequate education and training is required for communication and dissemination of information. A considerable (27.2%) of the sample population had a household income of at most R500, an amount that will make one less concerned about sanitation and hygiene.

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5.4. Phylogenetic analysis of sequences

We sequenced the 18S rRNA of 36 *Cryptosporidium* isolates (33 isolates from animals and 3 from humans). Of the 36 isolates, only 27 sequences (24 sequences from animals and 3 sequences from humans) were used in the final alignments and subsequent phylogenetic analysis. Phylogenetic analysis of the 18SrRNA gene of various isolates of *C. parvum, C. andersoni, C. suis, C. galli, C baileyi and C. scrofarum* in the present study indicated that the *Cryptosporidium* parasites belong to a multispecies complex that can be divided into different groups which for the purpose of this study we named them clusters 1 through to 3. The phylogenetic relationship inferred by neighbour-joining method in figure 4.8 demonstrates the clusters. Cluster 2 consisted only of isolates of *C. suis* and *C. scrofarum* from cattle and goats; cluster 3 consisted of *C. andersoni* from cattle and goats. Important to note is cluster 2 which groups all the porcine genotypes isolated from cattle and goats in one. This may be an indication the host predilection for these isolates as demonstrated by Bodager *et al.*, (2015). Phylogenetic analysis was inconsistent in separating the human isolates from the animals in cluster 1. In fact, it showed a 59% bootstrap value between the animals and the 3 human isolates.

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5.5. CONCLUSIONS AND RECOMMENDATIONS

Interest in *Cryptosporidium* was heightened in the veterinary field not only because animals were seen as a source of infection for humans, but also because the organism could cause economic losses in production animals and was proving to be very difficult to control (Frazer *et al.*, 2011). Infections have now been reported in most domestic animals as well as in a large variety of wild and captive animals (Samra *et al.*, 2013) since its first identification by Tyzzer (Tyzzer, 1912).

The presence of *Cryptosporidium* has been proven to exist in the Limpopo province long before this study ensued. However, previous studies have not fully deliberated on the eminent risks of zoonosis in these parts of South Africa. Considering that the majority of people in the Vhembe and Mopani regions live in poverty conditions under less acceptable sanitary environments, the role of *Cryptosporidium* as one of the causative agents of diarrhoea should not be taken lightly. This study therefore concludes that:

- *Cryptosporidium* is common among the inhabitants of the Vhembe and Mopani regions and this could probably account for the persistent diarrhoeal episodes.
- Cattle are in abundance in these areas and interaction with humans poses great risks of cross-transmission because cattle and humans are hosts to a lot of *Cryptosporidium spp.*
- Animals are left to mix and mingle hence the detection of previously described single-host bound species of *Cryptosporidium* are reported in unfamiliar hosts.

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RECOMMENDATIONS

The present study constitutes the third recorded report on the occurrence of *C. suis* in cattle. Thusly, the findings communicated by this study should provide grounds for re-classification of *C. suis* and *C. scrofarum* from being obligate porcine genotypes.

Further research to look into the cross-transmission of *Cryptosporidium* among animals and humans is suggested. Moreover, previous knowledge on the host range of *Cryptosporidium* spp. needs to be reviewed. Further, more characterization at DNA sequence level may need to be conducted to account for the transmission trend change.



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