Epidemiological Studies of Cryptosporidiosis

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ABSTRACT

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An interpretive overview of the literature on intestinal cryptosporidiosis in humans and domestic mammals (Chapter 1) is followed by two studies of the population genetic structure of the protozoan parasites *Cryptosporidium parvum* and *Cryptosporidium hominis* (Chapter 2), five epidemiological studies of cryptosporidiosis in foals, calves and humans in New Zealand (Chapter 3), and an investigation of a serendipitous outbreak of cryptosporidiosis among a class of veterinary students, which occurred at the end of 2006 (Chapter 4).

The analysis of the population genetic structure of *C. parvum* and *C. hominis* indicates the existence of a significant genetic segregation of geographically separated parasite populations, consistent with allopatry. The results do not conform to a simplistic model that considers all *C. parvum* as multi-host anthropozoonotic agents, and provide statistical support to the idea of the occurrence of anthroponotic cycles that do not involve cattle. Rather than conforming to a rigid paradigm of either a clonal or a panmictic species, data are consistent with the co-occurrence of clonal and recombinatorial diversification in *C. hominis*, and perhaps *C. parvum*.

The results of the epidemiological studies in New Zealand suggest cryptosporidiosis caused by *C. parvum* is relatively common in young foals and calves. In the time and space frames underlying these studies, humans, calves, and foals were infected with a genetically homogeneous *C. parvum* population. This feature is in accordance with previous reports that have indicated *C. parvum* as the dominant species in humans during the peaks of incidence of cryptosporidiosis in winter and spring, and support the view that the peaks are in large part attributable to direct and/or indirect zoonotic transmission of *C. parvum*.

Finally, the outbreak of cryptosporidiosis among a class of veterinary students highlighted the potential hazard for explosive large-scale outbreaks in New Zealand. The results of the investigation were consistent with point-source exposure and zoonotic transmission of a rare *C. parvum* subtype through direct contact with calves during a practicum.

PREFACE

With the advent of HIV-AIDS and the re-emergence of the importance of infectious diseases at the end of the 20th century, several microorganisms not previously known to cause disease, including members of the genus *Cryptosporidium*, were recognised as novel aetiological agents in humans and animals. In the inaugural issue of the journal *Emerging Infectious Diseases* in 1995, Dr. David Satcher, Director of the US Centers for Disease Control and Prevention, included *Cryptosporidium* in a group of microorganisms which in his opinion were the "major etiologic agents" identified since 1973.

Cryptosporidium is a genus of protozoan parasites first described in animals in 1907. Despite the early description of these parasites, cryptosporidiosis, that is, the disease caused by members of the genus, was initially described in 1971 in a heifer and then in 1976 in a 3-year old girl from a farm, both in the US. The most common clinical presentation of cryptosporidiosis in developed countries is of self-limiting diarrhoeal illness. However, in HIV-positive or otherwise immunocompromised patients, cryptosporidiosis may manifest as a chronic debilitating or fulminant disease. Conversely, in many developing regions of the world the infections with *Cryptosporidium* are associated with persistent childhood diarrhoea, high mortality, malnourishment, and stunted growth.

Cryptosporidiosis is a notifiable disease In New Zealand. According to a recent Public Health Surveillance Report, the rate of notifications of cryptosporidiosis in the trimester October-December 2008 was of >2 cases per 10,000 population, similar to the rate of salmonellosis and giardiosis over the same period (http://www.surv.esr.cri.nz/PDF_surveillance/, accessed April 2009). In addition to the endemism of cryptosporidiosis, *Cryptosporidium* parasites pose a significant hazard due to the resistance of the oocysts to chlorination and the potential to cause massive water-borne disease outbreaks, such as the epidemic in Milwaukee, Wisconsin in 1993, when an estimate of 400,000 people acquired an infection through the ingestion of contaminated municipal water supply. Thus, ensuring preparedness against outbreaks of cryptosporidiosis is critical.

Many *Cryptosporidium* taxa can infect both humans and animals, and infections in humans are often acquired zoonotically, through direct or indirect contact with the faeces of infected animals. Thus, understanding the biology and epidemiology of cryptosporidiosis in different host species is important, to both enhance the health of animals, and also to devise strategies for the control of the zoonotic spread of the parasites.

This PhD thesis includes an interpretive overview of the relevant literature, and accounts of eight epidemiological studies of *Cryptosporidium* infections in humans and animals undertaken between 2002 and 2007 by the author. A simple search in Medline between 1995-2008 using the keyword '*Cryptosporidium*' returned 2343 citations. Therefore, the overview of the literature

reports only those articles that in the author's view shaped the thinking in each particular area; however, further articles are cited in the sections for the individual studies. Six of the eight studies have been published in peer-reviewed journals. To integrate the individual studies into the whole picture and avoid corrupting the published manuscripts, each study is preceded by an Introduction. Some successive studies have been published in different years. Thus, the literature cited may differ between studies, mainly as a reflection of the progressive accumulation of publications on the same topics. The published manuscripts have been slightly modified. Modifications included the addition of cross-references and material and methods that - for conciseness - could not be included in the published manuscripts. In addition, the bibliography was re-formatted according to the requirements of the *New Zealand Veterinary Journal* and the relevant raw data were included in Appendices.

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The names of friends and colleagues who helped me to perform the studies have been included in the list of authors of the relevant published papers. Prof. Andy Tait, Wellcome Centre for Molecular Parasitology, Glasgow, United Kingdom, generously donated the raw data used in the study presented in Section 2.1. Prof. Anne Chao, National Tsing Hua University, Taiwan, calculated the 95% confidence intervals of the Chao1 and ACE1 richness estimates in the same study. The molecular characterisation of the *Cryptosporidium* isolates used in the study presented in Section 2.2 was performed by Dr. Sultan Tanriverdi, Division of Infectious Diseases, Cummings School of Veterinary Medicine, Tufts University, MA, USA. The studies performed in Chapters 3 and 4 utilised several molecular methods developed over the years at the Protozoa Research Unit, Massey University, Palmerston North, and compiled in the *Manual of Methods for Genotyping Cryptosporidium Oocysts from Faecal Specimens*, written in November 2002 by Mrs Kim Ebbett. Mr. Errol Kwan, Anthony Pita and the late Jim Learmonth performed some laboratory analyses reported in Sections 3.1-3.4. Assistance in the laboratory was also provided by Mr. Yi Shi, Animal Health Monitoring and Disease Prevention Unit, Urumqi City, Xinjiang, People's Republic of China, during his stay in New Zealand as a visiting scholar under te author's supervision in 2007-2008. Other people to whom I am very grateful are Mr. Graham Young, former bacteriologist at Gribbles Diagnostic Laboratories, Hamilton, Jan Bird, Microbiology Section Leader of Path Lab Waikato, and Chris Pickett, Hamilton Medical Laboratory, for providing *Cryptosporidium*-positive specimens from cattle and humans. Dr. Isobel Gibson, New Zealand Veterinary Pathology Ltd., kindly provided *Cryptosporidium*-positive specimens from foals. The sympathetic New Zealand farmers who allowed me to take samples from animals under their care and the veterinary students who provided faecal specimens and responded to the questionnaires reported in Chapter 4 are also thanked. Finally, I thank Prof. Saul Tzipori and the staff of the Division of Infectious Diseases, Cummings School of Veterinary Medicine, Tufts University, for hosting me for two months in 2008.

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For my beloved wife, Vicky

not by bread alone does man survive

Deuteronomy 8:3

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LIST OF ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
BLST	Bilocus sequence type
bp	base-pairs
CI	confidence interval/confidence limit
CIN	cefsulodin, irgasan and novobiocin agar
COWP	*Cryptosporidium* oocyst wall protein
GP60	*Cryptosporidium* surface GP45/15 glycoprotein (or 60-kDA glycoprotein)
HAART	Highly Active Antiretroviral Therapy
HIV	human immunodeficiency virus
HSP70	70 kDa Heat Shock Protein gene
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IVABS	Institute of Veterinary, Animal and Biomedical Sciences
LATU	Large Animal Teaching Unit
MLG	multilocus genotype
MU	Massey University
OPG	oocysts per gram of faeces
PAS	periodic acid-Schiff stain
PCR	polymerase chain reaction
poly T	polythreonine repeat
RFLP	restriction fragment length polymorphism
RNR	*ribonuclease reductase*
SIA	standardized index of association
SNP	single nucleotide polymorphism
UPGMA	Unweighted Pair Group Method with Arithmetic mean
UV	ultra violet
XLD	Xylose Lysine-dehoxycolate
ZN	Ziehl Neelsen
18S rRNA	small subunit 18S ribosomal RNA

CHAPTER 1

INTESTINAL CRYPTOSPORIDIOSIS IN HUMANS AND DOMESTIC MAMMALS: AN INTERPRETIVE OVERVIEW

1.1 CRYPTOSPORIDIOSIS IN THE HISTORICAL PERSPECTIVE

Cryptosporidium is a genus of protozoan parasites of amphibians, fish, reptiles, birds, mammals, and humans, first described by Ernest Edward Tyzzer (1875–1965) in 1907 (Tyzzer 1907). Using light microscopy, this eminent infectious disease researcher at Harvard University, Massachussetts, observed coccidian-like parasites in the gastric and intestinal cells of mice, which he then named *Cryptosporidium muris* and *Cryptosporidium parvum*, respectively (Tyzzer 1910, 1912). He proposed the name *Cryptosporidium* (from Greek κρυπτός, kryptos: hidden) because sporocysts were not seen in the oocysts (Tyzzer 1910).

Despite the early recognition of these new parasites by Tyzzer, the first description of the disease cryptosporidiosis in any host was recorded about 60 years later in a calf (Panciera 1971), and later in a 3 year-old girl from a farm who had symptoms of abdominal pain and diarrhoea (Nime et al. 1976). Until the 1980s *Cryptosporidium* parasites were not considered to be important pathogens in immunocompetent humans (Bird and Smith 1980), as cryptosporidiosis was mostly seen in immunodeficient patients. This was due to the fact that the diagnosis depended upon histopathological examination of intestinal tissues, a procedure that was performed only in the most severe cases (Meisel et al. 1976). With the emergence of the HIV-AIDS epidemic in the 1980s, histopathological tests were required more frequently to diagnose the disease in patients affected with AIDS (Casemore et al. 1984ab, 1985ab). Eventually, simple methods for the microscopic detection of the oocysts in faeces, for example those based on the cold Ziehl Neelsen staining of faecal smears, were adopted by human diagnostic laboratories (Garcia et al. 1983; Casemore et al. 1984ab). Interestingly, these methods were first devised for the diagnosis of cryptosporidiosis in calves (Pohlenz et al. 1978; Henriksen and Pohlenz 1981). The introduction of such simple, non-invasive and cost effective diagnostic procedures allowed cryptosporidiosis to also be included in the differential diagnosis of infectious diarrhoea in immunocompetent humans and in calves. Consequently, the rate of identification of *Cryptosporidium* increased, to eventually become one of the most common agents identified from cases of diarrhoea in humans and calves in more than 100 countries (reviewed by Fayer 2008).

The interest in *Cryptosporidium* research increased significantly after the realisation that these parasites can cause explosive water-borne diarrhoeal disease epidemics. The Milwaukee, Wisconsin, epidemic in 1993, in which more than 400,000 people are believed to have acquired the agent from a single drinking water plant source (MacKenzie et al. 1994), was a particular event that focused the attention on *Cryptosporidium*. This, and other outbreaks, prompted the

Director of the US Centers for Disease Control and Prevention to include *Cryptosporidium* in the list of agents defined by him as "major etiologic agents identified since 1973" (Satcher 1995).

1.2 IMPACT OF CRYPTOSPORIDIOSIS ON HUMAN AND ANIMAL HEALTH

The most common clinical presentation of cryptosporidiosis in immunocompetent patients in developed countries is of self-limiting diarrhoea. Some exceptional cases of gastrointestinal illness lasting up to four months have been described (Hunter and Nichols 2002; Warren and Guerrant 2008). Subclinical carriage of *Cryptosporidium* has been reported in humans (Siwila et al. 2007), and immunocompetent individuals can excrete oocysts in the faeces for up to seven weeks after infection (Steher-Green et al. 1987). However, in HIV-AIDS or otherwise immunocompromised patients cryptosporidiosis may be a chronic debilitating, or even a fulminant disease, and these patients might also suffer from extraintestinal infections (Chappell and Okhuysen 2002; Hunter and Nichols 2002; Tzipori and Ward 2002; Huang et al. 2004).

Specific anti-*Cryptosporidium* treatments are not available. Thus, cryptosporidosis was, until recently, a major debilitating disease and cause of mortality in HIV-positive patients. Encouragingly, the recent introduction of Highly Active Antiretroviral Therapy (HAART) protocols decreased the incidence, severity and mortality of cryptosporidiosis as well as other opportunistic infections in these patients (Maggi et al. 2000; Miao et al. 2000; Pozio and Morales 2005). However, the burden of cryptosporidiosis remains serious in immunosuppressed patients in countries that cannot afford HAART (Tzipori and Widmer 2008).

In many developing regions of the world cryptosporidiosis is associated with persistent childhood diarrhoea, malnourishment, stunted growth and high mortality (these aspects of human cryptosporidiosis have been reviewed by Dillingham et al. 2002, and Snelling et al. 2007). The reasons for the difference in the spectrum of severity of cryptosporidiosis between developed and developing regions are not understood because multifactorial epidemiological comparisons between regions are lacking. The higher prevalence of HIV-positive people in some developing regions could increase the overall risk of chronic cryptosporidiosis as compared with regions with lower HIV prevalence. For example, 67/76 (88%) of *Cryptosporidium*-positive children admitted with chronic diarrhoea to Mulago hospital, Kampala, Uganda, were HIV-positive (Tumwine et al. 2005). The cycle of malnourishment leading to immunosuppression could also explain the difference in the spectrum of severity of cryptosporidiosis between developing and developed regions. However, the effect of malnourishment is difficult to assess as recent observations in animal models indicate that malnourishment might be an effect, rather than a cause of chronic cryptosporidiosis (Coutinho et al. 2008). In one epidemiological study in Bangladesh, there was a significant difference in the IgA and IgM levels in patients with persistent diarrhea caused by *C. parvum*, as compared with those with acute diarrhea, which had greater IgA and IgM levels. The authors hypothesised

that a diminished mucosal IgA response may contribute to the persistence of the infections (Khan et al. 2004).

A high incidence of *Cryptosporidium* superinfections, as expected in heavily contaminated environments, can also provide a plausible explanation for the high rate of chronic infections observed in some regions where hygiene is poor. Indeed, as will be seen later in this overview, *Cryptosporidium* may diversify by recombination in the gastrointestinal tract of the host. In theory, superinfections with genetically heterogeneous parasites may facilitate the emergence of recombinant parasites, which may differ antigenically from their parental cells, a feature that could enable the evasion of the immune response of the host and enhance chronic infections. The study presented in Section 2.2 explores the rate of recombination in natural *C. parvum* and *C. hominis* populations.

Studies addressing the degree of protection conferred by previous exposure against re-infection with *Cryptosporidium* parasites in humans provided conflicting results. In one study with human volunteers, Okhuysen and colleagues (1999) found a partial resistance to re-infection in previously exposed persons. Conversely, in a different study, volunteers with pre-existing anti-*Cryptosporidium* antibodies shed less oocysts in the faeces than the seronegative, but on the other hand manifested a more severe disease (Chappell et al. 1999). It is plausible that the immune response developed in the course of cryptosporidiosis is stronger against homologous than heterologous species, or genotypes. This idea has been recently corroborated by Sheoran and colleagues (2008). These researchers showed that the immunity developed against *C. hominis* provided only partial protection against subsequent *C. parvum* infection in the gnotobiotic pig model. The effect of previous exposure on the susceptibility to *C. parvum* infection will be further explored in the study reported in Chapter 4.

A wide range of *Cryptosporidium* taxa have been identified in many host species (reviewed by O'Donoghue 1995 and Fayer 2008). However, so far a causative role in disease has been established only for *C. parvum* in calves and to a lesser extent lambs and kids, and perhaps for *Cryptosporidium andersoni* in juvenile and adult cattle.

Cryptosporidium parvum is an important causative agent of neonatal calf diarrhoea worldwide. Causality in calves has been confirmed by numerous observational studies, experimental infections, and therapeutic trials (Pohlenz et al. 1978; Tzipori et al. 1980b, 1983; Fayer and Ellis 1993; Naciri et al. 1993,1999; O'Handley et al. 1999; Castro-Hermida et al. 2002b; Grinberg et al. 2002; Sevinc et al. 2003). Significantly, results of recent molecular epidemiological studies indicated that *Cryptosporidium bovis*, which was described in 2005 (Fayer et al. 2005), is also widespread in young cattle (Fayer et al. 2007; Feng et al. 2007). The finding that *C. bovis* is highly prevalent in cattle is of considerable public health relevance, as this parasite, which is phenotypically-similar and thus, easily confused with *C. parvum*, has never been identifierd from

clinically overt infections in cattle or humans, and is therefore not considered either pathogenic or zoonotic.

Cryptosporidium andersoni infects the gastric glands of juvenile and adult cattle causing mild lesions of an uncertain pathogenic significance (see Section 1.6). It produces oocysts different from *C. parvum* in shape and size (Lindsay et al. 2000), and has been reported once in humans, in an HIV-positive person (Guyot et al. 2001).

1.3 THE TAXONOMIC CLASSIFICATION OF GENUS *CRYPTOSPORIDIUM*

There is lack of consensus on what constitutes a *Cryptosporidium* taxon. Thus, in this overview the specific terms of 'species' or 'genotype' will be used for those taxa which in the author`s opinion are widely accepted as such by the scientific community. Other *Cryptosporidium* genetic variants will be referred to by using the term 'taxon'. The term 'isolate' will be used to describe any *Cryptosporidium*-positive faecal specimen from an individual human or animal host, or genomic DNA extracted from such specimens.

Cryptosporidium are intracellular protozoan parasites that can be classified biologically as either intestinal (that is, parasites with a biological cycle that is completed in the cells of the intestinal tract of the host), or gastric (where the cycle is completed in the gastric cells) taxa. Some authors have indicated that there is high phylogenetic relatedness between taxa within each group, regardless of the host-range (Xiao and Ryan 2008). This highlights the shortcomings of the nomenclature based on the host species instigated by Tyzzer when proposing the name *C. muris* to the parasites he found in mice (Tyzzer 1910, 1912) and widely used in the 1970s (Barker and Carbonell 1974; Tzipori 1988). The sequencing of the genomes of *C. parvum* and *C. hominis*, the major intestinal *Cryptosporidium* of man and animals, has been completed (Abrahamsen et al. 2004; Xu et al. 2004), and that of the gastric *C. muris* is still in progress. According to some researchers, the completion of the sequencing of *C. muris* will represent a major advance, as it will enable the genomic comparison between the diverging gastric and intestinal species (Tzipori and Widmer 2008).

Many opinion leaders endorse the taxonomy indicating *Cryptosporidium* as a genus within the Phylum Apicomplexa, Class Coccidea, Order Eucoccidiorida, Family Cryptosporidiidae (Tzipori and Ward 2002; Fayer 2008). However, based on the nucleotide sequence of the small subunit ribosomal RNA gene, *Cryptosporidium* and the Gregarina appear to form a single clade, which is separated from the coccidia and the other members of the Phylum Apicomplexa (Carreno et al. 1999). In agreement with the apparent phylogenetic separation of *Cryptosporidium* from the coccidia, there are conspicuous phenotypic differences between these taxa, which had been reviewed by Barta (2007). These are the epicellular localization of *Cryptosporidium* within the cells, the apparent lack of a plastid, the endogenous sporulation, resistance to folate pathway antimicrobials, and lack of susceptibility to anticoccidial chemotherapeutic agents.

As said, in the 1970s the taxonomic nomenclature within the genus *Cryptosporidium* was largely based on the host-species from which the oocysts were recovered. However, as the oocysts of many intestinal *Cryptosporidium* are morphologically similar, and in many instances no host-specificity was observed, all intestinal cryptosporidium were subsequently considered to belong to a single species named *C. parvum* (Tzipori et al. 1980a). This view started to change in the 1990s, when the first papers reporting on the existence of genetic heterogeneity in *C. parvum* were published. Currently, the taxonomic classification within the genus *Cryptosporidium* is mainly based on the genotype, rather than on phenotype.

The first report of genetic heterogeneity in *C. parvum* isolates found in the literature was published in 1991. The paper described a study performed using southern blot hybridization of chromosomal restriction endonuclease digests (Ortega et al. 1991). The first application of the PCR technique to amplify *Cryptosporidium* gene sequences was published in the same year (Laxer et al. 1991). This work demonstrated the feasibility of using the PCR technique for *Cryptosporidium* research, which eventually resulted in the discovery of numerous genetic variants. The small subunit ribosomal RNA (18S rRNA) gene sequence is currently the most popular locus used for the taxonomic typing of *Cryptosporidium* isolates. In 1996, Zamani et al. (1996) indicated that the 18S rRNA gene of *C. parvum* is present in one heterogeneous and four identical copies in the genome. Heterogeneous copies of the 18S rRNA gene seem to occur also in other *Cryptosporidium* taxa, such as *Cryptosporidium felis* (Xiao et al. 1999). This locus is particularly useful for taxonomic typing, as it comprises a species-specific region. In addition, it is present in four identical copies in the genome of *C. parvum* and *C. hominis,* which should - at least in theory - enhance PCR sensitivity (Pevsner 2003; Riley 2004). Therefore, the 18S rRNA gene is usually among the first to be characterised when new *Cryptosporidium* parasites are found, and Caccio and colleagues (2005) recommended the inclusion of this gene in any molecular identification scheme for *Cryptosporidium*. Other coding or non-coding genes, such as the β-tubulin gene, the *Cryptosporidium* oocyst wall protein (COWP) gene, a polythreonin repeat (poly T), a thrombospondin-related protein, the *ribonuclease reductase* (RNR) gene and the so-called LIB13 marker, have also been used for taxonomic identification purposes (Carraway et al. 1996, 1997; Peng et al. 1997; Spano et al. 1998; Caccio et al. 1999, 2000, 2005; Tanriverdi et al. 2003). Intuitively, sequences of taxonomically informative loci should be conserved within a taxon, but differ between taxa. The differences between the taxa can be revealed by means of restriction fragment length polymorphism analysis (RFLP) or sequence analysis of the PCR amplicons.

So far, evidence for recombination between *Cryptosporidium* taxa in nature is lacking. Therefore, the same combination of alleles at all loci with taxonomic information content should be present in isolates belonging to the same taxon. However, for many *Cryptosporidium* taxa the nucleotide sequences of many loci are still unknown, and so identification schemes performed using several taxonomic loci can lead to ambiguous conclusions. For example, Elwin

and Chalmers recently reported on the finding of *Cryptosporidium* isolates in sheep showing the RFLP pattern of the 18S rRNA gene of *C. bovis*, and the COWP pattern of the *Cryptosporidium* 'cervine genotype' (Elwin and Chalmers 2008). The authors concluded that the isolates were mixtures of both taxa, and that preferential PCR amplification of the *C. bovis* 18S rRNA gene had occurred. In this author's view, such a conclusion is not supported as the COWP gene sequence of *C. bovis* is not known.

Different lists of *Cryptosporidium* taxa have been published in recent years (Xiao and Ryan 2004; Fayer 2004; Fayer 2008; Xiao and Fayer 2008). However, it is apparent that the taxonomy within genus *Cryptosporidium* is far from being resolved, and there is still a long list of genetic variants of uncertain taxonomic standing and biological significance. At the 6th Meeting on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases held in 2002, it was concluded that the following aspects should be described when suggesting a new *Cryptosporidium* species: (1), morphometric data on oocysts; (2), a genetic characterisation of the proposed species; (3), demonstrate natural, and when feasible experimental, host specificity; and (4), comply with International Code for Zoological Nomenclature (Fayer 2008). Notwithstanding this proposal there is still uncertainty and confusion as to what constitutes a valid *Cryptosporidium* taxon. Conflicting lists of "accepted", "recognised", or "valid *Cryptosporidum* species" have been recently published by different authors (Snelling et al. 2007; Xiao and Feng 2008; Fayer 2008; Xiao and Fayer 2008), and there are discrepancies between the lists suggested by the same authors in different papers. For example, in one recent paper, the number of species was set at 16 "recorded species" (Snelling et al. 2007), and in the same year other authors proposed 16 "valid" species and "over 30 genotypes" (Santin and Fayer 2007). A year later, Xiao and Feng (2008) indicated there are 16 "accepted" *Cryptosporidium* species and "about 50 genotypes", but in another paper Xiao indicated the number of "valid' species to be 18, and that there are "over 40 genotypes" (Xiao and Fayer 2008). While in 2008, Fayer suggested the existence of 16 species and more than 40 genotypes (Fayer 2008), in another paper this author indicated 16 species and only 11 genotypes (Xiao and Fayer 2008). Furthermore, the number of genetic variants and taxa recognised continues to increase. For instance, most recently, Ryan et al. (2008) proposed the new species name of *C. fayeri* (in honour of Fayer) for *Cryptosporidium* genetic variants isolated from the red kangoroo (*Macropus rufus*), and in the same year, Fayer et al. proposed the species name of *C. ryanae* (in honour of Ryan), as a new species name for the so-called *Cryptosporidium* deer-like genotype (Fayer et al. 2008).

According to Fayer, a genotype "is not a taxon", but rather a partial and temporary descriptor (Fayer 2008). In fact, the term 'genotype' has been used to acknowledge the uniqueness but also the incomplete knowledge on the new variant. Occasionally, new genotype names have been designated based on limited evidence. Such is the case of the *Cryptosporidium* 'horse genotype'. This descriptor was proposed by Xiao and Feng (2008) and Fayer and Xiao (2008)

based on a previous report of a new, partial (~470 base-pairs long) 18S rRNA gene sequence identified in one isolate from a Przewalski's wild horse (*Equus przewalski*) in a zoo (Ryan et al. 2003). The gene sequence of the 'horse genotype' has never been identified again in any other host, but nonetheless, it has been repeatedly claimed that horses are "well known to be infected with the horse genotype" (Xiao and Feng 2008; Fayer and Xiao 2008). The idea of the occurrence of a 'horse genotype' is further discussed in Section 1.5.4 and in the studies reported in Sections 3.2 and 3.3.

There are a number of objective reasons for the lack of a clear consensus of what constitutes a *Cryptosporidium* taxon. First, in many cases there is still uncertaintly on how to discern between the intra-taxon and inter-taxon genetic variation (Fayer 2008). Second, the requisite of a biological species, which implies a natural population reproductively isolated from other populations, is difficult to assess in *Cryptosporidium.* Indeed, although *in vitro Cryptosporidium* growth has been achieved (Current and Haynes 1984; Hijjawi et al. 2002, 2004), systems capable of yielding high numbers of *Cryptosporidium* oocysts *in vitro* have not been been developed. In addition, experimental animal models which support the growth of different species are often lacking. Consequently, the only experimental unit for cross-breeding experiments are the *Cryptosporidum* 'isolates', which are composed of a finite number of oocysts originating from one, naturally infected animal. However, as will be seen in the section describing the *Cryptosporidium* life cycle, the oocysts derived from an individual animal cannot be considered a single clone or strain, because they may represent mixtures deriving from assemblages of taxa infecting the same host, recombinants, or perhaps even reproductively separated lineages of the same taxon (Xiao et al. 2004). Unfortunately, the genetic identification using PCR-based gene amplification cannot resolve such a complex genetic make-up of the isolates, due to the problem of PCR amplification bias (Suzuki and Giovannoni 1996, Rochelle et al. 2000). This is somewhat different to the situation with bacteria, in which a single cell can be isolated and grown and its progenies considered as a single clonal lineage. Thus, the intra-isolate genetic diversity of *Cryptosporidium* is still unknown, and this limits the possibilities to meaningfully interpret the results of genetic cross-breeding studies using the isolates as the operational taxonomic unit. Lastly, as *in vivo* passages are needed for the propagation of *Cryptosporidium*, there is a great potential for cross contamination of the isolates, which is difficult to predict and monitor. This problem was well illustrated by the displacement of the original parasites of the widely used 'IOWA' *C. parvum* isolate with exogenous variants found in calves (Cama et al. 2006).

Of the several *Cryptosporidium* taxa identified in humans, *C. parvum* and *C. hominis* account for the majority of cases of cryptosporidiosis worldwide (reviewed by Xiao 2007, and Nichols 2008). Notwithstanding the problems concerning the taxonomy of genus *Cryptosporidium*, the differentiation of *C. parvum* and *C. hominis* into two different species seems to have reached a consensus. The species name *C. hominis* was initially proposed by Morgan-Ryan et al. (2002),

to differentiate the *C. parvum* 'human genotype' (synonym: 'type 1' or 'genotype H'), which cycles in humans, from the *C. parvum* 'cattle genotype' (synonym: 'genotype C', ' type 2', or 'cattle'/'bovine' genotype), infecting both humans and animals. The new proposed nomenclature preserved the species name of *C. parvum* to the 'cattle' genotype, while the new name of *C. hominis* was given to the 'human genotype'. The separation of these genotypes into two species is consistent with the conspicuous genetic and epidemiological differences existing between these taxa, and the apparent absence of recombinant genotypes, which is consistent with two reproductively isolated populations (Spano et al. 1998; McLaughlin et al. 2000). The species names of *C. parvum* and *C. hominis* will therefore be used throughout this thesis, except in the study presented in Section 3.2, which reports the results of a study performed in 2002, when the old name of *C. parvum* 'cattle' genotype was still widely use.

1.4 THE LIFE CYCLE OF THE INTESTINAL *CRYPTOSPORIDIUM* PARASITES

The present dissertation of the life cycle focuses on issues of genetic exchange and population genetic structure of intestinal *Cryptosporidium*, as these are further explored in the epidemiological study presented in Section 2.2.

The biological cycle of intestinal *Cryptosporidium* parasites was described before the different species could be differentiated based on the genotype. Except for some comparative notes (Tzipori 1988; Fayer 2008), authoritative descriptions of the cycle in the different host species, or of the different taxa, were not found in the scientific literature. In early descriptions of the life cycle, the name *C. parvum* was used to describe the cycle of both *C. parvum* and *C. hominis* (Tzipori and Griffiths 1998; Tzipori and Ward 2002). In a recent dissertation of the life cycle, Fayer (2008) used the general descriptor of *Cryptosporidium* spp.

The entire biological cycle of intestinal *Cryptosporidium* parasites is completed in the intestinal tract of the host, and culminates with the excretion of sporulated and fully infectious oocysts in the faeces (Figure 1.1). The oocysts are coated with a hard protective wall and are highly resistant in the environment (Ranucci et al. 1993; Spano et al. 1997; reviewed by Fayer 2008). The complex physical and chemical factors governing the decay in the infectivity of the excreted oocysts have been widely studied in the laboratory, and their description is beyond the scope of the present dissertation (reviewed by Fayer 2008 and Peng et al. 2008). However, it is assumed that some *Cryptosporidium* oocysts can remain infectious for long periods of time, even in what has been defined by Fayer as "harsh environments" (Fayer 2008).

Infections with *Cryptosporidium* are typically acquired through the ingestion of oocysts in water, food, or other ingesta. According to some authors, the cycle may also commence by an inhalation of the oocysts (Tzipori 1988; Fayer 2008). Each oocyst contains four haploid sporozoites, which are the basic replicating *Cryptosporidium* cells. Eight chromosomes have been described in *C. parvum* and *C. hominis* (Blunt et al. 1997; Xu et al. 2004). In the small

intestine, the sporozoites excyst along a suture at one of the poles of the oocyst, and the free sporozoites infect the enterocytes. It is thought that the invasion of the enterocytes is initiated by an adhesion of sporozoite surface lectins to the intestinal mucus followed by an interaction of ligand surface proteins with receptors present on the surface of the enterocytes, and finally, an internalisation of the sporozoite (Riggs et al. 1997; Cevallos et al. 2000; Smith et al. 2005a; Feng et al. 2006). As in other Apicomplexa, it is believed that the selection of the host cell, and contact and penetration of the sporozoite to the cell, is mediated by structures and macromolecules present in an apical complex (reviewed by Borowski et al. 2008).

Feng and co-workers (2006) reported that bile salts, which are normally present in the gut, significantly enhanced the initial invasion of cells by both *C. parvum* and *C. hominis in vitro*.
Once in the cell, the sporozoite develops within a parasitophorous vacuole located within the microvilli of the brush border. The cell membranes of the sporozoite and the enterocyte fuse at the basal zone of the parasitophorous vacuole, forming the feeder organelle (Theodos et al. 1998), and the sporozoite gradually transforms into a trophozoite. Then, in a process referred to as merogony (or schizogony) the trophozoite's nucleus undergoes multiple asexual divisions, to form a multinucleate meront (or schizont), which subsequently segregates into 6-8 uninucleate merozoites. The mature merozoites burst from the meront and abandon the cell, to infect new epithelial cells, in which they undergo a second round of merogony. Two types of meronts have been described for *C. parvum*: Type 1 meront, which is composed of of 6-8 merozoites, and Type II meront, with only four merozoites. It is believed that Type II meronts either differentiate into an intracellular microgamont, which releases several extracellular haploid microgametes, or into a macrogamont, which remains in the cell. By a poorly understood process, the microgamete penetrates first the host`s cell and then the macrogamont membranes, to fertilise the macrogamont, with the formation of a diploid zygote. Subsequently, the zygote undergoes a reductional division into two haploid progenies, which then divide again, conservatively, to generate four haploid sporozoites, while at the same time the oocyst cell wall is being formed. In this process, considered by many as a meiosis, chromosomal remodeling by recombination may occur if the gamonts are genetically-different, generating two pairs of progenies which differ genetically from each parental gamete.

While diversification by recombination has been documented in *C. parvum* in experimental infections (Tanriverdi et al. 2007), the contribution of this process to the genetic diversification of *C. parvum* and *C. hominis* in nature is not well understood. According to the above cycle, each oocyst may contain two pairs of genetically-heterogeneous sporozoites, which differ from the parental cells due to recombination. Moreover, the *Cryptosporidium* isolates, which are composed of a finite number of oocysts recovered from a single host, may themselves be composed of an assemblage of genetically-heterogeneous oocysts. Within-oocyst and between-oocyst heterogeneity are therefore the two possible sources of genetic variation within *Cryptosporidium* isolates. Interestingly, these sources of variation are not usually taken into

account, or discussed in molecular epidemiological studies of cryptosporidiosis, in which traditionally the operational taxonomic units of the analysis have been the 'isolates'. As said in Section 1.3, the intra-isolate genetic variation is difficult to assess using the PCR due to the potential for preferential amplification of the templates (Suzuki and Giovannoni 1996; Rochelle et al. 2000). The genotyping of single oocysts, as recently reported (Hashimoto et al. 2006), might in part address this problem, but cannot resolve the intra-oocyst genetic variation, which requires the use of PCR-free sequencing technologies that are just now becoming accessible.

At the end of the cycle, a fully infectious oocyst containing four naked sporozoites is released from the cell (Smith et al. 2005a). Some observations have suggested that *Cryptosporidium* oocysts exist in two forms, a thin walled and a thick walled variant (Current and Reese 1986; Tzipori 1988), leading to the belief that the thin walled oocysts may excyst in the intestinal tract of the same host causing autoinfections (Ridley and Olsen 1991; Templeton et al. 2004). As discussed in Section 1.4, these autoinfections may provide a plausible explanation for the occurrence of chronic infections in immunosuppressed individuals (Current 1988; Dillingham et al. 2002).

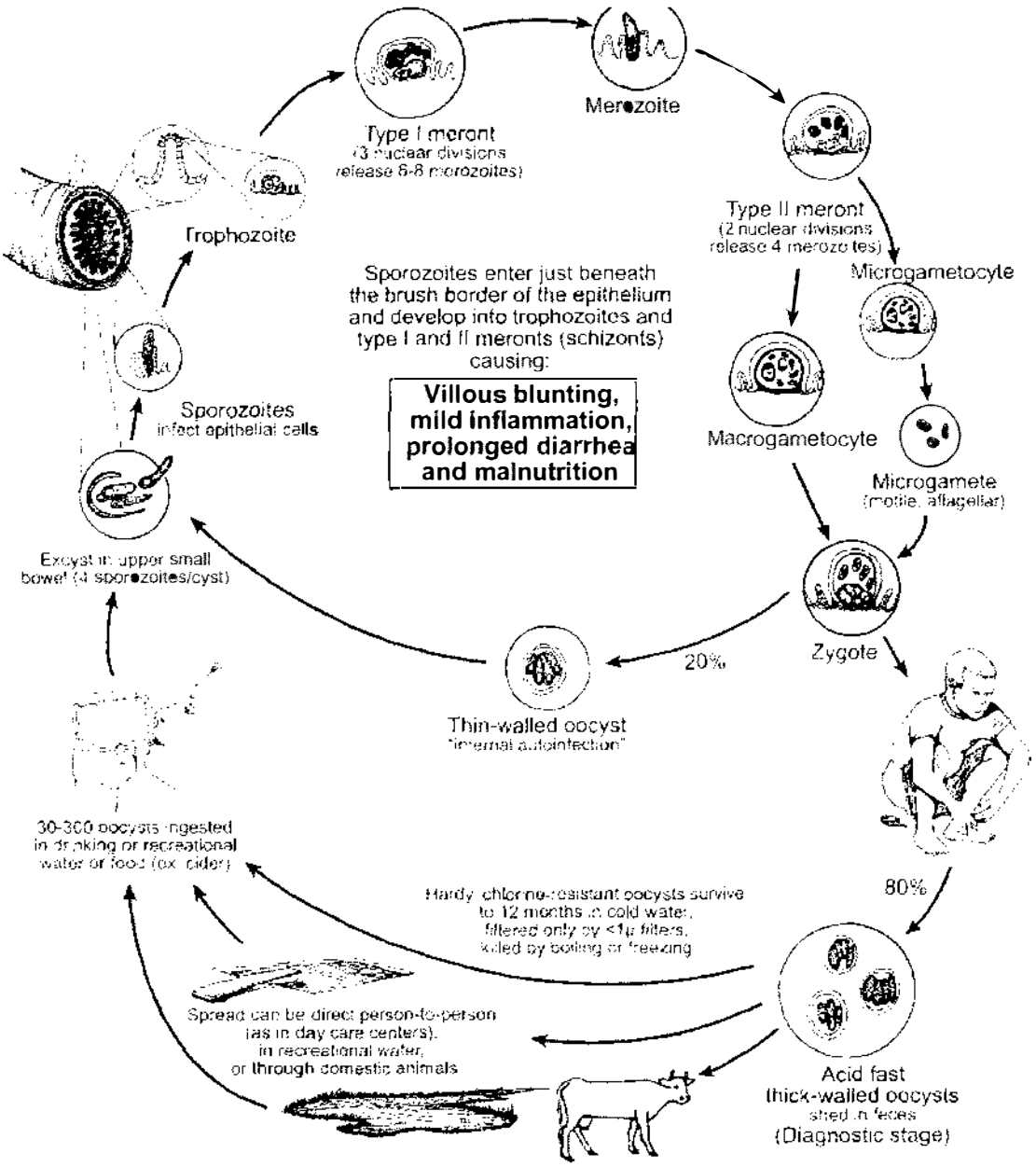

Figure 1.1: The intestinal and extraintestinal life cycle of *Cryptosporidium parvum*. (source: Dillingham et al. 2002). This figure will be incorporated in the thesis published online upon receipt of the copyright permission from the publisher.

1.5 PATHOGENESIS OF INTESTINAL CRYPTOSPORIDIOSIS

Intestinal *Cryptosporidium* are obligate intracellular parasites of the enterocytes. The invasion of the cell is believed to be initiated by an interaction between ligand macromolecules on the sporozoite's surface and its apical complex and the surface of the enterocyte, followed by the internalisation of the sporozoite. A number of putative ligands have been described in *C. parvum* sporozoites (reviewed by Tomley and Soldati 2001, and Borowski et al. 2008). Antibodies directed against some epitopes of these ligands were able to neutralise *C. parvum* infectivity both in mice and *in vitro* (Riggs et al. 1997, Tzipori and Ward 2002). One of the most widely studied *C. parvum* and *C. hominis* putative ligand protein is the 60 kDA surface glycoprotein (Cevallos et al. 2000; Strong et al. 2000), also known as the 'GP60' protein. As expected for a molecule under immunological pressure, the gene encoding the GP60 is highly polymorphic and comprises numerous non-synonymous polymorphisms (Strong et al. 2000). Such a polymorphism reduces the appeal of the GP60 as a potential immunising agent, but on the other hand enhances its usefulness as an epidemiological marker. Chapters 2 and 3 of this thesis report epidemiological studies using subtyping of the GP60 gene.

Within the enterocyte, the sporozoite carves a niche in a unique intracellular but extracytoplasmic location. Unlike other apicomplexan organisms, such as *Toxoplasma*, *Plasmodium*, *Eimeria* and *Cyclospora*, which develop in the cytoplasm surrounded by a parasitophorous vacuole, the *Cryptosporidium* sporozoite remains anchored to the host's cell membrane within the remnant of the microvillus (Griffiths et al. 1998). It has been hypothesised that in this location it can develop whilst protected from the host's cell cytoplasm, the immune system, and many chemotherapeutics (Griffiths et al. 1998; Theodos et al. 1998).

The pathological changes induced by *C. parvum* and *C. hominis* vary in severity, but are fairly similar in the various hosts. Histologically, using Giemsa stain the infecting organisms can be visualised as small basophilic bodies embedded in the microvilli. Other changes include the presence of blunt, fused intestinal villi combined with mucosal hyperplasia accompanied by a variable inflammatory response consisting of lymphoid cells, macrophages, and neutrophils infiltrating the lamina propria. The lesions are often found throughout the small intestine, but can also appear in the large intestine. They tend to be more severe in the distal jejunum and ileum (Laurent et al. 1999; Tzipori and Ward 2002; Stewart and Penzhorn 2004). Hyperaemia of the affected segments and stunting and fusion and/or cross bridging of the adjacent villi are often observed in calves, and have also been described in the horse (Kim 1990).

In HIV-positive patients, the morphologic changes seem to be correlated with the number of organisms present in the tissues (Genta et al. 1993). In immunocompetent hosts, intestinal cryptosporidiosis is typically a self-limiting disease lasting a few days. The short duration is generally presumed to be due to the mounting of an immune response, but perhaps also to the biological 'programming' of a limited number of merogony cycles, as only two types of meronts

are known to exist (Tzipori 1988). However, as previously stated, it has been hypothesised that the thin-walled oocysts excyst in the intestinal tract of the same host causing autoinfections. Autoinfections have been implicated as a leading cause of chronic debilitating cryptosporidiosis in malnourished children (Dillingham et al. 2002). While in animals, chronic *C. parvum* infections have been experimentally produced in mice (Ungar et al. 1990; Perryman and Bjorneby 1991), in nature they have only been described in immunodeficient Arabian horses (Snyder et al. 1978; Gibson et al. 1983).

The variability in infectivity between different *Cryptosporidium* isolates has been assessed in human volunteers (Okhuysen et al. 1999). However, the interpretation of the results of such studies is difficult, because *Cryptosporidium* oocysts do not survive freezing, and need to be passed *in vivo* in order for their viability to be maintained. Such passages may modify the genetic makeup of the isolate due to cross-contamination with wild oocysts or even internal recombination, which are difficult to assess or monitor.

1.6 INFECTIONS WITH *CRYPTOSPORIDIUM* IN DOMESTIC MAMMALS

1.6.1 Infections with *Cryptosporidium* in cattle (*Bos taurus*)

The most prevalent *Cryptosporidium* taxa found in cattle are *C. parvum*, *C. andersoni and C. bovis*. Other taxa, such as *C. hominis* (Smith et al. 2005b), the *Cryptosporidium* 'cervine' genotype and the 'deer-like genotype' (Fayer et al. 2005, 2006; Trotz-Williams et al. 2006; Feng et al. 2007; Feltus et al. 2008), *C. suis* and 'suis-like genotype' (Geurden et al. 2006), and *C. felis* (Bornay Llinares et al. 1999), have only been reported sporadically in cattle, but their impact on bovine health is unknown.

Cryptosporidium andersoni is a gastric species that infects the gastric epithelial cells of juvenile and mature cattle and causes mild dilation of the pyloric glands, hypertrophy of the gastric mucosa, and thinning of the epithelial lining, with little or no inflammation (Olson et al. 1997; Kváč et al. 2008). Animals infected with *C. andersoni* excrete oval oocysts 4x7 µm in diameter (Lindsay et al. 2000; Kváč et al. 2008). Some authors postulated that infections with *C. andersoni* may impair protein digestion and decrease productivity (Esteban and Anderson 1995). *C. andersoni* is not considered zoonotic, although it has been isolated from one HIV-positive patient (Guyot et al. 2001). No reports of *C. andersoni* infections in cattle in New Zealand were retrieved in the scientific literature consulted.

Conversely, *C. parvum* and *C. bovis* are considered intestinal parasites producing phenotypically similar, round oocysts 4-6 µm in diameter (Fayer et al. 2005; Fayer 2008). While *C. parvum* is a well-known pathogen of cattle, no reports of clinically overt infections with *C. bovis* were found in the scientific literature consulted. However, it should be remembered that *C. bovis* was only described for the first time in 2005 and consequently, much of the information about infections with this parasite is still unknown.

The first description of an infection with *Cryptosporidium* in cattle dates from 1971 (Panciera et al. 1971). A number of years later, the lesions in calves were described in more detail (Morin et al. 1976; Pohlenz et al. 1978). At the beginning of the 1980s, the aetiologic role of *Cryptosporidium* as a frank pathogen of cattle was debated due to the frequent co-infections with other enteropathogens and the mild histopathological lesions found in the course of infections (deGraaf et al. 1999). Indeed, Angus, from the Morendun Research Institute, Scotland, argued that "It seems probable that cryptosporidial infections represent a serious complication of virus-induced enteritis, particularly in young calves." (Angus 1983).

The first outbreak of calf diarrhoea in which *Cryptosporidium* was identified as the sole agent was published in 1980 (Tzipori et al. 1980b). Although 85% of the calves were affected, no mortality was recorded. Koch's postulates were fulfilled by Tzipori and co-workers in 1983 (Tzipori et al. 1983). Finally, in 1985, Upton and Current suggested the species name of *C. parvum* as a descriptor for the parasites producing 'small oocysts', distinguishing it from the 'large oocyst' type invading the gastric mucosa of cattle and at that time known as *C. muris*, but later re-classified as *C. andersoni* (Upton and Current 1985; Lindsay et al. 2000).

In the years that followed, the aetiologic role of *Cryptosporidium* was repeatedly corroborated by the results of experimental infections, observational studies, and therapeutic trials (Moore and Zeman 1991; Brenner et al. 1993; Fayer and Ellis 1993; Naciri et al. 1993, 1999; Moore et al. 2003; Grinberg et al. 2002; Joachim et al. 2003; Sevinic et al. 2003). Currently, *C. parvum* is considered among the commonest aetiological agents of neonatal calf diarrhoea worldwide (Olson et al. 2004; Fayer 2008). Conversely, the prevalence of *C. parvum* in post-weaned, juvenile, and adult cattle is low (Atwill et al. 1999; Atwill and DasPereira 2003; Fayer et al. 2006; Santin and Trout 2008; Santin et al. 2004, 2008). Moreover, because the oocysts of *C. parvum* and *C. bovis* are morphologically similar, it is likely that many parasites observed in the past in the faeces of juvenile and adult cattle were *C. bovis*, rather than *C. parvum*. In recent years, studies using some form of genotyping indicated that *C. parvum* is the commonest *Cryptosporidium* species of unweaned calves, while *C. bovis* is found at a greater prevalence in post weaned calves (Santin et al. 2004, 2008; Fayer et al. 2006; Geurden et al. 2006).

Infections with *C. parvum* in calves are typically acquired perinatally. The prepatent period ranges between 3 and 11 days (Anderson 1981, 1982; Fayer et al. 1998; Uga et al. 2000; Grinberg et al. 2002). In uncomplicated cases of cryptosporidiosis, the clinical presentation is fairly predictable and characterised by a profuse self-limiting diarrhoeal disease lasting a number of days (O'Handley et al. 1999; Uga et al. 2000; Grinberg et al. 2002). In addition to *C. parvum*, other viral, bacterial and parasitic pathogens, such as rotavirus, enterotoxigenic *Escherichia coli*, and coronavirus, are prevalent in calves during the first weeks of life. Some authors believe that co-infections with these agents increase the severity of cryptosporidiosis

(deGraaf et al. 1999; Olson et al. 2004), but no epidemiological studies supporting this view were found in the scientific literature consulted.

The faecal oocyst excretion curve in calves is predictable and bell-shaped. In general, infections are perinatal and the oocysts reach detectable numbers in the faeces 3-5 days post infection, concomitantly with the onset of diarrhoea. Their numbers peak a few days later, then rapidly decrease to undetectable levels (Anderson and Bulgin 1981; Fayer et al. 1998; Atwill et al. 1999; Uga et al. 2000; Castro-Hermida et al. 2002b; Grinberg et al. 2002; Figure 1.2). Oocysts numbers as high as 10^7 oocysts per g/ml (OPG) of faeces have been reported at the peak of excretion; diarrhoea, if it occurs, is generally concomitant with the shedding of oocysts (Fayer et al. 1998; Uga et al. 2000; Grinberg et al. 2002; Figure 1.2).

In neonatal calf cryptosporidiosis remission generally occurs, and death rates seem to be very low in well-managed farms (O'Handley et al. 1999; Uga et al. 2000; Grinberg et al. 2002). Consulatation of the scientific literature revealed only one report of high mortality in calves with cryptosporidiosis in one farm (Sanford et al. 1982). Evidence of differences in breed susceptibility to cryptosporidiosis is limited. Some opinion leaders have suggested that "when infection occurs in beef calves, it is usually more severe in these calves than in dairy" (Olson et al. 2004), although neither supporting data or references are provided. The study in New Zealand reported in Section 2.1, which was published in 2005, suggests a possible effect of the breed on the susceptibility of calves to *Cryptosporidium* infections. Interestingly, a similar effect has been suggested by the results of a subsequent molecular epidemiological study in the USA, in which none of the Jersey cattle surveyed were found to be carrying *C. parvum* (Starkey et al. 2006).

Unlike coccidian parasites, *Cryptosporidium* oocysts do not require particular environmental conditions to become infectious, as they are excreted fully infectious in the faeces. Each infected calf excretes hundreds of millions of oocysts during the patent period, readily contaminating the farm environment with oocysts that are fairly resistant to physical and chemical inactivants (Grinberg et al. 2002; reviewed by Fayer 2008). As a result, cryptosporidiosis can become a permanent problem on farms with anecdotal evidence suggesting that cleaning and chemical disinfection of the facilities provide little relief. Indeed, disease incidence risks of up to 100% have been recorded in herds with year round calving (Uga et al. 2000; Grinberg et al. 2002). The economic losses associated with calf cryptosporidiosis are mainly due to the increased labour needed to treat calves and the costs of diagnostic testing. Convalescent calves are believed to develop a protective immunity (Fayer 1998). Interestingly, chronic infections and stunting have not been recorded in calves, and there appear to be no significant studies providing evidence that cryptosporidiosis has a long-term effect on performance as measured, for instance, by body weight at weaning.

Since there is overwhelming evidence indicating a patent period of only a few days between the first and third week of life, purposive sampling of animals of this age is needed in order to assess the occurrence of *C. parvum* in cattle. In addition, the epidemiological term of 'prevalence' seems inappropriate to describe the rate of occurrence of *C. parvum* in cattle, as the infections are short lived. A more useful indicator might be farm-level prevalence, that is, the number of infected farms in a region, which can be measured by testing many calves between the first and third week of life on farms, as in the study presented in Section 3.4.

Cross sectional studies of calf cryptosporidiosis in different regions have shown a variable prevalence of infected farms, and of calves within farms (Genchi et al. 1984; Harp and Woodmansee 1989; Garber et al. 1994; Santin et al. 2004, Winkworth et al. 2008; Paul et al. 2008, Coklin et al. 2007). A few longitudinal studies from overseas applying repeated sampling on the same farms reported 100% farm-level prevalence (Castro-Hermida et al. 2002b; Trotz-Williams et al. 2005). At the animal-level, an infection incidence of 100% has been occasionally documented in longitudinal studies of calves on individual farms (Uga et al. 2000; Grinberg et al. 2002, Santin et al. 2008), and there is a general belief that most calves acquire *C. parvum* infections during the first month of life. This idea might be tested by the application of repeated sampling of cohorts of calves on a significant number of farms, but such studies are labour-intensive and were not found in the scientific literature, so the cumulative incidence of *C. parvum* infections in calves is not known.

Some farm management characteristics have been evaluated as potential risk factors for *Cryptosporidium* infections, with conflicting results. For example, some studies reported greater farm-prevalence in dairy herds than in open range cow-calf beef units (Olson et al. 1997; Kváč et al. 2006). Conversely, in Tennessee (USA), a greater prevalence of cryptosporidiosis was found in farms where calves were allowed to nurse with dams (Quigley et al. 1994), which was typical for open range cow-calf farms. In another study in the State of New York, a decreased risk of infection was found in farms with artificial feeding of calves (Mohammed et al. 1999). In a study in Spain, calf management practices had no effect on the prevalence of *C. parvum* infection (Castro-Hermida et al. 2002a), but in another study, a positive association between the herd size and the farm-level prevalence of *Cryptosporidium* was found (Garber et al. 1994). Collectively, these contrasting results indicate a complex multifactorial epidemiology compounded by regional factors.

Despite the wide variety of genetic variants of *C. parvum* found in nature, no studies comparing the effects or the infectious dose of different variants in cattle were found in the scientific literature. In one study using experimental infection, the duration of oocyst shedding was associated with the challenge dose, with larger doses leading to longer duration of the shedding of oocysts (Moore et al. 2003). It should be emphasised that the view that *C. parvum* is the only intestinal *Cryptosporidium* parasitising young cattle can no longer be universally applied, due to

the recent recognition of a wisdespread distribution of *C. bovis* in this host species. Previous estimates of the *C. parvum* prevalence should therefore be reassessed using genetic identification tools. Such a reassessment is important, as *C. bovis* is phenotypically indistinguishable from *C. parvum* but has never been found in humans, which challenges the idea that all *Cryptosporidum* infecting young cattle are potentially zoonotic.

Up until 2005, bovine cryptosporidiosis had only been occasionally reported in New Zealand. In a letter to the editor of the New Zealand Veterinary Journal, Townsend and Lance (1987) reported that 206 out of 550 (37%) calf diagnostic faecal specimens submitted to the Ruakura Animal Health Laboratory from July till December 1984 - 1986 were positive for *Cryptosporidium*, with the highest rate of infection seen in specimens from 4-14 days old calves. In 2003, Learmonth et al. reported the occurrence of *Cryptosporidium* in 7% of faecal specimens from cows (n=354) and calves (n=304) on 36 herds in the Waikato (Learmonth et al. 2003). However, the ages of the calves and the farm-level prevalence were not reported. Sections 3.4 and 3.5 of this thesis reports two epidemiological studies of *Cryptosporidium* in young cattle in New Zealand.

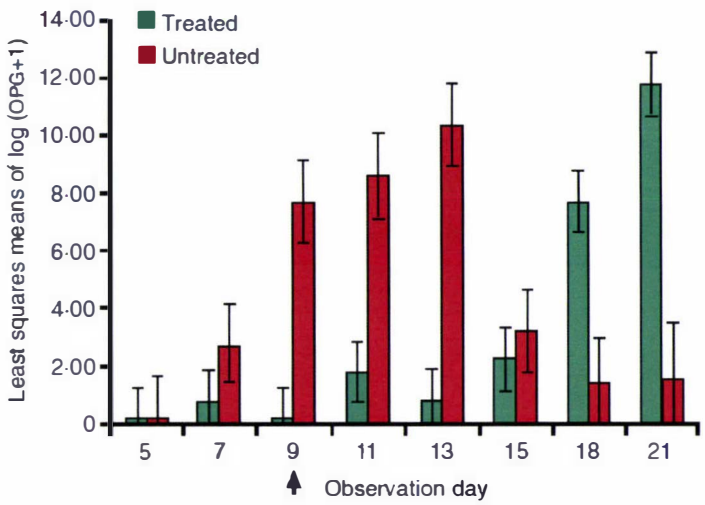

Figure 1.2. Least square means of the $\log_{10}$ (1+ number of *C. parvum* oocyst per gram of faeces) in 20 newborn calves affected with cryptosporidosis in a dairy farm in Israel. Red bars: 10 untreated calves; green bars: 10 calves treated with paromomycin sulphate between Days 1 and 9 of life. OPG+1= oocyst per gram of faeces +1; The calves' age in days are indicated on the X axis (from Grinberg et al. 2002). This figure will be incorporated in the thesis pubished on line upon receipt of the copyright permission from the publishers.

1.6.2 Infections with *Cryptosporidium* in small ruminants

Knowledge about the pathogenesis of *Cryptosporidium* infections in lambs and kids is scarce, and the impact of cryptosporidiosis on the health of small ruminants is not well defined. In a literature review, de Graaf suggested that *C. parvum* is an important pathogen of lambs and

kids (1999), but no supporting data were provided. Similarly, the public health significance of the isolates isolated from sheep and goats is not understood, as wide variation is reported in the prevalence of potentially zoonotic taxa in these host species.

Clinically-overt *Cryptosporidium* infections in small ruminants were first described in 1– 3 week old lambs (Barker and Carbonell 1974), and subsequently in a 2-week old kid with diarrhoea (Mason et al. 1981). Interestingly, Koch's postulates were fulfilled in specific-pathogen-free lambs using a calf isolate (Angus et al. 1982). Although the natural history of cryptosporidiosis in lambs and kids is not well understood, it appears to be similar to the disease observed in calves. Documented clinical signs include diarrhoea, depression and anorexia, accompanied by the excretion of faecal oocysts (Anderson 1982; Angus et al. 1982; Tzipori et al. 1982; Thamsborg 1990; Ortega-Mora and Wright 1994).

A variable prevalence of *Cryptosporidium* oocysts in faecal specimens from sheep has been reported in different studies, and were recently summarised by Santin and Trout (2008). However, there are conflicting reports about the genetic makeup and zoonotic potential of the *Cryptosporidium* parasites infecting lambs and kids. In 1998, Morgan et al. reported on the identification of *C. parvum* (the "calf group" in that paper) in a small number of goats and one lamb (Morgan et al. 1998). In a recent extensive molecular epidemiological study, *C. parvum* was the only species identified in 137 diarrhoeic lambs and 17 goat kids. All were under 21 days of age, and located on 71 sheep and 7 goat farms in the north-eastern region of Aragón, Spain (Quilez et al. 2008). In support of this finding, Mueller-Doblies and colleagues (2008) reported that *C. parvum* was the dominant species isolated from diarrhoeic lambs in the United Kingdom, but *C. bovis* and the 'cervine genotype' were also identified in some specimens. By contrast, the "cervine genotype" was the predominant genetic variant in a random sample of faecal specimens from subclinically infected lambs on ten farms in Belgium, while only *C. parvum* was identified in kids in the same region (Geurden et al. 2008). Conversely, Chalmers et al. reported a novel 'sheep genotype' in subclinically infected lambs (Chalmers et al. 2002). In a recent report, Pritchard and co-workers reported on the identification of *C. parvum* in 43 out of 48 oocyst-positive faecal specimens from lambs submitted to diagnostic laboratories in England and Wales for post morten examination (Pritchard et al. 2008). Other species sporadically isolated from lambs were *C. bovis* (Pritchard et al. 2008) and *C. hominis* (Ebeid et al. 2003; Giles et al. 2009). Interestingly, preweaned lambs in Western Australia were recently found to be infected with *C. bovis* (n = 52), the 'cervine genotype' (n = 10), and *C. parvum* (n = 2) when a genetic identification scheme using the 18S rRNA gene was used for the identification. However, when the same sample of faecal specimens was typed targeting a second locus (a C-type lectin-encoding gene which, according to the authors, is *C. parvum*-specific), 63 *C. parvum* were identified (Yang et al. 2008). It is unclear how the authors could differentiate between *C. bovis* and *C. parvum* using the above C-type lectin-encoding gene, as the sequence of this

gene in *C. bovis* is still unknown. Recently, Paoletti et al. (2009) identified *C. parvum* in 26/26 PCR-positive faecal specimens from lambs on six farms in central Italy.

1.6.3 Infections with *Cryptosporidium* in horses

Equine cryptosporidiosis was first reported in immunodeficient Arabian foals using microscopic parasitological methods, followed by a few descriptions of overt infections also in immunocompetent foals (Snyder et al. 1978; Gibson et al. 1983; Gajadhan et al. 1985; Coleman et al. 1989). A number of surveys indicate subclinical *Cryptosporidium* infections are relatively common in horses (Tzipori and Campbell 1981; Netherwood et al. 1994; Xiao and Herd 1994; Cole et al. 1998; Chalmers et al. 2005). However, reports confirming the causative role of these parasites in diarrhoea of horses are scant.

Early unsuccessful attempts to produce experimental disease in foals using calf *Cryptosporidium* isolates in the 1980s (Tzipori 1983) induced some authors to believe that horses are infected with unique *Cryptosporidium* variants (Saul Tzipori, personal communication to the author from 2002). This idea has been recently reiterated by other authors, who have suggested the existence of a *Cryptosporidium* 'horse genotype' (see Section 1.3). However, the *Cryptosporidium* 'horse genotype' has only been reported in one isolate from a Przewalski's wild horse (*Equus przewalskii*) in a zoo in the Czech Republic (Ryan et al. 2003).The original paper reporting this novel genetic variant included a number of discrepancies. For instance, while it was concluded that the isolate from the *E. przewalskii* was "most related" to *Cryptosporidium wrairi* based on its 18S rRNA gene sequence, the dendrogram presented in the same paper showed that the isolate clustered also with *C. parvum* (Ryan et al. 2003). Similarly, while it was acknowledged that the 70 kDa heat shock protein gene of the isolate from the Przewalski's wild horse was not determined (see Table 1 in Ryan et al. 2003), the authors concluded that a "high degree of sequence identity" with *C. wrairi* was also at this locus. In order to further investigate these discrepancies, this author retrieved the 18S rRNA gene sequence of the isolate from the Przewalski's wild horse from Genbank, dubbed the 'horse genotype' by Xiao and Feng (2008), and aligned it with the 18S rRNA gene sequence of *C. parvum* and *C. wrairi* published in the same database by Ryan et al. The aligned nucleotide sequence of *C. parvum*, *C. wrairi*, and the isolate from the Przewalski's wild horse, are shown in Figure 1.3. It is notable that there is a very small, similar number of polymorphisms between the sequence of the 'horse genotype' and *C. parvum* and *C. wrairi*, which is inconsistent with the idea that the former was mostly related to *C. wrairi.*

The first known outbreak of cryptosporidiosis in domestic foals incorporating clinical, epidemiological and pathological data, as well as the identification of the outbreak isolates as *C. parvum* 'cattle' genotype, is reported in Section 2.1 of this thesis. Follow up studies are reported in Sections 2.2 and 2.3.

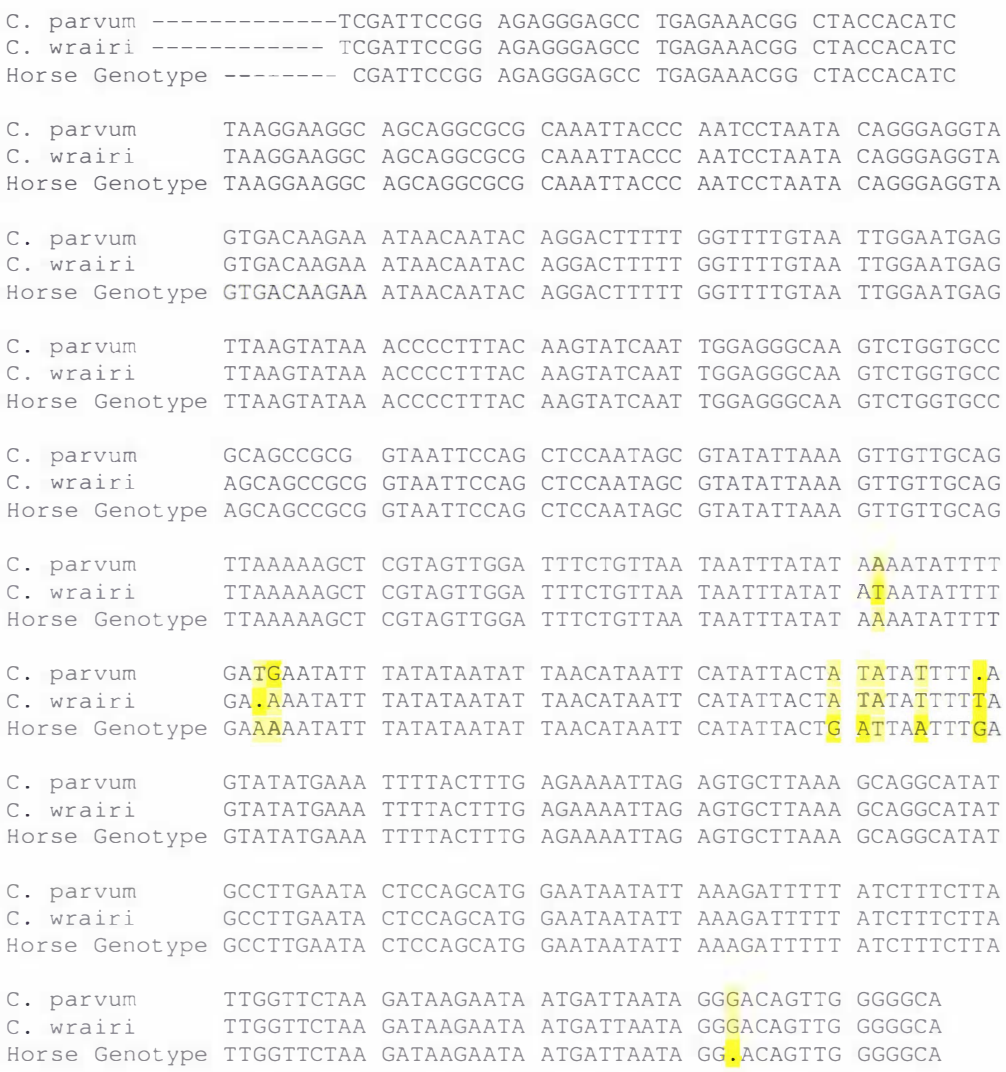

Figure 1.3 Nucleotide polymorphisms (in yellow) between the 18S rRNA gene sequence of the so-called 'horse genotype' (Genbank accession number AY273770), *C. parvum* (Genbank accession number AF093490), and *C. wrairi* (Genbank accession number AF115378). The *Cryptosporidium* 'horse genotype' gene sequence is reported in full, as originally reported in GenBank. For *C. parvum* and *C. wrairi*, only the segments overlapping the 'horse genotype' sequence are reported.

1.6.4 Infections with *Cryptosporidium* in cervids

Little is known about the epidemiology of *Cryptosporidium* infections in deer. Tzipori et al. reported an outbreak of diarrhoea in young red deer associated with the presence of *Cryptosporidium* oocysts in their faeces, and histopathological lesions consistent with cryptosporidiosis (Tzipori et al. 1981a). In 2002, an 18S rRNA gene sequence of a *Cryptosporidium* 'deer genotype' (synonym 'cervine genotype') was reported in deer (Genbank accession number AY120910), without specifying the species of deer (Xiao et al. 2002). The same genetic variant was later identified in a number of host species (summarised by Santin and Fayer 2007) including humans (Nichols 2008), and specifically, one person in New Zealand (Learmonth et al. 2004). Interestingly, a *Cryptosporidum* "deer-like genotype" was also

described, but this variant has only been reported in calves. The descriptor "deer-like" was proposed to account for the similarity between the 18S rRNA gene sequence of this variant and the above 'deer genotype'. Recently, the species name "*Cryptosporidium ryanae*" was proposed for the *Cryptosporidium* "deer-like genotype" (Fayer et al. 2008). The authors based the assignment of a species status to the *Cryptosporidium* "deer-like genotype" on the size of the oocysts (which measure 2.9-4 x 2.9-3.6 µm and are thus smaller than *C. parvum*), and the genetic differences from other known *Cryptosporidium* taxa at multiple loci.

In an epidemiological study of four farms in China, two out of 124 faecal specimens from farmed sika deer (*Cervus nippon Temminck)* contained *Cryptosporidium* oocysts (Wang et al. 2008). The isolates were assigned to the 'cervine genotype', although their 18S rRNA gene sequence differed slightly from the published sequence of this genotype. Subclinical *Cryptosporidium* infections in captive white-tailed deer (*Odocoileus virginianus*) have been reported; the ocysts recovered from the infected deer were infectious to neonatal mice and calves (Fayer et al. 1996). Asymptomatic shedding of *Cryptosporidium* oocysts has been reported in Ireland (Skerrett and Holland 2001), but the oocysts have not been identified. Cryptosporidiosis in farmed deer calves has been reported in New Zealand (Orr et al. 1985). Although there is anecdotal evidence of cryptosporidiosis being a significant clinical problem in young farmed deer in New Zealand, no reports were found in the veterinary literature. Only one report of an infection with a confirmed *C. parvum* in a deer was found in the scientific literature, but the species of deer was not specified (Sulaiman et al. 1998).

1.6.5 Infections with *Cryptosporidium* in dogs and cats

In 1979, Iseki proposed the name *Cryptosporidium felis* as a descriptor for the parasites found in cats in Japan (from Fayer 2008). Later, Sargent et al. (1998) described a *Cryptosporidium* "small oocyst type" in subclinically infected cats, and complemented the phenotypic findings with genotyping, resulting in the definition of a novel genotype in this host species. The first paper suggesting *C. felis* as a genetically-distinct species was published in 1998 (Morgan et al. 1998). As noted in Section 1.6.1, *C. felis* was also identified in one cow (Bornay Llinares et al. 1999). Santin and colleagues recently found *C. felis* and *C. muris* in cats in Bogota, Colombia (Santin et al. 2006). No other *Cryptosporidium* taxa are known to cycle in feline populations. In a literature review of 58 cases of human *C. felis* infection reported in different parts of the world, more than 80 % of cases occurred in HIV-positive patients (Raccurt 2007).

The first evidence of the cycling of *Cryptosporidium* parasites in dogs was provided by Tzipori and Campbell (1981), who reported the presence of anti-*Cryptosporidium* antibodies in 16/20 dogs. In 2000, Morgan and colleagues (2000) identified a novel *Cryptosporidium* variant in eight dogs from Australia and the US, which they dubbed the "dog genotype". This genotype was elevated to the species status by Fayer et al. one year later (Fayer et al. 2001).

Surveys aimed at assessing the prevalence of *Cryptosporidium* in canine and feline populations have been performed in different countries, with contrasting results (recently reviewed by Santin and Trout 2008). In a recent study, *C. canis* and *C. felis* were the main variants found in Australian dogs and cats, respectively (Palmer et al. 2008). In another survey in the USA *Cryptosporidium* oocysts were found in 30/250 domestic cats and all the successfully genotyped isolates were identified as *C. felis* based on the sequence of the 18S rRNA gene (Ballweber et al. 2009). As *C. canis*, *C. felis* and *C. muris* have only occasionally been found in humans, available epidemiological evidence would preclude dogs and cats as significant sources of zoonotic cryptosporidiosis in immunocompetent humans. However, the zoonotic species *C. parvum* has been sporadically identified in dogs Italy (Giangaspero et al. 2006), the USA (Fayer et al. 2001), and Czeck Republic (Hajdusek et al. 2004). Furthemore, the zoonotic potential of *Cryptosporidium* from cats has been suggested after one successful attempt to infect 6-weeks-old kittens by oral inoculation of an isolate recovered from an immunodeficient person (Current et al. 1983), and from one cat-to-human transmission event inferred in one case of cryptosporidiosis in a child following exposure to an infected cat (Egger et al. 1990). In addition, Xiao and colleagues reported an infection with *C. canis* in one girl, her brother and dog living in Lima, Peru. Both children had diarrhea, but the dog was asymptomatic (Xiao et al. 2007). No data on the pathogenicity of *Cryptosporidium* parasites in dogs and cats were found in the scientific literature.

1.6.6 Infections with *Cryptosporidium* in pigs

Although both *C. parvum* and *C. hominis* have been propagated in pigs (Widmer et al. 2000; Pereira 2002; Akiyoshi et al. 2003; Ebeid et al. 2003), knowledge about the prevalence and genetic makeup of these parasites in farmed pigs is scarce. Clinical signs of depression, diarrhoea and vomiting have been observed in piglets experimentally infected with oocysts originating from calves (Tzipori et al. 1981b). However, no clear association between infection and disease is known to exist, as surveys have reported subclinical infections in pigs (Guselle et al. 2003, Quilez et al. 1996).

In 2004, the species name of *C. suis* was suggested for a genetic variant found in pigs (Ryan, et al. 2004). Recently, a 22% infection rate with *C. suis* and the 'pig genotype II' was reported in 289 pigs located in four Western Australian facilities (Johnson et al. 2008). Xiao and colleagues (2008) found DNA sequences of *C. suis*, the 'pig genotype II', and *C. muris,* in 25 out of 56 pig slurry samples from 33 Irish farms. The *Cryptosporidium* 'pig genotype II' was found in 33/33 pigs from one facility in Canada (Guselle et al. 2003). Because the most common *Cryptosporidium* taxa found in pigs were only occasionally found in humans, some authors hypothesised that "domestic pigs do not pose a significant public health risk" (Johnson et al. 2008). However, the zoonotic *C. parvum* has also been identified in the Czech Republic (Kváč et al. 2008). Furthermore, piglets could be experimentally infected using isolates from calves (presumably *C. parvum*) (Tzipori et al. 1981b), suggesting there is no biological restriction for

the cycling of zoonotic *C. parvum* in pigs. No data about the pathogenicity of *Cryptosporidium* taxa occurring in pigs was found in the consulted literature.

1.7 GENETIC TYPING OF *CRYPTOSPORIDIUM*

Despite the recent improvements of *in vitro* cultivation (Hijjawi et al. 2002, 2004), *Cryptosporidium* are still hard to grow *in vitro* and their propagation depends on animal inoculation. Therefore, disease diagnosis depends on the direct detection of the oocysts or antigens in the faeces by microscopic techniques, or commercial kits, rather than the isolation of the agent. Because the oocysts of many intestinal *Cryptosporidium* are similar, and the phenotypic identification of the taxon of an isolate is not feasible, the generic term of cryptosporidiosis is commonly used in diagnostic practice. Fortunately, the genetic typability of isolates does not seem to be severely compromised if faeces are refrigerated, either with or without preservatives, which has enabled retrospective molecular epidemiological studies to be performed using extracted DNA from such faeces.

Although *Cryptosporidium* taxa are genetically similar, many polymorphic genetic loci, which can be used for the typing of clinical or environmental parasites, exist. As stated above, the first genetic polymorphisms between *Cryptosporidium* isolates were detected in the early 1990s (Ortega et al. 1991). Later, techniques involving the PCR amplification of genes followed by either an analysis for the presence of restriction fragment length polymorphisms (RFLP), or sequencing of the amplicons, were developed (Bonnin et al. 1996; Leng et al. 1996; Carraway et al. 1997). The high reproducibility and cost effectiveness of the PCR enabled the extensive use of molecular tools in epidemiological surveys, which resulted in the identification of a long list of *Cryptosporidium* taxa.

The first step of any genetic characterisation of a *Cryptosporidium* isolate is its assignment to a taxon. Genetic loci that are highly conserved within taxa, but on the other hand sufficiently polymorphic to allow differentiation between taxa, are used for this purpose. As stated above, Caccio and colleagues (2005) recommended the use of the 18S rRNA gene for all genetic identification schemes for *Cryptosporidium*. The advantages of using this gene for taxonomic purposes have been described in Section 1.3.

Once an isolate has been assigned to a taxon, genotyping beyond this level is not necessary unless further specific details are required. Conversely, if a differentiation between genetic variants within the same taxon is needed (for instance, to infer transmission routes or sources of infections), further subgenotyping (synonym: subtyping) at loci that are polymorphic within the taxa, such as micro and minisatellite repeats, is required. Micro and minisatellite repeats are sequence repeats of variable length found throughout the genomes of eukaryotes. They are usually composed of a variable number of repeats of 1-6 basepairs (microsatellites) or 10-500 basepairs (minisatellites) (Pevsner 2003). Micro and minisatellite sequences may exhibit length

polymorphisms due to either loss or gain of repeat units, presumably due to slippage during DNA replication (Jeffreys et al. 1985). As a result, alleles of different sizes can often be observed in eukaryotic organisms at mini and microsatellite loci, enabling the identification of unique genetic fingerprints. These loci are advantageous for epidemiological inference, thanks to their high degree of polymorphism.

Microsatellite and minisatellite length polymorphism analysis was first used for subtyping of *Cryptosporidium* in the early 2000s (Feng et al. 2000; Caccio et al. 2001). Because the genomes of *C. parvum* and *C. hominis* isolates have now been sequenced (Abrahamsen et al. 2004; Xu et al. 2004), it is possible to examine the genomic databases of these species for the presence of repeat sequences that are likely to show polymorphisms. The 70 kDa heat-shock protein (HSP70) gene of *C. parvum* and *C. hominis* contains such a polymorphic repeat (Khramtsov et al. 1995; Mallon et al. 2003). Another useful polymorphic protein-coding repeat is the polyserine repeat region of the *Cryptosporidium* 60 kDA surface glycoprotein gene. This gene codes a 60 kDa precursor protein that is cleaved into two sub-units, GP15 and GP40, and so it is also known as the 'GP60', 'GP40/15', or 'GP45/15' gene (Cevallos et al. 2000; Strong et al. 2000).

Both single-locus and multi-locus subtyping schemes have been used for the subtyping of *Cryptosporidium*. The single locus subtyping approach involves the characterisation of each isolate at a single polymorphic locus, whereas multilocus subtyping schemes use two or more loci. The single locus approach is useful for source tracking studies, and is advantageous, as compared with multilocus subtyping schemes, due to its simplicity. Indeed, a big disadvantage of the multilocus approach is that failure to characterise an isolate at all the loci could preclude it from the analysis. On the other hand, the multilocus approach is, intuitively, more discriminatory than any of the corresponding single locus schemes. In addition, as *C. parvum* and *C. hominis* undergo sexual recombination within the species as part of their life cycle (Tanriverdi et al. 2007), single-locus subtyping schemes are limited in their ability to capture phylogenetic relations between isolates.

1.8 *CRYPTOSPORIDIUM PARVUM* AND *C. HOMINIS* POPULATION GENETIC STRUCTURE

The genetic structures of local *C. parvum* and *C. hominis* populations have been studied in several locations using single or multiple genetic markers. Observations based on single loci (Peng et al. 1997; Widmer et al. 1998; Caccio et al. 2001) contributed to the proposal of elevating the genetically-distinct, anthroponotically transmitted *C. parvum* "human genotype" to a new species, which was named *C. hominis* (Morgan-Ryan et al. 2002).

Due to its extensive sequence polymorphism, the GP60 gene has been widely used for studying *C. parvum* and *C. hominis* populations (Leav et al. 2002; Alves et al. 2003, 2006; Sulaiman et al. 2005; Akiyoshi et al. 2006; Misic and Abe 2006; Xiao et al. 2006, Waldron et al. 2009). This

appoach has led to the identification of what has been designated as *Cryptosporidium* GP60 "subtypes", or "families" (Sulaiman et al. 2005). Multilocus subtyping schemes using unlinked loci (that is, loci that do not co-segregate during the meiotic process) are amenable to the study of the population genetic structure using allelle linkage disequilibrium analysis, which is not possible using the single locus approach. Such schemes have been applied to the study of *C. parvum* and *C. hominis* populations in Scotland (Mallon et al. 2003), India (Gatei et al. 2007a,b), and the Middle East (Tanriverdi et al. 2006). However, little is known about the population structure of *C. parvum* and *C. hominis* on a global scale.

Five molecular epidemiological studies of *Cryptosporidium* using single or multilocus subtyping approaches are reported in Chapters 2-4 of this thesis.

1.9 ZOONOTIC CRYPTOSPORIDIOSIS

As stated above, in the 1980s, intestinal *Cryptosporidium* parasites were still considered to be a single species known as *C. parvum* (Tzipori et al. 1980a), and cryptosporidiosis was mainly considered a zoonotic disease (Schultz 1983). However, at about the same time, the observation of human-to-human transmission events indicated a more complex epidemiology for human cyptosporidiosis (Casemore and Jackson 1984). Finally, in 1998, Awad-el-Kariem used isoenzyme electrophoresis to demonstrate the existence of genetically distinct "human and animal populations of *C. parvum*", a pattern later repeatedly confirmed genotypically using PCR, and eventually substantiated by the distinction into the species of *C. parvum* and *C. hominis*.

Since then, seven *Cryptosporidium* species and a number of other taxa have been identified in humans. Of these, *C. hominis* and *C. parvum* account for the vast majority of infections in immunocompetent and immunocompromised people, and other taxa have only been found sporadically, mainly in immunocompromised individuals. The taxa sporadically infecting humans have been repeatedly reviewed and are *C. meleagridis*, which infects mainly avian species, *C. canis*, found in dogs, *C. felis*, found in cats, *C. muris*, found in rodents, the *Cryptosporidium* 'cervine genotype', originally found in deer but also from sheep and cattle, the *Cryptosporidium suis*-like genotype, *Cryptosporidium andersoni* and the *andersoni*-like genotype, the 'chipmunk genotype', the 'skunk genotype' and *Cryptosporidium* 'monkey genotype' (Xiao and Fayer 2008; Nichols 2008; Xiao and Ryan 2008), and most recently the *Cryptosporidium* 'rabbit genotype' (Chalmers et al. 2009). Xiao and Fayer (2008) argued that, as the rare genetic variants found in humans were identified from microscopically-positive faecal specimens from clinically-affected individuals, they reflect active infections rather than a passive transit of oocysts in the gastrointestinal tract.

Although *C. hominis* is believed to cycle primarily among humans, it has also been reported in a dugong (*Dugong dugon*) (Morgan-Ryan et al. 2002), in non-human primates (Akiyoshi et al.

2003), cattle (Smith et al. 2005b), lambs (Giles et al. 2009), and one goat (Giles et al. 2009). In addition, *C. hominis* was propagated in gnotobiotic and conventionally reared piglets (Widmer et al. 2000; Ebeid et al. 2003), one lamb (Giles et al. 2001) and Mongolian gerbils (*Meriones unguiculatus*) (Baishanbo et al. 2005).

Conversely, *C. parvum* cycles in humans and other animals, in particular young cattle, and it is considered potentially zoonotic. There are reports of *C. parvum* infections in a wide spectrum of mammalian hosts, including horses. However, most studies have been based on microscopy, with no genetic characterisation of the isolates, and basically, the host-range of *C. parvum* is still unresolved.

Thus far, natural infections with *C. parvum* in animals have been confirmed by means of molecular tools in ruminants (cattle, sheep, goats, sika deer), pigs, dogs (see Section 1.5), two pet leopard geckos (*Eublepharis macularius)* (Pedraza-Díaz et al. 2009), and tortoises (one *Testudo graeca* and two *Testudo hermanni*) (Traversa et al. 2009). However, it should be noted that the genetic identification in the tortoises was performed by sequence analysis of the COWP gene, and the makeup of this gene in many *Cryptosporidium* taxa is unknown. The studies presented in Sections 2.1 and 2.3 of this thesis are the first known reports reports of clinically-overt infections with *C. parvum* in horses.

The majority of reports of *C. parvum* infections in animals concern cattle. While there is a large amount of evidence indicating *C. parvum* is the most prevalent species in pre-weaned calves, it has rarely been found in post-weaned calves. Conversely, the prevalence of *C. bovis* and *C. andersoni*, which seem to have little zoonotic significance, tends to increase in post-weaned and juvenile cattle (Santin et al. 2004; Fayer et al. 2006; Santin et al. 2008). It is widely accepted that young calves are mainly infected with *C. parvum*, and that these animals are a major source of zoonotic *C. parvum*. The possibility of a direct zoonotic transmission of *C. parvum* from cattle is supported by the results of transmission studies in human volunteers using bovine isolates, and from numerous case and case-control studies in people in several countries, including New Zealand (Pohjola et al. 1986; Reif et al. 1989; Millard et al. 1994; DuPont et al. 1995; Chappel and Okhuysen 2001; Stefanogiannis et al. 2001). However, the relative contribution of the oocysts originating from cattle as a source of human cryptosporidiosis is difficult to assess, as human-to-human and bovine-to-human transmissions can co-occur. Perhaps the most convincing data supporting a wide zoonotic transmission through the contamination of the ecosystem with bovine *C. parvum* oocysts was the sharp decrease in the number of notifications of human cryptosporidiosis in England and Wales during the 2001 foot and mouth disease epidemic. This decrease has been attributed to the restrictions on human movement in rural areas and the extensive culling of animals imposed during the epidemic, which reduced human exposure to livestock (Smerdon 2003; Sopwith et al. 2005). Interestingly, further epidemiological evidence supporting the widespread zoonotic transmission

of *C. parvum* originated from New Zealand. Here, human infections with *C. parvum* follow a seasonal pattern, with the number of notifications peaking every year in spring and early summer, soon after the calving season. Using faecal specimens submitted to diagnostic laboratories, Learmonth et al. (2003, 2004, 2005) elegantly showed that this peak is accompanied by a virtual substitution of the anthroponotic *C. hominis*, which is seen year round, with the potentially zoonotic *C. parvum* (Figure 1.4). Thus, it is possible that the synchronous presence of millions of highly susceptible newborn calves determines a massive amplification of *C. parvum*, contributing to the cattle-to human transmission of cryptosporidiosis at that time.

Until recently, a high prevalence of *C. parvum* infections in humans in a given region, as compared to *C. hominis*, was generally viewed as evidence of a widespread zoonotic transmission of cryptosporidiosis in the region (McLaughlin et al. 2000; Learmonth et al. 2003, 2004, 2005; Hunter and Thompson 2005). However, some researchers rejected this view, and suggested a more complex model, which contemplates the existence of anthroponotic *C. parvum* parasites that do not cycle in cattle (Mallon et al. 2003; Xiao et al. 2004). This concept is of considerable public health interest, as it implies that any barrier across the livestock–human interface would be ineffective in regions where anthroponotic *C. parvum* cycling prevails.

The anthroponotic model of transmission of *C. parvum* is supported by two lines of evidence. One line, proposed by Xiao et al. in a review of the literature (2004), is based on the observation of *C. parvum* isolates from humans carrying the GP60 allele family "*Ic*" (now nominated "*IIc*" following the acceptance of *C. parvum* and *C. hominis* as different species), which were never found in cattle. According to this argument, *C. parvum* carrying *IIc* GP60 alleles are anthroponotic and do not cycle in cattle. However, the literature supporting this model reveals a more complex picture.

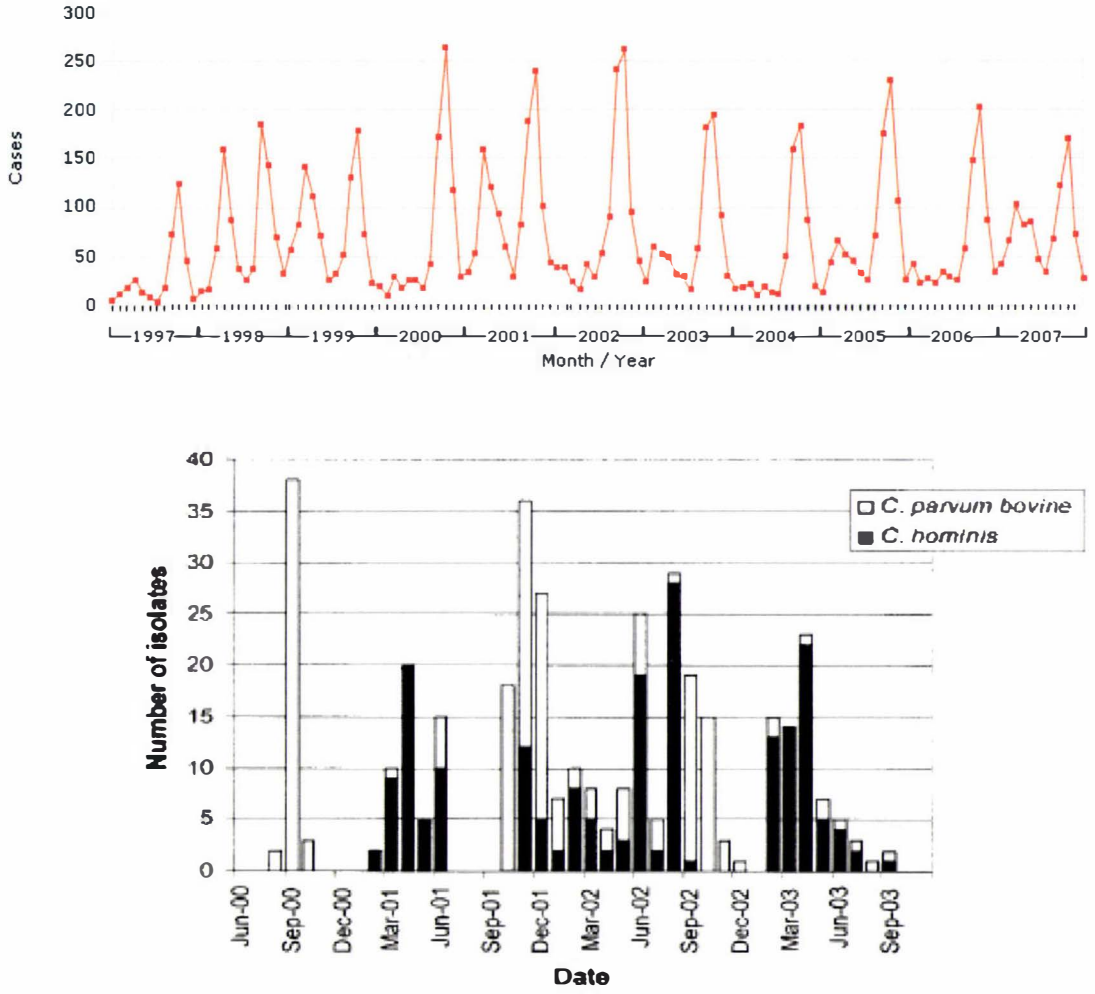

Figure 1.4 Upper graph: The number of cases of cryptosporidiosis notified in New Zealand between 1997 and 2007 (from: New Zealand Public Health Surveillance Report, Institute of Environmental Science and Research Ltd. http://www.nzpho.org.nz/NotifiableDisease.aspx, accessed October 2008); lower graph: the seasonal shifts between the number of *C. parvum* and *C. hominis* isolates identified in New Zealand between 2000 and 2003 (from: Learmonth et al. 2004). These figures will be incorporated in the thesis published on line upon receipt of the copyright permission from the publishers.

While it is indeed accurate that *IIc* alleles have been identified on a number of occasions in human *C. parvum* in some geographical regions (Leav et al. 2002; Alves et al. 2003, 2006; Xiao et al. 2004b; Gatei et al. 2007b; Waldron et al. 2009), their absence in the respective cattle populations could not be ruled out, as only a few or no bovine isolates from these regions were subtyped in the same studies. Moreover, most human *C. parvum* isolates carrying GP60 *IIc* alleles originated from HIV-positive patients, and so the generalisation to the population as a whole, is difficult. It should be also remembered that *C. parvum* is a genetically superdiverse species consisting of a large number of rare genetic variants (Mallon et al. 2003) and so a significant sampling effort would be needed in order to declare the bovine population as being free of the rare *C. parvum* GP60 *IIc* allele family.

A different line of evidence supporting the existence of anthroponotic *C. parvum* was provided by Mallon and co-workers (2003). These authors subtyped *C. parvum* isolates from humans and cattle in Scotland at seven polymorphic loci, and by doing so were able to define the multilocus genotype of each isolate. Then, the authors generated a pairwise genetic distance matrix of seven possible values (1-to-6 loci difference) between the multilocus genotypes, which was used to construct a dendrogram based on the Unweighted Pair Group Method with Arithmetic mean (UPGMA). Based on a simple visual inspection of the UPGMA dendrogram (reproduced in Figure 1.5), the authors concluded the presence of "human-only" multilocus genotype "sub-groups" in the sample. However, a re-analysis of the raw data provided by Mallon et al. by this author revealed numerous ties in proximity between the multilocus genotypes (that is, multilocus genotypes that are equidistant from multiple multilocus genotypes). These ties determined the presence of severe distortions in the dendrogram, which were probably not seen by the original authors (the concept of ties in proximity in cluster analysis and how they may distort dendrograms is well explained in MacCuish et al. 2001, and Arnau et al. 2005). For instance, multilocus genotype 21 (from the "human-only sub-group C") is a double locus variant of the other members of the same sub-group, but multilocus genotypes 19, 20, and 18 from the same "sub-group C" have double locus variants also in "sub-group B", some of which originated from bovine isolates (Figure 1.5). In addition, although the dendrogram would suggest the "human-only sub-group A" is a well-defined cluster, its components (multilocus genotypes 2, 3 and 57) are only weakly clustered. Indeed, the single and double locus variant networks of multilocus genotypes show that multilocus genotype 57 does not cluster with 2 and 3. Further exploration of the raw data provided by Mallon et al. indicated that multilocus genotype 57 is only a four-loci variant of 2 and 3.

Together, these results suggest that the concept of the existence of anthroponotic *C. parvum* requires corroboration. A study further addressing the question of the existence of anthroponotic *C. parvum* by a re-analysis of the data presented by Mallon et al. (2003) using a different analytical approach is reported in Section 2.1. Four studies addressing the zoonotic impact of cattle and horses in New Zealand are presented in Chapters 3 and 4.

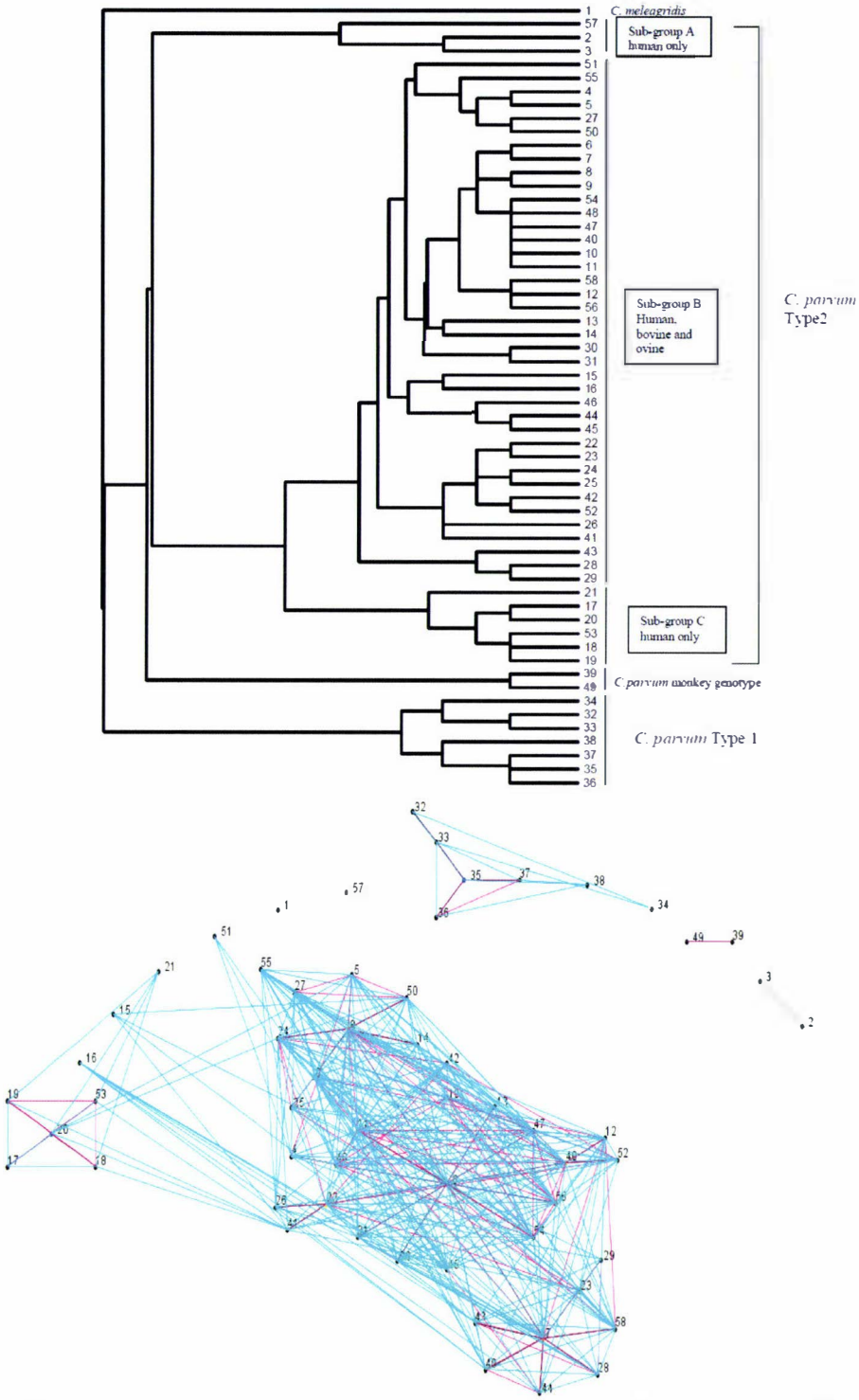

Fig. 1.5 Dendrogram showing the "human only sub-groups" of *C. parvum* multilocus genotypes in Scotland reported by Mallon et al. (2003) (above), and single and double locus variant networks (SDLVN) of the multilocus genotytpes (MLG), generated by the author using the data provided by Mallon et al. (below). In the networks, the multilocus genotypes are represented by dots linked to their single locus variants (purple lines) and double locus variants (blue lines). The numbers represent the MLG identifiers as defined by Mallon et al. Note the presence of numerous ties in proximity between MLGs of "sub-group C" and "sub-group B" in the dendrogram. The SDLVN were constructed using eBURST software (see Section 2.2). The upper figure will be incorporated in the thesis published on line upon receipt of the copyright permission from the publishers.

1.10 CONCLUDING REMARKS

Cryptosporidiosis is a disease of significant impact on both human and bovine health in both developed and developing countries. Ernest Edward Tyzzer first realised that while some *Cryptosporidium* parasites invaded the gastric epithelium, others were found in the enterocytes. He proposed the species name of *C. muris* for the gastric parasites, and *C. parvum* for the intestinal parasites of mice (Tyzzer 1910, 1912), forming the basis for the phenotypic taxonomic nomenclature which was accepted until recently. With the advent of cost effective PCR equipment and reagents in the 1990s, many laboratories around the world started genotyping isolates. As a consequence, a large amount of evidence indicating an extensive genetic diversity within genus *Cryptosporidium* rapidly accumulated. Nonetheless, the taxonomy within genus *Cryptosporidium* is far from being resolved and there is still no full consensus on what constitutes a *Cryptosporidium* taxon. These developments forced researchers to re-assess some of their established views, and perform new epidemiological research using molecular tools. Such a rapid evolution of thought will be apparent in some of the epidemiological studies that follow.

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CHAPTER 2

STUDIES OF THE POPULATION GENETIC STRUCTURE OF *CRYPTOSPORIDIUM PARVUM* AND *CRYPTOSPORIDIUM HOMINIS*

This chapter contains two studies of the population genetic structure of *C. parvum* and *C. hominis*. The first study (Section 2.1) is a re-analysis of the raw data previously published by Mallon et al. (2003a) (see also Section 1.9) using a different analytical approach based on diversity statistical tests. The study was possible due to the availability of unpublished post-code data generously supplied by Prof. Andy Tait, Wellcome Centre for Molecular Parasitology, Glasgow, United Kingdom. These data remain confidential. The second study (Section 2.2) is an original analysis of multilocus genotype data for *C. parvum* and *C. hominis* from seven countries provided by Drs. S. Tarniverdi and G. Widmer, Department of Infectious Diseases, Cummings School of Veterinary Medicine, Tufts University, MA, USA. All the laboratory work described in Section 2.2 was performed at Tufts. The author provided *C. parvum* DNA from New Zealand, performed the analysis of the data and wrote the manuscript, together with G. Widmer.

The above studies were published as:

Section 2.1: Grinberg A, Lopez-Villalobos N, Pomroy W, Widmer G, Smith H, Tait A. Host shaped segregation of the *Cryptosporidium parvum* multilocus-genotype repertoire. *Epidemiology and Infection* 136, 273-78, 2008

Section 2.2: Tanrıverdi S, Grinberg A, Chalmers RM, Hunter PR, Petrovic Z, Akiyoshi DE, London E, Zhang L, Tzipori S, Tumwine JK Widmer G. Inferences about the global population structure of *Cryptosporidium parvum* and *Cryptosporidium* hominis. *Applied and Environmental Microbiology*, 27, 7227–34, 2008

Both Sections are presented as published, except that their format has been modified as indicated in the Preface.

2.1 HOST-SHAPED SEGREGATION OF THE *CRYPTOSPORIDIUM PARVUM* MULTILOCUS GENOTYPE REPERTOIRE

2.1.1 Summary

Cattle are among the major reservoirs of *C. parvum* in nature. However, the relative contribution of *C. parvum* oocysts originating from cattle to human disease burden is difficult to assess, as various transmission pathways - including the human to human route, can co-occur. In this study, multilocus genotype richness of representative samples of human and bovine *C. parvum* are compared statistically using analytical rarefaction and nonparametric taxonomic richness estimators. Results indicate that in the time and space frames underlying the analysed data humans were infected with significantly wider spectra of *C. parvum* genotypes than cattle, and consequently, a significant fraction of human infections may have not originated from the local bovine reservoirs.

This study provides statistical support to the emerging idea of the existence of distinct anthroponotic *C. parvum* cycles that do not involve cattle.

2.1.2 Introduction

The multihost protozoan parasite *Cryptosporidium parvum* (formerly *C. parvum* 'Type 2') is a major cause of diarrhoea in humans and newborn calves, worldwide. Due to the high incidence of early calfhood infections and the large numbers of oocysts shed with faeces during natural infections (Ongerth and Stibbs 1989; Uga et al. 2000), newborn calves are considered among the most efficient amplifiers of *C. parvum* in nature. Direct calf-to-human *C. parvum* transmission has been repeatedly inferred from numerous case and case-control studies (Pohjola et al. 1986ab; Reif et al. 1989; Shield et al. 1990; Millard et al 1994; Stefanogiannis et al. 2001; Hunter et al. 2004; Hunter and Thompson 2005; Xiao et al. 2004). Yet, the relative contribution of the environmental dispersal of *C. parvum* oocysts originating from cattle to overall human morbidity is difficult to assess, as various transmission pathways, including the human-to-human route, can co-occur.

Based on molecular epidemiological data, some authors have argued for the existence of anthroponotic *C. parvum* that do not cycle in cattle (Xiao et al. 2004). In support of this idea, Alves et al. recently observed that HIV-positive humans in Portugal were infected with a wider spectrum of *C. parvum* genetic lineages than cattle (Alves et al. 2003, 2006). Such inference is of considerable biologic and public health interest, and challenges the generally held view that disease control measures should target livestock, in particular cattle, as the main reservoir for human infections. Yet, thus far this model is supported by nonstatistical inferences, and from the genetic characterisation of *C. parvum* isolates recovered from HIV-positive patients over long periods of time (Alves et al. 2003, 2006), and thus, its general validity needs to be corroborated.

In a study published in 2003, Mallon et al. applied a highly discriminatory multilocus genotyping scheme on a large battery of *C. parvum* clinical isolates from humans and cattle in the Scottish regions of Aberdeenshire and Dumfriesshire (Mallon et al. 2003a,b). Forty eight *C. parvum* multilocus genotypes (MLGs) were described, indicating an extensive genetic diversity of this parasite. Whereas a number of ubiquitous and highly abundant MLGs caused the majority of infections in both humans and cattle, there were many low abundance MLGs which were seen in one or both hosts or regions, featuring a superdiverse MLG distribution. Based on a dendrogram generated using the unweighted pair group method with arithmetic mean (UPGMA), the authors hypothesised that some *C. parvum* infecting humans might not cycle in cattle. Here, the results of an analysis of the MLG abundance data generated by Mallon et al. are presented. The analysis applies taxonomic diversity statistical methods to test the hypothesis that humans are infected with a wider spectrum of *C. parvum* MLGs than cattle. The results are discussed in an epidemiologic and public health context.

2.1.3 Materials and Methods

In this study, the *C. parvum* MLG abundance (i.e., the number of isolates in each MLG) of Aberdeenshire and Dumfriesshire originally reported by Mallon et al. (2003a,b) were used. The original data from Orkney and Thurso were not included, as no human isolates were originally typed in these regions. The aim of the analysis was to test the hypothesis that humans were infected with a wider spectrum of *C. parvum* MLGs than cattle. Hence, the MLG richness (i.e., the total number of MLGs) of the human and bovine *C. parvum* MLG assemblages were compared using established taxonomic diversity statistics, based on the working assumption that the isolates are independent (Krebs 1989). To conform to this assumption, it was necessary to remove the bovine duplicates with the same MLG, originating from the same farm, as within-farm enzootic *C. parvum* has been repeatedly documented using molecular tools (Peng et al. 2003; Tanriverdi et al. 2006; Trotz-Williams et al. 2006) and such duplicates could have biased the results. Therefore, the isolates' post-codes (most likely corresponding to the farm of origin) were retrieved, and a new dataset that included only one isolate per MLG-post-code combination was generated. Hence, data were subjected to the following comparisons:

1 - Comparison between human and bovine *C. parvum* MLG richness with no reference to the region of origin.

2 - Comparison between human and bovine *C. parvum* MLG richness in Aberdeenshire.

3 - Comparison between human and bovine *C. parvum* MLG richness in Dumfriesshire.

4 - Comparison between MLG richness of bovine *C. parvum* from Aberdeenshire and Dumfriesshire.

5 - Comparison between MLG richness of human *C. parvum* from Aberdeenshire and Dumfriesshire.

6 - Comparison between MLG richness of Aberdeenshire and Dumfriesshire, with no reference to the host species.

MLG richness was compared by means of analytical rarefaction and the total richness estimators Chao1 and ACE1. Rarefaction is a statistical method first described by Sanders (in Hughes et al. 2001) for estimating the number of taxa expected to be present in a random sample of any size taken from a given collection. The approach is useful to compare observed taxonomic richness among environments that have been unequally sampled. Indeed, observed taxonomic richness can fluctuate stochastically due to sampling variation and is sample-size dependant (Hughes and Bohannan 2005). In essence, the difference between taxonomic richness of samples taken from homogeneous (non-partitioned) populations should only reflect the combined effect of sampling variation and sample size difference. In our case, rarefaction answered the question: What is the expected number of MLGs - and variance – in a random sample of the size of the small subsample taken from the large sub-sample of each comparison?

Richness estimations by analytical rarefaction were calculated using PAST software (Hammer et al. 2005), which applies variance estimates given by Heck et al. (1975). Rarefaction curves of the sub-samples in each comparison were constructed increasing the sample size by one, each time, using the 'step by 1' procedure of the rarefaction menu of PAST. In addition, MLG richness of the human and bovine samples - and the 95% confidence intervals (CI) - were compared using the nonparametric total richness estimator Chao1 (Chao 1984) and the abundance coverage estimator ACE1 (Chao 1992), which return theoretical estimates of the total population richness, including unseen MLGs.

2.1.4 Results

Overall, 11 bovine duplicates were eliminated from the dataset. There were no missing post-codes of bovine *C. parvum* from Dumfriesshire, and 14 missing post-codes from Aberdeenshire, which proportionally correspond to the possible presence of only 2-3 bovine *C. parvum* duplicates for that region. No duplicates were seen in the human sample; there were 23 missing post-codes of human isolates. Yet, as will be discussed later, the possible presence of human *C. parvum* duplicates does not alter the inferences of this study.

The final dataset, which consists of 167 isolates, is shown in Table 2.1. Twenty five MLGs are represented in humans only, six in cattle only, and 17 MLGs were shared. Overall, and in each region separately, the human *C. parvum* sub-samples are larger than the bovine *C. parvum* sub-samples.

Nominal results of the analytical rarefaction, Chao1 and ACE1 total richness estimates and their 95% CI, are reported in Table 2.2 and Figure 2.1. Notice that, by rarefaction, the human sub-samples have greater MLG richness than the bovine subsamples, overall, and in each individual region (comparisons HB, HBA, and HBD as defined in Table 2.2). These features are not likely to be the result of stochastic sampling variation because the 95% lower confidence limits of the

MLG richness of the rarefied human sub-samples do not encompass the observed richness of the corresponding bovine sub-samples. Conversely, the lower 95% boundary of the calculated richness of the rarefied large subsamples in comparisons BB and HH largely overlap the observed richness of the small subsamples, indicating that there is no substantial difference in MLG richness between the regions in the human or bovine sub-samples (Table 2.2). The rarefaction curves are shown in Figure 2.1. Notice that at a sample size of 64 in comparison HB, the rarefaction curve for the bovine sample almost reaches the asymptote, whereas the curve for the human samples is still steep. This suggests that there is a significant number of unseen human *C. parvum* MLGs, but at the same time, bovine *C. parvum* MLGs were relatively well sampled, i.e. a further increase in the size of the bovine sample would not be expected to greatly increase the number of new MLGs. Interestingly, the rarefaction curves in contrast BB, HH, and AD largely overlap, which indicates that within each host, MLG richness does not differ between regions, nor does it differ among regions.

Chao1 and ACE1 total richness estimators of the human sub-sample are greater than the estimators for the bovine sub-sample. Interestingly, the 95% CIs of the Chao1 estimate of the human and bovine *C. parvum* samples do not overlap, and the CIs of ACE1 estimator overlap slightly.

	Aberdeenshire MLGs	Dumfriesshire MLGs	Total isolates
Human sample	2(1), 3(1), 4(1), 5(1), 6(8), 7(3), 8(14), 9(1), 10(3), 11(1), 13(2), 15(1), 17(1), 18(1), 19(1), 20(1), 21(1), 22(3), 23(1), 24(6), 25(5), 26(1), 27(1), 28(1), 29(1), 30(3). TOTAL ISOLATES: 64	6(8), 7(1), 8(6), 9(2), 10(1), 11(3), 22(4), 24(1), 25(1), 46(1), 47(1), 50(1), 51(1), 52(1), 53(3), 54(1), 56(1), 57(1), 58(1). TOTAL ISOLATES: 39	**103**
Bovine sample	6(9), 7(4), 8(7), 9(2), 10(2), 11(1), 12(2), 13(1), 14(1), 16(1), 22(4), 23(1), 24(1) 30(1), 31(1). TOTAL ISOLATES: 38	6(4), 7(1), 8(7), 9(3), 11(1), 22(4), 23(1), 24(2), 27(1), 48(1), 55(1). TOTAL ISOLATES: 26	**64**
Total Isolates	**102**	**65**	**167**

Table 2.1 Distribution of *C. parvum* multilocus genotypes (MLGs) in Scotland, stratified by region (Aberdeenshire or Dumfriesshire), and host species (human or bovine *C. parvum*). MLGs are represented with numbers, as in the original papers by Mallon et al. (2003a,b). MLG abundances are in parentheses.

Comparison	MLG richness in the small sub-sample	Richness (95% CI) of large samples rarefied at the respective small-sample size	Chao1 and ACE1 estimators (95% CI)
HB	18	26.8 (21.8-30.1)*	*Chao1* H: 324 (87-1653) *Chao1*B: 26 (20 - 56) *ACE1* H: 150 (72 - 396) *ACE1*B: 36 (22 - 96)
HBA	15	18.67 (15,2-22.08)*	NC
HBD	11	14.4 (11.7-17.2)*	NC
BB	11	12.1 (9.8-14.5)	NC
HH	19	19.9 (15.5-22.3)	NC
AD	23	23.5 (19.6-26.9)	NC

Table 2.2 Rarefaction, Chao1 and ACE1 richness estimators, by comparison. HH, human versus bovine *C. parvum*; HBA, bovine versus human *C. parvum* in Aberdeenshire; HBD, human versus bovine *C. parvum* in Dumfriesshire; BB, bovine Aberdeenshire *C. parvum* versus bovine Dumfriesshire *C. parvum*; HH, human Aberdeenshire *C. parvum* versus human Dumfriesshire *C. parvum*; AD, *C. parvum* from Aberdeenshire versus *C. parvum* from Dumfriesshire. The 95% confidence intervals not encompassing the observed richness of the respective small samples are indicated by asterisks. H: human *C. parvum* sample; B: bovine *C. parvum* sample. CI: confidence interval; NC: not calculated.

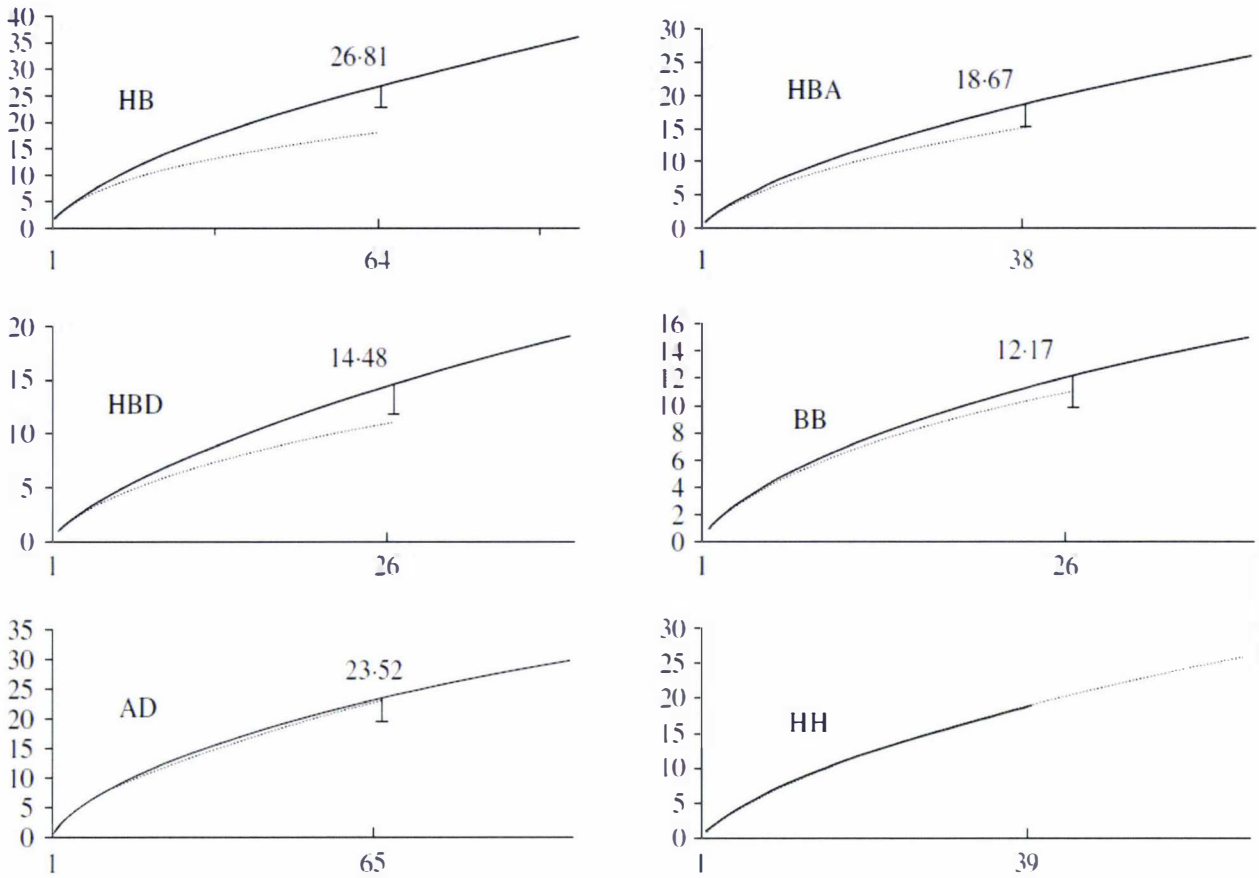

Figure 2.1 Rarefaction curves, by comparison. Sample sizes (starting from 1) are on horizontal axes and estimated multilocus genotype (MLG) richness on vertical axes. HB, human versus bovine *C. parvum*; HBA, human versus bovine *C. parvum* in Aberdeenshire; HBD, human versus bovine *C. parvum* in Dumfriesshire; BB, bovine Aberdeenshire *C. parvum* versus bovine Dumfriesshire *C. parvum*; AD, *C. parvum* from Aberdeenshire versus *C. parvum* from Dumfriesshire; HH, human Aberdeenshire *C. parvum* versus human Dumfriesshire *C. parvum*. Sub-sample sizes within each comparison are defined in Table 1. The long curves in each contrast represent either human or Aberdeenshire sub-samples. The calculated rarefied MLG richness are reported nearby the lower 95% confidence interval bars. Rarefaction curves in contrast HH overlap, so no error bar is provided.

2.1.5 Discussion

The study reported by Mallon et al. in two papers (2003a,b) is one of the most significant genetic comparisons between human and bovine *C. parvum* isolated from clinically overt infections so far published. The original authors explored patterns of population genetic structure using allele linkage statistics and phenetic clustering methods. Here, the MLG abundances were modelled using the diversity statistical approach, which allowed an estimation of total number of MLGs (seen and unseen MLG) as a function of the number of isolates in the sample. To comply with the working assumption of the approach, it was necessary to remove non-independent duplicates that might have inflated the MLG abundances. The most obvious of such duplicates were the bovine isolates of identical MLG, possibly originating from the same

farms. Indeed, without removing such clusters the difference between the MLG richness of the human and bovine samples would have been greater. Other levels of spatial clustering of MLGs, for example clustering due to animal trade between farms, could not be ruled out. Yet, such duplicates are also possible in the human sample, as the same MLG may have been transmitted to different households in the course of point-source outbreaks. Conversely, although the presence of post-code duplicates in the human sample were possible (as not all the human post codes were retrieved), this does not alter the results of this study but on the contrary, if present, such duplicates only increase the sample size of human *C. parvum* without adding new MLGs, leading to a more stringent statistical test for the comparisons between hosts.

One of the most important findings of the original study by Mallon et al. was that most infections were caused by a relatively small number of highly abundant and ubiquitous MLGs that were shared by both host species. Our results indicate that the MLG excess seen in the human sample cannot be discounted on the basis of sample sizes alone as that it is beyond the expected stochastic variation determined by sample size difference. Furthermore, a similar MLG excess was seen in the human sample in two different regions, but not between the sub-samples originating from the same host species but from different regions. This repetition in the two sub-samples provides a cross validation against random type-1 error (Hughes and Bohannan 2005). We therefore infer that in the time and space frames underlying the original study, humans were infected by a significantly wider spectrum of MLGs than cattle. These findings are in accordance with the inference by Alves et al. based on the genotyping of Cryptosporidium surface proteins in isolates recovered from HIV positive patients (Alves et al. 2003, 2006), and support its extension to the general population.

The occurrence of an excess of low abundance C. parvum MLGs that did not transcend the human boundary might indicate that certain MLGs infecting humans are not self-sustaining in cattle. Such an idea is in line with previous observations (Xiao et al. 2004), and with the hypothesis of the occurrence of 'human-only' MLGs formulated in the original study based on a simple inspection of a UPGMA dendrogram. Alternatively, it might merely reflect a wide reshuffling of the parasite's genetic repertoire across the human ecosystems via complex social networks, such as travel. Because this study analysed isolates collected from clinically overt cases, it could be claimed that some MLGs seen only in humans caused only subclinical infections or mild disease in cattle and thus, were not seen. Yet, such possibility is difficult to reconcile biologically. Indeed, unless the occurrence of host specificity is assumed, newborn calves – obviously lacking an acquired active anti-*Cryptosporidium* immunity – should be considered more susceptible to *Cryptosporidium* disease than adult humans, which were widely represented in this study (data not shown). Furthermore, this study analysed MLG richness differences, hence, the possibility that MLG richness of bovine *C. parvum* was underestimated is equally valid for the human sample.

In conclusion, in the time-space frame underlying this original study humans were infected with a wider spectrum of *C. parvum* genotypes than cattle, indicating that a significant fraction of human infections was likely to have been caused by parasites that did not originate from the regional bovine reservoirs. These results do not provide evidence of the occurrence of host specificity in *C. parvum*, which in the author`s view can only be obtained by cross transmission studies in cattle using putative human-only parasites. Yet, they do not conform to a simplistic model that considers all *C. parvum* as multihost anthropozoonotic agents, and support statistically the emerging concept of the occurrence of distinct cycles that do not involve cattle. Such a phenomenon should be taken into account when assessing the potential benefits of various barriers across the livestock-human interface on public health, as it is likely that such barriers would be ineffective in regions where anthroponotic *C. parvum* cycling is common. Further epidemiological studies in different geographical regions in which humans and newborn cattle share the same environment, and *in vivo* in cattle, are warranted.

2.2 INFERENCES ABOUT THE GLOBAL POPULATION STRUCTURES OF *CRYPTOSPORIDIUM PARVUM* AND *CRYPTOSPORIDIUM HOMINIS*

2.2.1 Summary

In the previous study, published multilocus genotype data of Scottish *C. parvum* from cattle and humans were analysed to explore for the presence of partitions in the genetic reprtoire of the parasite among the host species. In this study, a battery of *C. parvum* and *C. hominis* isolates from seven countries was genotyped using a nine-locus DNA subtyping scheme. To assess the existence of geographical partitions, the multilocus genotype data were mined using a cluster analysis based on the nearest-neighbor principle. Within each country, the population genetic structures were explored by combining diversity statistical tests, linkage disequilibrium, and eBURST analysis. For both parasite species, a quasi-complete phylogenetic segregation was observed among the countries. Cluster analysis accurately identified recently introduced isolates. Rather than conforming to a strict paradigm of either a clonal or a panmictic population structure, data are consistent with a flexible reproductive strategy characterized by the co-occurrence of both propagation patterns. The relative contribution of each pattern appears to vary between the regions, perhaps dependent on the prevailing ecological determinants of transmission.

2.2.2 Introduction

Cryptosporidium parvum and *C. hominis* are two species of ubiquitous protozoan parasites responsible for the vast majority of cases of intestinal cryptosporidiosis in humans. *Cryptosporidium parvum* infects humans and animals and is considered zoonotic. In contrast, *C. hominis* is thought to lack significant animal reservoirs and appears to be transmitted primarily among humans. Transmission is typically through the ingestion of water contaminated with oocysts, or direct contact with faeces. During most of their life cycle *C. parvum* and *C. hominis* are haploid and reside in the intestinal cells of the host, where they undergo two rounds of asexual multiplication. Thereafter, the differentiation and fusion of gametes leads to a transient diploid stage, followed by meiotic division. Meiotic recombination between genetically heterogeneous *C. parvum* genotypes has been documented in experimental infection (Tanriverdi et al. 2007), but the relative contribution of this process to the diversification of *C. parvum* and *C. hominis* in nature is not understood.

Ascertaining the population genetic structure of *C. parvum* and *C. hominis* is relevant to understanding the pathobiology of cryptosporidiosis and tracking sources of infection. *Cryptosporidium* parasite populations have been studied using PCR-based amplification of single or multiple genetic markers in Italy (Caccio et al., 2000, 2001), Denmark (Enemark et al. 2002), Scotland (Section 2.1; Mallon et al. 2003a,b), India (Gatei et al. 2007), Israel and Turkey (Tanriverdi et al. 2006), England and Wales (Hunter et al. 2007), France and Haiti (Ngouanesavanh et al. 2006), and India, Kenya, USA, and Peru (Gatei et al. 2006). Some

studies have used a gene encoding the sporozoite surface glycoprotein GP60 (Cevallos et al. 2000; Strong et al. 2000). This marker is appealing because of its extensive sequence polymorphism and functional relevance. However, PCR-based studies based on a single-locus are limited in their ability to capture the structure of a population, which is better explored using multilocus typing schemes. The multilocus approach was used to explore the population genetic structures of *C. parvum* and *C. hominis* in Scotland (Mallon et al. 2003a,b), Haiti (Ngouanesavanh et al. 2006), and in India, Kenya, USA, and Peru (Gatei et al. 2006).

The aim of the present study was to expand our understanding of the population structure of *C. parvum* and *C. hominis* to a global scale. Therefore, a large battery of archived *C. parvum* and *C. hominis* DNA samples from seven countries was genotyped at nine polymorphic loci, and the multilocus genotype (MLG) data were mined using cluster analysis, and combining diversity statistical tests with measures of linkage disequilibrium.

2.2.3 Materials and Methods

Parasite samples. DNA from 289 human *C. hominis* isolates from Uganda, the US, and the UK, and from 217 human and bovine *C. parvum* isolates from Uganda, New Zealand, Turkey, Israel, Serbia, and the US were initially included. DNA purification methods varied from country to country as described below. Archived DNA was stored at -20ºC.

Israel (IL): A total of 62 DNA samples from *Cryptosporidium parvum*-positive faecal specimens collected between March 2004 and April 2005 from 7-13 days-old calves on 14 farms (n=60), a horse (n=1) and a goat kid (n=1), were used. The collection sites, oocysts purification and DNA extraction method were previously described (Tanriverdi et al. 2006).

New Zealand (NZ): Twenty six isolates from humans (n=19) and calves (n=7) were used. The isolates originated from spontaneously reported cases diagnosed between August and November 2006 at medical or veterinary diagnostic laboratories in the Waikato region, North Island (n=13), and in the South Island (n=13). To purify the oocysts, approximately 5 g of faeces were suspended in water to a volume of 7 ml and strained with gauze. A volume of 3 ml of diethyl ether was added to extract the fat (Current 1990). Oocysts were captured with 50 µl of immunomagnetic beads (Dynabeads®, Dynal, Norway). Purified oocysts were suspended in 100 µl TE buffer and subjected to three cycles of freeze-thawing. DNA was extracted from the oocyst lysate using a commercial column DNA purification kit (HiPure™ template preparation kit, Roche Diagnostics, Indianapolis, Ind.) as previously described (Tanriverdi et al. 2006), and the DNA eluted in 50 µl distilled water.

Serbia (SRB): *C. parvum*-positive faecal specimens (n=50) were collected between April 2003 and September 2006 from calves 8-30 days of age raised on 10 farms located 18-45 km from the city of Belgrade. Oocysts were purified by sedimentation on sucrose gradients as previously described (Abe et al. 2002). Oocysts were lysed with three cycles of freeze-thawing, and DNA purified with DNA purification kits (QIAamp™ DNA Mini Kit, as previously reported (Misic and Abe 2006).

Turkey (TR): *Cryptosporidium parvum* oocysts were isolated between September 2001 and May 2005 from 15 faecal specimens from calves on 14 farms in the Kars region in northeastern Turkey. Oocyst purification and DNA extraction methods were previously described (Tanriverdi et al. 2006).

Uganda (UG): *Cryptosporidium*-positive faecal specimens (n=237; 62 *C. parvum*, 175 *C. hominis*) were collected from children under five years of age diagnosed with persistent diarrhoea at the Diarrhoea Treatment Ward of Mulago Hospital, in Kampala, Uganda, as part of a large cross-sectional survey that was previously described (Tumwine et al. 2003, 2005). The specimens were collected from November 1999 through January 2001, and from November 2002 through May 2003. Most specimens originated from patients residing in Kampala and its surroundings. DNA was extracted using the FastDNA® SPIN Kit for Soil (Qbiogene Inc., Carlsbad, California).

United Kingdom (UK): A total of 143 *C. hominis* DNA samples were selected from an archive composed of over 14,000 samples extracted from human isolates submitted between 2000 and 2005 to the UK *Cryptosporidium* Reference Unit by diagnostic laboratories throughout England and Wales. Of these, 113 samples were from sporadic cases in immunocompetent patients in the North West of England and Wales. These cases were previously included in a case control study which ran from November 2000 to February 2002 (Hunter et al. 2004). A further 21 *C. hominis* samples from sporadic cases were chosen from the national collection to include HIV-positive cases, and increase the geographical and age distribution, and nine samples were from cases involved in two outbreaks linked to water supplies. An outbreak was defined as two or more linked cases of cryptosporidiosis. Twenty-six sporadic cases had a history of being outside the UK in the two weeks before the onset of illness, of which eight had been in Pakistan. Other places were defined as Mediterranean countries (n=14), other European destinations (n=1), Africa (1), New Zealand (n=1) and one unknown. Oocysts were separated from faecal material by flotation on a saturated salt solution, then incubated at 100ºC for 60 min, digested with proteinase K, and the DNA extracted using DNA extraction kits as above (Elwin et al. 2001). Of all the samples from the UK, 105 were re-identified as *C. hominis* using the LIB13 marker (see below)

United States (US): Isolates from the US included *C. parvum* (n=10) and *C. hominis* (n=11). The *C. parvum* isolates were the reference isolate 'IOWA', three laboratory isolates used in human volunteer studies (Okhuysen et al., 1999, 2002), a bovine isolate from the state of Connecticut, as well as isolates from spontaneous infections and from HIV positive individuals enrolled in a clinical trial (Widmer et al. 1998). The *C. hominis* isolates were obtained from individuals enrolled in the same clinical trial, from two unrelated HIV-positive and two HIV-negative individuals.

Genotyping. The Lib13 species-specific marker (Tanriverdi et al. 2003) was used to discriminate *C. parvum* from *C. hominis* and rule out *C. meleagridis*, *C. muris*, and *C. andersoni*, except for the isolates from Uganda, which were typed using the COWP restriction fragment

length polymorphism (Spano et al. 1997). The UK isolates had all been previously identified at the COWP locus by PCR-RFLP and were additionally tested using the Lib13 marker. Nine mini- and microsatellite markers (MS) were used to subtype *C. parvum* and *C. hominis* as previously described (Tanriverdi et al. 2006; Tanriverdi and Widmer 2006). All genotyping work was performed at Tufts University. Markers are located on chromosome I (MSA, MSB), chromosome II (MSC), chromosome III (MSK), chromosome IV (MSE), chromosome V (MS9), chromosome VI (MSG), chromosome VII (1887), and chromosome VIII (TP14). Minisatellite MS9 and microsatellite TP14 were developed by Mallon et al. (2003a) and adapted as previously described (Tanriverdi and Widmer 2006). PCR amplifications were carried out in a real-time PCR machine (LightCycler® Roche Applied Science) using SYBR® Green I master mix (Roche Diagnostics) as described (Tanriverdi and Widmer 2006). Amplicons were fractionated on 15% native polyacrylamide gels and the bands visually scored in comparison to a set of representative alleles and to a 100-base-pairs ladder. The allele scoring method was validated by sizing selected markers on a capillary electrophoresis instrument (CEQ™ 8000, Beckman Coulter, Fullerton, CA). Alleles were numbered with a 3-digit code indicating their size in base-pairs.

Analysis of the data. Only isolates that amplified the Lib13 or COWP marker and all the nine loci were included in the analysis. The MLGs of isolates which included double banded loci were defined by omitting the large bands. This conversion was arbitrary, and was necessary because the species are haploid. No genotypes with three or more bands were observed. The possibility that some MLGs generated by the arbitrary omission of the large bands could have biased the inferences was ruled out by repeating the analyses after omitting double banded isolates from the dataset (see below). To eliminate possible bias introduced by resampling sub-structured populations, only one representative isolate per MLG-farm combination was left in the dataset as described in Section 2.1. This resulted in the removal of 65 *C. parvum* isolates. This procedure was not applied to human *C. parvum* and *C. hominis* as residence information was not available. However, most isolates from the UK originated from sporadic cases of cryptosporidiosis in patients residing at different addresses. For the analysis, MLG data were compiled in categorical matrixes composed of 11 columns, with *C. parvum* or *C. hominis* isolates characterised by a unique code, nine alleles, and a MLG identifier. All the differences between proportions were statistically assessed using the two-tailed Fisher's exact test.

The nine-locus subtyping scheme used in this study defines a genetic distance matrix of only nine possible values. Therefore, dendrograms were not used to assess global substructuring due to the occurrence of ties and potential for severe distortions. Instead, the extent of substructuring was assessed using a cluster analysis based on the Nearest Neighbor principle (Cover and Hart 1967). The pairwise Hamming distances (Hamming 1950), expressed as the number of loci by which the MLGs differ, were used as the genetic distances. For each *C. parvum* or *C. hominis* MLG, its nearest neighbors, defined as the MLGs at the shortest

Hamming distance, were retrieved manually. Within each country, the measure of clustering was the proportion of MLGs having all the nearest neighbors in the same country. The results of this cluster analysis were explored by inspecting the single locus variant networks generated using eBURST software as described below. As will be seen, the data did not require any statistical testing to be performed.

A combination of methods was used to explore the *C. parvum* and *C. hominis* population structure within each country. The genetic diversity of both species was assessed using MLG rank-abundance plots (Whittaker 1965), with MLG abundances scaled as a precent. In addition, multilocus genotype richness of *C. parvum* and *C. hominis* were statistically compared among countries by means of analytical rarefaction (Section 2.1; Hughes et al. 2001). Rarefaction is a statistical method for estimating the number of taxa expected to be present in a random sample of any size taken from a given collection. The approach is useful to compare taxonomic richness among environments, as the samples' taxonomic richness is dependent on sample size, and fluctuates stochastically due to sampling variation (Section 2.1). Rarefaction was performed using the individual-based rarefaction option in PAST software (Hammer et al. 2005). To assess whether the arbitrary omission of the large bands could have biased the shapes of the rarefaction curves, the analysis was repeated after omitting double-banded isolates.

Linkage disequilibrium across all loci was assessed using the standardized index of association (*SIA*) proposed by Habould and Hudson (Haubold and Hudson 2000). This index, a derivation of the Maynard Smith's index of association, fluctuates stochastically around zero in complete panmixia, but departs further from zero (unbound and in both directions) as linkage disequilibrium increases. The index, and its probability under a null model of panmixia, were calculated using LIAN software (http://adenine.biz.fh-weihenstephan.de/cgi-bin/lian/lian.cgi.pl, accessed July 2007), with 1000 allele randomisations. Linkage disequilibrium statistics were not calculated for *C. parvum* from the USA, Turkey, and Israel, due to the small sample sizes. These tests were repeated after omission of double-banded isolates from Uganda.

Finally, the population structures were explored by visual assessment of networks of single locus variants constructed using the eBURST software (Feil et al. 2004). Single locus variant eBURST networks are diagrams composed of MLGs depicted as dots, which are linked to their single locus variants by lines. A dot's diameter is proportional to the relative MLG frequency, so that the largest dots correspond to the most abundant MLGs and the smallest dots represent singletons.

2.2.4 Results

Genotyping. A total of 516 isolates were identified as *C. parvum* (n=227) or *C. hominis* (n=289) and genotyped. Out of 4,644 (516 isolates for each of the nine loci) PCRs, 230 (5%) failed to generate visible amplicons. The proportion of failing reactions was significantly greater

in C. hominis (7.0%) than in *C. parvum* (2.2%) (two-tailed Fisher's exact test; P=0.001). The same difference was observed between *C. parvum* and *C. hominis* isolates from Uganda, a country that provided human samples from both species which were processed in the same manner. Therefore, primer site polymorphisms or a lower *C. hominis* oocyst output, rather than different DNA extraction techniques or host species, may have contributed to this difference.

Among all PCRs, 30 *C. parvum* (1.4%) and 42 *C. hominis* (1.6%) isolates showed double bands. The sizes of the majority of the *C. parvum* (21/27 [78%]) and *C. hominis* (33/38 [87%]) bands which were found in double-banded reactions were also found individually in other isolates, suggesting that most double-banded reactions reflected mixed infections rather than PCR artifacts. The proportion of double-banded isolates was significantly greater in Ugandan *C. hominis* isolates, of which 18.2% had at least one double-banded locus, than in *C. hominis* isolates from the United Kingdom, of which only two (2%) were double-banded (two-tailed Fisher's exact test; P=0.00) (Table 2.3). The differences between the proportions of double-banded *C. parvum* isolates from various countries were also significant. However, there was no difference between the proportion of double-banded *C. parvum* and *C. hominis* isolates from Uganda (two-tailed Fisher's exact test; P= 0.24). After removing *C. parvum* replicates and incomplete *C. parvum* and *C. hominis* MLGs, the final data set was composed of 123 *C. parvum* and 190 *C. hominis* isolates (Appendix 2.2).

***Cryptosporidium hominis* and *Cryptosporidium parvum* geographic clustering**. Sixty nine unique *C. parvum* and 73 unique *C. hominis* MLGs were included in the cluster analysis (Appendix 2.2). No MLGs are shared between the two parasite species or between countries, in either *C. parvum* or *C. hominis*. Remarkably, with the exception of one *C. parvum* MLG from Uganda (UG27), which differs from its nearest neighbor at four loci, and one from Serbia (SRB46), which has equidistant nearest neighbors in Serbia and Israel, all the MLGs have their nearest neighbors within their own countries, indicating that more alleles are shared within each country than between the countries. A marked phylogenetic segregation is also evident in the single locus variant networks, all of which are composed of MLGs from only one country (Fig. 2.2). Significantly, the *C. hominis* isolates UK79, UK78, UK81, UK28, and UK29 are not linked to the large *C. hominis* cluster from the United Kingdom. Retrospective analysis of the patients' data indicated that all these MLGs originated from patients that had travelled to the United Kingdom from Pakistan less than two weeks prior to the isolation of the parasites. Together, the clustering of these isolates and the similar travel histories strongly suggested that these isolates were introduced. In contrast to other countries, *C. hominis* and *C. parvum* MLGs from the United States did not cluster, which is consistent with the disparate geographic and temporal origins of these samples, and are therefore not shown in Fig. 2.2.

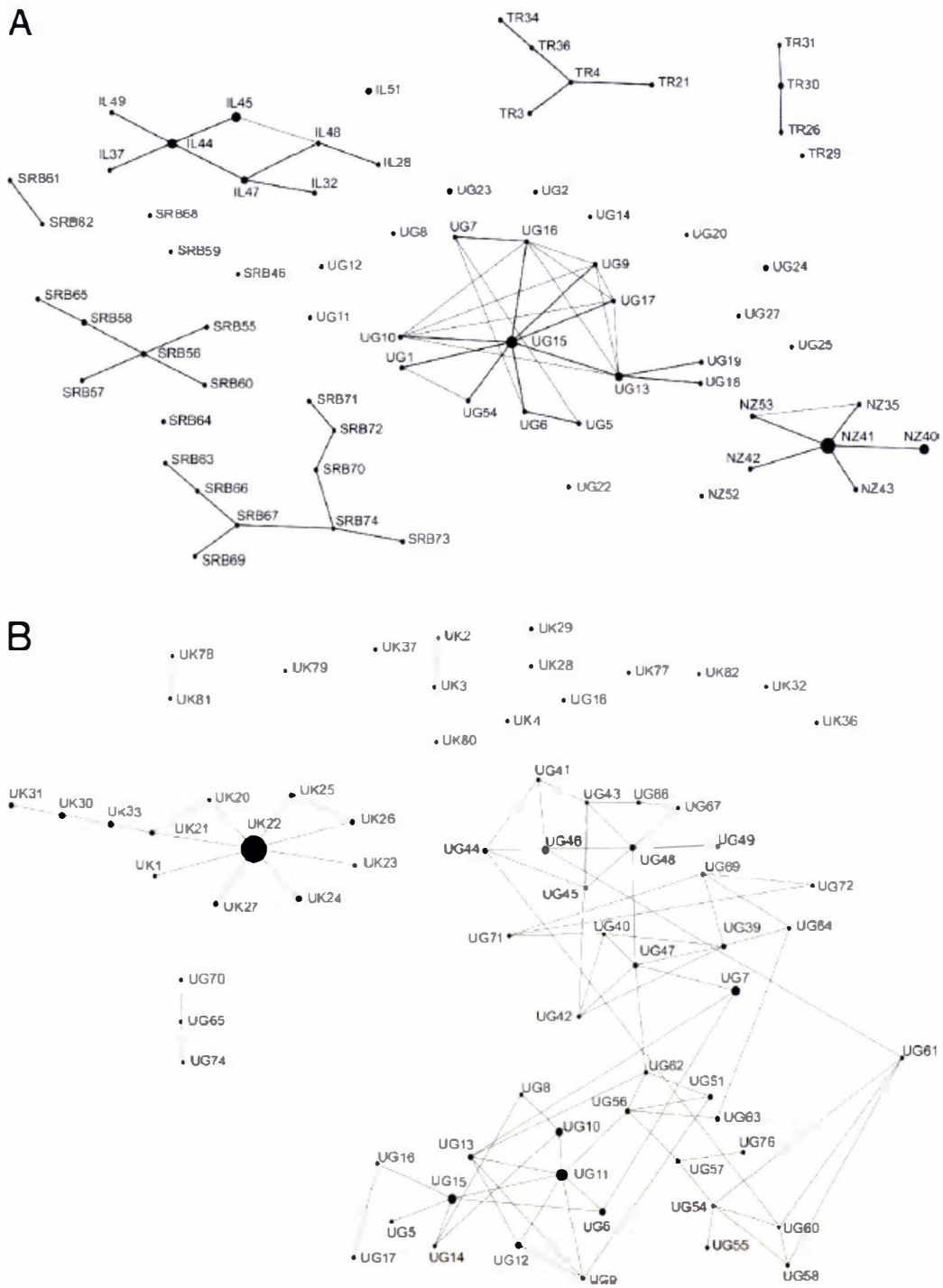

Figure 2.2 Single locus variant eBURST networks for *C. parvum* (A) and *C. hominis* (B). Each MLG is represented as a dot and designated by a unique identifier. Dot diameters are proportional to the number of isolates. For example, *C. hominis* UK22 was the most abundant MLG, found in 59 isolates, and the smallest dots represent singletons. Single-locus variants are connected by lines. IL, Israel; NZ, New Zealand; SRB, Serbia; TR, Turkey; UG, Uganda; UK, United Kingdom.

Population genetic structure. Figure 2.3 (left) shows the global MLG rank abundance curves for *C. hominis* and *C. parvum.* The plot reveals the presence of a small number of highly abundant MLGs and a large number of singletons in both species, consistent with previous data from Scotland (Mallon et al. 2003a,b; Section 2.1). In *C. hominis*, 59 out of 190 (31%) isolates belong to the most abundant MLG (UK22) (Appendix 2.2), whereas the most abundant *C. parvum* MLG (NZ41) accounts for 14/123 (11%) isolates. A comparison between *C. hominis* rank abundance plots from the United Kingdom and Uganda (Fig. 2.3, right) revealed a more even distribution in Uganda, where the most abundant MLG (UG11) accounts for only 10/85 (12%) of the isolates, compared with 51/74 (69%) in the United Kingdom (isolates from patients reporting travel excluded).

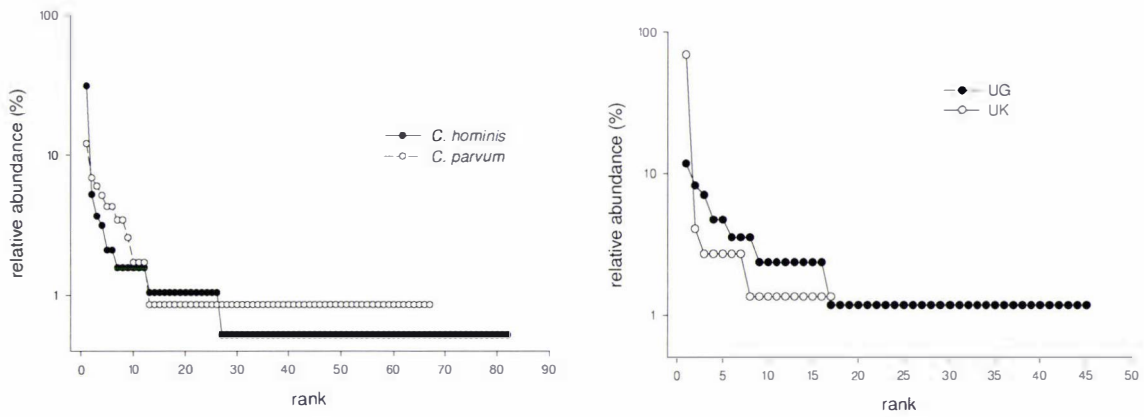

Figure 2.3 *C. parvum* and *C. hominis* MLG rank abundance plots. The relative abundance of each MLG is shown on the vertical axis as percent of all isolates. For each curve, the least abundant MLGs are singletons. Left: global analysis shows similar rank abundance for both species; Right: comparison of Uganda and UK *C. hominis* showing a more even MLG distribution in Uganda.

Rarefaction curves were used to compare *C. parvum* and *C. hominis* MLG richness levels among countries (Fig. 2.4). The MLG richness levels in the *C. hominis* and *C. parvum* samples from Uganda and *C. parvum* from Turkey and Serbia, countries with a large proportion of isolates with double-banded loci, are very similar, as indicated by similar rarefaction curves. In contrast, MLG richness in the samples from the United Kingdom, Israel, and New Zealand, countries where only a few double-banded genotypes were observed, is smaller, as indicated by lower curves approaching the asymptote. Rarefaction curves drawn without double-banded *C. parvum* and *C. hominis* isolates from Uganda and *C. parvum* isolates from Serbia and Turkey displayed very similar and overlapping curves (not shown), indicating that the arbitrary omission of large bands did not bias this result. As a substantial number of *C. parvum* and *C. hominis* isolates was collected from humans in Uganda as part of the same surveys, it was possible to compare MLG richness levels between *C. parvum* and *C. hominis* by using samples originating

from the same environment. Rarefied from a sample size of 85 to the *C. parvum* sample size of 36, *C. hominis* MLG richness in Uganda has a value of 24.2, which is the same as the MLG richness of 24 observed for *C. parvum* from the same country.

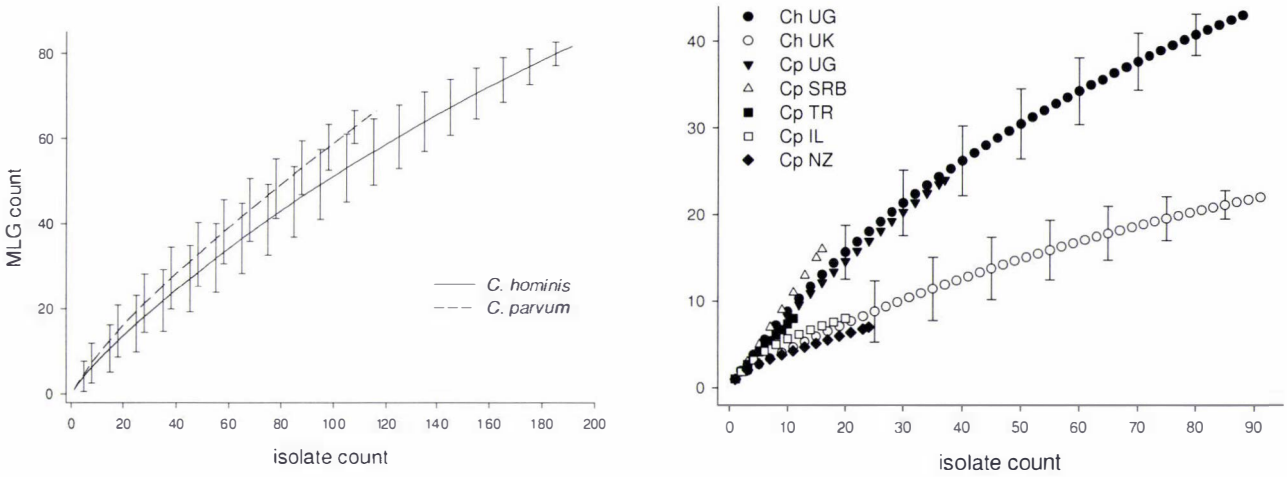

Figure 2.4 *C. parvum* and *C. hominis* analytical rarefaction curves. Left, global curves; right, country-specific curves. Ch, *C. hominis*; Cp, *C. parvum*. Error bars indicate the 95% confidence intervals for global *C. parvum* and *C. hominis* and *C. hominis* from Uganda and the UK. Isolates from the US were excluded. Note that the MLG richness in New Zealand is the lowest among the analysed countries, a result that will be re-addressed in the study presented in Section 3.5 and in Chapter 5.

In addition to showing a clear MLG segregation by country, as discussed above in the context of the cluster analysis, the eBURST diagrams (Fig. 2.2) also reveal differences between countries with respect to the parasites' population structure. The diagram of *C. hominis* from the United Kingdom has a star-like topology, with the most abundant MLG (UK22) connected to the greatest number of single-locus variants. This topology is reminiscent of eBURST networks of computer-simulated populations diversifying at a high mutation-to-recombination ratio (Turner et al. 2007). In samples drawn from such populations, most links between the single-locus variants mirror true clonal descent relations. Conversely, the eBURST diagram for *C. hominis* from Uganda is straggly and shows long chaining of single-locus variants, a feature typical of computer-simulated populations diversifying at a low mutation-to-recombination ratio, in which a significant proportion of links arise from recombination rather than true clonal descent (Turner et al. 2007). The eBURST diagram for *C. parvum* from Uganda is also straggly and shows MLG chaining. The exclusion of double-banded isolates from Uganda did not significantly affect the topologies of the eBURST diagrams (not shown), indicating that the omission of the large bands for the definition of the MLGs did not bias these results.

Interestingly, the MLGs with the greatest number of single-locus variants (UK22, UG11, UG15, IL-44, NZ41) were also the most abundant in all the countries where abundant MLGs were observed (Fig. 2.2 and Appendix 2.2). This pattern argues against a recent epidemic expansion of the abundant MLGs, as in such cases, all the MLGs were equally likely to expand epidemically and prevail in the sample. Instead, the pattern is consistent with a population originating from founders, as proposed for bacterial species by Feil and Spratt (2001). According to this structure, abundant genotypes show a greater degree of diversification than do rare genotypes, simply because they undergo more multiplications than rare genotypes.

In support of the differences between the eBURST network topologies and MLG richness levels between Uganda and the United Kingdom, there is strong statistical evidence of allele linkage disequilibrium in *C. hominis* isolates from the United Kingdom. Conversely, although "statistically significant," the *SIA* for *C. hominis* isolates from Uganda is close to zero (Table 2.3). Interestingly, the proportion of double-banded isolates of *C. hominis* is also significantly greater in Uganda than in the United Kingdom, indicating a higher rate of mixed infections in Uganda, but the *SIA* did not change much after eliminating the double-banded isolates (not shown). To further rule out an epidemic structure or a bias due to the presence of imported, thus reproductively isolated, MLGs in *C. hominis* isolates from the United Kingdom, linkage disequilibrium analysis was repeated by using one isolate per MLG, without the isolates from patients reporting travel. As expected for a basic clonal population structure (Smith et al. 1993), linkage disequilibrium persisted (*SIA*=0.1786; P= 0.01).

The population structure of *C. parvum* is less clear as the sample sizes from Israel, Serbia and Turkey are relatively small, and no *C. parvum* samples from the UK were available. Although there is statistical evidence for allele linkage disequilibrium in *C. parvum* from Uganda, the sIa is only 0.16 and the eBURST diagram is straggly and shows chaining of MLGs. On the other hand, there is no statistical evidence for linkage disequilibrium in *C. parvum* from New Zealand and Serbia (Table 2.3). However, the lack of linkage disequilibrium in these countries should not be viewed as evidence for complete panmixia, as it could be due to an underpowered statistical test due to the small sample size, or the presence of only four polymorphic loci in New Zealand *C. parvum* (Appendix 2.2). Indeed, the eBURST network for New Zealand *C. parvum* is star-like, with the most abundant MLG (NZ41) in the center, reminiscent of a clonal population structure. In contrast, the diagrams from Serbia and Turkey are straggly, consistent with the greater proportion of double-banded *C. parvum* isolates observed in these countries.

	Number of isolates with complete MLGs[a]	*SIA* (*p*-value)	Number (percentage) of double-banded isolates[b]
Uganda (UG)			
C. hominis	85	0.06 (p<0.001)	32 (18%)
C. parvum	36	0.16 (p<0.001)	7 (11%)
UK			
C. hominis	95	0.38 (p<0.001)	2 (2%)
New Zealand (NZ)			
C.parvum	24	0.03 (p<0.1)	0
Israel (IL)			
C. parvum	20	ND	1 (2%)
Serbia (SRB)			
C. parvum	23	0.016 (p<0.1)	7 (13%)
Turkey (TR)			
C. parvum	11	ND	5 (33%)

Table 2.3 *C. parvum* and *C. hominis* linkage disequilibrium and double-banded genotype statistics according to country of origin. *C. parvum* and *C. hominis* isolates from the United States were excluded. ND, not determined; *SIA*, standardised index of association.

[a] Isolates from USA excluded; [b] All typed isolates, including incomplete MLGs, were used to calculate the proportion of double-banded isolates.

2.2.5 Discussion

In this study, a battery of *C. parvum* and *C. hominis* isolates collected from seven countries was genotyped, enabling inferences on the parasites' global population structures to be made. The main aims were to assess whether *C. parvum* and *C. hominis* populations are geographically partitioned, and if so, compare the structure of different populations.

The results of the cluster analysis and the eBURST diagrams show a quasi-complete phylogenetic segregation among countries in both parasite species. Gene flow was therefore

not sufficient to erase genetic divergence among these geographically separated populations, which basically remained allopatric. This result is noteworthy considering that both species are cosmopolitan pathogens and may easily travel between countries as asymptomatic infections. Potentially, the ecology of cryptosporidiosis in different countries may have selected for phenotypes which are best adapted to each environment. This hypothesis would predict that imported parasites would be unlikely to spread if environmental factors or transmission patterns are unfavourable. This view is supported by the finding of a number of clustered *C. hominis* isolates from the United Kingdom, which were apparently introduced from Pakistan and did not cluster with the large network of MLGs from the United Kingdom. Analogous observations were reported in a recent MLG analysis of Scottish and Peruvian human isolates (Morrison et al. 2008) and are in accordance with the results of a study using sequence analysis of the GP60 locus (Chalmers et al. 2008). The diversity between local and imported MLGs may also explain the putative anthroponotic *C. parvum* reported by other authors (Mallon et al. 2003b; Hunter et al. 2007), as it is possible that "anthropogenic" isolates were in fact recently imported. Geographic segregation, however, was not reflected in the *C. parvum* sample from New Zealand, as no partition between North and South Island was observed and the isolates clustered in a single eBURST network (Figure 2.2). This interesting result suggests that intense gene flow, as might occur through animal and human movements, may mix the genetic repertoire of *C. parvum* even in the presence of significant geographical barriers, in this case the Cook Strait. To further evaluate this model, the same cluster analysis was performed with previously published *C. parvum* MLG data from Scotland (Mallon et al. 2003b) and no phylogenetic partition between the noncontiguous regions of Aberdeenshire and Dumfriesshire could be seen (Figure 1.5). The lack of partitioning in the Scottish data may be explained by the fact that the cattle population in Aberdeenshire experiences high movement due to the import of cattle from elsewhere in the United Kingdom, therefore preventing the establishment of settled and epidemiologically isolated herds (Gilbert et al. 2005). In contrast to these findings, *C. parvum* samples collected from individual farms in Israel and Turkey showed a marked segregation of the MLGs by farm and a microepidemic structure within the farms (Tanriverdi et al. 2006), indicating both micro- and macroecological factors impact *C. parvum* populations.

Because *C. hominis* is thought to propagate primarily in humans but *C. parvum* has a wider host range, it was interesting to compare the population structures of these species, to assess whether the differences in host range affect the population structures. *Cryptosporidium* parasites have been defined as being clonal or panmictic based on the results of linkage statistical tests emulating null models of random allele association (Mallon et al. 2003a,b; Ngouanesavanh et al. 2006). However, these tests are limited in their ability to describe populations propagating both clonally and by recombination, as in such cases the test statistic will be below or above the null-model rejection threshold in function of the contribution of each propagation pattern to the population structure (Smith et al. 1993). Therefore, in this study we used a combination of complementary analytical approaches. In contrast to reports of local

genetic homogeneity in *C. hominis* (Mallon et al. 2003a; Ngouanesavanh et al. 2006; Hunter et al. 2007), no evidence of difference in the genetic diversity of *C. parvum* and *C. hominis* was found in Uganda, a country that provided samples of both species. Furthermore, the eBURST topology and linkage disequilibrium statistics in Ugandan *C. parvum* and *C. hominis* are consistent with a similar population structure in both species. Conversely, the coherent differences between *C. hominis* from Uganda and the UK with respect to MLG richness, eBURST topology, and *SIA*, suggest a greater rate of recombination in *C. hominis* in Uganda than in *C. hominis* from the UK. The sample from Uganda showed greater MLG richness, as indicated by a significantly steeper rarefaction curve, as would be expected in a population that, in addition to accumulating variation by mutation, diversifies by recombination. Consistent with this result, there is a greater proportion of double-banded isolates in Uganda, suggesting that many infections were caused by genetically heterogeneous parasites, which enhances the opportunity for a diversification by recombination. On the other hand, the results of MLG diversity analysis, eBURST network topology, and linkage disequilibrium in the United Kingdom, converge to indicate a greater contribution of clonal propagation of *C. hominis* in this country, and perhaps also of *C. parvum* in New Zealand.

Therefore, rather than being species-specific, *C. parvum* and *C. hominis* populations appear to be shaped by local and host-related factors. The author postulates that frequent transmission in highly contaminated environments increases the probability of infections with genetically heterogeneous parasites, favouring recombination. In countries where the sanitary conditions are better and HIV is less prevalent (like in the UK), coinfections with heterogeneous parasites originating from environmental sources may be less frequent, and clonal propagation may prevail.

In summary, the results presented here indicate that although *C. parvum* and *C. hominis* are present worldwide, gene flow does not seem sufficient to erase genetic divergence among geographically separated populations, supporting a model of allopatric diversification. As a consequence, multilocus genotyping would enable in many cases the tracking of parasites to their country of origin. Rather than conforming to a strict paradigm of either clonal or panmictic species, the data are consistent with the co-occurrence of both propagation pathways. The relative contribution of each pathway appears to vary according to the prevailing ecological determinants of transmission, indicative of a high adaptive plasticity in *C. parvum* and *C. hominis*.

2.3 CONCLUDING REMARKS

The results of the studies presented in this Chapter indicate the existence of a conspicuous sub-structuring in space and host-range, of the genetic repertoire of *C. parvum* and *C. hominis*. These features support the idea of the existence of anthroponotic *C. parvum* parasites that do not cycle in cattle, and indicate the feasibility of tracking of *Cryptosporidium* parasites to their

country of origin using molecular tools. A combination of different analytical approaches, rather than linkage disequilibrium alone where results are open to multiple explanations, was used in the study presented in Section 2.2 to infer the population genetic structures of *C. parvum* and *C. hominis* at a global scale. The results converged in indicating that *C. hominis*, and perhaps *C. parvum,* are capable of diversifying both by mutation and recombination. The reliability of genetic markers as faithful indicators of ancestry (and their usefulness as epidemiological markers) may thus vary among regions in function of the relative contribution of recombination to the genetic diversification. The clinico-epidemiological implications of the bio-geographical sub-structuring of *C. parvum* and *C. hominis* still need to be established.

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CHAPTER 3

EPIDEMIOLOGICAL STUDIES OF CRYPTOSPORIDIOSIS IN DOMESTIC ANIMALS IN NEW ZEALAND

This chapter contains three epidemiological studies of cryptosporidiosis in New Zealand on horses (Sections 3.1- 3.3) and cattle (Sections 3.4 and 3.5). The studies on horses were initiated in 2002 with a letter submitted by the author to New Zealand equine practitioners. In that letter, the practitioners were asked to co-operate in identifying cases of cryptosporidiosis in foals in order to genetically characterise the *Cryptosporidium* isolates, given the lack of data available. Following this initiative, a phone call from a concerned clinician in the Waikato (Dr. Laurinda Oliver) was received, which prompted the outbreak investigation reported in Section 3.1. The molecular testing described in this section was kindly performed and described by the late Jim Learmonth. The subsequent studies reported in Section 3.2 and 3.3 were performed in 2006 and 2007. The studies on cattle were performed in 2002 (Section 3.4) and 2006 (Section 3.5). Except for some diagnostic testing performed in the study in Section 3.1, all the work for these studies was performed at Massey University.

Four of the above studies have been published as:

Section 3.1: Grinberg A, Oliver L, Learmonth JJ, Leyland M, Roe W, Pomroy WE. Identification of *Cryptosporidium parvum* 'cattle' genotype from a severe outbreak of neonatal foal diarrhoea. *Veterinary Record* 153, 628-30, 2003

Section 3.2: Grinberg A, Learmonth J, Kwan E, Pomroy W, Lopez Villalobos N, Gibson I, Widmer G. Genetic diversity and zoonotic potential of *Cryptosporidium parvum* causing foal diarrhoea. *Journal of Clinical Microbiology* 46:2396-98, 2008

Section 3.3: Grinberg A, Pomroy WE, Carslake H, Shi Y, Gibson I, Drayton B. A study of neonatal cryptosporidosis of foals in New Zealand. *New Zealand Veterinary Journal* 57, 284-289, 2009

Section 3.4: Grinberg A, Pomroy W, Weston JF, Ayanegui-Alcerreca A, Knight D. The occurrence of *Cryptosporidium parvum*, *Campylobacter* and *Salmonella* in newborn dairy calves in the Manawatu region of New Zealand. *New Zealand Veterinary Journal* 53, 315-20, 2005

The studies are presented as published, except that their format has been modified as indicated in the Preface.

3.1 IDENTIFICATION OF *CRYPTOSPORIDIUM PARVUM* 'CATTLE' GENOTYPE FROM A SEVERE OUTBREAK OF NEONATAL FOAL DIARRHOEA

3.1.1 Summary

A severe outbreak of diarrhoea in Thoroughbred foals accompanied by shedding of *Cryptosporidium*-like structures in the faeces of the affected animals was investigated. The outbreak occurred in 2002, in a commercial Thoroughbred broodmare farm in the Waikato region of New Zealand. Nine foals suffered from acute, mild, and severe disease accompanied by dehydration and weakness. Despite intensive treatments, two foals died from the disease and a third was euthanased due to severe condition. Six faecal samples from affected foals were sent to a veterinary diagnostic laboratory where they were tested for bacterial and viral enteropathogens and for *Cryptosporidium*. Five faecal samples were positive for *Cryptosporidium*. Postmortem examinations were performed on the euthanased foal and on the dead animals. Gross findings included extreme emaciation and dilated, thin-walled, fluid-filled intestines with no evidence of inflammation in the abdominal cavity. Tissues and a faecal specimen from the euthanised foal and three faecal specimens from other foals on the farm were submitted to Massey University. All four faecal specimens were negative for *Salmonella* species, *Yersinia* species, and group A rotavirus VP6 protein. Microscopically, round, acid-fast structures resembling *Cryptosporidium* oocysts were seen in the four specimens. Histopathologically, numerous round, PAS-negative, Giemsa-positive, *Cryptosporidium*-like organisms, 2-5 µm in diameter, were present at the apical border of villous epithelial cells throughout the intestine.

Cryptosporidium isolates recovered from the faeces of three foals were subjected to a multilocus genetic characterization. Sequence analysis of a 850 base-pair amplicon from the 18S rRNA gene revealed sequences identical to each other and to the *C. parvum* 'cattle' genotype sequences published at the GenBank accession number AF093490. The multilocus genotyping showed restriction fragment length features consistent with *C. parvum* 'cattle' genotype at the β-tubulin gene, the poly threonine repeat, COWP, and the RNR genes. This is the first report of an outbreak of cryptosporidiosis in foals which incorporated epidemiological, clinical and pathological data, as well as genetic characterization of the outbreak isolates.

3.1.2 Introduction

The intestinal protozoan parasite *Cryptosporidium parvum* has been intensively studied over the past decade, due to its major impact on human and animal health. So far, two genotypes of *C. parvum* have been extensively characterised: the 'human' genotype, primarily associated with infections in man, and the 'cattle' genotype, found in human beings and also in domestic livestock such as cattle, sheep and goats (Morgan et al. 2001; Akiyoshi et al. 2002). The genetic divergence between the two genotypes manifests at a number of genomic loci, including the 18S rRNA gene (Xiao et al. 2000), the *Cryptosporidium* oocyst wall protein (COWP) gene (Spano et al. 1997), the ribonuclease reductase (RNR) gene (Widmer et al. 1998), the

polythreonine repeat (poly T) (Carraway et al. 1997) and the β-tubulin gene (Caccio et al. 1999). Extensive within-genotype genetic heterogeneity also exists (Widmer et al. 2002; Mallon et al. 2003ab). Equine cryptosporidiosis was initially described in immunodeficient Arabian foals using morphometric and morphologic parasitological methods, followed by descriptions of the disease also in immunocompetent foals (Snyder et al.1978; Gibson et al. 1983; Gajadhan et al.1985; Coleman et al. 1989).

Whereas some surveys indicate that infections with *Cryptosporidium* species in horses are relatively common, reports confirming its role in foal diarrhoea are scarce and attempts to produce experimental disease have been unsuccessful (Tzipori and Campbell 1981; Tzipori 1983; Coleman et al. 1989; Xiao and Herd 1994; Netherwood et al. 1996; Cole et al. 1998). Moreover, the published data on the genetic makeup and biology of the equine isolates is scant and, strictly speaking, even their taxonomy within the genus of *Cryptosporidium* is still unresolved.

This study describes the results of an investigation of a severe outbreak of diarrhoea in thoroughbred foals, accompanied by the shedding of *Cryptosporidium*-like structures in the faeces of the affected animals. To the author's knowledge, this is the first report of an outbreak of cryptosporidiosis in foals, which incorporates epidemiological, clinical and pathological data, as well as the genetic characterisation of the outbreak isolates.

3.1.3 Materials and Methods

Outbreak characteristics. During the peak of the foaling season in 2002, there was a severe outbreak of foal diarrhoea in a commercial thoroughbred broodmare farm located in the Waikato region of New Zealand. According to the practitioner in charge, the outbreak lasted for approximately one month and, during that period, nine foals suffered from acute, mild to severe disease accompanied by dehydration and weakness. Approximately 30 foals were born on the farm during the same period. The index case and six other foals were aged between four and nine days at the onset of diarrhoea. The other two manifested the disease when they were three weeks of age, which was seven to 10 days after returning to the farm from a regional neonatal intensive care unit where they were sent soon after birth due to unrelated conditions. During the outbreak, two foals died from the disease and a third was euthanised due to its severe clinical condition despite intensive treatments, which included intravenous fluids, broad-spectrum antibiotics (including metronidazole), gastric protectants and anti-ulcer medication. Fresh cow colostrum was also given via a nasogastric tube to some of the foals as an external source of immunoglobulins. None of the affected foals was an orphan or on a foster mare. All mares had colostrum tested at the time of foaling and had adequate readings on a refractometer, with colostrum immunoglobulin G (IgG) levels corresponding to approximately 60 g/litre. The serum IgG levels of three affected foals were more than 800 mg/dl, indicating normoglobulinaemia. A fourth foal had an IgG level of 640 mg/dl, attributable to failure of passive transfer or to the

consumption of antibody over the course of the disease. Beef cattle co-existed with the foals and adult horses on adjacent paddocks of the same farm. Yearling cattle arrived every year from a constant external source and no cows calved on the farm. Some paddocks used by horses during the outbreak had been previously grazed by cattle. Foals aged up to one week were normally housed in a barn with a high animal turnover but, during the outbreak, all new foals were subsequently stabled in an alternative building and a strict isolation of the sick animals was promptly implemented. Six faecal samples from affected foals were sent to a commercial veterinary diagnostic laboratory where they were tested for bacterial and viral enteropathogens and *Cryptosporidium*. One of the samples had Gram-positive rods resembling *Clostridium* species, but it was negative for *Clostridium perfringens* on culture. Testing for *Clostridium difficile* toxin was not performed. Five faecal samples were positive for *Cryptosporidium* oocysts. Other bacterial or viral pathogens were not detected. Postmortem examinations were undertaken on the euthanased foal and on the two that died. Gross findings included extreme emaciation and dilated thin-walled, fluid-filled intestines with no evidence of inflammation in the abdominal cavity.

Laboratory methods. Pieces of small and large intestines, kidney, lung and liver from the euthanased foal were collected in 10 per cent formalin and submitted to Massey University, along with faecal samples from the euthanased foal and from three other affected foals which were still alive. The tissues were processed routinely for histopathology and all sections were stained with haematoxylin and eosin. Sections of intestine were also stained with periodic acid-Schiff (PAS) and Giemsa. The faecal samples were tested for the presence of several pathogens. For *Salmonella* species, the faeces were directly inoculated onto Xylose Lysine-dehoxycolate (XLD) plates and incubated at 37°C for 24 hours, or placed in selenite enrichment broth for 24 hours at 37°C with subsequent subculturing onto XLD and incubation as before. For *Yersinia* species, faeces were directly inoculated onto selective-differential media including cefsulodin, irgasan and novobiocin (CIN) and incubated at 37°C for 48 hours. These were placed in tubes containing phosphate-buffered saline (pH 7,3), and incubated up to 14 days at 2 to 4°C, with subsequent subcultures onto CIN plates, and then incubated as above. Virological procedures included testing for the faecal VP6 protein of group A rotavirus with commercial immunochromatographic kits, according to the manufacturer's instructions (Rota-Srip; Coris BioConcept, Gembloux, Belgium). For *Cryptosporidium* species, a method based on faecal oocyst sedimentation by centrifugation, followed by acid-fast stain of smears and screening with a light microscope at x400 was applied (the method is described in Section 3.4).

After completion of the diagnostic tests, the faecal specimens were mixed with equal volumes of 2 per cent potassium dichromate and conserved at 2 to 4°C for further genetic analyses. Two months later, *Cryptosporidium* isolates recovered from three faecal specimens were subjected to a multilocus genetic characterisation. Oocysts were concentrated from the faeces and most of the faecal debris was removed by the formol saline/ether method (Allen and Ridley 1970).

Methanol-fixed smears of the oocyst concentrates were stained with an immunofluorescent monoclonal antibody, according to the manufacturer's instructions (Merifluor C/G, Mediridian Bioscience, Cincinnati, Ohio, USA), and examined with an epifluorescent microscope at x200. The smears were positive for *Cryptosporidium* species and negative for *Giardia* species. To isolate the oocysts from the remaining faecal debris, an immunomagnetic separation kit was used according to the manufacturer's instructions (Dynal®, Invitrogen, Carlsbad, CA, USA). DNA was extracted by suspending the oocysts in 100 µl of TE buffer (10mM Tris hydrochloride, 1mM disodium ethylenediamine tetra-acetic acid, pH 8·5) containing 1 per cent Nonidet P40. To this, 20 µl of a 20 per cent suspension of Chelex 100 resin (Biorad Laboratories, Hercules, CA, USA) was added before five freeze/thaw cycles of two minutes in liquid nitrogen and two minutes in 95°C water. Cell debris was removed by centrifugation at 10,000 g for three minutes and the supernatant was collected and stored at 4 °C.

Each isolate was characterised at five genomic loci. A nested PCR-restriction fragment length polymorphism (PCR-RFLP) analysis was applied at the 18S rRNA gene as described by Xiao et al. (2000), followed by the sequencing in both directions of the 850 bp fragment of the secondary amplicon using an ABI Prism 377 autosequencer (Perkin Elmer, Waltham, MA, USA). The 18S rRNA gene sequences were aligned with other *Cryptosporidium* 18S rRNA gene sequences in Genbank using Clustal W software (Thompson et al. 1997).
PCR-RFLP methods were also applied at the β-tubulin gene (Caccio et al.1999), COWP gene (Spano et al. 1997), RNR gene (Widmer et al. 1998) and a *Cryptosporidium* poly threonine repeat (Poly T) (Carraway et al. 1997) using the restriction enzymes *Vsp*I (Promega, Madison, WI, USA), *Dde*I (Roche, Auckland, NZ), *Rsa*I (Invitrogen, Carlsbad, CA, USA), *Tsp*509 I (New England BioLabs, Ipswich, MA, USA) and *Rsa*I (Invitrogen, Carlsbad, CA, USA), respectively, according to the manufacturers' instructions. Restriction fragments were resolved on 3.5 per cent agarose and the gel was visualised by ethidium bromide staining. The DNA from a previously characterised *C. parvum* 'human' genotype isolate was included in the analysis as a control.

3.1.4 Results

The four faecal samples were negative for *Salmonella* spp. *Yersinia* spp. and group A rotavirus VP6. Microscopically, round, acid-fast structures resembling *C. parvum* oocysts were seen. Histopathologically, villi within the duodenum and ileum were moderately atrophic and occasional groups of villi were fused. There were mildly increased numbers of lymphocytes and plasma cells, with occasional neutrophils, in the lamina propria. Numerous round, PAS-negative, Giemsa-positive, *Cryptosporidium*-like organisms, 2 to 5 µm in diameter, were present at the apical border of villous epithelial cells throughout the intestine (Figure 3.1). These organisms were most numerous in the duodenum, where they extended to the base of crypts, but a few organisms were present in the colon. Within the jejunum there were occasional focal areas of vacuolation of epithelial cells at the tips of the villi. No other relevant histological

abnormalities were noted in the other tissues examined. The lesions were consistent with intestinal cryptosporidiosis (Kim 1990).

Sequence analysis of the 850 bp secondary amplicon from the nested PCR-RFLP of the 18S rRNA gene revealed that the three isolates from the foals were identical to each other and to the published sequences for the 'cattle' genotype (GenBank accession number AF 093490). The multilocus PCR-RFLP analysis showed restriction fragments consistent with *C. parvum* 'cattle' genotype at the *β-tubulin* gene, poly T, COWP, and RNR genes (Table 3.1; Figure 3.2).

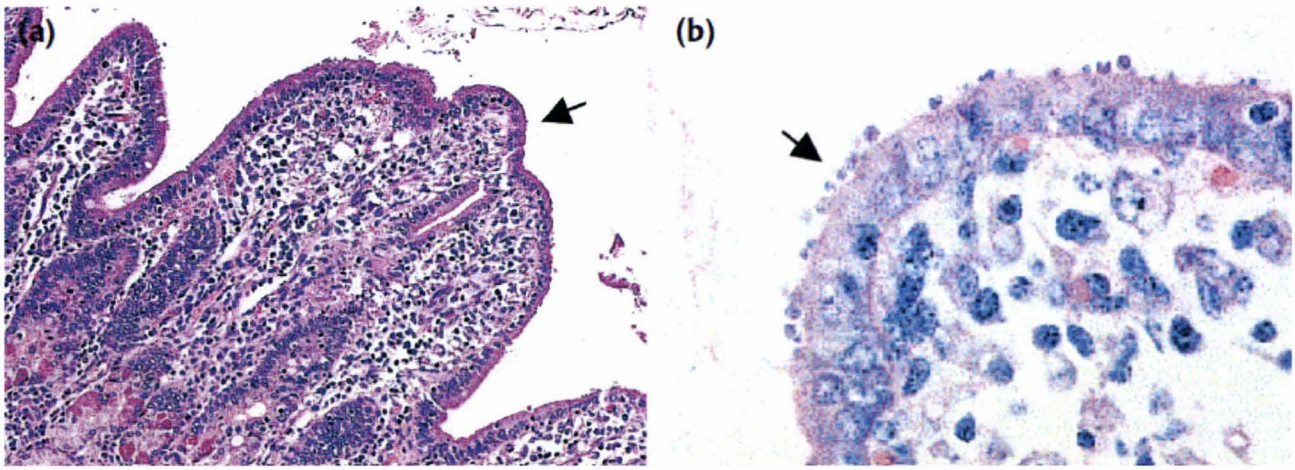

Figure 3.1 (a) Fused and atrophic duodenal villi (arrow) and mildly increased numbers of mononuclear inflammatory cells within the lamina propria. Haematoxylin and eosin, x100. (b) Protozoal organisms among microvilli on the luminal border of a duodenal villus (arrow). Giemsa, x 600

Locus	PCR length (in base-pairs)	Restriction endonuclease	'Human genotype' fragments size (in base-pairs)	'Bovine genotype' fragments size (in base-pairs)
Poly T	318	*RsaI*	318	45, 273
β-tubulin	592	*DdeI*	592	178, 414
RNR	441	*Tsp509 I*	180, 210	10, 47, 71, 96, 107, 110
COWP	550	*RsaI*	34, 106, 125, 285	34, 106, 410
18S rRNA	832	*VspI*	82, 86, 104, 560	82, 104, 645

Table 3.1 Restriction fragment length polymorphisms between *C. parvum* "cattle' and 'human' genotypes. The bibliographic references for each locus are provided in the text.

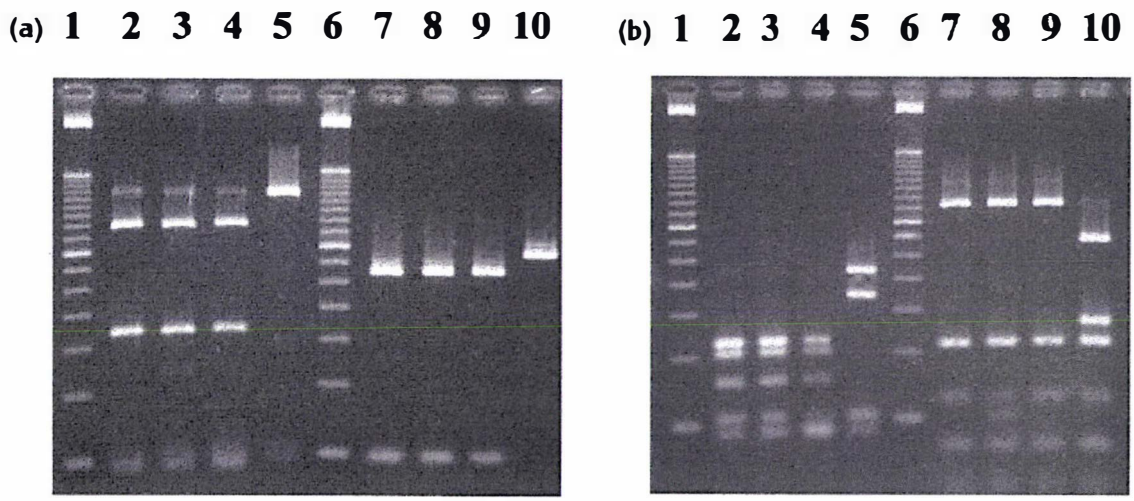

Figure 3.2 PCR-restriction fragment length polymorphism analysis of β-tubulin, poly T, RNR and COWP genes. (a) Lanes 1 and 6 50 bp ladder, Lanes 2 to 4 β-tubulin, horse isolates, Lane 5 β-tubulin, human genotype, Lanes 7 to 9 Poly T, horse isolates, Lane 10 Poly T, human genotype. (b) Lanes 1 and 6 50 bp ladder, Lanes 2 to 4 RNR, horse isolates, Lane 5 RNR, human genotype, Lanes 7 to 9 COWP, horse isolates, Lane 10 COWP, human genotype

3.1.5 Discussion

The results of this study indicate the possibility of the emergence and circulation of *C. parvum* 'cattle' genotype in foals, which might cause, or be a co-factor of, severe, life-threatening outbreaks of diarrhoea in apparently immunocompetent, normoglobulinaemic animals. This investigation did not include the whole range of possible causes of diarrhoea in newborn foals, and it is impossible to rule out completely co-morbidity with other causes. Nonetheless, the epizootological features and the histopathological findings were most consistent with cryptosporidiosis.

In this case, as in an outbreak previously reported by Konkle et al. (1997), there was a temporal-spatial link between the horses and calves. Human and calf cryptosporidiosis due to C. *parvum* 'cattle' genotype is commonly diagnosed in the Waikato district in New Zealand (Learmonth et al. 2001), a district also known for its intensive cattle-rearing industry and relatively high density of thoroughbred breeding premises. The same characteristics probably exist in other horse-rearing countries, yet, to the authors' knowledge, this is the first report confirming cryptosporidiosis in foals with identification of *C. parvum* 'cattle' genotype. This would suggest that the disease is being underdiagnosed in the field, or that the tests used by most veterinary diagnostic laboratories, while being well established for calves, are inadequate for diagnosing cryptosporidiosis in foals. Alternatively, it is plausible that, at present, the

susceptibility of horses to *C. parvum* 'cattle' genotype is limited to a relatively narrow spectrum of pathogenic horse-adapted subtypes, and that disease would only manifest following specific sporadic host-parasite encounters. This may also account for previous unsuccessful attempts to produce experimental disease in foals with bovine isolates (Tzipori 1983).

In order to test this hypothesis, a battery of horse isolates of *C. parvum* 'cattle' genotype from different outbreaks needs to be characterised by multilocus genetic typing of high discriminatory power, which may be followed by analysis of the population genetic structure (Aiello et al. 1999, Caccio et al. 2000, Mallon et al. 2003a,b). Such a study in horses would also further the general understanding of the molecular mechanisms of host adaptation in *C. parvum*.

3.2 GENETIC DIVERSITY AND ZOONOTIC POTENTIAL OF *CRYPTOSPORIDIUM PARVUM* CAUSING FOAL DIARRHOEA

3.2.1 Summary

Section 3.1 reported the first known report of an outbreak of cryptosporidiosis in foals which included the identification of the isolates as *C. parvum*. The author postulated that the susceptibility of horses to *C. parvum* may be limited to a relatively narrow spectrum of horse-adapted subtypes, and disease would only manifest following specific sporadic host-parasite encounters. Therefore, in the study presented in this section *Cryptosporidium* isolates from diarrhoeic foals in New Zealand (n=9) collected during the above outbreak and in 2006-2007, were initially again identified as *C. parvum*. Then, the isolates were subtyped at two polymorphic loci and compared with human (n=45) and bovine (n=8) isolates. Foal *C. parvum* were genetically-diverse, markedly similar to human and bovine isolates, and carried GP60 *IIa* A18G3R1 alleles, indicating a zoonotic potential.

3.2.2 Introduction

Intestinal *Cryptosporidium* parasites, in particular *Cryptosporidium parvum*, are common causes of diarrhoea in humans and animals worldwide. *C. parvum* is also a zoonotic species.

Diarrhoeal disease is a common clinical condition in newborn foals (Cohen 1994, Knottenbelt et al. 2005; Magdesian 2005; Crabbe 2007). While results of a number of studies suggest that intestinal carriage of *Cryptosporidium* is relatively common in horses (Xiao and Herd 1994; Cole et al. 1998; Hajdušeka et al. 2004; Majewska et al. 2004; Chalmers et al. 2005), reports confirming the role of *Cryptosporidium* in diarrhoea in foals are rare, and their pathogenic potential in these hosts is still debated (Wilkins 2004; Crabbe 2007). The idea of *Cryptosporidium* in foals being of low pathogenicity is difficult to reconcile with our previous report of an outbreak of foal diarrhoea in which *C. parvum* was identified as the sole agent (Section 3.1). Therefore, the author hypothesised that disease in foals may be underdiagnosed, or alternatively, may only manifest following specific encounters with a narrow spectrum of horse-adapted parasites (Section 3.1). However, at that juncture the hypothesis of the existence of horse-adapted *C. parvum* could not be corroborated, as highly-discriminatory molecular tools for the subtyping of the isolates were not widely available.

The aim of this study was to test this hypothesis using novel molecular tools. Therefore, *Cryptosporidium* isolates collected in New Zealand from diarrhoeic foals in 2002, 2006 and 2007 were genetically identified and subtyped by sequencing of the polymorphic regions of the sporozoite 60-kDa glycoprotein (GP60) and the 70 kDa heat shock protein (HSP70) genes. To infer about the possible routes of transmission and the zoonotic potential, the isolates from foals were compared with isolates from humans and cattle also isolated in New Zealand.

3.2.3 Materials and Methods

Parasites. *Cryptosporidium*-positive diagnostic faecal specimens collected in New Zealand from foals (n=9), humans (n=45), and cattle (n=8), were used in this study. The specimens from foals were collected during the foaling seasons of 2002 (n=3), 2006 (n=5), and 2007 (n=1). The specimens from 2002 originated from an outbreak of foal cryptosporidiosis in the Waikato region, which has been described in Section 3.1. Four specimens from 2006 originated from an outbreak of foal diarrhoea in a farm located in the North Island (see Section 3.3). One of these specimens originated from a foal that was hospitalised due to severe diarrhoea, and the other three were collected a week later by the first author, from 1-2 weeks-old diarrhoeic foals presented for examination during a visit to the farm. The fifth specimen from 2006 and the specimen from 2007 were diagnostic specimens from two and three weeks old diarrhoeic foals, and were donated by a commercial laboratory operating in the North Island. No additional information was provided on these sporadic specimens. The human specimens were collected between 2001 and 2003 as part of a different study (Learmonth et al. 2004). As only *C. parvum* was identified in foals (see below), *C. parvum*-positive human specimens were used. The bovine specimens originated from diarrhoeic calves on eight farms, and were collected between August -October 2006 by a veterinary laboratory operating in the North Island. All faecal specimens were stored between 2-4°C, with no preservatives. Faecal specimens collected in 2002 were preserved in 2 per cent potassium dichromate (Section 3.1).

***Cryptosporidium* identification and subtyping**. The identification of human *C. parvum* and foal *C. parvum* collected in 2002 has been previously done and described by the late Jim Learmonth and co-workers of the Protozoan Research Unit, Massey University (Learmonth et al. 2004; Section 3.1). The identification of *Cryptosporidium* from 2006 and 2007 was performed using a nested PCR followed by sequence analysis of a ~850 base-pair segment of the 18S rRNA gene (Xiao et al. 2000). Genomic DNA of the specimens from foals from 2006 was extracted from the oocysts as described in Section 3.1, with a modification consisting of the use of three freeze/thaw cycles of one minute in liquid nitrogen and water at 95°C. Genomic DNA of bovine specimens and the foal specimen from 2007 was extracted using a DNA extraction kit (DNA Stool Mini Kit, Qiagen, Hilden, GmbH). The nested PCR was performed in a thermo-cycler (GeneAmp 9700 Applied Biosystems, Foster City, CA), using the primers 5`-GTT AAA CTG CGA ATG GCT CA -3`(forward) and 5`-CCA TTT CCT TCG AAA CAG GA-3` (reverse) for the primary amplification of a ~1325 base-pairs region. The 20 µl reaction mixture consisted of 2 µl of 10X PCR buffer (Invitrogen, Carlsbad, CA, USA), 6 mM $MgCl_2$, 250 mM (each) deoxyribonucleoside triphosphate, 0.2 mM (each) primer, 2.5 units of Platinum® Taq (Invitrogen, Carlsbad, CA, USA), and 1 µl of DNA template. Thermo-cycling consisted of a hot start at 96°C for two minutes followed by 35 cycles of 94°C for 30 sec., 55° C for 30 sec., 72° C for 45 sec. and a final extension of 72° C for 5 min. The primary PCR product was diluted 1:10 with water prior to a secondary amplification with the primers 5'-CTC GAC TTT ATG GAA GGG TTG-3' and 5'- CCT CCA ATC TCT AGT TGG CAT A -3'. With the exception of 3 mM $MgCl_2$

the PCR and cycling conditions were the same as the primary round. This secondary PCR product was 850bp in length (Xiao et al. 2000). Molecular-grade water and a *C. parvum*-positive specimen from a calf were used as negative and positive controls. Amplicons were electrophoresed in 1.5% agarose, then stained with ethidium bromide and visualised under UV light. Products of the expected size were purified on a column (High Pure PCR Product Purification Kit, Roche Diagnostics, Mannheim, GmbH) and the DNA concentration was measured using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Wilmington, DE, USA). Amplicons were then sequenced in both directions using an ABI 3730 DNA analyser (Applied Biosystems, Foster City, CA).

Complementary sequences were aligned and edited; marginal segments that could not be accurately determined were trimmed and the final sequences aligned with *Cryptosporidium* 18S rRNA gene sequences in our database using ClustalX software (Thomson et al. 1997). For the subtyping, genomic DNA was extracted using extraction kits as above. Subtyping was achieved by means of sequencing two polymorphic loci. The first was a ~ 830 base-pairs region of the *Cryptosporidium* sporozoite GP60 gene. This locus was amplified by Jim Learmonth using the nested PCR described by Glaberman et al. (2000), except that the annealing temperature of the primary PCR of the present assay was 60°C. The second locus was a ~ 470 base-pairs region of the HSP70 gene that comprises a polymorphic repeat (Khramtsov et al. 1995; Mallon et al. 2003ab). Amplification of this locus was performed using a reaction developed by Mr. Errol Kwan, Protozoa Research Unit, Massey University.

The PCR mixture included 200µM of the primers 5'- CACCATCCAAGAACCAAAGG (forward), and 5'- GCCTAAAGGTAGAGTGTGCTTTTC (reverse), 1xPCR buffer (Invitrogen, Carlsbad, CA), 1.5mM MgCl2, 1mM dNTPs (Fermentas Lifesciences, GmbH), and 1 unit of taq polymerase (Platinum® Taq, Invitrogen, Carlsbad, CA), in a final volume of 20µl. Thermo-cycling consisted of 1 cycle of 96°C for 2 mins, 57°C for 2 mins, and 72°C for 2 mins, then 40 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec, and a final elongation step of 72°C for 2 mins. Water was used as a negative control in each batch testing. GP60 and HSP70 amplicons were electrophoresed, purified, and sequenced as above.

Final sequences were aligned with published GP60 and HSP70 gene sequences (Khramtsov et al. 1995; Strong et al. 2000). Due to the conserved nature of the HSP70 protein among eukaryotic organisms, the possibility that HSP70 genes from other organisms were amplified was checked by clustering the sequences on line with similar sequences deposited in GenBank using the neighbor-joining clustering algorithm of BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, accessed on January 2008) (provided by The National Center for Biotechnology Information of the National Institutes of Health, USA).

Initially, human and bovine *Cryptosporidium* were not sequenced at the HSP70 locus. However, isolates having GP60 sequences identical to the sequences in foal isolates were chosen at random and subtyped at the HSP70 locus, allowing a comparison between bilocus sequence types (BLST) from different hosts.

3.2.4 Results

All the 18S rRNA gene sequences of foal and bovine *Cryptosporidium* were indistinguishable from the *C. parvum* sequence deposited in GenBank under accession number AF093490 (Xiao et al. 2000). One foal sequence from 2006 initially differed by 1 base-pair, but re-extracted DNA yielded a sequence indistinguishable from AF093490. Six foal specimens amplified at both the GP60 and HSP70 loci, one at the GP60 locus only, one at the HSP70 locus only, and one could not be amplified at either locus. The edited GP60 and HSP70 sequences were longer than 710 and 420 base-pairs, respectively, and comprised the repeat regions of both loci. All foal, human and bovine *C. parvum* had GP60 *IIa* A18G3R1 alleles according to the nomenclature suggested by Peng et al. (2001) and Sulaiman et al. (2005). This nomenclature consists of the letter 'A' followed by the number of TCA codons, the letter 'G' followed by the number of TCG codons, and the letter 'R' followed by the number of ACATCA sequences at the end of the polyserine repeat.

There were five different GP60 sequences in foal *C. parvum*, differing by single nucleotide polymorphisms (SNP) but exhibiting more than 99% similarity with each other (Tables 3.2 and 3.3). The foal *C. parvum* GP60 sequences observed in this study have been deposited in GenBank under accession numbers EU483074 to EU483080.

There were five different HSP70 sequences *C. parvum* from foals, which differed with each other by SNPs and from the sequence reported by Khramtsov et al. (1995) by the number of minisatellite repeats and/or SNPs external to the repeat. Using Blast software, these sequences clustered only with *C. parvum* sequences. In each outbreak, at least one isolate differed from others at both loci. Eight subtyped foal *C. parvum* defined seven genetic variants (Table 3.3). Identical GP60 sequences were found in three *C. parvum* from foals, 41/45 from humans, and the eight from calves. Ten human *C. parvum* and the eight bovine *C. parvum* with this GP60 allele were also sequenced at the HSP70 gene, revealing the same BLST in two foal, 10 human and seven bovine *C. parvum.*

Bases position*	Codon change	Deduced amino acid change	*C. parvum* positive specimen in which the polymorphism was seen
419 - 421	TCA to TCG	serine to serine	1-6; 9
425 - 427	TCA to CCA	serine to proline	5
563-565	TCT to TCC	serine to serine	4
629-631	CAA to CGA	glycine to arginine	6
647-649	ACC to CCC	threonine to proline	6
671-673	AAA to AGA	lysine to arginine	5
698-700	ATG to GTG	metionine to valine	5
971-973	ACC to GCC	threonine to alanine	4
1070-1072	AGA to AGG	arginine to arginine	3

Table 3.2 Single nucleotide polymorphisms and deduced amino acid changes in the 60-kDa glycoprotein of *C. parvum* isolates from foals, as compared with the sequence reported by Strong et al in GenBank accession number AF022929. Differences in the number of serine repeats are not reported.

* According to Strong et al. (2000).

Isolate number*	GP60 allele designation	HSP70 allele designation	BLST designation
1/A (foal/Waikato/02)	1	1	1
2/1 (foal/Waikato/02)	1	2	2
3/2 (foal/Waikato/02)	2	3	3
4/74 (foal/Manawatu/06)	3	3	4
5/73 (foal/Manawatu/06)	4	DN	ND
6/48 (foal/Manawatu/06)	5	4	5
7/72 (foal/Manawatu/06)	DN	DN	ND
8/47 (foal/Waikato/06)	DN	5	ND
9/569 (foal/Waikato/07)	1	2	2
10-19 (human)	1	2	2
20-26 (bovine)	1	2	2
27 (bovine)	1	ND	ND

Table 3.3 Bilocus sequence types (BLST) of foal, human, and bovine *Cryptosporidium* parvum in New Zealand. Arbitrary numbers designate alleles and BLSTs. Isolates 9/569 and 8/47 are from the sporadic cases. BLST, bilocus sequence type; DN, did not amplify; GP60, *C. parvum* 60-kDa surface glycoprotein; HSP70, *C. parvum* heat-shock protein 70 gene; ND: not determined. *: these isolate numbers were used also in GenBank

3.2.5 Discussion

This is the first report describing the genetic diversity of *Cryptosporidium* parasites causing foal diarrhoea. The key findings of this study were the high genetic diversity of foal *C. parvum* and their similarity with human and bovine isolates.

Based on the identification of a novel partial 18S rRNA gene sequence in a Przewalski's wild horse (*Equus przewalskii*) in a zoo (Ryan et al. 2003), Xiao and Feng suggested that horses are infected with a *Cryptosporidium* 'horse genotype' (Xiao and Feng 2008), which has never been identified in other hosts. In addition, results of recent molecular studies support the possibility of the existence of host-restriction in *C. parvum* (Mallon et al. 2003a,b; Xiao and Feng 2008).

Thus, the mere identification of *C. parvum* in the 2002 outbreak did not allow conclusions to be drawn about the origin or zoonotic potential of foal *Cryptosporidium*. In this study, the isolates were identified, subtyped, and then compared with human and bovine isolates.

Conforming with the superdiverse genetic structure described for *C. parvum* populations in Section 2.1, eight foal isolates could be subdivided into seven genetic variants. Nonetheless, the dominant *IIa* GP60 allele and BLST were shared by the three host species. GP60 *IIa* alleles are also highly prevalent in human and bovine *C. parvum* in other countries (Sulaiman et al. 2005; Leoni et al. 2007). Therefore, the genetic repertoire of foal, bovine and human *C. parvum* largely overlap, and so foal *C. parvum* should be considered potentially zoonotic.

Lastly, in accordance with the results of waterborne outbreak investigations in humans (Mathieu et al. 2004; Glaberman et al. 2000; Leoni et al. 2007), we identified different *C. parvum* alleles among foals in two outbreaks, suggesting that genetically-heterogeneous parasite assemblages may be involved in outbreaks of cryptosporidiosis. This feature may hamper our ability to track infection sources using molecular tools.

3.3 A STUDY OF NEONATAL CRYPTOSPORIDIOSIS OF FOALS IN NEW ZEALAND

3.3.1 Summary

Sections 3.1 and 3.2 dealt with the molecular characterisation of *Cryptosporidium* parasites causing neonatal diarrhoea in foals. The study presented in the present Section deals with the clinico-epidemiological aspects of neonatal cryptosporidiosis in farmed foals in New Zealand. The *Cryptosporidium* isolates collected during this study were included in the study reported in Section 3.2.

To assess the occurrence of *Cryptosporidium* oocysts in diagnostic faecal specimens from foals, selected specimens received by a diagnostic veterinary laboratory in New Zealand between 2006 and 2007 were submitted to Massey University (MU) and tested microscopically for the presence of *Cryptosporidium* oocysts. Then, the *Cryptosporidium* parasites were genetically identified to taxon level. In addition, specimen submission data from the participating laboratory for 2005-2007 were examined.

Twelve faecal specimens from diarrhoeic foals were transferred to MU between 2006 and 2007, from which three tested positive for *C. parvum*. One *Cryptosporidium*-positive specimen identified in the course of the study originated from a foal that was hospitalised due to severe diarrhoea. This case triggered an on-site investigation of the broodmare farm that it came from, which revealed a high incidence of neonatal diarrhoea in foals. Four affected foals were examined during two visits to the farm. In all four cases, the disease was self-limiting and manifested during the second week of life, rasembling foal heat diarrhoea. A similar clinical manifestation had been observed by the owner and the veterinarian in charge in most of the foals born on the farm during the foaling season. *Cryptosporidium parvum* was the only enteropathogen found in the faeces of the 4/4 affected foals examined. The oocyst shedding was of short duration and intense in all cases, reminiscent of cryptosporidiosis in calves.

Specimen submission records for 2005-2007 indicated 67 faecal specimens were tested for *Cryptosporidium* by the participating laboratory, and 12 (18%) tested positive.

3.3.2 Introduction

Numerous microorganisms, parasites, and non-infectious factors have been associated with neonatal diarrhoea in foals (Magdesian 2005). However, the causal role of many of these agents is uncertain, and it has been estimated that the aetiological diagnosis of diarrhoea in foals remains elusive in about 50% of cases (Knottenbelt et al. 2005).

Cryptosporidium protozoan parasites, in particular *C. parvum*, are common causes of diarrhoea in humans and young calves worldwide. *Cryptosporidium parvum* is also a zoonotic species (Fayer 2008). Carriage of *Cryptosporidium* parasites appears to be relatively common in horses

(Tzipori and Campbell 1981; Coleman et al. 1989; Xiao and Herd 1994; Cole et al. 1998; Hajdušeka et al. 2004; Majewska et al. 2004; Chalmers et al. 2005). However, reports confirming the role of *Cryptosporidium* in diarrhoea in foals are scarce, and their pathogenic potential in immunocompetent animals has been debated (Wilkins 2004; Crabbe 2007). The concept of low pathogenicity for *Cryptosporidium* is also supported by early, unsuccessful attempts to produce disease in foals using isolates from calves (Tzipori 1983), and by at least one study which found no association between the presence of *Cryptosporidium* oocysts and diarrhoea in horses (Xiao and Herd 1994). Similarly, in a case control study performed in the United Kingdom, Netherwood and co-workers (1996) found *Cryptosporidium* oocysts in 17% of diarrhoeic foals and 20% of controls, but a statistically significant association between the presence of the oocysts and diarrhoea in only one of the multivariate statistical models examined. Further, data from the United States and the United Kingdom indicate that testing for *Cryptosporidium* is not widely performed by diagnostic laboratories, and the agent is not consistently included in the differential diagnosis of diarrhoea in foals in epidemiological studies (Anonymous 2007a,b,c, 2008a,b; Hollis et al. 2008; Roberts et al. 2008; Wohlfender et al. 2009).

The concept of a low pathogenicity of *Cryptosporidium* in foals is difficult to reconcile with the previous report of an outbreak of diarrhoea in foals in New Zealand in which *C. parvum* was identified as the sole agent (Section 3.1). In the present section, the results of a survey of the occurrence of *Cryptosporidium* parasites in diagnostic faecal specimens from diarrhoeic foals submitted to a diagnostic veterinary laboratory in New Zealand are presented. The results of the survey, and of an investigation of an outbreak of cryptosporidiosis in foals triggered during the same survey, are discussed in view of the author's previous findings.

3.3.3 Materials and methods

Study outline. Between 2005 and 2007, a collaborative study between Massey University (MU) and a diagnostic laboratory operating in the North Island of New Zealand was conducted. The study aim was to assess the occurrence of *Cryptosporidium* oocysts in diagnostic faecal specimens from foals, and genetically characterise *Cryptosporidium* isolates. Therefore, a subset of faecal specimens from foals received in 2006 and 2007 by the participating laboratory for a microbiological analysis for causes of diarrhoea was transferred to MU. The specimens were transferred at the discretion of the pathologist in charge, provided faecal material was available after the completion of the requested tests, and regardless of whether or not the testing for *Cryptosporidium* had been initially requested. In addition, the participating laboratory provided specimen submission data for 2005, 2006, and 2007. The results of the genetic characterisation of the isolates has been reported in Section 3.2.

On-site outbreak investigation. In November 2006, large numbers of *Cryptosporidium* oocysts were seen on direct faecal smears from one of the specimens transferred to MU. The

specimen originated from an 8-day-old Thoroughbred foal (index case) that had been hospitalised at MU due to severe diarrhoea. The foal was born on a broodmare farm in the lower North Island. The referring veterinarian reported that there had been a high incidence of neonatal diarrhoea on the farm, which prompted an outbreak investigation.

The farm was visited twice, on 5 and 12 December 2006. Three diarrhoeic foals (aged 6, 8, and 11 days at the first visit) and their dams, were presented for examination during these visits. Faecal and environmental specimens were collected at each visit, and rectal faecal specimens collected from the foals during the first (n=3), and second (n=3) visit to the farm, and from their mares (n=3) during the first visit. Faecal specimens from the hospitalised foal were taken 1, 4, 5, 6, 8, and 11 days following admission (n=6). In addition, a sample of bedding material from the foaling shed, and muddy soil from a paddock used to house mares and foals, were collected. All the samples were analysed for *Cryptosporidium* and other common enteropathogens of foals.

Laboratory methods. In order to increase the sensitivity of the test for the detection of *Cryptosporidium* oocysts, the faecal specimens submitted to MU were tested by means of two methods, with parallel interpretation. The first method included the simple staining of direct faecal smears using both the modified Ziehl-Neelsen (ZN) stain and a bivalent commercial immunofluorescence test kit for *Cryptosporidium* and *Giardia* (MeriFluor Crypto & Giardia; Meridian Bioscience, Cincinnati OH, USA). The stained smears were examined microscopically at 400x magnification, and were considered positive for *Cryptosporidium* if there were one or more round acid-fast oocysts of 4–6 µm diameter containing internal sporozoites (Ortega and Arrowood 2003), or apple-green fluorescent oocysts. The specimens that tested negative by this method were then subjected to an additional test, which differed in that it included separation of the oocysts from faecal debris by sedimentation prior to the preparation of the slides as previously described (Grinberg et al. 2002; see also Section 3.4).

The specimens collected from the hospitalised foal one day after admission and from the foals during the first visit to the farm, were tested for the presence of *Cryptosporidium* and *Giardia* as described above, and for ova of helminths, *Salmonella* spp., and Group-A rotavirus. Testing for *Salmonella* spp. was performed using an inoculation of a loopful of faeces onto xylose-lysine-deoxycholate agar and into selenite enrichment broth followed by subculture onto xylose-lysine-deoxycholate (Fort Richards Laboratories, Auckland, NZ). Media were incubated for 24 h at 37°C in aerobic conditions. Testing for Group-A rotavirus was performed using a commercial latex agglutination test kit (SerobactRotavirus; Medvet, Adelaide, Australia). The presence of ova of helminths in the faeces was assessed microscopically after salt flotation by the technician of the laboratory of parasitology of the Institute of Veterinary, Animal and Biomedical Sciences, MU. Specimens from mares, subsequent specimens collected from the hospitalised

foal on Days 4, 5, 6, 8 and 11 following admission, and from the foals during the second visit to the farm, were only tested for *Cryptosporidium*, as the other agents had already been excluded.

The samples of bedding material and soil were only tested for the presence of *Cryptosporidium* using the following method: A 5 g sample from each source was suspended in 50 ml tap water, sieved through a coarse sieve, and centrifuged at 900g for 10 minutes. The deposit was re-suspended in 3 ml water, and triplicates of 10 µl were deposited as drops on slides, stained for immunofluorescence, and screened microscopically as described above.

The taxonomic identification of the *Cryptosporidium* parasites was performed by means of sequence analysis of a species-specific region of the *Cryptosporidium* 18S small subunit ribosomal RNA (18S rRNA) gene, using the oocyst separation method, and nested PCR and sequencing procedures described in Section 3.2.3. In addition, DNA was extracted from the two environmental samples using a DNA extraction kit (DNA Stool Mini Kit; Qiagen, Hilden, Germany), and tested for the presence of the *Cryptosporidium* 18S rRNA gene using the same nested PCR and sequencing methods,

The number of *Cryptosporidium* oocysts per ml of faeces was estimated in the first faecal specimen collected from the hospitalised foal, and in one specimen collected from a diarrhoeic foal during the first visit to the farm. These specimens were thoroughly mixed, and triplicates of 10 µl were serially diluted in normal saline to a dilution of 10^{-2}. A 10 µl aliquot of this dilution was deposited as a drop on a slide and stained using the immunofluorescence kit. The oocysts on the entire surface of the drops were counted using the 400x magnification of an epifluorescent microscope, and the number of oocysts per ml of faeces extrapolated by multiplying the mean count of the triplicates by 10^4. An oocyst count could not be performed on the other specimens from foals on the farm due to the need to preserve the small amount of faecal material obtained for DNA extraction.

3.3.4 Results

Laboratory submission data for 2005-2007. A total of 131 specimens from foals with a history of diarrhoea and/or enteritis were received by the participating laboratory between 2005-2007. In many cases the specimens arrived with a request for testing but with no clinical history indicated. Routine testing for *Cryptosporidium* was only performed if specifically requested by the submitting veterinarian or pathologist in charge, by means of a ZN stain of faecal smears. Only 67 specimens were tested for *Cryptosporidium* and 12 (18%) tested positive. Twelve liquid faecal specimens, not all initially tested for *Cryptosporidium* by the participating laboratory, were transferred to MU in 2006 and 2007, and were used in this study in addition to the specimens collected during the on-farm investigation. Three of these specimens tested positive for *Cryptosporidium* at MU. Two of the *Cryptosporidium*-positive specimens were collected from foals in the Waikato aged 2 and 3 weeks, in 2006 and 2007; no

additional information on these specimens was available. The third *Cryptosporidium*-positive specimen originated from the hospitalised foal. Sequence analysis revealed that these isolates, and the additional isolates collected during the on-farm investigation (see below), were *C. parvum* (the molecular identification of these parasites is reported in Section 3.2).

Findings from the on-site investigation. The index case had a normal parturition and nursing history; 700 ml of the mare's colostrum was administered by stomach tube to the foal 1 hour after birth and the foal stood and nursed normally. Twelve hours prior to admission, the foal developed watery, malodorous, non-haemorrhagic diarrhoea. No abnormalities had been seen in either the mare or foal prior to this, and the mare had not shown any recent oestrous behaviour. The foal developed mild colic signs about 4 hours later, manifested by rolling and flank watching. At this time, lactated Ringers solution (1L, IV), flunixin meglumine (100mg IV), and trimethoprim and sulphadiazine (1440mg combined dose, IM), were administered by the referring veterinarian. The foal remained bright but did not nurse, and no improvement in the signs of colic was seen. Two hours before admission, hyoscine-n-butylbromide (40mg IV) and dipyrone (5000mg IV) were administered to no effect. On admission, the foal was depressed, and showed frequent flank-watching behaviour. Heart and respiratory rates and rectal temperature were within normal ranges. Intestinal sounds were normal, but there were frequent episodes of watery diarrhoea and faecal staining of the perineum. Ultrasonographic examination of the foal's abdomen showed mild gassy distension of the large intestine and hypermotility of the small intestine. Umbilical remnants appeared normal. Initial treatment included hydration treatment with IV boluses of lactated Ringer's solution supplemented with 2.5% glucose, and oral bismuth subsalicylate. A faecal specimen submitted for bacteriological culture to the participating diagnostic laboratory at admission revealed a light growth of a mixture of Gram-positive cocci and Gram-negative rods, but no pathogenic bacteria were isolated.

One day after admission, a faecal specimen was submitted to MU for an analysis for *Cryptosporidium* oocysts. At MU, numerous *Cryptosporidium*-like oocysts were seen on direct smears from this specimen and stained by both the modified ZN and the immunofluorescent stains. The same specimen was negative for *Salmonella* spp., *Giardia*, Group-A rotavirus, and ova of helminths. Two days after admission, isotonic fluids were administered via stomach tube, and omeprazole and live yoghurt treatments were started. Over the following 3 days the foal stabilised and faecal consistency gradually improved. Blood samples taken at admission and in the following days showed anaemia throughout the observation period, the exact cause of which could not be determined. A mild leucopenia, hyperfibrinogenaemia, and hyponatraemic metabolic acidosis, and elevated levels of aspartate aminotransferase and glutamate dehydrogenase were observed (Table 3.4). Additional faecal specimens taken on Days 4, 5, 6, after admission were positive for *Cryptosporidium*. The foal was discharged 12 days following admission, after two consecutive faecal specimens (for Days 8 and 11) were negative for

Cryptosporidium. Skin excoriations as a result of the diarrhoea were present on the perineal region and haunches on the day of discharge (Figure 3.3).

	Units	At admission	12 h post-admission	36 h post-admission	6 days post-admission	12 days post-admission	Normal rangea
RBC	$x10^{12}$	5.45*	4.79*	4.78*	5.6*	4.5*	6.9-11.8
HCT	%	21.6*	19.4*	18.9*	22*	18*	32 – 47
Hb	g/L	69*	-	-	-	-	102-154
PLT	$x10^9$	206	210	232	274	201	100 - 350
WBC	$x10^9$	7.9	4.4*	6.2	7.1	6.6	6.2–12.4
Neutrophils	$x10^9$	6.6	3.2*	5.0	4.1	5.2	4.1 – 9.1
Lymphocytes	$x10^9$	0.8*	0.9*	0.7*	2.4	1.1	1-3.1
Monocytes	$x10^9$	0.6*	0.3	0.5	0.6*	0.3	0.1 – 0.5
Fibrinogen	g/L	-	-	-	5.0*	-	1.5 – 4.2
IgG	g/dL	0.84	-	-	-	0.98	0.69-1.86
Glucose	mmol/L	6.6*	6.2*	-	-	-	3.3 – 5.6
CK	IU/L	-	-	-	193	-	141-4242
AST	IU/L	-	-	-	446*	-	69-316
GGT	IU/L	-	-	-	63	-	15– 63
GLDH	IU/L	-	-	-	308*	-	1-8
Bile acids	µmol/L	-	-	-	15	-	0 – 20
Total protein	g/L	45	42	50	53	-	42 – 66
Albumin	g/L	-	-	-	28	-	25 – 35
Globulin	g/L	-	-	-	25	-	13 – 36
Urea	mmol/L	5.7	4.3	-	1.7*	-	4.1 – 12.5
Creatinine	µmol/L	-	-	-	52*	-	106 – 312
Phosphate	mmol/L	-	-	-	1.86	-	1.2 – 2.2
Total Ca	mmol/L	-	-	-	2.94	-	2.4 – 3.3
Na	mmol/L	128	131	-	131	-	131– 141
K	mmol/L	3.4	3.6	-	4.2	-	3.0 – 4.6
Chloride	mmol/L	102	106	-	-	-	93 – 105
Venous pH	-	7.26*	7.21*	-	-	-	7.36- 7.43
$PVCO_2$	mm Hg	38.3*	41.9*	-	-	-	45 – 49
$VHCO_3$	mmol/L	17.2*	16.9*	-	-	-	22.3 – 25
Anion gap	mmol/L	-10*	-11*	-	-	-	+4 – -4

Table 3.4. Haematology, biochemistry and venous blood gas results from a hospitalised foal affected with cryptosporidiosis. Tests indicated by a dash were not performed.

[a] Normal ranges are those used by New Zealand Veterinary Pathology Ltd, New Zealand, for Thoroughbred foals aged 0-3 weeks. Asterisk denote values outside normal range. RBC= red blood cells; HTC= haematocrit; Hb=haemoglobin; PLT= platelets; WBC= white blood cells; CK= *Creatine kinase* AST= *Aspartate aminotransferase*; GGT= *Gamma Glutamyl transferase*; GLDH= *Glutamate dehydrogenase;* $PVCO_2$: carbon dioxide partial pressure; $VHCO_3$: venous bicarbonate concentration

The broodmare farm was visited twice, on 05 and 12 December 2006. The farm was located on agricultural land crossed by a stream. It was a new business in its first operational foaling season, established on land previously used to raise cattle and sheep. A small number of sheep and weaned beef cattle were grazing on the same facility during the visits.

Potable water was supplied to the animals, and they also had full access to a stream. Pregnant mares from different locations were transferred to the facility for foaling and breeding one month before the estimated date of foaling, and returned to their farms 2 months after foaling. Mares were allowed to graze up until 3-4 days before the expected foaling date, when they were moved to a foaling barn. Mares and neonates were moved back to a paddock as soon as possible after foaling. Forty-five foals, the majority of which were Thoroughbred, were born on the facility between August and November 2006. Diarrhoea had affected the majority of them throughout the season. Most cases were self-limiting, with an onset at about 6-8 days of age and a duration of 2-3 days. Some protracted cases were seen, and one foal died in October after suffering from diarrhoea. Management of diarrhoeic foals included their transfer, with their dams, to a section of the foaling shed also used for foaling, as no other facility for the sick animals was available (Figure 3.3). Treatment of diarrhoeic foals included the administration of oral electrolytes and a 3-day course of an oral co-trimoxazole.

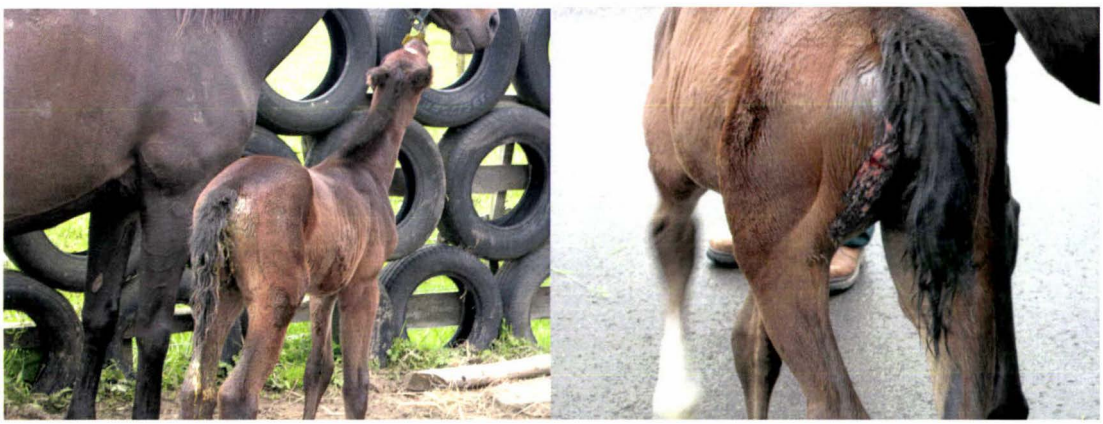

Figure 3.3 Foals with cryptosporidiosis. Left: a foal and the mare on the farm; Right: the hospitalised foal at the day of discharge. Notice the presence of excoriations in the haunches caused by the faecal soiling during the disease. These pictures were taken by BVSc student Abigail Deuel

The three diarrhoeic foals presented for examination during the visits to the farm were the last neonates of the season. They appeared bright but their tails were soiled with faeces. On the first visit, one foal passed liquid faeces, one a small amount of semi-liquid faeces, and the third did not pass faeces, although a small amount of faecal material could be extracted from the rectum. Their dams passed fully formed faeces. The rectal temperature of the 11-day-old foal was slightly elevated (39.4 ℃). As the other two foals were restless, their rectal temperatures were not measured. The same three foals were bright and passed firm faeces on the second visit.

Laboratory findings. The first faecal specimens collected from the foals on the farm were negative for *Salmonella* spp., *Giardia* spp, Group-A rotavirus, and ova of helminths, but had large numbers of *Cryptosporidium* oocysts, which were seen on direct smears by both the

modified ZN and immunofluorescent stains. There were >10^6 oocysts per ml of faeces in the first faecal specimen collected from the hospitalised foal and in one specimen collected from a diarrhoeic foal during the first visit to the farm. All the *Cryptosporidium* isolates were identified as *C. parvum* by sequence analysis, as described previously in Section 3.2. No oocysts were seen in the specimens from mares and foals taken during the second visit to the farm. Similarly, no oocysts were seen in the environmental samples. However, a *C. parvum* 18S rRNA gene sequence was amplified from DNA extracted from the soil sample, but not from the bedding material.

3.3.5 Discussion

Cryptosporidiosis in foals is a poorly characterised disease. In an earlier outbreak in 2002, the diagnosis of cryptosporidiosis was based on the isolation of *C. parvum* as the sole agent, in conjunction with conclusive histopathological findings (Section 3.1). In the outbreak described here, post-mortem findings were not available. Nonetheless, the same diagnosis was supported by the finding of a high incidence of self-limiting diarrhoea manifesting during the first and second weeks of life, and in particular the finding of the concomitant clinical and parasitological remission in the four foals, reminiscent of bovine cryptosporidiosis (Anderson 1981; Uga 2000; Grinberg et al. 2002).

According to the data reported in Sections 3.1 and 3.2, *C. parvum* has been identified in New Zealand from cases of diarrhoea in foals from two separate regions, during three non-consecutive foaling seasons. Furthermore, 18% of the specimens from foals tested for *Cryptosporidium* by the participating laboratory in 2005-2007 were positive for this agent. Collectively, these results suggest foal cryptosporidiosis caused by *C. parvum* is not uncommon in New Zealand.

In New Zealand, most registered foals are born on agricultural land in regions with large populations of cattle. Moreover, the foaling and calving seasons generally overlap (Rogers et al. 2007), and two independent studies converged in indicating *Cryptosporidium* parasites are present in about 40% of the dairy farms (Section 3.4; Winkworth et al. 2008). This could facilitate the transmission of *C. parvum* of bovine origin to foals as compared with other horse rearing countries. In fact, *C. parvum* isolates originating from foals, calves and humans in New Zealand are genetically-similar (Section 3.2). The finding in the study presented here of a *C. parvum* DNA signature in soil is evidence of a contaminated farm environment and supports this view. However, cryptosporidiosis in foals may also be common in other countries, as suggested by the presence of oocyst-like structures in the faeces of foals affected by foal-heat diarrhoea in Italy (Sgorbini et al. 2003).

It is intriguing that cryptosporidiosis is seldom reported in foals. There are remarkable similarities, in terms of disease onset, duration, and severity, between foal-heat diarrhoea and

the cases of cryptosporidiosis described previously (Section 3.1) and herein. Foal-heat diarrhoea is a common self-limiting condition of 1-2 week-old foals that has a poorly characterised aetiology (Magdesian 2005; Crabbe 2007), and it would seem possible that cryptosporidiosis in foals is underdiagnosed, or in many cases managed empirically as foal-heat diarrhoea. Indeed, the small number of specimens from foals tested for *Cryptosporidium* by the participating laboratory and overseas indicates the parasite is not consistently considered in the differential diagnosis by clinicians. The short patent period, as observed in this study in 4/4 foals, could also contribute to the underdiagnosis by increasing the likelihood of the agent not being present in specimens collected after any delay.

The presence of the hospitalised foal allowed clinical and haematological parameters of cryptosporidosis to be investigated, at least in this case. In addition to the alteration in generic markers of dehydration and inflammation, the foal suffered from anaemia throughout the observation period. However, as a similar clinical investigation could not be performed in the other foals, this finding needs to be corroborated.

Some authors have suggested that horses are infected with a so-called '*Cryptosporidium* horse genotype' (Xiao and Fayer 2008; Xiao and Feng 2008; Xiao and Ryan 2008; see also Section 1.6.3). Based on a single observation, which was not accompanied by any description of the infection with the same genotype in the horse, the 'horse genotype' has been classified by Fayer as a 'major' intestinal *Cryptosporidium* affecting the horse (Fayer 2008). The putative occurrence of the *Cryptosporidium* 'horse genotype' in equine populations is of significance, as unlike *C. parvum*, this genetic variant has not been identified in humans and therefore is not considered zoonotic. However, whereas *C. parvum* has been repeatedly identified in the domestic horse (*Equus caballus*) in New Zealand and overseas (Sections 3.1 and 3.2; Chalmers et al. 2005; Tanriverdi et al. 2006), the 'horse genotype' has only been reported in one Przewalski's wild horse (*Equus przewalskii*) in a zoo (Ryan et al. 2003). Thus, the idea of wide cycling of a *Cryptosporidium* 'horse genotype' in the domestic horse needs to be substantiated, and people handling diarrhoeic foals should be mindful of the zoonotic potential of cryptosporidiosis in foals caused by *C. parvum*.

In conclusion, the results of this study complement previous observations and suggest the incidence of clinically overt *C. parvum* infections in newborn foals in New Zealand may be underestimated. The author postulates that cryptosporidiosis may be underdiagnosed, perhaps accounting for a proportion of cases empirically diagnosed as foal-heat diarrhoea. It is advisable to take hygienic precautions when handling diarrhoeic foals, until *C. parvum*, which is a potentially zoonotic agent, is ruled out in the laboratory, preferably by multiple testing to enhance the agent's detection.

3.4 THE OCCURRENCE OF *CRYPTOSPORIDIUM PARVUM*, *CAMPYLOBACTER* AND *SALMONELLA* IN NEWBORN CALVES IN THE MANAWATU REGION OF NEW ZEALAND

3.4.1 Summary

In 2002, a cross-sectional study was conducted during the winter calving season, with the aim of determining the rate of occurrence of *Cryptosporidium* oocysts in faecal specimens taken from newborn dairy calves on 24 dairy farms in the Manawatu region of New Zealand.

Faecal specimens were collected from the rectums of 185 newborn calves from 24 dairy farms selected by convenience criteria. The specimens were tested microscopically for the presence of *Cryptosporidium parvum* oocysts, and bacteriologically for the presence of *Campylobacter* spp. and *Salmonella* spp. The identification of the *Cryptosporidium* oocysts to taxon level was not performed, as at that junction *C. parvum* was considered the only intestinal species infecting newborn calves (this matter will be further discussed in Section 3.5).

Infections with *C. parvum* were identified in 33/156 (21.1%) calves from 10 farms. More than 10^6 oocysts/gr of faeces were detected in calves from four farms. *Campylobacter* spp. were isolated from 58/161 (36%) calves from 18 farms; in particular, *C. jejuni* subsp *jejuni* was isolated from 11/161 (6,8%) calves from seven farms. *Salmonellae* were not detected.

These results indicate that despite the short, concentrated calving pattern and the long interval between calving seasons characterising most dairy farms in New Zealand, *C. parvum* is widespread among calves. *Campylobacter* spp, especially *C. jejuni,* colonise the intestinal tract of calves, early in life.

3.4.2 Introduction

The prevalence of human microbial enteropathogens in cattle has been the subject of intensive research in many countries, due to the risk of animal-to-human transmission. In New Zealand, the protozoan parasite *Cryptosporidium parvum* and bacteria belonging to the genus *Campylobacter* and *Salmonella* are among the microbial pathogens most commonly detected in cases of human gastrointestinal infections (Anonymous 2004).

A number of *Cryptosporidium* species have been reported to infect humans, but only the anthroponotic species *C. hominis* (Morgan-Ryan et al. 2002) (formerly known as *C. parvum*, 'human' genotype, or Type I) and the zoonotic *C. parvum* (formerly known as *C. parvum* 'cattle' genotype, or Type II) are widespread. Other species have only been reported sporadically in intestinal and non-intestinal infections in immunocompromised individuals (Xiao et al. 2004; Xiao and Ryan 2008). *Cryptosporidium parvum* is an obligate intestinal parasite and has a wide host range (Fayer 2004). The parasite is widespread in livestock and it is believed that livestock, especially cattle, function as natural reservoirs for human infections. Perhaps the most convincing data supporting this belief were the 35% reduction in laboratory notifications of human cryptosporidiosis during the 2001 foot-and-mouth disease epidemic in England and

Wales, attributed to a reduction in the cattle population and decreased direct and indirect exposure of humans to livestock (Smerdon et al. 2003).

In cattle, infections are predominantly perinatal, and a typical faecal oocyst shedding period lasts a number of days followed by the development of resistance to re-infection (Anderson 1981; Harp and Woodmansee 1990; Peeters et al. 1993; Fayer et al. 1998). In calves, *C. parvum* causes diarrhoea, as repeatedly indicated by results of observational studies, experimental infections, and therapeutic trials (Tzipori et al. 1983; Heine et al. 1984; Fayer and Ellis 1993; Naciri et al. 1993, 1999; Fayer et al. 1998; O'Handley et al. 1999; Castro-Hermida et al. 2002; Grinberg et al. 2002; Sevinc et al. 2003). Faecal counts as high as $10^6 - 10^7$ oocysts/g (OPG) were found in faeces of infected calves during the peak of excretion (Ongerth and Stibbs 1989; Fayer et al. 1998; O'Handley et al. 1999; Uga et al. 2000; Nydam et al. 2001). Most studies indicate peak shedding of oocysts during the second week after birth, and that adult cattle only sporadically shed low numbers of oocysts. Hence, 1 - 2-weeks-old calves are regarded among the most efficient amplifiers of *C. parvum* in nature, whereas adult cattle are, perhaps, maintenance hosts (Harp and Woodmansee 1990; Harp et al. 1996; Fayer et al. 1998; Atwill et al. 1999, 2003; de la Fuente et al. 1999, Sischo et al. 2000; Uga et al. 2000; Hoar et al. 2001; Nydam et al. 2001; Grinberg et al. 2002; Atwill et al. 2003; Atwill and das Pereira 2003; Sevinc et al. 2003; Fayer 2004). Given these characteristics, gauging the prevalence of *C. parvum* prevalence in newborn calves is important in assessment of the possible zoonotic impact of dairy farming.

Bacteria belonging to the genus *Campylobacter*, especially *C. jejuni* subsp. *jejuni* (*C. jejuni*), are important human pathogens causing mainly gastrointestinal illness (Nachamkin 2003). Although *C. jejuni* has caused mastitis experimentally and C. *jejuni* and *C. fetus* subsp *fetus* can sporadically cause abortion, only *C. fetus* subsp *veneralis*, the cause of bovine venereal campylobacteriosis, is considered a true pathogen of cattle. The other *Campylobacter* species are generally considered non-pathogenic commensals of the bovine gastrointestinal tract (Joens 2004) and are of mainly zoonotic importance.

There are about 2,500 serotypes of *Salmonella* and most of those infecting mammals belong to the species *S. enterica* subsp *enterica* (Libby et al. 2004). In the developed world, human salmonellosis tends to manifest clinically as a gastrointestinal illness accompanied by diarrhoea, fever, and abdominal cramps. Cases are mostly linked to the ingestion of contaminated food of animal origin (Bopp et al. 2003). Bovine salmonellosis manifests mainly as enteritis or a septicaemia (Libby et al. 2004). *Salmonella enterica* serotype Typhimurium was, by far, the serotype most frequently isolated from cases in humans and cattle in New Zealand in 2003 and 2004, and many of the isolates from both humans and cattle belonged to the same phagetype (Anonymous 2004). The bovine host-adapted *S. enterica* serotype Dublin (Libby et al. 2004) was not isolated from cattle in New Zealand in 2003 and 2004 (Anonymous 2004).

Although the presence of *C. parvum* in faecal samples from calves submitted to diagnostic laboratories in New Zealand has been repeatedly reported (Townsend and Lance 1987; Learmonth et al. 2004; Varney 2004), the prevalence of this parasite in dairy cattle in New Zealand is unknown. Extrapolating *C. parvum* prevalence data from overseas to cattle in New Zealand is difficult. Most existing prevalence data originate from countries where the dairy industry has a year-round calving pattern, whereas the vast majority of farms in New Zealand have a concentrated calving pattern, mostly in late winter. A sharp increase in the numbers of immunologically-naïve calves, and their virtual absence between calving seasons, might have a different effect on the epidemiology of this infection in New Zealand as compared to cattle-rearing countries in which there is unremitting enzootic cycling due to a continuous presence of susceptible calves.

The aim of the study presented here was to assess the occurrence of *C. parvum* among newborn calves in the Manawatu region of New Zealand, and to provide baseline data for future reference. Whilst assessment of *Campylobacter* and *Salmonella* was not the main purpose of the study, it was envisaged that by including these two zoonotic organisms, this study would also provide data on their impact on cattle in New Zealand.

3.4.3 Materials and methods

Study design and sampling strategy. A cross-sectional survey was conducted in the Manawatu region of New Zealand during the calving season, in August 2002. Dairy farms were selected by convenience criteria from the client list of a veterinary practice. Farms were eligible for inclusion in the study if at least 150 cows had been milked in the previous season, and approximately 60 farms fulfilled this criterion. No information on the occurrence of enteric pathogens and neonatal calf diarrhoea in previous calving seasons was considered. Farmers agreeing to participate were asked to allow all the calves between 9 and 15 days of age to be sampled. This purposive sampling was performed in consideration of the budget available, the estimated number of calves of this age that would be available for the sampling, and mainly given that longitudinal excretion studies in calves indicated a peak of prevalence of oocysts in faeces mostly coincidental with this age interval (Anderson 1981; Harp et al. 1996a; Fayer et al. 1998; Atwill et al. 1999; Uga et al. 2000; Grinberg et al. 2002). Faecal specimens were collected from the rectums of individual calves and transferred to clean watertight containers; gloves were changed between calves. Information recorded included gender and breed of the calves, and the type of flooring or bedding material.

Faecal specimens were categorised as 'solid' (when deposited in its container, the specimen conserved the original shape), 'semi-solid' (sample spreading across the bottom of the container but not liquid), and 'liquid' (sample had a liquid consistency). Specimens were transported on ice to the microbiology laboratory and were processed the same day for *Campylobacter* and *Salmonella*, and stored at 4ºC for up to 72 h until tested for *C. parvum*.

All procedures involving the sampling of the animals were approved by the Animal Ethics Committee of Massey University, Palmerston North, New Zealand.

Laboratory procedures for detection of *Cryptosporidium parvum.* One g of faeces was suspended in 10 ml of tap water and sieved through a 500 µm sieve. Then, oocysts were concentrated by centrifugation at 900 g and the pellet (of approximately one ml) was re-suspended in 4 ml normal saline. Ten microlitres of suspension was deposited as a drop on a slide, air-dried, and stained using the cold acid-fast staining technique. The entire area of the smear was examined using an optical microscope at 400x magnification, and acid-fast oocyst-like structures stained red were assessed morphologically and morphometrically at 1000x magnification. Samples were considered positive if they had at least one round acid-fast oocyst with a diameter of about 4 - 6 µm, and containing internal purple-stained sporozoites (Ortega and Arrowood 2003). Each oocyst detected represented an average of at least 500 oocysts in one gram of faeces.

Laboratory procedures for detection of *Campylobacter.* A 500 mg sample of faecal material was inoculated in cefoperazone-amphotericin-teicoplanin (CAT) broth prepared in-house (Atabay and Corry 1998), and incubated with loose caps for 48 h at 37°C in jars containing commercial kits for microaerobic, capnophilic atmosphere suitable for the growth of *Campylobacter* spp. (Mitsubishi Gas Chemical Co., Tokyo, Japan). Then, 5 µl were plated onto modified cefoperazone-charcoal-deoxycholate agar (mCCDA) and on commercial CAT agar plates (Fort Richards Laboratories, Auckland, New Zealand), and incubated as before. Plates were checked for growth every 48 h for a maximum of 8 days (Engberg et al. 2000). When growth was seen, one colony was tested for a Gram-stain reaction, and oxidase reaction using oxidase-detection strips (Oxidase strips, Oxoid Ltd., Hants, UK). Colonies of oxidase-positive, Gram-negative wavy bacilli resembling campylobacters were subcultured onto a 5% sheep blood agar plate prepared in-house and incubated at 37°C for 48 hours in jars, as before. Bacteria recovered from blood agar plates were suspended in nutrient broth containing 15% (Volume/Volume) glycerol, and frozen at -70°C for future reference. After 12 months, one frozen isolate from each positive sample was cultured for three cycles on 5% sheep blood agar plates, as before, and identified using the following tests (Nachamkin 2003): hippuricase activity using a fast test, as per the manufacturer's instructions (Becton Dickinson Microbiology Systems, Cockeysville MD, USA); susceptibility to nalidixic acid and cephalothin using the disc diffusion test; growth at 25°C and 42°C; catalase reaction, and production of indoxyl acetate as described by Popovic-Uroic et al. (1990).

Laboratory procedures for *Salmonella.* Swabs of faecal material were inoculated into tubes containing selenite enrichment broth (Fort Richards laboratories) and incubated aerobically at 37°C for 20-24 h. Five µl of broth were inoculated onto xylose-lysine-deoxycholate (XLD) plates

(Fort Richards Laboratories) and incubated as before. Further steps were not needed, as there was no growth consistent with *Salmonella* in the samples.

Statistical analysis. Assuming a perfect test, the 95% confidence interval (CI) of the proportion of *Cryptosporidium*-positive farms was calculated using the freeware WinEpiscope 2.0 (De Blas and Ortega 2000), option 'Sample Size to Estimate Percentage', a population size of 60, level of confidence set at 0.05, and finite population correction. Odds ratios (OR) and exact 95% CI, and two-tailed Fisher's exact tests, were used to assess associations between the presence of *Cryptosporidium* infections and liquid faeces on farms, and at the calf level. Calculations were performed using Episheet© 2002 freeware (Rothman 2002). The modest sample size precluded a multivariate analysis.

3.4.4 Results

A total of 185 calves from 24 farms were sampled, during 26 farm visits carried out between August 12 and September 3, 2002. Two farms, one testing positive and the other testing negative to *Cryptosporidium* at first instance, were re-sampled three weeks after the first sampling in order to re-asses their *Cryptosporidium* infection status. All the samples were tested for *Salmonella*, 162 for *Campylobacter* and 156 for *Cryptosporidium*, as some samples were of an insufficient quantity for testing according to the three different protocols. Most farms reported selling male calves at the age of about 1 week, hence, only 21/185 (11.4 %) calves were male. Management systems on farms included leaving calves with their mothers for 1 - 4 days, and then grouping them into pens in barns or paddocks, where they were fed colostrum and/or whole milk. Only one farm reported feeding calves with milk replacer. Calves of the same age were mostly kept together. A variety of flooring or bedding materials were reported (Table 3.5).

There were 33/156 (21.2%) *Cryptosporidium*-positive calves from 10/24 (42%) farms (95% CI= 26.3–56.8). Most infected farms had more than one calf infected. The within-farm prevalence of infected calves ranged from only one of seven to seven of seven calves testing positive (Table 3.4). There was interpretive uncertainty concerning only one farm, which was eventually considered positive by the presence of one positive sample containing only two oocysts on the entire slide. One farm where all calves tested negative on the first visit became infected and showed positive calves on a second visit about 3 weeks later, and was thus considered *Cryptosporidium*-positive. The other farm visited twice had infected calves on both occasions but it was counted as positive only once. Six infected calves from four farms were shedding more than 10^6 OPG faeces. Interestingly, none of the four farms rearing Jersey cattle had infected calves (Table 3.5). Of 10 farms where calves had liquid faecal samples, nine were *Cryptosporidium*-positive. The occurrence of liquid faeces on farms was positively associated with the finding of at least one *Cryptosporidium*-positive calf (OR=117, 95% CI= 4.8 – 5489; p<

0.01). Ten of 16 calves with liquid faeces were *Cryptosporidium*-positive, but only 10/33 *Cryptosporidium*-positive calves had liquid faeces. The association between the presence of liquid faeces and *Cryptosporidium* infections at the calf-level was significant (OR=8.47, 95% CI= CI=2.45–30.8; p<0.01).

Breed	Bedding type	No. *C. parvum*-positive samples/No. examined	No. *Campylobacter*-positive samples/No. examined	Number of liquid faeces
F	Wood chips	5/8	4/8	2
F	Paddock	0/7	5/7	0
F	Woodchips	0/9	1/9	0
J	Sawdust	0/8	7/13	0
J	Wooden slats	0/3	0/3	0
F	Sawdust	0/7	3/8	1
F	Sawdust	0/9	7/13	0
C	Concrete	1/7	2/7	1
C	Woodchips	3/8	10/11	1
nr	Saw dust	0/7	2/8	0
nr	Paddock	0/5	1/6	0
J	Paddock	0/3	0/3	0
C	Wooden slats	0/6	2/6	0
J	Woodchips	0/5	0/5	0
C	Wooden slats	6/6	5/9	2
F	Sawdust	4/10	2/10	4
C	Paddock	0/7	1/4	0
C	Straw	0/7	0/5	0
C	Sawdust	3/8	0/6	1
F	Sawdust	0/4	3/4	0
F	NR	3/6	1/4	2
F	Paddock	1/4	1/2	0
C	Sawdust	4/6	1/4	1
F	Woodchips	3/6	0/6	1

Table 3.5. Prevalence of *Cryptosporidium* parvum and *Campylobacter* spp among newborn calves from 24 dairy farms in the Manawatu region of New Zealand (each row represents one farm). F= Friesian; J= Jersey; C= cross-breed; NR= not recorded

Fifty-eight (36%) samples from 18 farms tested positive for *Campylobacter*. A wide range of *Campylobacter* phenotypes were detected; *C. jejuni* subsp. *jejuni* was isolated from 11 samples from 7 farms (Table 3.5). The most common phenotype was that of *C. sputorum-fecalis*, isolated from 30 samples from 11 farms. *C. hyointestinalis* subsp *hyointestinalis* phenotype was isolated in 10 samples from seven farms, *C. coli* phenotypes were isolated from three samples, and *C. lari* was isolated once. Colonies consistent with *Salmonella* were not observed on XLD agar plates.

3.4.5 Discussion

The prevalence of *C. parvum* in dairy calves in New Zealand has not been measured previously. In this study, a purposive sampling of newborn calves at the theoretical peak of the

patent period was applied to enhance herd-test sensitivity. More than 3 samples were tested in the majority of the *Cryptosporidium*-negative farms, and 8/10 *Cryptosporidium*-positive farms were declared positive by the presence of multiple infected calves (Table 3.4). Hence, test accuracy at the herd level was high. The overall farm-prevalence was 42%. Comparable percentages of *Cryptosporidium*-infected farms have been reported in countries with year-round calving (Genchi et al. 1984; Harp and Woodmansee 1990; Garber et al. 1994; Quilez et al. 1996). However, a prevalence >90% was reported in the Canadian states of Quebec and Ontario in cross-sectional studies, and in Galicia (Spain) in a longitudinal study (Ruest et al. 1998; Castro-Hermida et al. 2002; McAllister et al. 2005; Trotz-Williams et al. 2005). Although some inadvertent bias due to non-random recruitment of farms is possible, these results suggest that enzootic *C. parvum* cycles establish in a considerable proportion of farms, despite the short and concentrated period of calving and long time interval between calving seasons characteristic of most dairy farms in New Zealand. The proportion of *C. parvum* positive calves within farms was variable, in accordance with results described elsewhere (Santin et al. 2004). More than 10^6 OPG faeces were detected in six samples from four farms. Such high numbers are typically found during a small fraction of the patent period (Fayer et al. 1998; Uga et al. 2000; Grinberg et al. 2002). Hence, it is conceivable that in a longitudinal study, an excretion of this magnitude would have been observed, at some stage, in the majority of the positive calves. Given the high number of New Zealand dairy cattle, and in view of the fact that calves produce the vast majority of *C. parvum* oocysts shed on farms (Atwill and das Pereira 2003; Fayer 2004), these results indicate a massive natural amplification of *C. parvum* during the calving season. What remains unknown is whether infections on farms are initially acquired from environmental sources, or from infected reservoir-cows. Another question is whether or not calves on apparently negative farms become infected later in life, as calves raised in isolation remained susceptible to experimental infection for at least 3 months (Harp and Woodmansee 1990).

The possibility of cattle-to-human cycling of *C. parvum* is supported by the results of experimental infections in humans with cattle isolates (DuPont et al. 1995, Chappel and Okhuysen 2001), and by studies of the population genetic structure of *C. parvum* that have shown extensive sharing of genotypes between humans and cattle (Mallon et al. 2003ab). A number of findings converge in suggesting an epidemiological association between the occurrence of human cryptosporidiosis and the dynamics of the dairy cattle population in New Zealand: National notifications of cases of human cryptosporidiosis from the last 4 years denoted monthly fluctuations, and the number of notified cases peaked after the end of the calving season in spring (Anonymous 2004). Rivers impacted by dairy farms were found to be at a higher risk of being contaminated with faecal indicator bacteria (Donnison and Ross 1999), and results of a systematic survey indicate a widespread distribution of *Cryptosporidium* spp. oocysts in surface waters in New Zealand (Brown et al. 1998). Finally, molecular characterisation of the *Cryptosporidium* clinical isolates recovered from humans in 2001 and

2002 in New Zealand showed a distinctive virtual substitution of the anthroponotic species *C. hominis* with the zoonotic *C. parvum* after the end of the calving season in both years (Learmonth et al. 2004). Our study provides another line of evidence to tentatively explain this interesting shift: it is plausible that the significant expansion of the niche represented by the large increase in numbers of immunologically-naïve calves might allow a selective amplification of highly-infective clones, followed by their environmental dispersal via calf-related biomasses. However, humans can acquire *C. parvum* infection through a variety of routes, including ingestion of contaminated food and water, animal-to-human, and person-to-person (Guerrant 1997; Xiao et al. 2004) and at present, the idea of a massive cattle-to-human parasite cycling is speculative. To prove this concept, an analysis of the population genetic structure using a large number of isolates of *C. parvum* from human and cattle, and a highly discriminatory typing method, would be necessary. Such a study is presented in Section 3.5.

In this study, the probability of detecting cryptosporidial infections was significantly higher on farms in which at least one calf was suffering from liquid diarrhoea, and the association between the presence of liquid faeces and an infection was significant at the calf-level. However, this study was not designed to study the causes of diarrhoea, hence, these statistics are only reported for future reference.

A relatively high percentage of farms infected with *Campylobacter* spp, and especially with *C. jejuni*, the most important human pathogen of this genus (Nachamkin 2003), was observed in our study. *Campylobacter jejuni* is known to colonise the intestinal tract of asymptomatic cattle. However, most population-based studies were conducted using either samples from adult cattle, older calves, or without precise age-specification (Garcia et al. 1985; Meanger and Marshall 1989; Giacoboni et al. 1993; Atabay et al. 1998; Stanley et al. 1998; Busato et al. 1999; Hoar et al. 2001; Nielssen 2002; Savill et al. 2003; Sato et al. 2004; Bae et al. 2005; Wesley et al. 2000), and little is known about colonisation of the intestines by *Campylobacter* in the neonate calf. *Campylobacter jejuni* was found in a relatively high percentage of 4-week-old calves in an abattoir study (Grau 1988). The present study indicates that a wide range of *Campylobacter* species, in particular *C. jejuni*, colonise the intestinal tract of cattle early in life.

*Salmonella*e were not detected in this study. This is in agreement with reports from other countries indicating a very low rate of detection of intestinal carriage of *Salmonella* among healthy young calves (Lance et al. 1992; Busato et al. 1999; Naciri et al. 1999, McAllister et al. 2005) and consistent with the observation of a lower prevalence of carriage of *Salmonella* in unweaned calves than in adult animals observed by Huston et al. (2002). These low rate of subclinical carriage of *Salmonella* in newborn calves reinforces the diagnostic value of isolating of *Salmonella* from the faeces of newborn calves during disease outbreaks.

Calves infected with *C. parvum* and *C. jejuni* may be a source of human disease by direct transmission. Smith et al. (2004) concluded that calves were the reservoir of multiple enteric

pathogens, including *C. parvum* and *C. jejuni*, for children in the United States. Stefanogiannis et al. (2001) linked an outbreak of cryptosporidiosis in children in New Zealand to direct contact with calves during a farm visit. The infectivity of *C. parvum* and *C. jejuni* in humans is high (Okhuysen et al. 1999; Chappel and Okhuysen 2001; Nachamkin 2003). Thus, care should be taken to avoid unnecessary exposure of people to calves less than 4 weeks old on farms. An epidemiological study in the USA reported that the increased frequency of spreading manure on fields was a risk factor for detecting *Cryptosporidium* oocysts in streams (Sischo et al. 2000). At present, it seems advisable to avoid spreading calf manure and bedding on paddocks.

In conclusion, this study provides a snapshot of the occurrence of *C. parvum*, *Campylobacter* spp and *Salmonella* spp in the Manawatu region, which perhaps can be generalised to other regions in New Zealand with comparable cattle-rearing practices. Despite the theoretical sanitising effect of time between calving seasons in New Zealand, results confirm that newborn calves are amplifiers of *C. parvum*. Although results suggest an important role for *C. parvum* in the neonatal calf diarrhoea complex, its attributable impact in New Zealand needs to be established. *Campylobacter* spp, in particular *C. jejuni*, but not *Salmonella*, were also found to be widespread. Studies of alternative systems to reduce the environmental dispersal of viable enteropathogens from preweaned calf wastes at the end of the calving season are warranted.

3.5 PERSISTENT DOMINANCE OF TWO GP60 *IIa* ALLELES IN DIARRHOEAGENIC HUMAN AND BOVINE *CRYPTOSPORIDIUM PARVUM* IN NEW ZEALAND

3.5.1 Summary

The study presented in Section 3.4 provided an estimate of the dairy farm-prevalence of *C. parvum* in New Zealand. At that junction, the molecular characterisation of the isolates was not deemed necessary as *C. parvum* was considered the only taxon extensively parasitising cattle. The author has hypothesised that because New Zealand has a unique dairy environment, where millions of susceptible newborn calves are reared within a short time period during winter, *C.parvum* genetic lineages able to rapidly fill the niche represented by the transient presence of susceptible newborn calves, have become established.

In this study, 68 diarrhoeagenic human (n=20) and bovine (n=48) *Cryptosporidium* isolates collected during the annual peak of morbidity in winter and spring of 2006, were genetically-identified at the 18S rRNA gene and subtyped at the polymorphic region of the GP60 gene. All the identified isolates were *C. parvum.* Two dominant GP60 alleles belonging to the *IIa* A18G3R1 and *IIa* A19G4R1 alelle families, which persisted in New Zealand as the dominant *C. parvum* allele since 2002, were found in bovine and human *C. parvum.* The author postulates that the cyclic dominance of these subtypes in New Zealand is due to a rapacious phenotype, of an ability to rapidly fill the niche during the calving season, and survive between seasons. These results corroborate previous observations on the dominant role of *C. parvum* in humans during the seasonal peaks of morbidity, and provide the molecular epidemiological evidence to support the notion of widespread zoonotic transmission of this parasite during these peaks.

3.5.2 Introduction

Until recently, *C. parvum* was considered the sole intestinal *Cryptosporidium* species infecting young cattle and due to the high incidence of cryptosporidiosis and the large numbers of oocysts eliminated in the faeces, infected calves were considered among the major amplifiers of zoonotic *C. parvum* in nature (Harp and Woodmansee 1990; Harp et al. 1996; Grinberg et al. 2002). However, this view started to change as phenotypically-similar *Cryptosporidium* taxa of uncertain pathogenicity and zoonotic potential, such as *C. bovis*, the *Cryptosporidium* 'deer-like genotype', *C. suis*, *C. hominis*, and *C. suis*-like genotype, have been identified in cattle using molecular tools (reviewed by Santin and Trout 2008). Furthermore, results of some molecular epidemiological studies revealed the possibility of the existence of host-substructuring, and the occurrence of human *C. parvum* subtypes that do not cycle in cattle (Mallon et al. 2003a,b; Xiao and Fayer 2008; Section 2.1). Therefore, while in the past the mere identification of *C. parvum*-like oocysts in cattle was regarded as as compelling evidence for the zoonotic potential of these parasites, current epidemiological practice requires the gebetic identification of the isolates and their comparison with human parasites using molecular tools. This trend is clearly manifested by

the large number of molecular studies of *Cryptosporidium* in livestock using subtyping published in recent years (listed by Santin and Trout 2008).

Many studies have used the gene encoding the *Cryptosporidium* sporozoite surface glycoprotein GP60 (Cevallos et al. 2000; Strong et al. 2000) (see Sections 1.5 and 1.7). This locus is appealing because of its extensive sequence polymorphism, in particular the length polymorphism of a polyserine repeat. Many studies have revealed a variety of GP60 allelles in *C. parvum* from humans and cattle within different regions (Alves et al. 2003, 2006; Thompson et al. 2007; Broglia et al. 2008; Quilez et al. 2008; Zintl et al. 2009). However, it is somehow surprising that only a few studies have genetically-compared isolates collected from humans and animals over the same time and space frames (Mallon et al. 2003a,b; Section 2.1).

Human cryptosporidiosis has been a notifiable disease in New Zealand since 1996 (Learmonth et al. 2004). Since then, a significant association between the rate of notifications of cryptosporidiosis in humans and the dynamics of the livestock population have been recorded. For instance, every year the number of notified cases of cryptosporidiosis has peaked sharply after the end of the calving season in spring, and then abruptly dropped in the summer, coinciding with a sharp reduction in the number of immunologically-naive calves present (www.surv.esr.cri.nz/PDF_surveillance/AnnualRpt, accessed on 10 February 2009; Figure 1.4). Most interestingly, Learmonth et al. (2001, 2004) revealed a virtual substitution of the anthroponotic species *C. hominis* with *C. parvum* during the peaks of morbidity. However, except in the study presented in Section 3.2, no genetic comparisons between human and bovine *C. parvum* isolates were undertaken in New Zealand.

In the present study, *Cryptosporidium*-positive faecal specimens from diarrhoeic humans and calves collected over the same time period were genetically identified and subtyped by sequence analysis of the 18S rRNA gene and the *Cryptosporidium* sporozoite surface 60 kDA glycoprotein gene (GP60), to assess the possible role of cattle as a source of zoonotic cryptosporidiosis in the Waikato region. The results are discussed in the context of previous findings and the peculiar ecological conditions prevailing in the region.

3.5.3 Materials and Methods

Study location. This study used human and bovine *Cryptosporidium*-positive faecal specimens collected from overt infections in the region of Waikato, North Island, New Zealand. The Waikato is a mixed rural-urban region with *ca.* 4000 dairy farms and one million cows (2007/2008 New Zealand Dairy Statistics, Livestock Improvement Corporation Limited, www.lic.co.nz, downloaded on 10 February 2009). In 2006, the rate of notification of cryptosporidiosis in the Waikato was between 24.2 - 76.5 cases per 100,000 population, well above the national rate of 17.8 per 100,000 population (Notifiable and other Diseases in New Zealand, Annual Report 2006, www.surv.esr.cri.nz/PDF_surveillance/AnnualRpt, downloaded

on 10 February 2009). Results of previous molecular epidemiological studies indicated *C. parvum* as the predominant *Cryptosporidium* species in humans in that region (Learmonth et al. 2004).

***Cryptosporidium* positive faecal samples.** Forty eight bovine and 20 human faecal specimens were used in this study. The specimens were arbitrarily selected from a collection of bovine and human *Cryptosporidium*-positive specimens donated by veterinary and human diagnostic laboratories in the Waikato region. The bovine specimens originated from unweaned calves and were originally submitted by farmers or veterinary practitioners for analysis of causes of diarrhoea during the calving season between July-October 2006. No information about co-infections with other enteropathogens was available. Only one specimen per farm was included in this study. The human faecal specimens were originally submitted to two diagnostic laboratories for analysis of causes of diarrhoea between July-October 2006. No patient-level information was available. The bovine and human specimens were submitted on ice to Massey University and stored between 2 - 4°C with no preservatives, until analysed.

***Cryptosporidium* identification and subtyping.** Genomic DNA was extracted from the 68 specimens using a commercial DNA extraction kit (QIAamp® DNA Stool Mini Kit, Qiagen, Hilden, GmbH). The identification of *Cryptosporidium* was performed using a nested PCR followed by sequence analysis of a ~850 base-pair segment of the 18S rRNA gene described in Sections 3.1 and 3.3. Molecular-grade water was used as negative control. Forward and reverse sequences were aligned and edited with the aid of chromatograms. Proximal and distal sequence segments that could not be accurately determined were trimmed, and the edited sequences aligned with *Cryptosporidium* 18S rRNA gene sequences in GenBank using nucleotide alignment ClustalX software (Thompson et al. 1997). *Cryptosporidium* subtyping was performed by analysis of the sequence of the polymorphic region of the gene coding for the sporozoite GP60 gene (Cevallos et al. 2000; Strong et al. 2000) as described in Section 3.2.

3.5.4 Results

Thirty nine out of 48 (81%) bovine specimens yielded 18s rRNA gene amplicons of the expected size in gels. Fourteen of these (36%) yielded sequences that could be unambiguously edited. The 18s rRNA gene sequences of the remaining 25 amplicons could not be edited due to uneditable umbiguities in the chromatograms, or the absence of sequence signals. Conversely, 16 out of 20 (80%) human *Cryptosporidium*-positive faecal specimens yielded 18S rRNA gene amplicons of the expected sizes on gels, which could all be accurately edited. The 16 human sequences and 13/14 bovine sequences were identical to the sequence of the *C. parvum* 18S rRNA gene (GenBank accession number AF093490). One bovine sequence was identical to the polymorphic (or "Type B") copy of the same *C. parvum* gene (LeBlancq et al. 1997; see Section 1.3).

Unambiguous GP60 sequences could be generated for 32 (67%) bovine isolates (the GP60 sequences of eight bovine *C. parvum* isolates have been reported in Section 3.2). Thirty one out of 32 GP60 sequences of bovine isolates corresponded to known *C. parvum* alleles. Surprisingly, one isolate had a GP60 allele previously identified several times in *C. hominis* from humans. This *C. hominis* allele had been reported seven times in GenBank under the taxon name *C. parvum* 'human genotype' (accession numbers AF403169.1), and *C. hominis* (accession numbers AY382670, AY382673.1, AY382669.1, EF576980.1, EF591786.1, and EF591785.1). However, DNA extracted from the same isolate a number of months later yielded a GP60 sequence of *C. parvum* which was identical to the most abundant sequence found in this study (see below). Thus, the first *C. hominis* sequence may have been the result of an isolate identification error. Thirty eight out of 48 (77%) bovine isolates could be unambiguously identified as *C. parvum* at either the 18S rRNA gene, the GP60 locus, or both loci and ten bovine isolates (21%) could not be identified. Seven out of 20 (35%) human isolates yielded unambiguous GP60 sequences. All these human isolates yielded *C. parvum* 18S rRNA gene sequences.

There were three different GP60 sequences in the sample (Appendix 3.5). Twenty-nine bovine and six human sequences were identical to each other. According to the nomenclature suggested by Sulaiman et al. (2005), this allele belonged to the *C. parvum* GP60 allele family *IIa* A18G3R1. The same sequence was the dominant *C. parvum* allele identified in New Zealand from foals and humans between 2001-2007 (Section 3.2). A search in a database maintained by the Protozoa Research Unit of the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, New Zealand (PRU), revealed that the same *IIa* A18G3R1 allele has been identified in 165/263 (62%) of the isolates collected between 2002-2008, and genetically characterised by the Unit (Errol Kwan, personal cominication). A search in GenBank (website maintained by The National Center for Biotechnology Information (NCBI) of the National Institutes of Health, USA) revealed that this allele had also been identified from calves in Ontario, Canada (Trotz-Williams et al. 2006). Three *C. parvum* isolates from calves had GP60 sequences identical with each other and belonging to the *C. parvum* GP60 allele family *IIa* A19G4R1. A search in the PRU database indicated this allele as the second most frequent allele seen in human *C. parvum* in New Zealand, present in 64/263 (24%) isolates. The same allele has been found in bovine *C. parvum* in eastern USA (GenBank accession number DQ630516) and Canada (accession numbers AF164493.1 and DQ192504.1), and also in humans in Brasil (accession number AF164493.1). One human *C. parvum* isolate also had a GP60 sequence belonging to the GP60 allele family *IIa* A18G3R1 which differed from the sequence present in the other six human isolates at a single nucleotide external to the polyserine repeat. The difference was not likely to be an isolated editing error as it persisted after the author re-edited the sequence a year later. This allele has never been previously identified in New Zealand, and an on line search in GenBank did not return any identical sequence.

3.5.5 Discussion

In the present study, diagnostic faecal specimens were analysed in order to genetically-compare *Cryptosporidium* parasites from humans and calves collected during the peak of morbidity period in 2006 in the Waikato. The availability of isolates from both humans and cattle differentiates this study from other molecular epidemiological studies applying similar genetic typing methods which only used isolates from one host species.

Accumulated epidemiological data from a number of countries indicated that most *Cryptosporidium* infections in newborn calves are caused by *C. parvum*, whereas other taxa are rarely found in this age group (Santin et al. 2004, 2008; Trotz-Williams et al. 2006; Fayer et al. 2006; Geurden et al. 2006). The results of the present study support these results.

In the study in newborn calves in New Zealand presented in Section 3.4, the dairy farm prevalence of *C. parvum* was estimated to be ~40%. However, that study was performed in 2002, and at that juncture the genetic identification of the isolates was not carried out, so the presence of non-*parvum* taxa could not be established. Unlike the above study, the present study was not designed to estimate the farm-prevalence of *C. parvum*. Nevertheless, the identification of this species in 38 different farms in the Waikato supported the conclusion that there is a widespread presence of this parasite in the dairy econiche in New Zealand expressed in Section 3.4.

The exclusive finding of *C. parvum* in humans corroborated previous findings that indicated the dominant role of *C. parvum* in humans during the annual peaks of cryptosporidiosis notifications in spring (Learmonth et al. 2004). In the study presented in Section 2.1, a partition of the multilocus genotype repertoire of *C. parvum* between humans and cattle was identified using rarefaction analysis, which supported the notion of the existence of anthroponotic *C. parvum* cycles in Scotland. The studies presented in this section, and in Sections 2.2 and 3.2, failed to identify any genetic host-partition of the genetic repertoire of *C. parvum* in New Zealand. In fact, in the present study *C. parvum* isolates from humans and calves shared the most abundant GP60 alleles, and although in the study in Section 2.2 the sample-size of *C. parvum* was too small for an analysis using rarefaction, human and bovine isolates shared most of the multilocus genotypes, forming a single eBURST network (Figure 2.4). In addition, no putative anthroponotic *C. parvum* isolates carrying GP60 *IIc* alleles have been identified in this study, or stored in the database of the PRU. Collectively, these results do not support the existence of anthroponotic *C. parvum* cycles but rather suggest a significant role of the zoonotic transmission route in New Zealand.

The availability of the results of a study from previous years (Section 3.2), and the GP60 data stored by the PRU allowed an assessment of the temporal variation in the prevalence of the different *C. parvum* GP60 subtypes. The fact that the two most abundant alleles in 2006 were

also the dominant alleles in multiple host species since 2002 was striking. Despite the wide spectrum of *C. parvum* subgenotypes in circulation in humans in New Zealand, two subgenotypes - belonging to the allele families *IIa* A18G3R1 and *IIa* A19G4R1 - persistently dominated the samples. As hypothesised in the study presented in Section 3.4, the author postulates that the cyclic dominance of these subgenotypes is due to a rapacious phenotype, and an ability to rapidly fill the niche during the calving season and survive between seasons. The fact that this study analysed diagnostic specimens was a limitation. Further studies using representative samples of bovine *C. parvum* are needed in order to better capture the genetic diversity of this parasite species in cattle in New Zealand.

Finally, the pathogenic potential of the non-*parvum Cryptosporidium* cycling in cattle is not well understood. Apart from six studies analysing diagnostic specimens (Mallon et al. 2003a,b; Thompson et al. 2007; Broglia et al. 2008; Quilez et al. 2008; Soba and Logar 2008), all the molecular studies analysing *Cryptosporidium* isolates of bovine origin analysed specimens collected from animals of a wide age range and/or on a limited number of selected farms, with no clinical history indicated (McLauchlin et al. 2000; Learmonth et al. 2001; Alves et al. 2003; Peng et al. 2003; Santin et al. 2004; Neira-Otero et al. 2005; Roy et al. 2006; Geurden et al. 2006, 2007; Trotz-Williams et al. 2006; Langkjar et al. 2007; Xiao et al. 2007; Duranti et al. 2008; Feltus et al. 2008; Santin et al 2008; Soba and Logar 2008). The results of the present study are in accordance with the above six studies, indicating *C. parvum* as the sole diarrhoeagenic *Cryptosporidium* species in newborn calves.

3.6 CONCLUDING REMARKS

Chapter 3 reports five epidemiological studies of cryptosporidiosis in foals, cattle and humans in New Zealand. The combined results of studies 3.1, 3.2, and 3.3 indicate that clinically overt infections with *C. parvum* are relatively common in foals in New Zealand, and that the condition is likely to be underdiagnosed. The eight cases of cryptosporidiosis documented in foals in Sections 3.1- 3.3 had an onset between the first and third week of life. The disease was self-limiting and characterised by a short and intense oocyst shedding period, resembling neonatal cryptosporidiosis in calves. The infections were caused by *C. parvum* parasites genetically similar to bovine and human isolates, indicating the possibility of cross-transmission of *C. parvum* between these host species. Due to the potential for zoonotic transmission of *C. parvum*, it is advisable to take precautions when handling diarrhoeic foals and calves until this agent has been ruled out by the laboratory.

The results of the studies described in Section 3.4 - 3.5 indicate newborn calves are significant amplifiers of potentially zoonotic *C. parvum* in New Zealand, despite the short and concentrated calving pattern characterising dairy farming. Biomasses from calf-rearing operations should be adequately treated in order to avoid the contamination of the environment with infectious

oocysts. The finding of two persistently dominant GP60 *IIa* alleles in *C. parvum* warrants further investigation.

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CHAPTER 4

AN OUTBREAK OF CRYPTOSPORIDIOSIS AMONG A COHORT OF YOUNG ADULTS: MOLECULAR AND DESCRIPTIVE EPIDEMIOLOGY AND RISK FACTOR ANALYSIS

This chapter describes the investigation of an outbreak of gastrointestinal illness amongst a class of veterinary students at Massey University in 2006. The outbreak was investigated by combining traditional epidemiological and molecular techniques. Apart from a number of diagnostic tests performed by commercial laboratories as acknowledged in the text, all the laboratory analyses were performed at Massey University.

4.1 Summary

An explosive outbreak of gastrointestinal illness, which occurred in New Zealand in 2006 among a cohort of 96 veterinary students, instigated laboratory and questionnaire-based epidemiological investigations. There were 25 clinical cases among 80 respondents to the questionnaire (31% attack rate). *Cryptosporidium parvum* was isolated as the sole enteropathogen from four out of seven faecal specimens analysed. A rare GP60 *IIa* A21G4R1 allele was identified in two out of two *C. parvum* isolates successfully subtyped, indicating a point-source of infection. The results of the laboratory and epidemiological investigations ruled out a water-borne cause, leaving direct contact with newborn calves during a calf-handling practicum as the most likely point-source of exposure.The investigation failed to identify *C. parvum* in faecal specimens taken from calves on the farm that provided animals for the practicum, but revealed a DNA sequence very similar to the 18S ribosomal RNA gene of *Cryptosporidium bovis* in one specimen. Assuming that exposure to *C. parvum* occurred during the calf-handling practicum, the median incubation period was 5 days (range 0-11 days). All the cases were self-limiting and manifested with diarrhoea, abdominal discomfort, and in a small proportion of cases, vomiting. Among the putative risk factors analysed, originating from a rural background and previous contact with ruminants, were associated with a decreased risk of illness in males, but not in females.

4.2 Introduction

Protozoan parasites belonging to the genus *Cryptosporidium*, in particular *Cryptosporidium parvum* and *Cryptosporidium hominis,* are common causes of gastrointestinal disease in humans worldwide (Fayer 2008). Whilst *C. parvum* infects humans and young livestock and can be transmitted zoonotically, *C. hominis* is thought to cycle primarily among humans. Both species can be transmitted through the ingestion of water or food contaminated with oocysts. As the oocysts are excreted sporulated and fully infectious in the faeces, transmission through direct contact with infected hosts is also possible.

Cryptosporidiosis may manifest sporadically or as epidemic outbreaks. Outbreaks as a result of person to person transmission, ingestion of contaminated water supply or food, following exposure in swimming pools and water parks, and contact with farm animals, including among veterinary students, have been described (Alpert et al. 1986; Combee et al. 1986; Pohjola et al. 1986; Heijbel et al. 1987; Reif et al. 1989; Shield et al. 1990; Miron et al. 1991; Millard et al. 1994; Anonymous 2000; Hellard et al. 2000; Stefanogiannis et al. 2001; Preiser et al. 2003; Mathieu et al. 2004; Causer et al. 2006; Jones et al. 2006; Neira-Munoz et al. 2007; Turabelidze et al. 2007; Wheeler et al. 2007; Gait et al. 2008; Hajdu et al. 2008; Beaudeau et al. 2008; Hoek et al. 2008; Ethelberg et al. 2009). Although in many countries cryptosporidiosis is a notifiable disease, the outbreaks are often protracted and difficult to detect because of delays in the investigations (Miron et al. 1991; Millard et al. 1994; Hellard et al. 2000; Anonymous 2000; Preiser et al. 2003; Mathieu et al. 2004; Causer et al. 2006; Neira-Munoz et al. 2007; Turabelidze et al. 2007; Wheeler et al. 2007; Hajdu et al. 2008; Beaudeau et al. 2008, Valderrama et al. 2009). In addition, most previous cryptosporidiosis outbreak investigations have dealt with outbreaks in open communities (Alpert et al. 1986; Pohjola et al. 1986; Levine et al., 1988; Reif et al. 1989; Miron et al. 1991; Millard et al. 1994; Anonymous 2000; Hellard et al. 2000; Preiser et al. 2003; Mathieu et al. 2004; Causer et al. 2006; Neira-Munoz et al. 2007; Wheeler et al. 2007; Beaudeau et al. 2008; Hajdu et al. 2008; Hoek et al. 2008), requiring the use of case-control studies, which are more prone to uncontrollable confounding than cohort studies (Anonymous 2000; Crombie et al. 1981; Vonberg and Gastmeier 2007). Traditionally, the most compelling reason to investigate the outbreaks has been to determine the source of infection in order to prevent further transmission, and limited efforts have been directed towards the collection of clinico-epidemiological data during outbreaks. Little documented clinico-epidemiological information on infections in immunocompetent hosts exists (reviewed by Warren and Guerrant 2008), and knowledge about the natural history of cryptosporidiosis has mainly been obtained from experimental infection trials in human volunteers (Chappell et al. 1999, 2001; Okhuysen et al. 1999). Furthermore, apart from immunosuppression (Hunter and Nichols 2002), the constitutional risk factors for cryptosporidiosis are not understood.

In 2006, the author investigated an outbreak of gastrointestinal illness in a class of 96 veterinary students, in which *C. parvum* was isolated as the sole agent from multiple faecal specimens. The synchronous exposure of a cohort of young adults to wild *C. parvum* facilitated the collection of clinico-epidemiological data and an assessment for the presence of exposure and constitutional risk factors for cryptosporidiosis in the context of a retrospective cohort study. The epidemiological features of the outbreak and the results of a risk factor analysis are reported in this section.

4.3 Materials and Methods

Sequence of events. On October 9, 2006 (Day 13), three students from a class of 96 enrolled in the second year of the Bachelor of Veterinary Science program of Massey University, New

Zealand, reported a gastrointestinal illness to the regional public health service. As a result, faecal specimens from these students were submitted for analysis for *Campylobacter* and *Cryptosporidium* to a diagnostic laboratory associated with the same public body. The specimens tested negative for *Campylobacter* and one tested positive for *Cryptosporidium.* The presence of a single *Cryptosporidium*-positive specimen precluded the identification of an outbreak by the authorities at that stage. On October 10 (day 14), a concerned student from the same class informed a faculty member of the Institute of Veterinary, Animal and Biomedical Sciences (IVABS) of Massey University, about the occurrence of several cases of illness among students enrolled in the same class. The student inferred that the cases appeared to be linked to a practicum held on September 26 (Day 0) in the Large Animal Teaching Unit (LATU), in which the students handled young calves of less than one month of age. During the practicum, groups of students stayed in a calf barn for about 20 minutes to practise thoracic auscultations, as well as measuring the rectal temperature and respiration rate. No gloves were provided, but the students were able to wash hands at the end of the practicum. No other academic or recreational activities had occurred that could have resulted in exposure of the class to enteropathogens, and there were no other known cases of gastroenteritis among students from other classes during the same period. On October 11 (Day 15), an urgent request to conduct an outbreak investigation was solicited by the author. The request was approved by Massey University's Human Ethics Committee on October 13 (Day 17). The same day, the author addressed the class and invited the students to anonymously submit stool specimens to the microbiology laboratory of IVABS during the weekend of October 13-15. The diagnosis of cryptosporidosis was released the morning of Monday 16 October (Day 18). An environmental investigation at the LATU was instigated on October 17 (Day 21). On October 19 (Day 23), the author and a university health and safety officer delivered a questionnaire to the class. A second supplementary questionnaire was delivered on May 25, 2007.

Environmental investigation. The LATU was visited on October 17. No calves were present in the facility at the time of the visit. The facility received potable water from a non-artesian bore, which was maintained by university staff. The depth of the bore was 200 m below ground level. Water sanitation consisted of treatment with utraviolet light at the point of use. A 100L water sample was collected at one of the drinking water taps and filtered on-site using a commercial filter (FiltaMax®, Idexx Laboratories, USA). Flow rate was adjusted to 2L/min through the filter. The sample was tested for the presence of *Cryptosporidium* oocysts as described below.

The dairy farm from which the newborn calves were obtained was visited the same day in order to assess whether *C. parvum* was cycling in calves. The farm was situated about 5 km from the teaching facility. The calves actually used for the practicum had been sold, and thus could not be traced. As the calving season had ended in September, other calves less one month of age were not present on the farm. However, faecal specimens from nine calves older than 30 days of age were collected and submitted on ice to the microbiology laboratory of IVABS.

Microbiological analysis. Smears from the human and bovine stool specimens were examined microscopically for the presence of *Cryptosporidium* oocysts and *Giardia* using a commercial immunofluorescence test kit (MeriFluor C/G, Mediridian Bioscience, Cincinnati, Ohio, USA). In addition, the human specimens were tested for the presence of *Salmonella* spp., group A rotavirus, norovirus genogroups 1 and 2, and *Campylobacter* spp.

Testing for *Salmonella* spp. and group A rotavirus was performed as previously described (Section 3.1). Testing for norovirus genogroups 1 and 2 was performed by means of reverse transcriptase real-time PCR at the New Zealand Norovirus reference laboratory (Institute of Environmental Science and Research, Porirua City). Testing for *Campylobacter* was performed only in three human specimens that had been submitted to the diagnostic laboratory associated with the public health service (see above) using routine diagnostic procedures.

Testing for *Cryptosporidium* oocysts in drinking water was performed by Mr. Anthony Pita, Protozoa Research Unit, Massey University. Briefly, the filter was taken apart and the foam disks were homogenised with elution buffer. The eluant was collected and centrifuged at 1500g or 15 min. The pellet volume was recorded and supernatant aspirated from the tube until the volume above the pellet was 5 ml. Water was added so the pellet volume was 5% or less in a 10 ml sample. An anti-*Cryptosporidium* and anti-*Giardia* immunomagnetic separation kit (Dynabeads® GC-Combo IMS kit: Invitrogen, Carlsbad, CA, USA) was used for the separation of the oocysts. The sample was then dried onto a slide, stained with FITC labelled anti-*Giardia* and anti-*Cryptosporidium* antibodies (AquaGlo™ G/C kit, Waterborne Inc., New Orleans, LA, USA) and screened microscopically for the presence of apple-green oocysts using an epifluorescence microscope.

In October 2007, genomic DNA was extracted from the *Cryptosporidium*-positive human specimens and from the nine bovine specimens using DNA extraction kits as described in Section 3.5. For the taxonomic identification of *Cryptosporidium*, a fragment of the 18S ribosomal RNA (18S rRNA) gene was amplified using a nested PCR followed by sequence analysis. The identified *Cryptosporidium* were subjected to subtyping by means of PCR followed by sequencing of the polymorphic regions of the sporozoite 60-kDa glycoprotein (GP60) and the 70 kDa heat shock protein (HSP70) genes. The PCR and sequencing procedures for the 18S rRNA and HSP70 genes have been described in Sections 3.2 and 3.5. The GP60 sequences were aligned with other similar sequences reported in Genbank.

Questionnaires. Two questionnaires were delivered to the class on different dates and were anonymously completed in the classroom by interested students. The first questionnaire (Q1; Appendix 4.1) was delivered on 19 October 2006. The responses to Q1 were used to elicit the epidemic curve and the demographics and distribution of the clinical signs, and assess the significance of putative constitutional risk factors for illness, such as gender, nationality, chronic

use of medication, domestic background (rural or urban), and the frequency of previous physical contact with ruminants. A calendar reporting the dates of the main academic activities held between September 18 and October 18 was provided, in order to facilitate the correct identification of the dates of illness by the affected students. In order to minimise response bias, Q1 did not include specific questions addressing the calf-handling practicum. Thus, in order to assess the rate of attendance at the calf-handling practicum and the source of drinking water (tap water, other) consumed by the students during the practicum, a second supplementary questionnaire (Q2) was necessary. This questionnaire was delivered on 24 May 2007and included only three straightforward questions (Appendix 4.2).

Analysis of the data. Responses to Q1 and Q2 were manipulated using an electronic spreadsheet and used for the description of the demographics of the outbreak, the natural history of disease, the epidemic curve, and for a risk factor analysis. A case was defined as any student reporting abdominal discomfort, and/or diarrhoea, and/or vomiting between September 18 and October 18 2006, in Q1.

The statistical significance of the differences between proportions was assessed using the two-tailed Fisher's exact test. For the analysis of risk factors, the strength of association between exposure or constitutional variables and the outcome binary variable (case; non-case) was assessed using the relative risk (RR) and its probability under a null model of RR=1, and the 95% confidence interval (CI). Variables used were: gender, nationality (New Zealand citizens; non-New Zealanders), background (rural; urban), history of previous physical interaction with ruminants (no interaction; less than one-two interactions/year; more than one/two interactions/year), taking chronic medication (yes; no), attendance at the calf handling practicum (attended; not attended), drinking tap water at the same practicum (yes; no) (see Appendices 4.1-4.3). The presence of bivariate interactions between independent variables was explored by stratification of the data into 2x4 tables and calculation of the Wald's statistic for homogeneity of strata (Rothman et al. 2008). Adjusted Mantel-Haenszel relative risks (Mantel and Haenszel 1959) were calculated for the comparisons not showing heterogeneity of strata by the Wald's test (as indicated by $p > 0.05$). All the calculations were performed using Rothman's Episheet software (http://members.aol.com/krothman/episheet.xls, downloaded 27 August 2005). The modest sample size precluded a more complex multivariate analysis (see below).

4.4 Results

Response to the request for co-operation. Seven students submitted stool specimens to IVABS between 14-15 October 2006. One of the specimens, of a liquid consistency, had been collected by a student on October 8 and since then held in a home refrigerator. Three formed specimens collected on October 9 by the students who contacted the regional public health service (see above) were reclaimed by the same students and resubmitted to IVABS. An additional three formed specimens were dispatched to IVABS on October 15, with no

accompanying information. A total of 80 (83%) students completed Q1, and 64 (67%) completed Q2. Q1 was fully answered by 75 (93%) students (Appendix 4.3).

Microbiological findings. All the human specimens tested negative for group A rotavirus, *Salmonella* spp., norovirus and *Giardia*. Four specimens tested positive for *Cryptosporidium* oocysts by immunofluorescent stain. One *Cryptosporidium*-positive specimen had been collected on October 8, two on October 9, and one October 14. One *Cryptosporidium*-positive specimen collected on October 9 had previously tested positive also at the diagnostic laboratory (see above).

Only three out of four *Cryptosporidium*-positive specimens yielded editable 18S rRNA gene sequences. Two of the positive sequences were indistinguishable from the *C. parvum* 'Type A' 18S rRNA gene sequence reported in Genbank under accession number L16996 (Johnson et al. 1995); the third sequence was indistinguishable from the 'Type B' polymorphic copy of the 18S rRNA gene of *C. parvum* (Le Blanq et al. 1997).

The GP60 locus could be amplified from two *Cryptosporidium*-positive human specimens and the HSP70 from all four specimens. All the edited GP60 and HSP70 sequences were longer than 740 and 380 base pairs, respectively, and comprised the polymorphic repeat regions (Appendix 4.4). The two GP60 sequences were indistinguishable, and belonged to the allelic group *IIa* A21G4R1 (according to the nomenclature proposed by Sulaiman et al. 2005). There were no previous reports of this GP60 sequence in New Zealand, and a search in Genbank revealed that it had only been reported in some calves in the United States (GenBank accession number DQ630514). Most interestingly, the same sequence was also found in an isolate from an unpublished outbreak of cryptosporidiosis among veterinary students in the United Kingdom (GenBank accession number EU262602.1).

There were two different alleles at the HSP70 locus, differing by the addition of a single thymine downstream of the repeat region. One HSP70 allele was common to three isolates, and the second was only present in one isolate. The same polymorphism was conserved after re-editing of the second sequence in 2009, arguing against an initial editing error (Appendix 4.4).

All the bovine specimens from the source farm were negative for *Cryptosporidium* oocysts, but one specimen yielded an amplicon of a sequence that was 99% similar to the 18S rRNA gene of *C. bovis* (Santin et al. 2004) (Appendix 4.4). An identical sequence was not retrieved in Genbank. No *Cryptosporidium* oocysts were observed in drinking water collected during the visit to the large animal teaching facility.

Clinico-epidemiologal features of the outbreak. The demographic characteristics of the class, as elicited from the responses to Q1, are reported in Table 4.1. Seventy percent of the

respondents to Q1 were female and 28% were non-New Zealand citizens. These values were very similar to those of the entire class roll (not shown). The range and mean of the age of the students were very similar in both genders. There was no statistically-significant difference between the proportions of males and females coming from a rural or urban background (two tailed Fisher's exact p=0.3). The proportions of males and females that had never handled ruminants prior to the calf-handling practicum were not significantly different (p=0.19). In both genders, the proportion of students that had previously handled ruminants more than once-twice was greater in students from a rural background (not shown). A greater proportion of males had previously handled ruminants more than once-twice a year than females (p=0.0096).

There were 25 cases among the 80 respondents to Q1 (31%), and 15 among the 64 respondents to Q2 (23%). The difference in the proportion of cases between Q1 and Q2 were not statistically significant (two-tailed Fisher's exact p=0.35). Nine out of 25 (36%) cases sought medical advice during the course of the illness. All the respondents to Q2 had attended the calf-handling practicum and none reported having drunk tap water at LATU, ruling out a water-borne source of infection there.

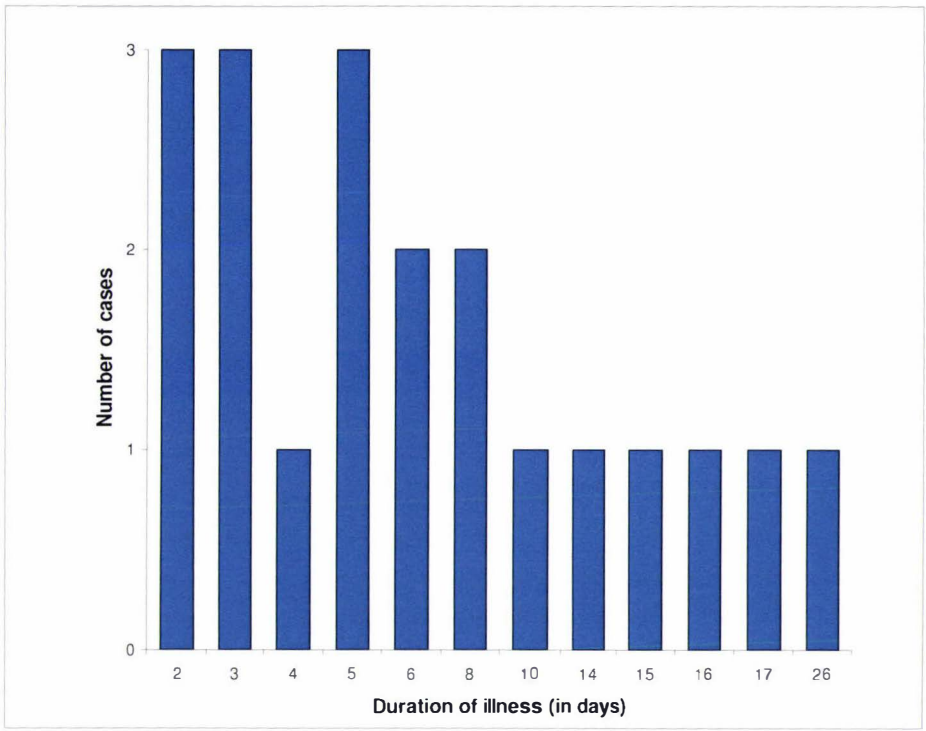
The duration of illness and distribution of the clinical signs, as elicited by the responses to Q1, are reported in Figure 4.1. Notice that the median duration of the illness was 5-6 days (range 2-26 days), and that the majority of cases suffered from diarrhoea and abdominal discomfort, whilst vomiting was only reported in six cases. The epidemic curve is reported in Figure 4.2. Only 2 out of 25 (8%) cases reported an illness starting before the date of the calf-handling practicum; the median and mode of the date of onset of the illness coincided on Day 5 after the same practicum.

Risk factor analysis. The results of the analysis for the presence of risk factors are summarised in Table 4.2. There were no differences in the proportion of cases between males and females (p=0.8), New Zealand citizens and non-New Zealanders, overall (p=0.3), and in each gender separately. In both genders, the habit of not washing hands after practicums was not significantly associated with an increased risk of illness (p=0.4 in females and 0.6 in males). No significant difference in the risk of illness was observed between students from rural or urban background (p=0.3). However, there was a reduction of the risk of illness in males from rural background as compared to their urban counterparts (RR=0.00; p=0.008), as well as compared to rural and urban females (the statistical comparisons between rural males and rural and urban females not shown but the data are reported in Table 4.1). In agreement with this finding, there was a greater risk of illness in males that had not handled ruminants (p=0.01), or handled ruminants up to twice a year (p=0.01) as compared with their counterparts, but these associations were not seen among females. However, as said, a greater proportion of males from rural background had reported handling ruminants more than once-twice a year than females (Table 4.2).

Five students reported using chronic medication, of which three were defined as cases (Appendix 4.3). Interestingly, these three cases spontaneously reported the chronic use of anti-asthma inhaled steroids (not shown).

	Males	Females
Age range (mean)	19-34 (22.5)	18-37 (22.5)
Number of students that sought medical advice during the illness/total respondents	1/24	8/56
Number of students that submitted a faecal specimen/total respondents	1/24	6/56
Smokers	0	1
Students of rural background/urban background	11/23	19/56
Number of students that never handled ruminants/total respondents	7/24	25/56
Number of students that handled ruminants more than once-twice a year/total respondents	13/24	13/56*

Table 4.1 Demographic characteristics of the outbreak of cryptosporidiosis among a class of veterinary students reported in Section 4.1. Asterisks denote a significant difference between the proportions in both genders (Fisher's exact test, $p<0.05$)



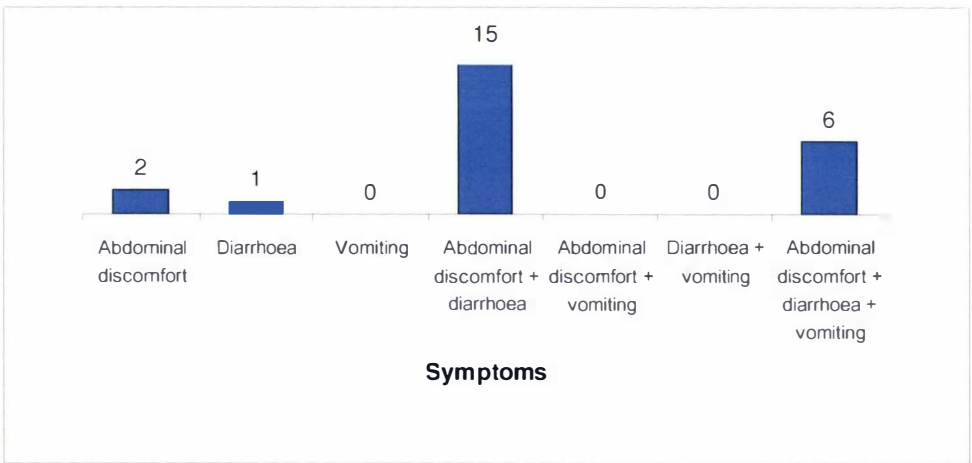


Figure 4.1 Duration of illness (upper graph) and distribution of symptoms (lower graph) in the outbreak of cryptosporidiosis, as elicited from the responses to questionnaire Q1. The number of respondents in the lower graph is reported above each bar.

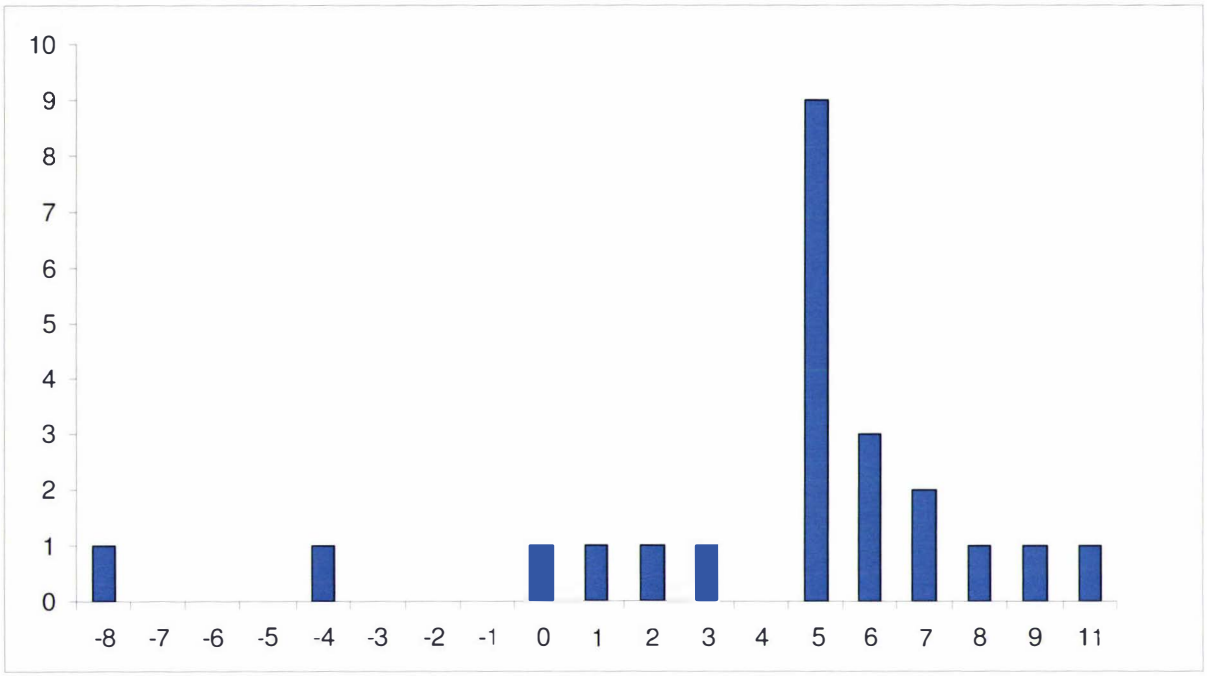


Figure 4.2 Epidemic curve of the outbreak of cryptosporidiosis as elicited by the responses to Q1

Putative risk factor (questionnaire number)	Factor present	Factor absent	RR (CI; p-value)	χ2 Wald test
	Number of cases/total respondents	Number of cases/total respondents		
Exposure factors				
Attended the calf-handling practicum (Q2)	15/64	n/a	n/a	n/a
Drank tap water during the calf-handling practicum (Q2)	0/0	15/64	n/a	n/a
Do not wash hands after practicums (Q1)				
all	4/13	21/67	0.98 (0.4 - 2.4;0.9)	
females	2/4	16/52	1.6 (0.5 - 4.7; 0.4)	p=0.30
males	2/9	5/15	0.66 (0.1 - 2.7; 0.6)	
MHrr			1.03 (0.4 - 2.5; 0.95)	
Constitutional factors				
Gender = male	7/24	18/56	0.9 (0.4 - 1.9; 0.8)	n/a
Non New Zealander (Q1)				
all	9 /23	16 /57	1.4 (0.7 - 2.7; 0.33)	
females	5 /15	13 /41	1.0 (0.4 - 2.4; 0.9)	p=0.22
males	4 /8	3 /16	2.7 (0.7- 9.1; 0.12)	
MHrr			1.4 (0.7-2.7; 0.33)	
Rural background (Q1)				
all	17/49	7/30	1.5 (0.7 - 3.15; 0.3)	
females	7/19	11/37	1.2 (0.5 - 2.7; 0.6)	n/c
males	0/11	6/12	0; p=0.008	
MHrr			0.7 (0.3-1.4; 0.31)	
Never handled ruminants (Q1)				
all	13/32	12/48	1.6 (0.9 - 3.1; 0.14)	
females	8/25	10/31	1 (0.5 - 2.1; 0.98)	p=0.025
males	5/7	2/17	6 (1.5 - 24; 0.01)	
MHrr			not calculated	
Handled ruminants up to twice a year, or never (Q1)				
all	20/54	5/26	1.9 (0.8 - 4.6; 0.1)	
females	14/43	4/13	1.05 (0.4 - 2.7; 0.9)	p=0.07
males	6/11	1/13	7 (1 - 50; 0.01)	
MHrr			1.8 (0.8 - 4; 0.11)	

Table 4.2 Risk factor analysis of the outbreak of cryptosporidiosis reported in Section 4.1. RR, relative risk; MHrr, Mantel-Haenszel relative risk; CI, 95% confidence intervals; n/a, not applicable;n/c=not calculated due to a value of zero in the data

4.5 Discussion

The present outbreak provided a unique opportunity to perform the investigation in the context of a retrospective cohort study. As in other investigations of outbreaks of cryptosporidiosis (Reif et al. 1999; Anonymous 2001; Mathieu et al. 2004; Wheeler et al. 2007), the availability of only four laboratory-confirmed cases was a limitation, which could have had implications on the specificity of the case definition. For instance, two cases reported an illness before the

practicum (Figure 4.2) but nevertheless, were included in the analysis. Their inclusion was necessary because the same students may have also suffered from cryptosporidiosis. On the other hand, the fact that the investigation could be performed with only short delay was a strength, which most likely prevented recall bias in Q1.

The finding of *C. parvum* as the sole agent in multiple specimens corroborated the occurrence of an outbreak caused by this parasite species. The outbreak was initially overlooked by the health authority, as only one out of three faecal specimens from the students tested positive for *Cryptosporidium* at the diagnostic laboratory, which precluded the identification of a cluster of cases. In this case, the authorities may have over-emphasized the value of the laboratory test results. To the author's knowledge, the diagnostic laboratory used a commercial antigen-detection kit for the analysis of cryptosporidiosis, whereas the the same specimens were tested using immunofluorescence at MU. Due to the potential for massive outbreaks of cryptosporidiosis, it would be prudent to assess the sensitivity of the diagnostic methods employed by the diagnostic laboratories in New Zealand.

The results of the molecular analysis of the *C. parvum* isolates supported the hypothesis of a point source oubreak, as in New Zealand the likelihood of finding such a rare GP60 allele in isolates from individuals acquiring infections from independent sources would have been very small. The results of the responses to Q2 and the water testing ruled out a water-borne outbreak, leaving the direct contact with newborn calves at the practicum as the only likely route of transmission.

Excluding the two cases with an onset on Days (-4) and (-8) (which were probably unrelated to the outbreak), if transmission during the calf-handling practicum is assumed, the median and mode of the incubation period coincide at five days post transmission, and the range is between 0-11 days post-exposure (Figure 4.1). These values fit the median and range observed in experimental infection trials in human volunteers for which most current knowledge of the natural history of cryptosporidiosis is derived (Chappell et al. 1999; Chappell and Okhuysen 2006; Okhuysen et al. 1999), and those extrapolated in an early retrospective outbreak investigation (Millard et al. 1994). The self-limiting diarrhoeal illness accompanied by abdominal discomfort and in a small proportion of cases also vomiting (Figure 1) were consistent with the results of previous retrospective studies in immunocompetent populations (Wolfson et al. 1985; Mac Kenzie et al. 1994; Mathieu et al. 2004; Anonymous 2001; Causer et al. 2006). However, vomiting had been reported by 82% of the cases in one outbreak of cryptosporidiosis from fresh-pressed apple cider (Millard et al. 1994).

As in other published cryptosporidiosis outbreak investigations (Hellard et al. 2000; Anonymous 2001; Turabelidze et al. 2007), an environmental source of infection could not be microbiologically traced. Unfortunately, calves less than one month of age were not available for

sampling, and so *C. parvum* could not be detected, as in calves the oocyst shedding period is usually short and concentrated in the first three weeks of life (see Section 1.6.1). The finding of a nucleotide sequence mostly similar, but not identical to the sequence of the 18S rRNA gene of *C. bovis* in calves was not surprising, given the extensive genetic diversity revealed in genus *Cryptosporidium* since the advent of molecular tools for the genetic characterisation of the isolates. This sequence remains of uncertain taxonomic positioning and pathogenic significance. Also the single nucleotide polymorphism observed in the HSP70 locus was not surprising. Indeed, genetic differences between *Cryptosporidium* isolates had been documented in previous outbreaks in humans (Glaberman et al. 2002; Leoni et al. 2007), and in our previous investigations of enzootic cryptosporidiosis in foals (see Section 3.2). In this case the genetic polymorphism in the HSP70 locus may have reflected a PCR or sequencing artefact, or the presence of genetically heterogeneous parasites.

In the present outbreak, no significant associations between any exposure or constitutional factor and the risk of illness were observed at the bivariate level, but some associations reached a statistical significance after stratification of the data. In particular, males coming from a rural background had a lower risk of illness than urban males and rural and urban females (Table 4.2). This risk-reduction should not be viewed as evidence of an effect of gender on the susceptibility to *C. parvum,* as this effect was confounded by the presence of a greater proportion of males reporting frequent contact with ruminants than females (Table 4.1). A plausible explanation for the risk-reduction in males from rural background could be that of the presence of a greater rate of immunity to *C. parvum* in males, acquired through previous exposures during repeated contacts with ruminants. Unfortunately, the modest size of this cohort precluded a more exhaustive multivariate analysis to disentangle the relationships between the variables, as initial exploration of logistic regression models including the independent variables of gender, background and the frequency of handling ruminants, resulted in an unbalanced design, perhaps due to the small size of the cohort (not shown). It is, however, noteworthy, that the effect of previous exposure on the susceptibility to cryptosporidiosis is not well understood. So far this effect has only been investigated by means of experimental infections in human volunteers, with conflicting results. As would be expected, volunteers experimentally pre-exposed to *C. parvum* showed a partial resistance to re-infection in one study (Okhuysen et al. 1999). However, in another experimental infection study, volunteers with pre-existing anti-*Cryptosporidium* antibodies exhibited a more severe disease than their sero-negative counterparts (Chappell et al. 1999).

In conclusion, an explosive outbreak of cryptosporidiosis in New Zealand caused by a rare subgenotype of *C. parv*um is reported. The attack rate was 31%. The infections were most likely transmitted through direct contact with young calves. The illness was self-limiting in all cases, with a median incubation period of 5 days. Symptoms included diarrhoea and abdominal discomfort, and vomiting in a limited proportion of cases. A significant disease-risk-reduction in

males coming from a rural background, attributable to an immunity acquired through previous exposures to *C. parvum* in the farm environment, was observed. To the author's knowledge, this is the first study in which a constitutional risk factor for cryptosporidiosis other than immunosuppression was identified. However, due to the modest size of the cohort, this study can be viewed as a hypothesis-generating rather than testing, and so the results should be corroborated by large-scale epidemiological studies.

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CHAPTER 5

GENERAL DISCUSSION

The first publications by Tyzzer defined most of the knowledge about the biology of *Cryptosporidium* parasites, whilst the recent advent of molecular tools for the genotyping of the isolates is currently enabling a clearer understanding of the epidemiology of cryptosporidiosis. The studies presented in this thesis were performed during a period of rapid expansion in the use of molecular tools for the study of *Cryptosporidium*, and applied a variety of such tools.

Chapter 2 reports two studies that analysed multilocus genotype data to explore the population genetic structure of *C. parvum* and *C. hominis.* For the analysis of the data, classic analytical techniques of population molecular genetics and rarefaction analysis - a statistical test traditionally used in ecological and paleontological studies - were combined. Conversely, Chapter 3 reports straightforward studies that applied simple genotyping schemes for epidemiological source-tracking. Ultimately, the epidemiological and molecular expertise acquired by the author over the years was used to investigate an outbreak of cryptospridiosis in humans in New Zealand.

The studies presented in Chapter 2 contribute to the discussion on the population structure of *C. parvum* and *C. hominis* in several ways. Firstly, the results of the study in Section 2.2 provide statistical support to the emerging idea of the existence of anthroponotic *C. parvum* cycles that do not involve local cattle reservoirs in Scotland. Unfortunately, the modest sample size precluded a comparative analysis between human and bovine *C. parvum* in New Zealand using rarefaction analysis. Also the use of different genetic markers by the research groups in Scotland (Wellcome Centre for Molecular Parasitology) and the USA (Tufts University) precluded a direct comparison between the samples from Scotland and New Zealand. However, anthroponotic *C. parvum* cycles could not be identified in New Zealand. Indeed, a striking homogeneity in the genetic repertoire of *C. parvum* was seen in this country using multilocus genotyping, with bovine and human isolates from North and South Island composing a single star-like eBURST network (Section 2.2). This homogeneity was later corroborated by comparative analysis of human, bovine and equine isolates using single and bilocus genotyping schemes in the studies presented in Sections 3.2- 3.5. In addition, putative anthroponotic *C. parvum* parasites carrying GP60 alleles belonging to the allele family *IIc* could not be identified in New Zealand.

Secondly, the study in Section 2.2 provides new data on the structuring role of geography in *C. parvum* and *C. hominis* populations. On the whole, the results show that gene flow is not sufficient to erase genetic divergence among geographically separated parasite populations, which basically remain allopatric. This result was somewhat surprising because *C. parvum* and *C. hominis* are cosmopolitan microorganisms small enough to be transported mechanically, or

through overt or subclinical infections, thus enabling mixing of oocysts from different sources. In the same study, the clear identification of five *C. hominis* isolates introduced to the UK from Pakistan via travel also demonstrate the feasibility of tracking *C. parvum* and *C. hominis* parasites to their country of origin using multilocus genotyping. The author hypothesises that the ecology of cryptosporidiosis may have selected for the best-adapted parasites in each environment, and that imported parasites would be unlikely to spread if environmental factors are unfavourable.

Lastly, the results of the study in Section 2.2 are inconsistent with a strict categorization of *C. parvum* and *C. hominis* as either clonal or panmictic. Instead, they support the notion of the co-occurrence of both mutational and recombinatorial diversification, with geography playing an important structuring role. In some regions where sanitary conditions are good (as in the UK), selfing seems to prevail, giving rise to what is often referred to as a "clonal" population, characterized by the occurrence of a small number of multilocus genotypes and a highly abundant putative founder type. Conversely, in some developing regions (as in Uganda) the genetic diversification of *C. parvum* and *C. hominis* seems to be accelerated by recombination between genetically-heterogeneous gametes. This may be due to suboptimal sanitation and high HIV prevalence, which enhance infections with environmental oocysts originating from multiple sources. The process generates a so-called 'panmictic' population, characterized by the occurrence of a large number of multilocus genotypes and the absence of an obvious founder type. These differences in the structures of *C. parvum* and *C. hominis* populations between countries may plausibly explain - at least in part - the severity of cryptosporidiosis observed in some developing regions, as the accelerated diversification by recombination could enhance the frequent appearance of novel, highly virulent genetic variants. However, the clinico-epidemiogical implications of these differences remain to be established, taking into account confounding factors related to the host, such as the higher prevalence of HIV-related immunosuppression in some developing countries.

It is relevant to highlight here the limitations of the above and previously published studies of population genetic structure of *C. parvum* and *C. hominis*. In all these studies, the structures have been inferred based on the observed genetic variation between isolates, as revealed following PCR amplification of multiple polymorphic markers. However, this approach may introduce bias, as the true genetic variation in *Cryptosporidium* is between the sporozoites, within the isolates. Unfortunately, this variation is difficult to assess using PCR, due to the problem of preferential amplification of the templates, as discussed in Section 1.3.

The studies presented in Sections 3.1-3.3 defined the basic molecular and clinico-epidemiological aspects of cryptosporidiosis in foals and, to the author's knowledge, represent the most comprehensive series of studies of cryptosporidiosis in these hosts reported in the literature. The study in Section 3.1 represents the first known description of the disease in foals

which includes clinico-epidemiological and pathological data, as well as the genetic identification of the isolates as *C. parvum*. At the time the study was done, genetic subtyping tools for *Cryptosporidium* were still embryonic and so the characterisation of the isolates was limited to the definition of the taxon. In the following years, the suggestion that a *Cryptosporidium* 'horse genotype' exists and the notion of the occurrence of anthroponotic *C. parvum* cycles triggered the study presented in Section 3.2. The genetic characterisation of nine *Cryptosporidium*-positive specimens collected from foals for that study resulted in the identification of *C. parvum* in all cases. Therefore, the notion of the occurrence of a *Cryptosporidium* 'horse genotype' in horses could not be validated. After identifying *C. parvum* as the dominant species parasitising young foals in New Zealand, it was necessary to perform a comparative genetic study of the isolates from foals, calves, and humans, in order to infer the transmission routes and assess the zoonotic potential of cryptosporidiosis in foals. Such a study was then feasible, as numerous polymorphic loci in the genome of *C. parvum* had already been reported. The results of the genetic comparison of diarrhoeagenic *C. parvum* isolates from foals, humans and cattle indicated the three host-species were infected with genetically-similar parasites, and so cryptosporidiosis in foals should be considered potentially zoonotic. Finally, the study presented in Section 3.3 was motivated by the lack of knowledge about the incidence of cryptosporidiosis in foals. The detection of a new outbreak in a broodmare farm situated near Massey University in the course of the study facilitated a longitudinal observation of four affected foals. The results indicate that the cryptosporidiosis in foals is likely to be more common than thought. The observed short patent period and self limiting nature of cryptosporidiosis in foals suggest the condition may be underdiagnosed, and may account for a proportion of cases empirically diagnosed as foal-heat diarrhoea. In the two documented outbreaks in foals, the animals co-existed with ruminants on the same land. However, newborn calves are considered the main amplifiers of *C. parvum* in nature, but such animals were not present on the farms during the foaling season, and no specimens from older ruminants were analysed. In addition, an environmental source of infection, such stream water, was also likely. Further studies to assess the impact of cryptosporidiosis in neonatal diarrhoea of foals in New Zealand are warranted.

The study presented in Section 3.4 was motivated by the paucity of data on the dairy-farm prevalence of *C. parvum* in New Zealand, which contrasted with the huge importance of dairying in this country. Despite the short and concentrated calving season, *Cryptosporidium* oocysts were detected in 40 percent of the surveyed farms. However, it is acknowledged that the phenotypic identification of the parasites in this study did not stand the test of time. In fact, a novel 18S rRNA gene sequence, most similar to the sequence of *C. bovis*, was identified in a faecal specimen from a calf in the successive study reported in Section 4, indicating that non-*parvum Cryptosporidium* parasites do cycle in young cattle in New Zealand. Nevertheless, a large amount of data from overseas indicate *C. parvum* as the most common taxon cycling in unweaned calves and so it is likely that most parasites isolated in the study in Section 3.4 were

indeed *C. parvum*. Although not designed to estimate the farm level prevalence, the successive finding of *C. parvum* in a large number of farms in the Waikato in the study presented in Section 3.5 clearly indicates that this potentially zoonotic species is widespread in cattle in New Zealand.

Many questions related to the incidence of infections with *C. parvum* in cattle and the immunity developing as a result of cryptosporidiosis remain unanswered. Likewise, little is known about the effect of the age on the susceptibility to *C. parvum* infections in calves which are not exposed to the infection in the perinatal period. In previous studies (which were not included this thesis), this and other authors have observed an incidence of cryptosporidiosis in 100% of the calves on farms with year round calving (see Section 1.6.1). However, the same may not necessarily occur in New Zealand, where the short and concentrated caving season might prevent - at least in theory - the accummulation of the pathogen in the farm environment.

The absence of oocysts in the samples from farms rearing Jersey cattle in the study presented in Section 3.4 was intriguing. To the author's surprise, this seemingly minor finding has been later corroborated in a study in the USA (Starkey SR, Zeigler PE, Wade SE, et al. Factors associated with shedding of *Cryptosporidium parvum* versus *Cryptosporidium bovis* among dairy cattle in New York State. JAVMA 229, pp. 1623–26, 2006), which triggered a successive letter to the editor of JAVMA by the author (JAVMA 23, p. 339, 2007). The finding of low infection rates in Jersey cattle in independent studies in different countries would suggest an effect of the breed on the susceptibility to *C. parvum*, which warrants further investigation.

The study presented in Section 3.5 represents the first systematic genetic comparison between human and bovine *C. parvum* in New Zealand using highly discriminatory sub-typing tools. The results of the study indicate a significant homogeneity of the genetic repertoire of bovine and human *C. parvum.* Despite the wide spectrum of *C. parvum* GP60 alleles in circulation, two alleles - belonging to allele families *IIa* A18G3R1 and *IIa* A19G4R1 - persistently dominated the samples, each year. As hypothesised in the study presented in Section 3.4 using microscopy-only, the author postulates that the cyclic dominance of these subgenotypes is due to the selection of a 'rapacious' phenotype, able to rapidly fill the niche during the calving season and survive between seasons. The pathways of survival of *C.parvum* in the New Zealand ecosystem between the calving seasons remain to be established.

Finally, Chapter 4 reported the investigation of a serendipitious outbreak of a gastrointestinal illness among a cohort of young adults. *Cryptosporidium parvum* was the only enteropathogen identified from multiple faceal specimens. The explosive nature of the outbreak and the high attack rate observed underscore the potential public health hazard posed by *C. parvum* in New Zealand. As in many other investigations of outbreaks of cryptosporidiosis published in the scientific literature, the source of infection could not be traced, presumably due to the delay in

the investigation. Indeed, the outbreak was initially overlooked by the local health authorities based on the laboratory test results, which precluded the occurrence of a cluster of cases. However, a point source infection could be determined following the identification of a rare subgenotype of *C. parvum* in two isolates. Also the circumstantial evidence and the epidemic curve strongly suggested a point-source zoonotic transmission through direct contact with calves during a calf-handling practicum. It is hoped that the lessons learned during the investigation of this outbreak will enhance preparedness.

In addition to the valuable data on the natural history of cryptosporidiosis obtained during the investigation, a history of previous interactions with ruminants in the farm environment was significantly associated with a reduction of the risk of cryptosporidiosis in males. This result reinforces the intuitive notion that exposure to *C. parvum* confers some protective immunity. However, in this study the effect of previous interactions with ruminants on the susceptibility to cryptosporidiosis may have been confounded, and should therefore be corroborated in large-scale epidemiological studies.

Many important questions on cryptosporidiosis remain unanswered. Future studies of the population genetic structure of *Cryptosporidium* should address the question of the intra-isolate genetic diversity, which is technically difficult to assess using PCR. In the author's opinion, this diversity could be captured using increasingly-accessible PCR-free sequencing technologies. Large-scale epidemiolgical studies are needed in order to assess the impact of cryptosporidiosis, and other infections, on the health of newborn foals. A re-assessment of the farm-prevalence of *C. parvum* in New Zealand using molecular tools, as well as incidence studies with longitudinal studies of large cohorts of calves in multiple farms would also be necessary in order to define the farm-level prevalence and animal-level incidence of cryptosporidiosis caused by *C. parvum* in New Zealand, and elsewhere. Such studies, if adequately powered, could also assess the presence of risk factors for *C. parvum* infections at the farm-level. Finally, studies assessing the potential zoonotic impact of *Cryptosporidium* parasites cycling in other dominant domestic and wild species in New Zealand, such as sheep, deer, possums and birds, are also warranted.

APPENDICES

APPENDIX 2.1

Multilocus genotyping results of the study reported in Section 2.2.

MLG_ID: identifier of the multilocus genotype; eBURST code: code used in the single locus variant networks using eBURST software.

Numbers represent the allele sizes in base-pairs. The markers are those described in Section 2.2. Notice that the isolates from the USA were not included in the eBURST diagrams.

These data are also available as an on-line supplement to the published paper at http://aem.asm.org/

	Marker										
	MSA	MSB	MSC	MSK	MSE	MS9	MSG	1887	TP14	MLG_ID	eBURST code
Isolate ID	*C. hominis*										
11766	100	266	168	205	250	382	298	171	179	1	UK1
3957	100	274	168	175	280	382	298	184	192	2	UK2
3010	100	274	168	175	306	382	298	184	192	3	UK3
2588	136	308	209	175	166	394	228	171	179	4	UK4
918	139	156	168	174	250	382	283	177	182	5	UG5
1428	139	165	168	174	214	382	283	177	182	6	UG6
1467	139	165	168	174	214	382	283	177	182	6	UG6
1473	139	165	168	174	214	382	283	177	182	6	UG6
922	139	165	168	174	214	382	316	177	182	7	UG7
996	139	165	168	174	214	382	316	177	182	7	UG7
1042	139	165	168	174	214	382	316	177	182	7	UG7
1209	139	165	168	174	214	382	316	177	182	7	UG7
1327	139	165	168	174	214	382	316	177	182	7	UG7
1414	139	165	168	174	214	382	316	177	182	7	UG7
1513a	139	165	168	174	235	382	283	174	182	8	UG8
1303	139	165	168	174	244	382	274	177	182	9	UG9
1500	139	165	168	174	244	382	283	174	182	10	UG10
1511	139	165	168	174	244	382	283	174	182	10	UG10
1519	139	165	168	174	244	382	283	174	182	10	UG10
1528	139	165	168	174	244	382	283	174	182	10	UG10
1187	139	165	168	174	244	382	283	177	182	11	UG11
1232	139	165	168	174	244	382	283	177	182	11	UG11
1259	139	165	168	174	244	382	283	177	182	11	UG11
1291	139	165	168	174	244	382	283	177	182	11	UG11
1308	139	165	168	174	244	382	283	177	182	11	UG11
1422	139	165	168	174	244	382	283	177	182	11	UG11
1430	139	165	168	174	244	382	283	177	182	11	UG11
1450	139	165	168	174	244	382	283	177	182	11	UG11
1485	139	165	168	174	244	382	283	177	182	11	UG11
1552	139	165	168	174	244	382	283	177	182	11	UG11
785	139	165	168	174	244	382	298	177	182	12	UG12
1241	139	165	168	174	244	382	298	177	182	12	UG12

Colors code	
C parvum	*C hon*
	UK
UG	UG
US	US
TR	
NZ	
IL	
SRB	

1371	139	165	168	174	244	382	298	177	182	12	UG12
1001	139	165	168	174	244	382	316	177	182	13	UG13
1319	139	165	168	174	244	382	316	177	182	13	UG13
1548	139	165	168	174	250	382	283	174	182	14	UG14
1124	139	165	168	174	250	382	283	177	182	15	UG15
1281	139	165	168	174	250	382	283	177	182	15	UG15
1333	139	165	168	174	250	382	283	177	182	15	UG15
1344	139	165	168	174	250	382	283	177	182	15	UG15
1407	139	165	168	174	250	382	283	177	182	15	UG15
1478	139	165	168	174	250	382	283	177	182	15	UG15
1558	139	165	168	174	250	382	283	177	182	15	UG15
1481	139	165	184	174	250	382	283	177	182	16	UG16
798	139	165	184	174	250	382	307	177	182	17	UG17
1479	139	165	187	174	195	484	283	194	185	18	UG17
Tu728	139	245	168	205	244	382	334	177	192	19	
4259	139	266	168	205	239	382	298	171	179	20	UK20
4250	139	266	168	205	244	382	298	171	179	21	UK21
4331	139	266	168	205	244	382	298	171	179	21	UK21
2945	139	266	168	205	250	382	298	171	179	22	UK22
2998	139	266	168	205	250	382	298	171	179	22	UK22
3000	139	266	168	205	250	382	298	171	179	22	UK22
3009	139	266	168	205	250	382	298	171	179	22	UK22
3015	139	266	168	205	250	382	298	171	179	22	UK22
3090	139	266	168	205	250	382	298	171	179	22	UK22
3229	139	266	168	205	250	382	298	171	179	22	UK22
3441	139	266	168	205	250	382	298	171	179	22	UK22
3504*	139	266	168	205	250	382	298	171	179	22	UK22
3554*	139	266	168	205	250	382	298	171	179	22	UK22
3590	139	266	168	205	250	382	298	171	179	22	UK22
3670	139	266	168	205	250	382	298	171	179	22	UK22
3690*	139	266	168	205	250	382	298	171	179	22	UK22
3765*	139	266	168	205	250	382	298	171	179	22	UK22
3796	139	266	168	205	250	382	298	171	179	22	UK22
3801*	139	266	168	205	250	382	298	171	179	22	UK22
3804	139	266	168	205	250	382	298	171	179	22	UK22
3870*	139	266	168	205	250	382	298	171	179	22	UK22
3887	139	266	168	205	250	382	298	171	179	22	UK22
3889*	139	266	168	205	250	382	298	171	179	22	UK22
3948	139	266	168	205	250	382	298	171	179	22	UK22
3999	139	266	168	205	250	382	298	171	179	22	UK22
4016	139	266	168	205	250	382	298	171	179	22	UK22
4034	139	266	168	205	250	382	298	171	179	22	UK22
4048	139	266	168	205	250	382	298	171	179	22	UK22
4088	139	266	168	205	250	382	298	171	179	22	UK22
4107	139	266	168	205	250	382	298	171	179	22	UK22
4117	139	266	168	205	250	382	298	171	179	22	UK22
4118	139	266	168	205	250	382	298	171	179	22	UK22
4121	139	266	168	205	250	382	298	171	179	22	UK22
4141	139	266	168	205	250	382	298	171	179	22	UK22
4206	139	266	168	205	250	382	298	171	179	22	UK22

4223	139	266	168	205	250	382	298	171	179	22	UK22
4239*	139	266	168	205	250	382	298	171	179	22	UK22
4252	139	266	168	205	250	382	298	171	179	22	UK22
4258	139	266	168	205	250	382	298	171	179	22	UK22
4273	139	266	168	205	250	382	298	171	179	22	UK22
4293	139	266	168	205	250	382	298	171	179	22	UK22
4310	139	266	168	205	250	382	298	171	179	22	UK22
4375	139	266	168	205	250	382	298	171	179	22	UK22
4384	139	266	168	205	250	382	298	171	179	22	UK22
4410	139	266	168	205	250	382	298	171	179	22	UK22
4430	139	266	168	205	250	382	298	171	179	22	UK22
4511	139	266	168	205	250	382	298	171	179	22	UK22
4512	139	266	168	205	250	382	298	171	179	22	UK22
4526	139	266	168	205	250	382	298	171	179	22	UK22
4569	139	266	168	205	250	382	298	171	179	22	UK22
4580	139	266	168	205	250	382	298	171	179	22	UK22
4591	139	266	168	205	250	382	298	171	179	22	UK22
4635	139	266	168	205	250	382	298	171	179	22	UK22
4655	139	266	168	205	250	382	298	171	179	22	UK22
4660	139	266	168	205	250	382	298	171	179	22	UK22
4663	139	266	168	205	250	382	298	171	179	22	UK22
4665	139	266	168	205	250	382	298	171	179	22	UK22
4670	139	266	168	205	250	382	298	171	179	22	UK22
4686	139	266	168	205	250	382	298	171	179	22	UK22
4762	139	266	168	205	250	382	298	171	179	22	UK22
11110	139	266	168	205	250	382	298	171	179	22	UK22
11425	139	266	168	205	250	382	298	171	179	22	UK22
2696*	139	266	168	205	250	382	298	171	192	23	UK23
3812	139	266	168	205	250	382	298	184	179	24	UK24
4301*	139	266	168	205	250	382	298	184	179	24	UK24
5863	139	266	168	205	250	382	298	184	179	24	UK24
3499*	139	266	168	205	250	382	316	171	179	25	UK25
4691	139	266	168	205	250	382	316	171	179	25	UK25
3227	139	266	168	205	250	382	325	171	179	26	UK26
4771	139	266	168	205	250	382	325	171	179	26	UK26
4498	139	266	168	205	250	460	298	171	179	27	UK27
11173	139	266	168	205	250	460	298	171	179	27	UK27
6642*	139	274	152	175	250	382	298	171	192	28	UK28
5276*	139	274	152	175	306	382	298	171	192	29	UK29
3871*	139	274	152	205	244	382	298	171	179	30	UK30
4140*	139	274	152	205	244	382	298	171	179	30	UK30
4222*	139	274	152	205	244	382	298	171	179	30	UK30
4238	139	274	152	205	244	382	316	171	179	31	UK31
4312	139	274	152	205	244	382	316	171	179	31	UK31
5139*	139	274	168	175	268	382	274	184	192	32	UK32
4540	139	274	168	205	244	382	298	171	179	33	UK33
4690	139	274	168	205	244	382	298	171	179	33	UK33
4733	139	274	168	205	244	382	298	171	179	33	UK33
KJHIV	139	276	168	135	244	382	274	177	179	34	
172	139	276	168	205	244	382	274	177	179	35	

4776	139	285	152	175	280	382	298	184	179	36	UK36
4938	139	286	168	175	280	382	325	184	192	37	UK37
478	139	291	168	174	214	382	286	177	182	39	UG39
584	139	291	168	174	214	382	286	177	182	39	UG39
717	139	291	168	174	214	382	298	177	182	40	UG40
187	139	291	168	174	214	382	307	174	185	41	UG41
696	139	291	168	174	214	382	307	177	182	42	UG42
26	139	291	168	174	214	382	307	177	185	43	UG43
194	139	291	168	174	214	382	315	174	185	44	UG44
240	139	291	168	174	214	382	315	174	185	44	UG44
164	139	291	168	174	214	382	315	177	185	45	UG45
214	139	291	168	174	214	382	316	174	185	46	UG46
235	139	291	168	174	214	382	316	174	185	46	UG46
257	139	291	168	174	214	382	316	174	185	46	UG46
271	139	291	168	174	214	382	316	174	185	46	UG46
448	139	291	168	174	214	382	316	177	182	47	UG47
674	139	291	168	174	214	382	316	177	182	47	UG47
104	139	291	168	174	214	382	316	177	185	48	UG48
295	139	291	168	174	214	382	316	177	185	48	UG48
2027	139	291	168	174	214	382	316	177	185	48	UG48
54	139	291	168	174	214	472	316	177	185	49	UG49
212	139	291	168	174	239	382	298	177	187	50	
536	139	291	168	174	244	382	274	177	182	51	UG51
667	139	291	168	174	244	382	274	177	182	51	UG51
NEMC1	139	291	168	174	244	382	274	177	187	52	
502	139	291	168	174	244	382	274	177	192	53	
196	139	291	168	174	244	382	283	174	185	54	UG54
198	139	291	168	174	244	382	283	174	187	55	UG55
591	139	291	168	174	244	382	283	177	182	56	UG56
682	139	291	168	174	244	382	283	177	182	56	UG56
278	139	291	168	174	244	382	283	177	185	57	UG57
285	139	291	168	174	244	382	283	177	185	57	UG57
251	139	291	168	174	244	382	286	174	185	58	UG58
Tu71	139	291	168	174	244	382	298	177	192	59	
211	139	291	168	174	244	382	315	174	185	60	UG60
203	139	291	168	174	244	382	316	174	185	61	UG61
451	139	291	168	174	244	382	316	177	182	62	UG62
369	139	291	168	174	248	382	283	177	182	63	UG63
420	139	291	168	174	248	382	283	177	182	63	UK63
329	139	291	168	174	248	382	286	177	182	64	UK64
220	139	291	168	174	248	382	297	174	185	65	UK65
2010	139	291	168	174	248	382	307	177	185	66	UK66
335	139	291	168	174	248	382	316	177	185	67	UK67
H39	139	291	168	174	250	382	274	150	179	68	
464	139	291	168	174	250	382	286	177	182	69	UG69
181	139	291	168	174	250	382	297	174	185	70	UG70
449	139	291	168	174	250	382	298	177	182	71	UG71
684	139	291	168	174	250	382	325	177	182	72	UG72
9897	139	291	182	135	268	382	274	174	187	73	
131	139	291	182	174	248	382	297	174	185	74	UG74

ECHIV	139	291	182	174	250	382	274	177	187	75	
94	139	291	187	174	244	382	283	177	185	76	UG76
2969	139	297	152	175	268	382	298	184	192	77	UK77
3004*	139	300	168	175	226	382	298	184	192	78	UK78
3334*	139	300	168	175	244	382	334	184	192	79	UK79
3555*	139	300	168	175	256	382	274	194	192	80	UK80
3351*	139	300	168	175	280	382	298	184	192	81	UK81
3423	230	322	209	205	250	460	298	165	230	82	UK82

	C. parvum										
1224	187	279	187	232	195	534	229	194	213	1	UG1
Tu114	188	274	209	232	195	528	229	194	213	2	UG2
TK41	188	276	225	198	214	454	179	150	220	3	TR3
TK46	188	276	225	198	214	460	179	150	220	4	TR4
2545	188	279	187	205	195	504	229	194	213	5	UG5
1222	188	279	187	205	195	534	229	194	213	6	UG6
2428	188	279	187	205	195	540	229	194	213	7	UG7
9	188	279	187	232	195	382	229	194	185	8	UG8
2815	188	279	187	232	195	484	229	194	213	9	UG9
2036	188	279	187	232	195	492	229	194	213	10	UG10
2687	188	279	187	232	195	504	229	174	213	11	UG11
1902	188	279	187	232	195	522	193	194	213	12	UG12
30	188	279	187	232	195	528	229	194	213	13	UG13
128	188	279	187	232	195	528	229	194	213	13	UG13
1600	188	279	187	232	195	528	229	194	213	13	UG13
2809	188	279	187	232	195	528	229	194	213	13	UG13
2631	188	279	187	232	195	534	229	174	187	14	UG14
114	188	279	187	232	195	534	229	194	213	15	UG15
1510	188	279	187	232	195	534	229	194	213	15	UG15
1601	188	279	187	232	195	534	229	194	213	15	UG15
1976	188	279	187	232	195	534	229	194	213	15	UG15
2088	188	279	187	232	195	534	229	194	213	15	UG15
2335	188	279	187	232	195	534	229	194	213	15	UG15
2556	188	279	187	232	195	534	229	194	213	15	UG15
2581	188	279	187	232	195	534	229	194	213	15	UG15
323	188	279	187	232	195	540	229	194	213	16	UG16
125	188	279	187	232	195	546	229	194	213	17	UG17
2553	188	279	187	232	235	528	229	194	213	18	UG18
1512	188	279	225	232	195	528	229	194	213	19	UG19
1302	188	279	232	232	195	472	229	174	213	20	UG20
TK13	188	322	225	198	214	460	179	150	220	21	TR21
Tu154	188	324	209	232	195	472	229	174	187	22	UG22
216	188	333	187	232	195	466	193	174	187	23	UG23
2100	188	333	187	232	195	466	193	174	187	23	UG23
170	188	333	187	232	195	484	229	194	187	24	UG24
2032	188	333	187	232	195	484	229	194	187	24	UG24
1862	188	345	187	205	166	492	193	174	213	25	UG25
TK31	206	274	232	198	214	474	179	150	220	26	TR26
Tu131	206	322	209	232	214	528	229	150	220	27	UG27

Is46	206	324	225	198	214	454	193	150	223	28	IL28
TK55	206	324	232	198	214	474	175	150	223	29	TR29
TK8	206	324	232	198	214	474	179	150	220	30	TR30
TK115	206	324	232	198	214	474	179	150	220	30	TR30
TK12	206	324	232	198	214	474	185	150	220	31	TR31
Is7	207	324	225	198	214	454	193	150	220	32	IL32
Cath	207	324	232	198	214	460	179	150	220	33	
TK32	230	274	225	198	214	460	179	150	220	34	TR34
SI1071442	230	274	225	205	214	460	193	150	223	35	NZ35
TK39	230	276	225	198	214	460	179	150	220	36	TR36
Is1	230	322	225	198	214	447	193	150	220	37	IL37
CISD	230	322	225	198	214	454	193	150	223	38	
MD	230	322	225	198	214	460	193	150	223	39	
JRL-HIV	230	322	225	198	214	460	193	150	223	39	
16W	230	322	225	198	214	460	193	150	223	39	
TAMU	230	322	225	198	214	460	193	150	223	39	
UCP	230	322	225	198	214	460	193	150	223	39	
ICP	230	322	225	198	214	460	193	150	223	39	
220	230	322	225	198	214	460	193	150	223	39	
Hhu1	230	322	225	205	214	460	179	150	223	40	NZ40
Hhu5	230	322	225	205	214	460	179	150	223	40	NZ40
Hbo4	230	322	225	205	214	460	179	150	223	40	NZ40
Hbo5	230	322	225	205	214	460	179	150	223	40	NZ40
Hbo6	230	322	225	205	214	460	179	150	223	40	NZ40
Hhu4	230	322	225	205	214	460	193	150	223	41	NZ41
Hhu6	230	322	225	205	214	460	193	150	223	41	NZ41
Hhu7	230	322	225	205	214	460	193	150	223	41	NZ41
Hbo1	230	322	225	205	214	460	193	150	223	41	NZ41
SI7595062	230	322	225	205	214	460	193	150	223	41	NZ41
SI6139326	230	322	225	205	214	460	193	150	223	41	NZ41
SI1113730	230	322	225	205	214	460	193	150	223	41	NZ41
SI7595147	230	322	225	205	214	460	193	150	223	41	NZ41
SI6141328	230	322	225	205	214	460	193	150	223	41	NZ41
SI6142922	230	322	225	205	214	460	193	150	223	41	NZ41
SI1102421	230	322	225	205	214	460	193	150	223	41	NZ41
SI1074114	230	322	225	205	214	460	193	150	223	41	NZ41
SI6138937	230	322	225	205	214	460	193	150	223	41	NZ41
SI4128821	230	322	225	205	214	460	193	150	223	41	NZ41
Hbo2	230	322	225	205	214	460	193	150	236	42	NZ42
Hhu3	230	322	225	205	214	472	193	150	223	43	NZ43
Is3	230	324	225	198	214	447	193	150	220	44	IL44
Is23	230	324	225	198	214	447	193	150	220	44	IL44
Is27	230	324	225	198	214	447	193	150	220	44	IL44
Is28	230	324	225	198	214	447	193	150	220	44	IL44
Is49	230	324	225	198	214	447	193	150	220	44	IL44
Is50	230	324	225	198	214	447	193	150	223	45	IL45
Is53	230	324	225	198	214	447	193	150	223	45	IL45
Is58	230	324	225	198	214	447	193	150	223	45	IL45
Is60	230	324	225	198	214	447	193	150	223	45	IL45
Is62	230	324	225	198	214	447	193	150	223	45	IL45

Sr9	230	324	225	198	214	447	193	170	213	46	SRB46
Is8	230	324	225	198	214	454	193	150	220	47	IL47
Is16	230	324	225	198	214	454	193	150	220	47	IL47
Is34	230	324	225	198	214	454	193	150	220	47	IL47
Is47	230	324	225	198	214	454	193	150	223	48	IL48
Is13	230	324	225	198	214	460	193	150	220	49	IL49
IOWA	230	324	225	198	214	460	193	150	223	50	
Is15	230	324	225	198	214	474	179	150	223	51	IL51
Is59	230	324	225	198	214	474	179	150	223	51	IL51
SI1121192	230	340	225	205	214	385	333	150	223	52	NZ52
SI 4.2c	230	340	225	205	214	460	193	150	223	53	NZ53
1504	238	279	187	232	195	534	229	194	213	54	UG54
Sr17	238	322	225	198	214	447	193	150	236	55	SRB55
Sr5	238	322	225	198	214	447	193	170	236	56	SRB56
Sr71	238	322	225	198	214	447	193	170	236	56	SRB56
Sr69	238	322	225	198	214	447	229	170	236	57	SRB57
Sr70	238	322	225	198	214	454	193	170	236	58	SRB58
Sr80	238	322	225	198	214	454	193	170	236	58	SRB58
Sr117	238	322	225	198	214	454	206	150	236	59	SRB59
Sr8	238	324	225	198	214	447	193	170	236	60	SRB60
Sr90	238	324	225	198	214	472	229	150	236	61	SRB61
Sr95	238	324	225	198	214	472	229	170	236	62	SRB62
Sr43	248	322	225	198	214	415	229	150	236	63	SRB63
Sr112	248	322	225	198	214	447	220	150	236	64	SRB64
Sr83	248	322	225	198	214	454	193	170	236	65	SRB65
Sr42	248	324	225	198	214	415	229	150	236	66	SRB66
Sr19	248	324	225	198	214	447	229	150	236	67	SRB67
Sr24	248	324	225	198	214	472	206	150	236	68	SRB68
Sr33	248	340	225	198	214	447	229	150	236	69	SRB69
Sr102	252	322	225	198	214	447	229	150	236	70	SRB70
Sr106	252	322	225	198	214	454	229	150	213	71	SRB71
Sr104	252	322	225	198	214	454	229	150	236	72	SRB72
Sr61	252	324	225	198	214	447	193	150	236	73	SRB73
Sr58	252	324	225	198	214	447	229	150	236	74	SRB74

APPENDIX 3.5

The GP60 sequences identified in bovine and human *C. parvum* in the study in Section 3.5

>*IIa* A18G3R1, bovine and human *C. parvum*

ATTTAAAGGATGTTCCTGTTGAGGGCTCATCATCGTCATCGTCATCGTCATCATCATCATCATCATCAT
CATCATCATCATCATCATCAACATCAACCGTCGCACCAGCAAATAAGGCAAGAACTGGAGAAGACGCA
GAAGGCAGTCAAGATTCTAGTGGTACTGAAGCTTCTGGTAGCCAGGGTTCTGAAGAGGAAGGTAGTG
AAGACGATGGCCAAACTAGTGCTGCTTCCCAACCCACTACTCCAGCTCAAAGTGAAGGCGCAACTAC
CGAAACCATAGAAGCTACTCCAAAAGAAGAATGCGGCACTTCATTTGTAATGTGGTTCGGAGAAGGTA
CCCCAGCTGCGACATTGAAGTGTGGTGCCTACACTATCGTCTATGCACCTATAAAAGACCAAACAGAT
CCCGCACCAAGATATATCTCTGGTGAAGTTACATCTGTAACCTTTGAAAAGAGTGATAATACAGTTAAA
ATCAAGGTTAACGGTCAGGATTTCAGCACTCTCTCTGCTAATTCAAGTAGTCCAACTGAAAATGGCGG
ATCTGCGGGTCAGGCTTCATCAAGATCAAGAAGATCACTCTCAGAGGAAACCAGTGAAGCTGCTGCA
ACCGTCGATTTGTTTGCCTTTACCCTTGATGGTGGTAAAAGAATTGAAGTGGCTGTACCAAACGTCGA
AGATGCATCTAAAAGAGACAAGTACAGTTTGGTTGCAGACGATAAACCTTTCTATACCGGCGCAAACA
GCGGCACTACCAATGGTGTCTACAGGTTGAATGAGAACGGAGACTTGGTTGATAAG

> *IIa A1*8G3R1, human *C. parvum*

TGTTCCTGTTGAGGGCTCATCATCGTCATCGTCATCGTCATCATCATCATCAtCATCATCATCATCATCA
TCATCATCAACATCAACCGTCgCaCCAgcAAATaAGGCAAGAaCTGgAGAAGACGcAGAAGGCAGTCAA
GATTCTaGTGGTaCTGAaGCTTCTGGTAGCCAGGGTTcTGAAGAGGAAGGTagTGAAGACGATGGCCA
AACTagTGCTGCTTCCCaACCCACTACTCCaGCTCAAAGtGaAGGCGCAACTACCGAAACCATAGAAgC
TACTCCAAAAGAAGAATGCGGcaCTTcaTTTGTaATGTGGTTcGgAGAAgGtACCCCaGCTGCGACATTG
AaGtGTGGtGCCTACACTATCGTCTATGCACCTATAAAAGACCAAAcAGATCCCGCACCAAgATATATCT
CTGGTGAAGTTACATCTGTAACCTTTGAAAAGAGTGATAATACAGTTAAAATCaAGGTTAACGGTcAGG
ATTTCAgCaCTCTCTCTGCTAATTCAAGTAgTCCAACTGAAaATGGcGGATCTGCGGGTcAgGCTTCATC
AaGATCAAGAAGATcACTCTCAGAgGAAACCagTGAAGCTGCTGCAACCGcCgATTTGTTTGCCTTTACc
CTTGATGGTGGTAAAAGAATTGAAGTGGCTGTACCAAACGTCGAAGATGCATCTAAAAGAGACAAGTA
CAGtTTGGTTGCAGACGATAAACCTTTCTATACCGGCGCAAACAGCGGcACTACcaATGGTGTCTACAG
GTTGAATGAgAACGGAGAC

>*IIa* A19G4R1, bovine *C. parvum*

CTTTAAaGGATGTTCCTGTTGAGGGCTCATCATCGTCATCGTCATCGTCATCGTCATCATCATCATCAT
CATCATCATCATCATCATCATCAACATCaaCCGTCgCACCAGCaAATaAGGcAAGAaCTGGAGAAGA
CGCAGAAGGCAGTCAAGATTCTaGTGGTACTGAAGCTTCTGGTAGCCAGGGTTCTGAAGAGGAAGGT
aGTGAAGACGATGGCCaAACTagTGCTGCTTCCCaACCCACTACTCCAGCTCAAAGTGaAGGCGCAAC
TACCGAAACCATAGAAGCTACTCCAAAAGAAGAATGCGGcaCTTcaTTTGTaAtGTGGtTcGgAGAAGGTA
CCCCAGCTGCGACATTGAaGtGTGGTGCCTACACTATCGTCTATGCACCTATAAAAGACCAAAcAGATC
CCGCACCAAgATATATCTCTGGTGAAGTTACATCTGTAACCTTTGAAAAGAGTGATAATACAGTTAAAA
TCaAGGTTAACGGTCAGGATTTCAgCACTCTCTCTGCTAATTCAAGTAGTCCAACTGAAaATGGcGGAT
CTGCGGGTcAGGCTTCATCAAGATCAAGAAGATcACTCTCAGAGGAAACCAGTGAAGCTGCTGCAACC
GTCGATTTGTTTGCCTTTACCCTTGATGGTGGTAAAAGAATTGAAGTGGCTGTACCAAACGTCGAAGA
TGCATCTAAAAGAGACAAGTACAGTtTGGTTGcAGACGATAAAcCTTTCTATACCGGCGCAAACAGCGG
cACTACcaATGGTGTCTACAGGTTGAATGAgAACGGAGACTTGG

APPENDIX 4.1

Questionnaire Q1 (See Section 4.3).

1 - Did you experience one of the following in the last four weeks? (tick the box)

Abdominal discomfort--- ☐

Diarrhoea---------- ☐

Vomiting---------- ☐

None of the above--------- ☐

2 – If you answered yes to Q1, please circle the date when you first experienced the symptoms and the date when you first felt healthy (the dates of Mechanisms of Disease Case Scenarios are given for your orientation):

Date	**Mechanisms of disease Case Scenario**
Monday 18/09/06----------	A cat having difficulty breathing
Tuesday 19/09/06	
Wednesday 20/09/06	
Thursday 21/09/06	
Friday 22/09/06	
Saturday 23/09/06	
Sunday 24/06/06	
Monday 25/09/06 ----------	A calf with severe diarrhoea
Tuesday 26/09/06 ----------	Visit to LATU (calf handling)
Wednesday 27/09/06	
Thursday 28/09/06	
Friday 29/09/06	
Saturday 30/09/06	
Sunday 01/10/06	
Monday 02/10/06 ----------	A thirsty, balding portly poodle
Tuesday 03/10/06	
Wednesday 04/10/06	
Thursday 05/10/06	
Friday 06/10/06	
Saturday 07/10/06	
Sunday 08/10/06	
Monday 09/10/06----------	Ill thrift in a group of lambs
Tuesday 10/10/06	
Wednesday 11/10/06	
Thursday 12/10/06	
Friday 13/10/06	
Saturday 14/10/06	
Sunday 15/10/06	
Monday 16/10/06----------	Dog with hindlimb lameness
Tuesday 17/10/06	
Wednesday 18/10/06/Today	

3 – Did you seek medical advice? Circle either **Yes** or **No**

4 – Did you submit faecal samples for analysis? **Yes** **No**

3 – Gender: **Male** ☐ **Female** ☐

4 – Age:

5 –Background: **NZ Australia American Asian Other**

6 - In general, how do you quench your thirst during practical sessions?

Bottled water **Tap water** **Soft drink** **Other**

7 – Are you a smoker? **Yes** **No**

8 - Is it your habit to wash your hands after practical classes where animals are handled? **Yes** **No**

9 - Are you on any medication for acute or chronic disorders (e.g. insulin, thyroxine). **Yes** **No**. If yes, could you specify?

10 – Did you grow up in: **Rural** **Urban**

11 -Before starting the Veterinary course, how often did physically handled ruminants:

Never **Once/twice a year** **Monthly** **Weekly/more**

Thank you very much for your help,

APPENDIX 4.2

Questionnaire Q2 (See Section 4.3)

Please tick the appropriate box!

	Did you attend last year's practical "A calf with severe diarrhoea" at LATU?		Did you drink tap water at LATU on that day?		Did you declare suffering from abdominal discomfort and/or diarrhoea and/or vomiting in the questionnaire?	
	YES	NO	YES	NO	YES	NO
1						
2						
3						
4						
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APPENDIX 4.3

Class responses to questionnaire Q1 (See also Tables 4.1 and 4.2)

Key to appendix:

Abdom: abdominal discomfort: yes= 1; no= 0

Diarrhoea: yes= 1; no= 0

Vomiting: vomiting. Yes= 1; no= 0

Date first: the date of the onset of the symptoms

Date healthy: last day of symptoms

Gender: 1= male; 0= female

Nationality: New Zealand= 1; Australian= 2; USA= 3; Asia=4; other= 5

Tap: drinking tap water during practicals. Yes= 1; no= 0

Wash hands: washing hands after practical classes where animals are handled. Yes= 1; no=0

Chronic medic: taking chronic medication. Yes= 2; no= 0

Rural/urban: rural= 0; urban=10,

Handled ruminants: no previous physical interaction with ruminants= 0; previous physical interaction with ruminants= 1

Frequency handled ruminants: up to once-twice/year= 0; more than once-twice/year= 1

Diseased	Abdom	diarrhoea	vom	Date first	Date healthy	gender	nationality	Tap	wash hands	chronic medic	Rural/urban	Handled calves	Frequen handle ruminan
0	0	0	0			0	3	0	1	0	1	1	0
0	0	0	0			0	3	0	1	0	1	1	0
0	0	0	0			0	1	0	1	0	0	1	1
0	0	0	0			0	1	1	1	1	1	1	0
0	0	0	0			0	5	1	1	0	0	0	0
1	1	1	1	28/09/2006	6/10/2006	1	5		1	0		1	0
0	0	0	0			1	1	0	0	0	1	0	0
0	0	0	0			0	3	0	1	0	1	0	0
1	1	0	0	1/10/2006	5/10/2006	0	5	0	0	0	0	1	0
1	1	1	0	1/10/2006	17/10/2006	0	1	0	1	1	0	1	1
0	0	0	0			0	3	1	1	0	1	0	0
1	1	1	0	2/10/2006		0	5	1	1	0	0	1	0
0	0	0	0			0	1	1	1	0	1	0	0
1		0	1	26/09/2006	28/09/2006	0	1	0	1	0	1	0	0
0	0	0	0			0	1	1	1	0	1	1	0
1	1	1	0	1/10/2006	4/10/2006	0	1	1	1	0	1	0	0
0	0	0	0			0	1	0	1	0	1	0	0
1	1	1	0	7/10/2006	13/10/2006	0	1	0	1	0	1	0	0
0	0	0	0			0	1	0	1	0	1	0	0
1	0	1	0	3/10/2006	6/10/2006	0	1	1	1	0	1	0	0
0	0	0	0			1	1	0	1	0	1	1	0
1	1	1	0	2/10/2006	10/10/2006	0	1	0	1	0	0	1	1
0	0	0	0			0	4	0	1	0	1	0	0
0	0	0	0			0	1	1	1	0	0	1	1
0	0	0	0			0	1	0	1	0	0	1	0
0	0	0	0			0	1	0	1	0	0	1	1
0	0	0	0			0	1	0	1	0	0	1	1
0	0	0	0			0	1	1	1	0	1	1	1

0	0	0	0			1	1	0	1	0	0	1	1
0	0	0	0			0	1	0	1	0	0	1	0
0	0	0	0			0	4	0	1	0	1	0	0
0	0	0	0			0	1	0	1	0	1	1	0
0	0	0	0			0	1	0	1	0	0	1	1
0	0	0	0			0	1	0	1	0	1	0	0
0	0	0	0			1	1	0	0	0	1	1	0
0	0	0	0			0	1	0	1	0	1	1	0
0	0	0	0			0	1	0	1	0	0	1	1
1	1	1	0	5.10.2006	8.10.2006	1	1	1	1	0	1	0	0
0	0	0	0			1	2	0	0	0	0	1	1
0	0	0	0			0	1	0	1	0	1	0	0
0	0	0	0			1	1	0	1	0	0	1	1
0	0	0	0			1	1	1	0	0	0	1	1
0	0	0	0			1	1	1	0	1	0	1	1
0	0	0	0			1	5	0	0	0	0	1	1
1	1	1	0			1	3	0	1	0	1	0	0
1	1	1	1	1.10.2006	19.10.2006	0	1	1	1	0	1	0	0
1	1	1	0			1	4	0	0	0	1	1	1
0	0	0	0			0	1	1	1	0	1	0	0
1	1	1	0	29.09.2006	14.10.2006	0	1	0	1	0	0	1	1
1	1	1	0	3.10.2006	8.10.2006	0	1	0	1	1	1	0	0
0	0	0	0			1	1	1	1	0	0	1	1
0	0	0	0			1	1	0	1	0	0	1	1
1	1	0	0	18.09.2006		0	1	0	1	0	1	1	0
0	0	0	0			0	1	0	0	0	1	0	0
0	0	0	0			0	1	0	1	0	1	1	0
1	1	1	0	4.10.2006	9.10.2006	1	1	0	1	1	1	0	0
0	0	0	0			1	1	1	0	0	0	1	1
0	0	0	0			1	1	0	1	0	0	1	1
0	0	0	0			0	1	1	1	0	1	1	0
0	0	0	0			0	1	0	1	0	0	1	1
1	1	1	0	1.10.2006	7.10.2006	0	1	0	1	0	0	1	1
1	1	1	1	2.10.2006	17.10.2006	0	3	1	1	0	1	1	0
0	0	0	0			0	3	0	1	0	1	0	0
1	1	1	1	1.10.2006	16.10.2006	0	3	0	1	0	1	1	0
0	0	0	0			0	1	0	1	0	1	0	0
1	1	1	1	1.10.2006	4.10.2006	0	5	0	1	0	0	1	0
1	1	1	0	1.10.2006	12.10.2006	1	5	1	0	0	1	0	0
0	0	0	0			1	4	0	1	0	1	1	0
0	0	0	0			0	1	0	1	0	0	1	0
0	0	0	0			0	1	0	1	0	1	0	0
0	0	0	0			1	1	1	1	0	0	1	1
1	1	1	0	22.09.2006		0	1	1	1	0	1	0	0
0	0	0	0			1	1	0	1	0	1	1	1
0	0	0	0			0	1	0	1	0	1	1	0
0	0	0	0			0	5	0	1	0	1	1	1
0	0	0	0			0	1	1	0	0	0	0	0
1	1	1	1	1.10.2006	6.10.2006	1	1	1	1	0	1	0	0
0	0	0	0			1	4	0	1	0	1	0	0
1	1	1	0	27.09.2006	29.09.2006	0	1	0	0	0	1	0	0
0	0	0	0			0	3	0	1	0	1	0	0

APPENDIX 4.4

GP60 and HSP70 sequences of *C. parvum* isolates from affected students and the 18S rRNA sequence found in a calf during the outbreak investigation reported in Section 4.1.

Key: HSP70_1_student: sequence of the C. parvum HSP70 gene found in 3 students; HSP70_2_students: sequence of the C. parvum HSP70 gene found in one student; 18S_rRNA_ calf: f the 18S rRNA gene sequence found in one calf.

>HSP70_1_student

ATCTGAAATTGATGAGGCTGAGAAGAAGATCAAGGATGCTCTTGACTGGCTCGAGCACAACCAAACTGCTGAAAAGGACGAGTTTGA
ACATCAACAAAAGGAGATTGAAACTCATATGAATCCACTCATGATGAAGATCTACTCTGCTGAGGGTGGTATGCCAGGTGGAATGCC
AGGTGGTATGCCAGGCGGTATGCCAGGTGGAATGCCAGGTGGTATGCCAGGTGGAATGCCAGGCGGTATGCCAGGTGGTATGCCA
GGTGGTATGCCAGGTGGTATGCCAGGATCTAATGGTCCAACTGTTGAAGAGGTCGACTAATTATTTTAGTCACCAAAAAAACTCACTC
AAAATGGAAAGTTAAGAACTATTTACACACTTTCAATTTCTAGTTATTTTTTACCAAAATAAGAAGAAAAGCACACTCTCCCTTTAGG

>HSP70_2_student

ATGAgGCTGAGAaGAAGATCAAGGATGCTCTTGACTGGCTCGAGCACAACcAAACtGctGAAAAGGACGAGTTTGAACATCAACAAAAG
GAgATTGAAACTCATATGAATCCACTCATGATGAAGATCTACTCTGCTGAGGGTGGTATGCCAGGTGGAATGCCAGGTGGTATGCCA
GGCGGTATGCCAGGTGGAATGCCAGGtGGTATGCCAGGTGGAATGCCAgGCGGTATGCCAGGtGGTATGCCAGGtGGTATGCCAGGt
GGTATGCCAgGATcTAatGgtCCAaCTGTTGaAgagGtcGACTAATtATTTTAgTCACCtAaaAAAACTcaCTCAAAAtggAAAGtTAAgAACTAttT
ACACACtTtCAaTTTCTagTTaTTTTTTACCAAA

>GP6_student_1

GGATGTTCCTGTTGAGGGCTCATCATCGTCATCGTCATCGTCATCGTCATCATCATCATCATCATCATCATCATCATCATCATCAT
CATCAACATCAACCGTCGCACCAGCAAATAAGGCAAGAACTGGAGAAGACGCAGAAGGCAGTCAAGATTCTAGTGGTACTGAAGCTT
CTGGTAGCCAGGGTTCTGAAGAGGAAGGTAGTGAAGACGATGGCCAAACTAGTGCTGCTTCCCAACCCACTACTCCAGCTCAAAGT
GAAGGCGCAACTACCGAAACCATAGAAGCTACTCCAAAAGAAGAATGCGGCACTTCATTTGTAATGTGGTTCGGAGAAGGTACCCCA
GCTGCGACATTGAAGTGTGGTGCCTACACTATCGTCTATGCACCTATAAAAGACCAAACAGATCCCGCACCAAGATATATCTCTGGT
GAAGTTACATCTGTAACCTTTGAAAAGAGTGATAATACAGTTAAAATCAAGGTTAACGGTCAGGATTTCAGCACTCTCTCTGCTAATTC
AAGTAGTCCAACTGAAAATGGCGGATCTGCGGGTCAGGCTTCATCAAGATCAAGAAGATCACTCTCAGAGGAAACCAGTGAAGCTGC
TGCAACCGTCGATTTGTTTGCCTTTACCCTTGATGGTGGTAAAAGAATTGAAGTGGCTGTACCAAACGTCGAAGATGCATCTAAAAGA
GACAAGTACAGTTTGGTTGCAGACGATAAACCTTTCTATACCGGCGCAAACAGCGGCACTACCAATGGTGTCTACAGGTTGAATGAG
AACGGAGACTTGG

>GP6_student_3

GAaCTTTAAGGatGTTCCTGTTGAGGGCTCATCATCGTCATCGTCATCGTCATCGTcATCAtCATCATCATCATCATCATCATCATCATCA
TCATCATCaTCAaCATCaaCCGtCGCACCAgcAAATaAgGcAAGAaCTGgAGAAGACGcAGaAGGcAGTCAaGATTCTaGTGGTaCTGAaG
CTtCTGGTAgCcAGGGTtcTGAAGAgGaAGGTagTGAAGACGATGGCCaaACTagTGCTGCTTCCCaACCcACTACTCCaGCTCAAaGTGa
AGGCGCaACTACCGaAACCATAGAAGCTACTCCAAAAGAAGaATGCGgcaCTTcaTTTGTaAtGTGGtTcGgAGAAGGtACCCCaGCTGCG
ACATTGAaGtGTGGtGCCTACACTATCGTCTATGCACCTATAAAAGACCAAACAGATCCCGCACCAAGATATATctCTGGTGAAGtTACAT
CTGTAACCTTTGAAAAgAGTGATAATACAGTTAAAATCaAGGTTAACGGTCAgGATTTCAgcACTCTCTCTGCTAATTCAAGTAgTCCAA
CTGAaaATGGcGGATCTGCGGGTcAGGCTTCATCAaGATCAAGAAGATCACTCTCAGAGGAAACCaGTGaAGCTGCTGCaACCGtCgAT
TTGtTTGCCTTTACcCTTGATGGTGGtAAAAGAaTTGAAGTGGCTGTACCAaACGtCGAAGATGCATCTAAAAGAGAcAAGTACAGttTGG
TTGcAGAcGATAaAcCTTTCTATACCGGcGCAAACAGCGGcACTACcaATGGTGTCTACAGGTtGAATGAgAACGGAgACTT

>18S_rRNA_ calf

TTTGGTGACTCATAATAACTTTACGGATCACATTATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGG
CCTACCGTGGCTATGACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAG
GCAGCAGGCGCGCAAATTACCCAATCCTAATACAGGGAGGTAGTGACAAGAAATAACAATACAGAGCCTTACGGTTTTGTAATTGGA
ATGAGTTAAGTATAAACCCCTTAACAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCACGGTAATTCCAGCTCCAATAGCGT
ATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTAATTTTCTGTTAATTTTTATATACAATGCTACGGTATTTATATAATATTAACATAA
TTCATATTACTTTTTAGTATATGAAACTTTACTTTGAGAAAATTAGAGTGCTTAAAGCAGGCTATTGCCTTGAATACTCCAGCATGGAAT
AATATTAAGGATTTTTATTCTTCTTATTGGTTCTAGAATAAAAATAATGATTAATAGGGACAGTTGGGGGCATTTGTATTTAACAGTCAG
AGGTGAAATTCTTAGATTTGTTAAAGACAAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGG
ATCGAAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCAACTAGA