PREVALENCE, CHARACTERIZATION AND TRANSMISSION OF *CRYPTOSPORIDIUM* SPECIES BETWEEN ANIMALS AND HUMANS ON DAIRY FARMS IN ZAMBIA

BY

JOYCE SIWILA

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DEPARTMENT OF CLINICAL STUDIES

LUSAKA

DECLARATION

I, **Joyce Siwila** do hereby declare that this dissertation represents my own work and that it has never been previously submitted for the award of a degree or any other qualification at this university or any other university.

Signature: ---------- Date: 05/10/2006

CERTIFICATE OF APPROVAL

This dissertation of **Joyce Siwila** is approved as fulfilling the requirements for the award of the Degree of Master of Science in Veterinary Parasitology of the University of Zambia.

Dr. Isaac K. Phiri 09/10/06

(Supervisor) Signature Date

DR. A.M. NAMBOTA 09/10/06

(Chair, Board of examiners) Signature Date

ABSTRACT

In the last two decades, *Cryptosporidium* infection has increasingly become important as a cause of diarrhoea in humans especially in children under the age of 5 years and in the immuno-compromised, particularly those with Human Immunodeficient Virus or acquired immunodeficiency syndrome (HIV/AIDS). This is especially so in developing countries such as Zambia where there is high HIV/AIDS prevalence, which is currently estimated to be 16% among adults of reproductive age.

There are, however, no known reports in Zambia of whether farm workers who are constantly in touch with animals are at risk of getting *Cryptosporidium* infection directly from these animals and information on genotypes involved is also lacking. The absence of such important information led to the formulation of this study. The aims of this study were to determine the presence of *Cryptosporidium* infection in dairy calves and in lambs and goat kids on selected dairy farms on one hand and in humans working with animals and members of their households on the other hand, using a copro-antigen enzyme linked immunosorbent assay (ELISA). Management factors and potential risk factors of infection were also investigated through questionnaires to find out if there was any possible association with the occurrence of *Cryptosporidium* infection in animals and humans. Genotypes of parasites isolated were also determined.

The faecal samples were collected per rectum from 207 calves, 39 lambs and 14 kids, aged six weeks and below belonging to 20 dairy farms in Lusaka and Central provinces between October 2004 to March 2005. Stool samples were also collected from 289 humans of various ages ranging from 0 to 60 years after informed

consent. The faecal consistency was noted for each sample after which the samples were frozen until analyzed.

The copro-antigen ELISA analysis showed an overall *Cryptosporidium* prevalence of 33.8% in calves. When the farms were divided into large-scale and small-scale dairy farms based on the total number of animals reared on the farm, the prevalence was found to be 39.02% for the large-scale farms and 13.95% for the small-scale farms. The prevalence in lambs was 2.6% while that for the kids was 7.1%.

Out of the 289 human stool samples from 89 families that were analyzed, 6.2% were positive for *Cryptosporidium*. Ten (5.7%) of these were males while 8 (7.2%) were females. Eight of the positive males were farm workers while two were non-farm workers. All the positive females were non-farm workers. The highest number of positive individuals was in the age range of 21-30 years. The positive humans were from eight farms only out of the 20 farms sampled.

Results from the questionnaire survey on calf management factors and their association with infection with *Cryptosporidium,* indicated a significant difference in prevalence between individually housed calves and those that were housed in groups (χ^2=33.420, P<0.0001). The prevalence was higher in individually housed calves (52.4%) as compared to those housed in a group (16.3%). The frequency of bedding removal was also significantly associated with the prevalence of cryptosporidial infection (χ^2=38.875, P<0.0001). Bottle/bucket feeding was also associated with the infection (χ^2=13.034, P=0.04) as opposed to suckling. *Cryptosporidium* infection was also significantly associated with faecal consistency, with prevalence of 51.4%, 38.6% and 10% for normal, watery and pasty faeces, respectively (χ^2=9.228, P=0.010).

Questionnaires were administered to 89 households to determine if contact with animals, occurrence of diarrhoea in the home, water source and sharing water source with animals were associated with cryptosporidial infections in farm workers and their families. The individuals that had contact with neonatal calves were 2.091 times more likely to be infected with *C. parvum* than those that did not have contact. Those that had diarrhoea in the home were also 2.295 times more likely to have the infection but the association in both cases was not significant. Age was also not significantly associated with *Cryptosporidium* infection (χ^2=8.825, P=0.184).

Twenty positive calf samples, one lamb and one kid samples were analyzed using Polymerase Chain Reaction (PCR). Amplification products using the Heat Stock Protein (HSP-70) gene and the 18S rDNA gene were obtained for 20 isolates from calves and for the lamb and kid samples. After sequence analysis, *Cryptosporidium parvum* bovine genotype, *C. bovis* and a deer-like genotype were identified from calf isolates. The lamb sample revealed *C. parvum* bovine genotype while the kid sample revealed *C. suis*.

Amplification products for the HSP-70 gene and 18S rDNA gene were also obtained for nine and 14 human samples, respectively. Four samples did not amplify on both the 18S rDNA gene and the HSP-70 gene. Sequence analysis on the amplified samples revealed *C. parvum* bovine genotype and *C. hominis*. These genotypes were found in both the farm workers and non-farm workers.

From this study, it can be concluded that *Cryptosporidium* infections are prevalent in Zambian dairy cattle, sheep and goats. It can also be concluded that *Cryptosporidium* infection is prevalent in humans working and living on dairy farms. The *C. parvum* bovine genotype was found in humans, an indication that humans are at risk of infection and do get infected with the animal type of *Cryptosporidium*

parasites. However, the strength of association between the potential risk factors and the occurrence of cryptosporidiosis could not be conclusively established. In view of this, it is, therefore, recommended that further studies be done to include other areas of the country and further genotyping of *Cryptosporidium* isolates from humans also be expanded.

DEDICATION

I dedicate this dissertation to my loving mother Alice Nakaonga Siwila who has been and continues to be my source of strength in striving to be the best and for making sure I lacked nothing in my quest to be the best in my career; and to my husband for being my guide and for always being there for me.

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ABBREVIATIONS

%	Percentage
+ve	Positive
-ve	Negative
χ^2	Chi- Square
µl	Microlitre
bp	base pair
BSA buffer	Bovine Serum Albumin buffer
CI	Confidence interval
g	gram
HSP 70	70-Kilodalton Heat Stock Protein
Km^2	Square Kilometer
ml	Milliliter
nm	Nanometer
No.	Number
OR	Odds ratio
P	Probability
rDNA	ribosomal Deoxyribonucleic acid
rpm	Revolutions per minute
SSU rRNA	Small subunit ribosomal Ribonucleic acid
Taq	*Thermus aquaticus*
UNAIDS	United Nations AIDS
UNAID	United Nations AID

CHAPTER ONE

INTRODUCTION

Cryptosporidiosis is mainly an enteric infection caused by a protozoan parasite of the genus *Cryptosporidium* and is mainly spread by the faecal-oral route. It is characterised by a typically mild infection in healthy persons but can be very severe in patients who are immuno-compromised such as those with acquired immune-deficiency syndrome (Leach *et al.,* 2000). *Cryptosporidium parvum* is the single most important species that affects both animals and humans.

Cryptosporidiosis caused by *C. parvum,* is of great concern as a zoonosis especially in developing countries such as Zambia due to the high prevalence of human immuno-deficiency virus/acquired immune-deficiency syndrome (HIV/AIDS) cases, poor sanitation and nutritional status, thus increasing the percentage of population at risk. *Cryptosporidium* has become a leading cause of persistent diarrhoea and continues to be a major threat to human health due to lack of effective therapy. For the last 20 years or more, *Cryptosporidium* has increasingly been associated with diarrhoea outbreaks in both animals and humans (O'Donoghue, 1995). Cryptosporidiosis can also be very severe especially in very young animals and humans. In immuno-compromised individuals, a very severe, prolonged and life-threatening diarrhoea can result from the infection (Jonas *et al.,* 1983; Malebranche *et al.,* 1983). In immuno-competent individuals on the other hand, the infection generally results in an acute, self-limiting diarrhoea (Fayer *et al.,* 1997).

In Zambia, the sero-prevalence of HIV/AIDS was estimated at 25% (Kelly *et al.,* 1997). In a later study carried out during 2001-2002, HIV/AIDS infection rate

was reported to be 16% among adults of reproductive age (USAID, 2003). *Cryptosporidium parvum* was found to be the most common parasite associated with chronic diarrhoea in HIV infected individuals (Conlon *et al.,* 1990). This HIV-related cryptosporidiosis was also more likely to be persistent (Nchito *et al.*, 1998). In other studies done in Lusaka, cryptosporidiosis was found more frequently in HIV-seropositive (14%) than HIV-seronegative (8%) children with diarrhoea (Chintu *et al.,* 1995). Amadi *et al.* (2001) also found *C. parvum* to be the most common intestinal infection in HIV sero-positive children and cryptosporidiosis was one of the most common conditions highly associated with their mortality in Zambia.

Cryptosporidium parvum cattle genotype and *C. hominis*, also known as *C. parvum* human genotype, are responsible for the majority of human infections (Morgan-Ryan *et al.*, 2002). However, humans especially those infected with HIV/AIDS, appear to be susceptible to other *Cryptosporidium* species including *C. canis*, *C. felis, C. meleagridis* and novel genotypes being isolated from wildlife (Pieniazek *et al.,* 1999; McLauchlin *et al.*, 2000; Morgan *et al.,* 2000; Guyot *et al.*, 2001; Pedraza-Diaz *et al.,* 2001; Xiao *et al.*, 2001; Cassio *et al.*, 2002; Ong *et al.,* 2002). Despite these advances in identifying *Cryptosporidium* parasites at species level in different parts of the world, there is no information about the species and strains of *Cryptosporidium* that are isolated from humans in Zambia.

In Zambia, high prevalence rates in the dairy herds (42.8%) have been reported as compared to 8% in beef cattle and 6.2% in traditional cattle (Goma, 2005). The presence of the pathogen in both animals and humans in the country raises concern that animals may act as a potential source of environmental contamination and direct infection to humans. In fact, high prevalence rates of *C.*

parvum in cattle in areas where human cryptosporidiosis is also found (Yu *et al.*, 2004) might suggest a possible correlation and/or zoonotic inter-transmission of *C. parvum* types between animals and humans. At risk are people who work closely with animals such as veterinarians, veterinary students, dairy farmers and other farm workers (Lengerich *et al.*, 1993). However, in spite of the apparent zoonotic potential, no studies had been done in Zambia to determine whether there is any common link between the animal and human cryptosporidiosis. Furthermore, no work has been done to determine if Zambian farm workers who closely work with animals and the people that live closely with them are at great risk of infection with *C. parvum*. In other parts of the world, transmission of the bovine genotype of *C. parvum* from calves to humans has been established (Miron *et al.*, 1991; Lengerich *et al.*, 1993).

This absence of data on the prevalence of *Cryptosporidium* in farm workers, the lack of information on the *C. parvum* genotypes infecting humans and the little information on the species in animals stimulated the formulation of this study. The study was carried in commercial dairy farms in Lusaka and Central Provinces of Zambia because these have been found to have a high prevalence of *Cryptosporidium* (Goma, 2005). The present study was restricted to certain area in the two provinces due to financial restrictions. All the selected farms also had boreholes as a water source to reduce variability. In a study to determine the presence of cryptosporidiosis in adults and its relationship to oocyst contamination of drinking water in Lusaka, borehole water was found to have low exposure to *Cryptosporidium parvum* and the prevalence of *C. parvum* in persons served by these boreholes was also low (Kelly *et al.*, 1997). This water contamination could probably have been due

to sewage seepage in these densely populated areas in which the study was done. Genotypic analysis of some of the human isolates from individuals in other parts of the world who developed cryptosporidiosis after consuming contaminated water has actually indicated consistency with a human origin (Peng *et al.,* 1997; Leav *et al.,* 2003).

The main objective of the present study was to determine the possibility of transmission of *C. parvum* infection between animals and humans on dairy farms in Zambia and to identify the potential transmission risk factors. The potential risk factors for transmission were identified through questionnaires which were administered to both farm owners and the farm workers. The specific objectives were: to determine the prevalence of *C. parvum* in dairy cattle, sheep and goats; to determine the prevalence of *C. parvum* in farm workers and members of their households on the selected farms; to characterize and identify the genotypes of *C. parvum* found in both animals and humans and finally to identify the potential risk factors for the transmission of *C. parvum* between animals and humans.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical background of cryptosporidial infections

Cryptosporidium (C. *muris*) was first recognised by Tyzzer (1907) in the gastric glands of mice. The genus *Cryptosporidium* was named at the turn of the 20th century but was only recognised as a potential cause of disease when it was found to be associated with diarrhoea in turkeys (Slavin, 1955). Cryptosporidiosis was subsequently recognised in cattle in the 1970s (Panciera *et al.*, 1971). Currently, *Cryptosporidium parvum* is considered an important primary entero-pathogen in ruminants, causing neonatal diarrhoea outbreaks (de Graaf *et al.,* 1999). Infections have also been reported in most domestic animals as well as in a large variety of wild and captive animals (O'Donoghue, 1995; Ramirez *et al.*, 2004). Most infections described in these animals are attributed to bovine sources of *C. parvum*. Among cattle, calves are more susceptible shortly after birth and remain so for several months (Xiao and Herd, 1994a).

The first case of cryptosporidiosis in a human was described in the United States of America by Nime *et al.* (1976) in a 3 year 5 months old girl who developed a self-limiting enterocolitis. At this time, it was considered to be a rare opportunistic infection of immuno-compromised individuals. Little was known about *Cryptosporidium* in humans until the early 1980s when it was identified as a marker for AIDS in humans (Current *et al.*, 1983). To date, cryptosporidiosis has continued to be associated with HIV/AIDS and other forms of immuno-suppression (Muriuki *et al.*, 1997).

Contaminated water represents the major source of *Cryptosporidium* infections in humans. However, direct transmission of the bovine genotype of *C. parvum* from calves to humans has been established (Miron *et al.*, 1991; Lengerich *et al.*, 1993). The zoonotic potential of *Cryptosporidium* was first recognised in the 1980s due to frequent reports of intestinal disease in animals and humans (Anderson, 1998). Since then, it has been described throughout the world (Casemore, 1990; Current and Garcia, 1991; Tzipori and Griffiths, 1998). In tropical countries, it has been associated with 4-27% of all incidents of diarrhoeal illness in children (Thamlikitkul *et al.*, 1987; Geyer *et al.*, 1993; O'Donoghue, 1995) and has been shown to be associated with diarrhoea persistence and increased mortality (Molbak *et al.*, 1993). In addition, large urban waterborne outbreaks have brought the organism to the attention of the public (MacKenzie *et al.*, 1994).

2.2 Parasite and host range

Cryptosporidium is a protozoan parasite that belongs to the Phylum Apicomplexa, subphylum Sporozoa and class Coccidia (Urquhart *et al.*, 1987). *Cryptosporidium* taxonomy is, however, still controversial (Xiao *et al.*, 2004). The classification of *Cryptosporidium* in the class of Coccidia is being reconsidered as it is now thought to have a closer affinity with the Gregarines than with the Coccidia based on the 18S rDNA phylogenetic analysis (Carreno *et al.*, 1999; Hijjawi *et al.*, 2002).

Among the apicomplexan parasites of medical and veterinary interest, *Cryptosporidium* is the least studied (Widmer, 2004). There are more than 20

Cryptosporidium species that have been described in various animals (O'Donoghue, 1995) but the validity of many of these species has not been confirmed (Xiao *et al.,* 2002). The parasite is ubiquitous in nature and the lack of differentiation between the species and strains has made it difficult to track down the sources of human and animal infections (Joachim, 2004). Generally, the species of this genus have been identified based on oocyst morphology, host range and site of infection. Oocyst morphological features have been used more often than any other single characteristic to designate genus and species. Most oocysts measure 4 - 6 microns, appear nearly spherical and have obscure internal structures (See Figure 2.1) (Fayer *et al.,* 2000).

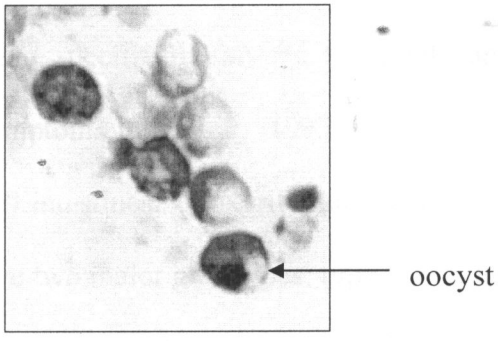

Figure 2.1 *Cryptosporidium* oocysts after Ziehl-Neelsen stain (source: www.dpd.cdc.gov/dpdx)

Some species are relatively well defined by their morphological and biological characteristics (Joachim, 2004). However, there were difficulties earlier because oocysts of a given species were found to be morphologically indistinguishable from those of another species or genus (Morgan *et al.,* 1999a). Moreover, cross transmission among several hosts occurs, thus, determination of a species is dependent on the interpretations and experience of the researcher (Morgan

et al., 1999a). The application of sensitive molecular approaches has shown that there is considerable evidence of genetic heterogeneity among isolates of *Cryptosporidium* from different species of vertebrates and there is mounting evidence suggesting that a series of host adapted genotypes/strains/species of the parasite exist (Morgan *et al.,* 1999a). In recent years, the molecular characterization of *Cryptosporidium* has helped to clearly identify and validate the existence of multiple species in each vertebrate class (Xiao *et al.,* 2004).

Thirteen (13) "valid" species of *Cryptosporidium* are currently recognized based on oocysts morphological differences, host, site of infection and genetic differences (Hijjawi *et al.*, 2002; Morgan-Ryan *et al.*, 2002; Thompson, 2003; Olson *et al.*, 2004). These are shown in Table 2.1.

The species *C. parvum* specifically infects mammals and is endemic in calves, with or without symptoms (Juranek, 1995). It mainly parasitizes the microvillar border of the small intestines. Deoxyribonucleic acid (DNA) sequencing has revealed *C. parvum* to have two major genotypes: type I, a human genotype (now known as *C. hominis*) that is occasionally identified in other animals and type II (now known as *C. parvum*), which can infect humans, livestock and wild animals (McLauchlin *et al.,* 2000).

Table 2.1 Recognised *Cryptosporidium* species and their host range as reported in some literature.

Number	Species	Host range	Reference
1	*C. parvum*	Mouse	Tyzzer (1912)
		Cattle	Panciera *et al.* (1971)
		Humans	Nime *et al.* (1976)
		Sheep	Barker and Carbonell, (1974)
		Pig	Kennedy *et al.* (1977)
		Goat	Tzipori *et al.* (1982)
		Horse	Snyder *et al.* (1978)
2	*C. wrairi*	guinea pigs	Vetterling *et al.* (1971)
3	*C. felis*	Cats	Iseki, (1979)
		Humans	Pieniazek *et al.* (1999)
4	*C. muris*	Mouse	Tyzzer, (1907)
		Humans	Katsumata *et al.* (2000)
5	*C. meleagridis*	Poultry	Slavin, (1955)
		Humans	Morgan *et al.* (2000)
6	*C. baileyi*	Chickens	Current *et al.* (1986)
7	*C. andersoni*	cattle, other livestock, rodents	Lindsay *et al.* (2000)
8	*C. canis*	Dogs	Fayer *et al.* (2001)
		Humans	Morgan *et al.* (2000)
9	*C. serpentis*	Snakes	Levine, (1980)
		Lizards	Xiao *et al.* (2004)
10	*C. saurophilum*	Reptiles	Koudela and Modry, (1998)
11	*C. nasorum*	Fish	Hoover *et al.* (1981)
12	*C. molnari*	Marine fish	Alvarez-Pellitero and Sitja-Bobadilla (2002)
13	*C. hominis*	Humans	Morgan-Ryan *et al.* (2002)

Currently, 5 genotypes of *Cryptosporidium* parasites (*C. parvum* human and bovine genotypes, *C. canis*, *C. meleagridis* and *C. felis*) have been explicitly found in humans (Pieniazek *et al.,* 1999; Morgan *et al.,* 2000; Xiao *et al.,* 2000; Pedraza-Diaz *et al.*, 2001). More recently, a novel cervine *Cryptosporidium parvum* genotype has been described in humans (Ong *et al.,* 2002). It is, however, also presumed that *C. andersoni* (syn. *C. muris* "bovine" genotype), which infects adult ruminants and parasitizes the abomasum, can also infect humans. Katsumata *et al.* (2000) detected oocysts of *Cryptosporidium* whose morphology resembled that of *C. muris* found in the stool of two healthy girls in Surabaya, Indonesia. Gatei *et al.* (2002) described a case of *Cryptosporidium muris* in an HIV-infected person in Nairobi, Kenya, an indication that *C. muris* can infect humans. This, and the report of possible asymptomatic *C. muris* infection in a healthy person (Katsumata *et al.,* 2000) suggest that this may be yet another *Cryptosporidium* species with a zoonotic potential.

In regular infections of immuno-competent patients, two different transmission cycles have been postulated, one involving the anthroponotic *C. hominis* (formerly named *C. parvum* type I or the human genotype of *C. parvum*), and the other one involving the bovine type of *C. parvum* (type II) in a zoonotic cycle (Monis and Thompson, 2003). *Cryptosporidium hominis* seems to be highly adapted to its host and displays little genetic variation, while *C. parvum* bovine type has a broad host range and can be subdivided into several genotypes (Xiao *et al.,* 2002). However, recent studies have shown that calves, lambs and piglets can also be infected with *C. hominis* (Akiyoshi *et al.,* 2002). In immuno-compromised patients especially those with AIDS, other species such as *C. andersoni*, *C. felis*, *C. canis, C.*

muris and the avian species *C. bailey* and *C. meleagridis* have also been described (Morgan *et al.,* 1999; Pieniazek *et al.,* 1999). There is increasing evidence, however, that many species and genotypes can infect both immuno-compromised and immuno-competent hosts (Xiao *et al.,* 2001; Chalmers *et al.,* 2002).

2.3 Life cycle

The life cycle of *Cryptosporidium* is direct and monoxenous and follows the patterns described for other enteric coccidia (Figure 2.2). It includes a merogonic cycle with two generations of meronts, a gametogonic cycle with macrogametes, microgametes and zygote, as well as a sporogony (Fayer *et al.*, 1990). The life cycle is completed within the gastrointestinal tract primarily the small intestine and colon of the host, with the developmental stages being associated with the luminal surface of the mucosal epithelial cells (Ramirez *et al.*, 2004).

Following ingestion (and possibly inhalation) of oocysts by a suitable and susceptible host, excystation occurs and this is triggered by various factors including carbon dioxide, temperature, pancreatic enzymes and bile salts (O'Donoghue, 1995). The sporozoites are released and mainly invade and attach to the epithelial cells of the gastrointestinal tract and sometimes other tissues such as the respiratory tract (Juranek, 1995). Invasion of the epithelium of other structures like the biliary tract and the conjunctiva of the eye have been reported (Fayer *et al.,* 2000; Mosier and Oberst, 2000). The parasite develops inside the epithelial cell along the brush border, although on the edge of the host cell cytoplasm and separated from it by a feeder organelle. This intracellular extracytoplasmic location is unique for this coccidia and

might play a major role in the failure of many antimicrobial agents to inhibit the growth of *Cryptosporidium* (Tzipori and Griffiths, 1998). It could also be one of the reasons why some researchers think the classification of *Cryptosporidium* should be reconsidered (Hijjawi *et al.*, 2002). In the epithelial cells, the parasites undergo asexual multiplication (schizogony and merogony) producing trophozoites which increase in size and then differentiate into meronts. There are two types of meronts, Type I meronts which contain 8 merozoites and Type II meronts which contain 4 merozoites. These asexual stages are produced in cycles and can spread infection to contiguous epithelium unless they are limited by an effective immune response (Chappell and Okhuysen, 2002).

Sexual multiplication (gametogony) then takes place producing microgamonts (male) and macrogamonts (female). Many microgametocytes are released from a single mature microgamete and these fertilize macrogametes. Upon fertilization, a zygote forms and this undergoes the asexual process of sporogony to produce oocysts. The oocysts sporulate within the infected host. Two different types of oocysts are produced, the thick-walled type, which is commonly excreted from the host and the thin-walled which is primarily involved in autoinfection. The oocysts are infective upon excretion, thus permitting direct and immediate faecal-oral transmission. The sporulated oocysts contain four sporozoites when excreted in faeces by the infected host (Urquhart *et al.*, 1987).

Cryptosporidium has some unique features which distinguish it from other enteric protozoa, some of which have been outlined above. These include its unusual location in the cell (intracellular but extracytoplasmic), its ability to auto-infect, its

innate antimicrobial resistance and its general lack of host specificity especially for *C. parvum* (Ramirez *et al.*, 2004).

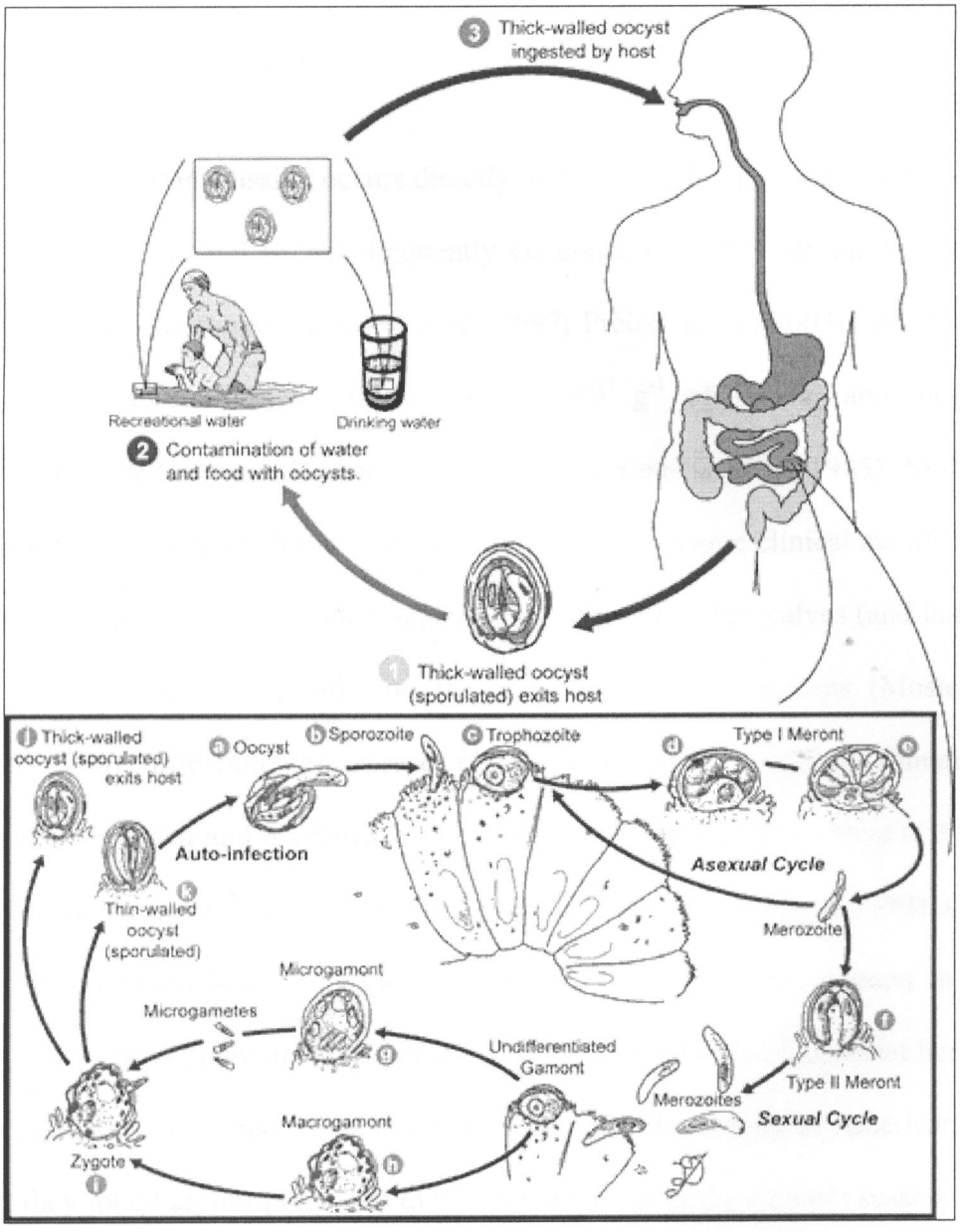

Figure 2.2 Schematic drawing of the life cycle of *Cryptosporidium parvum* and *C. hominis* (source: www.dpd.cdc.gov/dpdx).

2.4 Transmission and risk factors

2.4.1 Ruminants

In cattle, transmission occurs directly or indirectly by the ingestion of oocysts from an infected animal or very frequently via contaminated water and food or can be from animal to animal (Graczyk *et al.,* 1997; Preiser *et al.*, 2003). Infected new born calves excrete millions of oocysts (10^6-10^7 g^{-1} of faeces) and these are considered to be the most dangerous source of infection (Current, 1985). Normally, the periods of oocysts shedding coincide with periods of severe clinical manifestation leading to greater environmental contamination. Thus, it makes calves (and lambs) a more important source of infection for other animals and humans (Mosier and Oberst, 2000). Infection can spread rapidly from calf to calf if animals are communally housed and overcrowded or from cow to calf via the udders if they are contaminated with calf faeces in the area where the dams lie (Angus, 1990). Garber *et al.* (1994) found that *Cryptosporidium* infections were more common in dairy farms with multiple cow-maternity facilities. These authors also found that herd size and season were other risk factors of transmission. In their study in American dairy farms, they found cryptosporidiosis to be more common in the summer season. Mann *et al.* (1986), on the other hand, found a higher prevalence in winter and spring in Canadian beef herds. This is the period related to calving season and consequently the period with the greatest number of calves in the high risk group (1-3 week old). Stress on cattle and environmental conditions mentioned previously may increase the disease incidence during the calving season (Ramirez *et al.,* 2004). Another risk factor identified for increasing the probability of calves shedding *Cryptosporidium*

oocysts is frequent bedding changes as personnel and equipment used for the removal of the bedding can actually become a vehicle for spreading the infection (Sischo *et al.,* 2000). Drinking water source could also be a risk factor for cryptosporidiosis. Herds with well-water as their principle water sources are associated with lower concentrations of *Cryptosporidium* contrary to herds with access to dug-out (ponds) and run-off water sources (Heitman *et al.,* 2002). This could be due to contamination of dug-out water sources by direct defaecation by an infected animal into the water source, resulting in subsequent infections in other members of the herd.

In small ruminants, infection is more common in animals under a month old (Ortega-Mora and Wright, 1994). Transmission occurs mainly by ingestion of oocysts eliminated in the faeces of infected neonates or asymptomatic adult carriers (Xiao *et al.,* 1993). Adult sheep can act as asymptomatic carriers shedding small numbers of oocysts to the environment. This shedding was shown to increase in number in the perinatal period and thus contributing to maintaining the infection between lambing periods (Xiao *et al.,* 1994b). About 75-100% of lambs born in this environment are said to become infected in the first few weeks of life.

2.4.2 Humans

A single oocyst is sufficient to produce infection and disease in susceptible hosts (Pereira *et al.,* 2002a). Routes of transmission can be person to person through direct or indirect contact, animal to human, waterborne through drinking water or recreational water, food borne and possibly airborne (Fayer *et al.*, 2000). The best-

documented routes of transmission are waterborne, foodborne and person-to-person spread (Leav *et al.,* 2003). The majority of documented outbreaks have been attributed to contaminated drinking water supplies. An example is the 1993 massive outbreak that occurred in Milwaukee in United States of America (USA) in which more than 400,000 people developed cryptosporidiosis after consuming contaminated water (Mackenzie *et al.,* 1995). A study in Zambia by Nchito *et al.* (1998) also showed that transmission of *C. parvum* is at least, in part, determined by contamination of drinking water with oocysts. Although the source of contamination remains uncertain, recent genotypic analysis of four of the isolates indicates consistency with a human origin (Peng *et al.,* 1997). However, one outbreak in Canada was attributed to cattle faeces contaminating the watershed (Peng *et al.,* 1997). Due to this possible route of infection, studies were undertaken to establish the role cattle rearing facilities have on contaminating source watersheds (O'Donoghue, 1995; Graczyk *et al.*, 2000; Heitman *et al.*, 2002). In urban populations, person-to-person transmission is now recognised as an important means of transmission (Meinhardt *et al.,* 1996).

Populations at increased risk of cryptosporidiosis include household and family as well as sexual partners of infected patients, health-care workers, day-care centre personnel, users of communal swimming pools and travellers to regions of endemicity (Meinhardt *et al.*, 1996). The sources of contamination certainly vary depending on the climate, population density, farming practices and hygienic standards regarding wastewater management and food processing. However, farm animal and human sewerage discharges are generally considered the major sources of surface water contamination with *C. parvum* (Meinhardt *et al.*, 1996).

The most common risk factors associated with the human cryptosporidiosis are concurrent immunosuppressive disease, age, travel (particularly international travel) and direct and indirect contact to farm animals. Immuno-suppression due to a variety of causes such as but not limited to HIV infection and AIDS, drugs, organ transplantation and cancer chemotherapy predispose individuals to chronic and debilitating cryptosporidiosis (Farthing, 2000). According to Casemore (1990), the peak incidence of cryptosporidiosis is in children aged 1-5 years. In developing countries, it has been reported to be endemic and the most common cause of persistent diarrhoea among children (Leav *et al.,* 2003). Young age is probably a surrogate for inadequate hygiene, faecal incontinence and the need for more assistance during illness (MacKenzie *et al.,* 1995). Close contact between humans especially under poor hygienic conditions, as described for highly endemic subtropical and tropical regions such as Brazil also favour transmission (Newman *et al.,* 1994; Pereira *et al.,* 2002b). Sexual behaviour is also a significant risk factor for cryptosporidial infection in homosexual men (Hellard *et al.,* 2003). These authors found that insertive anal sex and attending a sex venue one or more times increased the odds of having cryptosporidiosis.

Contact with animals is another important risk factor for transmission of *Cryptosporidium*. The transmission of *Cryptosporidium* from calves to humans has been well established, with documented outbreaks among veterinary hospital staff and visitors to infected farms. Evans and Gardner (1996) reported an outbreak of cryptosporidiosis among 43 school children and four staff members after a week's holiday at a rural farm. *Cryptosporidium* oocysts were detected in six of the 29 and one of the four staff members that were ill. Preiser *et al.* (2003) also reported an

outbreak of cryptosporidiosis among students working with calves as part of their science program. Infections with the cattle or animal-adapted genotype (genotype II) of *C. parvum* were also found in a human population that shares habitats with the free-ranging gorillas in Uganda (Nizeyi *et al.,* 2002b). A high prevalence was found in park workers (21%) that were frequently in touch with the animals compared to 3% prevalence in the local community (Nizeyi *et al.,* 2002b).

Cryptosporidium oocysts can travel a considerable distance following runoff (Mawdsley *et al.,* 1997) and are infectious to a wide range of animals, thus having potential excretors (Tzipori and Griffiths, 1998). As a result, this parasite undoubtedly has an exceptional capacity to disseminate (de Graaf *et al.*, 1999). Several factors are responsible for the translocation and spread of parasites and these include the ubiquitous distribution and high resistance of the oocysts against physical and chemical inactivation, the low number of oocysts required to initiate infection, the high reproductive potential and the availability of susceptible hosts (Joachim, 2004). Cockroaches, flies and beetles have also been implicated in disseminating the parasite in the environment (Fayer *et al.*, 2000; Graczyk *et al.*, 2000). *Cryptosporidium* like-oocysts were found in intestinal tracts of cockroaches (*Periplaneta americana*) examined from a household in USA where a child had cryptosporidiosis leading to suggestions that the cockroaches were probably involved in disseminating the oocysts (Zerpa and Huicho, 1994 in Fayer *et al.*, 2000).

Fayer *et al.* (2000) suggested the possibility of airborne transmission following many reports in humans of coughing and respiratory symptoms in persons with cryptosporidiosis. Serious respiratory cryptosporidiosis has been reported in immuno-compromised individuals (Fayer *et al.,* 2000) and oocysts have been

isolated in sputum from a patient with a persistent productive cough, an indication of respiratory involvement (Moodley *et al.*, 1991).

Unpasteurised milk may be a potential risk to humans due to faecal contamination especially during the milking process (Gelletli *et al.*, 1997). Many smallholder dairy farms in Zambia hand-milk their animals and it is during this process that faecal contamination may occur if the animal defaecates in the process of milking. These small holder dairy farms are becoming increasingly important sources of milk leading to increased risk of human infections with cryptosporidiosis if strict hygiene is not followed and if the milk is consumed unpasturised. Gelletli *et al.* (1997) reported a cryptosporidiosis outbreak which was associated with milk from a local, small-scale milk producer in the United Kingdom who was using a not so effective on-farm pasturiser. The outbreak involved 50 school children.

2.5 Effects of *Cryptosporidium* on the host

Cryptosporidium parvum and *C. andersoni* (*previously C. muris*) are the main species in ruminants, with *C. parvum* infecting the distal small intestine and *C. andersoni* the abomasum (de Graaf *et al.*, 1999). In humans, especially the immuno-suppressed, various species and genotypes of *Cryptosporidium* can cause infection.

The mechanism by which *Cryptosporidium* causes disease remains elusive but the infection begins when the ingested oocysts release sporozoites, which subsequently attach to and invade the intestinal epithelial cells. This attachment to and invasion of the host cells are crucial primary events in the pathogenesis (Leav *et al.*, 2003). The parasite has a particular predilection for the jejunum and the terminal

ileum. This point is very important because diagnostic evaluations such as endoscopy may miss the site of infection. Immuno-compromised people, particularly those with deficiencies in cell-mediated immunity are especially susceptible to *Cryptosporidium* infection and experience the most severe consequences of this illness (Leav *et al.*, 2003).

The pathophysiology is characterised by secretion of excess fluid into the intestinal lumen, failure of the colon to reabsorb water adequately, malabsorption from decreased surface area and maldigestion from altered digestive enzymes in the mucosal epithelium (Anderson, 1998). The major organ parasitized is the gastrointestinal tract but developmental stages of the pathogen have been reported in the respiratory tract and biliary tree (Baron *et al.*, 1994). The parasite elicits a local inflammatory response, and increased production of prostaglandins and several cytokines, particularly interferon γ (IFN- γ) has been described (Chen *et al.*, 2002). These inflammatory mediators could be responsible for altering solute transportation in the intestinal epithelial cells and thus leading to osmotic diarrhoea. The presence of enterotoxins during the course of cryptosporidial infection has been hypothesized but has never been conclusively demonstrated (Chen *et al.*, 2002; Leav *et al.*, 2003).

2.5.1 Clinical features

2.5.1.1 Humans

In essence, all humans are susceptible to *Cryptosporidium* infection although individuals with serological evidence of previous infection seem to be more resistant (Chappell *et al.*, 1999). Although the infection does not have a predilection for any

particular age group, it is more common among children in developed and developing nations (Leav *et al.,* 2003). However, a small peak in laboratory-confirmed incidence of cryptosporidiosis has been described in young adults aged 20-40 years and this was attributed to familial contact with children or occupational exposure (Casemore, 1988). Clinical infection was observed to be less common after the age of 40 years. The distribution of cryptosporidiosis cases by sex indicates that males and females appear to be equally susceptible to infection (Fayer and Ungar, 1986; Casemore, 1988).

The clinical signs associated with the disease in humans include watery diarrhoea which is associated with abdominal cramps, tenesmus, anorexia, nausea, vomiting, depression and weight loss (Leav *et al.,* 2003). The symptoms are similar in adults and children although cryptosporidiosis acquired during infancy may have permanent effects on growth and development (Molbak *et al.,* 1993). In a study by Guerrant *et al.* (1999), cryptosporidiosis and persistent diarrhoea among children were correlated with subsequent impairments in physical fitness and diminished cognitive function.

After an incubation period of seven to 10 days, more than 90% of infected patients present with acute watery diarrhoea that lasts approximately two weeks accompanied by nausea, vomiting and cramp like abdominal pain. About 36% of the patients also develop fever. In immuno-competent individuals these clinical signs are usually transient and fluid therapy is rarely necessary. The disease typically lasts from four to seven days, although it can extend up to a month in a small percentage of individuals (Chappell and Okhuysen, 2002). During this acute stage in the immuno-competent individual, the frequency of stool passage is mostly about six

times a day, but may range from two episodes to 20. However, many patients also experience intermittent loose stools for a period of weeks following the acute stage (Casemore, 1988; HMSO report, 1990). It is important to note that many exposed patients are asymptomatic. In a study of experimental infection in humans, Dupont *et al.* (1995) observed that a third of the subjects who ingested oocysts had no diarrhoea.

In immuno-compromised patients, especially those with AIDS, a very severe chronic diarrhoea frequently develops producing excreta with 10 litres or more of liquid stool each day which may contain more than 10^{10} oocysts. This leads to considerable fluid and weight loss and even death (Casemore, 1988; Casemore, 1992; Farthing, 2000). In patients with advanced AIDS, the illness has a highly variable presentation and Manabe *et al.* (1998) described these patterns as transient infection, intermittent relapsing infection, cholera-like infection, and chronic infection. In addition to the upper intestines, the parasite also infects other organs such as the stomach, pancreas, liver and bile ducts. Cholangitis is often observed in persons with more severe immunosuppression with a $CD4^+$ T cell count of ≤ 50 cells/mm^3 and such patients normally have a poor prognosis (Chen *et al.*, 2002). Gastric cryptosporidiosis and pancreatitis have also been described (Leav *et al.*, 2003). These HIV infected patients can have chronic diarrhoea that lasts for more than two months, shedding oocysts during the entire period. This eventually leads to severe dehydration, weight loss and malnutrition, extended hospitalisations and even mortality (Farthing, 2000).

2.5.1.2 Animals

In calves, oocyst excretion has been described as early as three days of age. This means that calves are already susceptible to infection during or shortly after birth (Snodgrass *et al.*, 1980; Xiao and Herd, 1994a). One to three week old, symptomatic calves have been found to shed up to 10^6 - 10^7 oocysts per gram of faeces for 3-12 days (Casemore *et al.*, 1997). In most calves, diarrhoea begins 3-5 days post infection (PI) and lasts for 4-17 days PI (Fayer *et al.*, 1998). The predominant signs of cryptosporidiosis are those of enterocolitis and hence are not pathognomonic. Specifically, the most consistently reported signs attributed to *C. parvum* infection in calves, lambs and kids include diarrhoea, increased frequency of defecation, tenesmus, anorexia, weight loss and depression (Tzipori *et al.*, 1983; de Graaf *et al.*, 1999). Dehydration is noted occasionally. The diarrhoea is commonly described as profuse, watery and yellow in colour. High mortality is not a common feature of *C. parvum* infections unlike other enteric pathogens, although morbidity can be very high (O'Donoghue, 1995). However, high mortality due to cryptosporidiosis has been reported in calves, even in the absence of other entero-pathogens although infection with *C. parvum* is usually transient (Sanford and Josephson, 1982). This is in contrast to *C. andersoni* infection, which causes chronic gastric cryptosporidiosis in older cattle (Anderson, 1987). These infected adult cattle generally shed oocysts intermittently and usually at low levels. Esteban and Anderson (1995) found that chronic infection with *C. andersoni* reduced milk production in dairy herds in which up to 15% of the cows in some milking strings were shedding oocysts of the parasite. These cows were chronically parasitized and

produced about 13% less milk than did their unaffected pen mates, which were matched for age and lactation.

2.5.2 Pathology

The pathological findings associated with *Cryptosporidium* are mild to moderate villous atrophy, villous fusion and changes in the surface epithelium (de Graaf *et al.*, 1999). Furthermore, infiltration of mononuclear cells and neutrophils was seen in the lamina propria (Sanford and Josephson, 1982). In children, infections lead to invasion and destruction of the intestinal epithelium causing disturbances of resorption and brush border enzymes (Farthing, 2000). The histopathological features of cryptosporidiosis include a minimal inflammatory infiltrate and blunting of the villi. More extensive inflammatory changes such as disruption of the epithelial cell barrier and more extensive infiltration of the lamina propria with inflammatory cells are seen in immuno-deficient patients (Lumadue *et al.*, 1998).

In ruminants, pathological changes due to *C. parvum* are mostly confined to the intestinal tract, particularly the distal small intestines. In the initial stages of the disease, infections are seen throughout the small intestines and in the later stages they progress to the large intestines, although in this region no lesions are seen microscopically (Tzipori *et al.*, 1983). The gross pathological findings include congestion of the intestinal mucosa, a distended intestinal tract with either gas or yellow mucoid or watery material. The mesenteric lymph nodes have been found to be enlarged and oedematous (Tzipori *et al.*, 1983). The histopathological lesions include shortened and blunted villi, infiltration of lamina propria with mononuclear

cells, neutrophils and some eosinophils as well as metaplasia of the surface epithelium. The metaplasia is evidenced by an increased number of mitotic enterocytes. Also seen is bridging and fusion of adjacent villi and degeneration or sloughing of enterocytes (Sanford and Josephson, 1982; Anderson, 1987). Large numbers of cryptosporidial organisms are seen embedded in the enterocytes. Pathological lesions due to *C. andersoni* are confined mostly to the abomasum where it mostly lodges. Histopathological lesions include an increase in abomasal mucosal thickness, widening of gland lumens and atrophy of the epithelium (Anderson, 1987).

2.6 Public health and economic importance of cryptosporidiosis

The many reports of increased prevalence of *Cryptosporidium* infections in both animals and humans emphasize the need for development of effective control measures for individual animals. This is very cardinal because some of the *C. parvum* isolates have been found to be transmissible from animals to man. Human infections have been associated with exposure to infected animals particularly calves and lambs (Ungar, 1990a; Casemore, 1990). This is why there is need for persons at risk to avoid contact with animal faeces (Willocks *et al.,* 1998). Horses and horse stables have also been linked to these infections.

Since the AIDS pandemic has the greatest impact on those in the productive years (UNAIDS, 2000) and cryptosporidiosis has been found to have devastating effects on AIDS patients (Farthing, 2000), the spread of the infection is therefore having devastating consequences at the household, community and sectoral level,

with significant negative consequences for the national economy as a whole. This therefore, means the health sector is stretched and needs more funds to provide long term treatment which is usually symptomatic for the persistent diarrhoea in these patients. This also causes serious impact on the macro economy of the country as it affects the country's gross domestic product (GDP) (UNAIDS, 2000).

2.6.1 Public health significance

Cryptosporidiosis, long considered to be a veterinary disease, has only recently emerged as a serious human problem (Guerrant, 1997). The zoonotic potential of *C. parvum* was recognised in the early 1980s when it was frequently reported as a gastrointestinal disease of animals and humans and some isolates of *C. parvum* were found to be transmissible from animals to humans (Current *et al.*, 1983; Anderson, 1998). The parasite continues to be a major threat to human health because: firstly the current methods for water purification are ineffective for its removal from the public water supply and secondly, there is no effective therapy for cryptosporidiosis (Leav *et al.*, 2003).

Although the infection is common in humans, it is only a very serious condition in immuno-compromised individuals and children between 1 to 5 years old (Leav *et al.*, 2003). In Zambia, the prevalence of *C. parvum* in children was found to be 18% (Nchito *et al.*, 1998) to 26% (Amadi *et al.*, 2001). Chintu *et al.* (1995) reported that cryptosporidiosis was found more frequently in HIV sero-positive (14%) than HIV sero-negative (8%) children with diarrhoea in Zambia. In Rwanda, *Cryptosporidium* was found to be a frequent cause of diarrhoeal disease especially in

children, in whom it was present as a sole pathogen in 8.3% of those with diarrhoea (Bogaerts *et al.,* 1984).

Many reports of zoonotic transmission have been documented with direct contact of ruminants and humans being the most common (Morgan *et al.,* 1999b; Mosier and Oberst, 2000). The other important mode of zoonotic transmission which has raised a lot of public concern and research is the contamination of water for human consumption by oocysts from livestock and wild animals. The dairy industry waste has been incriminated as the major livestock source of contamination for water source leading to human outbreaks of the disease (Peng *et al.*, 1997; Anderson, 1998). However, suggesting animals as the major source of human infections would not be correct as human infections do occur even where there is no association with animals (Anderson, 1998). There is emerging evidence of person-to-person transmission of cryptosporidiosis in hospital environments (Meinhardt *et al.,* 1996) and so other sources of contamination like human sewerage cannot be ruled out (Anderson, 1998). *Cryptosporidium hominis* was in fact detected in drinking water and mineral water by nested PCR (Nichols *et al.,* 2003). Mineral water is normally laboratory tested.

2.6.2 Economic importance in the livestock industry

Since the first time *C. parvum* was identified in a calf with diarrhoea (Panciera *et al.*, 1971), it has become increasingly important as a sole pathogen or a concurrent infection with other entero-pathogens in calf-hood diarrhoea (Faubert and Litvinsky, 2000). The infection leads to weight loss (Tzipori *et al.,* 1983). The

parasite has also been found as one of the common leading causes of enteropathies in lambs and kids (Xiao *et al.*, 1993; de Graaf *et al.*, 1999). The direct economic losses arise mostly from cost of drugs, fluid therapy, veterinary assistance and increased labour costs (de Graaf *et al.*, 1999). Although high mortalities are not a common feature of the disease, Sanford and Josephson (1982) reported a high mortality in 42 scouring calves. Mortalities due to cryptosporidiosis contribute to economic losses *but to a lesser degree as compared to other enteropathogens and in Belgium, they are estimated at 5-10% (de Graaf et al., 1999). The losses are primarily indirect from* weight loss and retarded growth in calves, lambs or kids (de Graaf *et al.*, 1999).

The economic significance of cryptosporidiosis in ruminants is compounded by the fact that there is no effective treatment (O'Donoghue, 1995). In adult cattle, *C. andersoni* has been found to reduce weight gains in feedlot cattle and reduce milk production in lactating cows (Anderson, 1998). Anderson (1987) found that *C. andersoni* was the only pathogen found in the gastric glands of 88 feedlot cattle that had a reduced weight of 0.5kg per day, whereas Esteban and Anderson (1995) found that cows shedding *C. andersoni* oocysts produced significantly less milk, approximately 3.2 kg/day (13%), as compared to the non-shedders in five herds of cattle. All these losses do constitute a significant loss in income for the dairy farmers as well as the nation as a whole.

2.7 Prevalence of *Cryptosporidium*

2.7.1 Humans

It is difficult to compare the prevalence rates and occurrence of human cryptosporidial infections from available data because the examined populations as well as the techniques used to detect the infection vary between studies (Joachim, 2004). However, the prevalence of cryptosporidial infections in humans is documented worldwide with most infections being due to *C. parvum*. In some studies, prevalence is higher in the developing countries than in developed ones (Leav *et al.*, 2003). This could be attributed to poor sanitation, contaminated water supplies, overcrowding or greater contact with domestic animals (O'Donoghue, 1995). An extensive review of cases of diarrhoea demonstrated that the incidence of *Cryptosporidium* infection among immunocompetent patients from developing countries was 6.1% (Leav *et al.*, 2003). The prevalence rate from various reports, excluding AIDS patients and outbreak situations, range from 0.1 to 27.1% in developed countries (mean of 56 surveys = 4.9%) and 0.1 to 31.5% in less developed countries (mean of 48 surveys = 7.9%) (O'Donoghue, 1995).

Generally, both *C. hominis* and the animal genotype of *C. parvum* occur frequently in humans in Europe (Joachim, 2004). In the United Kingdom, 97% of cases in humans are due to *C. parvum,* 1% *C. meleagridis,* 0.2% *C. felis* and less than 0.1% *C. canis*, while the remainder have not yet been characterised (Pedraza-Diaz *et al.*, 2001). Typing of 39 out of 49 confirmed cases of human cryptosporidiosis in Northern Ireland in 1998 revealed *C. parvum* genotype II in 87.2% of the samples, the rest consisted of *C. hominis* (Lowery *et al.*, 2001). In Denmark, Enemark *et al.*

(2002) reported the distribution of different genotypes in humans to be 41% for *C. parvum* zoonotic animal genotypes and 57% for *C. hominis* and one case of *C. meleagridis*. *Cryptosporidium hominis* seems to be correlated with travel while *C. parvum* animal genotype is correlated with contact to farm animals (McLauchlin *et al.,* 2000). In Germany, *Cryptosporidium* seems to be a regular souvenir from travels as concluded from the history of 795 travellers returning from developing countries, 2.8% of which had *Cryptosporidium*-associated diarrhoea (Jelinek *et al.,* 1997). Appendix one shows the prevalence of *Cryptosporidium* species in immuno-competent individuals and in patients with HIV from selected parts of the world (extracted from Olson *et al.,* 2004).

AIDS and other conditions of immunosuppression increase the prevalence of cryptosporidiosis and the range of parasite genotypes involved (Griffiths, 1998). In a study in Italy (Gomez-Morales *et al.,* 1992), antibodies against *Cryptosporidium* were detected in 95% of AIDS patients compared with 5.3% of a healthy control population. In another study in Italy, during a waterborne outbreak involving 1731 individuals, 30.7% of the HIV positive individuals were affected with *Cryptosporidium* in comparison with 13.6% of HIV-negative ones (Pozio *et al.,* 1997). In Africa, some reported prevalence rates are 20.5% (n=2446) in children in Uganda (Tumwine *et al.*, 2003), 9% (n=82) in adults presenting with diarrhoea or HIV/AIDS in Zimbabwe (Gumbo *et al.*, 1999) and 24.8% (n= 101) in children in South Africa (Leav *et al.*, 2002). In two studies in Zambian children, the prevalence rate was found to be 18% (Nchito *et al.,* 1998) and 26% (Amadi *et al.,* 2001), being more frequent in HIV sero-positive (14%) than HIV sero-negative (8%) Zambian children (Chintu *et al.,* 1995). Among some major reasons contributing to the high

prevalence of cryptosporidiosis in sub-Saharan Africa is probably the high incidence of HIV/AIDS with an equivalent of 70% of the world's cases (UNAIDS, 2002). Zambia's HIV/AIDS estimated prevalence is about 15.6% among adults and is among the highest in Sub-Saharan Africa. Since 1994, HIV/AIDS in Zambia has become prevalent in both rural and urban areas, affecting 10.8% of the rural and 23.1% of the urban population (Central Statistic Office, Zambia, 2001-2002).

2.7.2 Ruminants

The prevalence of *C. parvum* in cattle ranges from 1 to 80% around the world, with higher rates in calves. In a survey conducted in the USA involving 15 dairy farms in seven states, the prevalence in pre-weaned calves aged five days to two months was observed to be 50.3% (Santin *et al.*, 2004). In 20 Canadian dairy farms in British Columbia, Olson *et al.* (1997b) reported a prevalence of 59% in calves (newborn to 6 months). In another study carried out in Central Mexico to determine the prevalence in calves (1 to 30 days old) on 31 dairy farms, a prevalence of 25% was found (Maldonado-Camargo *et al.*, 1998).

In Europe, prevalence studies have also been done and some prevalence results reported in calves are 80% in Britain (Scott *et al.*, 1995), 17.9% and 43.4% in two different surveys in France (Lefay *et al.*, 2000), 47.9% in Spain (Castro-Hermida *et al.*, 2002a) and 21.5% in Germany (Joachim *et al.*, 2003).

There are reported prevalence rates in different parts of the Asian continent. These include 20% in 60 cattle of Basrah in Iraq (Mahdi and Ali, 2002), 12% in 50 calves (0 to 2 months) in Tokachi District in Hokkaido Japan (Sakai *et al.*, 2003) and

100% in 84 dairy cattle in Gokseong-gun, Korea (Yu *et al.*, 2004). In the Korean study, the prevalence in beef cattle in Chungju-si region was 8.9% (n=754) and 98.2% (n=107) in Gokseong-gun region.

In Africa, very few documented data is available on the prevalence of *C. parvum* in ruminants. However, few studies have been done in East Africa where prevalence of 5.3% (n=486) in Tanzania and 38% (n=50) in Uganda (Mtambo *et al.*, 1997; Nizeyi *et al.*, 2002a) have been reported. In Zambia, the prevalence of *C. parvum* in cattle was found to be 42.8% in dairy cattle, 8% in beef cattle and 6.2% in traditional cattle (Goma, 2005). This study showed a high prevalence in animals kept under intensive management system.

In sheep, *C. parvum* is one of the leading causes of neonatal diarrhoea and the lambs tend to shed large numbers of oocysts (de Graaf *et al.*, 1999). The prevalence varies from country to country. Two studies in Spain have revealed a prevalence of 40.2% in diarrhoea outbreaks in lambs (Pilar-Izquierdo *et al.*, 1993) and 59% in a cross sectional study in Zaragoza (Causapé *et al.*, 2002). In Canada, the prevalence in 89 sheep of varying ages was found to be 23% (Olson *et al.*, 1997a). In Egypt, Abd-El-Wahed (1999) reported a prevalence rate of 68.3% when he examined faeces of 120 lambs. In Zambia, the prevalence was 18.4% (n=152) (Goma, 2005).

Data available on the prevalence of *C. parvum* in goats around the world is scarce. However, *C. parvum* infections in kids can be a serious problem in herds (flocks) with high prevalence (see Table 2.2). A study of diarrhoeic kids in Spain showed a prevalence of 42% (Muñoz *et al.*, 1996). In an outbreak of diarrhoea on a goat farm in Oman, morbidity approaching 100% in kids less than six months with high mortality despite intensive supportive therapy were reported (Johnson *et al.*,

1999). In France, Chartier *et al.* (2002) found a prevalence of 58.9% in 137 kids using an Enzyme Linked Immunoabsorbent Assay (ELISA) for oocyst detection. In Sri Lanka, Noordeen *et al.* (2001) reported a prevalence rate of *Cryptosporidium* infection in 3 agro-climatic zones to be 28.5% from 1020 goats using the modified Ziehl-Neelsen staining technique.

Table 2.2 Prevalence of *Cryptosporidium parvum* in goats in different countries

Country	Prevalence (%)	Reference
Zambia	5.7	Goma, 2005
Oman	100	Johnson *et al.*, 1999
France	58.9	Chartier *et al.*, 2002
Spain	42	Muñoz *et al.*, 1996

2.8 Diagnostic techniques

Cryptosporidiosis has been detected wherever humans or juvenile animals congregate. Severe outbreaks occur where calves, lambs, kids or children are grouped (Anderson, 1998). A variety of methods are available for the detection of *Cryptosporidium* (Arrowood, 1997). The most commonly employed technique for identifying *C. parvum* infection in live individuals is the detection of oocysts in faecal samples (O'Donoghue, 1995). Direct microscopic staining methods, immunological based methods and molecular techniques are some of the methods used (Fayer *et al.*, 2000).

2.8.1 Direct staining techniques and microscopic examination

Smears are made either directly from faeces or after performing a concentration technique on the faecal samples after which direct or differential staining techniques are used to stain the *Cryptosporidium* oocysts. Safranin-methylene blue stain (Baxby *et al.*, 1984) and Ziehl-Neelsen stain (Henricksen and Pohlenz, 1981) are the commonly used stains in the visual detection of oocysts under the microscope. The major difficulty with this direct microscopy method is the difficulty in distinguishing oocysts from other small particles in the faecal and environmental specimens. The method is also time consuming, tedious and requires experienced technicians to accurately identify oocysts (Morgan *et al.*, 1998). The oocysts stain variably under the acid-fast stain, depending on their age and viability (O'Donoghue, 1995). There are few or no morphometric differences on which to differentiate the species as the oocysts are usually small (4-6 μm) and have obscure internal structures (Fayer *et al.*, 2000). These authors also found that the oocysts of a given species could be morphologically indistinguishable from those of another species or genus.

The most widely used test is the modified acid-fast or Kinyoun stain (Leav *et al.*, 2003). Weber *et al.* (1991) found that the Kinyoun acid-fast staining, after concentration by the formalin-ethyl acetate, recovered 60% of oocysts compared to 90% in an immuno-fluorescent technique, using watery stool specimen seeded with 5,000 oocysts per gram. On the other hand, in formed stool, it was found that the immuno-fluorescent and modified acid-fast staining technique had 100% detection rates in specimens seeded with 50,000 and 500,000 oocysts per gram of faeces. From these findings, Weber *et al.* (1991) concluded that although the immuno-fluorescent

method is more sensitive than the acid-fast method, both these conventionally used methods have low sensitivity as they require the presence of large numbers of oocysts to identify infection. However, the method (Kinyoun acid-fast staining) is widely used for diagnosis of clinical cases and in surveys (Arrowood, 1997) but interpretation of stained smears requires experience because other organisms in the stool may also stain acid fast (Leav *et al.,* 2003).

2.8.2 Immunodiagnostic methods

The last 10 years have seen the development of highly sensitive and specific enzyme immuno-assays (EIA) and these have become widely accepted for screening stool (Anusz *et al.*, 1990; Newman *et al.*, 1993; Katanik *et al.*, 2001). Most of these are in form of commercial kits and are of high specificity and sensitivity compared to direct microscopic examination. The sensitivity of these commercial EIA has been reported to be 100% and specificity ranges from 98.6 to 99.5% based on comparison with the modified acid-fast staining technique (Katanik *et al.*, 2001). In a study which compared the Kinyoun acid-fast staining technique and a commercially produced enzyme immunoassay, McCluskey *et al.* (1995) found an overall agreement of 72% between the two tests, with the EIA being more sensitive. However, according to Kehl *et al.* (1995) the sensitivity of the two microscopic methods, the immuno-fluorescence (Meriflour kit) and the modified acid-fast stain of 96.4% is comparable to the EIA commercial packs (ProSpect and Colour Vue) of 94.5%. The commercial kits are becoming widely used for screening stool (Katanik *et al.*, 2001). They are also easy to use, quick and there is no need for an experienced

technician to identify the parasite (Fayer *et al.*, 2000). However, they are quite expensive (Mosier and Oberst, 2000).

The wall of the oocysts contains antigen that may stimulate an antibody response in immunized animals and such antibodies can be labelled to aid identification of oocysts using immunodiagnostic methods. However, many of these oocyst wall antigens are conserved within the genus *Cryptosporidium* and appear across species (Fayer *et al.*, 2000) and therefore cannot reliably differentiate between species.

Some immunologic based techniques that are used to detect infection in live hosts include immuno-fluorescence (IF) with monoclonal antibodies, Enzyme immunoassays (enzyme-linked immunosorbent assay – ELISA, copro-antigen ELISA), latex agglutination reactions, reverse passive haemagglutination (RPH) immuno-serology using IF detection and ELISA, and solid phase immuno-chromatographic assays (Fayer *et al.*, 2000). Although some researchers have used the immunologic based techniques to determine prevalence in certain populations, they have limitations in that they only suggest exposure to the parasite during some time in the individual's life and not depicting the actual disease situation (O'Donoghue, 1995).

2.8.3 Molecular methods

Over the past 15 years, there has been a dramatic evolution in molecular approaches to study parasites and parasitic diseases (Zarlenga and Higgins, 2001). *Cryptosporidium* and cryptosporidiosis have been studied and this has been brought

about through the development of new applications of the polymerase chain reaction (PCR). Saiki *et al.* (1985) defined PCR as an in vitro method of producing large amounts of specific DNA fragment of defined length and sequence from small amounts of a complex template. The PCR reaction repeatedly amplifies selected specific DNA sequences in a complex DNA mixture (Saiki *et al.,* 1985).

In PCR, the target DNA is copied by a thermostable DNA polymerase enzyme, in the presence of selected nucleotides and primers. Through multiple cycles of heating and cooling in a thermocycler to rounds of target DNA denaturation, primer hybridisation and primer extension, the target DNA is amplified exponentially (Yang and Rothman, 2004). To increase the sensitivity and specificity, a double amplification step can be done with appropriately designed nested primers (Erlich *et al.,* 1991). This way of amplifying rare sequences from a mixture has vastly increased the sensitivity of genetic tests (Darnell *et al.*, 1990) and the ability to selectively amplify a specific region of the genome from a small amount of DNA has made the PCR technique particularly useful as a diagnostic tool (Monis and Andrews, 1998).

A number of PCR techniques have been developed to detect small numbers of oocysts within environmental and faecal samples and to distinguish between different *Cryptosporidium* species and *C. parvum* genotypes (Mosier and Oberst, 2000). Some of the techniques used are random amplified polymorphic DNA (RAPD), sequence analysis and restriction fragment length polymorphism (RFLP). Sequence analysis provides the most complete and reliable data as it examines all bases at a particular locus but it is more costly and time consuming than RFLP, which examines bases at particular restriction sites within the locus (Fayer *et al.,*

2000). In recent years, researchers have also developed PCR-based techniques for differentiating *C. parvum* of human origin and *C. parvum* of animal origin (Sulaiman *et al.,* 1999). The techniques are based on the polymorphic nature of *C. parvum* strains that infect humans and most animals at the B-tubulin, oocyst wall protein (COWP), dihydrofolate reductase (DHFR), thrombospondin-related adhesive protein 1 (TRAP-C1), thrombospondin-related adhesive protein 2 (TRAP-C2), internally transcribed spacer 1 (ITS1), polythreonine (Poly-T), small-subunit (SSU) rRNA and unidentified genomic sequences (See Table 2.3).

Table 2.3 Some PCR types, their target gene and size of product used in molecular typing of *Cryptosporidium*

Type of PCR	Target	Size of product	Reference
Nested PCR-RFLP	SSU rRNA	~825	Xiao *et al.*, (1999)
PCR-RFLP	TRAP-C1	1200	Spano *et al.*, (1998)
PCR-RFLP	TRAP-C2	369	Sulaiman *et al.*, (1998)
PCR-RFLP	Poly-T	318	Carraway *et al.*, (1997)
Nested PCR-RFLP	DHFR	408	Gibbons *et al.*, (1998)
PCR-RFLP	COWP	553	Spano *et al.*, (1997)
Genotype-specific PCR	ITS1 of rRNA	513(human),519(bovine)	Carraway *et al.*, (1996)
PCR-RFLP	SSU rRNA	556	Awad-El-Kariem *et al.*, (1994)

The advantage of molecular techniques over the other methods is that they are rapid, highly sensitive and specific and also provide information on genetic variability of *Cryptosporidium* isolates that could be used in their speciation (Morgan *et al.*, 1998; Xiao *et al.*, 1999). The method, however, has several limitations. It can give false positives by detection of naked nucleic acids, non-viable micro-organisms and other forms of laboratory contamination (Fayer *et al.*, 2000), which could be from exogenous DNA. It can also give false negatives which could be due to the relatively small sample volume permissible for PCR reactions and also problems associated with PCR processing (Yang and Rothman, 2004). Sample processing problems that may lead to false positives include: inadequate removal of PCR inhibitors in the sample, ineffective release of DNA from the raw sample or poor DNA recovery after extraction and purification steps (Yang and Rothman, 2004). To prevent laboratory contamination, all solutions, glassware and plasticware must be specially treated and gloves should be worn (Gasser, 1999) as hands are a major source of contamination.

2.9 Management and control of cryptosporidiosis

2.9.1 Drug therapy

No specific treatment that has been found to be fully efficacious against cryptosporidiosis (Current *et al.*, 1986; Fayer and Ungar, 1986; Ungar *et al.*, 1990b). Oral or intravenous rehydration remains the single most important symptomatic

treatment to diminish clinical signs of disease in humans and animals (Ramirez *et al.*, 2004).

A number of drugs have been tried in humans and animals both as potential preventatives and curatives with little success (Moon *et al.*, 1982; Joachim *et al.*, 2003). In initial therapeutic studies, two drugs, furazolidone and spiramycin, appeared to afford some benefit in alleviating diarrhoea and in reducing oocyst shedding in some cases. In more recent years, drugs such as co-trimoxazole, paromomycin, halofuginone lactate and β-cyclodextrin have been tested in experimental infections, therapeutically or prophylactically (Naciri *et al.*, 1993; O'Donoghue, 1995; Castro-Hermida *et al.*, 2001; Grinberg *et al.*, 2002). All of these drugs have been shown to reduce the prepatent or patent period, severity of clinical signs and/or oocysts shedding but none eliminated the parasite completely.

In immuno-compromised patients infected with *Cryptosporidium*, orally administered human serum immunoglobulin or bovine colostrum with specific antibodies for the parasite have been found to be beneficial (Pickering, 2000). Paromomycin and, recently, nitazoxanide have been reported to have some clinical efficacy. Nitazoxanide now represents a significant advance in therapeutic approaches to cryptosporidiosis and has now been registered in the United States for treating childhood cryptosporidiosis (Smith and Corcoran, 2004). Protracted cryptosporidiosis in immuno-deficient patients may respond to one or more weeks of daily spiramycin treatment (Portnoy *et al.*, 1984). Control of viraemia with highly active antiretroviral therapy and concomitant increase in $CD4^+$ T cells remains the most effective medical intervention for cryptosporidiosis in AIDS patients (Maggi *et al.*, 2000).

In an immuno-competent host, the disease is usually self-limiting and recovery is uneventful (Preiser *et al.*, 2003). Effective management of the disease requires more epidemiologic information on the sources of infection, methods of spread and relative risk for different populations (Mosier and Oberst, 2000). Vaccines always present an attractive strategy to battle infectious diseases. However, the lack of understanding of the host's immune response to the infection and the mechanisms utilized by the parasite to invade the gastrointestinal tissues and evade host immunity have been barriers to the development of effective therapy and prophylaxis for cryptosporidiosis (Ramirez *et al.*, 2004).

2.9.2 Prevention and control

In the absence of effective, specific therapy against infection with *Cryptosporidium*, preventive measures are of great importance in controlling this parasite. This was proven by the marked decline in human cases of cryptosporidiosis during the 2001 epidemic of Foot and Mouth Disease (FMD) the United Kingdom. Hunter *et al.* (2003) and Smerdon *et al.* (2003) found that limiting human access to the country side, containment of animals and restriction of livestock movement for trade or pastures, and extensive slaughtering of FMD-infected animals not only ended the FMD epidemic but also resulted in a significant reduction (81.8%) in reported cases of human cryptosporidiosis.

Because the major source of human infection is contamination of water supplies, implementation of measures to decrease the spread of parasitic oocysts in the environment is critical. Patients with underlying immune system weakness are at

greater risk from the most severe complications of cryptosporidiosis. Prevention of cryptosporidiosis transmission is dependent on hygienic measures in any setting (Ramirez *et al.,* 2004). Hygienic preventive measures that should be employed include extensive hand washing, avoiding direct contact with stool from animals or humans, avoiding accidental ingestion of water used in recreational activities and taking measures to ensure the safety of drinking water. *Cryptosporidium* species can be removed from drinking water by boiling for one minute (Leav *et al.*, 2003).

In animals, preventive measures should include management practices and hygienic measures. Management practices that help prevent and control cryptosporidiosis include reduction in the stocking rate, individual housing of calves, separation of healthy animals from the sick ones during diarrhoea outbreaks and administration of appropriate amounts of colostrum to neonates (Heath, 1992; Atwill *et al.*, 1999; de Graaf *et al.*, 1999).

Hygiene at feeding times especially for dairy calves separated from dams at birth, is an important factor in preventing calves from getting the infection (Heath, 1992; Mtambo *et al.*, 1997). Hygienic measures that should be employed include regular cleaning of calf pens with running water and disinfection of the calf pens. This reduces the number of oocysts (eggs) contaminating the environment that could possibly be ingested by the calves. Fifty percent (50%) ammonia, 3% hydrogen peroxide, 10% formalin, Expor (chlorine dioxide based) and Oo-cid (two phase product producing ammonia) are some of the disinfectants that have been found to be effective against oocysts but prolonged use of these concentrated disinfectants in confined areas is not advisable and is impractical in many situations (O'Donoghue, 1995). Measures that reduce transmission should also be encouraged. These include

limiting the number of animals enclosed in the same facilities (i.e. reduced stocking density), keeping young animals separated from adults, minimizing contact between personnel and calves, and maintaining a short calving period (Hoar *et al.,* 2001).. Cattle facilities should also be located away from streams, lakes, dams and rivers whenever possible and waterways should be fenced-off in pasture lands to prevent possible run-off into these water sources. Frequent manure spread on pasture lands has been associated with detection of oocysts in streams (Sischo *et al.,* 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study areas

Zambia, a southern African country, lies between longitudes 22°E and 34°E and latitudes 8°S and 18°S. It is a land-locked country and shares borders with eight other Southern African countries (Angola, Malawi, Tanzania, Democratic Republic of Congo, Angola, Namibia, Botswana, Zimbabwe and Mozambique). It occupies an estimated area of seventy-five million hectares (752 610 km^2). The country is divided into nine provinces namely the North-western, Western, Southern, Lusaka, Central, Copper belt, Luapula, Northern and Eastern Provinces. The Lusaka Province is one of the major livestock keeping areas in Zambia. Others are the Central, Eastern, Western and Southern provinces.

Commercial dairy cattle in Zambia are kept under controlled grazing. The production units are characterised by use of sophisticated machinery and substantial amounts of hired human labour. However, small-scale dairy farms have also become important in the country and these normally do not have sophisticated machinery and rely mostly on human labour. This study was done on selected commercial dairy farms in the Lusaka and Central Provinces. Figure 3.1 shows the areas in which in the study farms are located. A preliminary survey was done from August to September 2004 on the selected farms to confirm presence of *Cryptosporidium* in the animals. A total of 20 dairy farms were included in the study based on the willingness of the farmer to participate.

Besides dairy cattle, the farms also had either sheep or goats. The presence of a borehole as a source of drinking water on the dairy farms was also one of the selection criteria. This was done to reduce variability but on two farms the animals and humans also had access to stream water. The main study commenced in October 2004. From October 2004 to March 2005, 207 calf, 39 lamb and 14 kid faecal samples and 289 human stool samples were collected.

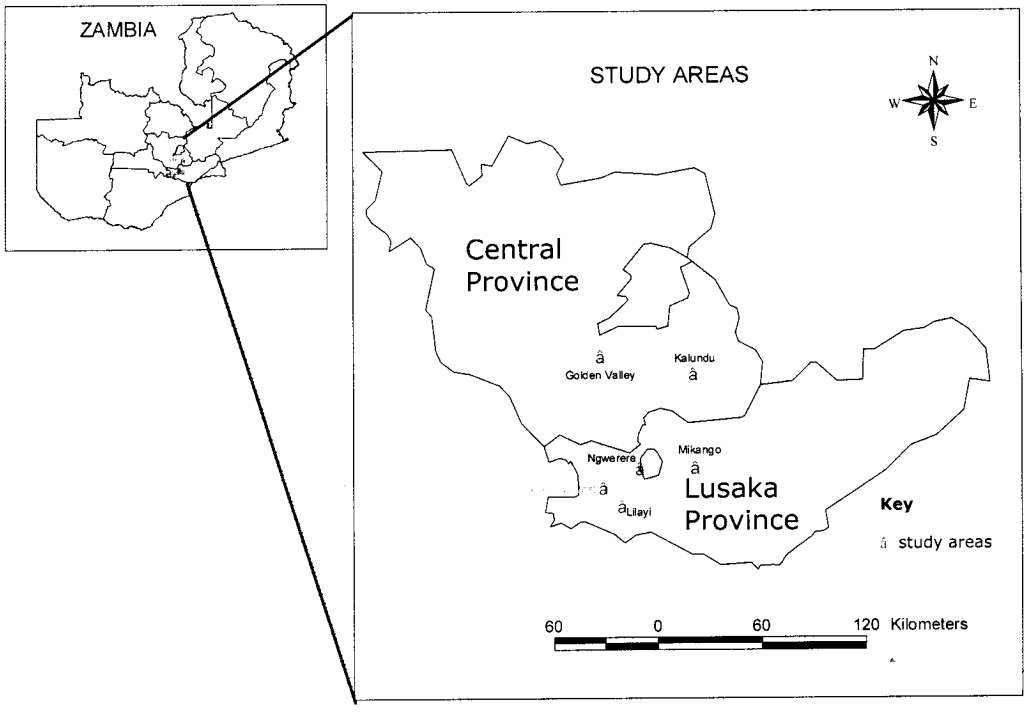

Figure 3.1 Map showing the study areas (source: Anonymous, 2005)

3.2 Study design

A cross-sectional study was conducted on 20 dairy farms. The study was divided in two main phases. The first phase was a preliminary study to confirm the presence of *C. parvum* infection on the selected farms. In addition, a questionnaire was administered to the farm owners in order to collect general information on their farms and animals (Appendix 2). The information collected included age, sex, herd size, occurrence of diarrhoea or mortality due to diarrhoea and the general farm management such as animal housing and waste management. During the first part of the second phase, *Cryptosporidium* positive farms were visited every two weeks for a

period of six months (October 2004 – March 2005). A questionnaire was also administered to the human subjects and information on their age, sex, possible history of diarrhoeal illness, direct/indirect contact with a symptomatic patient or animal, water sources, was obtained (Appendix 3). The questionnaires were administered the same day that the stool specimens were collected. This was done to find out some risk factors that maybe associated with prevalence of *C. parvum* in humans working in the dairy farms. During the second part of the second phase (April 2005 – June 2005) the genotypes of *C. parvum* positive samples were determined using Polymerase Chain Reaction (PCR) to determine whether there was any similarity between the animal and the human infection. The genotypic characterization was done at the Faculty of Veterinary Medicine, Department of Virology, Parasitology and Immunology, Gent University, Belgium.

3.3 Animal study population

Our target population was all calves and all lambs less than six weeks of age and all kids found at the time of the farm visit. Goats were not reared or kept on all farms and, where present, they were in small numbers (average was 25, all ages included).

3.4 Human study population

The human subjects were divided into animal workers (with direct contact with the animals) and their relatives (with no direct contact with the animals). Only the farm workers working with calves and members of their households were recruited in the study. All the children and 50% of the adults, if they were more than

10 in the home, were sampled. Those that were less than 10 in the household were all sampled. From the 20 farms visited, 289 human samples were collected. These comprised 175 males and 111 females. Ninety-eight (98) of these were children less than 10 years old. The sexes of three individuals were not stated.

3.5 Ethical clearance

Ethical clearance for human sampling was sought from and was approved by the University of Zambia Research Ethics Committee before the study commenced. Upon arrival on a farm, the study was explained to the farm owners and permission was sought to conduct the study on the farm. The study was also explained to the farm workers and the heads of the households. Permission was sought to interview them and collect samples. The respondents were advised that the study was expected to provide information on the presence, magnitude and causal factors of cryptosporidial infection, which would enable the planning of future intervention and control measures of the infection.

3.6 Sample collection

A single faecal sample was collected from each animal neonate per rectum using clean disposable, latex examination gloves. For each sampled animal, the faecal consistency (that is, whether normal, watery or pasty) was recorded on pre-designed laboratory forms immediately after collection. The animal's identification number (ID), age and sex were also recorded. The samples were then transported in *cooler boxes packed with ice packs to the laboratory at the University of Zambia*

where they were put in clean tight - screwed plastic containers. The containers were appropriately labelled and the samples were preserved and stored at -20°C until analysis.

The human stool samples were collected in universal plastic containers or bottles normally provided at medical centres. After obtaining informed consent, each human subject was provided with a container to put in their stool. Sample transportation to the laboratory and storage was similar to that of the animal samples. All the samples were analysed by ELISA and PCR.

3.7 Copro-antigen Enzyme Linked Immunosorbent Assay – ELISA

A specific anti-*Cryptosporidium* monoclonal antibody based commercial kit (Techlab, Inc., Blacksburg, Va) was used to detect oocysts in the collected faecal samples. This kit uses antibodies against *Cryptosporidium* to detect the parasite in the faeces. The kit contains 96-well micro-titre plate sensitized by specific antibodies for an antigenic determinant of *Cryptosporidium*. The antibodies allow specifically the capture of the corresponding pathogen, if it is present in the faecal samples. The reagents provided in the kit include a positive control, detecting antibody, conjugate, substrates A and B, a stop solution (containing sulphuric acid) and wash buffer concentrate. The buffer concentrate (50 ml) was diluted with 950 ml distilled water before use.

Faeces (approximately 0.1 g) were diluted with 0.4 ml dilution buffer to make a 1:5 dilution ratio and vortexed. After vortexing, 0.1 ml of the mixture was added to the test well on the microtitre plate and incubated at room temperature for 1 h. The

plate was washed with the buffer five times and slap-dried on a paper towel until there was no buffer in the wells after which the detecting antibody was added to the wells. The plate was then incubated at room temperature for 20 min. After this incubation step, the plate was again washed five times with buffer. After washing, the conjugate, a peroxidase labelled anti-*Cryptosporidium* specific monoclonal antibody, was added to the wells. The plate was again incubated at room temperature for 10 min, washed again as described before and then the enzyme substrate (hydrogen eroxide) and chromogen (tetramethylbenzidine) were added (The chromogen tetramethylbenzidine has advantages of being more sensitive than the other peroxidase chromogens and is not carcinogenic). Enzymatic reaction was stopped by acidification by the stop solution. The plate was then read spectrophotometrically using an ELISA reader (Labsystems Multiskan EX Finland) at 450 nm. A sample was considered positive if the absorbance exceeded 0.150.

3.8 Polymerase chain reaction (PCR) and genotyping of oocyst identified samples

3.8.1 Deoxyribonucleic acid (DNA) extraction

DNA was extracted from all human samples that were positive on ELISA and from 21 calf, 1 lamb and 1 kid positive samples. Frozen DNA samples were transported to the Faculty of Veterinary Medicine, Gent University, Belgium on ice for PCR analysis.

The protocol for isolation of DNA from stool for pathogen detection (QIAGEN, Germany) was used to extract the DNA from the ELISA positive stool

samples from both animals and humans. Before starting, wash buffers AW1 and AW2 were prepared as per instructions. For each sample, about 180-220 g of stool was weighed and placed into a labelled 2 ml micro-centrifuge tube using a spatula and placed on ice. For liquid samples, 200 µl was pipetted into a micro-centrifuge tube on ice. To each stool sample, 1.4 ml stool lysis buffer ASL was added and the mixture was vortexed for a min until the entire sample was thoroughly homogenized. The suspension was placed in liquid nitrogen for five min and then heated at 95°C for five min in a water bath. The freeze-thaw cycle was repeated five times. After the last heating, the suspension was vortexed for 15 sec and centrifuged at 14000 rpm in a microcentrifuge for a min to pellet the stool particles. The supernatant (~1.2 ml) was pipetted into a new, labelled micro centrifuge tube and the pellet discarded. One InhibitEX tablet (inhibitors adsorb to the InhibitEX tablet) was added to each sample and the mixture vortexed immediately and continuously until the tablet was completely suspended. The suspension was incubated at room temperature for a min to allow inhibitors to adsorb to the InhibitEX matrix after which it was centrifuged at 14000 rpm for three min to pellet inhibitors bound to InhibitEX. All the supernatant was then pipetted into a new 1.5 ml micro centrifuge tube and the pellet discarded. The sample was centrifuged again at 14000 rpm for three min and 200 µl of the supernatant from this last centrifugation was pipetted into a new 1.5 ml micro-centrifuge tube containing 15 µl proteinase-K. Two hundred microlitres buffer AL was added to the mixture and the mixture was vortexed for 15 sec followed by an incubation at 70°C for 10 min. After incubation, 200 µl of 96% ethanol was added to the lysate and mixed by vortexing to remove condensed drops from the inside of the tube lid. A new QIAamp spin column placed in a 2 ml collection tube was labelled

and the complete lysate was applied directly into the spin column without moistening the rim. The spin column with the lysate was centrifuged at 14000 rpm for a min. After this, the QIAamp spin column was placed into a new collection tube and the tube containing the filtrate was discarded. Five hundred microlitres of wash buffer buffer AW1 was added to the QIAamp spin column and centrifugation done at 14000 rpm for a min. The spin column was again placed into a new collection tube and the one with the filtrate was discarded. The QIAamp spin column was carefully opened, 500 µl buffer AW2 added to it and the tube centrifuged at 14000 rpm for three min. After this, the tube containing the filtrate was discarded and the QIAamp spin column was placed into a new, labelled 1.5 ml micro-centrifuge tube. Fifty µl elution buffer AE was pipetted directly into the QIAamp membrane and incubation was done at room temperature for a min. The mixture was then centrifuged at 14000 rpm for a min to elute DNA. After centrifuging, the eluate was placed back into the QIAamp membrane and centrifuged again at full speed for one min to improve the yield. This final eluate was stored at -20°C until analyzed.

3.8.2 Polymerase Chain Reaction (PCR)

The isolates of *Cryptosporidium* from the 21 calves, 1 lamb, 1 kid and 18 humans were sequenced at two loci, the Heat Stock Protein (HSP-70) gene and the 18S rDNA gene after PCR amplification.

3.8.2.1 HSP-70 gene amplification

The primers described by Morgan *et al.* (2001) were used to genetically characterize and identify the *C. parvum* genotypes on the selected number of positive faecal samples. A two step nested PCR was used to amplify the HSP-70 gene from genomic DNA of the *Cryptosporidium* isolates for nucleotide sequencing. This involved a primary and secondary PCR amplification. For the primary amplification, the forward primer HSPF4 (5'-GGT GGT GGT ACT TTT GAT GTA TC-3') and the reverse primer HSPR4 (5'-GCC TGA ACC TTT GGA ATA CG-3') were used to amplify a 448 bp PCR product. The PCR reaction consisted of 2.5 µl of each extracted DNA, 0.5µl deoxynucleoside triphosphate (dNTP), 0.5 µl HSPF4, 0.5 µl HSPR4, 2.5 µl BSA standard buffer, 18.25 µl PCR water and 0.25 µl *Taq* polymerase in PCR eppendorf tubes making a total volume of 25 µl. Forty (40) PCR cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec were carried out in a PTC-100 Peltier thermal cycler (MJ Research) with an initial hot start of 94°C for five min and a final extension of 72°C for 10 min. For the secondary PCR amplification, the primers HSPF3 (5'-GCT GST GAT ACT CAC TTG GGT GG-3') and HSPR3 (5'-CTC TTG TCC ATA CCA GCA TCC-3') were used. The PCR reaction consisted of 2.5 µl of the primary PCR product for each sample, 0.5 µl dNTP, 0.5 µl HSPF3, 0.5 µl HSPR3, 2.5 µl BSA Buffer, 18.25 µl PCR water and 0.25 µl *Taq* polymerase. Forty (40) PCR cycles were repeated and the condition for the secondary PCR was the same as for the primary PCR. The amplification products were then visualised on 1.5% agarose gel with ethidium bromide staining. Purified water was used as a negative control. The PCR products that amplified were then purified using QIAquick PCR Purification Kit (QIAGEN, Germany) and stored at -20°C until analysed.

3.8.2.2 18S rDNA gene amplification

The primers described by Ryan *et al.* (2003) were used to identify the *C. parvum* genotypes. A two-step nested PCR protocol was used to amplify the 18S rDNA gene. For the primary PCR, a PCR product of 763 base pairs was amplified using the forward primer 18SiCF2 (5'-GAC ATA TCA TCC AAG TTT CTG ACC-3') (base pair position 292) and the reverse primer 18SiCR2 (5'-CTG AAG GAG TAA GGA ACA ACC-3') (base pair position 1007). The PCR reaction mixture consisted of 2.5ul of each extracted DNA, 0.5 µl of deoxynucleoside triphosphate (dNTP), 0.5 µl 18SiCF2, 0.5 µl 18SiCR2, 2.5 µl BSA buffer, 18.25 µl PCR water and 0.25 µl *Taq* polymerase making a volume of 25 µl. Forty-five (45) PCR cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec were carried out in a PTC-100 Peltier thermal cycler (MJ Research) with an initial hot start of 94°C for five min and a final extension of 72°C for 10 min. For the secondary PCR amplification, a fragment of approximately 587 base pairs was amplified using the forward primer 18SiCF1 (5'-CCT ATC AGC TTT AGA CGG TAG G-3') (base pair position 289) and the reverse primer 18SiCR1 (5'-TCT AAG AAT TTC ACC TCT GAC TG-3') (base pair position 851). A dilution of 1/10 for each primary PCR product was made using distilled water. 2.5ul of the diluent for each sample was pipetted into a clean sterile PCR epperndorf tubes. The PCR reaction consisted of 2.5 µl diluent of primary PCR product, 0.5 µl dNTP, 0.5 µl 18SiCF1, 0.5 µl 18SiCR1, 2.5 µl BSA Buffer, 18.25 µl PCR water and 0.25 µl *Taq* polymerase were then added to each PCR product. The conditions for the secondary PCR were identical to those for the primary PCR. The secondary PCR products were visualised on 1.5% agarose gel

after ethidium bromide staining, purified using QIAquick PCR Purification Kit and stored at -20° C until analysed.

3.8.2.3 DNA purification

The principle of the QIAquick system for purifying DNA is that buffers provided are optimized for efficient recovery of DNA and removal of contaminants. The DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities are efficiently washed away, and the purified DNA is eluted with tris buffer or water.

The procedure involved mixing 20 µl of PCR product with 100 µl of buffer PB. To bind the DNA, this mixture was then pipetted into a QIAquick spin column placed in a 2 ml collection tube and centrifuged for 60 sec at 13200 rpm. The flow-through was discarded and the QIAquick column was placed back into the same collection tube. Buffer PE (0.75 ml) was added to the QIAquick column to wash and the tube was centrifuged for 60 sec at 13200 rpm. The flow-through was discarded and the QIAquick column was placed back into the same tube and centrifuged for an additional one min to get rid of residual ethanol from buffer PE. The QIAquick column was placed into a clean, labelled 1.5 ml micro centrifuge tube, 30 µl PCR water added to the centre of the QIAquick membrane and the column was centrifuged for one min at 13200 rpm to elute DNA. The purified DNA was sequenced directly in both directions.

3.7.3 Sequence reaction

Two PCR reactions per purified PCR product were done: one using the forward primer (HSPF3 for HSP-70 PCR product and 18SiCF1 for 18S rDNA product) and one using the reverse primer (HSPR3 and 18SiCR1 for the HSP 70 and 18S rDNA PCR products, respectively). Big dye terminator (2 µl), 1 µl 5X buffer, 1 µl primer and 4 µl DNAse- and RNAse-free water was added to 2 µl of each purified DNA sample making a volume of 10 µl. Forty (40) cycles of 95°C for 30 sec, 55°C for 10 sec, 60°C for four min were carried out in a PTC-100 Peltier thermal cycler (MJ Research) with an initial hot start of 95°C for five min. The sequence reaction products were then purified of Dye-terminator using the DyeEx spin kit prior to sequence analysis.

3.8.3.1 Removal of dye-terminator prior to sequence analysis

DyeEx spin kits (DyeEx^{TM} 2.0 Spin Kits) have been designed for fast and easy removal of unincorporated dye terminators from sequencing reactions. The principle of the DyeEx spin kit is that the gel-filtration chromatography separates molecules based on molecular weight. It uses gel-filtration material consisting of spheres with uniform pores. When sequencing reactions are applied on DyeEx modules, dye terminators diffuse into the pores and are retained in the gel-filtration material, while the DNA fragments are excluded and recovered in the flow-through.

The spin column with the sequence reaction product of each sample was gently vortexed to re-suspend the resin. The bottom closure of the spin column was then snapped off and the column placed into a 2 ml collection tube. The cap of the

column was loosened to quarter turn to avoid a vacuum inside the spin column. This was then centrifuged at 3000 rpm for three min. After centrifuging, the spin column was transferred to a clean, labelled micro-centrifuge tube and 10 µl of DNAse- and RNAse-free water was added to the sequence reaction to adjust the volume to 20 µl for easier handling and more reproducible pipetting. The sequence reaction was then applied to the gel bed of the spin column taking care not to touch the gel bed with the pipette tip. This was centrifuged for three min at 3000 rpm. Post-centrifugation the spin column was removed from the micro-centrifuge tube and discarded. The eluate was submitted for sequencing.

3.8.3.2 Sequencing

Sequencing was done using the Big Dye Terminator V3.1 Cycle sequencing kit (Applied Biosystems). Sequencing reactions were analysed on a 3100 Genetic Analyzer (Applied Biosystems) and assembled with the program Seqman II (DNASTAR, Madison WI, USA). The identity of the DNA sequences was confirmed by challenging the NCBI BLAST database (www.ncbi.nlm.nih.gov).

3.9 Statistical data analysis

Data were entered and analysed in the computer using the SPSS software (Statistical Package for Social Sciences) for windows (version 11.0). The statistical tests used to analyse the data were the Chi-square (χ^2) test and the Java Stat -2- Way Contingency Table Analysis. The results were considered significant at $P < 0.05$.

CHAPTER FOUR

RESULTS

4.1 *Cryptosporidium* copro-antigen ELISA results in ruminants

A total of two hundred and seven (207) calf, 39 lamb and 14 goat kid faecal samples were collected from 20 dairy farms. The commercial copro-antigen ELISA (Techlab, Inc., Blacksburg, Va) was used to identify the samples that were positive for *Cryptosporidium*. Out of the 207 calf faecal samples, 70 were positive giving an overall point prevalence of 33.8% (Table 4.1). Thirty-six (17.4%) of these were in the age range of 11-20 days with highest point prevalence observed at approximately 14 days. The prevalence was also high (8.7%) in calves aged less than 10 days old as compared to only 1.0% of those over one month old (See Figure 4.1). The difference in prevalence rates in the various age groups was significant (χ^2=37.800, P<0.0001; standard deviation =1.260).

Table 4.1 Prevalence of *Cryptosporidium* in ruminants at surveyed farms on the copro-antigen ELISA assay

	Number sampled	Positive	% Positive
Calves	207	70	33.8
Lambs	39	1	2.6
Goat kids	14	1	7.1

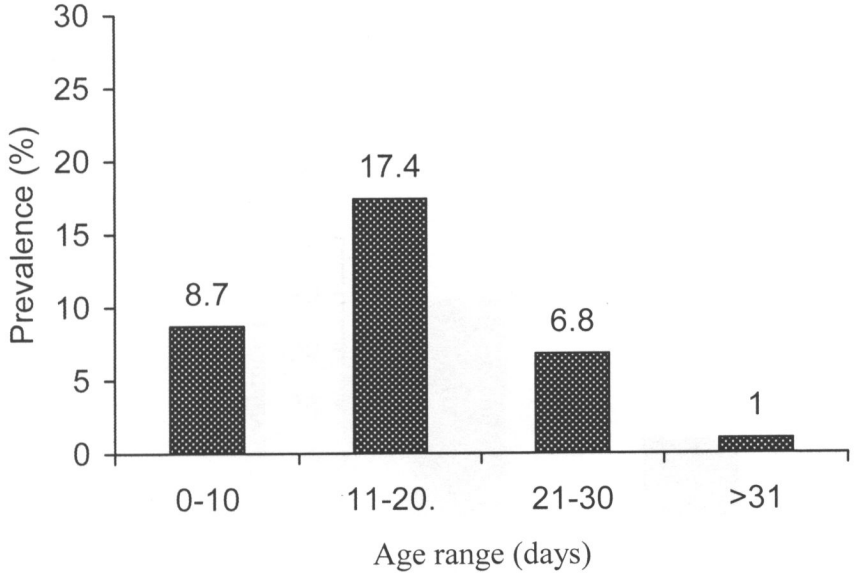

Figure 4.1 Age distribution of calves that were positive on the *Cryptosporidium* copro-antigen ELISA assay

Each of the sampled dairy farms had one or more calves shedding *C. parvum* oocysts, an indication that *Cryptosporidium* infection was prevalent on dairy farms. The dairy farms were grouped into small scale and large scale based on the total number of animals kept on the farm. Small scale farms had less than or equal to 300 cattle while large scale farms had more than 300 cattle on the farm. According to these groups, prevalence was found to be higher in animals from large scale farms (39.02%, n=164) than those from small scale farms (13.95%, n=43). The difference in prevalence between the two groups was significant (χ^2, P=0.004). The large scale farms were 3.9 times more likely to have *Cryptosporidium* positive calves than the small scale farms (Odds Ratio (OR) = 3.947; 95% Confidence Interval (CI) = 1.612-9.623).

Out of all the 70 positive calves, only four were from farms where humans were also positive.

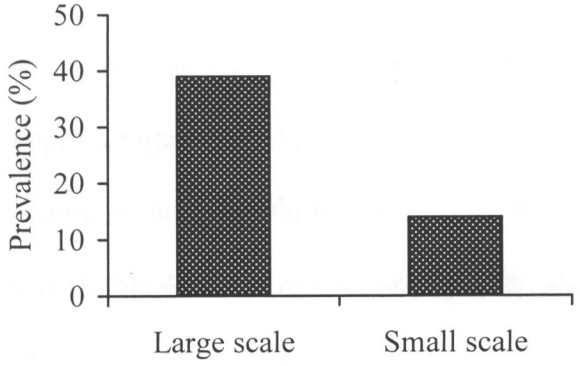

Figure 4.2 Farm prevalence of *Cryptosporidium* infection by farm size grouped into large-scale farms (n=164) and small-scale dairy farms (n=43.)

The overall prevalence of *Cryptosporidium* infection in lambs and kids was very low (Table 4.1). Out of the 39 lambs sampled, only one was positive giving a prevalence of 2.6%. The ages of the lambs ranged from 4-42 days and all those sampled had normal faecal consistency. All the lambs in all the sampled farms were housed in groups and their housing had only soil or concrete floor. The lambs were not bottle fed but suckled *ad-libitum* and were not separated from the dam.

The prevalence in kids was 7.1% (n=14). The kids, just like the lambs, were housed in groups and their housings had no bedding. The kids also suckled from their dams *ad-libitum*.

4.2 *Cryptosporidium* copro-antigen ELISA results in humans

During the same period of October 2004 to March 2005, a total of 289 human stool samples were collected from 18 farms. The workers and families from two farms refused to be included in the study due to personal reasons. A single sample was collected from each individual. Out of these 289 human samples, 18 were found to be positive by the copro-antigen ELISA, giving an overall prevalence of 6.2%. A total of eight farm workers and 10 family members were found to harbour *Cryptosporidium* oocysts (Table 4.2). There was, however, no statistical difference in prevalence between the farm workers and the non farm workers (χ^2=2.439, P=0.118).

The positive humans were from eight of the 18 sampled farms. These positive human samples were from 14 families (15.7%) out of 89 families that were sampled. The farm distribution of the positive families was that one farm had four positive families, three farms had two families each and four farms had only one positive family each. Four out of eight of these human positive farms had no calf that was positive for *Cryptosporidium,* that is, only humans were positive on these farms.

Table 4.2 The prevalence of *Cryptosporidium* among farm workers (n=82) and their family members (non farm worker) (n=207) on the copro-antigen ELISA assay

	Farm workers	Family members	Total
Males	8 (9.8%)	2 (0.9%)	10 (3.5%)
Females	0	8 (3.9%)	8 (2.7%)
Total	8 (9.8%)	10 (4.8%)	18 (6.2%)

Ten (5.7%) of the positive individuals were males and 8 (7.2%) were females. Out of the 10 males, 8 were farm workers (Table 4.2) while all the positive females were just family members of the farm workers. Only two households had two members of one family being positive whereby one was a farm worker and the other a non-farm worker.

The majority of the positive individuals were in the age range of 21-30 years for both males and females (Table 4.3). Among the children (n = 98), only one child in the 0-10 years age range was found positive. None of the adults over 50 years were positive. The sexes of three individuals tested were not stated and these were also negative on ELISA. None of the positive humans was clinically sick. The distribution of the prevalence rates in the different age groups is shown in Figure 4.3 below. There was no statistical difference among the different age groups (χ^2=8.825, P=0.184).

Table 4.3 Age and sex distribution of *Cryptosporidium* positive humans from 18 dairy farms (Males n=175; Females n=111) after copro-antigen ELISA assay

Age (years)	Male No (%)	Female No (%)
0-10	1 (0.6)	0 (0)
11-20	2 (1.1)	3 (2.7)
21-30	4 (2.3)	4 (3.6)
31-40	2 (1.1)	1 (0.9)
41-50	1 (0.6)	0 (0)
>50	0 (0)	0 (0)
Total	**10 (5.7)**	**8 (7.2)**

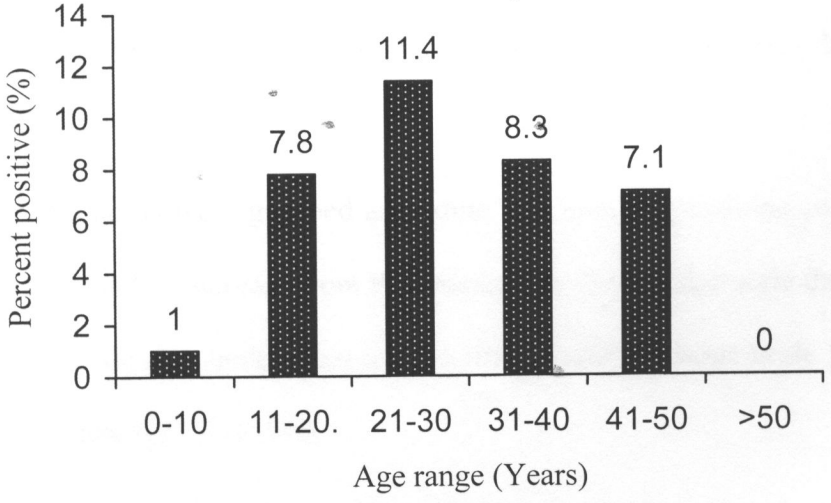

Figure 4.3 Prevalence of *Cryptosporidium* in humans in the different age groups [0-10 years (n=98), 11-20 years (n=64), 21-30 years (n=70), 31-40 years (n=36), 41-50 years (n=36) and > 51 years (n=4)]

4.3 Questionnaire surveys

4.3.1 Ruminants

A total of 20 farm questionnaires were administered to the farm owners (see Appendix 4). Information regarding type of animals kept on the farm, total number of animals, management (type of husbandry, typing of housing and frequency of cleaning, feeding) and presence of diarrhoea among other things was obtained.

Table 4.4 summarises the livestock management factors that were evaluated. The dairy farms in the study were either under intensive management with zero grazing (n=3) or semi-intensive management, that is, grazing plus concentrate feeding (n=17). The prevalence of *Cryptosporidium* in farms with intensive management system was 27.6% while for semi-intensive farms it was 35.6% but the difference between the systems was not significant (χ^2=0.705 P=0.401; OR = 0.691, CI = 0.341-1.403).

When the farms were grouped according to animal populations on the farm, prevalence was found to increase from the small scale to the large-scale dairy farms. The difference in the prevalence between the small-scale and large-scale farms was significantly different (χ^2, P=0.004).

The prevalence in calves housed individually was higher (52.4%; n=105) than those that were housed in groups (16.3%; n=86). The difference between the groups was significant (χ^2=33.420, P<0.0001). For the calves that were sometimes grouped and sometimes individually housed, the prevalence was very low (6.2%; n=16). The frequency of bedding removal or cleaning was also evaluated. It was found that there were significant differences in the prevalence of *Cryptosporidium* (χ^2=38.875,

$P<0.0001$) in the different categories of cleaning the calf pens. The prevalence was higher in pens that had their bedding removed every day than those that had their bedding removed several times a week or weekly or in pens that had no bedding at all (Table 4.4).

On feeding, calves that were bottle-fed or bucket-fed were found to be more at risk of developing cryptosporidiosis than those that suckled and were bottle-fed at the same time ($\chi^2=13.034$, $P=0.001$, OR = 4.634).

Table 4.4 Management factors evaluated to determine their association with *Cryptosporidium* infections in calves from 20 dairy farms

Factor	Total	No. positive (%)	P-value	χ^2 value
Husbandry system				
Intensive	47	13 (27.6)	0.401	0.705
Semi-intensive	160	57 (35.6)		
Housing				
Individual	105	55 (52.4)		
Group	86	14 (16.3)	0.000	33.420
Individual +group	16	1 (6.2)		
Bedding removal				
Daily	107	57 (53.3)		
Several times/week	29	6 (20.7)	0.000	38.875
Weekly	7	3 (42.8)		
No bedding/no cleaning	31	4 (12.9)		
Calf feeding				
Bottle/bucket fed	166	65 (39.1)	0.001	13.034
Suckle+bottle/bucket fed	41	5 (12.2)		

Out of the 207 calves sampled, 190 had boreholes only as a source of drinking water whilst 17 had both borehole and stream water. Of the 190 calves drinking borehole water, 69 (36.3%) were found positive for *Cryptosporidium* while only one (5.9%, n=17) calf was positive from those that also had access to stream water. The difference between the two groups was significant (χ^2=5.169, *P*=0.023).

Faecal consistency (recorded on pre-designed laboratory forms) was also evaluated for each sampled calf. Of the 70 positive calves, 27 (38.6%) had watery faeces (diarrhoea), seven (10%) had pasty faeces and 36 (51.4%) had normal faecal consistency (Figure 4.4). Diarrhoea was more common in calves less than three weeks old, mostly those between 10 and 20 days of age. There was a significant difference amongst the three groups of samples (χ^2=9.228, *P*=0.010).

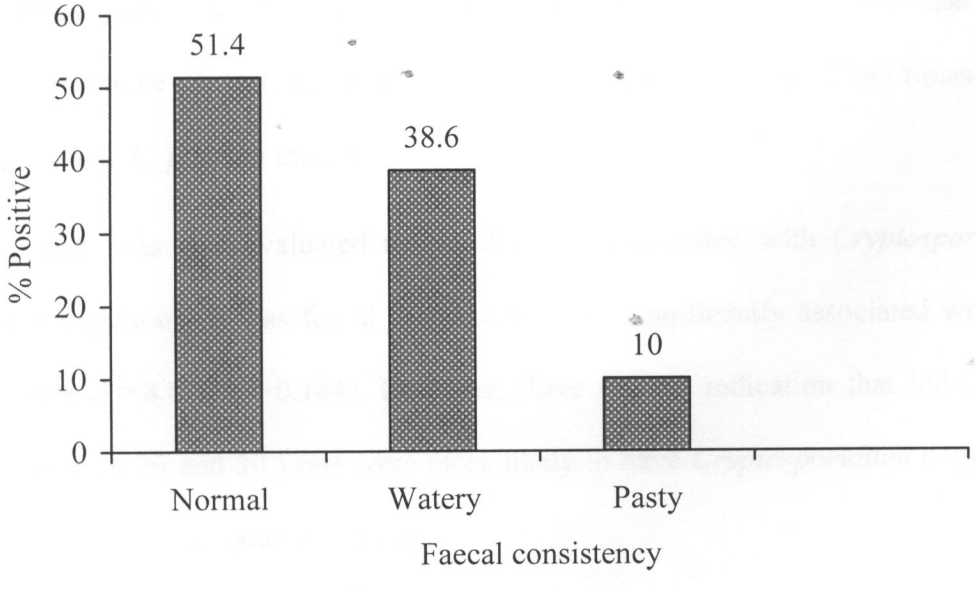

Figure 4.4 Distribution of *Cryptosporidium* positive calves (copro-antigen ELISA) according to faecal consistency

4.3.2 Humans

A questionnaire was administered to each of the 89 households (families). Age, sex and the other evaluated factors for each individual are listed in Table 4.5 and appendix 4. From the findings (Appendix 4), only 9.9% of individuals who had contact with neonates were positive for *Cryptosporidium* and 11.8% of those that had diarrhoea cases in the home were also positive for *Cryptosporidium*. Furthermore, 6% of individuals who shared water source with animals were also positive.

None of the factors investigated above were found to be significantly associated with *Cryptosporidium* infection (Table 4.5). Contact with neonates was also not significantly associated with the infection (χ^2=2.319, *P*=0.128). The type of contact was also not significantly associated with *Cryptosporidium* infection, (χ^2=3.730, *P*=0.444) as shown in Table 4.6. The individuals that had contact with neonates, however, were 2.091 more likely to be infected with *C. parvum* than those that did not have contact. The homes that had diarrhoea were also 2.295 times more likely to have *C. parvum* infection.

Age was also evaluated to see if it was associated with *Cryptosporidium parvum* infections. It was found that age was not significantly associated with the infections (χ^2=8.825, *P*=0.184). However, there was an indication that individuals aged between 20 and 30 years were more likely to have *Cryptosporidium* oocysts in their faeces than any other age group.

Table 4.5 Evaluation of the association of selected risk factors with the occurrence of *Cryptosporidium* infections in respondents from 89 households in dairy farms.

Risk factor	OR	95% CI	P-value	χ^2 value
Sex	0.803	0.138 -1.023	0.654	0.201
Contact with neonates	2.091	0.818-5.349	0.128	2.319
Diarrhoea in the home	2.295	0.748-7.110	0.155	2.022
Water source	0.377	0.138-1.023	0.056	3.657
Share water source with animals	0.451	0.067-2.930	0.457	0.554

Table 4.6 Evaluation of the possible association of age and type of contact with animals as risk factors for acquiring *Cryptosporidium* infection by humans on the dairy farms

Factor	Respondents	Positive	P-value	χ^2 value
Type of contact				
Feeding	9	1		
Cleaning pens	4	0		
Feeding & cleaning pens	66	7	0.444	3.730
Others (e g. Milking)	4	0		
No contact	206	10		
Age range (years)				
1-10	98	1		
11-20	64	5		
21-30	70	8		
31-40	36	3	0.184	8.825
41-50	14	1		
>51	4	0		
Unknown	3	0		

4.4 Molecular characterization and sequencing

4.4.1 Amplification of *Cryptosporidium* isolates

Twenty one calf samples, one lamb and one goat kid samples were run on PCR and amplification products were obtained for 20 calf samples, for the lamb and the goat kid. The PCR products were visualised on 1.5% agarose gel after Ethidium bromide staining and pictures taken. Figures 4.5, 4.6 and 4.7 shows HSP-70 gene PCR amplification products and Figures 4.10-4.12 show amplification products for the 18S rDNA gene. Eighteen human *Cryptosporidium* isolates were also run on PCR and amplification products were visualised as for the ruminant samples. The results are shown in Figures 4.7, 4.8 and 4.9 for the HSP-70 gene and Figures 4.10 and 4.11 for the 18S rDNA gene.

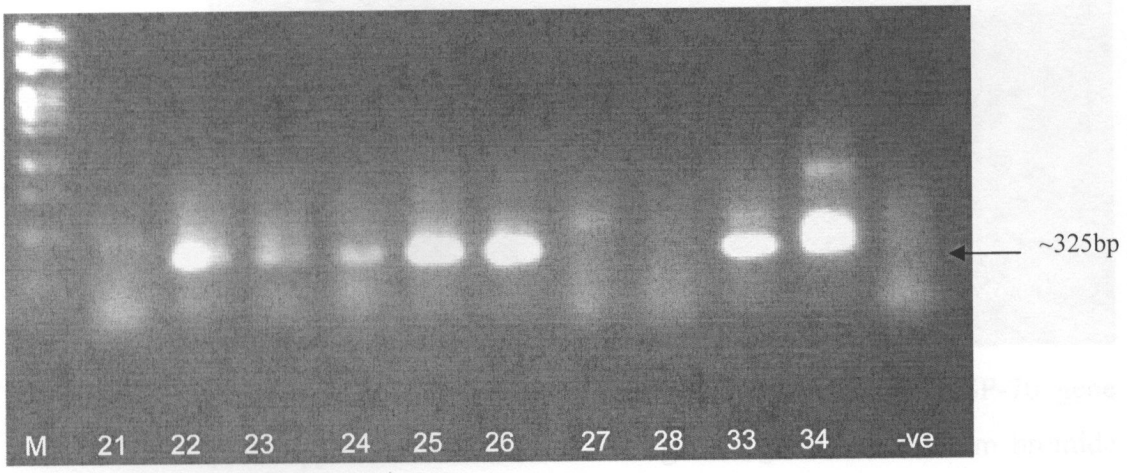

Figure 4.5 PCR amplification products of the *Cryptosporidium* HSP-70 gene (~325base pairs) as visualised on 1.5% agarose gel after ethidium bromide staining. [Goat sample (21), calf samples (22 – 28, 33, 34), M is the molecular marker and -ve is negative control]

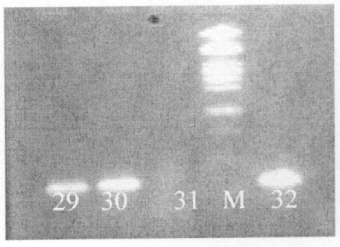

Figure 4.6 PCR amplification products of the *Cryptosporidium* HSP-70 gene as visualised on 1.5% agarose gel after ethidium bromide staining [Calf samples 29-32 and M is molecular marker]

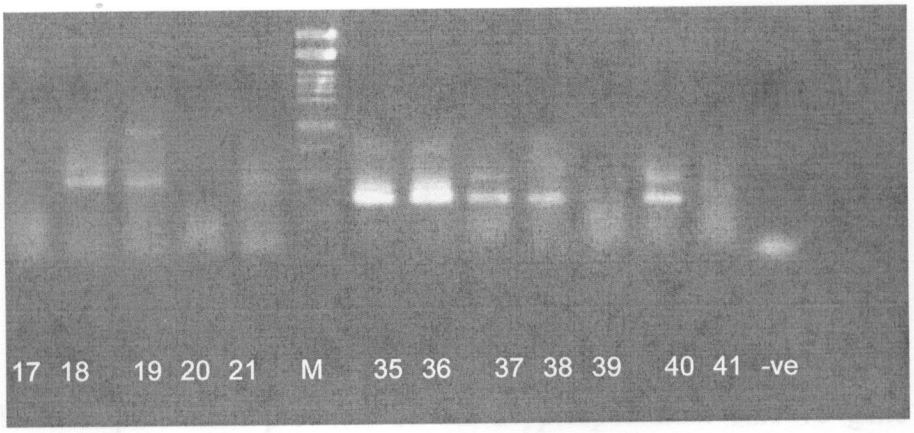

Figure 4.7 PCR amplification products of the *Cryptosporidium* HSP-70 gene (~325bp) as visualised on 1.5% agarose gel after ethidium bromide staining [Calf samples (35-41), with no amplification on samples 39 and 41, human samples (17-20) with amplification of non-*Cryptosporidium* isolate on sample 18 and 19 and goat sample (21). M is the molecular marker and -ve is negative control]

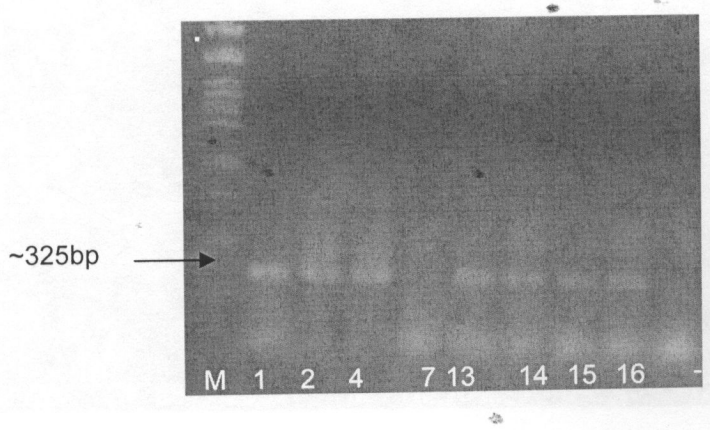

Figure 4.8 PCR amplification products of the *Cryptosporidium* HSP-70 gene (~325bp) as visualised on 1.5% agarose gel after ethidium bromide staining. [Human samples (1, 2, 4, 7, 13-16), M is the molecular marker and -ve is negative control]

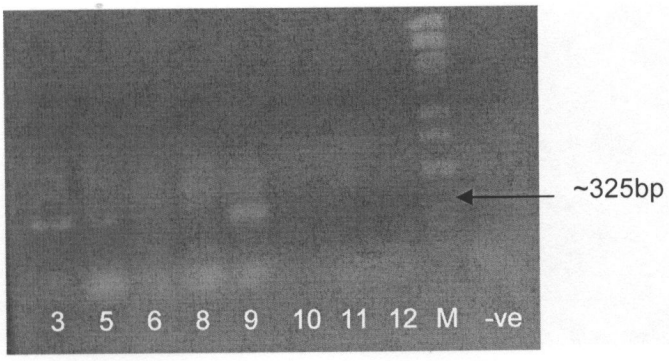

Figure 4.9 PCR amplification products of the *Cryptosporidium* HSP-70 gene (~325bp) as visualised on 1.5% agarose gel after ethidium bromide staining. [Calf sample (6), human samples (3, 5, 8, 9, 10, 11, 12), with no amplification on samples 10-12, M is the molecular marker and -ve is negative control]

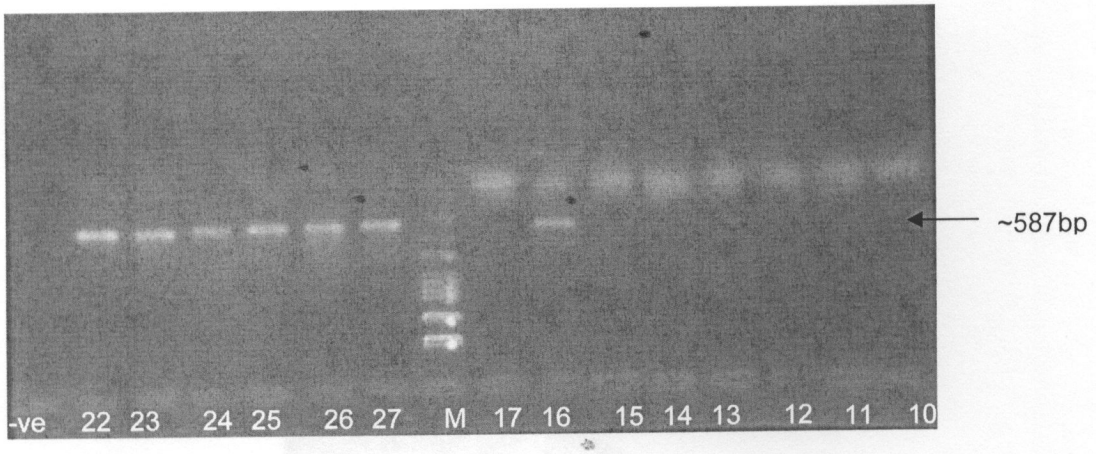

Figure 4.10 PCR amplification products of the *Cryptosporidium* 18S rDNA (~587bp) as visualised on 1.5% agarose gel after ethidium bromide staining. [Calf samples (22-27), human samples (10-17) with the only amplification on sample 16. M is the molecular marker]

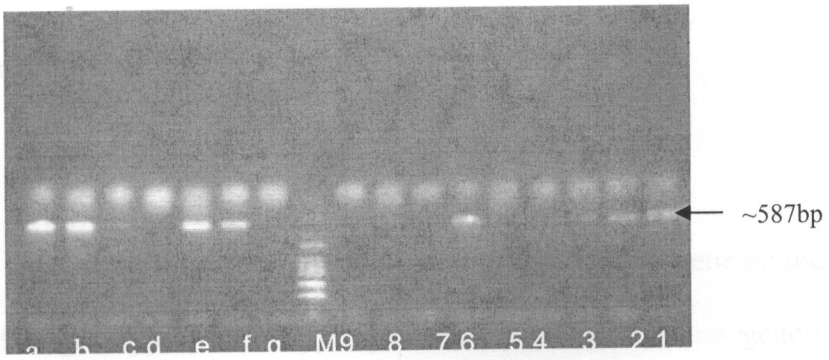

Figure 4.11 PCR amplification products of the *Cryptosporidium* 18S rDNA (~587bp) as visualised on 1.5% agarose gel after ethidium bromide staining. [Calf samples a-g (34, 33, 32, 31, 30, 29, 28), human samples (1-9) and M is the molecular marker]

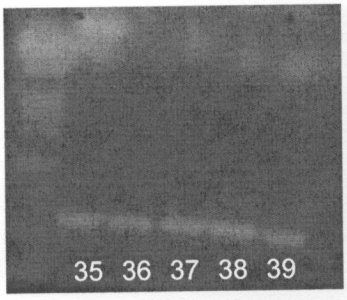

Figure 4.12 PCR amplification products of the *Cryptosporidium* 18S rDNA (~587bp) as visualised on 1.5% agarose gel after ethidium bromide staining. [Calf samples 35-39]

4.4.2 Sequencing

4.4.2.1 Ruminants

Sequence analysis of the 325 base-pair region of the HSP-70 gene on the calf samples revealed 3 genotypes, the *Cryptosporidium parvum* "bovine genotype", *Cryptosporidium bovis* (formerly *Cryptosporidium* bovine B), and a cervid genotype (*Cryptosporidium* deer genotype). Sequences were obtained for 20 samples out of the 21 calf DNA samples analysed. Sequence analysis of the 587 base pair region of the 18S rDNA gene revealed the same genotypes after comparing with sequence information from GenBank. The sequence for one sample could not be obtained. Sequence analysis of the lamb sample revealed *C. suis,* a pig genotype and kid isolate was *C. parvum* "bovine genotype". Details of the genotyping results are tabulated in Table 4.7.

Table 4.7 Genotypes of *Cryptosporidium* isolates from DNA extracted from faecal samples of ruminants from the dairy farms

Sample ID	Host	HSP-70 gene	18S rDNA genotype
J1	calf	*C. parvum* bovine genotype	*C parvum* bovine genotype
J6	calf	*C. bovis*	*C. bovis*
J21	kid	*C. parvum* bovine genotype	*C. parvum* bovine genotype
J22	calf	*C. bovis*	*C. bovis*
J23	calf	Deer-like genotype	Deer-like genotype
J24	calf	*C. bovis*	*C. bovis*
J25	calf	*C. parvum* bovine genotype	*C. parvum* bovine genotype
J26	lamb	*C. suis*	*C. suis*
J27	calf	*C. parvum* bovine genotype	*C. parvum* bovine genotype
J28	calf	-	*C. bovis*
J29	calf	*C. parvum* bovine genotype	*C. parvum* bovine geotype
J30	calf	*C. parvum* bovine genotype	*C. parvum* bovine genoytpe
J31	calf	*C. bovis*	*C. bovis*
J32	calf	*C. parvum* bovine genotype	*C. parvum* bovine genotype
J33	calf	*C. parvum* bovine genotype	*C.* parvum bovine genotype
J34	calf	*C. parvum* bovine genotype	*C. parvum* bovinegenotype
J35	calf	*C. parvum* bovine genotype	*C. parvum* bovine geotype
J36	calf	*C. parvum* bovine genotype	*C. parvum* bovine genotype
J37	calf	*C.bovis*	*C. parvum* bovine genotype
J38	calf	*C. bovis*	*C. bovis*
J39	calf	*C. parvum* bovine genotype	*C. parvum* bovine genotype
J40	calf	*C.* bovis	*C. bovis*
J41	calf	*C. parvum* bovine genotype	-

4.4.2.2 Humans

All 18 positive human isolates were run on PCR and genotyped. Three DNA samples failed to amplify on both the HSP-70 gene and the 18S rDNA gene and could not be sequenced. Sequence analysis of the 325 base pair region of the HSP-70 gene on the amplified samples revealed 2 genotypes, the *C. parvum* "bovine genotype" and *C. hominis*, (human genotype). Analysis of the 587 base pair region of the 18S rDNA gene revealed the same genotypes of *C. parvum* bovine genotype and *C. hominis* (details in Table 4.8). The genotypes were found in both the animal workers and in some of their relatives.

Table 4.8 Human isolates sequenced using the HSP-70 gene and 18S rDNA gene and the genotypes identified in Farm workers (W) and Farm workers' relatives (R)

Sample ID	Host age (years)	HSP-70 gene	18S rDNA gene	W/R*
J2	29	*C. hominis*	*C. hominis*	R
J3	10	*C. parvum*	*C. parvum*	R
J4	27	*C. parvum*	*C. parvum*	W
J5	25	*C. parvum*	*C. parvum*	R
J7	15	*C. hominis*	*C. hominis*	R
J8	27	*C. parvum*	*C. parvum*	R
J9	42	*C. parvum*	*C. parvum*	W
J10	35	-	-	R
J11	25	-	-	W
J12	20	-	-	R
J13	30	*C. parvum*	*C. parvum*	R
J14	19	*C. parvum*	*C. parvum*	W
J15	17	*C. parvum*	*C. parvum*	R
J16	30	*C. hominis*	*C. hominis*	W
J17	29	*C. parvum*	*C. parvum*	W
J18	35	*C.parvum*	*C. parvum*	W
J19	13	*C. parvum*	*C. parvum*	R
J20	31	*C. parvum*	*C. parvum*	W

*W = Worker R = Relative

Four out of the eight human positive farms had at least a calf positive for *Cryptosporidium*. The genotypes isolated from both humans and calves on these farms were then compared for similarity. Both the human and bovine genotypes were identified in the humans (Table 4.9). Out of the six farm workers five had *C. parvum* bovine genotype while one had *C. hominis*. *C. parvum* was also identified in non-farm workers. In farm 2, J3 and J4 (Table 4.9) were husband and wife, the husband being a farm worker. The finding of the same genotype in the two suggests that indirect transmission could have occurred from the farm worker to his wife.

Table 4.9 *Cryptosporidium* genotypes isolated from humans [Workers (W), Relatives (R)] and calves

Farm	Human ID	Genotype	R/W	Calf ID	Genotype
1	J7	*C. hominis*	R	J2	*C. bovis*
2	J2	*C. hominis*	R	J1	*C. parvum*
2	J3	*C. parvum*	R		
2	J4	*C. parvum*	W		
2	J5	*C. parvum*	R		
2	J20	*C. parvum*	W		
3	J9	*C. parvum*	W	J25	*C. parvum*
3	J10	-	R		
4	J16	*C. hominis*	W	J22	*C. bovis*
4	J17	*C. parvum*	W	J23	Deer genotype
4	J18	*C. parvum*	W	J24	*C. bovi*

CHAPTER FIVE

DISCUSSION

5.1 *Cryptosporidium* infection in ruminants

It was found in this study that *Cryptosporidium* infection was not localized to specific farms but present on all the 20 farms. The study also found that the overall point prevalence of *Cryptosporidium* in dairy calves, lambs and kids from the dairy farms was 33.8%, 2.6% and 7.1%, respectively. The study population included all calves 0-6 weeks old irrespective of clinical status. This, therefore, represents a serious problem on the dairy farms. The prevalence rate in the dairy calves in this study is similar to that reported by Santin *et al.* (2004) who reported a prevalence of 35.5% in pre-weaned calves (5 days to 2 months old) in the USA after examination of samples by immuno-fluorescence microscopy. The prevalence in dairy cattle tends to be higher than in beef cattle or other systems probably due to the intensive husbandry systems that are employed on dairy farms. A prevalence study done in the Lusaka, Central, Eastern and Southern provinces of Zambia (Goma, 2005) reported a prevalence of 42.8% in dairy calves after carrying out copro-antigen ELISA on 250 dairy calf faecal samples. This is slightly higher than the prevalence recorded in this study. The reason for this difference could be due to differences in the sample size, hygiene standards and the locations of the farms. The season of sampling could also be a reason for the differences in the prevalence since in this present study, sampling was done for six months from October 2004 to March 2005 while in the previous study by Goma (2005), sampling was done throughout the year from August 2003 to August 2004.

In this current study, age was strongly associated with *Cryptosporidium* infection ($P<0.0001$) with the highest prevalence (17.4%) being observed in calves aged 11-20 days old. These findings are in agreement with those of Maldonado-Camargo *et al.* (1998) in which they reported the maximum shedding of oocysts to be at about 15 days of age in dairy calves in central Mexico. A total of 8.7% of calves of 2-10 days old were also found to be shedding *Cryptosporidium* oocysts in their faeces indicating that calves were infected shortly after birth. The prevalence declined in calves over one month old (1%). These findings are similar to most studies that have reported the highest prevalence in animals of less than a month of age (Quilez *et al.*, 1996; Sischo *et al.*, 2000; Goma, 2005). However, the duration of oocysts excretion varies from animal to animal (Fayer *et al.*, 1998) and does depend largely on the immuno-competence of the animal itself (Q'Donoghue, 1995).

In this study, it was observed that after grouping the farms into large- and small-scale dairy farms, there was a significant difference in the prevalence of *Cryptosporidium* in the two farm groups ($P=0.004$) with a higher prevalence observed in large-scale farms as compared to the small-scale farms. These findings are similar to Garber *et al.* (1994) who found that the large-scale farms in the USA were 3.9 times more likely to have *Cryptosporidium* positive calves than the small-scale farms. The reason for this difference could be due to high stocking rates on large-scale farms as this has been reported to be a risk factor for *Cryptosporidium* infection in calves (Garber *et al.*, 1994). This is however, in contrast to a study in Galicia, Spain, by Castro-Hermida *et al.* (2002a), who found that smaller farms had a higher prevalence of *Cryptosporidium* infection than medium- or large-scale farms.

They attributed this situation to poor housing and hygienic conditions in rural Galicia as compared to conditions on the larger farms.

In this present study, the association between faecal consistency and *Cryptosporidium* infection was also analyzed. The prevalence was higher in calves with normal faecal consistency (51.4%) than in calves with diarrhoea (38.6%) and those with pasty faeces (10%). The difference amongst the three groups was statistically significant (P=0.01). The findings in this study are in contrast to a report by Mtambo *et al.* (1997) who found a significantly higher prevalence in diarrhoeic than non-diarrhoeic calves in Morogoro region, Tanzania. Lefay *et al.* (2000) also reported a higher prevalence in diarrhoeic than non-diarrhoeic calves in France. Goma (2005) also found that faecal consistency was significantly associated with *Cryptosporidium* infection in the dairy sector in Zambia. However, McCluskey *et al.* (1995) observed that oocyst shedding does occur in asymptomatic calves, a situation also observed in this study. The calves may shed oocysts prior to manifestation of clinical signs as was observed in 17 out of the 32 calves studied by Castro-Hermida *et al.* (2002b) in Spain. Finding of *Cryptosporidium* oocysts in calves without signs of neonatal diarrhoea indicates that cryptosporidial infection is not always associated with clinical disease in cattle. However, the periods of greater oocyst shedding normally coincide with periods of severe clinical manifestation (Mosier and Oberst, 2000). This was observed in this study in individual farms that had a high number of diarrhoeic calves corresponding with a high prevalence of *Cryptosporidium* infection.

5.2 *Cryptosporidium* infection in humans

To the best of my knowledge, this is the first report of *Cryptosporidium* infection in dairy farm workers and their families on dairy farms in Zambia. This study observed that the overall point prevalence was 6.2%, 9.8% of these were farm workers while 4.8% were family members of the farm workers (relatives). The prevalence rate was high in the farm workers as compared to the family members suggesting that working with animals possibly increases the chances or risk of infection with *Cryptosporidium*. These results are comparable to the prevalence found in animal handlers in a study in Iraq in which Mahdi and Ali (2002) investigated the prevalence of cryptosporidiosis among animal handlers. They reported a prevalence of 5% among the animal handlers.

In the present study, there was no statistical difference in the prevalence rates of *Cryptosporidium* between the males and females (P=0.654). This is in agreement with the findings of Casemore (1988) and Fayer and Ungar (1986), who reported that males and females appear to be equally susceptible to infection. It was also observed in this study that individuals aged between 20 and 30 years were more likely to have *Cryptosporidium* oocysts in their faeces than any other age group. However, there was no significant difference between the different age groups (P=0.184). This is in agreement with the findings of Casemore (1988) in which it was reported that a small peak of laboratory-confirmed cryptosporidiosis does occur in young adults of 20-40 years. The high prevalence of *Cryptosporidium* in adults of 20-30 years recorded in this study is, however, in contrast to reports that the infection is more common among children than in adults in both developed and developing countries (Allam *et al.*, 2002; Leav *et al.*, 2003).

5.3 Questionnaire surveys in ruminants

The prevalence in intensively managed farms (with zero grazing) was 27.6% while that for semi-intensive farms (with access to pasture) was 35.6%. There was, however, no significance difference between the systems. These findings are similar to the findings of Castro-Hermida *et al.* (2002a) in which they reported a prevalence of 45.9% and 50.4% in intensively and semi-intensively managed calves, respectively, with no statistical difference between the two management systems in north-western Spain. The results in this study are also in agreement with the findings of Kaminjolo *et al.* (1993) who also found no statistical difference in the prevalence between the different cattle husbandry systems in Trinidad and Tobago.

It was also noted in this study that the prevalence in individually housed calves was higher than those that were housed in groups. The difference between the groups was significant ($P<0.0001$). We found this strange as one would have expected prevalence to be higher in calves housed in groups due to overcrowding. The high prevalence in individually housed calves could have been due to mechanical transmission through cleaning and feeding of the calves by the same infected personnel.

The frequency of bedding removal or cleaning of the calf pens was also significantly associated with the presence of *Cryptosporidium* infection in calves. The prevalence was higher in pens that were cleaned every day than those that had their bedding removed weekly or a several times a week. Frequent bedding removal has been reported to increase the probability of calves shedding *Cryptosporidium* oocysts as the personnel and equipment or materials used for cleaning act as vehicles for spreading the infection (Sischo *et al.*, 2000). Furthermore, in many of the dairy

farms included in this study, the personnel that fed the calves also cleaned the calf pens. The dairy farms that housed their calves individually also removed or cleaned the bedding everyday. It is, therefore, possible that oocysts were mechanically transferred from shedding calves to the newborn calves through materials used for cleaning and through feeding since the same people cleaned and fed the calves. The findings in this study are, however, in contrast to findings in other studies done by Santin *et al.* (2004) and Maldonado-Camargo *et al.* (1998) in which there was no significant differences between type of housing, cleaning and *Cryptosporidium* infection. A possible explanation could be that individuals or personnel in the studies by these authors were restricted to their sections unlike the situation in this present study in which personnel that fed the calves also cleaned the pens. Garber *et al.* (1994) on the other hand found that cleaning or bedding removal significantly decreased the risk of infection.

The overall point prevalence of *Cryptosporidium* infection in lambs and kids in this study was found to be 2.6% and 7.1%, respectively. This prevalence is much lower than that observed in a study by Goma (2005) who reported a prevalence rate of 18.4% in 152 lambs. In Canada, Olson *et al.* (1997b), after examining 40 lambs found a prevalence of 23% using immunofluorescent microscopy. In Spain, a much higher prevalence was observed (59%, n=583) in lambs (Causapé *et al.*, 2002). The lower prevalence in the present study could have been due to a smaller sample size and differences in management systems. Goats and sheep are normally extensively managed in Zambia. The sample size for both lambs and kids was small and so was the number of positive animals and therefore the factors could not be compared statistically. The prevalence observed in kids is also lower than that reported

prevalence in studies done in other parts of the world. In France, a prevalence of 58.5% (n=137) was reported (Chartier *et al.*, 2002).

5.4 Questionnaire survey in humans

This study found that individuals that had contact with the animal neonates were 2.091 more at risk of having *Cryptosporidium* infection than those that did not have contact with the animals. This was also seen in the prevalence rate in the farm workers which was higher than that observed in the farm workers' family members who had no direct contact with the neonates. Although the statistical association between the farm workers and the non-farm workers was not significant *(P*=0.128) in this study, the importance of cattle as a risk factor for cryptosporidiosis has been highlighted (Roy *et al.*, 2004). The findings in this study are in agreement with those of Lengerich *et al.* (1993) in which they assessed the risk of exposure to *Cryptosporidium* among 70 dairy farmers and 50 non-dairy farmers in Wisconsin using a self administered questionnaire and the ELISA test. They found that the risk of being seropositive for *Cryptosporidium* was about 1.9 times higher for dairy farmers that had contact with animals than for other persons. Similarly, Roy *et al.* (2004), in the USA, also found that contact with cattle was a risk factor for cryptosporidiosis (OR 3.5; CI 1.8 to 6.8). An outbreak of cryptosporidiosis among 43 children and four staff after a week's holiday at a rural farm found illness to be 3.8 times more likely in those who handled calves than those who did not (Evans and Gardner, 1996). Miron *et al.* (1991) also described *Cryptosporidium* infection in young infants and children in which the source of infection was most likely infected calves.

The risk of being seropositive for *Cryptosporidium* was about 2.295 times higher for households that had cases of diarrhoea than in those that had no cases of diarrhoea in this study. The association of *Cryptosporidium parvum* with diarrhoea in Zambia has been highlighted by some authors (Conlon *et al.,* 1990; Nchito *et al.,* 1998). Conlon *et al.* (1990) found that *C. parvum* was the most common parasite associated with diarrhoea while Nchito *et al.* (1998) reported a prevalence of 18% in children with diarrhoea. From the observations it can, therefore, be said that *Cryptosporidium* in humans is likely to be associated with diarrhoea.

The water source for the farms in this study was borehole water, although some households on two farms also had access to stream water. The prevalence in individuals who used borehole water only was 67% while for the families that used both stream water and borehole water was 33%. Although there was no significant difference between the two sources (P=0.056), contaminated drinking water is a known risk factor (Roy *et al.,* 2004). For instance, in the United States of America, a number of drinking waterborne outbreaks have been reported including the 1993 massive Milwaukee outbreak that affected more than 400, 000 people (Mackenzie *et al.,* 1994; CDC, 2002). In a study conducted in Lusaka to determine cryptosporidiosis in adults and its relationship to oocyst contamination of drinking water, Kelly *et al.* (1997) found that not only was there a clear and significant relationship between water contamination and the prevalence of the infection but also that the prevalence of *C. parvum* is determined at least to some degree by the intensity of contamination of the water supply.

Sharing water source with animals was not associated with increased risk of *Cryptosporidium* infection (χ^2, P=0.457). Most of the farms in the study used

borehole water for both animal and human consumption and borehole water has been found to have very low levels of oocyst contamination (Kelly *et al.,* 1997). As borehole water is normally piped and has less exposure to environmental contamination, this could be the reason for the lack of association with cryptosporidial infection in this study. However, cattle have been implicated in the contamination of water which led to an out break of human cryptosporidiosis in USA (Peng *et al.,* 1997).

5.5 Molecular characterization of *Cryptosporidium* isolates from ruminants and humans

In this study, molecular characterization of the *Cryptosporidium* isolates from the calves revealed three genotypes on both the HSP-70 gene and 18S rDNA. These were *C. parvum* bovine genotype, *C. bovis* and a deer-like genotype. As shown in the results, the bovine genotype *Cryptosporidium parvum* was the most common genotype identified. Twelve out of the 20 amplified samples were identified as *C. parvum* genotype on the HSP-70 and 11 on the 18S rDNA. These findings are in agreement with those from other studies in other countries that have found *C. parvum* 'bovine genotype' to be the most common species in calves of less than 2 months old (Becher *et al.*, 2004; Santin *et al.*, 2004). Goma (2005) also reported *C. parvum* to be the most common genotype isolated from cattle in Zambia. *Cryptosporidium bovis* (which was previously named Bovine B genotype by Santin *et al.* (2004) was also identified in 7 isolates on both the HSP-70 gene and the 18S rDNA gene. This genotype has been found in pre and post-weaned cattle in other studies after sequencing the 18S rDNA gene of *Cryptosporidium* (Xiao *et al.*, 2002;

Santin *et al.*, 2004). Fayer *et al.* (2005) renamed this genotype *C. bovis*. *Cryptosporidium bovis* is thought to be host specific for cattle as *C. hominis* is to humans (Fayer *et al.*, 2005). A deer-like genotype was identified in one calf. This genotype has been identified in calves in other studies (Santin *et al.*, 2004; Fayer *et al.*, 2005). The findings in this study confirm the validity of *C. parvum, C. bovis* and deer-like genotype at three independent loci. These results, therefore, provide evidence for the genetic distinctiveness of these different genotypes infecting calves.

Cryptosporidium suis was identified in the lamb isolate. *Cryptosporidium parvum* bovine genotype was identified in the kid isolate. Goma (2005), in the study to find out the prevalence of *Cryptosporidium parvum* infections in cattle, sheep and goats in Zambia found *C. parvum* bovine genotype to be the most common genotype. The sample size was small and so cannot say which genotype was the most common in this study.

Genetic sequencing and analysis of the human isolates revealed *Cryptosporidium parvum* 'bovine genotype' and *C. hominis* (*C. parvum* human genotype) and former was the most common genotype identified from the humans on both genes. The two genotypes were isolated from both farm workers and their family members. No studies have been done before to characterize the *Cryptosporidium* species isolated from humans in Zambia to enable comparison with the present results in this study. The present findings are, however, in agreement with the findings from other parts of the world (McLauchlin *et al.*, 2000). These authors genetically typed 1705 human faecal samples and found a higher proportion of *C. parvum* 'bovine genotype' (61.5%) as compared to *C. hominis* (37.8%). On the other hand, Gatei *et al.* (2003) reported *C. hominis* to be the most prevalent genotype

occurring in over 75% of isolates from humans whether HIV-infected or not. *Cryptosporidium hominis* is thought to be the most prevalent genotype in humans in most parts of the world except in some parts of the United Kingdom (Xiao *et al.*, 2000). In addition, most genotyping studies in most parts of the world have been on *Cryptosporidium* isolates from HIV-infected individuals and so the results are biased towards the HIV-infected individuals. In this study, the HIV status of the subjects was not known and so the results are not biased. However, this present study was localized and therefore, cannot conclusively say that *C. parvum* bovine genotype is the most common genotype in humans in the whole of Zambia but it was the commonest genotype isolated in both animals and humans studied here.

The association between the infection in animals and in humans in this study could not be established from the possible risk factors analyzed. This could have been due to the small sample size. However, it is still possible that the bovine genotype isolated in humans could have been from the farm animals as this genotype has been correlated elsewhere with contact to farm animals (McLauchlin *et al.*, 2000). The role of farm animals as the source of infections was also highlighted by Hunter *et al.* (2003) who found that during the period of Foot and Mouth outbreaks in the United Kingdom, when access to farm areas was restricted, the number of human cryptosporidiosis cases and infections with the bovine genotype were dramatically reduced. Joachim (2004) also emphasized the importance of direct or indirect contact to farm animals as the most common risk factors associated with *Cryptosporidium* infection.

Cryptosporidium parvum bovine type was also the commonest genotype isolated from farms where both calves and humans were positive in this study. The

high number of farm workers with *C. parvum* bovine genotype indicates the possibility of transmission from the calves to humans on these farms even though the sample was small. The correlation of *C. parvum* infection with contact to farm animals (McLauchlin *et al.*, 2000) does support the findings in this study.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

To the best of my knowledge, this study is the first report of *C. parvum* infections in farm workers in Zambia and in Africa.

Among the ruminants, the observed prevalence was highest in the dairy calves and low in lambs and kids. The large-scale dairy farms showed higher prevalence than the small scale farms. Watery and normal faeces were associated with infection in dairy calves. In humans, there was no significant difference between the infection in males and females and also between the different age groups. Individuals who had contact with the neonates were more likely to have the infection than those who did not have contact.

Molecular characterization of calf samples showed that the majority of samples were *C. parvum* bovine genotype which is of public health significance. In the lamb, *C. suis* was identified and this is an indication of cross transmission between species. *Cryptosporidium suis* is a pig genotype (also called *C. parvum* pig genotype). In humans, *C. parvum* bovine genotype and *C. hominis* were identified. This is the first report of characterization of *Cryptosporidium* genotypes in humans in Zambia. The identification of this *C. parvum* bovine genotype in humans indicates that it is possible to get infected with the animal strain of *Cryptosporidium* parasite.

6.2 Recommendations

Based on the observations and the results obtained in this study, the following recommendations are made:

The study on the relationship of *Cryptosporidium* between humans and animals in Zambia, especially in the dairy industry, be expanded to include other areas of the country as the present study was limited to areas around Lusaka due to financial constraints. This will increase the sample size and will help to conclusively determine the relationship between animal and human infection and the possible source of *Cryptosporidium* infections.

Further genotyping of a large sample of isolates in humans is needed as genotyping in this study was carried out on a limited number of samples. It is also important that genotyping of isolates from HIV/AIDS patients be done to be able to know what genotypes are prevalent in that group in Zambia.

The high prevalence in dairy calves and the finding of the *C. parvum* bovine genotype in humans entails a serious risk of infection for humans especially those in high risk groups such as those with HIV/AIDS. It is, therefore, recommended that animal handlers be educated on the possibility of acquiring infections from contact with calf faeces and the possible subsequent transmission to their family members. This education can be done by extension workers, veterinarians and health workers.

Although borehole water is generally less contaminated than surface water, it is possible that it could be contaminated by *Cryptosporidium* oocysts filtering through the soil from farm animal manure. Assessment and testing of borehole water

on the dairy farms for the presence of *Cryptosporidium* oocysts is, therefore, recommended.

In animals, control measures such as good management and hygiene practices should be employed and targeted especially against animals of one to four weeks of age, as infections are concentrated in this age range. Since there is no specific therapy against *Cryptosporidium*, supportive treatment should be given to sick neonates and these should be isolated from the healthy ones.

In the absence of effective specific therapy against the infection, preventive measures are key to stopping the spread of infection. Therefore, strict hygienic measures should be practiced by humans to prevent infection and these should include avoiding direct contact with animals or faeces and/or respiratory excretions from animals and humans, extensive hand washing after handling animals and human excretion and taking measures to ensure safety of drinking water. Finding the parasite in people drinking borehole water but with no contact with animals may mean that these individuals could have got the infection from the water.

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Appendix 1: Prevalence of *Cryptosporidium* spp. in immuno-competent and patients with HIV in different parts of the World

Location	No. of samples	Prevalence (%)						References
		C. hominis	*C. parvum*	*C. meleagridis*	*C. canis*	*C. felis*	*C. muris*	
USA	10	5(50)	1(10)	0	1(10)	3(30)	0	Pieniazek *et al.*, 1999
Various	22	7(31.8)	8(36.4)	2(9.1)	0	5(22.7)	0	Morgan *et al.*, 2000b
Peru (children)	85	67(78.8)	8(9.4)	7(8.2)	2(2.3)	1(1.2)	0	Xiao *et al.*, 2001
UK	1711	651(38.0)	1055(61.7)	5(0.3)	0	0	0	McLauchlin *et al.*, 2000
Thailand (HIV)	29	24(82.7)	0	3(10.3)	0	1(3.5)	1(3.5)	Tiangtip *et al.*, 2002
Northern Ireland	39	5(12.8)	34(87.2)	0	0	0	0	Lowery *et al.*, 2001
France	57	18(31.6)	29(50.9)	3(5.3)	0	6(10.5)	1(1.7)	Guyot *et al.*, 2001
Denmark	44	25(56.8)	18(40.9)	1(2.3)	0	0	0	Enermark *et al.*, 2002
Thailand (HIV)	34	17(50)	5(14.7)	7(20.6)	2(5.9)	3(8.8)	0	Gatei *et al.*, 2002
New Zealand	66	1(1.5)	65(98.5)	0	0	0	0	Learmonth *et al.*, 2003
Various (HIV)	63	47(74.6)	14(22.2)	1(1.6)	0	0	1(1.6)	Gatei *et al.*, 2003
Canada	35	19(54.3)	14(40)	2(5.7)	0	0	0	Olson *et al.*, 2004

Appendix 2: Survey on ruminant cryptosporidiosis

GENERAL FARM AND FARM MANAGEMENT INFORMATION

A FARM IDENTITY

Farm Owner:.....

Date:.....

Farm name:. .....Village:.....

District:.....

B HERD COMPOSITION

1. *What types of animals are kept at the farm? (Circle letter)*

   (a) Cattle.....

   (b) Sheep.....

   (c) Goats.....

2. *What is the total number of animals?*

   (a) Cattle.....

   (b) Sheep.....

   (c) Goats.....

3. *What is the total number of:*

   (a) Calves.....

   (b) Lambs.....

   (c) Kids.....

C MANAGEMENT HUSBANDRY

1. *Type of husbandry*

   (a) Intensive.....

   (b) Semi-intensive.....

   (c) Extensive.....

   (d) Free range.....

2 *Type of housing*	*Calves*	*Lambs*	*Kids*
(a) Individual housing.....			
(b) Group housing.....			

3. *Type of flooring* *Calves* *Lambs* *Kids*

(a) Concrete floor.....

(b) Slotted floor.....

(c) Soil.....

(d) Other (specify).....

4. *Type of bedding* *Calves* *Lambs* *Kids*

(a) None.....

(b) Straw/hey.....

(c) Sand.....

(d) Sawdust.....

(e) Other (Specify).....

5 *How often is the bedding removed or cleaned?* *Calves* *Lambs* *Kids*

(a) Daily.....

(b) Several times/week.....

(c) Weekly.....

(d) Less than monthly.....

(e) Monthly.....

6. *How are the neonates fed?* *Claves* *Lambs* *Kids*

(a) Suckle.....

(b) Bottle fed.....

7. *What is the drinking water source?*

(a) Borehole.....

(b) Stream water.....

(c) Pool/dam water.....

D DISEASE

1. *Is there a diarrhoea problem in the (indicate yes or no)*

(a) Calves.....

(b) Lambs.....

(c) Kids.....

2. *Is there mortality due to diarrhoea (indicate yes or no)?*

(a) Calves.....

(b) Lambs.....

(c) Kids.....

Appendix 3: Survey on Human Cryptosporidiosis

Please note that all the information will be treated as confidential

Farm name:.....

Farm worker's ID:.....Sex:.....Age:.....

Number in the household:.....

Household member ID:.....Sex:.....Age:.....

Name of interviewer.....

Q1
Do you have contact with calves, lambs or kids (1) Yes (2) No

Q2
If yes, what type of contact is it?
(1) Feeding the calves, lambs or kids
(2) Cleaning calf pens
(3) Others specify.....

Q3
Does any of your family members have contact with animals? (1) Yes (2) No

Q4
If yes, what type of contact is it? Specify.....

Q5
Have you had any diarrhoea cases in the home? (1) Yes (2) No

Q6
If yes, how many family members were affected? Specify.....

Q7
Did the affected person have any prior contact with a symptomatic patient or animal?
(1) Yes (2) No

Q8
What is the source of water? (1) Dam (2) Stream (3) Borehole

Q9
Do you share water source with the animals? (1) Yes (2) No

Appendix 4: *Cryptosporidium* risk factors evaluated in the household questionnaire for transmission of *Cryptosporidium* from animals to humans, total number sampled, number positive and the and percentage of those positive

Factor	Total No. sampled	No. positive (%)
Contact with animals (animal workers)		
Yes	81	8 (9.9)
No	1	0 (0)
Contact with animals (family members)		
Yes	2	0 (0)
No	205	10 (4.9)
Diarrhoea cases in the home		
Yes	34	4 (11.8)
No	255	14 (5.5)
Diarrhoea plus contact with animals		
Yes	0	0 (0)
No	34	4 (11.8)
No diarrhoea	255	14 (5.5)
Water source		
Borehole only	240	12 (5.0)
Stream and borehole	49	6 (12.2)
Share water source with animals		
Yes	281	17 (6.0)
No	8	1 (12.5)