

**THE PREVALENCE OF *CRYPTOSPORIDIUM PARVUM*
INFECTIONS IN CATTLE, SHEEP AND GOATS IN ZAMBIA**

BY

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*A dissertation submitted to the University of Zambia in fulfilment of the
requirements for the award of the degree of Master of Science in Veterinary
Medicine.*

**THE UNIVERSITY OF ZAMBIA
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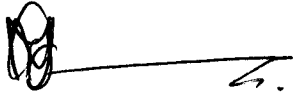
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DECLARATION

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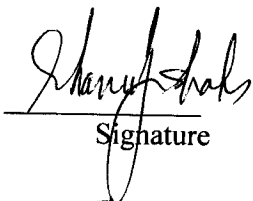
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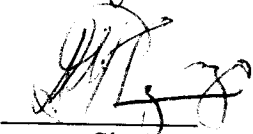
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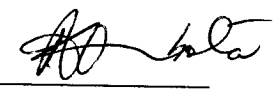
CERTIFICATE OF APPROVAL

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ABSTRACT

Cryptosporidium parvum has become increasingly important in ruminants in recent years as an etiological agent of neonatal diarrhoea complex causing significant economic losses. *Cryptosporidium parvum* “bovine genotype” found in ruminants is also zoonotic causing life threatening diarrhoea in immunocompromised individuals and children below 5 years. In developing countries like Zambia, infections with *C. parvum* are prevalent in humans due to high prevalence of HIV/AIDS, poor sanitation and use of contaminated drinking water. Reported prevalence of *C. parvum* in humans ranges from 18 to 30%. However, it is not known if the parasite occurs in Zambian livestock and whether the genotypes found are of public health significance. Therefore the objectives of this study were; to determine the prevalence of *C. parvum* in calves in 3 cattle husbandry systems, determine prevalence in small ruminants and find out the genotypes involved. The association between management factors and infection were also investigated in the dairy sector. Furthermore the conventional microscopic method of oocyst detection was compared to that of coproantigen ELISA (Techlab).

During the period August 2003 to July 2004, faecal samples were collected per-rectum from lambs, kids and calves (dairy, beef and traditional) aged 3 months and below. The study area included 4 of the 9 provinces of Zambia. For each faecal sample the consistency was recorded and all samples were analysed using the coproantigen ELISA (Techlab) commercial kit.

A total of 744 calves were sampled, out of these 250 were from dairy, 238 from ranch beef and 256 from the traditional husbandry systems. A total of 37, 26 and 91 farms were sampled from the dairy, beef and traditional husbandry systems, respectively. After coproantigen ELISA examination an overall prevalence 19.2% was obtained and specific prevalence in dairy, beef and traditional husbandry systems were 42.8%, 8.0% and 6.2%, respectively. The farm prevalence was 75.7% dairy, 42.3% beef and 15.4% traditional husbandry systems. Comparison of prevalence in the 3 husbandry systems revealed that the dairy sector (intensive management) had a significantly higher prevalence than the beef and traditional (extensive management) sector (χ^2 , $P < 0.001$). When farms were grouped according to animal population, the prevalence was found to be increasing from the small-scale to the medium-scale and large-scale farms. There was significant difference in prevalence among the 3 farm sizes in the dairy (χ^2 , $P < 0.001$) and traditional (χ^2 , $P < 0.001$) husbandry systems. Infection with *C. parvum* was significantly associated with liquid faeces only in the dairy sector (χ^2 , $P = 0.02$).

A questionnaire survey was conducted on 27 dairy farms to determine the association of infection with 3 risk factors namely, general management, maternity management and calf housing and hygiene. Only 1 of the management risk factor (intensive or semi-intensive) was found to be significantly associated with infection (χ^2 , $P = 0.004$).

In lambs and kids the prevalence on coproantigen ELISA was 18.4% (n=152) and 5.7% (n=105), respectively. Both the sheep and goat farms were under semi-

extensive management. Of the 16 sheep farms sampled, 37.5% had one or more lambs shedding *C. parvum* oocysts, while of the 12 goat farms sampled, 41.7% had one or more kids shedding oocysts. In sheep, infections were significantly associated with faecal consistency, with prevalence of 40%, 34.8% and 14.5% for liquid soft and solid faeces (χ^2 , $P=0.03$), respectively. In goats there was no association between infection and faecal consistency (χ^2 , $P=0.4$).

A total of 432 calf faecal samples were also analysed using the modified Ziehl Neelsen staining technique to evaluate this test against the coproantigen ELISA (Techlab). The sensitivity and specificity were determined to be 58% and 84.3%, respectively with a kappa value of 0.41. The oocyst detection level of the 2 tests was significantly different (χ^2 , $P<0.0001$).

A total of 55 samples comprising 5 sheep, 5 goat and 45 cattle which were positive on the coproantigen ELISA (Techlab), were examined by Polymerase Chain Reaction (PCR) for amplification of the Heat Shock (HSP-70) gene of *Cryptosporidium*. Amplification products were obtained for 45 isolates, whereas 9 isolates failed to amplify. Sequence analysis was performed on a sub sample of 10 isolates from cattle and sheep. Analysis revealed *C. parvum* “bovine genotype” and a novel Cervid genotype.

It can be concluded from the present study that *C. parvum* infections are prevalent in cattle, sheep and goats in Zambia with high levels in dairy calves and lambs. The

study has also observed that prevalence in calves is higher in the intensive (dairy) than extensive husbandry systems (ranch beef and traditional). In view of the lack of effective treatment against cryptosporidiosis, control measures targeting management practices and hygiene measures are recommended in the dairy sector. Furthermore, the “bovine genotype” of *C. parvum* found in the samples that were analysed is of public health significance. Therefore, it is suggested that farm owners and animal handlers/workers be educated on the risk of infection with the parasite. It is suggested that further studies be conducted to elucidate on the risk that dairy cattle and sheep pose in transmitting the infection to humans, especially those living in close proximity to these animals. Although there was good agreement between the microscopic method and the coproantigen ELISA, the latter is preferable as it is fast, easy to read and a number of samples can be done simultaneously.

DEDICATIONS

I dedicated this dissertation to my late mother **Lydia Kanandwe Goma** for striving to make me the very best that I could be in word and deed and to God without whom all things would not be.

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ABBREVIATIONS AND SYMBOLS

%	Percentage
=	Equal to
>	Greater than
≥	Greater than or equal to
<	Less than
≤	Less than or equal to
+ve	Positive
χ^2	Chi-square
<i>P</i>	Probability
SD	Standard deviation
CI	Confidence Interval
OR	Odds ratio
μl	Microlitre
ml	Milliliter
nm	Nanometer
pM	Parts per million
g	grams
bp	Base pair
rpm	Revolutions per minute
°C	Degrees Celsius
IgG	Immunoglobulin G
IFN- γ	Interferon γ .
IL-12	interleukin-12
TNF- α	Tumour Necrosis Factor alpha
N/n	Sample size or number examined
No.	Number
ELISA	Enzyme-Linked Immunosorbent Assay
EIA	Enzyme immunoassays
IF	Immuno-fluorescent
RPH	Reverse Passive Haemagglutination
IFA	Immuno-fluorescence assay
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Polymorphism
H ₂ SO ₄	Sulphuric acid
ETEC	<i>Enterotoxigenic Escherichia. Coli</i>
HIV/AIDS	Human Immuno-deficiency Virus/ Acquired Immuno Deficiency Syndrome
CDC	Centres for Disease Control
UNAIDS	United Nations AIDS

CSO	Central Statistics Office
USA	United States of America
UK	United Kingdom
DNA	Deoxyribonucleic acid
rDNA	ribosomal Deoxyribonucleic acid
rRNA	ribosomal Ribonucleic acid
SSU rRNA	Small subunit ribosomal Ribonucleic acid

CHAPTER ONE

1.0 INTRODUCTION

The major diseases affecting neonatal ruminants are grouped as diarrhoea, pneumonia and septicemia (Heath, 1992). In cattle husbandry, calf diarrhoea has been estimated to account for approximately 75% of the mortalities in the dairy calves of 3 weeks and below (Fuente *et al.*, 1998). Although there are a number of enteropathogens implicated in neonatal diarrhoea complex, *Cryptosporidium parvum*, is among the top 4 enteropathogens associated with the reported diarrhoea worldwide (Moore and Zeman, 1991; Fuente *et al.*, 1998; Castro-Hermida *et al.*, 2002b,). The diarrhoea due to enteric cryptosporidiosis lasts from a few days to approximately 2 to 3 weeks. However, in neonates and immunocompromised individuals, the diarrhoea is profuse and life-threatening leading to dehydration, weight loss and mortalities. *Cryptosporidium* species infect a wide range of vertebrates, however, the most common and significant manifestation of disease is reported in mammals, particularly ruminants and humans (Mosier and Oberst, 2000). Ruminants are affected mainly by 2 species namely, *Cryptosporidium parvum* (*C. parvum*) and *Cryptosporidium andersoni* (*C. andersoni*) formerly known as *C. muris*. The “bovine genotype” of *C. parvum* is most common among ruminants especially in cattle and sheep (Fayer *et al.*, 1997) and is zoonotic.

For the last two decades the disease has been gaining importance mainly because it is increasingly found as the sole etiological agent or in association with others in diarrhoea outbreaks in livestock and humans (O'Donoghue, 1995). In the livestock

industry, the importance stems from the fact that there is no effective treatment of cryptosporidiosis in neonatal ruminates and direct economic losses arise due to symptomatic treatment of diarrhoea. The indirect losses include weight loss, reduced weight gains and mortalities. In lactating cows, economic losses are due to reduction in milk production caused by *C. andersoni* (Esteban and Anderson, 1995).

In humans, *Cryptosporidium* has become increasingly important as it has emerged as one of the most important protozoan zoonosis with domestic animals being implicated to transmit the disease to humans through direct contact and food and water contamination (O'Donoghue, 1995; Anderson, 1998). However, the exact role played by domestic and wild animals in transmission of the disease remains unclear (Anderson 1998; de Graaf *et al.*, 1999). The other reason why the disease has gained prominence in public health is due to massive municipal water borne outbreaks in developed countries with the largest outbreak having occurred in Milwaukee, USA in 1993. It is due to this reason that some countries have declared the disease notifiable (Weir, 2001). In developing countries especially those in the sub-Saharan Africa, including Zambia, the disease is very prevalent in humans (Martins and Guerrant, 1995). The high prevalence among the human populations in these countries is due to poor sanitation, use of contaminated drinking water and the high number of HIV/AIDS cases, which creates a large susceptible population (Martins and Gurrant, 1995; Fayer *et al.*, 2000).

Although there are several reports on the occurrence of *C. parvum*, studies have mainly been done in Europe, North America and Australia with prevalence varying

from 1 to 62% (Scott *et al.*, 1995; Maldonado-Camargo *et al.*, 1998; Lefay *et al.*, 2000; Hoar *et al.*, 2001; Castro-Hermida *et al.*, 2002b). It has been stated by Anderson (1998) that investigators at the Caine Veterinary Centre in Idaho, USA and other diagnostic laboratories cannot find a dairy or calf rearing facility without the parasite. In African countries on the other hand, data on the presence of the parasite in livestock is limited with two reports from Eastern Africa namely Tanzania (Mtambo *et al.*, 1997) and Uganda (Nizeyi *et al.*, 2002) with prevalence of 5.3% and 38%, respectively. Although data on the prevalence of *Cryptosporidium* in livestock on the continent is scanty, research reports in humans are many (Martins *et al.*, 1995). Prevalence rates have been shown to range from 3.1 to 17.6% in Guinea Bissau (Molbak *et al.*, 1990), 7.5% in Burkina Faso (Nacro *et al.*, 1998) and 25% in Uganda (Tumwine *et al.*, 2003). In Zambia, the prevalence ranges from 18-32% (Conlon *et al.*, 1990; Nchito *et al.*, 1998; Amadi *et al.*, 2001).

In Zambia, the livestock industry is well established with ruminants (cattle, sheep and goats) being the major livestock kept. Cattle are the most important with a large population being kept under the traditional husbandry system, although the commercial dairy and ranch beef systems are more productive (Chilonda *et al.*, 1999). One of the frequently reported diseases among young ruminants in Zambia is diarrhoea (James, 1992). *Cryptosporidium* has been identified in diarrhoea cases in young ruminants around the world but little data is available on the presence of the parasite in ruminants in Africa and Zambia in particular. This absence of data in ruminants and the increasing importance of the parasite as a zoonotic pathogen especially in HIV/AIDS patients stimulated the formulation of this study.

The general objective of this cross sectional study was to establish the existence of *Cryptosporidium parvum* oocysts and the level to which they occur in Zambian ruminants. In order to achieve this, the prevalence of *C. parvum* and comparison of prevalence in 3 cattle husbandry systems namely, dairy, beef and traditional was determined, so as to identify the husbandry system that poses the highest risk for human transmission. Prevalence of *C. parvum* in small ruminants (sheep and goats) was also determined. Genotypic characterization of the *Cryptosporidium* identified was carried out in order to determine its' public health significance. It was also considered important to evaluate the sensitivity and specificity of the modified acid fast method compared to the commercial coproantigen (Techlab) ELISA kit in detecting the presence of *Cryptosporidium*.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Brief historical background of cryptosporidiosis

Cryptosporidium was first discovered in 1907 (Tyzzer, 1907) in the gastric glands of mice. Since then, there has been increasing interest in the disease both in the medical and veterinary fields. The parasite was first found to be pathogenic in 1955 when it was identified in turkeys with diarrhoea (de Graaf *et al.*, 1999) but it was not until the early 1970s when it was first identified in diarrhoeic calves, that it was recognised as a pathogen in ruminants (Panciera *et al.*, 1971; Baker and Carbonell, 1974). *Cryptosporidium* gained popularity in veterinary science in the 1980s when it was frequently reported as an aetiological agent in calf diarrhoea either alone or in association with other enteropathogens. The first human cases in which *Cryptosporidium* was involved on the other hand were diagnosed in 1976 (Meisel *et al.*, 1976; Nime *et al.*, 1976). There were increasing human reports of *Cryptosporidium* in the 1980s especially in immunocompromised individuals with Human Immuno-deficiency Virus/ Acquired Immuno Deficiency Syndrome (HIV/AIDS). This coincided with the increased incidence in ruminants and the escalation of the HIV/AIDS epidemic. Although cryptosporidiosis was initially thought to be an opportunistic infection, it has now been found to occur commonly among immunocompetent individuals although the diarrhoea in most of these cases is self-limiting (Meinhardt *et al.*, 1996).

2.2 Taxonomy

Cryptosporidium species are protozoan parasites that belong to the phylum *Apicomplexa* (Urquhart *et al.*, 1996). The parasite has been found in reptiles, birds, mammals and humans (Lindsay and Blagburn, 1990; Mosier and Oberst, 2000). The parasite has been reported in 152 species of mammals from 14 orders (Fayer *et al.*, 2000). Most of these reports are in humans and ruminants (Fayer *et al.*, 1997).

According to Xiao *et al.* (2000a), the classical definition of species is “groups of interbreeding natural populations that are reproductively isolated from other groups”, but this definition is difficult to apply to *Protozoa*. Generally, the species of the genus *Cryptosporidium* have been identified based on oocyst morphology, biological characteristics (host range) and site of infection. Morphological differentiation is mostly based on microscopic examination of the oocysts. This has been the most commonly used feature to designate genus and species than any other characteristic (Morgan *et al.*, 1999). This method has limited use because most oocysts are similar in size and shape and have obscure internal structures (Fayer *et al.*, 2000).

During the early 1980s, it was suggested that *Cryptosporidium* should be considered a monotypic (single species) genus because a number of researchers involved in cross transmission studies found infections in various animals were readily transmissible to other domestic and laboratory animal species (Snodgrass *et al.*, 1984; Koudela and Jiri, 1997). Furthermore, the first immuno-serological study detected specific antibodies in humans and animals sera using parasite antigens from

a single lamb (O'Donoghue, 1995). This immuno-serological finding was misleading because there are no antibodies to reliably differentiate species (Fayer *et al.*, 2000). It is reported by Xiao *et al.* (2000a) that more recent cross transmission studies support the multiple species concept of *Cryptosporidium* in different classes of vertebrates as oocysts in one vertebrate class will not readily infect another class. Differences in infection within vertebrate classes have also been observed, especially with the *C. parvum* which infects a wide range of mammals. This then raises the question: "is *C. parvum* a single species with a wide range of biological characteristics or is it a taxon actually consisting of multiple species with oocysts that are indistinguishable from one another?" (Morgan *et al.*, 1999). Furthermore, host specificity as a method of species identification has problems because although a parasite can infect a particular host species in the laboratory, it may not be the natural host of that parasite. Therefore, Xiao *et al.* (2000a) states that results of cross transmission should be used only as indicators of existence of biological difference between the *Cryptosporidium* parasites under study.

Molecular studies have in the recent years provided a better tool to help in the speciation of the genus *Cryptosporidium* (Morgan *et al.*, 1998; Xiao *et al.*, 1999). DNA-sequence analysis suggests that the genus is complex and may contain over 20 different species (Pedraza-Diaz *et al.*, 2001). Phylogenetic trees which are based on differences in the sequence of the small subunit (SSU) rRNA gene nucleotide show that *Cryptosporidium* can be divided into 2 groups: the gastric and intestinal parasites with the gastric having larger oocysts (Xiao *et al.*, 2000a). In this regard there is an agreement among phylogenetic analysis, oocyst morphology and infection sites. Xiao

et al. (2000a) state that this agreement among the three methods is similar to the observation in different avian *Eimeria* species, which further supports the identity of these *Cryptosporidium* parasites as distinct species. The genetic distance (2.40-7.34 nucleotide changes per 100 base pairs) between some species of *Cryptosporidium* have been found to be comparable to those in between other well-established Apicomplexan genera and species. Fayer *et al.* (2000) on the other hand states that differences in an enzyme structure or in one or a few base pairs in a single gene containing over 1000 base pairs may not constitute sufficient basis for a speciation. The present dilemma in taxonomy can be overcome by relying on a combination of data from all three tools namely morphometrics, host specificity and molecular techniques (Xiao *et al.*, 1999; Fayer *et al.*, 2000).

Due to the aforesaid problems in taxonomy, there are varied opinions on the number of species in the genus *Cryptosporidium*. More than 23 species have been named but most researchers do not accept them as they were mainly arrived at based on host occurrence. O'Donoghue (1995) suggested that there are 6 valid species. Later, Morgan *et al.* (1999) when reviewing the taxonomy of the genus suggested 8 species as valid. More recently, Fayer *et al.* (2000) reported that there are currently 10 valid species of the genus namely: *C. andersoni*, *C. baileyi*, *C. felis*, *C. meleagridis*, *C. muris*, *C. nasorum*, *C. parvum*, *C. saurophilum*, *C. serpentis* and *C. wrairi*. There is a more recent specie that was identified from HIV-positive individuals designated *C. parvum dog genotype* (Pedraza-Diaz *et al.*, 2001). The common species in birds are *C. meleagridis* and *C. baileyi* and in reptiles *C. serpentis* and *C. saurophilum*, while the specie found in fish is *C. nasorum* (Fayer *et al.*, 2000). In mammals, the common

species are *C. muris*, *C. parvum* and to a lesser extent *C. wrairi*. The main species in ruminants are *C. andersoni* (formerly known as *C. muris*), and *C. parvum* whereas *C. wrairi* occurs in guinea pigs (O'Donoghue 1995; Anderson, 1998). *Cryptosporidium andersoni* parasites the abomasum, mostly in adult cattle whereas *C. parvum* infects the small intestines mainly in neonates (de Graaf *et al.*, 1999).

Cryptosporidium parvum has two major genotypes namely genotype I (anthroponotic, human) and genotype II (calf, cattle, bovine) which is zoonotic. A recent study has proposed that genotype I be designated species status as *C. hominis* n. sp (new species), based on biological and molecular data (Morgan-Ryan *et al.*, 2002). The biological differences were in the host range (*C. hominis* oocysts were not infective to cattle while *C. parvum* genotype II is known to be infective) and pathogenicity (by assessing parasite associated lesions in gnotobiotic pig model with *C. hominis* parasitizing the ileum and colon whereas *C. parvum* was in the entire length of the small and large intestines). Molecular and phylogenetic analysis of 16 unlinked loci in this study supported the species status of *C. hominis*.

2.3 Parasite and life cycle

Cryptosporidium has endogenous and exogenous developmental stages that are both microscopic in size. Endogenous development occurs in the epithelium of the gastrointestinal tract of the host after ingestion of the fully sporulated oocysts (exogenous stage). Invasion of epithelium of other organs like the conjunctiva of the eye, biliary and respiratory tract have been reported (Fayer *et al.*, 2000; Mosier and Oberst, 2000).

The exogenous oocysts contain 4 sporozoites (which are not enclosed in sporocytes), residuum made up of numerous small granules and a membrane bound globule. The shape of the oocysts is mostly spherical with some being ovoid and elliptical with shape indices length /width ranging from 1.0 to 1.4. The sizes of oocysts from different host species vary in the range 4.5 to 7.9 μm in length by 4.2 to 6.5 μm in width (O'Donoghue, 1995). The exogenous development starts with the sporozoites attaching to and developing within the brush border of the microvilli cell surface to form merozoites. The sporozoites are 2 to 6 μm in diameter and the shape is elliptical (Symth, 1994.) The ultrastructure of *Cryptosporidium* zoites consists of a pellicle, micronemes and several electron dense bodies some of which are similar to rhoptries and subpellicular microtubles (Symth, 1994; O'Donoghue, 1995). The zoites differ from other enteric sporozoan in that they lack polar rings, a typical conoid, mitochondria or microspores although it has been suggested that the electron dense collar may be analogous to an anterior polar ring (O'Donoghue, 1995).

As depicted in Figure 1.1, the monoxenous life cycle of *Cryptosporidium* begins by the ingestion of the sporulated oocysts by a susceptible host (Juranek, 1995). In vitro studies have indicated that excystation is triggered by various factors including carbon dioxide, temperature, pancreatic enzymes and bile salts. The released sporozoites then attach to the epithelial cells of the intestinal microvilli and they become enclosed within a parasitophorous vacuole (O'Donoghue, 1995). They are then said to be intracellular but extracytoplasmic. The organisms become rounded and develop attachment organelles to form trophozoites.

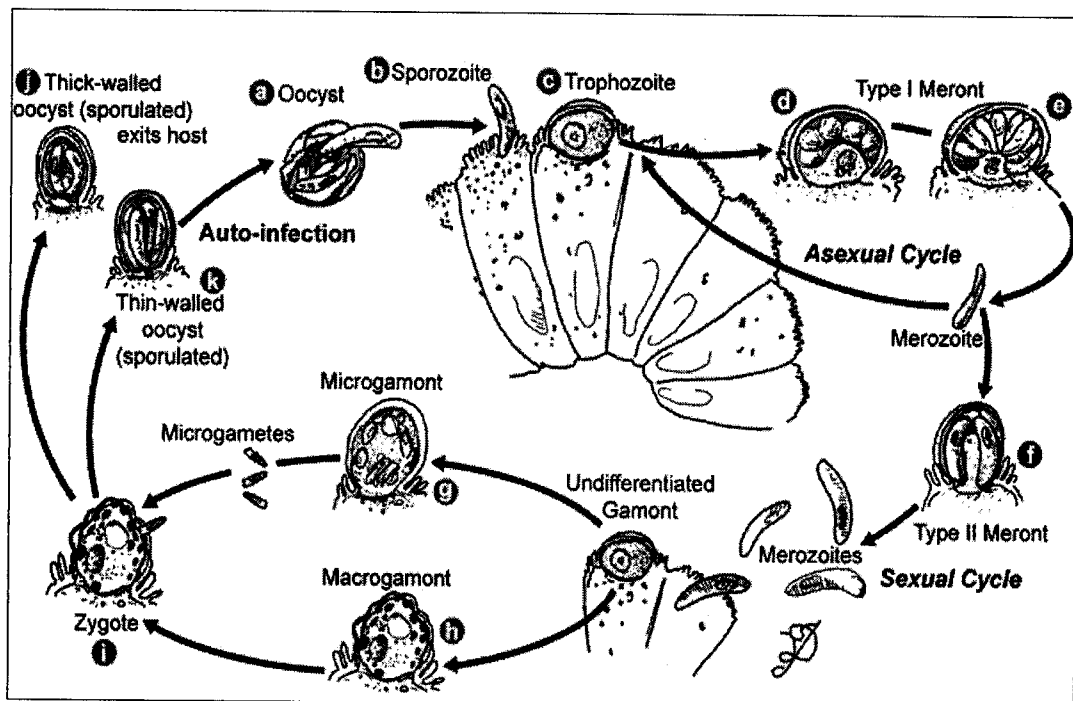


Figure 1.1 Schematic diagram of *Cryptosporidium* life cycle. (Source: Juranek D.D, 2000)

The asexual phase of division, referred to as merogony (formerly shizogony) then takes place. Initially, the trophozoites increase in size to form meronts that undergo nuclei division by rapid succession of mitoses (Symth, 1994). *Cryptosporidium* has two generations of meronts: Type I (first generation) meronts which contain 8 merozoites and Type II (second generation) meronts which contain 4 merozoites. The sexual phase of division known as gametogony then takes place with the production of microgamonts (male) and macrogamonts (female), which differentiate into microgametocytes and macrogametocytes, respectively (Symth 1994; O'Donoghue 1995). Many microgametocytes are released from a single mature

microgametocyte and these fertilize macrogametes, which remain attached to the epithelium producing zygotes. The zygote then undergoes further asexual development referred to as sporogony yielding sporulated oocysts, which are immediately infective upon excretion. Unique to *Cryptosporidium* is that there are two types of oocysts produced; thin and thick walled with the most being thick walled (Urquhart *et al.*, 1996). The thick walled oocysts are passed in the faeces whereas the fragile thin walled type rupture within the same host releasing sporozoites which begin the cycle anew (Smyth, 1994.) These auto-infective oocysts and recycling type I meronts are believed to be the means by which persistent chronic infections may develop in the hosts without further exposure to exogenous oocysts and the entire life cycle maybe completed in as little as two days (O'Donoghue, 1995).

2.4 Clinical manifestation

The clinical presentation of *Cryptosporidium* infections are usually complicated by concurrent infections by other pathogens (Moore and Zeman, 1991; Fuente *et al.*, 1998). It is important to note, however, that not all cryptosporidial infections necessarily result in clinical signs. 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, yellow in colour and watery. High mortality is not a common feature of *C. parvum* infections unlike other enteric pathogens, although morbidity can be very high (O'Donoghue,

1995). Some researchers have reported high mortality rates in some outbreaks of cryptosporidial diarrhoea in cattle and goats (Sanford and Josephson, 1982; Johnson *et al.*, 1999).

2.5 Pathophysiology

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 progressed 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 eosinophiles and metaplasia of the surface epithelium as 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. On the other hand, 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).

The diarrhoea due to *C. parvum* has been suggested to be mediated 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 exact mechanisms however, by which the infection causes diarrhoea are however not clearly elucidated because little is known about specific parasite and host molecules involved in the process (Leav *et al.*, 2003). The parasite supposedly elicits a local inflammatory response, increasing the production of prostaglandins and several cytokines, particularly Interferon γ (IFN- γ). These inflammatory mediators may consequently alter solute transportation in the intestinal cells, leading to osmotic diarrhoea (Leav *et al.*, 2003). It has been hypothesized that there is production of enterotoxins during the course of cryptosporidial infection, although it has never been conclusively demonstrated (Chen *et al.*, 2002; Leav *et al.* 2003).

2.6 Risk factors for infection and development of cryptosporidiosis

The severity of the diarrhoea differs from clinical, sub-clinical and asymptomatic in individual animals and in between herds depending on a number of factors. These could be individual host, herd management or parasite factors. These factors also predispose to infection and determine the duration of the prepatent and patent periods.

2.6.1 Individual host factors

In the individual animal, the most important predisposing factors to infection, severity of clinical signs and duration of oocysts shedding are age and immunity and to a certain extent nutrition (especially observed in humans) (Mosier and Oberst, 2000). Individual animal traits seem to be more important in determining the

outcome of infection. According to a study by Fayer *et al.* (1998), it was found that, despite the attempt to reduce variability by using only male calves from the same herd, at the same age, housed and fed similarly and given the same number of inoculum from the same isolate of *C. parvum*, great variation was observed in the severity and duration of the diarrhoea and oocysts shedding. The idea of age related resistance has been suggested because of the consistent observation of severe disease in calves, lambs and kids of 1 to 4 weeks and children of 1 to 5 years (O'Donoghue, 1995). In support of age related resistance, experiments in mice have shown that 3 weeks old mice are resistant to infection even without prior exposure to the parasite (Fayer *et al.*, 1998). Moiser and Obsert (2000) state that the intestinal mucosa in neonates has carbohydrates which the sporozoite lectins attach to but the composition of the carbohydrate mucosa changes with age. Development of mature intestinal flora has also been said to be an important factor in the development of the disease. In an experiment where germfree and non-germfree mice were infected at 3 weeks of age, it was found that the germfree mice developed disease and shed oocysts longer than non-germfree ones (Harp *et al.*, 1990). However, in another study carried out by Harp *et al.* (1990) to try and determine the effects of age and previous exposure on resistance of calves to *C. parvum* found 2 out of 7 calves first exposed to the parasite at 3 months developed diarrhoea, while all the 7 calves shed oocysts in their faeces. The calves had some rumenal function as measured by biochemical and microbiological analysis but not the typical level found in a mature bovine. According to these data, Harp *et al.* (1990) states that, it still remains to be determined whether a calf with mature rumen function but with no previous exposure to *C. parvum* would be resistant to challenge with the parasite. The age related

resistance seen in young ruminants and humans may also be influenced by immunological immaturity (Moiser and Obsert, 2000).

The most important immunological response for preventing or resolving cryptosporidial infection is cell mediated. The observation of severe disease in immunocompromised individuals (especially those with HIV/AIDS) who have a low CD4⁺ T cell counts and murine models which were lacking CD4⁺ T cells, have confirmed the central role these cells play in adaptive immunity (Leav *et al.*, 2003). In calves there is substantial increase in CD4⁺ and CD8⁺ T cells, although the levels are much lower than those reported in adult cattle (Fayer *et al.*, 1998), explaining the age related susceptibility from an immunological point of view while supporting the view that T cell subsets protect against *C. parvum* infection. The CD4⁺ T cells are thought to mediate protection by cytotoxicity or help production of neutralizing antibodies (Moiser and Obsert, 2000). The cytokines interferon gamma (IFN- γ), interleukin-12 (IL-12) and Tumor Necrosis Factor alpha (TNF- α) appear to be produced in increasing amounts and seem to be protective in experimental models (Fayer *et al.*, 1998; Moiser and Obsert, 2000; Leav *et al.*, 2003).

Humoral immune responses to *Cryptosporidium* infections have been reported but the exact role the antibodies play in host defence against infection is unclear (Mosier and Oberst, 2000). Administration of high tittered hyperimmune bovine colostrums has been shown to reduce the patent periods, oocyst shedding and to decrease the severity of diarrhoea in calves (Perryman *et al.*, 1999). Mosier and Oberst (2000), however state that, to obtain beneficial effects, anti-cryptosporidial antibodies need

to be present in concentrations that are 10 to 2000 times higher than in normal colostrum. Based on the available information, the benefits of normal colostrum in providing protection against infection appear minimal although it does contain anti-cryptosporidial properties.

Innate immune mechanisms involving the structure of the intestinal mucosa (as mentioned previously) also play an important role in the resistance to infection.

2.6.2 *Parasite factors*

The parasitological determinant of severity of disease and oocysts shedding patterns stems from the hypothesis that there is varying virulence of *Cryptosporidium* strains (Pozio *et al.*, 1992; O'Donoghue, 1995). Conclusive evidence to support this claim has yet to be established (Anderson, 1998). On the other hand, it has been observed that mixed infection diarrhoea outbreaks tend to present with more severe clinical signs (Moore and Zeman, 1991; Fuente *et al.*, 1998). Therefore, one important factor which determines whether an infection results in clinical or sub-clinical presentation is the presence of more than one enteropathogen (Fuente *et al.*, 1998).

2.6.3 *Management under which the hosts are kept*

Along with parasite and host factors, the farm management is considered a major risk factor for propagation of the infection. Since most herds have been exposed to the parasite, management of a herd determines the impact of the disease (Heath, 1992). Management factors can vary for the various diarrhoeal diseases and *Cryptosporidium* in particular. These factors are mainly those that involve young ruminants housing, hygiene and rearing. An association between the size of the farm

(stocking density) and the risk of infection has been established (Garber *et al.*, 1994; Atwill *et al.*, 1999b) due to the higher number of animals in a smaller place, and thus a higher convergence of large numbers of oocysts in a large susceptible population (Atwill *et al.*, 1999b). Thus, individual or group housing is an important factor determining infection rates. The other important management risk factors for predisposing to infection and determine severity of clinical manifestation include type of housing floor, feeding of the calves, source of drinking water, number of times neonate pens are cleaned and whether or not disinfectants are used during cleaning (Heath 1992; Garber *et al.*, 1994; Mtambo *et al.*, 1997; Castro-Hermida *et al.*, 2002b). How much standard colostrum is given during the first 24 hours after birth does not appear to reduce the possible risk of infection but may reduce severity of clinical manifestation (Harp *et al.*, 1990; Anderson, 1998).

2.7 Transmission in ruminants

Transmission of *C. parvum* is by direct contact from one host to another by ingestion of oocysts and indirectly either by environmental contamination or ingestion of contaminated feed and water (Fayer *et al.*, 2000). In ruminants transmission is mostly through direct contact between neonates and contact with contaminated environment (O'Donoghue, 1995; Koudela and Jiri, 1997). Calves have been found to shed oocysts as early as 3 days of age, which peaks at 1 to 2 weeks with the highest amounts on day 13 (Xiao and Herd, 1994; de Graaf *et al.*, 1999). Infected newborn calves can excrete $1 \times 10^6 - 1 \times 10^7$ oocysts per gram of faeces while lambs can exceed 2×10^9 per day (de Graaf *et al.*, 1999). The periods of oocyst shedding coincide with the periods of severe clinical manifestation leading to enormous environmental

contamination, making calves and lambs therefore a more important source of infection for other animals and humans (Mosier and Oberst, 2000). Shedding of oocysts does occur in calves older than 5 months and in adult cattle and ewes but only in low amounts (Henriksen and Krogh 1985; Xiao and Herd, 1994). Therefore, older animals are thought to be reservoirs of infection for the young (Scott *et al.*, 1995). It is also thought that the low excretion of oocysts by older animals provides the initial infection but amplification of the infection occurs in the neonates providing more lethal doses to subsequent newborns (Hoar *et al.*, 2001).

Peri-parturient or post-parturient rise in oocysts excretion in cattle, sheep and mares has been described by some researchers (Xiao and Herd 1994; Anderson, 1998; Ortega-Mora *et al.*, 1999; Faubert and Litvinsky., 2000). This rise in oocysts/egg count has been observed in other parasite infections. In the case of *C. parvum*, this rise in oocyst shedding before or during parturition is considered to be an important source of infection for the neonates. Faubert and Litvinsky (2000) studying the transmission of *C. parvum* in calves found that there was an average of 125 and 250 oocysts excretion during the peri-parturient and post-parturient periods from 148 cows with a sudden rise during parturition that reached 500 oocysts. They concluded that this rise in oocysts excretion by cows does contribute to calf infections resulting in the high prevalence among calves seen in this study. Results from other investigations (Scott, 1995, Hoar *et al.*, 2001), contradict the peri-parturient/post-parturient rise in oocyst production possibly due to less sensitive diagnostic techniques and lesser quantity of faecal material used in the studies.

Transmission of *C. parvum* from wild rodents to calves has been suggested. This is because wild red mice found around barns and fields on farms were found to harbour *C. parvum* oocysts, which were found infectious to calves (Mosier and Oberst, 2000).

2.8 Transmission in humans

In humans the transmission routes for *C. parvum* are person-to-person, animal to person, waterborne and foodborne by contamination with cow dung or human stool (Fayer *et al.*, 2000).

The most common mode of transmission is the direct human-to-human contact (Juranek, 1995). This is seen more commonly where children are grouped together such as in day-care centres. The reasons put forward for this are careless nappy changing or intimate play between children (Cordell and Addiss, 1994). The direct animal to human transmission was established in the 1980s and 1990s when dairy farmers and vet students working with calves would contract the disease (Anderson *et al.*, 1982; Lengerich *et al.*, 1993). Some outbreaks have been reported among school children that undertook a school farm visit (Shield *et al.*, 1990). Transmission of the disease from pets does occur especially in immunocompromised individuals but there is weak epidemiological data to support this route of infection in the normal population (Anderson, 1998; Robertson *et al.*, 2000).

Indirect food-borne transmission is difficult to document and it is therefore greatly under reported because individual cases and small group outbreaks are less likely to

be recognized. Foods can be contaminated directly by faeces or by food-handlers (Fayer *et al.*, 2000). An outbreak of cryptosporidiosis involving 50 school children was associated with milk from a local, small-scale producer in the United Kingdom using an on-farm pasteurizer. The pasteurizer on this farm was found to be faulty at the time that the children were infected. It was therefore postulated that drinking unpasteurized milk poses a potential risk to humans due to faecal contamination (Gelletli *et al.*, 1997).

The major mode of transmission that has evoked public and international concern is indirect waterborne transmission through public water supply (Meinhardt *et al.*, 1996; Haas, 2000; Rose *et al.*, 2002). This is because the oocysts are not killed nor removed by conventional water purification methods (Meinhardt *et al.*, 1996). There are more than 6 well-documented waterborne outbreaks in the USA and United Kingdom (Juraneck, 1995). This has led the Centres for Disease Control (CDC) in the USA to set up guidelines for the detection of oocysts in drinking water, while some national Governments have declared the disease notifiable (Rose *et al.*, 2002; Weir, 2001). Most of these waterborne outbreaks are due to human excreta contamination but 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). The current strategy for eliminating *Cryptosporidium* species from the public water supply involves preventing contamination of water source, physical removal of the organisms and chemical or physical disinfection. However, these methods are only

capable of reducing the number of oocysts not eliminating the parasite from the water supply (Leav *et al.*, 2003). Therefore, more sophisticated methods such as filtration, ultraviolet light irradiation and ozone treatment are more effective but have not been widely used because of financial and health related concerns (Fayer *et al.*, 2000). Other modes of indirect waterborne transmission are through recreational water namely swimming pools and fountains (Fayer *et al.*, 2000).

Cockroaches, flies and beetles have been implicated in disseminating the parasite in the environment (Fayer *et al.*, 2000, Graczyk *et al.*, 2000). Intestinal tracts of cockroaches (*Periplaneta americana*) were examined from a household where a child had cryptosporidiosis. *Cryptosporidium* like oocysts were found and it was suggested that the cockroaches were probably involved in disseminating the oocysts (Zerpa and Huicho, 1994 cited by Fayer *et al.*, 2000).

The theory of airborne transmission has been suggested because of the occurrence of serious respiratory cryptosporidiosis in immuno-compromised individuals and avian species (Lindsay and Blaghurn, 1990; Griffiths, 1999). *Cryptosporidium* oocysts were detected in dust from window frames and beams that were located 3 and 6 meters off the floor respectively. The oocysts were thought to have arrived there possibly through air borne transmission or flies that had contact with contaminated faecal material (Faubert and Litvinsky, 2000).

2.9 Prevalence of *Cryptosporidium parvum*

Cryptosporidium parvum infections in ruminants have been reported worldwide from the continents of Africa, Australia, Europe, Asia and North and South America. Most of these reports are from the developed countries with majority being in *Bos Taurus* rather than *Bos Indicus* breeds of cattle, which include Asian and African breeds (Fayer *et al.*, 2000). However, breed and sex predisposition to the disease is said not to exist. The majority of *C. parvum* investigations are associated with outbreaks of diarrhoea in the susceptible age range namely neonates. Accurate determination of the prevalence of infection in animal or human populations is difficult because some surveys will depend on faeces submitted to diagnostic laboratories which have an inherent sampling bias favouring clinical infections. Furthermore, surveys in asymptomatic individuals may under-estimate the prevalence through failure to detect oocysts, which are excreted sporadically or in low numbers. Although serology may give a better indicator of exposure, antibody titres can not be used to distinguish between patent and past infections nor clinical and asymptomatic infections (O'Donoghue, 1995). Survey data on prevalence of *C. parvum* from around the world shows a lot of variations because of variable age ranges of sampled animals, the sensitivity of the method used for the detection of oocysts in faeces, the amount of faecal material used in diagnosis and the number of times each individual animal is sampled (Fayer *et al.*, 1998; Lefay *et al.*, 2000).

2.9.1 Prevalence in ruminants

The prevalence of *C. parvum* in cattle ranges from 1 to 80% around the world, with higher rates in calves (see Table 2.1). In a survey conducted in the USA involving 15

dairy farms in 7 states, the prevalence in pre-weaned calves (5 days to 2 months) was observed to be 50.3 % (Santin *et al.*, 2004). In 20 Canadian dairy farms in British Columbia, Olson *et al.* (1997a) 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) on 31 dairy farms, a prevalence of 25% was found (Maldonado-Camargo *et al.*, 1998). Some prevalence in European 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.*, 2002b) and 21.5% in Germany (Joachim *et al.*, 2003).

In Asia there are reported prevalence of 20% in 60 cattle of Basrah in Iraq (Mahdi and Ali, 2002) and 12% in 50 calves (0 to 2 months) in Tokachi District in Hokkaido Japan (Sakai *et al.*, 2003). While in Gokseong-gun Korea the prevalence in 84 dairy cattle was found to be 100%. In the same study the prevalence in beef cattle in Chungju-si region was 8.9% (n=754) and 98.2% (n=107) in Gokseong-gun region (Yu *et al.*, 2004). In Africa very few documented data is available on the prevalence of *C. parvum* in ruminants. However, there are two studies from East Africa with reported prevalence of 5.3% (n=486) and 38% (n=50) in Tanzania and Uganda, respectively, (Mtambo *et al.*, 1997; Nizeyi *et al.*, 2002). In Zambia, there is no available literature on the existence of the disease in ruminants or any other animal species.

Table 2.1 Prevalence of *Cryptosporidium parvum* in cattle from different countries in the world.

Country	Farm Type	No. Sampled	Prevalence %	Reference
USA	Dairy ¹	503	50.3	Santin <i>et al.</i> , 2004
Canada	Dairy ¹	386	59	Olson <i>et al.</i> , 1997
Mexico	Dairy ¹	512	25	Maldonado-Camargo <i>et al.</i> , 1998
Britain	Dairy and beef ³	-	80	Scott <i>et al.</i> , 1995
France	Dairy ¹	1628	17.9	Lefay <i>et al.</i> , 2000
	dairy/suckler ²	440	43.4	Lefay <i>et al.</i> , 2000
Germany	Dairy ¹	873-	21.5	Joachim <i>et al.</i> , 2003
Spain	Dairy ¹	535	47.5	Castro-Hermida <i>et al.</i> , 2002b
	Beef ¹	309	48.5	Castro-Hermida <i>et al.</i> , 2002b
Iraq	- ³	60	20	Mahdi and Ali, 2002
Japan	- ¹	50	12	Sakai <i>et al.</i> , 2003
Korea	Dairy ¹	84	100	Yu <i>et al.</i> , 2004
	Beef ¹	863	20.2	Yu <i>et al.</i> , 2004
Tanzania	Dairy ¹	486	5.3	Mtambo <i>et al.</i> , 1997
Uganda	Dairy ³	50	38	Nizeyi <i>et al.</i> , 2002

Note: ¹= cross sectional study, ²= diarrhoea outbreak study, ³other

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). There are variable ranges of prevalence from country to country (see Table 2.2). Two studies in Spain have revealed a prevalence of 40.2% in diarrhoeic 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.*, 1997b). In Egypt, Abd-El-Wahed (1999) reported a prevalence of

68.3% when he examined faeces of 120 lambs. In all these studies coproscopy was used as the detection method with the modified Zeihl Neelsen staining technique.

Table 2.2 Prevalence of *Cryptosporidium parvum* in lambs of less than 3 months of age from different countries.

Country	Type of study	No. Sampled	Prevalence %	Reference
Spain	2	-	40.2	Pilar-Izquierdo <i>et al.</i> , 1993
	1	583	59	Causapé <i>et al.</i> , 2002
Canada	1	89	23	Olson <i>et al.</i> , 1997
Egypt	1	120	68.3	Abd-El-Wahed , 1999

Note: 1= cross sectional study, 2= diarrhoea outbreak, 3=other

There is less data available on the prevalence of *C. parvum* in goats around the world. This could be due to the fact that goats are of lesser importance in developed countries as compared to other domesticated ruminants. Nevertheless, *C. Parvum* infections in kids can be a serious problem with high prevalence (see Table 2.3). A study of diarrhoeic kids in Spain observed 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 6 months was reported with high mortality despite intensive supportive therapy (Johnson *et al.*, 1999). In France, Chartier *et al.* (2002) using an enzyme linked immunoabsorbent assay (ELISA) for oocyst detection, found a prevalence of 58.9% in 137 kids. While in Sri Lanka, Noordeen *et al.* (2001) studying the prevalence of *Cryptosporidium* infection in 3 agroclimatic zones reported a prevalence of 28.5% from 1020 goats using the modified Ziehl Neelsen staining technique.

Table 2.3 Prevalence of *Cryptosporidium parvum* in goats in different countries.

Country	Type of study	No. Sampled	Prevalence %	Reference
Spain	2	-	42	Muñoz <i>et al.</i> , 1996
Oman	3	-	100	Johnson <i>et al.</i> , 1999
France	3	137	58.9	Chartier <i>et al.</i> , 2000
Sri Lanka	1	1020	28.5)	Noordeen <i>et al.</i> , 2001

Note: 1= cross sectional study, 2= diarrhoea outbreak, 3=other

2.9.2 Prevalence in humans

The prevalence of cryptosporidial infections in humans is well documented with most of these infections being due to *C. parvum*. In the UK, 97% of cases in humans are due to *C. parvum*, 1% *C. meleagridis*, 0.2% *C. felis* and less than 0.1% *C. dt* (dog type), while the remainder have not yet been characterised (Pedraza-Diaz *et al.*, 2001). Prevalence is higher in the developing countries than in developed countries (Leav *et al.*, 2003). The prevalence 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). Poor sanitation, contaminated water supplies, overcrowding or greater contact with domestic animals could be the reasons for the observed difference (O'Donoghue, 1995). In Africa, some reported prevalence 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 was found to be 18% (Nchito *et al.*, 1998) and 26%

(Amadi *et al.*, 2001). Among the major reasons contributing to the high prevalence of cryptosporidiosis in sub-Saharan Africa is the high incidence of HIV/AIDS with an equivalent of 70% of the world's cases (UNAIDS, 2002). Zambia's HIV/AIDS estimated prevalence of 15.6% among adults 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 (CSO, 2001-2002).

2.10 The importance of cryptosporidiosis

2.10.1 Economic importance

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 enteropathogens in calf-hood diarrhoea (Faubert *et al.*, 2000) which leads to weight loss (Tzipori, 1983). According to data from the Veterinary and Agrochemical Research Centre in Belgium on the proportional occurrence of *C. parvum* and viral enteropathogens in samples from calves with diarrhoea over a 10 year period, *Cryptosporidium* was found to be increasingly important with 30.7% as a sole pathogen and 8.9% in association with other pathogens (de Graaf *et al.*, 1999). The parasite has also been found as one of the common leading causes of enteropathies in lambs and kids (Xiao and Herd, 1993; 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). 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 high mortalities in forty two scouring calves. These mortalities do contribute to economic losses but to a lesser degree, which in Belgium is estimated at 5-10% (de Graaf *et al.*, 1999). The indirect losses are primarily from weight loss and retarded growth in calves, lambs or kids (de Graaf *et al.*, 1999). In adult cattle *C. andersoni* (formerly known as *C. muris*) 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) studying *C. andersoni* in five herds of cattle found that cows shedding *C. andersoni* oocysts produced significantly less milk, approximately 3.2kg/day (13%) as compared to the non-shedders. All these losses do constitute a significant loss in income for the dairy farmers.

2.10.2 Public health importance

The zoonotic potential of *C. parvum* was recognised in the early 1980s when it was frequently reported as a gastro-intestinal 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). 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. Malnutrition has also been found to compound the problem especially in children in third world countries (Moisier and Oberst, 2000).

Many reports of zoonotic transmission have been documented with direct contact of ruminants and humans being the most common, (Mosier and Oberst, 2000, Morgan *et al.*, 1999). The other important mode of zoonotic transmission that has already been alluded to which has raised a lot of public concern and research is the contamination of water for human domestic use 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). In the largest waterborne outbreak of disease reported in Milwaukee in 1993 that affected an estimated 403,000 people with approximately 100 deaths (Leav *et al.*, 2003), *C. parvum* was found to be the causative agent. The source of contamination was postulated to be from human faecal material, because genotypic characterisation of 4 isolates was found to be human (Peng *et al.*, 1997). But the author of the original paper on the outbreak states that other potential sources including cattle farms and slaughterhouses could not be ruled out as genotyping was done on very few samples to make a conclusive thesis (Health Stream Article, 1997). 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).

2.11 Diagnostic methods

The most commonly employed technique for identifying *C. parvum* infection in live individuals is the detection of oocysts in faecal samples (O'Donoghue, 1995). A variety of methods are available such as microscopic staining methods,

immunological-based detection methods and molecular techniques (Fayer *et al.*, 2000).

2.11.1 *Them microscopic methods*

In coproscopy, the methods include making of faecal smears either directly or after performing a concentration technique (with the most common being the sucrose gradient) on the faecal samples and employing direct or differential staining techniques for *Cryptosporidium* oocysts. The stain of choice for most diagnostic laboratories is the modified acid-fast staining.

The direct smear method is less sensitive than if a concentration step was incorporated into the procedure. Therefore, the direct smear method tends to underestimate the number of oocysts detected especially if excretion rates are low (Scott *et al.*, 1995). Microscopy in general is problematic because it is difficult to distinguish oocysts from other small particles in faecal and environmental specimens (Fayer *et al.*, 2000). It is also time consuming, tedious and requires experienced microscopists to accurately identify oocysts (Morgan *et al.*, 1998) because the oocysts stain variably under the acid fast stain depending on their age and viability (O'Donoghue, 1995). Waber *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. Whereas, 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 faeces. He then

concludes 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 large numbers of oocysts to identify infection. Arrowood and Sterling (1989) found that microscopic acid fast method was of low specificity (52%) compared to monoclonal antibody based techniques but Quílez *et al.* (1996) reports a specificity of 92.9% in bovine faeces with sensitivity of 79.3% with a commercial direct Immuno-fluorescent as the standard. In another study by Ignatius *et al.* (1997) it was found that the new enzyme immunoassays did not exhibit higher sensitivities in detecting low numbers of oocysts than the routinely used Immuno-fluorescent and modified acid-fast staining methods. Due to the conflicting reports on the sensitivity of immuno-detection methods over microscopy, and that the microscopic method does not need complex equipment and is cheap, it is still widely used in surveys and diagnostic laboratories.

2.11.2 Enzyme Immuno Assay methods

In the past decade highly sensitive and specific enzyme immuno-assays (EIA) have been developed and these have become widely accepted for screening stool (Anusz *et al.*, 1990; Newman *et al.*, 1993; Katanika *et al.*, 2001). The sensitivity of the commercial enzyme immuno-assays has been reported to be 100% and specificity to be from 98.6 to 99.5% based on comparison with the modified acid-fast staining technique (Katanika *et al.*, 2001). In a study by McClusky *et al.* (1995) to compare the Kinyoun acid-fast staining technique and a commercially produced enzyme immunoassay, an overall agreement of 72% was found, with the EIA being more sensitive. Although according to Kehl *et al.* (1995) the sensitivity of the two

microscopic methods, Immuno-fluorescence (Meriflour kit) and modified acid-fast stain of 96.4% is comparable to the EIA commercial packs (ProSpect and Colour Vue) of 94.5%. The advantages of these commercially available packs is that they are easy to use with ease of interpretation of results, quick and there is no need of a well-experienced technician to identify the parasite (Kehl *et al.*, 1995; Fayer *et al.*, 2000). Unfortunately, some of these are expensive making it impractical for some clinical laboratories to use them as routine procedures (Mosier and Oberst, 2000). Furthermore, Fayer *et al.* (2000) reports that these immunological detection methods can be problematic due to non-specificity of antibodies, because of cross reactivity with other microorganisms.

Some immuno-serology tests used in live hosts include Immuno-fluorescence, ELISA and solid and phase immuno-chromatographic assays (Fayer *et al.*, 2000). Although some researchers have used this technique to determine prevalence in certain populations, it has limitations in that it only suggests exposure to the parasite during some time in the animal's life and not depicting the actual disease situation (O'Donoghue, 1995).

2.11.3 Polymerase Chain Reaction based techniques

A number of Polymerase Chain Reaction (PCR) techniques have been developed to identify *Cyptosporidium* to molecular level. Some of the techniques used are random amplified polymorphic DNA (RAPD), sequence analysis and restriction fragment polymorphism (RFLP). According to Fayer *et al.* (2000) sequence analysis provides the most complete and reliable data as it examines all bases at a particular locus but

is more costly and time consuming than RFLP, which examines bases at particular restriction sites within the locus. The advantage of molecular techniques is that they are rapid, highly sensitive and accurate and also provide information on genetic variability of *Cryptosporidium* isolates so that different species can be identified (Morgan *et al.*, 1998; Xiao *et al.*, 1999). The method has restrictions as false positives can occur from detection of naked nucleic acids, non-viable microorganisms, and laboratory contamination (Fayer *et al.*, 2000).

2.12 Therapeutic management of cryptosporidiosis

Currently there is no effective treatment for cryptosporidiosis in humans and livestock (Juranek, 1995; O'Donoghue, 1995). Therefore treatment is mostly symptomatic by intravenous fluid and electrolyte replacement (de Graaf *et al.*, 1999). A number of drugs have been tried in humans and animals both as potential preventatives and curatives with minimal success (Moon *et al.*, 1982; Joachim *et al.*, 2003). In the 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. However, more studies are required to ascertain the therapeutic value of most of these drugs at non-toxic dose levels.

Although hyperimmune bovine colostrum has been reported to protect calves against infection, a study by Harp *et al.* (1996) revealed that feeding of colostrum every 6 hours for 2 weeks did not protect calves against *C. parvum*. The investigators also found that vaccination or probiotic treatment were not effective in preventing *C. parvum* infection in calves under field conditions and stated that further work is necessary to develop effective prophylaxis against *C. parvum*.

2.13 Prevention and control

Prevention and control of *C. parvum* infection like other enteric pathogens lies in good management and hygiene practices on the farm. Management practices that reduce the stocking rate, do not group house calves, separate the healthy and sick animals during outbreaks of diarrhoea and administer appropriate amounts of colostrums to neonates, help to reduce infection and outbreaks of enteric diseases and cryptosporidiosis in particular (Heath, 1992; Atwill *et al.*, 1999a; de Graaf *et al.*, 1999). Hygiene at feeding times especially for dairy calves separated from dams at birth is an important factor in calves getting the infection (Heath, 1992; Mtambo *et al.*, 1997). Regular cleaning of the calf housing units reduces the number of oocysts contaminating the environment that could possibly be ingested by calves (Maldonado-Camargo *et al.*, 1998; Atwill *et al.*, 1999a; Castro-Hermida *et al.*, 2002b). Disinfection of calf pens has been shown to reduce contamination with oocysts. Most commercial disinfectants based on aldehyde, chlorine and ammonia are ineffective when following the manufacturers' recommendations. According to O'Donoghue (1995) the disinfectants which have been found to be effective following relative short exposure periods are 50% ammonia, 3% hydrogen peroxide,

10% formalin, Exspor (Chlorine dioxide based) and Oo-cid (two phase product producing ammonia). O'Donoghue (1995) further states that although higher concentrations and longer exposures do kill oocysts in some cases, the prolonged use of concentrated disinfectants in confined areas is inadvisable and impractical in many situations.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

During the period from August 2003 to August 2004 a total of 744 calf, 154 lamb and 105 kid faecal samples were collected from 4 provinces of Zambia namely; Central, Eastern, Lusaka and Southern (see Figure 3.1). These provinces together with Western province which was not included in the study are the major livestock keeping areas in Zambia. The provinces are divided into veterinary camps for purpose of livestock disease management by the Ministry of Agriculture and Co-operatives. All the veterinary camps sampled in this study are in the same agro-ecological zones (region 2) which comprises grasslands and woodlands with 600 to 800mm annual rainfall.

3.2 Farms sampled

Farms were selected based on the willingness of the farmer to participate and the presence of at least 1 calf, lamb or kid aged 3 months and below at the time of sampling. Calves from 3 husbandry systems namely dairy, beef ranch and traditional husbandry systems were sampled. The dairy farms were intensively managed whereas the beef ranches were extensively managed. Feed supplementation and rigorous disease control were practiced on dairy farms and ranches. Majority of the ranches practice synchronized breeding. The traditional husbandry system in Zambia consists of extensive management but unlike on the ranches, the cattle graze

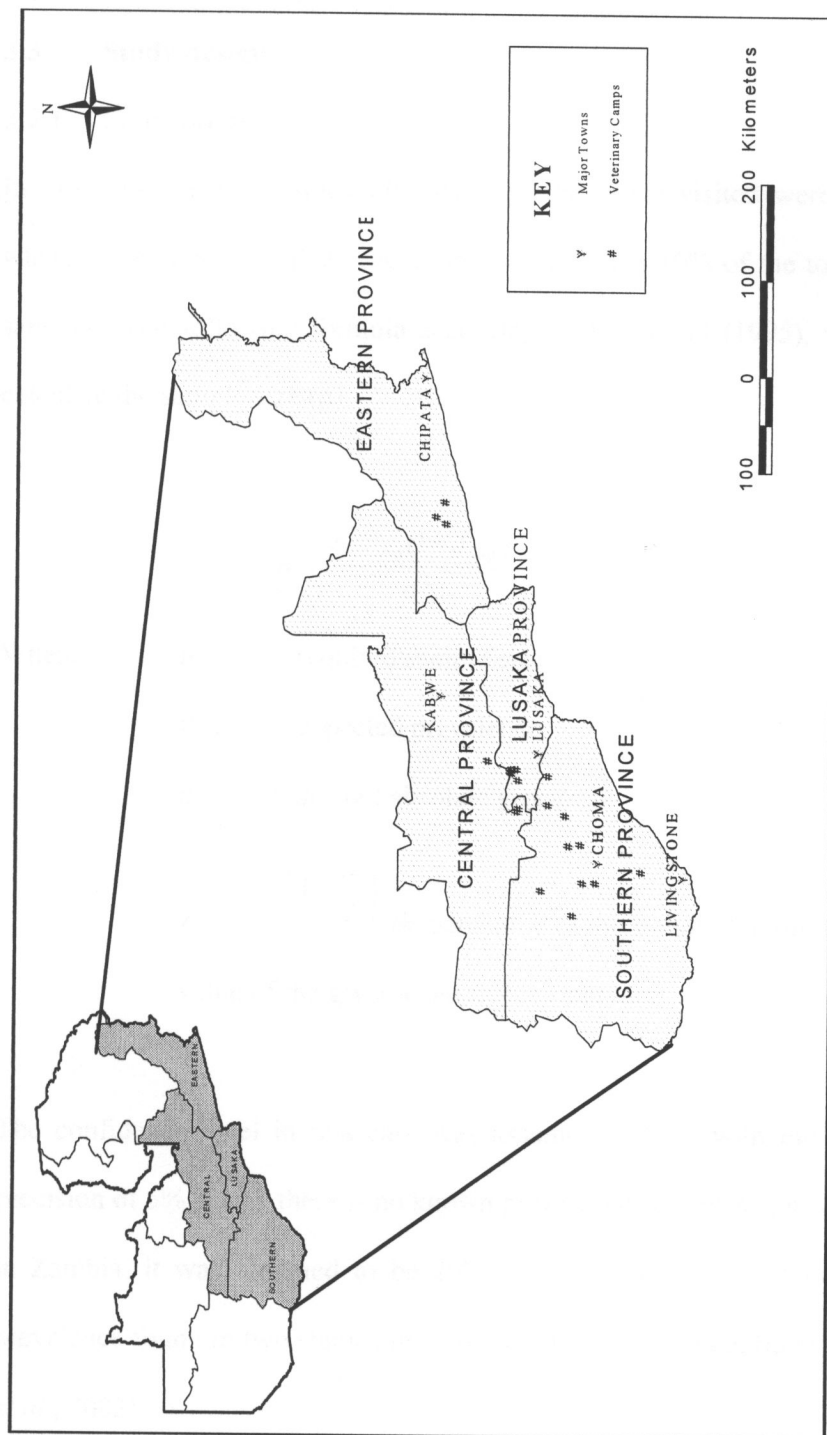


Figure 3.1 Map showing study areas in Central, Eastern, Lusaka and Southern provinces of Zambia. (Source: Annon, 2004)

communal native pastures walking over 9 hours a day. Little or no disease control is practiced in the traditional husbandry system.

3.3 Study design

3.3.1 Calculation of sample size

In this cross-sectional study all calves on the farms visited were sampled except where the number of calves was more than 20, then 10% of the total herd size was sampled. The following formula according to Thrusfield (1995), was employed to calculate the sample size (n).

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

Where n = required sample size

P_{exp} = expected prevalence

d = desired absolute precision

$Z = \left(1 - \frac{\alpha}{2}\right)$ th percentile of the standard normal distribution or Z value of the given Confidence Interval.

The confidence level in this case was assumed at 95% with the desired absolute precision of 5%. Since there is no known prevalence of *Cryptosporidium* in livestock in Zambia, it was assumed to be 20%, which is the approximate average of the prevalence found in two studies in cattle in East Africa (Mtambo *et al.*, 1997; Nizeyi *et al.*, 2002).

The calculated sample size was:

$$n = \frac{1.96^2 \times 0.20 (1-0.30)}{0.0025}$$

$$n = \underline{247}$$

Therefore a total of 247 faecal samples were to be collected from dairy, ranch and traditional cattle husbandry systems. A total of 250 dairy, 238 ranch and 256 traditional calves were sampled with an age range of 0.3 to 94 days with a mean age of 23 days (SD $\pm$ 15.2). For lambs and kids a total of 152 and 105 samples were collected, respectively. The samples could not be based on those calculated for calves as the goats and sheep populations in the country are much lower than for cattle (Appendix 1). The age ranges were 2 to 49 days with mean 21.7 days (SD $\pm$ 8.5) for sheep and 7 to 56 days with mean 20.2 days (SD $\pm$ 9.0). A total of 16 sheep and 12 goat farms were sampled.

3.3.2 Sampling methodology

Faecal samples were collected per-rectum from sheep, goats and cattle aged 3 months and below using clean disposable latex gloves. For each faecal sample, the consistency was recorded. Faecal smears of approximately 2 cm in diameter were made in duplicate for each sample collected and about 5-10g of faecal material was stored in a freezer at -20°C for ELISA and PCR analysis.

A questionnaire was administered in the dairy sector to find out some risk factors that may be associated with prevalence of *C. parvum* in Zambian dairy farms. The

questionnaire captured data on general calf management, maternity management, calf housing and hygiene practices (Appendix 2). Of the 37 dairy farms sampled, 10 were not included in the questionnaire survey due to lack of adequate data. The diagnostic test used for analysis of the question data was the ELISA.

3.4 Coprological oocyst detection methods

3.4.1 Microscopic technique

Microscopy with the Modified Ziehl Neelsen staining technique of Garcia (2001) (see Appendix 3) was performed on 483 calf faecal samples. Faecal smears were made on frosted glass slides and the slides air dried and fixed in Methanol for 3 minutes. They were then stained with carbofuschin for 5 minutes after which they were washed with tap water. After the water had stopped dripping by placing the slide rack on filter paper, the smears were decolorized with 95% acid alcohol. Methylene-blue was used to counter stain for 30 seconds and then the slides were washed with tap water and left to air dry. Examination of the slides was carried out using a calibrated light microscope at x1000 magnification under oil immersion. Bright red and pale stained, spherical and sub-spherical, refractile wall oocysts were observed and noted as positive (see Appendix 4). However, some oocysts did not pick up the stain. The oocysts were measured and the number per smear recorded.

3.4.2 Enzyme Linked Immunosorbent Assay

A coprologic Enzyme Linked Immunonosorbent Assay (ELISA) employing a specific anti-*Cryptosporidium* monoclonal antibody (commercial kit, Techlab ELISA) was used for oocyst detection in all the faecal samples collected. The ELISA

was used as the gold standard in this study. The test consists of 96-well microtitration plates coated with specific antibodies for an antigenic determinant of *Cryptosporidium* (Appendix 5). The reagents provided in the kit were wash buffer concentrate, substrate A and B, conjugate, detecting antibody, stop solution and positive control solution. The 50ml wash buffer concentrate was diluted with 950ml-distilled water to make the wash solution that was also used as the negative control.

Approximately 0.1g of the frozen faecal samples were placed in 15ml round bottomed test-tubes using a stainless steel spatula. To the thawed sample 0.4ml of the diluted wash solution was added to make a 1:5 dilution. The sample was vortexed and 0.1ml of the prepared specimen was added to the test well. The wells were then incubated for 1 hour to allow the antibodies to specifically capture the corresponding oocyst antigen. The contents of the well were then discarded and the wells were washed 5 times using the wash solution in a squirt bottle with a fine tipped nozzle. After each wash the plate was slapped dry on a paper towel. A drop of detecting antibody was then added to each well and incubated for 20 minutes. The plates were then washed five times as described above. One drop of the conjugate solution was then added to each well and incubated for 10 minutes. The washing procedure was repeated and 1 drop of substrate A was added to each well followed by a drop of substrate B. The wells were incubated for 5 minutes during which time the plate was tapped 3 times to mix the contents of the wells. All incubations were carried out at room temperature. To stop the reaction one drop of the stop solution was added to each well. The absorbency was measured at 450nm wavelength using a labsystems ELISA reader.

3.5 Genotypic characterization of oocysts identified

3.5.1 DNA extraction

A total of 55 samples comprising 45 calves (25 dairy, 11 beef, 9 traditional), 5 lambs and 5 kids were analysed. The QIAamp DNA stool Mini kit was used for DNA extraction. The protocol employed was the one for isolating DNA from pathogens in stool as can be seen in Figure 3.2. The following reagents are provided with the kit, Proteinase K, inhibitEx tablets QIAamp spin columns, 2ml collection tubes, buffers ASL, AL, AW1 and AW2. Buffers AW1 and AW2 are supplied as concentrates and before use, they were mixed with 25ml and 30ml of 100% ethanol, respectively. Approximately 0.2 to 0.4g (or 200µl if stool was liquid) was put in a 2ml micro-centrifuge tube. The tube was then put in liquid nitrogen for 5 minutes and then in a water bath at 95°C for 5 minutes, this was repeated 3 times, to lyse the oocysts. Then 1.4 ml of buffer ASL was added to the sample and vortexed continuously for 1 minute until thoroughly homogenized. The suspension was then heated in a water-bath at 95°C for 5 minutes to lyse the sporozoite cells, after which it was vortexed for 15 seconds and centrifuged at 14,000rpm (full speed) for 1 minute to pellet stool particles. After centrifugation 1.2ml of the supernatant was pipetted into a new 2ml micro-centrifuge tube and the pellet discarded. A tablet of InhibitEX was added to the sample and vortexed immediately and continuously for 1 minute until the tablet was completely suspended. The suspension was incubated for 1 minute at room temperature to allow inhibitors to adsorb to the InhibitEX matrix. The sample was then centrifuged at full speed to pellet inhibitors bound to InhibitEX. The supernatant was pipetted to a new 2ml micro-centrifuge tube and the pellet discarded. The supernatant was then spun at full speed for 3 minutes, to pellet any remaining

material from the Inhibitor tablet. Fifteen microlitres of Proteinase K were pipetted into a new 1.5ml micro-centrifuge tube to which 200µl of the sample supernatant was added. Then 200µl of buffer AL was added to the solution and vortexed for 15 seconds, after which it was heated in a water-bath at 70°C for 10 minutes. Then 200µl of ethanol (100%) was added to the lysate and vortexed. The lysate was then transferred into a new labelled QIAamp spin column placed in a 2ml collection tube and centrifuged for 1 minute at full speed. The QIAamp spin column was then placed in a new collection tube and the tube containing the filtrate discarded. Buffer AW1 (500µl) was added to the QIAamp spin column and centrifuged at full speed for 1 minute. The QIAamp spin column was then placed in a new 2ml collection tube and the collection tube containing the filtrate was discarded. Buffer AW2 (500µl) was then added to the spin column and centrifuged at full speed for 3 minutes. The spin column was transferred to a new labelled 1.5ml micro-centrifuge tube and 200µl of AE buffer was added directly onto the QIAamp membrane. The spin column was then incubated for 1 minute at room temperature and then centrifuged at full speed for 1 minute to elute the DNA. The elute was stored at -20°C till PCR was performed.

3.5.2 *Polymerase Chain Reaction technique*

A total of 55 samples found positive on the coproantigen ELISA were examined using a two step nested PCR protocol, to amplify the Heat Shock Protein 70 (HSP-70) gene. The samples were from cattle (dairy, beef and traditional husbandry systems), sheep and goats (Table 4.7). A nested PCR reaction, previously described by Morgan *et al.* (2001), was used to amplify the *C. parvum* genome. For the primary

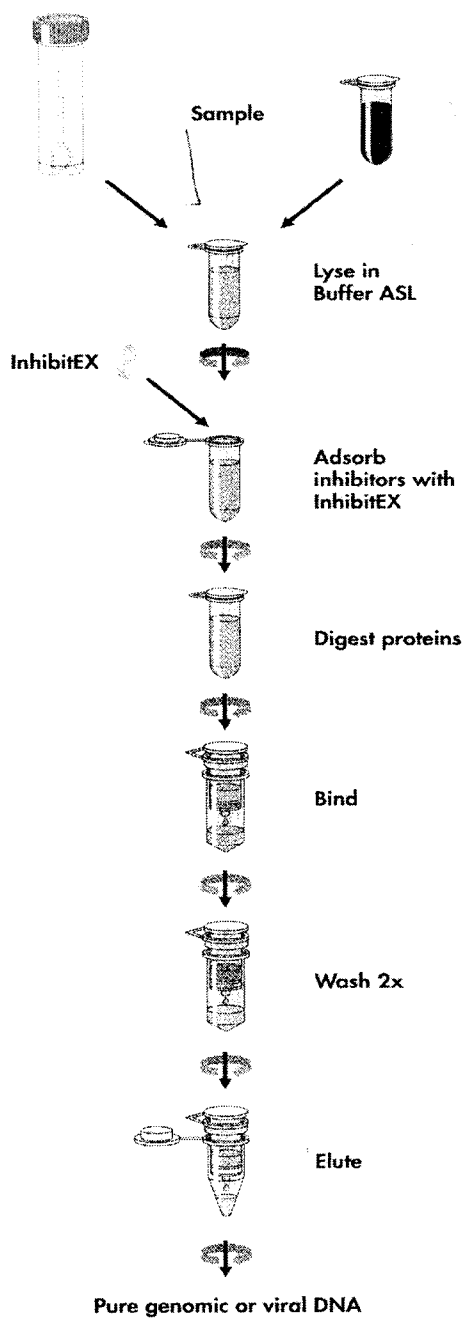


Figure 3.2 The QIAamp DNA Stool Mini kit procedure for extraction and purification of DNA from stool sample.

amplification, a PCR product of 448 bp was amplified using the oligonucleotide primers HSP-F4 (5'-GGT GGT GGT ACT TTT GAT GTA TC-3') and HSP-R4 (5'-GCC TGA ACC TTT GGA ATA CG-3'). For the secondary PCR, the oligonucleotide primers HSP-F3 (5'-GCT GST GAT ACT CAC TTG GGT GG-3') and HSP-R3 (5'-CTC TTG TCC ATA CCA GCA TCC-3') were used. The reaction mixture consisted of a master mix containing Taq DNA polymerase, dNTP mixture, Bovine Serum Albumin, standard Taq buffer, 10 pM of each primer and 2.5µl of template DNA in total of 25µl. For the secondary reaction, 2.5µl of a 1/10 dilution of the amplification product of the first reaction was used. Subsequent steps were initial denaturation for 5 min at 94°C, followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 56°C and an extension for 30 s at 72°C. Amplification products of the secondary reaction were subsequently visualized on 1.5 % agarose gel with ethidium bromide and sequenced. A positive and negative control sample was included in each PCR reaction. The positive control consisted of a plasmid containing the 325 bp rDNA product. This was originally cloned from a *C. parvum* DNA sample isolated from a calf. The negative control was purified water.

3.6 Statistical data analysis

The statistical methods used included Chi-square and Fisher's exact test for testing bivariate association between a hypothesized factor and the risk of *C. parvum* infection. The results were considered significant at $P < 0.05$. A multivariate logistic regression analysis was also carried out to compare prevalence in the three cattle husbandry systems and some hypothesized risk factors found to be significant on

Chi-square test or Fisher's exact test. The independent variables included in the model were type of husbandry system, age, faecal consistency and farm size with the dependant variable being the infection status of the calves (positive or negative). The results were expressed as odds ratio (OR) at 95% confidence interval (CI 95%). The sensitivity and specificity of the microscopic method against the ELISA was calculated using a two by two contingency table. The computer softwares Epi-Info and SPSS (Statistical Package for Social Sciences) for windows (version 11.0:1998) were employed for data analysis.

CHAPTER FOUR

4.0 RESULTS

4.1 *Cryptosporidium parvum* infections in cattle

4.1.1 *Prevalence in the three cattle husbandry systems*

During the period from August 2003 to April 2004, a total of 744 calves were sampled from 37 dairy farms, 26 ranches and 91 traditional farms. Two methods were used for oocyst detection, microscopy with the modified Ziehl Neelsen staining technique and the commercial coproantigen Techlab ELISA. The ELISA technique was carried out on samples from all the farms, whereas the microscopic method was performed on 483 samples from 23, 13 and 61 farms from the dairy, beef and traditional husbandry systems, respectively.

The overall prevalence on ELISA from the 3 husbandry systems after testing 744 samples was 19.2% and specific prevalence in the dairy, beef and traditional husbandry systems were 42.8%, 8.0% and 6.2%, respectively. After microscopic examination of 483 faecal samples, an overall prevalence of 23.4% was recorded and prevalence in the 3 husbandry systems was 47.9% for dairy, 14.7% for beef and 4.7% for traditional. The prevalence of *C. parvum* in the three cattle husbandry systems based on both tests is shown in Figure 4.1. The difference in prevalence in the three systems was significantly different (χ^2 , $P < 0.001$) on the ELISA as well as microscopy.

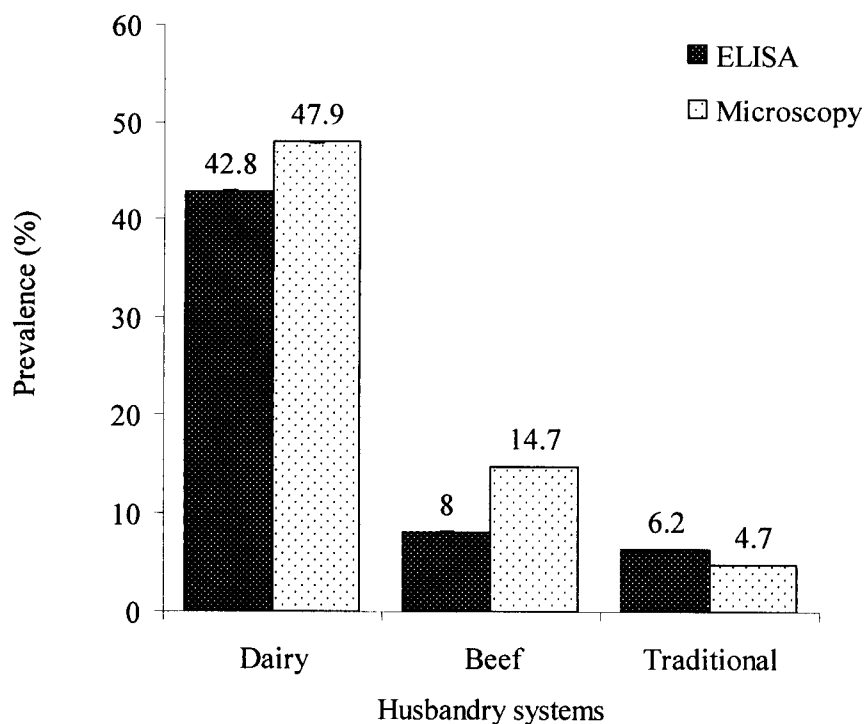


Figure 4.1 Prevalence of *Cryptosporidium parvum* in calves in three husbandry systems using the ELISA [dairy (n=250), beef (n=238) and traditional (n=256)] and microscopy with the modified Ziehl Neelsen techniques [dairy (n=171), beef (n=163) and traditional (n=149)].

The farm prevalence based on ELISA was 75.7% dairy, 42.3% beef and 15.4% traditional husbandry systems. Microscopic examination revealed prevalence of 51.3%, 19.2% and 7.1%, for the three husbandry systems respective. Farm prevalence was significantly different (χ^2 ; $P= 0.001$) among the three husbandry systems based on both tests (Figure 4.2).

It was found that the prevalence in the dairy husbandry system was significantly higher than the beef sector (OR: 8.6; CI 95%: 5.09-14.6). Thus the dairy calves were 8.6 times more likely to be infected than those in the beef sector. Comparison of the dairy husbandry system with the traditional system revealed that calves in the dairy

sector were 11.2 times more likely to be infected with *C. parvum* (OR: 11.2; CI 95%: 6.4-19.1). However, there was no significant difference in prevalence between the beef and traditional husbandry system (OR: 1.3; CI 95%: 0.66-2.57).

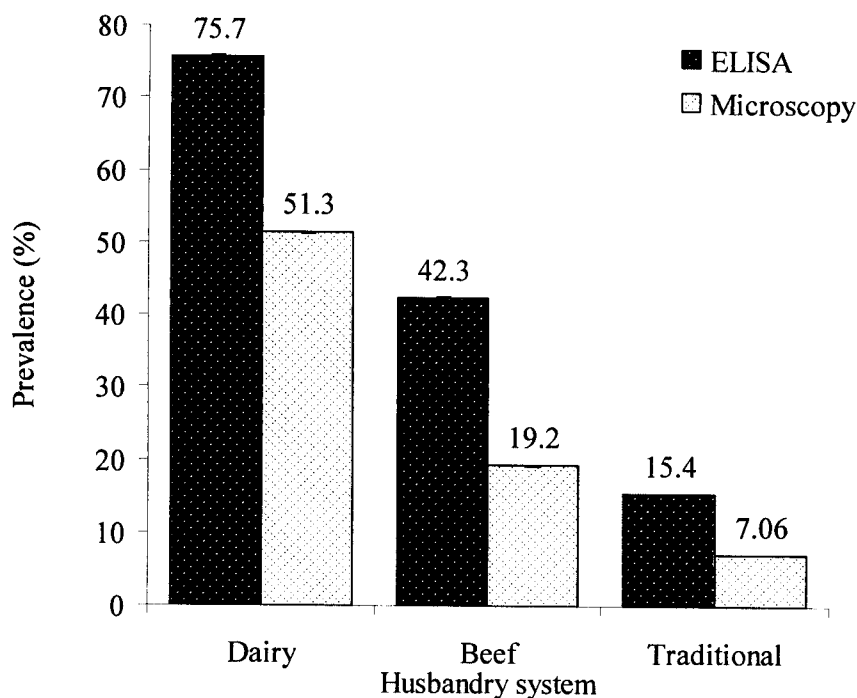


Figure 4.2 Farm prevalence of *Cryptosporidium* in the three cattle husbandry systems based on ELISA [dairy (n=37), beef (n=26) and traditional (n=91)] and Microscopy [dairy (n=23), beef (n=13) and traditional (n=61)].

When the farms in the three husbandry systems were grouped according to animal populations on the farm, prevalence was found to be increasing from the small to the large-scale farms (Figure 4.3). The overall difference in prevalence among the three farm sizes from the three husbandry systems was significant (χ^2 , $P<0.001$). However, it is only in the dairy (χ^2 , $P<0.001$) and traditional (χ^2 , $P=0.004$) sectors where this difference was significant. The calves on the small-scale farms were found to be 67%

less likely (OR: 0.33; CI 95%: 0.18-0.62) to be infected with *C. parvum* than those on the large-scale farms (OR: 1.07; CI 95%: 0.7-1.65). The risk of infection on the medium-scale farms was not significantly different from that on the large-scale farms.

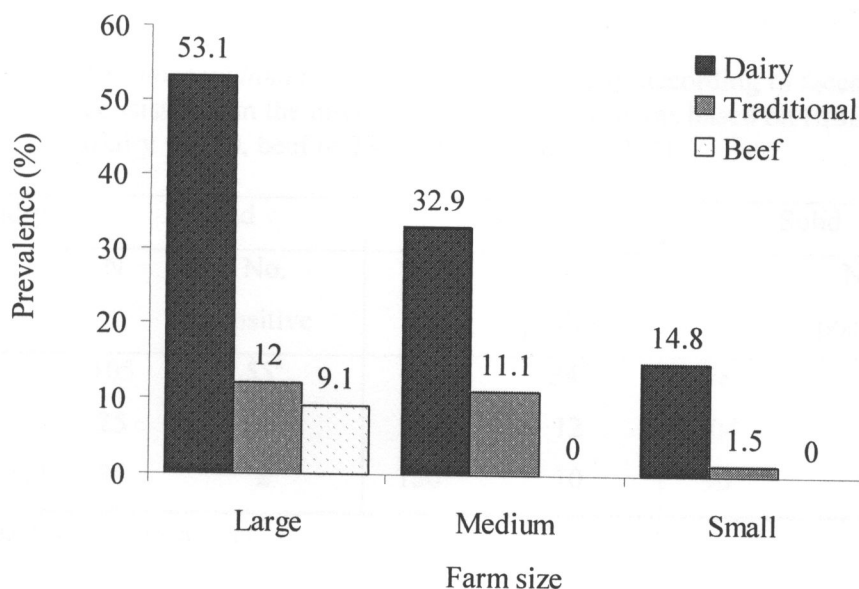


Figure 4.3 Prevalence of *Cryptosporidium parvum* by farm size in the three cattle husbandry systems based on ELISA (small=1-50 animals, medium=51-150 animals, large=151-1600 animals).

The analysis of *C. parvum* infections according to faecal consistency are presented in Table 4.1 and Figure 4.4. From a total of 740 calves whose faecal consistency was recorded, 164 (22.2%) had liquid faeces of which 57 (34.8%) were positive for *C. parvum*, 324 (43.8%) had soft faeces with 56 (17.3%) positive and 252 (34.1%) had solid faeces with 29 (11.5%) positive. Overall, there was a significant difference in the infection rate among the calves with liquid, soft and solid faeces (χ^2 , $P < 0.001$). When statistical analysis was performed for samples from calves in each husbandry

system, it was found that parasitization with *C. parvum* was only significantly associated with faecal consistency in the dairy sector (χ^2 , $P= 0.02$). Furthermore, there was a significant difference in prevalence of *C. parvum* between calves passing out liquid and soft faeces as well as the liquid and solid faeces. There was no significant difference in prevalence between calves passing out soft and solid faeces.

Table 4.1 *Cryptosporidium parvum* infections in calves according to faecal consistency in the three cattle husbandry systems based on ELISA (dairy n=250, beef n=236 and traditional n=254).

Husbandry System	Liquid		Soft		Solid	
	N	No. positive	n	No. positive	n	No. positive
Dairy	105	55 ^a	87	34 ^b	58	18 ^b
Beef	25	0	107	12	104	7
Traditional	34	2	130	10	90	4

^a shows significant difference from ^b

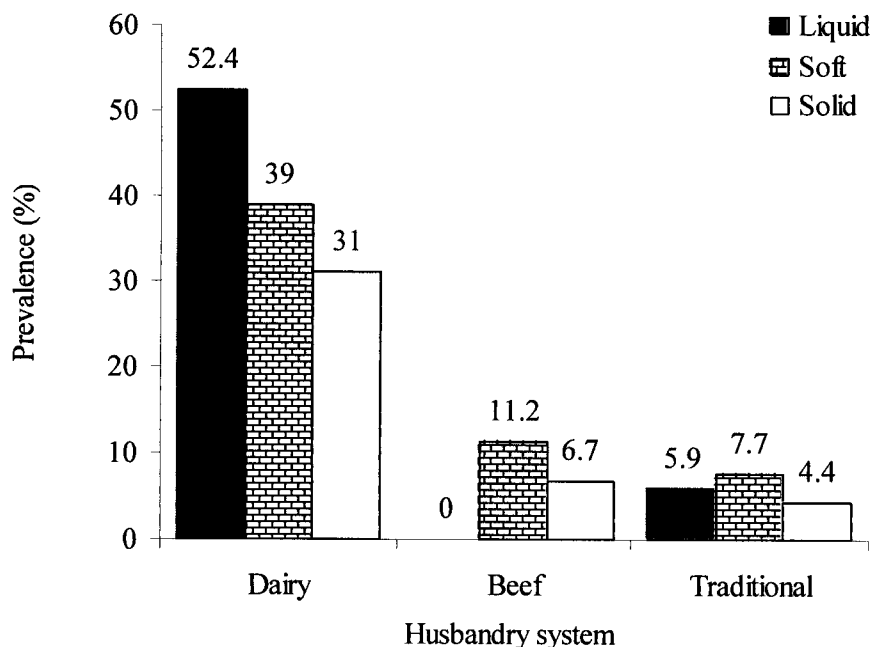


Figure 4.4 Prevalence of *Cryptosporidium parvum* according to faecal consistency in the three cattle husbandry systems based on ELISA (dairy n=250, beef n=236 and traditional n=254).

Generally, there was increasing rate of infection with age up to 3 weeks, after which there was a decrease (Figure 4.5). The majority of infections with *C. parvum* were in the age range of 1 to 4 weeks in all the three husbandry systems but association between age and infection was significant only in the dairy (χ^2 , $P=0.02$) and the beef (χ^2 , $P=0.04$) sectors. Using the multivariate logistic regression analysis with reference age as 5 to 12 weeks, showed that calves of 1 week and below were 50% less likely to be infected by *C. parvum* (OR: 0.50; CI 95%: 0.3-0.8) whereas those of 3 weeks (OR: 1.70; CI 95%: 1.1-2.6) and 4 weeks (OR: 1.63; CI 95%: 1.04-2.6) were 70% and 63%, respectively more likely to be infected. The risk of infection for calves of 2 weeks comparable to those aged 5 to 12 weeks (OR: 1.47; CI 95%: 0.97-2.3).



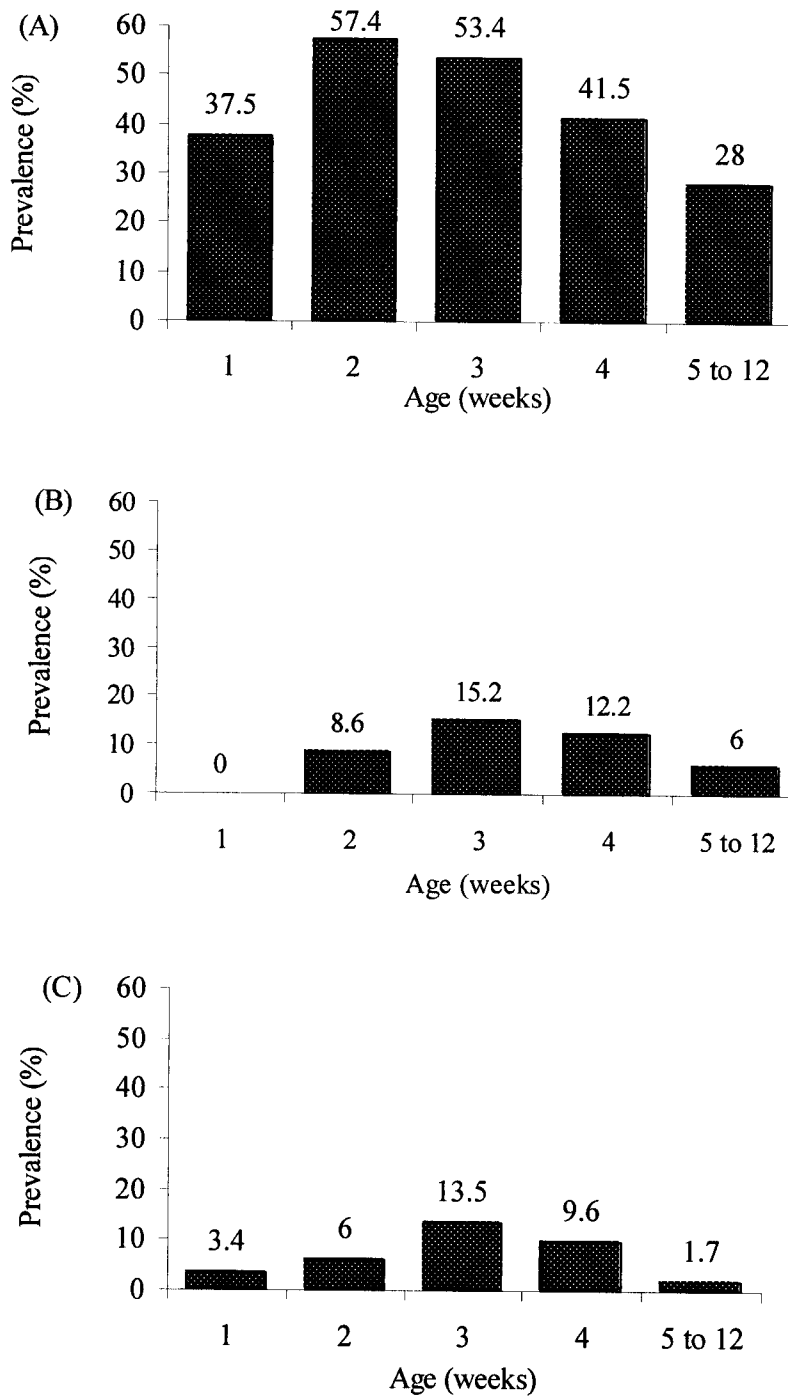


Figure 4.5

Prevalence of *Cryptosporidium parvum* by age in weeks in calves in A) dairy (n=244), B) beef (n=238) and C) traditional (n=256) husbandry systems using ELISA.

4.1.2 Questionnaire survey in the dairy management system.

Results of analysis of association between two general management factors (type of farm and presence of small ruminants) and occurrence of *C. parvum* in faecal samples from calves are presented in Table 4.2. Only type of farm was found to be significantly associated with occurrence of *C. parvum*. The intensively managed farms were found to be 20.8 times more likely to have infected calves than those that were semi-intensively managed.

Table 4.2 General management factors from respondents in 27 dairy farms evaluated for association with the occurrence of infections with *Cryptosporidium parvum*.

Factor	Respondents	Positive	Odds ratio	P-value
Type of farm				
Intensive	14	13	20.8	0.004
Semi-intensive	13	5		
Presence of small ruminants				
Yes	7	6	-	0.4
No	20	12		

A total of three maternity management factors were evaluated for association with *C. parvum* infection and results of analysis are shown in Table 4.3. None of these factors were found to be significantly associated with *C. parvum* infections.

Table 4.3 Maternity management factors from respondents in 27 dairy farms evaluated for association with the occurrence of *Cryptosporidium parvum* infections in calves.

Factor	Respondents	Positive	Odds ratio	P-value
Calf is separated from cow at birth				
Yes	6	6	-	0.07
No	21	12		
Calves having access to calving area				
Yes	13	6	-	1.0
No	1	1		
How much colostrums is fed to calves in the first 24 hours				
1 to 4l	6	6	-	0.07
Ad lib	21	12		

Calf housing and hygiene factors whose association with *C. parvum* infection were evaluated were type of housing and cleaning of calf pens as shown in Table 4.4. None of the factors were found to be significantly associated with *C. parvum* infection on the farms.

Table 4.4 Housing and hygiene factors from respondents in 27 dairy farms evaluated for association with *Cryptosporidium parvum* infection.

Factor	Respondents	Positive	Odds ratio	P-value for factor
Type of housing				
Individual	13	11	-	0.1
Group	14	7		
Individual housing				
Inside	2	2	-	1.0
Outside	12	10		
Group housing				
Inside	6	4		0.6
Outside	8	3		
How often is bedding removed/floor cleaned				
Daily	14	12	-	0.1
Not daily	6	3		

4.2 Prevalence in small ruminants

A total of 152 Black-headed dorper lambs and 105 crossbreed kids were sampled with prevalence of 18.4% and 5.7%, respectively. Although sheep had a higher overall prevalence than goats, farm prevalence for sheep (37.5%) was lower than that for goats (41.7%) but this difference was not significant ($P=0.1$).

Out of the total of 257 faecal samples from the goats and sheep, only 3.9% (10) were liquid, 16.7% (43) were soft and 79.4% (204) were solid (Table 4.6). Infections with

C. parvum were significantly associated with faecal consistency (χ^2 , $P=0.03$) for sheep but not for goats (χ^2 , $P=0.4$)

Table 4.6 Prevalence of *Cryptosporidium parvum* in small ruminants according to faecal consistency [lambs (n= 152) and kids (n=105)]

Ruminant Specie	Liquid		Soft		Solid	
	n	No. positive (%)	n	No. positive (%)	n	No. positive (%)
Sheep	5	2 (40)	23	8 (34.8)	124	18 (14.5)
Goats	5	0	20	0	80	6 (7.5)

In sheep infections with *C. parvum* were in the age range of 1 to 3 weeks with majority of them being in lambs aged 3 weeks (Figure 4.6). In goats infections were in the age range of 1 to 4 weeks. For both sheep (χ^2 , $P=0.06$) and goats (χ^2 , $P=0.43$) age was however not significantly associated with infection.

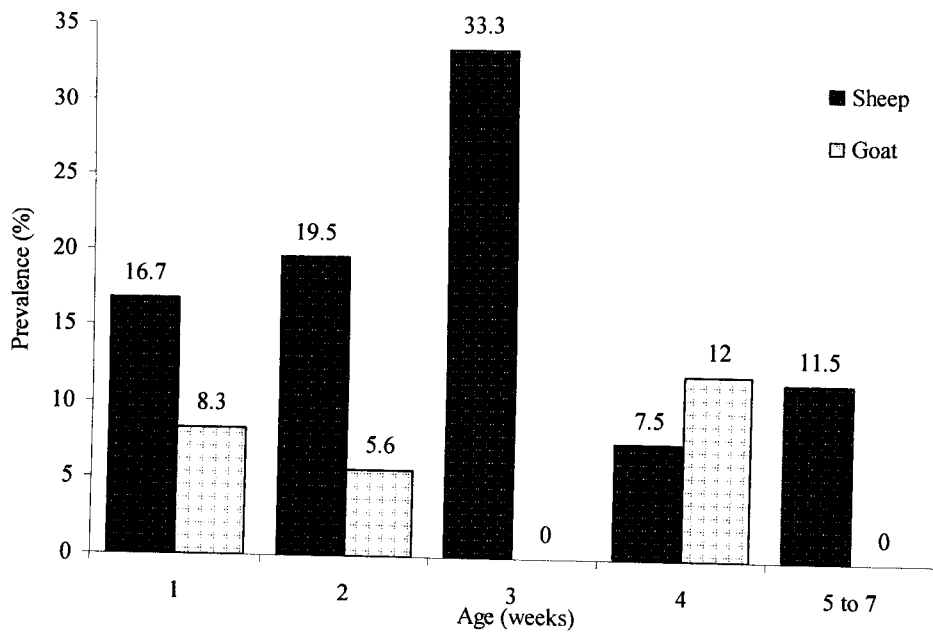


Figure 4.6 Prevalence of *Cryptosporidium parvum* in sheep (n=152) and goats (n= 105) according to age in weeks based on ELISA.

4.3 Sensitivity and specificity of modified Ziehl Neelsen acid fast staining technique compared to the ELISA (Techlab) technique

A total of 432 faecal samples from calves were tested using both the modified Ziehl Neelsen acid fast staining technique and the Techlab *Cryptosporidium* ELISA kit. A total of 58 specimens were positive on both microscopy and ELISA while 280 were negative when tested by both methods. The microscopic method had 52 false positive results using the ELISA as standard reference.

The sensitivity and specificity of the modified Ziehl Neelsen acid-fast staining technique using the ELISA as the reference method was 58% and 84.3%, respectively. The overall agreement of the microscopy and ELISA tests for oocyst

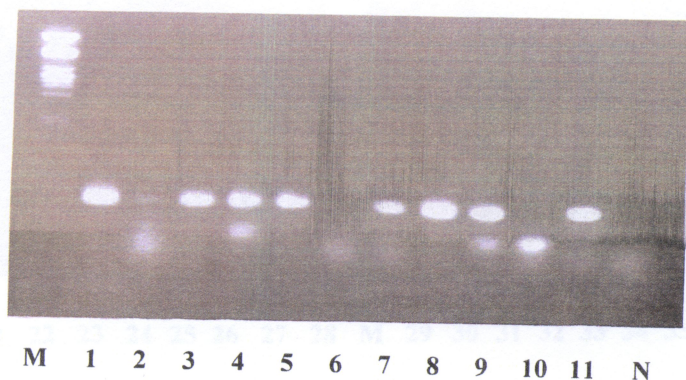
detection was 78.2% with a Kappa statistic of 0.41. The oocyst detection levels of the tests was significantly different (χ^2 , $P<0.0001$).

**4.4 Molecular characterization of the *Cryptosporidium* isolates by
Polymerase Chain Reaction amplification and sequencing**

Amplification products were obtained from 46 of the 55 samples (Table 4.7). Pictures of amplification products on 1.5% agarose gel stained with ethidium bromide can be seen in Figure 4.7.

Table 4.7 PCR amplification of 55 samples that tested positive on coproantigen ELISA from cattle (dairy, beef and traditional), sheep and goats

Cattle				Small Ruminants	
PCR Analysis	Dairy	Beef	Traditional	Sheep	Goats
Amplified	24	9	7	3	3
Not amplified	1	2	2	2	2
Total	25	11	9	5	5



(B)

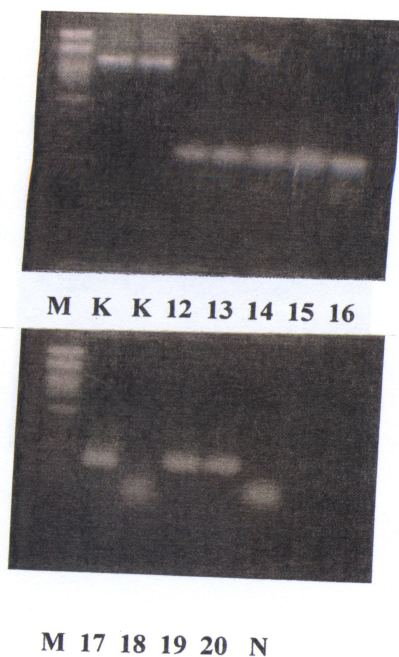
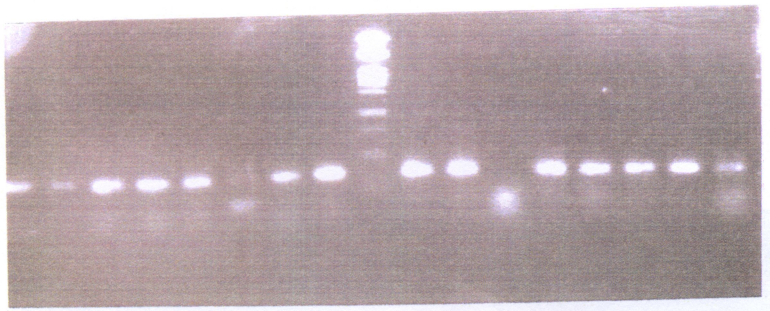


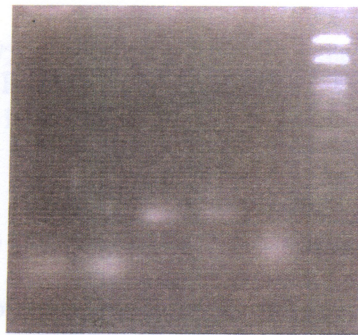
Figure 4.7 Detection of *Cryptosporidium* HSP-70 gene by PCR on 1.5% agarose gel. A) Lanes: M, molecular marker (100bp ladder); 1 to 11 isolates from beef cattle; N, negative control. B) Top row Lanes: M, molecular marker (100bp ladder) K, non *Cryptosporidium* isolate; 12 to 16 isolates from dairy cattle; Bottom row Lanes: M molecular marker (100bp ladder); 17 to 20 isolates from dairy cattle; N negative control.

(C)



21 22 23 24 25 26 27 28 M 29 30 31 32 33 34 35 36

(D)



37 38 39 40 N M

Figure 4.8 Detection of *Cryptosporidium* HSP-70 gene by PCR on 1.5% agarose gel. c) Lanes: M, molecular marker (100bp ladder); samples 21 to 28 left of marker and 29 to 36 right of marker isolates from dairy cattle. d) Lanes: 37 to 40 isolates from traditional cattle; N, negative control; M molecular marker (100bp ladder)

(E)

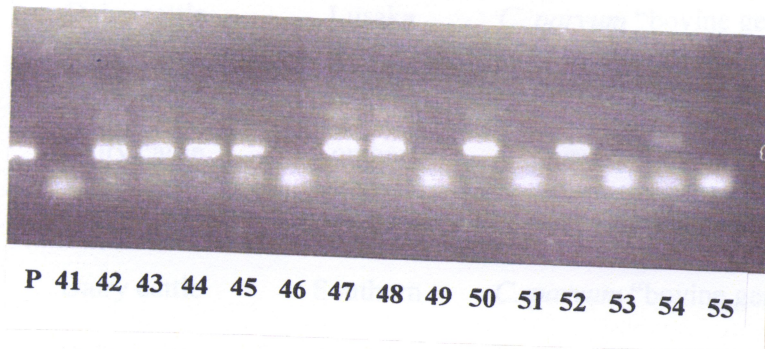


Figure 4.7 Detection of *Cryptosporidium* HSP-70 gene by PCR on 1.5% agarose gel. e) Lanes: P, positive control; 41 to 45 isolates from traditional cattle; 46 to 50 from sheep; 51 to 55 isolates from goats.

A sub-sample of 10 amplicons from cattle and sheep was sequenced for genotyping. Sequence analysis of the 325 bp region of the HSP-70 gene revealed 2 genotypes, the *C. parvum* “bovine genotype” and a novel “Cervid genotype” (see tale 4.8). Only partial sequencing of 108 bp out of 325 bp was achieved for sample 17 from a dairy calf. A weak match of 50-80 bp out of 108 bp was obtained, which showed the isolate to be of the “bovine genotype”.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Prevalence of *Cryptosporidium parvum* in cattle

The present study has revealed for the first time the presence of *C. parvum* infections in cattle in Zambia. This is evidenced by the prevalence found in dairy cattle of 42.8%, in beef cattle of 8.0% and in traditional reared cattle of 6.2%. The high prevalence found in the dairy calves indicates a serious problem particularly because the calves were randomly selected.

The prevalence of *C. parvum* recorded from the dairy sector in this study is similar to that observed in Spain by Castro-Hermida *et al.* (2002b) who examined 535 dairy calves of 3 weeks old using microscopy with acid fast staining and found a prevalence of 47.7%. Also in agreement with data from the present study are the findings of Santín *et al.* (2004) in the U.S.A who found a prevalence of 50.3% (n=503) in 2 months old dairy calves using an immunofluorescent staining procedure. The prevalence of *C. parvum* in dairy calves recorded in the present study is however, much higher than that observed in neighbouring Tanzania where a prevalence of 16.3% was found after examining 116 dairy calves of less than 3 months of age (Mtambo *et al.*, 1997). They used acid fast staining with microscopic examination for oocysts detection after an oocyst concentration technique was performed and all positive samples were confirmed with a coproantigen ELISA (Pathasure-Cambridge Veterinary Sciences, Littleport,UK). The reason for the difference in prevalence between the Tanzanian study and present one could be due to

sample size, spectrum of farms sampled, hygiene on farms and diagnostic technique employed.

Prevalence of *C. parvum* according to age in the dairy sector revealed that majority of infections were in calves aged between 2 and 3 week. Age was significantly associated with infection (χ^2 , $P= 0.02$). It is clearly shown that peak shedding of oocysts occurred in the second week of age, with a decline thereafter. These findings are similar to those reported by Santín *et al.* (2004) who observed that in pre-weaned dairy calves, the majority of those infected were aged between 1 to 3 weeks with a peak in the second week (O'Donoghue, 1995). The observation of peak oocyst shedding in the second week was attributed to most infections occurring in the first week after birth and is consistent with the life cycle of the organism, which needs 2 to 4 days from infection to shedding of oocysts in the faeces. The patent period (duration of oocysts excretion) varies from animal to animal from several days to several months (Fayer *et al.*, 1998). However, the most important factor determining the longevity of oocyst shedding is the immuno-competence of the animal (O'Donoghue, 1995).

In the beef husbandry system in the current study a prevalence of 8% was obtained, which was comparable to the 10.3% prevalence found in California in the USA in beef calves of less than one month raised on ranches after microscopic examination using Immunofluorescent staining (Atwill *et al.*, 1999a). In Canada, a cumulative prevalence of 5% was reported after examining 20 ranch beef calves (Ralston *et al.*, 2003). Although multiple samples were analysed in the study in Canada, the

prevalence was still lower than what was found in the present study. The reason for the lower prevalence could be the limited sample (20) and herd (1) size. On the other hand, very high prevalence of 48.5% after examining 459 beef calves using immunofluorescent staining has been reported in Spain by Castro-Hermida *et al.* (2002b). This high prevalence in the Spanish study unlike in the present study could be due to difference in management practices as the ELISA and Immunofluorescent staining diagnostic techniques (used in these two studies) have been found to be comparable (Kehl *et al.*, 1995). Most probably the Spanish beef calves in the study by Castro-Hermida *et al.* (2002b) were raised under semi-intensive or intensive management unlike those in Zambia, which were raised extensively on ranches. Shedding of *C. parvum* oocysts by beef calves in the current study started in the second week, with peak prevalence in the third week and age was significantly associated with infection (χ^2 , $P= 0.04$). In a study carried out by Atwill *et al.* (1999a), they found maximum prevalence in calves from beef herds at 2 months of age. They suggested that the delay in infection might have been due to passive immunity the calves acquired from their mother as the calves stayed with the dams for a longer period than those in the dairy sector. However, other studies have shown that passive immunity acquired through colostrums and maternal milk does not provide protection in calves and mice experimentally infected by *C. parvum* even though the milk does contain anti-cryptosporidial properties (Moon *et al.*, 1988; Harp *et al.*, 1990). The lack of infections in the first week in our study could have been due to delayed period of picking up the infection in these extensively managed calves as the dose of infection is lower because of dispersed oocysts in faeces over a larger area.

The prevalence of 6% obtained for traditional calves in this study can not be compared to any data, as this is the first report of *C. parvum* infections in traditionally reared cattle in Africa to the best of our knowledge. However, the low prevalence observed is as expected since these calves are raised under extensive conditions with communal grazing of expansive pastures. Prevalence by age for the traditional sector showed that peak infection occurred in the third week, which is similar to that observed in beef calves. However, age was not significantly associated with infection. The reason for the high infections in the third week could be similar to those for beef calves as both systems practice extensive management. The majority of infections were in the 2 to 4 weeks age range and this is in agreement with other researchers that cryptosporidiosis is more of a problem in neonates than adult cattle (Garber *et al.*, 1994; Harp *et al.*, 1996; Maldonado-Camargo *et al.*, 1998).

This study has shown that the dairy husbandry system had a significantly higher prevalence than the beef and traditional systems (χ^2 , $P < 0.001$), whereas there was no significant difference in prevalence between the beef and traditional husbandry systems. Statistical analysis using multivariate logistic regression showed that the dairy calves were 8.6 and 11.2 times more likely to have an infection than those in the beef or traditional husbandry systems. Few studies have been carried out to compare prevalence in different management systems, but generally studies in the dairy sector have shown higher prevalence levels than beef animals in ranches. The results of this study conform to the observations that there is higher prevalence levels

in the intensive (dairy) than the extensive (ranch beef and traditional) management systems (Atwill *et al.*, 1999a; Santín *et al.* 2004).

One of the major reasons for higher infection rates in intensively managed cattle compared to those extensively managed is greater stocking levels. The studies on the risk factors for *Cryptosporidium* infection in calves have shown that higher stocking increases the chances for *C. parvum* infection (Garber *et al.*, 1994, Atwill *et al.*, 1999b). This is because infected calves produce large numbers of oocysts (1×10^5 to 1×10^6) which in a limited area ensures high levels of environmental contamination. Heath, (1992) states that the management system that allows the convergence of a large number of animals and infectious agent predisposes to enteropathological infections. The higher prevalence in the dairy sector compared to the beef and traditional sectors in this study, is mainly due to the higher stocking densities. Whereas, the lower prevalence in the beef and traditional husbandry systems could be attributed to the lower infection pressure due to dispersion of oocysts over a larger area. It is possible that these sparsely distributed oocysts in the pastures could also have had limited infectivity as they were exposed to environmental stressors like direct sunlight, higher temperatures and drying which reduce viability (Walker *et al.*, 2001).

When Castro-Hermida *et al.*, (2002b) compared prevalence in intensively (45.9%) and semi-extensively (50.4%) managed calves, they found that there was no difference in prevalence in the two systems. Contrary to the results in the current study, a study in Trinidad and Tobago (Kaminjolo *et al.*, 1993) reported prevalence

of 7.7% (21/273), 18.2% (2/11) and 21.4% (3/14) in the intensive, semi-intensive and extensive management systems, respectively, in calves. The reason they put forward for these findings was the paucity of specimens obtained from extensive (16 of 683) and semi-intensive (118 of 683) husbandry systems, compared with 549 of 683 from the intensive husbandry system..

The association of infections with higher stocking levels is also supported by our findings when farms in the three systems were grouped into small (less than 50 animals), medium (51 to 150 animals) and large (above 151 animals). There was a significant difference among the three groups of farm sizes in the dairy (χ^2 , $P < 0.001$) and traditional (χ^2 , $P = 0.004$) husbandry systems with rates of infection increasing from the small to the large farms. The trend was more marked in the dairy husbandry system. This could have been due to continual housing of calves in a limited area as well as due to less hygienic conditions on the big farms as there are more calves to look after. This pattern of infection among small, medium and large-scale farms has also been observed by Gaber *et al.* (1994) and Mtambo *et al.* (1997). However, Castro-Hermidda *et al.* (2002b) in Spain found that smaller farms had a higher prevalence than medium and large scale farms. They stated that this could be due to the particular circumstances found in rural Galicia, where housing and hygiene conditions on large farms are better than those on smaller farms.

Difference in prevalence in the three husbandry systems could also have been due to the difference in the length of the calving season. Studies on the risk of infection with *C. parvum* in beef cattle have shown that the length of the calving season is

positively associated with the proportion of positive animals in a herd (Hoar *et al.*, 2001). Atwill *et al.* (1999b) also found similar results with the probability of shedding *C. parvum* oocysts increasing six to twelve-fold as duration of calving season for cows in the herd increased from 0 to a median of 60 days and up to year round. Therefore, long calving seasons provide a prolonged supply of susceptible animals (calves) thereby providing a suitable environment for infections to be sustained throughout the year, like is the case in dairy animals. In Zambia, most beef ranches have synchronized breeding with two major breeding seasons namely, the summer (August to October) and the winter (April-June) calving seasons. That means a susceptible population exists only for a limited period of time during the year and the possibility of these animals picking up the infection will depend on the environmental oocyst load shed by the adult animals. Thus, the lower prevalence in beef calves as compared to the dairy sector could have been due to the lower stocking levels and the limited susceptible population at risk because of a shorter calving season. In the traditional husbandry system on the other hand, there are calves born through out the year but the number of calves (population at risk) at any given time throughout the year, which does not favour propagation of the infection.

Analysis of the association between *C. parvum* infection and faecal consistency was performed in each husbandry system to determine whether the parasite could be contributing to calf-hood diarrhoea in the studied areas. It was found that faecal consistency was significantly associated with infection in the dairy sector (χ^2 , $P=0.02$). A prevalence of 52.4% for calves with liquid faeces was recorded, while for soft and solid faecal consistency it was 39% and 31%, respectively. This agrees with

other findings that prevalence is higher in diarrhoeic than in non-diarrhoeic calves (Quilez *et al.*, 1996; Lefay *et al.*, 2000). In a study of cryptosporidiosis in calves on one farm, it was observed that all of the 32 calves tested suffered from bouts of diarrhoea, which lasted between 5 and 12 days (Castro-Hermida *et al.*, 2002a). However, even though oocyst shedding has been associated with incidences of diarrhoea, it can occur in asymptomatic calves as reported by McCluskey *et al.* (1995) and as observed in the present study. It is difficult to say whether the calves that were positive with soft and solid faeces, in the present cross sectional study, were actually asymptomatic or had had diarrhoea during the earlier course of their infection and were in the process of recovery. It is also possible that some could have been shedding oocysts prior to the manifestation of the diarrhoea as was observed in 17 of the 32 calves sampled by Castro-Hermida *et al.* (2002a). Although other aetiological agents of calf enteropathies were not investigated in the present study, *C. parvum* can be considered as one of the contributors to neonatal diarrhoea complex in dairy farms in Zambia.

5.2 The risk factors in the dairy husbandry system

Under the general management factor, only type of farm (intensive or semi intensive) was found to be associated with infection. Farms with intensive management were 20.8 times more likely to have a calf infected with *C. parvum* than those under semi intensive management.

Maternity management factor results showed that there was no increased risk of infection whether calves were separated from cows at birth or not, or whether calves had access to calving area or not. Scott *et al.* (1995) investigating the prevalence of *C. parvum* in two herds of apparently healthy adult cattle on two farms found no relationship between the presence of oocyst-excreting adults and the development of infection in calves. These results suggest that there are other factors responsible for infections in the young calves than contact between calf and cow. On the other hand, Quigley *et al.* (1994) found that there was an increased risk of infection if calves were left to suckle from the cow while Mohammed *et al.* (1999) found that there was a decreased risk of *C. parvum* infection when calves were hand feed. The explanation put forward by Mohammed *et al.* (1999) for this observation was that hand feeding eliminates the contact between the calf and cow and the time the calf spends in the maternity area so that transmission of infection from the post-parturient dam is reduced. Analysis to determine whether feeding of different amounts of colostrum in the first 24 hours afterbirth had an association with infection revealed no association. This is in agreement with earlier reports that indicated feeding of colostrums does not reduce the risk of infection although colostrum does have anti-cryptosporidial properties (Moon *et al.*, 1988; Quigley *et al.*, 1994).

None of the factors examined under calf housing and hygiene were found to be significantly associated with infection. The type of housing, whether individual or group, was not found to be associated with prevalence of *C. parvum*. Whether these individual or group housed calves were kept inside or outside was also not associated with prevalence of *C. parvum*. Studies by Santín *et al.* (2004) in the U.S.A and

Maldonado-Camargo *et al.* (1998) in Mexico, agree with these findings that there is no clear association between housing and prevalence of *Cryptosporidium* infection. In contrast to these findings, Mohammed *et al.* (1999) in a study in dairy cattle in New York found that group housing animals increase the risk of infection with *C. parvum* because of increased contact between animals. The possible reason for lack of association between group or individual housing with infection in our study could be that despite calves being individually housed, contact between calves was possible. Furthermore, personnel cleaning and feeding the calves could have aided in transmission of the disease as they moved from pen to pen, irrespective of the type of housing.

The frequency of bedding removal and cleaning did not affect the prevalence of *C. parvum* infection. This is in contrast to a study by Garber *et al.* (1994) in the U.S.A who found that cleaning/disposal of beddings significantly decreased the risk of infection. The possible reason for the lack of association between infection and frequency of floor cleaning in our study could be due to the mode of cleaning. It was observed that most of these farms did not use high-pressured hose that would ensure removal of oocysts from the cracks/grooves in the floors. Flies have been implicated in the transportation of oocysts (Faubert and Litvinsky, 2000). A number of these dairy farms had problems in controlling flies, thus it is possible that flies could have been involved in the transmission of the infections from calf to calf.

5.3 Prevalence of *C. parvum* in small ruminants

Cryptosporidium parvum, which has emerged as one of the principle enteropathogens in lambs and kids in recent years, is present in the small ruminant population in Zambia. The prevalence obtained based on results of the antigen-ELISA (Techlab) analysis in lambs and kids were 18.4% (n=152) and 5.7% (n=105) respectively, with farm prevalence of 37.5% (n=16) in sheep and 41.7% (n=12) in goat farms.

The prevalence of *C. parvum* in lambs in the present study is similar to that observed by Olson *et al.* (1997b) of 23% when they examined 40 lambs of less than 6 months from 6 provinces in Canada using the microscopic method with immunofluorescent staining. Similar results of 14.7% prevalence have been reported in Spain, when they examined 2204 lambs from 97 randomly selected sheep farms (Matos-Fernandez *et al.*, 1993). More recently, Abd-El-Wahed (1999) in Egypt and Causapé *et al.* (2002) in northeastern Spain using the microscopy with an acid fast stain observed a much higher prevalence in lambs of 68.9% (n=120) and 59% (n=583), respectively. The explanation for the lower prevalence obtained in the present study could be due to differences in management where the intensive management tends to create favourable conditions for endemic infections. In Zambia, sheep are reared semi-extensively. The sheep are housed at night or put in paddocks, this creates higher stocking levels and increases contact between animals which favour transmission of the infection.

Cross sectional studies of infections with *C. parvum* in kids have revealed prevalence of 19% (n=42) and 58.5% (n=137) in Italy and France, respectively (Rossanigo *et al.*, 1987; Chartier *et al.*, 2002). These prevalence are much higher than that observed in the current study of 5.7%. The reason for the lower prevalence obtained in this study could be due to the semi-extensive management system practiced for the sampled goats which was similar to sheep.

Goats in Zambia prefer to browse than graze unlike sheep which tend to graze. It has been observed that goats are reluctant to graze or browse on vegetation which is below their necks from the ground (Nalubamba, 1996). This means that adult goats then pick up fewer oocysts from faeces thereby having a lower level of oocyst shedding, which in turn would cause less environmental contamination for the young kids. This feeding habit of goats could explain in part the lower prevalence of *C. parvum* infections in goats than sheep in the present study. On the other hand, the hardness of goats and greater resistance to disease could also account for their lower infection rates compared to sheep.

The other possible explanation for the lower prevalence in our study for both sheep and goats could be due to differences in herd size of the sampled animals, as it is significantly associated with infection (Ortega-Mora and Wright, 1994; Causapé *et al.*, 2002). From the lambs and kids sampled 3.3% and 4.8% presented with liquid faeces at the time of sampling, respectively. The small numbers of diarrhoeic animals at the time of sampling could also account for the lower prevalence observed because studies both in sheep and goats have shown that prevalence is higher in diarrhoeic

than non-diarrhoeic animals (Muñoz *et al.*, 1996; de Graaf *et al.*, 1999). Infections with *C. parvum* were significantly associated with faecal consistency only in lambs, where those with liquid, soft and solid faeces had 20%, 18.6% and 11.8% prevalence, respectively. It cannot be definitively concluded that *C. parvum* was causing the diarrhoea in lambs in the current study because the presence of other enteric pathogens was not investigated. However, these findings indicate that it is a contributor to diarrhoea in lambs in Zambia. In goats, all the positive samples were of solid faecal consistency and this is in agreement with other studies which have reported low rates of infection among non-diarrhoeic animals (Current, 1985; Sanford, 1982).

5.4 Sensitivity and specificity of Modified Ziehl Neelsen acid fast staining technique against the coproantigen ELISA (Techlab) technique

In this study the sensitivity and specificity of the microscopic method was tested against the coproantigen ELISA (Techlab). The Ziehl Neelsen acid-fast technique had a sensitivity and specificity of 58% and 84.3%, respectively when compared to the commercial Techlab *Cryptosporidium* ELISA kit with a kappa value of 0.41 ($P < 0.0001$). These results are difficult to compare to other studies because other studies evaluated different ELISA kits against microscopy with either an acid-fast staining technique or monoclonal antibody immunofluorescence staining technique. However from the manufacturer's users manual for the Techlab ELISA, it is reported that in a study carried out to determine the performance of the kit, the acid-fast microscopic method had a sensitivity and specificity of 97% and 76.4%. Similar results were found by Newman *et al.* (1993) when they evaluated a commercial

ELISA (LMD Laboratories, Carlsbad, California.) against microscopic examination using the modified acid fast and auramine stains after a concentration procedure. They obtained sensitivity and specificity of 98.2% and 94.8%, respectively with a Kappa value of 0.77. Our results on the other hand are comparable to those found by Mc Cluskey *et al.* (1995) when comparing the acid fast staining technique and a commercially produced ELISA (Pathasure Cryptosporidium Test kit, Innovet, West Palm Beach, FL, USA) on un-concentrated bovine faeces. They observed a sensitivity of 50.3% and specificity of 90.8% with a kappa value of 0.42 ($P < 0.01$). Kehl *et al.* (1995) found the sensitivity (96%) and specificity (99%) of the acid-fast technique comparable to two enzyme immunoassays (EIA), ProSpect Microtiter assay (Aleon, Inc., Mountain View, Calif.) and Color Vue Cryptosporidium assay (Seradyn, Indianapolis, Ind.) and an immunofluorescent assay (Meridian Diagnostics, Cincinnati, Ohio).

It can be seen from the above studies that the acid-fast staining technique is comparable to the immunologically based techniques when a concentration technique is employed on the faecal specimen prior to microscopic examination. This could explain the lower sensitivity and specificity observed in the current study, as no concentration technique was performed.

The kappa statistics is a measure of agreement of diagnostic tests with the agreement owing to chance alone removed. Kappa values greater than 0.75 are considered excellent, those from 0.4 to 0.75 intermediate to good and those less than 0.4 poor (Landis and Koch, 1977 cited by McCluskey *et al.*, 1995). The intermediate

agreement between the tests observed in our study is due to the high number of positives found on microscopy than ELISA. Presence of debris in the faeces (that could have picked up the stain) and experience of the microscopist could have lead to misinterpretation of the results in the microscopic method.

Faecal consistency has been found to affect the level of oocysts detection for both microscopy and immunologically based techniques. In a study by Waber *et al.* (1991) a sensitivity of 100% was observed on both the modified cold Kinyoun acid-fast staining technique and monoclonal antibody immunofluorescence technique when human faeces seeded with 10,000 oocysts per gram were concentrated and tested. The sensitivity of the two tests dropped drastically to 0% and 60% for the Modified cold Kinyoun staining technique and immunoflourescence technique when solid faeces of the same oocysts concentration were used. In diarrhoea the faeces are watery and almost devoid of debris, making oocysts detection easier. In the solid or formed faeces particles are compacted obscuring and binding to oocysts. In view of this it is possible that the prevalence of *Cryptosporidium* in ruminants in Zambian could have been under-estimated in the present study.

The ELISA in our study was used as the gold standard because not only has it got a high specificity and sensitivity, it is fast, easy to read and a number of samples can be done simultaneously. However, there could have been false negatives especially in faeces with low oocyst numbers because detection levels of immunological tests tend to decrease with low oocyst concentrations (Waber *et al.*, 1991; Ignatius *et al.*, 1997; Kvác *et al.*, 2003) leading to an underestimation of prevalence. Furthermore, a

single faecal sample analysis was carried out, which could have further led to under-estimation of prevalence. A single sample analysis has been observed to give lower rates than multiple sampling on alternative days (Fayer *et al.*, 2000).

5.5 Molecular characterization of the *Cryptosporidium* isolates

Cryptosporidium parvum “bovine genotype” (referred to as *C. parvum* by others), was the most common genotype identified and was found both in cattle and sheep samples. These findings are in agreement with other studies that have found *C. parvum* “bovine genotype” to be the predominant species in calves of less than 2 months (Beacher *et al.*, 2004; Santin *et al.*, 2004). Another genotype *Cryptosporidium* “Bovine genotype B” that has been found in pre and post weaned cattle in other studies (Xiao *et al.*, 2002; Santin *et al.*, 2004), was not identified in the sequenced isolates in the present study. This could be because this genotype is less prevalent than *C. parvum* “Bovine genotype” (Xiao *et al.*, 2002). According to Xiao *et al.* (2002), it is possible that immunity induced by *C. parvum* “bovine genotype” has limited the prevalence of *Cryptosporidium* bovine genotype B in cattle populations. The other reason for the absence of this genotype in this study could be due to the limited number of samples that were sequenced. *Cryptosporidium* bovine genotype B is thought to be a cattle genotype like *C. hominis* is in humans.

The two sheep isolates in the present study were both found to be *C. parvum* “bovine genotype”. This is in agreement with other workers who have found neonatal lambs and kids (like calves) to be commonly infected by *C. parvum* “bovine genotype”

(Fayer *et al.*, 1997). Although no isolates from goats were sequenced at the time of writing this dissertation, it is most likely that they were infected by *C. parvum* genotype as the goats in this study lived in close proximity to cattle, making it most likely that they were infected by the same genotype.

The identification of *Cryptosporidium* Cervid genotype in 2 calves in the dairy and traditional husbandry systems is of particular interest as this is, to the best of our knowledge, the first report of this genotype in cattle. The Cervid genotype was first identified by Xiao *et al.* (2000b) in storm water samples from lower New York State. In the following year, Perez and Le Blancq (2001) identified it in white tailed deer-derived isolates and referred to it as genotype 3. Subsequently, the Cervid genotype has been identified in humans in Canada (Ong *et al.*, 2002), in a lemur in the USA (da Silva *et al.*, 2003) and more recently in a blesbok, sheep and a nyala in the Czech Republic (Ryan *et al.*, 2004). Ryan *et al.* (2004) states that morphologically the Cervid genotype is similar to *C. parvum* but genotypically it is very distinct sharing only 92.7 to 94.2% similarity at the HSP-70 locus.

In North America, the livestock industry especially the dairy has been incriminated in some waterborne outbreaks in humans. However, there is lack of conclusive evidence to support this claim (Peng *et al.*, 1997). In Europe on the other hand zoonotic sources of infection involving livestock appear to be more common in water-borne outbreaks (Becher *et al.*, 2004). In Africa and Zambia in particular, it is not known if zoonotic transmission of *Cryptosporidium* does occur and to what extent. The identification of the bovine genotype in this study is important in that, it

is zoonotic and can cause life threatening diarrhoea in immuno-compromised individuals especially those with HIV/AIDS and children less than 5 years of age. Given the poor state of sanitation and poor quality of drinking water in many parts of Zambia, there is a possible threat of transmission of the parasite from animals to humans through food and water. The country has also got a high prevalence of HIV/AIDS that could make diarrhoea caused by *C. parvum* life threatening in many individuals.

5.6 Conclusion and recommendations

This study has shown that *Cryptosporidium parvum* is prevalent in neonatal cattle, sheep and goats in Zambia. To the best of our knowledge this study is the first report of *C. parvum* infections in traditional cattle and small ruminants in Africa. The observed prevalence was highest in the dairy calves and lambs and lowest in traditional calves and kids. Furthermore, the intensive dairy husbandry system had a significantly higher prevalence than the extensive beef and traditional husbandry systems. The big farms showed higher prevalence than the small farms. Liquid faeces were associated with infection in lambs and dairy calves, thus *C. parvum* can be considered to be an important contributor to neonatal diarrhoea complex in these species. Molecular characterization showed that the majority of samples were *C. parvum* “bovine genotype” which is of public health significance. This study reports for the first time in cattle in Africa, the occurrence of the Cervid genotype. The identification of this genotype in a variety of hosts including humans indicates that it could also be of public health importance. It was also determined that the

microscopic method had a low specificity and sensitivity compared to the coproantigen ELISA (Techlab).

Based on the findings of this study the following recommendations are made:

- It is recommended that more studies be carried out to determine the prevalence of *C. parvum* in livestock in Africa and in Zambia in particular, and the importance of the parasite as an aetiological agent of neonatal diarrhoea.
- Further genotyping of isolates of *Cryptosporidium* in ruminants in Zambia is needed as genotyping in the present study was carried out on only a limited number of samples.
- It is also recommended that further studies be conducted to find out the relationship of the parasite between humans and animals in Zambia especially in the dairy industry.
- The high prevalence of *Cryptosporidium* in dairy calves and lambs is a serious risk of infection for humans especially those with HIV/AIDS. It is recommended that animal handlers particularly those on dairy farms, be educated on the possible dangers of infections from calves.

- Although ground water generally is less contaminated than surface water, it is possible that it could be contaminated through *C. parvum* oocysts filtering through the soil from ruminant faeces. An assessment of the borehole water on the large dairy farms is advisable to find out if contamination of the underground water is occurring
- Control measures involving good management and hygiene practices should target animals 1 to 4 weeks of age, as infections are concentrated in this age range. Furthermore supportive treatment should be given to sick neonates. Control of flies and rodents could be of some value as these have been implicated in the transmission of the infection.

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APPENDICES

APPENDIX: 1

ESTIMATED RUMINANT POPULATIONS IN ZAMBIA FROM 1995 TO 2000

Ruminant	1995	1997	2000
Cattle			
Commercial dairy	66,700	70,000	-
Commercial beef	926,00	970,000	927,829
Traditional	1,649,000	1,700,000	1,831,022
Total	2,642,200	2,740,000	2,620,987
Sheep	57,242	84,284	61,268
Goats	565,886	722,072	886,987

Source: Ministry of Agriculture and Co-operatives, National Epidemiology and information unit.

APPENDIX: 2

QUESTIONNAIRE: INVESTIGATION OF CRYPTOSPORIDIUM IN CATTLE

Farm Owner: _____ Date: _____

Farm Name: _____

District: _____

A. HERD COMPOSITION

1.) What types of animals are kept on the farm?

☐ Cattle ☐ Goats ☐ Sheep ☐ Poultry ☐ Pigs ☐ Dogs and Cats

2.) What type of production system is it? ☐ Beef ☐ Dairy ☐ Mixed

3.) Total Number of animals _____

a. Neonates (< 3 month) _____ b. Adult ____ pregnant cows

B. MANAGEMENT/HUSBANDRY

1.) Breed: _____

2.) Type of husbandry:

☐ Intensive ☐ Semi-intensive ☐ Extensive ☐ Free range ☐ Tethering

3.) Type of housing for

	Neonates (<3months)	Weaners	Adults (parturition area)
Individual housing	outside / inside	outside / inside	outside / inside
Group housing	outside / inside	outside / inside	outside / inside

3.) Do calves have access to the parturition area? ☐ Yes ☐ No

6.) How often is: the bedding removed or floor cleaned?

Daily ☐ Several times/week ☐ Weekly ☐ Monthly ☐ Less than Monthly.

7.) Are the neonates immediately separated from the dam?

☐ Yes

☐ No

8. If yes to question 7, how long do they stay with the dam?

☐ 24hrs

☐ 48hrs

☐ 72hrs

☐ More

9. How much colostrum do the neonates receive in the first 24 hours?

☐ 1L

2 ☐ L

☐ 3L

☐ 4L

10. What is the drinking water source?

	Neonates (< 1month)	Weaners	Adults
Well/Borehole water	☐	☐	☐
Dam water	☐	☐	☐
Stream/River water	☐	☐	☐

C. DISEASE

1. What is the major disease encountered in your calves < 1month?

☐ Diarrhoea ☐ Weight loss ☐ Respiratory diseases ☐ Skin disease ☐ Other

2. What treatment course do you follow?

☐ Do nothing ☐ Medication ☐ Call Veterinarian ☐ Other (specify)

APPENDIX: 3

MODIFIED ZIEHL NEELSEN ACID FAST STAIN

A. REAGENTS

Fixing solvent

Methanol (absolute)

Carbofuschin

Basic Carbofuschin 1g

Absolute Alcohol 10mls

5 mls Phenol in distilled water 100mls

The Carbofuschin was dissolved in the alcohol and the phenol in the distilled water was added.

Decolourizer

Ethanol (95%) 97mls

Concentrated Hydrochloric acid (HCl) 3mls

The Hydrochloric acid was added to the ethanol slowly.

Counterstain

Methylene blue 0.3g

Distilled water 100mls

The methylene blue powder was dissolved in the distilled water.

B. SMEAR PREPARATION AND STAINING PROCEDURE

A smear was made with a splinter on a frosted glass slide, and then the following steps were performed:

- Fixing in Methanol for 2 minutes.
- Staining in Carbofuschin for 5 to 10 minutes.
- Washing with tap water.
- Decolourizing with the acid-alcohol.
- Counter stained with Methylene blue for 30 seconds.

APPENDIX 4

**VARIBLY STAINED OOCYSTS OF CRYPTOSPORIDIUM FROM DIARY
CALVES**

