Investigating the Public Health Significance of

*Cryptosporidium* in the Environment

Thesis submitted in accordance with the requirements of the
University of Wales College of Medicine
for the degree of Doctor of Philosophy

Guy Robinson

2005
This thesis is dedicated to the following people who have continually shown me their love and support:
Quirien, Noor, Caroline, Annelies & Oma
Aunty May & Uncle Roy
My ever-supportive parents, Carla & Brian
&
My wonderful wife, Rebeca

Thank You
Summary

The high-resolution molecular characterisation of the Cryptosporidium species and subtypes ubiquitous in environmental samples can provide important information regarding their potential public health significance. The purpose of this study was to develop, evaluate and apply sensitive screening and DNA recovery methods to environmental Cryptosporidium for molecular characterisation. A literature review was undertaken to identify methods of subtyping Cryptosporidium species recovered from environmental samples. Prior to molecular characterisation, the recovery and detection of oocysts from water is by immunomagnetic separation and immunofluorescence microscopy. However, this method is not currently suited for screening large numbers of faecal samples. A commercially available faecal parasite concentrator protocol was modified and evaluated for the enhanced detection of Cryptosporidium oocysts. Once recovered from samples, DNA must be released from the oocyst bound sporozoites before molecular methods can be applied. Commonly used oocyst disruption methods were identified and using samples containing high numbers of oocysts, evaluated by microscopy and a SYBR Green real-time polymerase chain reaction (PCR) developed from the internal stage of a previously published nested small subunit ribosomal DNA PCR. The eight best methods were then evaluated with low numbers of oocysts in the presence of immunomagnetic beads to replicate field samples, using the published nested PCR and SYBR Green real-time PCR. The enhanced screening and optimal DNA recovery combined with microsatellite multilocus fragment analysis was applied in a study investigating the species and subtypes of Cryptosporidium recovered from water, non-clinical farmed and wild animal faeces as well as clinical human and cattle samples from within a single water catchment. Several human pathogenic Cryptosporidium subtypes were identified in addition to the unprecedented finding of Cryptosporidium andersoni as the predominant species in the catchment surface water. This study demonstrated the potential application of the developed methodology in the public health investigation of environmental Cryptosporidium.
DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed ................................................................. (candidate)

Date 12/1/06

STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged giving explicit references. A bibliography is appended.

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I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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Acknowledgements

The work carried out would not have been possible without the help and support of the following people to whom I extend my gratitude: The National Public Health Service for Wales (NPHS) for funding this three year PhD position; Alison Weightman from the library at the University of Wales College of Medicine for her advice whilst developing the systematic search strategy for the literature review; Dr. Jason Sawyer and the staff at the Biotechnology Department in Veterinary Laboratories Agency (VLA) Weybridge for their technical advice and hospitality in allowing me the full use of their department whilst developing the SYBR Green I real-time PCR; John Watkins and Carol Francis at the Centre for Research into Environment and Health (CREH) Analytical Ltd. for the training provided in the laboratory in Leeds and for their help during the sampling and processing of samples for the Caldew study; Dr. Carl Stapleton for the Geographical Information System (GIS) analysis and production of some of the figures; The four Caldew farmers for their time and patience during the sampling of their stock and for the local knowledge regarding wildlife activity on their land; The United Kingdom Water Industry Research (UKWIR) and Drinking Water Inspectorate (DWI) for part funding the work carried out during the Caldew study; Staff at the Molecular Diagnostics Unit in the NPHS Microbiology Cardiff Laboratories, in particular Dr. Sally Corden, Catherine Moore for their advice and Dr. Diana Westmoreland for allowing me to use the facilities to carry out subtyping analysis; Staff at the NPHS Microbiology Swansea for their support and full use of the laboratories over the three years, in particular the directors, Dr. David Joyson, Dr. Anne Lewis and the late Dr. Phillip Thomas; All of the staff at the NPHS Cryptosporidium Reference Unit (CRU) in Swansea not only for their advice, support and use of their facilities, but also for their friendship and good sense of humour.

From the CRU, I would like to particularly thank: David Gomez and Rachael Seymour for their assistance when collecting and processing the samples for the Caldew study; Anne Thomas for her discussions regarding parasitology and her help with the microscopic screening of the 1,387 faecal samples collected in the Caldew catchment; Phill Tynan (based in NPHS Microbiology Rhyl) for his assistance with
some of the Caldew microscopy; Dr. Kristin Elwin and Dr. Stephen Hadfield for their stimulating discussions and advice regarding the molecular biology of Cryptosporidium.

I would also like to thank my two supervisors, Professor Stephen Palmer from the University of Wales College of Medicine and Professor David Kay from the University of Wales Aberystwyth, for their advice and supervision during this PhD.

Finally, I would like to extend my warmest gratitude to Dr. Rachel Chalmers from the CRU for her continued supervision and support throughout the three years that I was working in her unit and whose suggestions and advice have been invaluable during this time.
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<td>Acquired Immunodeficiency Syndrome</td>
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<tr>
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<td>DABCO</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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DDBJ  DNA DataBank of Japan
Defra  Department for Environment, Food and Rural Affairs
DHFR  Dihydrofolate reductase
DIC   Differential Interference Contrast
DMSO  Dimethyl Sulphoxide
DNA   Deoxyribonucleic Acid
DPGE  Denaturing Polyacrylamide Gel Electrophoresis
DWI   Drinking Water Inspectorate
EA    Environment Agency
EDTA  Ethylenediaminetetraacetic Acid
EIA   Enzyme Immunoassay
ELISA Enzyme Linked Immunosorbent Assay
EMBL  European Molecular Biology Laboratory
EPA   Environmental Protection Agency
ESWTR Enhanced Surface Water Treatment Rules
FE    Formol-Ether
FEA   Formol-Ethyl Acetate
FISH  Fluorescence In Situ Hybridisation
FMD   Foot and Mouth Disease
FWAG  Farming and Wildlife Action Group
FYM   Farm Yard Manure
g    Gravitational force
GIS   Geographical Information System
GLDH  Glutamate Dehydrogenase
gp    Glycoprotein
GPI   Glucose Phosphate Isomerase
ha  Hectares
HAART  Highly Active Anti-Retroviral Therapy
HCl  Hydrochloric Acid
Hfr  Holstein Friesian
HIV  Human Immunodeficiency Virus
HK  Hexokinase
HMA  Heteroduplex Mobility Assay
HPA  Health Protection Agency
hsp  Heat shock protein
HV  High Volume
ICZN  International Commission of Zoological Nomenclature
ID  Infectious Dose
IF  Immunofluorescence
IFAT  Immunofluorescence Antibody Test
IgG  Immunoglobulin G
IMS  Immunomagnetic Separation
ITS  Internal Transcribed Spacer
k  Kilo
l  Litre
L-dsRNA  Large Double Stranded RNA
LSU rRNA  Large Subunit Ribosomal RNA
μ  Micro
m  Milli
M  Molar
Mab  Monoclonal Antibody
MAFF  Ministry of Agriculture, Fisheries and Food
MCLG  Maximum Contaminant Level Goal
MDH  Malate Dehydrogenase
MEEGID VI  6th Meeting on Molecular Epidemiology and Evolutionary Genetics in Infectious Disease
MgCl₂  Magnesium Chloride
MLFT  Multilocus Fragment Type
mZN  Modified Ziehl-Neelsen
NaCl  Sodium Chloride
NaHCO₃  Sodium Bicarbonate
NaOH  Sodium Hydroxide
NCBI  National Center for Biotechnology Information
opg  Oocysts per gram
OS  Ordnance Survey
PA  Phenol-Auramine
PAGE  Polyacrylamide Gel Electrophoresis
PCR  Polymerase Chain Reaction
PDB  Protein Data Bank
PHLS  Public Health Laboratory Service
Poly-T  Polythreonyl repeat
PWS  Private Water Supply
RAPD  Random Amplified Polymorphic DNA
RFLP  Restriction Fragment Length Polymorphism
RNA  Ribonucleic Acid
RNR  Ribonucleotide Reductase
RT-PCR  Reverse Transcription PCR
SDS  Sodium Dodecyl Sulphate
<table>
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<tr>
<td>S-dsRNA</td>
<td>Small Double Stranded RNA</td>
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<td>Safe Drinking Water Act</td>
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<td>SIGLE</td>
<td>System for Information of Grey Literature in Europe</td>
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<td>Single Strand Conformation Polymorphism</td>
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<td>Small Subunit Ribosomal RNA</td>
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<td>TRAP</td>
<td>Thrombospondin-Related Adhesive Protein</td>
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<tr>
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<td>Ungar <em>Cryptosporidium parvum</em></td>
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<td>USA</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
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<td>WHO</td>
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Section 1

Introduction

and

Literature Review
Chapter 1 – Introduction

Since its first discovery in mice (Tyzzer, 1907), the apicomplexan protozoan Cryptosporidium has been shown to be the causative agent of gastro-intestinal disease (cryptosporidiosis) in a wide range of hosts including humans. The public health significance of Cryptosporidium is apparent with the large number of mainly sporadic cases of cryptosporidiosis per year (a mean of 4482 reported cases per year in England and Wales from 1994-2003, which equates to 8.5 cases per 100,000 people, HPA data) and additionally lies in the severe consequences of infection for groups of immunocompromised patients, the lack of curative anti-parasitic treatment and the potential for large-scale outbreaks to occur. Infected individuals generally present with profuse watery diarrhoea, stomach cramps and on some occasions, vomiting, anorexia and fever (Palmer and Biffin, 1990). Although in immunocompetent individuals human cryptosporidiosis is generally self-limiting, lasting less than two weeks, in individuals with impaired T cell function, and in particular those with acquired immunodeficiency syndrome (AIDS) or congenital immune deficiencies (e.g. severe combined immune deficiency or CD40 ligand deficiency), infections can be fatal (Hunter and Nichols, 2002).

Until recently, most of the epidemiological studies of Cryptosporidium have studied the genus rather than individual species or subtypes. Whilst this has provided information regarding the public health and environmental impact of Cryptosporidium in general, it is impossible to make informed decisions regarding individual species without the characterisation of recovered isolates. To achieve this, molecular methods have been developed that allow the high-resolution characterisation of Cryptosporidium and can be used to increase our understanding of the ecology, epidemiology and the different risks to public health that these individual species and subtypes of this ubiquitous parasite pose. The work undertaken for this thesis has involved the development and application of methodologies to improve the investigation of Cryptosporidium present in environmental samples such as water and faeces from wild or farmed animals and to determine the public health significance of these isolates.
1.1 – Historical Background (Figure 1.1)

The first description of Cryptosporidium was probably by Clarke in 1895, who likely misidentified the merozoites of Cryptosporidium by referring to them as a “swarm spore” stage of Coccidium falciforme (Eimeria falciformis) (Tyzzer, 1910). However, it was 12 years later that Ernest E. Tyzzer published the first preliminary report of Cryptosporidium muris and with the exception of calling it extracellular, described the life cycle stages found in the peptic glands of the common mouse, Mus musculus (Tyzzer, 1907). Then in 1910, Tyzzer further described and illustrated in detail these various life cycle stages and proposed the new genus and species of Cryptosporidium muris for this parasite (Tyzzer, 1910). In addition to the morphological descriptions, infectivity through oral inoculation of oocysts was also demonstrated by in vivo studies with mice (Tyzzer, 1910). Two years later, he described a protozoan that appeared to be smaller in size than C. muris and developed in the intestine of mice instead of the stomach was published (Tyzzer, 1912). Following infectivity studies with this parasite to confirm the size and developmental location, a new species named Cryptosporidium parvum was proposed (Tyzzer, 1912).

In addition to the discovery of these two species, Ernest Tyzzer also reported the first description of Cryptosporidium in birds (Tyzzer, 1929). He briefly described the occurrence of a Cryptosporidium species, with biological characteristics resembling that of C. parvum, in chickens and considers the possibility of cross infection between mice that were evidently contaminating feed and chickens. However, he did not attempt to confirm this by experimental infection and the species identified in the chickens may therefore not have been C. parvum. The next species of Cryptosporidium that was identified and is still considered valid today was Cryptosporidium meleagridis, which was described 26 years later from turkeys (Slavin, 1955). This was also the first time that Cryptosporidium was found to be pathogenic. Slavin described the various developmental stages, which were indistinguishable from those described from C. parvum by Tyzzer in 1912, and found that the infection was associated with increased morbidity and mortality in 10- to 14-day old turkey poults (Slavin, 1955). Despite the potential economic impact of these Cryptosporidium infections in farmed animals it was not until 1971 when C. parvum was found to be associated to diarrhoea in a calf (Panciera et al., 1971) that veterinary workers became more interested. Also in 1971, a further species, Cryptosporidium
Figure 1.1: A timeline summarising the discovery of individual species and important events involving Cryptosporidium
wrair, was described from the small intestine of guinea pigs (*Cavia porcellus*) (Vetterling et al., 1971). Another species of veterinary and economic importance was identified in 1986, when *Cryptosporidium baileyi* was discovered in the small and large intestines as well as the sinuses, trachea and conjunctiva of chickens (*Gallus gallus*) (Current et al., 1986).

Soon after the increased interest from the veterinary perspective, two separate groups identified *Cryptosporidium* for the first time in humans (Nime et al., 1976; Meisel et al., 1976). By 1980 two more species had been identified, *Cryptosporidium felis* from the domestic cat (*Felis catus*) (Iseki, 1979) and *Cryptosporidium serpents* from snakes, which was first reported by Brownstein et al. (1977) but named by Levine (1980). In 1980, *C. parvum* was identified as serious, highly contagious, primary cause of acute diarrhoea outbreaks amongst several animal species (Tzipori and Griffiths, 1998). However, the public health significance of cryptosporidiosis became apparent in 1982 and 1983, when *Cryptosporidium* was found to cause fatal disease in individuals with AIDS (Ma and Soave, 1983; Current et al., 1983; Forgacs et al., 1983; Anon., 1982a) and as a common cause of acute diarrhoea in immunocompetent individuals (Jokipii et al., 1983; Current et al., 1983; Tzipori et al., 1983). Following this realisation of the risk to public health, the amount of *Cryptosporidium* research increased, as seen by the number of publications since 1982 (Figure 1.2). In 1984, the first waterborne outbreak was identified and *Cryptosporidium* became recognised as a waterborne pathogen (D'Antonio et al., 1985). However, eleven years later the importance of waterborne transmission was highlighted when a waterborne outbreak in Milwaukee (Wisconsin, USA) was estimated to have affected 403,000 people in 1993 (Mackenzie et al., 1994). Since then, waterborne *Cryptosporidium* has been associated with a number of outbreaks and epidemiological studies have been undertaken to clarify the species of public health significance and their routes of transmission.

The development of molecular methods has helped further characterise and clarify the multispecies nature of *Cryptosporidium* (Table 1.1). In 2002 at the 6th Meeting on Molecular Epidemiology and Evolutionary Genetics in Infectious Disease (MEEGID VI), the criteria for naming *Cryptosporidium* species were discussed and four basic requirements, including genetic characterisation, were proposed (described in more
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Table 1: Proposed species of Cuproporphyra and their current status.
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<td>Y</td>
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<td><strong>Table 1:</strong> cont. Proposed species of <em>Cyprinidogaster</em> and their current status</td>
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detail in 1.2 – Taxonomic Classification). Differences between the valid species described prior to the development of molecular characterisation methods (C. muris, C. parvum, C. meleagrisidis, C. wrairi, C. felis, C. serpentinis and C. baileyi) have since been confirmed with these tools and so far a further six have now been proposed and considered valid with the criteria proposed at MEEGID VI. These are Cryptosporidium saurophilum (Koudela and Modry, 1998), Cryptosporidium galli (Pavlasek, 1999; redescribed by Ryan et al., 2003), Cryptosporidium andersoni (Lindsay et al., 2000), Cryptosporidium canis (Fayer et al., 2001), Cryptosporidium hominis (Morgan-Ryan et al., 2002) and Cryptosporidium suis (Ryan et al., 2004). Two Cryptosporidium species from fish, Cryptosporidium mohnari (Alvarez-Pellitero and Sitja-Bobadilla, 2002) and Cryptosporidium scophthalmi (Alvarez-Pellitero et al., 2004), are also considered valid based on distinctive biological differences, but have yet to be characterised using molecular tools (Table 1.1).

1.2 – Taxonomic Classification

Knowledge of the taxonomy and systematics of Cryptosporidium can assist in our understanding of the biology, epidemiology and public health significance of Cryptosporidium spp. Knowing exactly which isolate has been recovered can reveal potential sources of infection, routes of transmission, those infectious to humans, the burden of disease or virulence associated with the different species and enable important public health decisions to be made (Xiao et al., 2000c).

When Tyzzer first identified C. muris he was uncertain of it’s systematic position, because with “its possession of an organ of attachment and of iodophilic granules, it resembles the gregarines”, but “in its morphology, in the lack of motion in the adult, and in sexual dimorphism it resembles the coccidia” (Tyzzer, 1907). However, when he further described the parasite in 1910 and officially proposed the new genus and species Cryptosporidium muris, it was placed in the class Sporozoa, subclass Telosporidia, order Coccidiomorpha, suborder Coccidia and within the family Eimeridae (Tyzzer, 1910). The definition of organisms within Eimeridae was:

“Coccidia with macrogametes and microgametes of approximately equal size, which ripen apart from one another; no ‘Syzygian’ formation before copulation: each microgametocyte gives rise to numerous microgametes
which move through the agency of two free flagella. The agametes
divide directly into young agametes, the microgametocytes directly into
microgametes” (Tyzzer, 1910)

No flagella were demonstrated with the microgametes of C. muris but all the other
requirements of the family were met and as two other coccidia at the time were found
to be extracellular the definition of the group had been modified so they were no
longer defined as intracellular parasites (Tyzzer, 1910). Despite classifying
Cryptosporidium in this way he also stated once more that “it would appear that the
mode of life of this species is essentially that of a gregarine” as it “undergoes the
greater part of its development either attached to the surface of the epithelium or free
in the lumen of the gastric glands” (Tyzzer, 1910).

The classification of Cryptosporidium has changed slightly as the several different
systems have been introduced since 1907, but the most current taxa to be published by
the Society of Protozoologists is (Lee et al., 2000):

   Phylum Apicomplexa Levine, 1970
   Class Conoidasida Levine, 1988
   Subclass Coccidiasina Leuckart, 1879
   Order Eucoccidiorida Leger & Duboscq, 1910
   Suborder Eimeriorina Leger, 1911
   Family Cryptosporidiidae Leger, 1911
   Genus Cryptosporidium Tyzzer, 1907

However, with the increase in molecular characterisation and phylogenetics it has
become apparent that members of the genus Cryptosporidium should not be grouped
in the subclass Coccidiasina, as they form distinct clades separate from these
organisms (Relman et al., 1996; Barta et al., 1997; Cai et al., 1992; Lopez et al.,
1999; Carreno et al., 1998; Morrison and Ellis, 1997; Tenter and Johnson, 1997;
Carreno et al., 2001; Barta, 2001; Slapeta et al., 2003). In 1999, Carreno et al. used
the SSU rRNA gene to study the phylogenetic position of five different
Cryptosporidium species. All of the Cryptosporidium species formed a clade that was
more closely related to the gregarines (in subclass Gregarinasina) than the coccidia (in
subclass Coccidiasina) in the genera *Eimeria*, *Sarcocystis* and *Toxoplasma* (Carreno et al., 1999). In addition to the molecular data demonstrating a distance between *Cryptosporidium* and the coccidia and a closeness to the gregarines, there is also biological evidence that supports these findings (Hijjawi et al., 2002). There are differences in the life cycle of *Cryptosporidium* compared to coccidian organisms, such as the intracellular but extracytoplasmic location on the host’s epithelial cells, the attachment of a feeder organelle at the base of the parasitophorous vacuole and the formation of both thick-walled and thin-walled oocysts. Carreno et al. (1999) also described some differences from the gregarine life cycle, with *Cryptosporidium* lacking syzygy and extracellular trophozoite/gamont stages. However, Hijjawi et al. (2002) have since identified stages similar to these in the *Cryptosporidium* life cycle. Cross-reactivity between the gregarine, *Monocystis*, and a fluorescent labelled anti-*Cryptosporidium* monoclonal antibody used for microscopic detection has also been reported to occur (Bull et al., 1998), which highlights the need to evaluate *Cryptosporidium* detection methods with gregarine species. These findings could have a public health impact, as it may explain why anti-coccidial treatments have proven to be ineffective and may suggest future drug targets.

The taxonomy at the species level of *Cryptosporidium* has also changed over the years (sometimes incorrectly) as our understanding of the biology of these parasites has evolved. The differentiation between species of *Cryptosporidium* can be problematic due to the similar morphology and infection sites as well as the lack of host specificity in some cases. Initially, Tyzzer identified two species of *Cryptosporidium* based on the biological differences between *C. muris* and *C. parvum* (Tyzzer, 1907; Tyzzer, 1912). Subsequently, the misidentification of sporocysts from *Sarcocystis* spp. or developmental stages of gregarines resulted in a number of new species of *Cryptosporidium* being proposed and later designated nomen nudum based on the rules of the International Commission of Zoological Nomenclature (ICZN)(Table 1.1). Once the differences between these parasites were recognised, the concept of strict host specificity was erroneously applied and again several new species were proposed (Xiao et al., 2004). However, through experimental studies it became apparent that *Cryptosporidium* could be transmitted from one host species to another and several of the proposed new species were synonymised with others (Table 1.1). This led to further errors in the taxonomy as it was proposed that *Cryptosporidium*
was a single-species genus (Tzipori \textit{et al.}, 1980a), or that only four species (\textit{C. muris, C. meleagridis, C. crotali} and \textit{C. nasoritis}) should be considered valid for those found in mammals, birds, reptiles and fish respectively (Levine, 1984). In 1985, obvious biological differences between \textit{C. muris} and \textit{C. parvum} meant that they were again separated into individual species (Upton and Current, 1985). The subsequent development of molecular and phylogenetic analysis has validated the existence of various distinct \textit{Cryptosporidium} species (Xiao \textit{et al.}, 2004). However, some of the previously described species of \textit{Cryptosporidium} were not adequately described and no molecular characterisation has yet been possible, resulting in them being designated \textit{nomen dubium} (Table 1.1).

The definition of a new \textit{Cryptosporidium} species must be carefully undertaken using both molecular and biological criteria (Egyed \textit{et al.}, 2003). Biological criteria alone cannot be used as the morphology is often similar and most individual species are known to infect more than one host species. Molecular data alone are also insufficient to define separate species, as it is impossible to know what level of genetic difference defines a new species. Following the MEEGID VI meeting in 2002, guidelines were suggested for the naming of new \textit{Cryptosporidium} species: 1) morphometric studies of oocysts, 2) genetic characterisation of common loci, 3) demonstration of natural and, if possible, experimental host specificity, and 4) compliance with the ICZN (Xiao \textit{et al.}, 2004).

Host specificity varies greatly in \textit{Cryptosporidium} species with some (e.g. \textit{C. parvum}) found to infect numerous host species and others (e.g. \textit{C. wrairii}) that are currently thought to have a very narrow host range. In addition to the host specificity of a particular \textit{Cryptosporidium} species there are also a number of “host-adapted” genotypes that were initially grouped with \textit{C. parvum} or \textit{C. muris} due to the similar morphology, but have displayed some genetic differences during the gradual development of some host specificity (Xiao \textit{et al.}, 2004). These host-adapted genotypes are usually named after the initial host from which they were recovered (e.g. goose genotype, duck genotype, deer genotype, bear genotype, etc). When the required criteria have been assessed they may then be elevated to new species (as with \textit{C. parvum} genotype 1 to \textit{C. hominis}, dog genotype to \textit{C. canis} and pig genotype I to \textit{C. suis}) (Ryan \textit{et al.}, 2004; Fayer \textit{et al.}, 2001; Morgan-Ryan \textit{et al.}, 2002). However,
although these species or genotypes have been named after a particular host, this does not necessarily mean that they exclusively infect that animal or even that the named species is the major host. For example, *C. meleagridis* was named as it was first recovered from turkeys, however since then it has been identified as causing numerous mammalian infections and through phylogenetic analysis is thought to possibly be a mammalian parasite that subsequently transferred its host range to birds (Xiao et al., 2002b).

The two predominant species of public health significance are *C. parvum* and *C. hominis*, which have been found to cause the majority of human infections. However, *C. meleagridis*, *C. felis* and *C. canis* have also been identified as causing human infections worldwide, and to a lesser extent *C. muris*, *C. suis* and the *C. parvum* cervine genotype, regardless of the host’s immune status (Morgan et al., 2000; Pieniazek et al., 1999; Alves et al., 2001b; Alves et al., 2001a; Gatei et al., 2002b; Tiangtip and Jongwutiwes, 2002; Alves et al., 2003b; Xiao et al., 2001a; Gatei et al., 2003; Yagita et al., 2001; Pedraza-Diaz et al., 2001c; Pedraza-Diaz et al., 2001b; Pedraza-Diaz et al., 2001a; Pedraza-Diaz et al., 2000; Gatei et al., 2002a; Ong et al., 2002; Palmer et al., 2003; Xiao et al., 2002a).

1.3 – Life Cycle
From the first discovery of *Cryptosporidium* the majority of the developmental stages were described (Tyzzer, 1907; Tyzzer, 1910) and our understanding of the life cycle (Figure 1.3) has changed very little since then (Current and Garcia, 1991; O'Donoghue, 1995; Fayer et al., 1990).

Members of the genus *Cryptosporidium* are monoxenous and the life cycle begins when the host ingests sporulated oocysts. If the site of infection is the gastrointestinal tract then pancreatic enzymes and bile salts may enhance excystation, although it can also occur in the warm aqueous environment of extraintestinal sites (Fayer et al., 1997). When conditions are favourable, the four infectious sporozoites emerge through the suture in the oocyst wall (Reduker et al., 1985b; Reduker et al., 1985a) and attach by the anterior end to the surface of an epithelial cell. A parasitophorous vacuole encloses the sporozoite leaving it extra-cytoplasmic but intracellular and a feeder organelle develops between the parasite and the cytoplasm of the epithelial cell
Figure 1.3: The life cycle of *Cryptosporidium*. Oocysts (A) are ingested by the host; Sporozoites (B) excyst and invade the brush border of epithelial cells; The sporozoite develops into a trophozoite (C); The trophozoite undergoes merogony to form a type I meront (D and E); Merozoites (F) are released and undergo merogony again to form further type I meronts or type II meronts (G); Merozoites (H) are released from the type II meronts and form either microgamonts (I) or macrogamonts (K); Microgametes (J) are released from the microgamont and fertilise a macrogamont to form a zygote; The zygote undergoes sporogony to produce either thin-walled oocysts (L) that autoinfest the host or thick-walled oocysts (M) that are shed in the faeces ready to infect a new host.
(Fayer et al., 1997). Each attached sporozoite then develops into a spherical
trophozoite, which undergoes asexual division called merogony and forms a type I
meront containing eight merozoites (O'Donoghue, 1995; Fayer et al., 1997). These
eight merozoites are released and attach again to the surface of an epithelial cell
where they undergo merogony once more and either form a further type I meront, or a
type II meront. Two types of meront have been identified in the life cycle of C.
wrairi, C. felis and C. parvum (Current and Reese, 1986; Iseki, 1979; Vetterling et al.,
1971), three in C. baileyi (Current et al., 1986) and only one in C. meleagris
d (Slavin, 1955). A type II meront contains four merozoites that when released attach
to the epithelium but instead of developing into further meronts they initiate the
sexual reproduction (gametogony) by forming the micro- and macrogamonts (Gobel
and Brändler, 1982). Each macrogamont differentiates to form up to 16
microgametes, which when released, locate and fertilise a unicellular
macrogametocyte that has developed from a macrogamont, producing a zygote. The
zygote undergoes two asexual cycles of sporogony to produce an oocyst with either a
thick wall or a thin wall, both of which contain four sporozoites (Current and Reese,
1986). Unlike the majority of coccidian parasites Cryptosporidium is able to
autoinfect the host, with the thin-walled oocysts excysting once separated from the
epithelium and releasing the four sporozoites for the cycle to start again (Current and
Reese, 1986). This autoinfection and the recycling type I meronts could explain the
persistent chronic infection that can occur in hosts and the low infective dose seen in
some studies (DuPont et al., 1995; Okhuysen et al., 1999; Jokipi and Jokipi, 1986).
The thick-walled oocysts are excreted from the host in the faeces and as already
sporulated are immediately infective.

Attempts to culture Cryptosporidium by replicating this life cycle in vitro have not
been particularly successful, often resulting in the incomplete cycle or infections not
being maintained for more than a few days (Hijjawi, 2003). The complete
development of all the parasite stages has been achieved, but the numbers of
sporulated oocysts produced have been low and failed to maintain the intensity of
infection (Hijjawi, 2003). Recently, an extracellular gamont stage in the life cycle has
been identified (Hijjawi et al., 2002) and subsequently the complete development was
been described in a completely cell-free culture producing a significant increase in
sporulated oocysts (Hijjawi et al., 2004). This is a highly significant development for
the future of *Cryptosporidium* research and has potential uses in evaluating drugs on various life cycle stages, assessing viability of detected oocysts, or even amplifying recovered organisms prior to characterisation.

### 1.4 – Epidemiology, Clinical Features & Treatment

There have been many epidemiological studies of *Cryptosporidium* and several review articles summarise the major aspects of this topic (Meinhardt *et al.*, 1996; Fayer *et al.*, 2000a; Casemore, 1990). Until recently only the epidemiology of the genus *Cryptosporidium* was being studied, as species differentiation techniques were not being applied. However, differences in the public health significance occur between and also within *Cryptosporidium* species, demonstrating the need for high-resolution characterisation of the strains in question. The age and geographic distribution, seasonality, natural reservoirs, routes of transmission, infectivity and clinical features can all differ between strains.

#### 1.4.1 – Sources, Natural Reservoirs and Transmission

The number and types of natural reservoirs also varies between the species of *Cryptosporidium*. There are many animals that have been identified as natural hosts of *C. parvum*, whereas host-adapted species such as *C. hominis* or certain subtypes may be restricted to a much narrower host range. Molecular characterisation of these isolates can suggest sources of infection or environmental contamination.

Several direct and indirect routes of transmission have been identified. The direct routes are by faecal-oral contact from animal-to-animal, animal-to-human (zoonotic), human-to-human (anthroponotic) and human-to-animal. The identification of a *C. hominis* infection in a human would suggest an anthroponotic route whereas a *C. parvum* infection could be zoonotic or anthroponotic (although certain subtypes may suggest one or the other). The identification of *C. meleagridis, C. felis, C. canis, C. muris, C. suis* or the cervine genotype in humans demonstrates that farm animals, domestic pets and wildlife can be potential zoonotic sources (Xiao *et al.*, 2004).

Initially, direct routes of transmission were identified for the zoonotic cycle, involving contact with infected animals. This route of transmission has been implicated in human infections following educational farm visits and occupational exposure, although epidemiological data suggests that those repeatedly exposed, such as
farmers, can develop immunity (Casemore, 1990). When visiting farms, veterinary students and particularly young children, who are at increased risk due to immature immune systems, are often in contact with the animals most prone to shedding *Cryptosporidium* (the young livestock such as lambs and calves) thus exposing them to greater risk of infection. The direct routes of transmission in the anthropogenic cycles occur when there is greater contact with faecal material (e.g. infections caught in hospitals, nursing homes, day-care centres and when changing dirty nappies). The risk associated with these routes can be reduced or prevented by less contact with infected animals or individuals, and through good hygiene procedures following exposure. A number of indirect routes of both zoonotic and anthropogenic transmission have also been identified and involve contact with *Cryptosporidium* contaminated faecal material away from the source. For the zoonotic cycle, indirect routes have included dirty clothing and footwear worn on farms or in the countryside, and via environmental contamination (Casemore, 1990). For example, transmission was thought to have occurred with young children of mechanical-digger operators, whose clothes and footwear became infected whilst working on farms (Casemore, 1987). Potentially zoonotic *Cryptosporidium* can be transmitted easily through environmental routes as wild and farmed animal faeces are commonly found to contaminate fences, soil, fields (particularly with crops or vegetables growing) and watercourses. Indirect routes of anthropogenic cryptosporidiosis have been linked to infected food handlers contaminating their products during preparation (Quiroz *et al.*, 2000) and also through contact with environmental contamination. Environmental contamination from human sources usually occurs following the release of untreated sewage, by accident or as overflow following storm events. Once again molecular characterisation can assist in the study of these routes of transmission. When the massive waterborne outbreak occurred in Milwaukee, the source of the contamination to the water supply was unknown and agricultural run-off, abattoir waste and sewage overflow were all considered (Mackenzie *et al.*, 1994). However, subsequent molecular analysis has since demonstrated that the outbreak was caused by *C. hominis*, which indicates a human source and suggests that agricultural run-off or abattoir waste was unlikely (Peng *et al.*, 1997).
1.4.2 – Age, Geographical and Seasonal Distribution

Unlike many other host species, cryptosporidiosis occurs in humans of all ages, although symptomatic infections are more common in young children, probably due to immature immune systems and increased faecal-oral transmission (Ungar, 1990b). The number of laboratory confirmed cases of cryptosporidiosis is highest in children aged 1-5 years with a smaller secondary peak occurring in adults aged 20-40 years (Palmer and Biffin, 1990). A recent case-control study has shown that this secondary peak is associated with *C. hominis* and not *C. parvum* (Hunter et al., 2004b).

Disease from *Cryptosporidium* spp. has been reported worldwide, with varying prevalences in different countries (Meinhardt et al., 1996). Molecular characterisation of the *Cryptosporidium* causing disease can identify differences in the geographic distribution that were previously hidden. For example, in England and Wales the species of *Cryptosporidium* causing human disease varies between regions (Figure 1.4) and may be due to local differences in the amount of agriculture or the density of human population in the area.

The seasonality of cryptosporidiosis also varies between countries, but in England and Wales as a whole two peaks of cryptosporidiosis occur, in the spring and in the autumn (Casemore, 1990). The epidemiological importance of these two peaks did not become apparent until molecular methods were used to characterise the predominant species causing each peak (Figure 1.5). The primary peak in the spring is caused by *C. parvum* and coincides with calving, lambing and periods of heavy rainfall (McLauchlin et al., 2000; Anon., 2002b). However, the secondary peak is predominantly associated with *C. hominis* and coincides with increased travel abroad (McLauchlin et al., 2000; Anon., 2002b).

1.4.3 – Infectious Dose and Duration of Infection

The infectious dose of oocysts, the duration and severity of illness are all influenced by several factors including the species and strain of parasite, as well as the age and immune status of the host. In immunocompetent individuals, cryptosporidiosis can cause a self-limiting gastroenteritis. However, in immunodeficient individuals (e.g. those with a HIV infection, other immunosuppressive disease or treatment) a *Cryptosporidium* infection can be prolonged, extra-intestinal and life threatening.
Figure 1.4: The geographical distribution of *C. hominis* and *C. parvum* cases reported in 2000 (used with permission from Dr. Rachel Chalmers)
Figure 1.5: The seasonal distribution of disease caused by *C. hominis* and *C. parvum* in England and Wales during 2000

(used with permission from Dr. Rachel Chalmers)
Research has shown that individuals with a CD4 count of $>200$/mm$^3$ do not suffer more severe or prolonged cryptosporidiosis than otherwise healthy individuals and that those with a CD4 count of $<50$/mm$^3$ are at greatest risk (Hunter and Nichols, 2002).

In humans, experimental transmissions have been undertaken with *C. parvum* and the infectious dose was seen to be low, particularly when there was no prior evidence of infection. DuPont *et al.* (1995) successfully infected seronegative healthy volunteers with oocysts of *C. parvum* (Iowa strain) and found that the ID$_{50}$ for this strain was 132 oocysts, with just 30 oocysts capable of causing infection in one of the subjects. This ID$_{50}$ was reduced to 83 oocysts when presumed infections (symptomatic but with no oocysts detected) were included and the data reassessed (Chappell *et al.*, 1999), and the ID$_{100}$ reduced from 1000 to 500 oocysts (Okhuysen *et al.*, 1998). Okhuysen *et al.* (1999) found that even fewer oocysts were required to infect 50% of healthy adults depending upon the strain of *C. parvum* used. They found that the TAMU strain had the lowest ID$_{50}$ at 9 oocysts followed by Iowa and UCP at 87 and 1042 oocysts respectively. Increased levels of anti-*Cryptosporidium* IgG through previous infections also has a major effect on the infectious dose as the Iowa ID$_{50}$ in these individuals was seen to be 1,880 oocysts (a 23-fold increase), or 7,638 oocysts (57 times higher) when only those seen to be shedding oocysts were included (Chappell *et al.*, 1999). Although the infective dose needed to cause infection in these individuals was higher, the severity of disease was greater with significantly longer duration of illness and number of unformed stools (Chappell *et al.*, 1999). In the same way that these different host-parasite factors influence human infections with *C. parvum*, other species and strains of *Cryptosporidium* may also affect the course of anthroponotic infections. The only report of experimental transmission of *C. hominis* to humans is by Cynthia Chappell in a poster presented at the EPA Science Forum 2004. They found that the infectious dose of *C. hominis* was similar to the most infectious *C. parvum* isolate, but the course of infection was more severe with longer duration of illness and oocyst shedding.

The prepatent period (from the time of ingestion until the time when the first oocysts can be detected in the host’s faeces) and patent period (from the time oocysts are first detected until no more oocysts are detected in the host’s faeces) of a *Cryptosporidium*
infection also vary depending upon the parasite species or subspecies, host age and immune status, as well as the infectious dose. Therefore, measurement of these periods is not only influenced by time taken for a particular species or subspecies to infect and start producing oocysts, but also by the sensitivity and specificity of the detection method. The infectivity studies described above demonstrated that different strains of *C. parvum* can influence the prepatent period, as can the individual being infected (Okhuysen et al., 1999), although in these studies some individuals were presumed to be infected due to clinical symptoms though no oocysts were detected.

The incubation period is the time from ingestion of oocysts to the appearance of the first sign or symptom of disease and is another measurement of the establishment of infection. However, in *Cryptosporidium* infections there are many reports of asymptomatic carriage as well as clinical disease. DuPont et al. (1995) reported a mean and median incubation period for diarrhoeal disease due to *C. parvum* Iowa strain at 9 and 6.5 days respectively (range 4-22 days) and symptoms lasted for 58-87 hours (mean = 74 hours). In addition to those with diarrhoeal disease, 4 of the volunteers in this study had mild enteric symptoms lasting 1-4 days (with incubation periods of 2, 5, 31 and 6 days respectively) and 7 were asymptomatic whilst still shedding detectable levels of oocysts, but the prepatent periods for these individuals were not reported. Also using the *C. parvum* Iowa strain, Chappell et al. (1999) found that volunteers with previous exposure to *Cryptosporidium* had mean and median incubation periods for diarrhoeal illness of 5.5 and 5 days (range 3-12) respectively and it lasted for 30-336 hours (mean = 145.1 hours). Okhuysen et al. (1999) reported the variation that different strains of *C. parvum* have on the prepatent period, the incubation period and duration of illness in human infections. For the TAMU, Iowa and UCP *C. parvum* strains, the mean prepatent period was 4, 7.7 and 7 days respectively, the mean incubation period was 5, 9 and 11 days respectively and the mean duration of illness was 94.5 (6-195), 64.2 (6-223) and 81.6 (6-193) hours respectively (Okhuysen et al., 1999). Other studies have also reported incubation periods using natural infections and the average is approximately 7 days with a range of 5 to 28 days (Ungar, 1990b). From one in depth study, the mean duration of illness was 12 days, but ranged from 2 to 26 days (Jokipi and Jokipi, 1986). Once the clinical symptoms have ended, oocysts can still be shed in faeces for some time afterwards and reports into the duration of this are dependent on the method of
detection. Jokipii and Jokipii (1986) extensively screened 50 otherwise healthy individuals with cryptosporidiosis and found that oocysts were shed, sometimes intermittently, for a mean of 7 days (range 1-15 days) after symptoms ceased with an occasional positive stool up to 85 days afterwards. All of these studies have demonstrated wide periods of infection, illness and oocyst shedding, reflecting the influence that the many parasite and host factors have on the course of infection. Further studies are required to determine whether the different species that have been identified as causing human infections, some of which appear more host-adapted (e.g. *C. hominis*) than others, are more virulent or pathogenic.

1.4.4 – Clinical Symptoms

The severity of a *Cryptosporidium* infection can vary greatly from asymptomatic shedding of oocysts to severe, life-threatening disease. The main acute clinical feature of cryptosporidiosis is watery diarrhoea, sometimes with mucus present, which also varies in severity. However, other symptoms include abdominal cramps, anorexia, nausea, vomiting, slight fever and general malaise, which can lead to dehydration, malnutrition and weight loss (Ungar, 1990b). However, extra-intestinal infections do occur, particularly in immunocompromised individuals, and include respiratory, hepatobilary and pancreatic cryptosporidiosis (Arrowood, 1997; Hunter and Nichols, 2002). In addition to the extensive reports and reviews that describe these acute clinical features, a recent case-control study examined the medium-term health effects of a *C. parvum* or *C. hominis* infection in immunocompetent patients who had previously been diagnosed with sporadic cryptosporidiosis (Hunter *et al.*, 2004a). Hunter *et al.* (2004a) demonstrated that recurrence of gastrointestinal symptoms was common following infection with either species, but following infections with the host-adapted *C. hominis*, other non-gastrointestinal symptoms (i.e. joint or eye pain, recurrent headaches, dizziness and fatigue) were significantly higher than in the controls or those infected with *C. parvum*.

1.4.5 - Treatment

One of the major public health problems associated with cryptosporidiosis, particularly in immunocompromised patients, has been the lack of effective curative treatment. Whilst management of the gastrointestinal symptoms in treating the self-limiting disease in immunocompetent patients is possible, the major challenge is with
the severe infections in immunocompromised individuals. Smith and Corcoran (2004) reviewed the efficacy of antiparasitic drugs, immunotherapy and the role of highly active anti-retroviral therapy (HAART) in the treatment of cryptosporidiosis. A number of drugs, such as paramomycin and nitazoxanide have been used with varying success, often with promising results in the immunocompetent but poor efficacy in those with weakened immune systems (Amadi et al., 2002; Hewitt et al., 2000). Amadi et al. (2002) demonstrated that nitazoxanide resolved diarrhoea in 56% of HIV-negative patients compared to 23% receiving a placebo (p=0.037), but no benefit was observed following primary treatment in the HIV-positive group included in this study. Nitazoxanide (marketed as Alinia) is currently the only drug licenced by the FDA for the treatment of cryptosporidiosis. The use of HAART has been used with some success in patients with HIV, reducing the length and severity of infection. This is due to increasing the patient’s immune system by suppressing the virus and boosting the levels of CD4 lymphocytes (Schmidt et al., 2001). However, the presence of protease inhibitors in these drug cocktails is thought to have a direct affect on cryptosporidial infections (Mele et al., 2003). Mele et al. (2003) demonstrated that treatment with indinavir, an aspartyl protease inhibitor, without any other drug combinations gave a marked reduction in the number of *C. parvum* parasites *in vitro* and *in vivo* using mice. Hommer et al. (2003) also demonstrated the successful inhibition of *Cryptosporidium* development with indinavir and other protease inhibitors, and found that the combination of indinavir or ritonavir with paramomycin gave increased inhibition.

1.5 – Recovery of *Cryptosporidium* spp.

The high detection rate of *Cryptosporidium* infections in clinical samples is related to the large numbers of oocysts that are often present. However, there are occasions when the numbers of oocysts can be low, such as in non-symptomatic infection, during intermittent shedding, as an infection is subsiding or in environmental samples. It is then necessary to concentrate the samples and recover any oocysts present, prior to detection.

1.5.1 – Faeces

The concentration method with the highest recovery rates prior to detection in faeces is immunomagnetic separation (IMS) due to the *Cryptosporidium*-specific antibodies
used to bind the oocysts to the magnetic beads (Pereira et al., 1999; Rochelle et al., 1999a; Davies et al., 2003). In addition to the high recovery rates, IMS also separates the oocysts from any molecular inhibitors that may be present in the sample. However, the high cost of IMS reagents (~£20 per sample) makes this method unaffordable for studies with high numbers of samples or in routine public health laboratories and therefore cheaper methods are required. Several methods have been used when screening for Cryptosporidium, including sucrose flotation, formol-ether (FE) sedimentation, formol-ethyl acetate (FEA) sedimentation, saturated salt flotation, zinc sulphate flotation and calcium chloride flotation (Sheather, 1923; Casemore, 1991; Casemore et al., 1985; Young et al., 1979; Ritchie, 1948). The FE or FEA sedimentation techniques have been reported to result in a significant loss of oocysts (Casemore et al., 1985). This is due to the density of Cryptosporidium oocysts, which is different from the ova and cysts that the method was originally developed for, resulting in them becoming trapped in the ether or ethyl acetate plugs and therefore failing to sediment (Arrowood, 1997). The FE method was therefore modified by Casemore et al. (1985) to separate the oocysts from the sample, whilst leaving the ether plug and much of the debris behind. The oocysts could then be allowed to sediment in formol water without the losses. This method, closely followed by FEA sedimentation, was found to give the greatest recovery of all the concentration techniques and of the flotation methods, the saturated salt technique recovered the most oocysts (Casemore et al., 1985). Further comparison studies have also found FEA sedimentation to give greater oocyst recovery than the other methods (McNabb et al., 1985; Mtambo et al., 1992; Bukhari and Smith, 1995). Kuczynska and Shelton (1999) found that FEA sedimentation gave much lower recoveries than most methods and that saturated sodium chloride flotation resulted in the most oocysts. However, this could be due to the losses, mentioned above, from oocysts getting trapped and not sedimenting (Casemore et al., 1985; Arrowood, 1997). Kvac et al. (2003) found the Sheather sucrose flotation method to provide the best results, but they did not include FE or FEA in their comparison of methods. Several commercially available faecal parasite concentration kits have been developed based on the sedimentation methods and evaluated for use with Cryptosporidium (Zierdt, 1984; Perry et al., 1990). Zierdt (1984) found that the Fecal Parasite Concentrator (Evergreen Scientific) combined with acid-fast (AF) microscopy or sucrose flotation increased the sensitivity compared to the standard FEA sedimentation and AF microscopy. Perry et al. (1990) compared
the Fecal Parasite Concentrator (Evergreen Scientific) with three other commercial 
kits (Fecal Concentrator Kit, Remel; Para-Pak Macro-Con, Meridian Diagnostics; 
Trend FeKal CON-Trate, Trend Scientific) and the standard FEA method. All of the 
commercial methods concentrated *Cryptosporidium* oocysts to a greater extent than 
the standard FEA method when detected by AF microscopy.

1.5.2 – Water

In addition to the direct transmission of *Cryptosporidium* through contact with 
infected faecal material, indirect transmission is also common, particularly through 
contaminated water sources (Rose *et al.*, 1997; Smith and Rose, 1998; Fayer *et al.*, 
2000a; Rose *et al.*, 2002; Fayer, 2004). This became particularly apparent in 1993 
following the massive waterborne outbreak of *Cryptosporidium* in Milwaukee, which 
reportedly affected approximately 403,000 people (Mackenzie *et al.*, 1994).

However, this number was calculated from a retrospective telephone-based survey, 
which may have over-estimated the number by 10-100 times (Hunter and Syed, 
2001). To screen water samples for the presence of *Cryptosporidium*, large volumes 
that potentially contain few oocysts must be collected and therefore concentration is 
required prior to detection. Different methods have been used for this and include 
various filtration, centrifugation and flocculation techniques (reviewed by Quintero-
Betancourt *et al.*, 2002; Zarlenaga and Trout, 2004). Standard operating protocols 
have been published both in the USA and the UK for the concentration, purification 
and detection of *Cryptosporidium* oocysts for monitoring their presence in water 
supplies (USEPA, 2001; DWI, 2005). These involve concentrating all of the 
particulate matter in the sample by filtration, elution and centrifugation followed by 
oocyst purification from the particulate matrix by immunomagnetic separation. The 
recovery efficiencies of these methods vary depending upon the sample as factors, 
such as the particulate matter, can inhibit or even assist the recovery process, 
particularly at the filtration stage (Zarlenaga and Trout, 2004; Feng *et al.*, 2003). 
Therefore, the US and UK standard operating protocols have quality control 
acceptance criteria of 11-100% and more than 30% recovery efficiency respectively 
for samples spiked with known numbers of oocysts (USEPA, 2001; DWI, 2005).
1.6 – Detection of *Cryptosporidium* spp.

The detection of *Cryptosporidium* in samples is important, not only in identifying the cause of an illness but also in epidemiological surveys, environmental or disease surveillance and drinking water monitoring (Percival *et al.*, 2004). Different microscopic, molecular and immunological methods have been developed to detect the presence of *Cryptosporidium*, in faeces and other matrices such as food or water, and depend upon visualising oocysts, or detecting *Cryptosporidium* antigens or DNA.

1.6.1 – Evaluating Detection Methods

The method of detection is chosen by consideration of performance and convenience criteria. The performance criteria describe the technical limitations of the method and are defined as (Last, 2001; European co-operation for Accreditation, 2002):

- **Sensitivity**: “the probability of obtaining a true positive result”
- **Specificity**: “the probability of obtaining a true negative result”
- **Positive Predictive Value**: “probability that a positive result is true”
- **Negative Predictive Value**: “probability that a negative result is negative”
- **Repeatability**: “closeness of the agreement between the results of successive measurements of the same measurand under the same conditions of measurement”
- **Reproducibility**: “closeness of the agreement between the results of measurements of the same measurand carried out under changed conditions of measurement”

The positive and negative predictive values must be used with caution as they are influenced by the prevalence of the organism and can only be extrapolated to populations with a similar prevalence. The convenience criteria describe factors that may influence the choice of methods based upon the circumstances in question and include the cost, turn-around time, facilities or equipment required and expertise to perform the method or interpret the results.

1.6.2 – Microscopic Detection Methods

Due to the relatively low cost, the majority of initial screening involves staining faecal smears and examining by microscopy for oocysts of 4-6μm or 7-8μm x 5-6μm
depending upon the species (Table 1.1). In 2002, a survey of the laboratories serving England and Wales identified staining by either the modified Ziehl-Neelsen method (Figure 1.6 and 1.7) or auramine phenol prior to microscopy were the most common methods of screening (Chalmers et al., 2002c). Various adaptations of carbol fuchsins based AF methods, such as Ziehl-Neelsen, Kinyoun and dimethyl sulfoxide-carbol fuchsin, have been described by different authors for the detection of Cryptosporidium but no comprehensive comparisons have been made between these different protocols (Bronsdon, 1984; Garcia et al., 1983; Ma and Soave, 1983; Stibbs and Ongerth, 1986; Henriksen and Pohlenz, 1981; Casemore, 1991; Casemore et al., 1985; Casemore et al., 1984). There are also several variations on the auramine protocols using different counter-stains but again no comprehensive comparisons between the methods have been undertaken (Casemore et al., 1985; Casemore et al., 1984; MacPherson and McQueen, 1993; Garcia et al., 1983; Stibbs and Ongerth, 1986; Arrowood and Sterling, 1989; Chalmers et al., 2002c). Identification of oocysts by microscopy in wet preparations (Jokipii et al., 1983; Ma and Soave, 1983) and following the use of Romanowsky stains (Pohlenz et al., 1978; Tzipori et al., 1980b) have been described as insensitive for Cryptosporidium detection (Casemore et al., 1985). A safranin-methylene blue staining method was described by Baxby et al. (1984), who claimed that this method was more sensitive than the Ziehl-Neelsen methods, but despite this, other methods remain more widely used in the literature. In addition to these chemical stains, immunofluorescent (IF) stains that use Cryptosporidium-specific antibodies with a fluorescent label (Figures 1.8a and 1.9a) have also been developed (Garcia et al., 1987; Garcia et al., 1992; McLauchlin et al., 1987; Sterling and Arrowood, 1986; Stibbs and Ongerth, 1986). The immunofluorescent stains are often used in combination with 4',6-diamidino-2-phenylindole (DAPI), which stains the DNA within oocysts allowing the presence of up to 4 sporozoites to be identified (Figures 1.8b and 1.9b). In addition to the use of DAPI, internal structures can be observed using differential interference contrast (DIC) microscopy (Figures 1.8c and 1.9c). Several studies have compared the use of some of the staining protocols for the detection of Cryptosporidium oocysts (Baxby et al., 1984; Stibbs and Ongerth, 1986; Garcia et al., 1983; Rusnak et al., 1989; Arrowood and Sterling, 1989; Garcia et al., 1987; Garcia et al., 1992; MacPherson and McQueen, 1993; Alles et al., 1995; Mtambo et al., 1992; Quilez et al., 1996), the
Figure 1.6: *Cryptosporidium parvum* oocysts stained by modified Ziehl-Neelsen using a x100 objective.

Figure 1.7: *Cryptosporidium andersoni* oocysts stained by modified Ziehl-Neelsen using a x100 objective.
Figure 1.8: A Cryptosporidium parvum oocyst using a x100 objective: A) with the wall stained by a fluorescein-labelled antibody; B) with DAPI staining of four sporozoite nuclei; C) with sporozoites visible by DIC microscopy.
Figure 1.9: *Cryptosporidium andersoni* oocysts using a x100 objective: A) with the walls stained by a fluorescein-labelled antibody and showing one with the suture open; B) with DAPI staining of four sporozoite nuclei within each oocyst; C) with sporozoites and oocyst residual bodies visible by DIC microscopy.
majority of which demonstrated the superior sensitivity of using IF staining techniques for detection particularly when fewer oocysts were present.

The US and UK standard operating protocols for monitoring Cryptosporidium in water supplies use immunofluorescence microscopy to detect oocysts following filtration and immunomagnetic separation (USEPA, 2001; DWI, 2005). In addition to increased sensitivity, immunofluorescent methods can also improve specificity due to the Cryptosporidium specific antibodies that are used in the stain, resulting in fewer false positives (Stibbs and Ongerth, 1986; Rusnak et al., 1989; Arrowood and Sterling, 1989; Garcia et al., 1987; Garcia et al., 1992; Garcia and Shimizu, 1997). Whilst this is an advantage in reducing the misidentification of artefacts that can occur with the chemical stains, other parasites that may be present in samples will be missed when using immunofluorescent stains (Arrowood, 1997). A further disadvantage with using immunofluorescent stains is the added cost involved in reagents and specialist microscopy facilities that are required. However, it may be argued that the added cost of the reagents may be saved in the reduced labour costs due to faster screening.

The sensitivities of all these techniques are dependent on the number of organisms present in the sample, as even the least sensitive methods will appear to have a high sensitivity with samples containing large numbers of oocysts. Therefore, the threshold of detection is an important means of evaluating the best techniques for screening samples that could potentially contain few oocysts. Realising this, Weber et al. (1991) determined the threshold of detection for an AF method and an IF method following concentration by the standard FEA sedimentation method (Young et al., 1979). In their study the threshold of detection in watery stool with a sensitivity of 100%, was 10,000 oocysts gram⁻¹ (opg) for both methods but for formed stools this rose to 50,000 opg by IF and 500,000 opg for the AF method. Another important issue that was raised by that study is the different thresholds with variation in faecal consistency. In fluid stool, the sensitivity of the IF method was reduced to 90% and for the AF staining this was reduced to 60% for 5,000 opg, but in the formed stool no oocysts were detected by AF in samples with less than 100,000 opg and the IF sensitivity was reduced further to 20% at 5,000 opg (Weber et al., 1991). The loss of a substantial number of oocysts was also recorded at each stage of the FEA method. However the number of oocysts observed by IF in concentrated samples was seven
times higher than in non-concentrated samples (Weber et al., 1991). Weber et al. (1992) published a new method using the FEA stage in a single step followed by salt flotation and a final washing stage. This new method with IF screening improved the sensitivity in watery faeces from 90% to 100% at 5,000 opg and in formed faeces the threshold of detection with a sensitivity of 100% was lowered from 50,000 opg, as found previously, to 10,000 opg. The sensitivity of the new method with 5,000 opg of formed faeces was also increased from 20% to 80% by IF (Weber et al., 1992). However, despite the increased sensitivity and threshold of detection in this new method, extra steps were involved which would likely result in oocyst losses (during the salt flotation and washing stages). During the evaluation of this new method, the authors also tested the Sheather’s sugar and zinc sulphate flotations prior to IF screening, but found that these methods were not as sensitive as the FEA and could not detect any oocysts at 50,000 opg in the formed stools (Weber et al., 1992). Clavel et al. (1996) found that an extended centrifugation step increased the sensitivity when screening by mZN. However, this centrifugation step was found by Weber et al. (1992) to increase the background debris so much that screening by IF was not possible. The Sheather’s sucrose flotation method was used more successfully by Deng and Cliver (1999) following an initial clean-up stage similar to the modification of the formol-ether method used by Casemore et al. (1985) and involved a slow and short centrifugation to remove some of the faecal debris from samples. Deng and Cliver (1999) used this method to determine a threshold of detection of 200 opg with three replicates, but did not state the source of the faeces or whether it was formed or fluid. The thresholds of detection of the AF and IF methods without prior concentration of equine faeces of typical consistency were examined by Cole et al. (1999) who achieved 100% sensitivity in three replicates at $5 \times 10^5$ opg for both AF and IF.

1.6.3 – Enzyme Immunoassay Detection Methods
The first enzyme immunoassays (EIA) for Cryptosporidium detection in faecal samples were described in 1990 (Robert et al., 1990; Ungar, 1990a; Anusz et al., 1990). Robert et al. (1990) described a microplate EIA for the detection of bovine cryptosporidiosis, but the sensitivity was comparable to sucrose flotation and worse than modified Ziehl-Neelsen staining, with a threshold of detection at $>10^6$ oocysts per ml$^{-1}$. Ungar (1990a) described a microplate EIA which also had a low sensitivity
(82.3%) when compared to AF or IF microscopy. However, the method developed by Anusz et al. (1990) could detect a threshold of 3x10^5 oocysts ml^{-1} of faeces, which was better than AF staining (10^6 oocysts ml^{-1}) but not as good as IF (10^3 oocysts ml^{-1}). However, since these first generation assays a number microplate and cartridge based kits have been developed and are now commercially available.

Siddons et al. (1992) described the evaluation of a microplate EIA compared to phenol-auramine (PA) staining of faecal and water concentrates and found the EIA to be more sensitive than the PA, which was confirmed by IF. An evaluation of a different microplate EIA (LDM Laboratories) demonstrated a sensitivity of 66.3% and specificity of 99.8% using AF microscopy as a gold standard (Newman et al., 1993), but in a separate study (Rosenblatt and Sloan, 1993) the sensitivity was much higher (93%) when IF was the gold standard. The ProSpecT microplate EIA (Alexon Inc.) was compared to AF staining by Dagan et al. (1995) and reported to have a sensitivity and specificity of 98% and 98% respectively. However, despite the AF positive samples being confirmed by IF, 13 EIA positive but AF negative samples were not, so it is not possible to compare the EIA with IF and it is possible that these were false positives. Instead, the authors tested these false positives with a Cryptosporidium-specific antigen inhibition test and concluded that the results were not false positives (Dagan et al., 1995), but they were assuming that the immunoassay was not cross-reacting with something present in those samples.

Kehl et al. (1995) evaluated two EIAs (ProSpecT, Alexon Inc.; Color Vue, Serady), MERIFLUOR IF (Meridian Diagnostics) and AF staining to detect Cryptosporidium in faeces during a waterborne outbreak. All of the methods showed a high level of sensitivity (94-96%), specificity (98.6-100%) and predictive values (96-100%), but due to convenience criteria such as cost, ease of use and hands-on time, the IF assay was preferred (Kehl et al., 1995). Graczyk et al. (1996) studied the sensitivities and specificities of the ProSpecT EIA, and two IF tests (MERIFLUOR, Meridian Diagnostics Inc. and Hydrofluor, Ensys Inc.) with different species of Cryptosporidium as until this point only C. parvum had been used to evaluate the kits. The two IF assays had much superior sensitivities (only C. baileyi and five of seven C. spp from lizards gave negative results) than the EIA when tested with different species, although all the tests were positive with the C. parvum samples. Ignatius et
al. (1997) compared three EIAs (ProSpecT microplate assay, Alexon; Cryptosporidium Antigen EIA, Novum; Cryptosporidium Antigen Test, Merlin) with IF (Monofluo, Diagnostics Pasteur) and AF staining for samples containing low numbers of oocysts. They concluded that the sensitivity of the Novum EIA was significantly worse than the other methods in the presence of higher numbers of oocysts and the Merlin EIA sensitivity was significantly worse than the ProSpecT, IF and AF methods at low numbers of oocysts. In addition to three IF stains, Garcia and Shimizu (1997) also evaluated two EIAs, Meridian Premier (Meridian Diagnostics Inc.) and ProSpecT Microplate Assay (Alexon Inc.). In this study the authors found the EIAs to perform within the sensitivity and specificity stated by the manufacturers (91% and 99% respectively for Meridian and 97% and 100% respectively for Alexon) with only two false negatives for the ProSpecT assay and one for the Premier test which were positive by the three IF stains (Garcia and Shimizu, 1997).

The ProSpecT Rapid cartridge based EIA was evaluated by Parisi and Tierno (1995) and found to have a sensitivity of 100% and specificity of 98.5% when compared with AF staining. Chan et al. (2000) evaluated the cartridge based immunoassay ColorPAC Rapid (Becton Dickinson), which had a sensitivity of 100% and specificity of 98.7%. The false positives that were obtained with this kit could not be confirmed by trichrome, iron-haematoxylin or AF staining or by the ProSpecT microplate assay (Alexon-Trend Inc.), suggesting the results could either be true false positives or the sensitivity of the ColorPAC assay may be slightly better than these other tests (Chan et al., 2000). Garcia and Shimizu (2000) also evaluated the ColorPAC Rapid Assay (Becton Dickinson) and compared it with the ProSpecT Rapid cartridge based assay (Alexon-Trend Inc.) The ColorPAC assay was be slightly better than the ProSpecT Rapid test but neither had 100% sensitivity as confirmed by IF (Garcia and Shimizu, 2000). The ColorPAC Rapid was also compared by Katanik et al. (2001) but this time with the ProSpecT microplate assay (Alexon-Trend Inc.). Although both assays were found to demonstrate sensitivities of 100%, the cartridge based ColorPAC Rapid Assay (Becton Dickinson) had a specificity of 99.5% and was preferable based on convenience criteria than the microplate ProSpecT assay (Alexon-Trend Inc.), which had a specificity of 98.6%. The authors suggested that the three false positive results for the ProSpecT kit were possibly due to a cross-reaction in stools containing a large amount of blood (Katanik et al., 2001), although no cross reaction was seen with the
same antibodies used in the ProSpecT Rapid assay when tested against various parasites and human cells (Garcia and Shimizu, 2000). However, despite the 100% sensitivity of the ColorPAC Rapid and ProSpecT microplate assays, the samples were only screened by microscopy if there was a discrepancy between two kits, which means that if both kits gave a false positive result then it was counted as a Cryptosporidium positive sample (Katanik et al., 2001).

An other immunoassay cartridge (Triage Micro Parasite Panel®, Biosite Diagnostics Inc.) was evaluated in two studies and found to have sensitivities of 100% and 100% and specificities of 99.8% and 99.5% when compared with AF microscopy (Sharp et al., 2001; Garcia et al., 2000). However, Garcia et al. (2000) used IF microscopy to confirm the false positives produced by the Triage® panel as true, which suggests that the Triage® cartridge is more sensitive than the AF microscopy. As not all the samples were tested by IF, the sensitivity and specificity of this method cannot be compared with the Triage® assay. Bialek et al. (2002) found the Optimum T® EIA (Merlin) with uncentrated faeces to be equally as sensitive as the Crypto-Cel IF test (Cellabs Diagnostics) with ether sedimentation concentrated faeces. Diarrhoeic human faecal samples and known positive calf samples were used in that study, but the threshold of detection was not determined. Johnston et al. (2003) compared the immunochromatographic assay ImmunoCard STAT (Meridian Bioscience Inc.), the ProSpecT microplate assay (Alexon-Trend Inc.), the MERIFLUOR immunofluorescence stain (Meridian Biosciences) and an AF stain to detect Cryptosporidium in 246 human faecal samples. Using the IF as a gold standard the sensitivities and specificities of the ImmunoCard STAT, ProSpecT and AF respectively were 70.3% and 99.5%; 67.6% and 99.0%; 78.4% and 100%, suggesting that the microscopic methods were superior to the immunoassays, which struggled to detect samples with low parasite numbers (Johnston et al., 2003). Garcia et al. (2003) also evaluated the ImmunoCard STAT assay on human faecal samples that had been tested by trichrome, iron-haematoxylin, AF or IF and found the sensitivity, specificity, positive predictive value and negative predictive value of the ImmunoCard STAT (98.8%, 100%, 100% and 99.7% respectively) to be higher than Johnston et al. (2003).
There is an increased risk of obtaining false positives with non-microscopic immunoassays, as the organism of interest is not actually seen and a positive signal could be the result of cross-reactivity with something else in the sample (Doing et al., 1999; Miller et al., 1999; Anon., 2004; Anon., 2002a). However, if the sensitivity and threshold of detection of the microscopic method is not sufficient to detect low-level infections, then false negatives could occur. This may explain the result of a recent study in Brazil, where only three of the seven samples positive by the ProSpecT microplate assay (Alexon-Trend) were also positive by five low sensitivity microscopic techniques (Silva et al., 2003). The use of an EIA with a high sensitivity may be warranted when a large number of samples must be tested, e.g. in an outbreak situation or epidemiological study. Further tests, such as microscopic examination and molecular characterisation, should identify any false positives resulting from initial screening with a method of low specificity, however false negatives from a method of low sensitivity would be missed.

1.6.4 – Molecular Detection Methods
A number of different molecular methods have been developed that can detect the presence of Cryptosporidium (Wiedenmann et al., 1998; Xiao and Ryan, 2004). The majority of these methods involve the amplification of Cryptosporidium nucleic acid, usually by polymerase chain reaction (PCR), to a level that can be detected. Due to this amplification process, PCR-based methods can often detect much lower parasite numbers than the more traditional microscopic techniques. A few of these protocols have got exceptional thresholds of detection and can even detect a single oocyst (Wiedenmann et al., 1998). Therefore, even when low numbers of oocysts are present, the sensitivity of the PCR can be very high. In addition to the high sensitivity, the primers that are used in these assays, which can be genus or species specific, reduce the chance of getting false positive samples, so with well-designed primers the specificity is also high.

Although a PCR may have a high sensitivity and specificity even when low numbers of oocysts are present, there are factors associated with testing both environmental and faecal samples that can seriously reduce the performance of a PCR detection method. The initial factor influencing the use of PCR is releasing the DNA from the oocysts that are naturally tough and resistant to many chemical and environmental
exposures. There is currently no standardised method for this and most researchers use a slightly different protocol (discussed further in Chapter 4 – Maximising the recovery of DNA for molecular characterisation from oocysts of Cryptosporidium).

In addition to the inadequate recovery of DNA from oocysts, the assay result can be affected by the many PCR inhibitory substances present in clinical and environmental samples (Abu Al-Soud and Radstrom, 1998; Wilson, 1997). These substances include bile salts and complex polysaccharides in faeces (Lantz et al., 1997; Monteiro et al., 1997), phenolic compounds, humic acids, heavy metals and small solutes in environmental samples (Tebbe and Vahjen, 1993; Tsai and Olson, 1992; Sluter et al., 1997) and their removal is vital prior to detection. A further disadvantage to molecular detection is the high cost that can be involved and access to specialist equipment, although more laboratories are being equipped with this type of technology. Until cost effective standardised procedures are accepted for the optimum extraction of DNA from oocysts and purification to remove inhibitors, these molecular methods cannot be used in place of microscopic screening.

1.7 – Molecular Characterisation of Cryptosporidium spp.

Whilst molecular methods are not currently suitable for the routine detection of Cryptosporidium, they are useful in the characterisation of detected populations that are often morphologically identical. With the information that these tools can provide, not only can the population genetics and taxonomy of Cryptosporidium be studied, but also which species, genotypes or subtypes are of public health importance and the differences in their transmission and epidemiology (e.g. geographic, temporal, zoonotic potential)(Xiao and Ryan, 2004).

1.7.1 – Evaluating Molecular Characterisation Methods

Performance and convenience criteria can also be used to evaluate these molecular methods used for characterisation of recovered organisms. In addition to the sensitivity and specificity, performance criteria for typing systems have been previously published and include typability, reproducibility and discriminatory power (Struelens et al., 1996). Although these criteria were initially published for use with bacterial typing systems, the same principles apply for Cryptosporidium typing.
The typability (T) is the proportion of strains than can be assigned a specific pattern or marker (type) by the method (Struelens et al., 1996). However, negative results must be treated with caution as they may not reflect the typability of the method, but the sensitivity, which as discussed above can vary greatly with molecular methods due to numerous extrinsic factors. The reproducibility (R) of a typing method is the ability to assign the same type to a strain on repeat testing and includes in vitro, in vivo and inter-centre reproducibility (Hunter, 1991). In vitro reproducibility reflects the reliability of the method and involves typing the strains by the same method a few months apart. In vivo reproducibility reflects the stability of the marker used in the typing method when passaged through hosts between testing and inter-centre reproducibility demonstrates that independent users can obtain the same type results when using the methods (Hunter, 1991). The discriminatory power (D) of a typing method is the ability to distinguish between two strains randomly chosen from a population of unrelated strains (Hunter and Gaston, 1988). In order to compare these performance criteria for different methods in an unbiased and precise manner more, than 100 epidemiologically unrelated strains must be used in the evaluation of each method (Struelens et al., 1996).

1.7.2 – Methods and Targets

The vast majority of the molecular methods currently used to differentiate between Cryptosporidium species or genotypes are based on PCR followed by restriction fragment length polymorphism (RFLP) analysis or DNA sequencing (Jiang and Xiao, 2003). Higher resolution subtyping methods that distinguish between strains within the same species are starting to become more commonly used now and are described in much greater detail below (see Chapter 2 – Review of subtyping methods and implications for the investigation of environmental transmission).

Many of the PCR-RFLP or sequencing methods were developed to differentiate between C. parvum and C. hominis, due to their predominance in human infections and several loci have consistently demonstrated polymorphisms between these two species (Table 1.2). However, whilst all of these loci have been used to differentiate between C. parvum and C. hominis, there are other species of public health importance (see 1.2 – Taxonomic Classification) that also need to be detected and
<table>
<thead>
<tr>
<th>Locus Product</th>
<th>References</th>
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<tr>
<td>Small-subunit (SSU) rRNA</td>
<td>Awad-El-Kariem et al., 1994; Carraway et al., 1996; Leng et al., 1996b; Morgan et al., 1997; Morgan et al., 1998; Xiao et al., 1998; Kimbell III et al., 1999; Ong et al., 1999; Xiao et al., 1999a; Xiao et al., 1999b; Xiao et al., 2000a; Sturbaum et al., 2001; Xiao et al., 2001a; Xiao et al., 2001c; Ward et al., 2002; Nichols et al., 2003</td>
</tr>
<tr>
<td>Cryptosporidium oocyst wall protein (COWP)</td>
<td>Spano et al., 1997; Spano et al., 1998a; Widmer et al., 1998b; Homan et al., 1999; McLauchlin et al., 1999; McLauchlin et al., 2000; Pedraza-Diaz et al., 2001b; Amar et al., 2004</td>
</tr>
<tr>
<td>Thrombospondin-related adhesive protein 1</td>
<td>Peng et al., 1997; Spano et al., 1998a; Spano et al., 1998b; Sulaiman et al., 1998; McLauchlin et al., 1999; Elwin et al., 2001</td>
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<tr>
<td>(TRAP-C1)</td>
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<tr>
<td>Thrombospondin-related adhesive protein 2</td>
<td>Peng et al., 1997; Sulaiman et al., 1998; Ong et al., 1999; Elwin et al., 2001</td>
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<tr>
<td>(TRAP-C2)</td>
<td></td>
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<tr>
<td>β-tubulin</td>
<td>Widmer et al., 1998a; Caccio et al., 1999; Rochelle et al., 1999b; Sulaiman et al., 1999a</td>
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<tr>
<td>Internal transcribed spacer 1 (ITS1)</td>
<td>Carraway et al., 1996; Spano et al., 1998a; Morgan et al., 1999; Ong et al., 1999</td>
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<tr>
<td>Polythreonine repeat (Poly-T)</td>
<td>Carraway et al., 1997; Spano et al., 1998a; Widmer et al., 1998b</td>
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<tr>
<td>Dihydrofolate reductase (DHFR)</td>
<td>Vasquez et al., 1996; Gibbons et al., 1998b; Ong et al., 1999</td>
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<tr>
<td>Ribonucleotide reductase (RNR)</td>
<td>Spano et al., 1998a; Widmer et al., 1998b</td>
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<td>Acetyl-CoA synthetase</td>
<td>Morgan et al., 1998; Morgan et al., 2000</td>
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<tr>
<td>70kDa heat shock protein (hsp70)</td>
<td>Morgan et al., 2000; Gobet and Toze, 2001</td>
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<tr>
<td>Surface protein p23</td>
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<td>60 kDa glycoprotein (gp60)</td>
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<tr>
<td>900 kDa glycoprotein (gp900)</td>
<td>Sturbaum et al., 2003</td>
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<tr>
<td>Actin</td>
<td>Sulaiman et al., 2002</td>
</tr>
<tr>
<td>Undefined genomic sequences</td>
<td>Bonnin et al., 1996; Morgan et al., 1997; Guyot et al., 2002</td>
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characterised. It is not clear from the development of most of these methods whether the complete range of Cryptosporidium species can be differentiated. A recent study compared 10 commonly used protocols to amplify and distinguish between some of the species that are known to infect humans (C. hominis, C. parvum, C. meleagrisidis, C. felis, C. canis, C. muris and C. suis)(Jiang and Xiao, 2003). The PCR methods that they chose targeted the genes coding for COWP (Pedraza-Diaz et al., 2001b; Homan et al., 1999), hsp70 (Gobet and Toze, 2001), TRAP-C1 (Spano et al., 1998b), TRAP-
C2 (Elwin et al., 2001), DHFR (Gibbons et al., 1998b), SSU rRNA (Sturbaum et al., 2001; Ward et al., 2002; Xiao et al., 2001a) and an undefined genomic region (Guyot et al., 2002). All of these methods (except for Ward et al. (2002) who used DNA sequencing) used RFLP to differentiate between species and all positive products from the COWP, TRAP-C2 and DHFR loci were additionally sequenced. The three SSU rRNA gene-based tools amplified DNA from all species, but only the Xiao and Ward methods could differentiate between all of the species (although it was difficult to sequence C. felis due to heterogeneous copies of the gene). The TRAP-C2, COWP, DHFR and Guyot protocols only amplified DNA from C. hominis, C. parvum and C. meleagris and all three could be differentiated at the COWP, DHFR genes and the undefined genomic locus, but the TRAP-C2 method could only distinguish C. parvum from the other two species. The TRAP-C1 and hsp70 methods produced non-specific amplification and were not reliable in this study to detect or differentiate any of the species. Therefore, the results of this study demonstrated that of the loci tested only the SSU rRNA gene was capable of detecting and differentiating all of the human pathogenic Cryptosporidium species. However, the COWP, DHFR genes and genomic sequence can be used to detect mixed infections with C. hominis, C. parvum or C. meleagris in samples that test positive for C. felis, C. canis, C. muris or C. suis by one of the SSU rRNA gene tools (Jiang and Xiao, 2003).

The threshold of detection and primer cross-reactivity for 11 of the species/geneotype differentiation methods were examined by Sulaiman et al. (1999b). The protocols that they compared targeted genes coding for SSU rRNA (Awad-El-Kariem et al., 1994; Leng et al., 1996b; Xiao et al., 1999a), ITS1 of rRNA (Carraway et al., 1996), COWP (Spano et al., 1997), DHFR (Gibbons et al., 1998b), Poly-T (Carraway et al., 1997), TRAP-C1 (Spano et al., 1998b), TRAP-C2 (Sulaiman et al., 1998) and two undefined genomic regions (Bonnin et al., 1996; Morgan et al., 1997). With the exception of Bonnin’s genomic region (which only amplified C. parvum), all of the other methods amplified DNA from both C. parvum and C. hominis. In addition, the three SSU rRNA and the COWP gene-based tools amplified DNA from C. muris and C. serpentis. However, the methods by Awad-El-Kariem and Leng targeting the SSU rRNA gene also amplified DNA from 2 species of Eimeria that were tested, indicating that the primers are not specific for the genus Cryptosporidium. The threshold of detection was 100 oocysts for the Bonnin and ITS1 methods, 20 oocysts for the
Morgan and TRAP-C1 protocols, 10 oocysts for Poly-T and COWP, 5 oocysts for TRAP-C2 and a single oocyst for the Xiao SSU rRNA and DHFR techniques. The other two SSU rRNA gene-based methods were not assessed for their threshold, as they were not *Cryptosporidium* specific. The two methods that could detect a single oocyst in this study were both nested PCRs, explaining the lower detection limits than the others that were all based on a single PCR (Sulaiman *et al.*, 1999b).

From these two comparative studies (Sulaiman *et al.*, 1999b; Jiang and Xiao, 2003) it is clear that with the correct primers, the SSU rRNA gene clearly provides a efficient target for a species/genotype differentiation tool and by using a nested PCR for the amplification the threshold of detection is very low. The only other gene target in these studies that was amplified with any other *Cryptosporidium* species was the COWP gene using the method of Spano *et al.* (1997) which in the original paper also differentiated *C. wrairi*. Xiao *et al.* (2000b) identified extensive sequence variation in the COWP gene between different species and genotypes of *Cryptosporidium*, resulting in a number of RFLP patterns, using the primers described by Spano *et al.* (1997), but the efficiency of amplification with these primers was poor with some species. A more recent publication also used the COWP gene as a target and efficiently amplifies DNA from at least 10 of the different *Cryptosporidium* species using a nested PCR with 23 different primers (6 as external forward, 6 as external reverse, 5 as internal forward and 6 as internal reverse)(Amar *et al.*, 2004). However, this method could not differentiate between all of the species as although unique RFLP patterns are obtained for *C. hominis*, *C. wrairi*, *C. parvum*, *C. canis* and *C. baileyi*, both *C. felis* and *C. meleagrisidis* produce the same pattern as did *C. muris*, *C. andersoni* and *C. serpentis*. This target does potentially provide a non-SSU rRNA gene-based method of distinguishing between several different species or genotypes of *Cryptosporidium*, but currently the SSU rRNA methods are superior. No comprehensive evaluations of the typability, reproducibility and discriminatory power have been carried out for any of these typing methods and loci.

**1.8 – Public Health Issues**
The use of molecular methods in the study of epidemiology of *Cryptosporidium* has advanced our knowledge of the geographical and temporal differences in the transmission of *C. parvum* and *C. hominis*, and given us a greater appreciation of the
public health importance of other species, “host-adapted” genotypes and subtypes (Xiao and Ryan, 2004). To prevent Cryptosporidium infections, the sources and routes of transmission must be determined so that interventions can be put in place. Molecular epidemiology will help to provide us with this information required to develop strategies and make important public health decisions.

1.8.1 – Sporadic Cryptosporidiosis

Cases of human cryptosporidiosis can be classified as either sporadic or outbreak-related. Sporadic cases are more common than outbreak-related cryptosporidiosis (Evans et al., 1998; Djuretic et al., 1996), but it is often hard to identify the source or route of transmission involved with sporadic disease.

Case-control studies have been undertaken to determine risk factors associated with sporadic cryptosporidiosis (Goh et al., 2004; Robertson et al., 2002; Hunter et al., 2004b; Khalakdina et al., 2003). The identification of risk factors can lead to control measures, such as good hygiene practices, particularly after contact with animals, diarrhoeic people, and before eating. Robertson et al. (2002) found that swimming pools and direct contact with people with diarrhoea were major risk factors for sporadic cryptosporidiosis in two Australian cities and did not find any significant risk associated with the consumption of tap water. Interestingly, the consumption of raw vegetables was identified as protective against sporadic infection (Robertson et al., 2002). Khalakdina et al. (2003) also supported the lack of association between drinking tap water and sporadic infections in a small study in San Francisco.

In contrast to these studies, Goh et al. (2004) undertook a study in North Cumbria from 1996-2000 and identified a significant dose-response relationship between consuming cold unboiled municipal tap water and an increased risk of contracting sporadic cryptosporidiosis. This relationship was significant with the amount drunk at home, but not at work, nursery or school. A subsequent publication demonstrated that the introduction of membrane filtration at two water treatment works supplying two thirds of this population was highly effective at reducing the risk of sporadic cryptosporidiosis during 2000 to 2002 (Goh et al., 2005). Other risk factors for sporadic disease that were identified included contact with farms, which increased with short farm visits or the frequency of visits and direct contact with animals (Goh
et al., 2004). However, they also identified a significantly lower risk of sporadic
disease associated with consuming lettuce, tomatoes, mixed salad and cream (Goh et
al., 2004). The species of Cryptosporidium causing infection was only determined in
37.5% (57/152) of the enrolled cases, meaning that these risk factors cannot be
determined for individual species. However, of the 67 cases that were included in
species differentiation, 83.6% (56/67) were C. parvum, 1.5% (1/67) was C. hominis
and 14.9% (10/67) could not be typed (Goh et al., 2004).

The importance of using molecular species differentiation in epidemiological studies
was highlighted in a large case-control study undertaken to investigate the aetiology
and epidemiology of sporadic cryptosporidiosis in the northwest of England and in
Wales (Hunter et al., 2004b). When all of the cases were grouped together without
species differentiation the risk factors of sporadic cryptosporidiosis were travel
outside the UK, contact with a diarrhoeic person or cattle and toileting contact with a
child under 5 years old. In addition, the number of glasses of unboiled tap water
consumed was statistically significant, but there was no association with drinking tap
water in general or the use of water filters. The authors felt that the risk identified
with the number of glasses consumed could be linked to the person drinking more
during illness or recall bias. Once again, protective factors appeared to be eating raw
vegetables and tomatoes, as well as washing fruit and vegetables before consumption
and strangely, eating ice cream. When separated into different species, the risk
factors associated with a C. hominis infection were travel outside the UK and contact
with dirty nappies, with negative association identified with sitting or sleeping on the
ground outside, eating fresh fruit and the likelihood of washing fruit and vegetables
prior to eating (Hunter et al., 2004b). Risk factors with C. parvum infections were
direct contact with farm animals and negative associations were eating raw tomatoes
and vegetables (Hunter et al., 2004b).

Although the clinical symptoms for both species had no significant differences in this
report (Hunter et al., 2004b), a follow-up study identified that the medium-term health
effects of C. hominis infections included non-gastrointestinal symptoms that were not
identified with C. parvum infections (Hunter et al., 2004a) (see 1.4 – Epidemiology,
Clinical Features & Treatment).
1.8.2 – Outbreaks and Incidents Associated with Environmental Contamination

A number of routes of transmission have been associated with outbreaks including municipal and private drinking water supplies, swimming pools and other recreational waters, consumption of contaminated food products, contact with farm animals and contact with infected humans, including transmission in hospital and child-care centres (Casemore et al., 1997; Rose et al., 1997; Fayer et al., 2000a; Meinhardt et al., 1996; Rose et al., 2002). Preventative measures for the spread of cryptosporidiosis include water treatment and monitoring, educating parents to reduce faecal accidents in pools and good hygiene practices during the production of food and drink. However, breaches of these control measures do occur and large-scale outbreaks can happen.

Whilst foodborne cryptosporidiosis does occur, it is the waterborne nature of this parasite that has been most studied due to the sheer scale of some of the reported outbreaks. Some of the larger outbreaks in the USA, Canada and Japan have affected an estimated 15,000, 14,500 and >9000 people, respectively, but the largest waterborne outbreak recorded for any pathogen was in Milwaukee, where an estimated 403,000 individuals were ill due to Cryptosporidium infections (Mackenzie et al., 1994; Fayer, 2004; Fayer et al., 2000a).

The occurrence of Cryptosporidium spp. has been demonstrated in various types of water, including waste (26-100%), surface (4-100%), drinking (4-40%) and ground water (10-22%) (Rose et al., 2002; Smith and Rose, 1998; Rose et al., 1997; Hancock et al., 1998). The number of Cryptosporidium oocysts in these water systems can vary greatly and concentrations (in oocysts per 100 litres) have been reported from 3-3.8x10^5 in wastewater, 0.5-1.36x10^2 in drinking water and 0.12-4.8x10^4 in surface waters (Smith and Rose, 1998; Rose et al., 2002). The environmental contamination of these waters can be from either point or diffuse sources (Smith, 1998). The point sources are particular places where the contamination can be traced back to, such as a sewage treatment works, an abattoir or a farmyard. Whereas the diffuse sources describe contamination that cannot necessarily be traced back to a particular point such as agricultural animal waste that is spread on fields as fertiliser or faeces from wildlife reservoirs, which can run-off into surface waters and drinking water reservoirs following rainfall events (Kistemann et al., 2002). The concentration of
oocysts present in samples from point sources are often much higher than samples contaminated by diffuse sources (Smith, 1998), but as the source of contamination is often not known at the beginning of an investigation, methods of recovering and detecting the low numbers of oocysts are required (see 1.5 – Recovery of Cryptosporidium spp. and 1.6 – Detection of Cryptosporidium spp.).

The detection of Cryptosporidium in environmental samples only tells us that there is contamination but does not normally indicate the source. However, molecular characterisation is required if public health decisions are to be made about the presence of the oocysts. Factors affecting these public health decisions include, whether the oocysts are viable, how infectious they are and most importantly, are the species infectious to humans. Genus specific techniques cannot answer this, as not all species of Cryptosporidium that are morphologically indistinguishable from C. parvum or C. hominis are infectious to humans.

One source of environmental contamination is the widespread infection of Cryptosporidium in wild animals (over 155 different species of mammal alone), including many “host-adapted” genotypes and species that may or may not be infectious to humans, but the extent of this contamination is still unknown (Fayer, 2004; Xiao and Ryan, 2004). The “host-adaptive” nature of most Cryptosporidium spp. suggests that most are unlikely to have high infectivity to humans, and most of those found in reptiles and wild mammals have never been detected in humans (Xiao et al., 2004). Therefore, using molecular tools, it is possible for us to assess the human infection potential of oocysts recovered from the environment and make accurate risk assessments of contamination in water (Xiao et al., 2004). Xiao et al. (2000a) examined storm water samples from a stream that contributes to the New York City water supply and identified 12 wildlife genotypes of Cryptosporidium, none of which have ever been detected in humans. A second study by Xiao et al. (2001c) analysed raw surface water and found four different Cryptosporidium spp., two of which were human pathogens, and from wastewater they identified seven different species, six of which are known to be pathogenic to humans. Molecular methods have implications in water monitoring and outbreak investigations through tracing sources of contamination and linking (or excluding) cases of human illness to outbreaks.
1.8.3 – Drinking Water Regulations

Due to the potential for large-scale waterborne outbreaks of cryptosporidiosis to occur there are guidelines and regulations that aim to control the quality of drinking water. The drinking water regulations vary between countries and use different approaches in Cryptosporidium monitoring and legislation (Percival et al., 2004).

In the United States, the US Environmental Protection Agency (EPA) establishes national drinking water regulations under the Safe Drinking Water Act (SDWA). The “Enhanced Surface Water Treatment Rules” (ESWTR) were introduced to control Cryptosporidium in public water systems that use surface or ground water under the direct influence of surface water. The “Interim ESWTR” and “Long Term 1 ESWTR” were introduced to control systems that serve populations of >10,000 and <10,000, respectively. The key provisions of these rules include a Maximum Contaminant Level Goal (MCLG) of zero and at least 2-log (99%) removal or inactivation of Cryptosporidium in systems using filtration only (USEPA, 1998; USEPA, 2002). The methods used to monitor for the occurrence of Cryptosporidium in raw surface water are described in Method 1622: Cryptosporidium in Water by Filtration/IMS/FA (USEPA, 2001).

In England and Wales, water undertakers must follow the Water Supply (Water Quality) Regulations 2000 and the Water Supply (Water Quality) Regulations 2001 (Wales) respectively. These regulations state that water undertakers supplying from English or Welsh sources must carry out a risk assessment to identify if there is any significant risk from Cryptosporidium (DWI, 1999) and the results submitted to the Secretary of State or the National Assembly for Wales. If the risk assessment identifies a significant risk then the water undertaker must use a process for treating water, which secures that the average number of Cryptosporidium oocysts per 10 litres of water is less than one. If no risk is identified during the risk assessment then the water undertaker does not have to comply with this treatment standard. In the UK, legislation does not allow for the inactivation of Cryptosporidium with the use of treatments such as UV radiation, as following this form of treatment the oocysts will still be detected by the continuous monitoring and the water company would therefore be liable to prosecution if a number greater than 1 oocyst per 10 litres were detected. Water undertakers required to meet the treatment standard must monitor the
effectiveness of the treatment by continuously sampling at least 40 litres per hour and determining the concentration of oocysts by following the standard operating procedures (DWI, 2005).

In Australia, the government has not imposed mandatory drinking water regulations as they felt the cost-health benefits of such systems are disproportionate and the monitoring is not based on risk to public health, such as viability and infectivity (Fairley et al., 1999a; Fairley et al., 1999b). In addition to the high cost, by the time that monitoring has detected *Cryptosporidium*, the contaminated water has already been supplied to the consumer. Instead, they produced guidelines that provide a framework for the good management of drinking water (Australian Government, 2004). The framework is based upon risk-analysis from catchment-to-tap and recommends the use of multiple barriers to minimise the risk of *Cryptosporidium* contamination of drinking water. There is no guideline value for the presence of *Cryptosporidium* and routine monitoring is not recommended, although investigative testing in response to events that increase the risk of contamination is recommended. The principle reason that the Australian guidelines state for not setting a value for the level of *Cryptosporidium* in drinking water is due to the lack of methodology to identify human infectious strains. This approach is in line with the current WHO Guidelines for Drinking Water Quality, which are also a framework for safe drinking water from source-to-consumer based on risk management strategies (World Health Organisation, 2004).

Both the US and the UK water regulations monitor using genus specific methods and can therefore not make any accurate public health based decisions based on these results. More discriminatory methods are required to assess the risks to humans from any *Cryptosporidium* present in the water supplies.

1.9 – Aims and Objectives of the Work Undertaken

Recent findings in *Cryptosporidium* research have shown that there is much genetic diversity within the genus, with the identification of multiple species and subtypes. Due to these advances in the taxonomy and systematics, the public health significance of *Cryptosporidium* is also becoming clearer. It is now known that certain species, host-adapted genotypes and subtypes are anthroponotic, whereas others have never
been found in humans. That does not mean that these species will never be found in people, but the large number of human derived *Cryptosporidium* that have so far been characterised by molecular methods in different studies worldwide suggest that they are not of major public health significance. During surveys, surveillance and regulatory monitoring, oocysts are often detected in environmental waters that may be used for drinking or for recreational purposes, but with the genus specific methods that are often used, the risk to human health cannot be determined. Wildlife and agricultural animals act as reservoirs for *Cryptosporidium*, but the extent of environmental contamination from these sources are still unclear. All of these uncertainties can be addressed using higher resolution molecular characterisation. Before these methods can be used to characterise *Cryptosporidium* the oocysts must be detected, recovered and the DNA extracted. The number of *Cryptosporidium* oocysts in environmental water and asymptomatic or post-symptomatic faecal samples can be low. Efficient methods are available for the concentration and detection of oocysts from water samples, but very high thresholds of detection have been found with faecal samples so more sensitive methods are required for screening these samples. Following the detection and recovery of oocysts, DNA must be extracted from the environmentally tough oocysts and particularly when low numbers are present, the optimal method is required to release the maximum amount of nucleic acid. However, most researchers appear to use slightly different approaches, as there is no standardised method to obtain the optimum yield of DNA.

Aim of this Thesis: To elucidate the public health significance of environmental *Cryptosporidium* spp. by developing, optimising and applying methods for the detection, recovery and characterisation of low numbers of oocysts.

Objective 1: To identify, through a review of the literature, the high resolution characterisation methods enabling discrimination between isolates of the same *Cryptosporidium* species and how these methods may be used to study the molecular epidemiology and environmental transmission into human populations (Chapter 2).
Objective 2: To develop and evaluate an improved method for detecting low numbers of Cryptosporidium oocysts in faeces when screening the large numbers of samples collected during studies (Chapter 3).

Objective 3: To compare a number of approaches of oocyst disruption and identify the optimal method for the maximum release of quality DNA, particularly from low numbers of oocysts, prior to characterisation by molecular methods (Chapter 4).

Objective 4: Apply the developed and optimised methods to investigate the occurrence and variation of Cryptosporidium species and subtypes in surface water, non-clinical faecal samples from farmed and wild animals and clinical samples from humans and cattle within the Caldew water catchment during and following the FMD restocking period (Chapter 5).
Chapter 2 – Review of subtyping methods and implications for the investigation of environmental transmission

2.1 – Introduction

*Cryptosporidium* is most often differentiated to the species level using molecular techniques, such as PCR-RFLP of various genetic loci (see 1.7 – Molecular characterisation of *Cryptosporidium* spp.), which provides some information on the source of infection (e.g. the detection of *C. hominis* indicates a high likelihood of a human source). However, with the application of recently developed subtyping techniques, it is possible to differentiate isolates further to better understand the population structure (Mallon *et al.*, 2003a; Mallon *et al.*, 2003b) and the relationships between isolates and the infections they cause. Subtyping information is also useful as a tool for more accurately tracing the sources of human and animal infections and environmental contamination. Additional benefits of these molecular techniques potentially include the identification of virulence markers and thus virulent strains of *Cryptosporidium*. With access to this kind of data more effective control measures can be developed to protect the public from *Cryptosporidium* infections. However, this is a rapidly developing field and it is helpful to identify the state of the technology for subtyping isolates from environmental samples by means of a comprehensive review.

Systematic principles were used in developing the search strategy for the review. The strategy avoids selection bias by using specific search terms with inclusion criteria and attempts to retrieve all the publications that fit these criteria through electronic and “grey literature” (i.e. that not published in peer review journals) searches and manually searching through each paper to identify those papers not retrieved previously (Knipschild, 1994).
Objective 1: To identify, through a review of the literature, the high-resolution characterisation methods enabling discrimination between isolates of the same Cryptosporidium species and how these methods may be used to study the molecular epidemiology and environmental transmission into human populations.

Objective 1a: To develop an effective search strategy to identify all publications (peer-reviewed and grey literature) describing the development and application of subtyping methods.

Objective 1b: To collate and review the publications returned by the search strategy and identify which publications meet the inclusion criteria.

Objective 1c: To analyse the information from within the included publications in order to identify and compare the different methods of subtyping Cryptosporidium spp.

Objective 1d: To explore the potential for using subtyping methods in characterising Cryptosporidium from environmental samples and their usefulness in public health situations.

2.2 – Search Strategy and Method Evaluation
Electronic searches of 21 online databases that cover a wide range of disciplines were undertaken, including those containing unpublished grey literature (System for Information on Grey Literature in Europe, Conference Papers Index)(Table 2.1). Since there is a close connection between the ecology of Cryptosporidium, agriculture and the water industry, the search strategy included agricultural, environmental and engineering databases in addition to biological and medical databases. The electronic searches were not limited by either document type or language and included the full time-span covered by each database from its inception up to the end of December 2003.
Table 2.1: The databases used to search for literature on subtyping *Cryptosporidium*

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<th>Gateway</th>
<th>Time Period Searched</th>
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<td>Ovid</td>
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<tr>
<td>Medline</td>
<td>Ovid</td>
<td>1966-2003</td>
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<tr>
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<td>Ovid</td>
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<td>AGRICOLA</td>
<td>CSA</td>
<td>1970-2003</td>
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<tr>
<td>Aquatile</td>
<td>CSA</td>
<td>1960-2003</td>
</tr>
<tr>
<td>Aquatic Sciences &amp; Fisheries Abstracts (ASFA)</td>
<td>CSA</td>
<td>1978-2003</td>
</tr>
<tr>
<td>Biological Sciences</td>
<td>CSA</td>
<td>1982-2003</td>
</tr>
<tr>
<td>Biology Digest</td>
<td>CSA</td>
<td>1989-2003</td>
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<td>Biotechnology &amp; Bioengineering Abstracts</td>
<td>CSA</td>
<td>1982-2003</td>
</tr>
<tr>
<td>Conference Papers Index</td>
<td>CSA</td>
<td>1982-2003</td>
</tr>
<tr>
<td>Environmental Sciences &amp; Pollution Management</td>
<td>CSA</td>
<td>1981-2003</td>
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<td>Oceanic Research</td>
<td>CSA</td>
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<td>Clinical Medicine</td>
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<tr>
<td>Physical, Chemical &amp; Earth Sciences</td>
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<tr>
<td>Science Citation Index Expanded</td>
<td>ISI CCC</td>
<td>1997-2003</td>
</tr>
<tr>
<td>System for Information on Grey Literature in Europe (SIGLE)</td>
<td>ISI Web of Science</td>
<td>1981-2003</td>
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<td></td>
<td>SilverPlatter</td>
<td>1980-2003</td>
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</tbody>
</table>

The search terms in the strategy were designed to cover all possibilities, including the varying terminology used by different scientific groups, common subtyping methods and national spelling differences. Thus, the search term “Cryptosporidi” and a wildcard character (*) was used to capture all records pertaining to the genus, and 20 search terms were included using the “AND” Boolean operator (Table 2.2). For this review, an “isolate” is defined as “a population of organisms recovered from a single sample and characterised to an accepted species” and “subtyping” was defined as “characterising a *Cryptosporidium* isolate to a resolution high enough for differentiation between different isolates of the same species”.

All retrieved references were entered into Reference Manager 9 for Windows (Research Information Systems), and any duplicates (identified by title, primary author and year) retrieved from different databases, deleted. The references remaining in the database were then screened and those not relevant to the subtyping of *Cryptosporidium* were discarded. The inclusion criterion for papers was the
Table 2.2: Terms used in the search strategy and their respective references

<table>
<thead>
<tr>
<th>Search Term Reference</th>
<th>Search Terms</th>
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<tr>
<td>2</td>
<td>Cryptosporidi* AND typing</td>
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<td>3</td>
<td>Cryptosporidi* AND subtyp*</td>
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<td>4</td>
<td>Cryptosporidi* AND sub-typ*</td>
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<td>5</td>
<td>Cryptosporidi* AND subgenotyp*</td>
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<td>6</td>
<td>Cryptosporidi* AND sub-genotype</td>
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<td>7</td>
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<td>12</td>
<td>Cryptosporidi* AND fingerprint*</td>
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<td>13</td>
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<td>14</td>
<td>Cryptosporidi* AND microsat*</td>
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<td>Cryptosporidi* AND micro-sat*</td>
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<td>16</td>
<td>Cryptosporidi* AND telomer*</td>
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<td>17</td>
<td>Cryptosporidi* AND characteri*</td>
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<td>18</td>
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<td>19</td>
<td>Cryptosporidi* AND DNA</td>
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<tr>
<td>20</td>
<td>Cryptosporidi* AND RNA</td>
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</table>

* - Indicates a wildcard character that will include all word ending variations

description of characterising Cryptosporidium spp. to a level that could discriminate between isolates of the same species. The references cited in each of the included publications were then hand-searched to identify any additional papers that may have been missed by the electronic searches.

As discussed previously (see 1.7.1 – Evaluating Molecular Characterisation Methods), the sensitivity and specificity are not the only criteria important in evaluating typing methods or schemes, as the user is not trying to diagnose or detect the organism, but instead characterising those present in the sample. Therefore, although it is useful to know the limit of detection for the method or scheme, the evaluation of both the performance (efficacy) and convenience (efficiency) criteria is most important (Struelens et al., 1996). In order to establish more information about the evaluation of each subtyping method used in the publications retrieved for this review, a record was made of any evidence provided in each publication of sensitivity (proportion of known positive samples to give a positive result) or typability (T), reproducibility (R) and discriminatory power (D) as defined by Struelens et al. (1996).
2.3 – Approaches and Targets Used to Subtype Cryptosporidium spp.

The initial search strategy yielded 13,652 references from the 21 databases searched (Table 2.3), of which 12,132 duplicates were identified and subsequently deleted. Following the removal of a further 1,474 papers that did not meet the inclusion criterion, 46 remained and were included in the study. No additional references were identified from the hand searching of cited references. Of the 46 papers describing the subtyping of Cryptosporidium spp., 43 described the use of PCR based methods that targeted different genetic loci or generated fingerprints using arbitrary primers, whereas the remaining three examined the electrophoretic properties of different proteins (Table 2.4). DNA sequence analysis of one or more genetic loci was the most commonly used analytical tool to subtype Cryptosporidium isolates (35/46 papers). As the polymorphic potential of various gene loci has been widely used to develop subtyping tools for Cryptosporidium, the results of this review are structured to examine the different loci and the methods that have been used to reveal the variation within species.

2.3.1 – The gp60 Locus

The most common genetic locus and method of analysis identified was DNA sequencing of a region that codes the 60-kDa glycoprotein (gp60; synonymous with gp15 or gp15/45/60) (Table 2.4). Of the 13 papers using this method (Table 2.4 and 2.5), all subtyped isolates recovered from human or animal faeces but four also described the subtyping of isolates recovered from environmental samples (Glaberman et al., 2001a; Glaberman et al., 2002; Peng et al., 2003b; Zhou et al., 2003). Strong et al. (2000) first applied the method and identified four alleles (different forms of the same gene) from within 16 C. hominis samples (Ia, Ib, Ic and Id) and one allele from 13 C. parvum isolates (allele II). So far, six major C. hominis, five C. parvum and six C. meleagris alleles have been proposed, although allele Ic has since been re-classified as a C. parvum allele (Alves et al., 2003b) and further groups may yet be identified (Table 2.5). Within the alleles, further sequence variations that discriminate between isolates have been described (Glaberman et al., 2001a; Glaberman et al., 2002; Leav et al., 2002; Sulaiman et al., 2001; Peng et al., 2003a; Peng et al., 2003b; Peng et al., 2001; Alves et al., 2003b; Wu et al., 2003; Zhou et al., 2003). Leav et al. (2002) tested the accuracy of the method to some extent by amplification and cloning of the fragments from three isolates using
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Table 2.3: The number of references retrieved from the databases with each search term.
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<th>Ingenta Online Articles</th>
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Table 2.3 cont. The number of references retrieved from the databases with each search term.
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<tr>
<td>Alizio et al., 1999; Suhirmann et al., 2001</td>
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<td>Chevalier et al., 2003; Peng et al., 2003</td>
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</tr>
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<td>Chevalier et al., 2003; Suhirmann et al., 2001; Peng et al., 2003</td>
<td>0 GS1, MS6</td>
</tr>
<tr>
<td>Di Giovanni et al., 1999; Treccevalier et al., 2003</td>
<td>0 GS1, MS6</td>
</tr>
<tr>
<td>Presto et al., 2003; Nair et al., 2003; Gibbons-Mahar et al., 2003; Cattaneo et al., 1999; Corvec et al., 2001; Gibbons-Mahar et al., 2002; Chevalier et al., 2003; Peng et al., 2003</td>
<td>0 GS1, MS6</td>
</tr>
<tr>
<td>Zhou et al., 2003</td>
<td>0 GS1, IF07 &amp; IF007</td>
</tr>
<tr>
<td>2003a; Sinigato-Pizzirane et al., 2003; Wu et al., 2003; Alves et al., 2003b; Peng et al., 2003b; Peng et al., 2003c; Peng et al., 2003d; Peng et al., 2003e; Peng et al., 2003f</td>
<td>0 GS1, IF07 &amp; IF007</td>
</tr>
<tr>
<td>Chevalier et al., 2002; Levey et al., 2002; Suhirmann et al., 2002; Chevalier et al., 2002</td>
<td>0 GS1, IF07 &amp; IF007</td>
</tr>
<tr>
<td>Suhirmann et al., 2002; Chevalier et al., 2002</td>
<td>0 GS1, IF07 &amp; IF007</td>
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</tbody>
</table>

**Table 2.4**: Methods and loci used to sub-type *Cyprisporidiuim spp.*
<table>
<thead>
<tr>
<th>Locus Type</th>
<th>Location (if applicable)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inter-simple sequence (ISS)</td>
<td></td>
<td>1.5 Upp. dna unit</td>
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<tr>
<td>SSV RNA locus in the dna unit</td>
<td></td>
<td>1.5 Upp. dna unit</td>
</tr>
<tr>
<td>Cpgo locus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLG locus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extran-chromosomal s-drna</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extran-chromosomal s-drna</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TR205-2 locus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TR205-2 locus</td>
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<td></td>
</tr>
<tr>
<td>TR205-2 locus</td>
<td></td>
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<td>TR205-2 locus</td>
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<tr>
<td>TR205-2 locus</td>
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<tr>
<td>TR205-2 locus</td>
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<td></td>
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Table 2.4 cont: Methods and Local used to Subtype C. Pyrogenium spp.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample Type</th>
<th>Year</th>
<th>Size</th>
<th>ML-PCR</th>
<th>RAPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Million et al., 2003</td>
<td>1 (R)</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fan et al., 2002</td>
<td>1 (R)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fan et al., 2002</td>
<td>1 (R)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fan et al., 2000</td>
<td>1 (R)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fan et al., 2002</td>
<td>1 (R)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fan et al., 2000</td>
<td>1 (R)</td>
<td>2</td>
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<td>Million et al., 2002</td>
<td>1 (R)</td>
<td>3</td>
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<td>Million et al., 2002</td>
<td>0</td>
<td>1</td>
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</tr>
<tr>
<td>Million et al., 2002</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4 cont.: Methods and Local used to Sustain *Cryosporium* spp.

- **AV-PCR/RAPD**
  - ML-PCR: MSS ≠ M12
  - RAPD: ML-mutant
  - ML-PCR: MSS ≠ M12
  - RAPD: ML-mutant

- **Microbial Pathogens**
  - ML-PCR: MSS ≠ M12
  - RAPD: ML-mutant

- **Fungal Size Analysis**
  - ML-PCR: MSS ≠ M12
  - RAPD: ML-mutant

- **Other Pathogens**
  - ML-PCR: MSS ≠ M12
  - RAPD: ML-mutant
Table 2.5: A description of the allele groups identified at the gp60 locus

<table>
<thead>
<tr>
<th>Species</th>
<th>gp60 Allele</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptosporidium hominis</td>
<td>Ia</td>
<td>Strong et al., 2000; Glaberman et al., 2001a; Peng et al., 2001; Sulaiman et al., 2001; Glaberman et al., 2002; Leav et al., 2002; Peng et al., 2003a; Zhou et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Ia1</td>
<td>Wu et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Ia2</td>
<td>Wu et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Ib</td>
<td>Strong et al., 2000; Glaberman et al., 2001a; Peng et al., 2001; Sulaiman et al., 2001; Glaberman et al., 2002; Leav et al., 2002; Alves et al., 2003b; Peng et al., 2003a; Wu et al., 2003; Zhou et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Ic*</td>
<td>Strong et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Id</td>
<td>Strong et al., 2000; Peng et al., 2001; Sulaiman et al., 2001; Leav et al., 2002; Peng et al., 2003a</td>
</tr>
<tr>
<td></td>
<td>Ie</td>
<td>Peng et al., 2001; Sulaiman et al., 2001; Alves et al., 2003b; Peng et al., 2003a; Wu et al., 2003</td>
</tr>
<tr>
<td></td>
<td>If**</td>
<td>Leav et al., 2002; Alves et al., 2003b; Wu et al., 2003</td>
</tr>
<tr>
<td>Cryptosporidium parvum</td>
<td>Ila</td>
<td>Strong et al., 2000; Glaberman et al., 2001a; Peng et al., 2001; Glaberman et al., 2002; Alves et al., 2003b; Peng et al., 2003b; Zhou et al., 2003</td>
</tr>
<tr>
<td></td>
<td>Ilb</td>
<td>Peng et al., 2001; Alves et al., 2003b</td>
</tr>
<tr>
<td></td>
<td>Ic*</td>
<td>Peng et al., 2001; Alves et al., 2003b; Peng et al., 2003a</td>
</tr>
<tr>
<td></td>
<td>Ild</td>
<td>Alves et al., 2003b</td>
</tr>
<tr>
<td></td>
<td>Ile</td>
<td>Peng et al., 2003a</td>
</tr>
<tr>
<td></td>
<td>Ila, Ilb and Ilc***</td>
<td>Wu et al., 2003</td>
</tr>
<tr>
<td></td>
<td>GPα, GPβ and GPγ****</td>
<td>Stantic-Pavlinic et al., 2003</td>
</tr>
<tr>
<td>Cryptosporidium meleagridis</td>
<td>1</td>
<td>Glaberman et al., 2001b</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Glaberman et al., 2001b</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Glaberman et al., 2001b</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Glaberman et al., 2001b</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Glaberman et al., 2001b</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Glaberman et al., 2001b</td>
</tr>
</tbody>
</table>

* - Strong et al. (2000) gave the name Ic to two isolates they said were C. hominis but sequence data at two other loci showed C. hominis/C. parvum hybrid sequences. Since then all isolates identified within this allele group have been C. parvum. Alves et al. (2003b) proposed that the allele name be changed to IIc.

** - This allele group was initially named Ie but was changed by Alves et al. (2003b) to If as it was genetically distinct from other isolates that were grouped as Ie.

*** - Wu et al. (2003) identified three C. parvum alleles, which they named Ila, Ilb and Ile. However, as far as can be determined they have not grouped isolates under these alleles by comparison with the other published II alleles.

**** - Stantic-Pavlinic et al. (2003) did not compare alleles with those already named and therefore used a different nomenclature.
different primers. The sequence identity within each isolate was found to be identical. However, no systematic evaluation of the gp60 sequencing subtyping method has been undertaken. In addition to the DNA sequence analysis approach described in all of the papers subtyping Cryptosporidium at the gp60 locus, Wu et al. (2003) described two further techniques: a PCR-RFLP analysis using either Rsal or Alul to digest the PCR fragments and create restriction fragment profiles and a PCR-RFLP-single strand conformation polymorphism (SSCP) assay, where the restriction fragments from either Rsal or Alul are denatured and allowed to form various conformations that create different banding patterns by electrophoresis. By PCR-RFLP analysis they identified five subtypes (two C. parvum and three C. hominis) in a set of 28 C. parvum and 13 C. hominis isolates and using the PCR-RFLP-SSCP assay, identified 7 subtype alleles (three C. parvum and four C. hominis) in the same 41 isolates, all of which corresponded to sequence analysis.

2.3.2 – The rDNA Unit

The rDNA unit that codes for the cytoplasmic rRNA in C. parvum has been described by Le Blancq et al. (1997). There are five copies per genome, shared over at least three chromosomes. The rDNA unit consists of five sequential loci: 5’ small subunit rRNA (SSU rRNA, synonymous with 18S rRNA), internal transcribed spacer 1 (ITS1), 5.8S rRNA, internal transcribed spacer 2 (ITS2), and large subunit rRNA (LSU rRNA) 3’. Morgan et al. (1999) used nucleic acid sequence analysis to examine the ITS1-5.8S rRNA-ITS2 region but the degree of variation between 8 isolates was minimal and mainly identified differences between species. The SSU rRNA locus was examined in 7 of the 46 references included in this study, by sequence analysis in six and by SSCP analysis in one (Table 2.4). In addition to the investigation of C. parvum and C. hominis, Guyot et al. (2001) describe an isolate similar to C. andersoni that differed from a published sequence by 3 point mutations and Glaberman et al. (2001b) also used sequence analysis to identify 2 subtypes of C. meleagridis.

However, the usefulness of this locus for subtyping Cryptosporidium spp. for ecological or epidemiological purposes is questionable due to limited variation within C. parvum and C. hominis isolates and the poor accuracy due to intra-isolate heterogeneity (Carraway et al., 1994; Glaberman et al., 2001b; Sulaiman et al., 2001; Gibbons-Matthews and Prescott, 2003). The reason for this intra-isolate variation could be as a result of the five copies per genome and may indicate that heterogeneous
copies exist within the same isolate. Le Blancq et al. (1997) identified four copies of a Type A sequence and one copy of a Type B sequence within the *C. parvum* genome, located on different chromosomes. However, Guyot et al. (2001) state that the Type B sequence reported on GenBank™ identifies the Moredun *C. parvum* isolate and these Type A and Type B sequences have been used to identify two separate *C. parvum* subtypes (Guyot et al., 2001; Navarro-i-Martinez et al., 2003). Although no systematic evaluation of this subtyping method has been described, the observed intra-isolate heterogeneity indicates that the reproducibility of subtyping *Cryptosporidium* at the SSU rRNA locus is naturally low.

The use of SSCP at the SSU rRNA locus was studied by Gasser et al. (2003) and the subtyping capabilities were found to be limited in a set of 108 *C. hominis* and 76 *C. parvum* isolates from human clinical cases, with only 1 isolate of *C. parvum* identified as a different subtype to the rest and no variation within *C. hominis*. However, greater discrimination was observed at the ITS-2 locus where 13 *C. hominis* and 10 *C. parvum* subtypes were identified in the same set of isolates. This locus was also examined using denaturing polyacrylamide gel electrophoresis (DPGE) which also identified 10 *C. parvum* subtypes but only 9 within the *C. hominis* isolates (Gasser et al., 2003). All 184 isolates tested were typable and the authors state that reproducible profiles were obtained on different days with amplicons also produced on different days. The discrimination between isolates was much better at the ITS-2 locus than the SSU rRNA locus and once more mutation scanning gel electrophoresis demonstrated its usefulness in subtyping studies.

### 2.3.3 – The hsp70 Locus

Six papers reported the DNA sequence analysis of part of the hsp70 locus to subtype *Cryptosporidium* isolates, although one study was reported in two papers (Table 2.4). Di Giovanni et al. (1999) and Le Chevallier et al. (2000) analysed a 361 bp fragment applied to 36 *C. parvum* isolates from environmental waters and identified 7 subtypes. More recently, Le Chevallier et al. (2003) identified 2 *C. hominis* and 3 *C. parvum* subtypes by analysis of a 346 bp fragment from 22 isolates, also recovered from environmental samples. Sulaiman et al. (2001) and Peng et al. (2003a) both detected 6 *C. hominis* subtypes from human samples (A-F and A, C, D, E, G and H, respectively) using the same primers. As with the gp60 locus, Glaberman et al.
(2001b) identified 6 *C. meleagridis* subtypes at the hsp70 locus, although, two isolates with different hsp70 subtypes had the same gp60 allele. No systematic evaluation of subtyping by analysis at the hsp70 locus has been undertaken, although discrimination between isolates appears to be high as several different subtypes were identified from relatively few isolates.

2.3.4 – The β-tubulin, TRAP-C2 and Poly-T Loci

Other genes that have been used to subtype *Cryptosporidium* spp. are those coding for β-tubulin, thrombospondin related adhesive protein 2 (TRAP-C2) and polythreoneine protein (Poly-T) (Sulaiman et al., 2001; Widmer et al., 1998a; Rochelle et al., 2000)(Table 2.4). Two *C. hominis* subtypes were detected by DNA sequence analysis of a 369 bp fragment of the TRAP-C2 locus, out of 62 human isolates and a sensitivity of 97% (Sulaiman et al., 2001). However, with just 2 subtypes identified, a low level of discrimination at this locus or the use of related samples is indicated.

Sulaiman et al. (2001) also detected 3 *C. hominis* subtypes at the Poly-T locus by sequence analysis of a 318 bp fragment in the same 62 isolates, indicating a slightly higher discrimination than the TRAP-C2 locus albeit with a slightly lower sensitivity of 82%. The β-tubulin locus was studied in two of the included references: Widmer et al. (1998a) investigated sequence variation in 14 isolates from both human and animal sources (5 *C. hominis* and 9 *C. parvum*) and Rochelle et al. (2000) investigated 11 human and animal derived isolates (4 *C. hominis* and 7 *C. parvum*). Substantial intra-isolate variation was detected, which made it difficult to define subtypes. Whilst the sensitivity for the β-tubulin sequence analysis was 100%, the reproducibility was poor due to the intra-isolate variation and the discrimination of the method to subtype between different isolates was also overshadowed by this heterogeneity.

In addition to sequence analysis, Widmer et al. (1998a) also identified 4 subtype patterns (2 *C. hominis* and 2 *C. parvum*) by RFLP analysis with the restriction enzyme *Tsp*509I in the 14 human and animal derived isolates studied. However, different patterns were identified from three different experimental calf passages of the same isolate, which the authors claim is due to the variable ratios of mixed β-tubulin alleles within isolates. However, this questions the reproducibility of the method and usefulness of the locus for subtyping purposes. An alternative explanation for the
variation could be natural selection by passage of a mixed type primary infecting inoculum.

2.3.5 – Microsatellite and Minisatellite Loci

From the 46 included references, 25 have used analysis either based on sequence or fragment size of repeating DNA sequence motifs called micro- or minisatellites. 22 different microsatellites and 3 minisatellites have been used to subtype Cryptosporidium spp. (Table 2.4 and 2.6). ML1 was most widely used as 7 of the included papers describe subtyping C. parvum and C. hominis by either sequence or fragment size analysis at this locus and 2 C. hominis and 5 C. parvum alleles published (Table 2.6). Of the 7 references, 5 used the ML1 marker to type C. parvum isolates from different hosts, however Enemark et al. (2002a) found that isolates of C. parvum porcine genotype could not be typed at this locus. Neither Enemark et al. (2002a) or Mallon et al. (2003a) could amplify a C. meleagridis isolate at this locus with the primers published by Caccio et al. (2000)(Table 2.6).

Sequence and fragment size analysis of the ML2 microsatellite has also been described (Caccio et al., 2001; Alves et al., 2003a)(Table 2.4). From two of the three papers using the ML2 marker, 9 alleles (1 C. hominis and 8 C. parvum) were described (Table 2.6). However, the third paper mentioned 8 alleles but did not provide further details so these could be the same as those described in the other studies (Caccio and Pozio, 2001). Alves et al. (2003a) described the formation of stutter bands with ML2 fragment analysis of C. parvum isolates, which occurred when the amplicon was greater than 200bp and hindered the allocation of a specific allele. However, this approach appeared to provide a higher level of discrimination than ML1 when Caccio et al. (2001) tested both methods on the same samples.

Feng et al. (2000) described the fragment size analysis of 9 different microsatellite loci (and sequence analysis of three of these) to subtype C. parvum (4E12, Cp273, 12C07, 2G04, 6B03) or both C. hominis and C. parvum (5B12, 1G09, 1F07, 7E1C)(Table 2.4 and 2.6). It is claimed by Feng et al. (2000) that loci 9B10, 2G04, 6B03 and 5D11 can all be used to subtype C. hominis but the paper shows that this variation is between C. hominis and an isolate that the authors claim is neither C.
<table>
<thead>
<tr>
<th>Locus</th>
<th>Reference</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>c001</td>
<td>2001</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c002</td>
<td>2002</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c003</td>
<td>2003</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c004</td>
<td>2004</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c005</td>
<td>2005</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c006</td>
<td>2006</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c007</td>
<td>2007</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c008</td>
<td>2008</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c009</td>
<td>2009</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
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<tr>
<td>c010</td>
<td>2010</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
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<tr>
<td>c011</td>
<td>2011</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c012</td>
<td>2012</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c013</td>
<td>2013</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
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<tr>
<td>c014</td>
<td>2014</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
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<td>c015</td>
<td>2015</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
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<td>c016</td>
<td>2016</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
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<tr>
<td>c017</td>
<td>2017</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
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<tr>
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<td>2018</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c019</td>
<td>2019</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c020</td>
<td>2020</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c021</td>
<td>2021</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c022</td>
<td>2022</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
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<td>c023</td>
<td>2023</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
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<td>2024</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
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<td>2025</td>
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</tr>
<tr>
<td>c026</td>
<td>2026</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c027</td>
<td>2027</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
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<td>2028</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c029</td>
<td>2029</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c030</td>
<td>2030</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c031</td>
<td>2031</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
<tr>
<td>c032</td>
<td>2032</td>
<td>C. porcellus spp. isolates from humans, animals, and water</td>
</tr>
</tbody>
</table>

Table 2.6: A description of the alleles identified at various microsatellite and minisatellite loci
<table>
<thead>
<tr>
<th>Alleles</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.A.</td>
<td>2 C. parvum parental isolates &amp; 2 progeny of mixed infection</td>
</tr>
<tr>
<td>1 and 3</td>
<td>1 C. parvum isolates from humans &amp; 2 C. parvum isolates from calves</td>
</tr>
<tr>
<td>1</td>
<td>2 C. parvum isolates from humans &amp; 8 C. parvum isolates from calves</td>
</tr>
<tr>
<td>2</td>
<td>6 C. hominis isolates</td>
</tr>
<tr>
<td>2</td>
<td>Several recombinations of mixed isolates following CCK mouse</td>
</tr>
<tr>
<td>2</td>
<td>2 parental C. parvum isolates from a human and a deer</td>
</tr>
<tr>
<td>4</td>
<td>1 C. parvum isolates from a human</td>
</tr>
<tr>
<td>5</td>
<td>4 C. parvum isolates from humans &amp; 8 C. parvum isolates from calves</td>
</tr>
<tr>
<td>5 and 7</td>
<td>2 parental C. parvum isolates from a human</td>
</tr>
<tr>
<td>1, 2, and 3</td>
<td>2 parental C. parvum isolates from a human and a deer</td>
</tr>
<tr>
<td>4, 5, and 6</td>
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<td>22 C. parvum isolates from humans</td>
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<td>11 C. hominis isolates</td>
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*Table 2.6 cont.: A description of the alleles identified at various microsatellite and minisatellite loci.*
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Table 2.6 cont.: A description of the alleles identified at various microsatellite and minisatellite loci.
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<th>Isolation Source</th>
<th>References</th>
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<td>(dop008)</td>
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<th>References</th>
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Table 2.6 cont.: A description of the alleles identified at various microsatellite and minisatellite loci.
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hominis or C. parvum. The sensitivity at loci 5B12, 1G09 and 1F07 was 100% but for the other loci it ranged from 37%-68%. Feng et al. (2002) also described fragment analysis of several of these loci (5B12, 4E12, 1F07, Cp273, 7E1C) as well as two others (Cp492, Cp358) (Table 2.4 and 2.6), to study the potential for genetic recombination between different C. parvum subtypes and produced multilocus fingerprints with a mixture of parental alleles. In vivo reproducibility was also tested in this study as each isolate was seen to be stable when passaged through several hosts. Widmer et al. (2000) stated that they focused on 14 microsatellite loci but only a few results for the loci 5B12 and 1F07 were presented (Table 2.6).

Mallon et al. (2003a; 2003b) describe a multilocus approach using 4 microsatellite loci (TP14, MS9, ML1 and GP15) and 3 minisatellite loci (MS5, MS12 and MS1) to subtype human and animal isolates of Cryptosporidium initially using sequence analysis and then fragment size analysis (Table 2.4 and 2.6). Between 5 and 20 alleles were identified at each individual locus (Table 2.6). However, when the multilocus approach was taken, 58 multilocus subtype fingerprints were identified (38 from the sequence analysis of 180 isolates and a further 20 from the fragment analysis of 242 isolates), which included 7 C. hominis subtypes, 48 C. parvum subtypes, 2 C. parvum 'monkey genotype' subtypes and 1 C. meleagris. The sensitivity of both of these approaches to C. parvum and C. hominis was 100% and the discrimination high, but the reproducibility of the method was not described.

Two microsatellite loci that have been used as subtyping markers, but did not appear to be very discriminatory, are MS1 (synonymous SSR-1) (Aiello et al., 1999; Sulaiman et al., 2001) and MS6 (Aiello et al., 1999) (Table 2.4 and 2.6).

One final paper subtyped isolates of Cryptosporidium using polyacrylamide gel electrophoresis (PAGE) of PCR fragments produced with a microsatellite and telomere anchor primer set (Blasdall et al., 2002) (Table 2.4). Using this approach the authors identified banding patterns that created fingerprints from specific isolates. Blasdall et al. (2002) stated that no identical patterns were identified from epidemiologically unrelated isolates but were seen in related isolates so from this description the discrimination of the method appears to be high. The authors tested the reproducibility of the method by examining multiple isolates from the same source.
collected at the same time and also a reference isolate (Iowa) that had been continually passaged for several years all of which gave identical fingerprints. The typability of this approach could not be assessed from this paper, as the authors did not state if all isolates had been assigned a type. No formal evaluation has been undertaken of any micro- and minisatellite markers as a Cryptosporidium typing tool.

2.3.6 – Extra-chromosomal Double-stranded RNA
In addition to genomic-based loci for distinguishing between isolates of the same Cryptosporidium spp., two extra-chromosomal virus-like double-stranded (ds) RNAs (large, L and small, S) have been identified and found to contain some variation (Khramtsov et al., 2000). Khramtsov et al. (2000) used the L-dsRNA to distinguish between all 12 isolates studied (5 C. parvum from calves, 2 C. parvum from humans and 5 C. hominis) and also found that there was variation between 11 by analysis of the S-dsRNA. Three other studies used sequence analysis of the S-dsRNA molecular to subtype Cryptosporidium and revealed good discrimination (Xiao et al., 2001b; Leoni et al., 2003a; Leoni et al., 2003b). Xiao et al. (2001b) identified 8 C. parvum and 10 C. hominis subtypes consisting of between 1 and 13 nucleotide differences in 61 samples (23 C. parvum from cattle, 5 C. parvum from humans and 33 C. hominis). However, none of these nucleotide changes were species-specific and phylogenetic analysis could not separate the two Cryptosporidium species. Leoni et al. (2003a) initially identified 5 C. hominis subtypes, 5 C. parvum subtypes and one common to both species, in 18 samples (8 C. parvum from humans and 10 C. hominis) by sequence analysis and 9 subtypes by a heteroduplex mobility assay (HMA). In a further study additional resolution was added to the HMA, which identified 26 subtypes (8 C. hominis, 12 C. parvum, 1 subtype common to both species, 4 C. felis and 1 C. meleagridis) in 265 of 407 samples (247 from humans and 18 from livestock) (Leoni et al., 2003b). In this study they also detected further heterogeneity within these subtypes by sequence analysis. Sensitivity was 100% for all but one of these studies using admittedly relatively low numbers of isolates, but when the locus was used on 407 isolates by HMA the sensitivity reduced to 65% but the level of discrimination remained high (Leoni et al., 2003b). Reproducibility was only tested with the heteroduplex mobility assay by testing PCR amplicons and cloned material with identical sequences but from different samples, testing PCR amplicons and cloned material from the same sample and multiple tests on the same cloned material.
(Leoni et al., 2003b). The reproducibility for each of these tests was 100% with the exception of one test where a PCR amplicon contained the same pattern as the cloned material from the same sample but also had an extra band.

2.3.7 – Multilocus Subtyping
As well as using an individual locus a subtype profile can be produced by examining a combination of several loci to provide greater discriminatory power. Widmer et al. (2000) demonstrated the importance of multilocus fingerprinting with the 5B12 and 1F07 microsatellite loci. Several of the retrieved papers used a microsatellite multilocus approach to increase the discriminatory power over a single locus study (Caccio et al., 2001; Feng et al., 2002; Widmer et al., 2000; Mallon et al., 2003a; Mallon et al., 2003b). This approach has also been taken to develop multilocus genotyping at other loci. Sulaiman et al. (2001) use the multilocus sequence analysis of one microsatellite (MS1) and the TRAP-C2, Poly-T, SSU rRNA, hsp70 and gp60 genes to increase the discriminatory power when subtyping 62 C. hominis isolates. Glaberman et al. (2001b) used combined analysis of DNA sequences of the SSU rRNA, gp60 and hsp70 loci on 11 C. meleagridis samples. This approach increased the discrimination as 7 different subtypes were identified as opposed to 6 at both the gp60 or hsp70 loci and 2 at the SSU rRNA locus.

2.3.8 – Arbitrarily Primed PCR (AP-PCR)
Three of the retrieved references described the use of PCR-based methods using arbitrary primers (AP) to produce unique fingerprints for different subtypes. Carraway et al. (1994) demonstrated that there was heterogeneity between all 5 animal derived C. parvum isolates and a C. muris isolate investigated. Shianna et al. (1998) could differentiate between all of the 20 isolates that they examined using a random amplified polymorphic DNA (RAPD) PCR, which uses shorter arbitrary primers. Although each isolate was distinguishable they could all be grouped into 4 subtype groups. The authors also acknowledge that it is possible that bacterial contamination of the samples could be the cause of the apparent differences between isolates. Giles et al. (2001) also used RAPD PCR to produce unique fingerprints for the 16 isolates that they tested. The in vitro reproducibility of this method was reported to be 100% by the authors although the data are not shown. However, the in vivo reproducibility was 0% as each isolate gave a pattern distinct from the initial
fingerprint once passaged through an animal host, implying that this approach is not useful in epidemiological studies.

2.3.9 – Electrophoretic Analysis of Proteins

Three papers described the identification of subgroups within *Cryptosporidium* species by studying the electrophoretic profiles of various proteins. Mead *et al.* (1990) examined the 2 dimensional gel electrophoresis patterns of sporozoite proteins, using 5 *C. parvum* isolates recovered from different hosts but amplified by passage through calves. Four subtypes were identified based on the isoelectric point mobility of a 106-kDa polypeptide (one of the subtypes lacked this peptide but had a 40-kDa peptide, which the others did not). Oocyst wall antigens were investigated by McLauchlin *et al.* (1998) to identify 8 subtypes by SDS-PAGE and Western blotting of 227 *C. parvum* (and probably *C. hominis*, as it was unclear at the time exactly what species the isolates were) isolates. In the third study, El-Ghaysh and Barrett (1999), distinguished between 3 *C. parvum* isolates from different geographic regions and hosts using enzyme electrophoretic variation. Different glucose phosphate isomerase (GPI) patterns were obtained for each isolate and the analysis of hexokinase (HK), malate dehydrogenase (MDH) and glutamate dehydrogenase (GLDH) differentiated between the UK isolate and the two from Egypt. However, despite the variation described in these papers it is difficult to know whether this is sub-species variation as the isolates have not been genetically characterised and could just be different species. Similarly in many of the early studies isolates were identified by microscopy as *C. parvum* but could be one of several species that have similar morphology (e.g. McDonald *et al.*, 1991; Nichols *et al.*, 1991; Griffin *et al.*, 1992; Nina *et al.*, 1992; Ogunkolade *et al.*, 1993).

Despite the identification of variation with these methods, they are not particularly useful when studying environmental isolates, or those from sub-clinical infections, as high numbers of oocysts are required. For example, Mead *et al.* (1990) used 2x10⁹ oocysts and McLaughlin *et al.* (1998) stated that 1x10⁴ oocysts are required.

2.4 – Subtyping Environmental Isolates

While the majority of papers described typing from oocysts recovered from human or animal faecal samples, only 7 of the retrieved publications applied the subtyping
methods to isolates recovered from environmental samples. Di Giovanni et al. (1999) and LeChevallier et al. (2000) collected raw source water and treatment plant filter backwash samples and recovered Cryptosporidium oocysts by immunomagnetic separation (IMS) before using cell culture and PCR (CC-PCR) followed by DNA sequence analysis at the hsp70 locus to subtype viable oocysts in the samples. The same approach was used by LeChevallier et al. (2003) on samples collected from surface water representing various watersheds. Caccio & Pozio (2001) also used IMS to recover Cryptosporidium oocysts from water samples, but characterised the isolates by DNA sequence analysis of 2 microsatellite loci (ML1 and ML2). Glaberman et al. (2002; 2001a) recovered isolates from a wastewater sample from a blocked drain and found they were indistinguishable from those causing human disease during a waterborne outbreak. Finally, Peng et al. (2003b) collected 16 water samples and managed to subtype two of the four from which C. parvum oocysts were recovered using IMS by DNA sequence analysis of the gp60 locus.

2.5 – Discussion

Reviews are an important means of gaining a comprehensive and minimally biased picture of the subject being studied. Whilst every attempt is made to remove bias from the review by using a systematic search strategy, there is likely to be some publication bias, which can be introduced by researchers or editors not publishing studies when they feel that the results are not significant. However, this tends to make the results of a review biased towards the positive results and for the purposes of this study, to identify the most useful methods for subtyping isolates, this positive bias should not be problematic. By retrieving all the relevant literature it is possible to identify what has been done as well as gaps or problems that are yet to be addressed or resolved. This study demonstrated that in order to fully cover a subject, access to multiple databases covering all the associated fields of study is necessary since none of the databases yielded all of the retrieved references. In addition, it highlights the wide range of methods to characterise Cryptosporidium spp. to the subtype level currently available to researchers and how these methods can be used to study isolates recovered from environmental samples. Indeed, while many approaches have been described, just 7 were applied to environmental samples. This is perhaps not surprising since clinical samples, containing high numbers of oocysts and perhaps having detailed patient and/or epidemiological data are a sensible starting point for
investigation of new techniques. Whereas environmental samples may contain low numbers, which presents further challenges in maximal oocyst recovery.

The majority of Cryptosporidium subtyping studies have used sequence analysis of particular genes or regions of polymorphic DNA. Although this is the highest resolution typing tool available to researchers, and may be regarded as a gold standard, there are several drawbacks to this approach. Sequence analysis is an expensive technique and despite computer technology to assist the analysis, it is highly skilled and time-consuming. In an outbreak situation where results from many samples are needed quickly, a sequenced based approach may therefore not be the most appropriate. Additionally, some of the loci that are examined in subtyping (e.g. multicopy genes such as SSU rDNA) can have substantial variation within individual organisms and between those from the same population. In Cryptosporidium research pure cultures are difficult to obtain, and the presence of mixtures of species or subtypes may not easily be determined by sequence analysis since only a single sequence is obtained from each sample. Whilst this may not cause a problem with clinical samples (although mixed infection do occur), it is not uncommon in for several species or subtypes to be present in environmental samples.

Several other alternative or precursor approaches to using sequence analysis include the use of fragment size analysis, mutation scanning gel electrophoresis or PCR-RFLP all of which have demonstrated reasonable discrimination at specific loci. The level of discrimination that is required in a subtyping technique is dependent on the hypothesis. Approaches that identify intra-isolate heterogeneity, such as sequence analysis of the SSU rRNA locus or β-tubulin locus (Carraway et al., 1994; Glaberman et al., 2001b; Sulaiman et al., 2001; Gibbons-Matthews and Prescott, 2003; Widmer et al., 1998a; Rochelle et al., 2000) would not be particularly useful in ecological studies, such as tracing the source of contamination or tracking the spread of particular isolates. On the other hand, these methods are useful in studying the population structure of Cryptosporidium spp.

Fragment size analysis has been primarily used to study polymorphisms at loci comprising tandem repeats such as microsatellite and minisatellite DNA (Enemark et
Using fragment size analysis is easier, faster and cheaper than DNA sequencing and depending upon the system used, both techniques can be carried out using the same equipment and software. Another advantage with fragment analysis over sequencing is the higher sensitivity for the detection of more than one subtype within a sample (Enemark et al., 2002a). However, as fragment analysis indicates only the size of the amplified product, prior species determination is necessary since some fragment sizes are common between species (Enemark et al., 2002a). Additionally, various subtypes may also have the same fragment sizes and would therefore be missed. The risk of this can be reduced by a multilocus approach since different fragment lengths will be generated by various microsatellite loci. Mutation scanning gel electrophoresis approaches such as SSCP and DGGE have shown very good typability and depending on the locus studied can be highly discriminatory (Gasser et al., 2003; Wu et al., 2003). There is also no need for prior species determination since the results have been found to be species-specific. The use of PCR-RFLP at the gp60 locus was also shown to be of use as a subtyping tool (Wu et al., 2003) although the discriminatory power was not quite as high as PCR-RFLP-SSCP. These methods, although as yet to be applied to environmental samples are also much quicker and cheaper than sequence analysis and like fragment analysis should be able to identify a mixture of isolates within samples. The level of discriminatory power seen by PCR-RFLP-SSCP is also equivalent to that of sequence analysis at the gp60 gene (Wu et al., 2003).

Another rapid approach to subtyping Cryptosporidium was with a HMA using the extra-chromosomal S-dsRNA, which gave good discrimination between isolates, but a sensitivity of 65% when a large number of isolates were tested (Leoni et al., 2003a; Leoni et al., 2003b). AP-PCR and RAPD-PCR are much simpler techniques that can be carried out in many laboratories. However, because the shorter arbitrary primers for these techniques are not specific for a particular region in the genome of the organism being studied the possibility of non-specific amplification of exogenous DNA in the sample is increased.

The choice of locus to examine is important as the amount of polymorphism within the region and also the type of variation may also influence the choice of analytical method. For example, a size-based analysis such as fragment size analysis would not provide much typing information if the locus examined was mainly polymorphic.
through nucleotide substitutions (e.g. the hsp70 locus). From the results of the
systematic literature search several different loci were identified for providing
subtyping information. Some of these presented several problems due to intra-isolate
heterogeneity, such as loci within the rDNA unit and the β-tubulin gene (Carraway et
al., 1994; Widmer et al., 1998a; Rochelle et al., 2000; Glaberman et al., 2001b;
Sulaiman et al., 2001; Gibbons-Matthews and Prescott, 2003). Also, the potential of
the β-tubulin, TRAP-C2 and Poly-T loci as well as most of the rDNA unit, for
subtyping appears to be quite small at present due to the low amount of discrimination
that was seen between isolates (Sulaiman et al., 2001). The one exception from the
rDNA unit was an SSCP analysis of the ITS2 locus, which provided a higher level of
discrimination between isolates tested (Gasser et al., 2003). The hsp70 locus has also
shown to be a reasonable locus to examine in subtyping studies of various
Cryptosporidium spp. and as many of the polymorphisms at this locus are nucleotide
substitutions (Di Giovanni et al., 1999) a sequence based or mutation scanning (e.g.
SSCP or DGGE) approach is more suited. The gp60 locus is at present the most
studied in subtyping analyses and appears to show a good level of discrimination
between isolates. The polymorphic regions within this locus include a polyserine
coding microsatellite, a hypervariable region and further single nucleotide
polymorphisms (Strong et al., 2000). The single nucleotide polymorphisms have
been exploited by sequence analysis, PCR-RFLP and PCR-RFLP-SSCP, all of which
have shown a high degree of discrimination. The microsatellite region within this
locus is one of the several micro- or minisatellite regions that have been used in
Cryptosporidium subtyping studies. A number of these microsatellite were seen to be
highly polymorphic between isolates and prove useful through sequence and fragment
size analysis. Mutation scanning electrophoresis may also provide a reasonably
inexpensive method to analyse these regions for inter-isolate variation. As well as
individual loci, the multilocus approach has shown its usefulness in increasing the
amount of information that can be obtained from each isolate and in turn provide a
higher level of discrimination between isolates. The approach chosen to carry out a
multilocus study would again be decided upon depending on the actual loci being
studied. However, due to the increased number of tests per sample, a cost and time
factor may play a role in the decision and methods other than sequence analysis that
can be faster and cheaper such as fragment analysis and SSCP may initially be more popular.

As well as the genomic loci that have been used to subtype Cryptosporidium, four studies characterised isolates by examining extra-chromosomal virus-like dsRNA and identified a number of subtypes (Khramtsov et al., 2000; Xiao et al., 2001b; Leoni et al., 2003a; Leoni et al., 2003b). However, the fact that the target is not part of the genome must be considered, as this approach types the virus-like dsRNA and not necessarily the Cryptosporidium isolate. The biological functions of these units are as yet unknown and therefore it may be difficult to draw conclusions about the biology of Cryptosporidium from these studies.

It has been demonstrated that each environmental sample has a different composition that can directly affect the detection of Cryptosporidium (Sluter et al., 1997). This highlights the need to assess the reproducibility of a particular test under various conditions, which are directly relevant to the samples being studied and the importance of quality control.

PCR-based techniques can be highly sensitive since the Cryptosporidium DNA in the sample is amplified and can theoretically be used when low numbers or even single oocysts are present. By contrast, enzyme or antigen based approaches to subtyping are only really suitable for samples with high numbers of oocysts (>1.2x10^7) (Awad-El-Kariem et al., 1995) and need to be evaluated with genetic analysis to confirm species or sub-species status. When testing clinical samples high numbers are usually present, but with environmental samples or those from subclinical/asymptomatic infections there may only be a low number of oocysts available. Thus, these phenotypic techniques are not currently applicable for routine water sampling or for tracking the source of infection or environmental contamination. However, since the genotypic techniques involve amplifying the particular DNA region of interest, they are broadly applicable to these purposes.

Almost all of the subtyping papers that were retrieved for this review examined isolates in clinical samples from both animals and humans where the recovery of oocysts was not a problem. A possible reason for so few studies into environmental
subtypes is the increased difficulty to recover oocysts and extract sufficient DNA from the samples. Five of the seven papers that subtyped Cryptosporidium from water used IMS to recover oocysts from the samples (Di Giovanni et al., 1999; LeChevallier et al., 2000; Caccio and Pozio, 2001; LeChevallier et al., 2003; Peng et al., 2003b). Di Giovanni’s and LeChevallier’s groups used the recovered oocysts to infect cell cultures before lysing the infected cells by freeze-thawing and finally extracting the DNA, whereas Caccio & Pozio and Peng et al. extracted the DNA directly from the oocysts. Where prior recovery by IMS was not used, wastewater in a blocked drain, which was likely to have contained a large number of oocysts was tested (Glaberman et al., 2002; Glaberman et al., 2001a). An additional problem that may be encountered when trying to subtype environmental Cryptosporidium (and indeed faecal isolates) is the presence of PCR inhibitors. However, the use of IMS to recover oocysts should leave behind any inhibitors that may have been in the samples (Johnson et al., 1995; Deng et al., 1997), and commonly used DNA spin-columns should also remove inhibitory components leaving a purified DNA suspension. As well as containing low numbers of oocysts, environmental samples are likely to be heterogeneous mixes. Therefore, the prior recovery of all species of Cryptosporidium is desirable and the subtyping method of choice should be able to distinguish between mixed isolates so that accurate fingerprints can be obtained. A further consideration when subtyping from environmental samples is the potential for the presence of other waterborne organisms that may also amplify. Most primer sequences are tested against a range of organisms as a measure of their specificity to Cryptosporidium. However, in the environmental samples there are a number of organisms that may not have been tested. For example, Sturbaum et al. (2002) used a primer set targeting the SSU rRNA locus that they had previously tested on several organisms and found to be specific for several Cryptosporidium spp., but in this study they found that the primers also amplified DNA from a dinoflagellate common in water.

The characterisation of Cryptosporidium spp. by high-resolution methods is becoming more important in our understanding of the ecology and epidemiology of this parasite. There are many reports of both sporadic cases and outbreaks of cryptosporidiosis throughout the world and the importance of this parasite to public health has been identified (Fayer et al., 2000a). The presence of Cryptosporidium spp. oocysts in environmental samples, through faecal contamination, is common (Rose, 1997), but
tracing the sources of these oocysts has been problematic. Contamination of surface water from farmland due to the presence of livestock, slurry spreading or wildlife has been implicated as the source of many clinical cases (Meinhardt et al., 1996), and due to the hardy nature of the Cryptosporidium spp. oocysts they can survive water treatments, such as chlorination, and be transmitted to the public through municipal supplies. Water companies try to reduce the risk of infection from their supplies by risk assessment and routine monitoring for Cryptosporidium. This monitoring involves filtering samples and using fluorescence microscopy to identify any oocysts that are morphologically similar to C. parvum or C. hominis (USEPA, 2001; DWI, 2005). Recently there have been several reports of species other than C. parvum or C. hominis causing clinical disease in humans, including C. meleagris, C. felis, C. canis and C. muris (Pieniazek et al., 1999; Morgan et al., 2000; Pedraza-Diaz et al., 2000; Chalmers et al., 2002b; Gatei et al., 2002a). Therefore, the use of techniques that characterise Cryptosporidium isolates to at least a species level are important to identify which oocysts are of public health significance. These methods for identifying species that cause human infection are readily used by research groups. However, the water industry uses genus-specific microscopy for monitoring, and if such data are to be applied to public health then further characterisation of oocysts is necessary.

When evaluating detection systems the important criteria include the sensitivity, specificity, repeatability and reproducibility of the techniques used (Feldsine et al., 2002). However, when evaluating typing systems other criteria must also be considered (see 1.7.1 – Evaluating Molecular Characterisation Methods), since the isolates have usually been identified as present in the sample and so measuring the probability of obtaining a false positive or negative is not important. In order to fully evaluate a typing system the isolates to be investigated need to be epidemiologically unrelated. The criteria for evaluating typing systems have been well described in the field of bacteriology and include the typability (proportion of isolates that are assigned a type), reproducibility (ability to assign the same type to an isolate on independent, separate assays) and discriminatory power (the probability of distinguishing between two unrelated isolates) of the system (Struelens et al., 1996). Only one of the papers that were retrieved in this study acknowledged the need to evaluate the typing methods using these criteria (Leoni et al., 2003b), but although a
previous study by these authors used epidemiologically unrelated samples (Leoni et al., 2003a), this second study did not.

With the recent advances in molecular technology, new techniques have been developed and targets identified that provide us with more detailed characterisation of organisms. As well as defining the species of Cryptosporidium, it is now possible to use accurate, high-resolution techniques to subtype isolates, which is extremely important in the study of the epidemiology and ecology of this parasite. The choice of subtyping approach is highly dependent on the hypothesis as some methods and targets are more suited to particular studies. Several of the targets (i.e. gp60, hsp70, ds-RNA and various microsatellites), either individually or particularly using a multilocus approach have shown to provide high levels of discrimination and may be useful in tracking studies and identifying risk factors to public health. This in turn will enable better control measures and management strategies to be developed for the protection of not only the human population but also wild and domestic animal populations.

2.6 - Conclusions

The search strategy that was developed was successful in the identification of peer-reviewed and grey publications that describe the development and application of Cryptosporidium subtyping methods (Objective 1a). The definition of inclusion criteria allowed these publications to be either accepted into the study or rejected following their collation and review (Objective 1b). From those included in the study, the different methods and loci used to subtype isolates of Cryptosporidium could be identified and compared for their potential use in examining the public health significance of environmental isolates (Objectives 1c and 1d).

To identify all of the publications describing the use of subtyping Cryptosporidium isolates a multiple database strategy was required. The most common method of subtyping Cryptosporidium was through DNA sequence analysis, which is the highest resolution method available. However, there are drawbacks to this approach such as problems in identifying mixed species or subtypes as well as the cost, skills and time required. Other approaches, such as fragment size analysis and mutation scanning gel electrophoresis have also demonstrated high-resolution characterisation and have the
added benefit of distinguishing mixed species and subtypes. The most promising highly polymorphic loci for subtyping Cryptosporidium from the literature included in the search were gp60 (the whole gene or just the microsatellite fragment) and various microsatellites (e.g. ML1 and ML2). The discriminatory power of the method was found to increase as more loci were added in a multilocus approach. However, the number of loci used must be balanced with the resolution that is required by the hypothesis due to the extra time and cost that is involved. These highly discriminatory subtyping methods can be used in the investigation of Cryptosporidium in the environment as well as within human and animal populations, leading to a greater understanding of their interactions.
Section 2

Development

and

Optimisation of Methodology
Chapter 3 – Detection of potentially low numbers of 

*Cryptosporidium* spp. oocysts

3.1 – Introduction

Before the subtyping methods described in the previous chapter can be used to characterise *Cryptosporidium* species, isolates must be detected in the sample. This chapter describes the development and evaluation of an improved method of concentration and detection of potentially low numbers of *Cryptosporidium* oocysts from faecal samples. This can be particularly important when screening animal populations, as it is common for wild and farmed animals (particularly adults) to demonstrate asymptomatic carriage, resulting in the shedding of low numbers of oocysts. These populations may be screened to include or exclude them as the source of environmental contamination (e.g. when identifying risks to a water catchment or the cause of a waterborne outbreak).

When low numbers of oocysts are present in samples it is necessary to concentrate them prior to detection (see 1.5 – Recovery of *Cryptosporidium* spp., 1.5.1 - Faeces). Whilst IMS is the most efficient method of oocyst concentration, the cost of reagents is prohibitive for routine and large-scale faecal screening. Other than IMS, Casemore *et al.* (1985) reported that their modification to the standard FE sedimentation method (to reduce oocysts being trapped in the ether plugs), closely followed by FEA sedimentation, gave the greatest oocyst recovery. The application of commercially available faecal parasite concentrators, based on the FE/FEA sedimentation techniques, has also been reported to increase the recovery of oocysts from faecal samples. However, these kits were developed for the recovery of other cysts and helminth ova and as with the standard sedimentation methods the density of oocysts can result in them becoming trapped in the ether/ethyl acetate plug (see 1.5 – Faeces).

Different microscopic, molecular and immunological methods have all been used to detect *Cryptosporidium* oocysts in stool samples (see 1.6 – Detection of *Cryptosporidium* spp.). The majority of initial screening involves examining faeces for the presence of oocysts by microscopy following acid-fast, auramine or
immunofluorescence staining (see 1.6.2 – Microscopic Detection Methods).

Microscopic methods of detecting Cryptosporidium can be time-consuming when screening large numbers of samples and due to the subjective nature, require experienced parasitologists. The development of enzyme immunoassays (EIA) for faecal detection of Cryptosporidium may provide a quicker, less subjective alternative to the microscopic methods. Since the first generation EIAs with low sensitivities a number of commercially available EIAs (enzyme-linked immunosorbent assays, immunofiltration or immunochromatographic assays) have been developed (see. 1.6.3 – Enzyme Immunoassay Detection Methods). Most studies found EIAs to be more sensitive than chemical staining methods, but IF microscopy was superior.

As the purpose of these methods are to screen faeces and detect Cryptosporidium infections, the sensitivity, specificity, positive and negative predictive values are important criteria (see 1.6.1 – Evaluating Detection Methods). The sensitivity of these techniques is closely related to the threshold of detection and the number of organisms in the sample. Although clinical samples often contain high numbers of Cryptosporidium, in non-symptomatic infections low numbers are common (Fayer et al., 2000b; Xiao et al., 1994; Kotler et al., 1990). Several studies have shown that microscopic methods with or without prior concentration can have high thresholds of detection resulting in low sensitivity, although concentration prior to detection always resulted in lower thresholds (Weber et al., 1992; Weber et al., 1991; Clavel et al., 1996; Deng and Cliver, 1999). One factor that is seen to affect the detection threshold is the consistency of faeces (Weber et al., 1992; Weber et al., 1991), which means that the sensitivity of a chosen method can vary depending on the status and source of the sample. Therefore, sensitive techniques with known low limits of detection in various sample types are required to screen for Cryptosporidium, particularly when there is the potential for low numbers to be present, for example in epidemiological surveys and screening of apparently healthy individuals.

Objective 2: To develop and evaluate an improved method for detecting low numbers of Cryptosporidium oocysts in faeces when screening the large numbers of samples collected during studies.
Objective 2a: To modify the commercially available Parasep® Faecal Parasite Concentrator that used the FEA sedimentation principle for the recovery of Cryptosporidium oocysts.

Objective 2b: To evaluate the use of IF microscopy and two commercially available EIAs for the detection of Cryptosporidium oocysts following the modified concentration method.

Objective 2c: To determine the threshold of detection for the modified concentration method with the different consistency faecal samples from sheep and cattle.

3.2 – Materials and Methods

3.2.1 – Preparation of faecal samples spiked with Cryptosporidium oocysts

3.2.1.a – Source of Cryptosporidium oocysts

Human faecal samples previously found to be positive for Cryptosporidium were selected for screening from the National Collection of Cryptosporidium Oocysts held at the Cryptosporidium Reference Unit (Swansea, UK). The samples were selected based on the volume available and physical nature. Diarrhoeic faeces were preferable as clinical disease was still present at the time of sampling and the numbers of oocysts present were likely to be higher. The selected faeces were screened to identify samples that contained high numbers of oocysts by microscopy following modified Ziehl-Neelsen staining. Air-dried faecal smears were fixed in methanol and stained with concentrated carbol fuchsin for 15-20 minutes. Slides were rinsed in tap water and decolourised with acid alcohol (1% HCl in Industrial Methylated Spirit) for 15-20 seconds, rinsed once more and counterstained with 0.4% malachite green for 30 seconds. Smears were thoroughly rinsed in tap water, allowed to air dry and at least 50 fields screened by bright field microscopy using a x40 objective (with more detailed examination and measurements using a x100 oil immersion objective) on a Nikon Eclipse E600 Microscope.
3.2.1.b – Preparation of stock oocyst suspensions

Samples that were found to contain high numbers of oocysts (more than 5-10 oocysts per field) were selected to produce stock oocyst suspensions by saturated salt flotation. Faeces of roughly the same size as a broad bean or 2ml if fluid, were emulsified in 8ml of saturated sodium chloride (specific gravity of 1.2) and mixed thoroughly on a vortex at maximum speed. Approximately 2-3ml of deionised water was carefully added to form a distinct layer above the faecal slurry and centrifuged (PK130, DJB Labcare) at 1,600g for eight minutes with the brake off. The deionised water layer was then gently swirled to draw the oocysts (at the interface of the two fluid layers) into the water phase, which was gently removed and dispensed into a new tube and made up to 10ml with deionised water. The suspensions were mixed by vortex to dilute any sodium chloride residue and centrifuged (PK130, DJB Labcare) at 1,600g for five minutes with the brake off. Supernatants were gently decanted and the pellet re-suspended in 1ml of deionised water.

Oocyst suspensions were enumerated using an improved Neubauer Haemocytometer. Prior to loading, the suspensions were thoroughly mixed on a vortex for 30 seconds to ensure a homogenous suspension. Suspensions were loaded into the chamber and the number of oocysts in the four 0.1μl corner squares (Figure 3.1) counted using differential interference contrast microscopy (DIC) with a x40 objective on a Nikon Eclipse E600 Microscope. Each suspension was counted ten times and the number of oocysts per ml calculated using the following formula.

\[
\text{Oocysts per ml} = \frac{\sum \text{Number of oocysts per 0.1}\mu\text{l square}}{\text{Number of squares counted}} \times 10^4
\]

Those found to contain >2x10⁴ oocysts ml⁻¹ and little faecal debris were kept as oocyst stock suspensions.

3.2.1.c – Source of faecal samples

Ovine and bovine faecal samples, previously been identified as negative by modified Ziehl-Neelsen staining, immunofluorescence microscopy (IFAT) and PCR, were identified from those stored at the Cryptosporidium Reference Unit (CRU) in
Figure 3.1: A diagrammatic representation of the improved Neubauer Haemocytometer grid. The four 0.1µl corner squares used for enumerating Cryptosporidium oocyst suspensions are shaded and an example of which oocysts are counted (oocysts touching the top or left central boundary are counted and those touching the bottom or right central boundary are not) is shown in the bottom-right square.
Swansea. One faecal sample from each animal species (representing a typical consistency) was selected for the spiking experiments. Ten replicates of each sample were screened by IFAT without concentration and a further ten replicates were concentrated using the formol-ethyl acetate protocol prior to IFAT screening (see 3.2.3 – Detection and enumeration of Cryptosporidium oocysts by immunofluorescence microscopy) to confirm their negativity.

3.2.1.d – Spiking faeces with enumerated oocysts
To minimise the loss of oocysts by transferring the spiked faeces, the mixing of oocysts with faeces was undertaken in the mixing chamber of the Parasep® faecal parasite concentrator (Intersep Filtration Systems). Stock oocysts were mixed on a vortex for 30 seconds to ensure a homogenous suspension. An aliquot containing the number of oocysts required for each experiment (Figures 3.3 and 3.4) was added to a 1g sample of sheep or cattle faeces in the mixing chamber and the suspension thoroughly mixed with the faeces using a wooden swabstick and by vortexing the sample at maximum speed. The samples were then left at 4°C overnight before mixing once more by maximum speed vortex.

3.2.2 – Formol-ethyl acetate concentration of Cryptosporidium oocysts from sheep and cattle faeces
A 6ml aliquot of 10% formol water was added to the 1g of faeces in the mixing chamber of a Parasep® faecal parasite concentrator (Intersep Filtration systems) and homogenised with a wooden swabstick. Ethyl acetate (2ml) was then added to the formal-faecal slurry and the Parasep® container sealed before thoroughly mixing the samples by vortex at maximum speed for 30 seconds. The Parasep® concentrator was then inverted and spun in a centrifuge (PK130, DJB Labcare) until 450g was reached, at which point the centrifuge was stopped with the brake off. The supernatant layer directly above the sediment was then carefully aspirated, dispensed into a clean 15ml tube and the remaining sample (ethyl-acetate, fatty plug and debris) disposed of in vermiculite (Figure 3.2). Deionised water was then added to the supernatant to a volume of 10ml and the sample spun in a centrifuge (PK130, DJB Labcare) at 1000g for 10 minutes. The majority of supernatant was then aspirated to leave a concentrated sample pellet. Following concentration, the dilution factor (D) was
calculated from the volume of the pellet (P) and the total volume of remaining supernatant and pellet (T) using the formula:

\[ D = \frac{P}{T} \]

The number of oocysts counted in each well \( (O_{\text{counted}}) \) was then adjusted for the dilution factor to give the total number of oocysts per well following concentration \( (O_{\text{total}}) \) using the formula:

\[ O_{\text{total}} = \frac{O_{\text{counted}}}{D} \]

Figure 3.2: A diagrammatic representation of the Parasep® faecal concentrator, used in the modified formol-ethyl acetate method, showing the oocyst-containing layer which is removed for further concentration.
3.2.3 – Detection and enumeration of Cryptosporidium oocysts by immunofluorescence microscopy

A thin layer of sample (faeces or faecal concentrate) was evenly smeared into a well of a 4-well slide (Hendley-Essex), allowed to dry at 40°C and fixed with 50μl of methanol. To each of the dry wells, 30μl of fluorescein-labelled Cryptosporidium specific monoclonal antibody (Mab) (TCS Water Biosciences, UK) was added and the slides incubated in a humid chamber at 37°C for 45 minutes. Excess Mab was aspirated from the side of each well, a single drop of 4’,6-diamidino-2-phenylindole (DAPI) (Sigma) added and incubated for two minutes at room temperature. The DAPI was aspirated as before and a single drop of water applied to each well before immediately being aspired and the slide allowed to air dry. A 10μl drop of glycerol mounting medium containing DABCO as an anti-fadant was added to each well and a 22mm x 50mm coverslip applied. Coverslips were sealed with clear nail varnish and slides stored in darkness at 4°C until screened using a Nikon E600 fluorescence microscope with a 450-490nm excitation filter (for fluorescein) and a 330-380nm excitation filter (for DAPI). Samples were examined using a x40 objective and the oocysts present in each sample enumerated.

3.2.4 – Detection of Cryptosporidium oocysts by enzyme linked immunosorbent assay (ELISA)

Two commercially bought Cryptosporidium ELISA kits (Bio-X Diagnostics, Belgium and Institute Pourquier, France) were tested as a method of detecting oocysts in sheep and cattle faeces concentrated by the formol-ethyl acetate protocol. These two kits were selected based on the belief that the detected antigen was not soluble. Each ELISA was undertaken as described in the manufacture instructions. Briefly, the faecal concentrate was diluted in the supplied dilution buffer and 100μl deposited into each well of the ELISA plate, including a positive and negative control. The plates were incubated for one hour at room temperature before the wells were washed three times with the supplied washing solution. A 100μl aliquot of conjugate was added to each well and the plate incubated again for one hour at room temperature before being washed three times as before. The addition of 100μl of substrate to each well was followed by a further 10 minute incubation in darkness at room temperature before
50μl of the supplied stop solution was added to each well. The optical densities of the samples were read at 450nm on an Anthos hII plate reader (Anthos Labtech).

3.2.5 – Evaluation of the formol-ethyl acetate concentration protocol for detection by immunofluorescence microscopy

3.2.5.a – Level of oocyst concentration achieved from sheep and cattle faeces
To assess the amount of concentration obtained by the formol-ethyl acetate protocol, 1g of faeces was seeded with 1x10^5 oocysts and the oocysts enumerated prior to and after concentration by immunofluorescence microscopy. Faeces from both the selected ovine and bovine samples were used and spiking was carried out in triplicate, whilst five replicates were screened from each spiked sample (Figure 3.3).

3.2.5.b – Limit of oocyst detection with sheep and cattle faeces
To determine the limit of detection for the formol-ethyl acetate concentration followed by immunofluorescence microscopy, 1g samples of faeces from both ovine and bovine sources were seeded with different numbers of oocysts (Figure 3.4). The different ranges of oocyst numbers used to spike the sheep and cattle faeces were identified through preliminary screening, as the limit of detection is known to vary in each type of faecal sample. Once again five replicates of each faecal sample were screened by immunofluorescence microscopy to determine the level at which all replicates were positive.

3.2.6 – Evaluation of the formol-ethyl acetate concentration protocol for detection by ELISA
To assess the potential detection by ELISA following the formol-ethyl acetate concentration, 1g samples of ovine and bovine faeces were seeded with different numbers of oocysts (Table 3.1). Five replicates of each sample were used in the ELISA. In addition to the seeded samples, positive control samples (one supplied with each kit, one known positive sample submitted to the CRU tested with and without concentration and purified suspensions of 10^5, 10^4 and 10^3 oocysts) and negative control samples (one known negative sample and one deionised water blank) were included.
**Figure 3.3:** An algorithm of the experiment to determine the level of concentration achieved using the formol-ethyl acetate protocol with sheep and cattle faeces.

- **Sheep Faeces**
  - 3 replicates spiked with $1 \times 10^3$ oocysts
  - 15 IFATs (5 from each replicate) for oocyst enumeration

- **Cattle Faeces**
  - 3 replicates spiked with $1 \times 10^3$ oocysts
  - 15 IFATs (5 from each replicate) for oocyst enumeration

**Formol-ethyl acetate concentration**

**Figure 3.4:** An algorithm of the experiment to determine the limit of oocyst detection in sheep and cattle faeces using formol-ethyl acetate concentration and immunofluorescence microscopy.
Table 3.1: Samples used to evaluate the use of ELISA following concentration by formol-ethyl acetate concentration

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of oocysts seeded into sample</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep faeces</td>
<td>2.5x10^3</td>
<td>5</td>
</tr>
<tr>
<td>Sheep faeces</td>
<td>2.0x10^3</td>
<td>5</td>
</tr>
<tr>
<td>Sheep faeces</td>
<td>1.5x10^3</td>
<td>5</td>
</tr>
<tr>
<td>Sheep faeces</td>
<td>1.0x10^3</td>
<td>5</td>
</tr>
<tr>
<td>Sheep faeces</td>
<td>0.5x10^3</td>
<td>5</td>
</tr>
<tr>
<td>Cattle faeces</td>
<td>2.5x10^3</td>
<td>5</td>
</tr>
<tr>
<td>Cattle faeces</td>
<td>2.0x10^3</td>
<td>5</td>
</tr>
<tr>
<td>Cattle faeces</td>
<td>1.5x10^3</td>
<td>5</td>
</tr>
<tr>
<td>Cattle faeces</td>
<td>1.0x10^3</td>
<td>5</td>
</tr>
<tr>
<td>Cattle faeces</td>
<td>0.5x10^3</td>
<td>5</td>
</tr>
<tr>
<td>Kit positive control</td>
<td>N/A</td>
<td>1 per plate</td>
</tr>
<tr>
<td>Non-concentrated positive faeces</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>Concentrated positive faeces</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>10^5 oocysts in suspension</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
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<tr>
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</table>

N/A – No oocysts added to the sample

3.2.7 – Statistical analysis

The descriptive statistics, one-way ANOVA and paired-sample t-test analyses were undertaken using SPSS 12.0.1 for Windows (SPSS Inc.). The one-way ANOVA generated a variation ratio (F) at the calculated degrees of freedom (df), which when compared with the critical value from an F-distribution table indicates whether the difference in mean counts were statistically significant (p<0.05). The paired-sample t-test generated a t-value (t) at the calculated degrees of freedom (df), which when compared with a t-distribution table indicates whether the difference in means was statistically significant (p<0.05).

3.3 – Results

3.3.1 – Evaluation of the formol-ethyl acetate concentration protocol for detection by immunofluorescence microscopy

3.3.1.a – Level of oocyst concentration achieved from sheep and cattle faeces

The formol-ethyl acetate protocol increased the concentration of oocysts in all of the samples (Table 3.2). The mean counts for the three replicates of each faecal sample from either a sheep or a cow were calculated prior to the concentration (Table 3.2) to confirm that the replicates were not statistically different from each other by one-way
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<th>Mean per Replicate</th>
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</table>

Table 3.2: Oocyte counts and means for the fecal samples prior to and following concentration using the formal-ethyl acetate protocol.
ANOVA (sheep replicates: F=0.058, df=2, p=0.994; cow replicates: F=0.350, df=2, p=0.712). Following the concentration, the mean counts for each replicate from either a sheep or cow were also found to be not significantly different when compared using one-way ANOVA (sheep replicates: F=3.778, df=2, p=0.053; cow replicates: F=2.650, df=2, p=0.111). The mean number of oocysts counted from either sheep or cow faeces was calculated prior to and following concentration (Table 3.2). The number of oocysts detected from both sheep and cattle faeces was greater following concentration (sheep faeces = 5.3-fold increase; cow faeces = 2.6 fold-increase).

These mean increases in concentration were both highly significant when compared using the paired-sample t-test (sheep faeces: t=−10.710, df=14, p=0.000; cow faeces: t=−26.629, df=14, p=0.000). In addition to this significant increase, when the counts are adjusted to remove any dilution effect between the concentrate and any remaining supernatant the level of concentration rises to 16.0-fold for typical sheep faeces and 5.9-fold for typical cattle faeces.

3.3.1.b – Limit of oocyst detection from sheep and cattle faeces
Each of the five replicates of sheep and cow faeces seeded with different concentration of oocysts were screened for the presence of oocysts (Table 3.3). For the seeded typical sheep faeces, the limit of detection with a sensitivity of 100% was 3\times10^3 oocysts g^{-1} (Table 3.3). The limit of detection with the same sensitivity for the seeded typical cow faeces was 2\times10^3 oocysts g^{-1} (Table 3.3). Oocysts were also detected in the faecal samples from both species at a level of 0.5\times10^3 oocysts g^{-1}, although the sensitivity was reduced to 60% with cow faeces and 20% with sheep faeces.

3.3.2 – Evaluation of the formol-ethyl acetate concentration protocol for detection by ELISA
The results from both kits that were tested gave the same results. All of the seeded samples that were concentrated and screened by ELISA were negative and therefore the formol-ethyl acetate method combined with ELISA detection had a sensitivity of 0% (Table 3.4). The positive controls supplied with each kit and the non-concentrated known positive sample were both positive. However, the concentrated known positive sample and the 10^2, 10^4 and 10^3 oocysts suspensions were all negative, as were the negative controls.
Table 3.3: Limits of oocyst detection for formol-ethyl acetate concentration of sheep and cattle faeces with immunofluorescence microscopy

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<thead>
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<th>Sample</th>
<th>Oocysts</th>
<th>Replicates</th>
<th>Sensitivity</th>
</tr>
</thead>
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<td>1</td>
<td>2</td>
</tr>
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<td>Positive</td>
<td>Positive</td>
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</tr>
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<tr>
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<td>0.5x10^3</td>
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</tr>
<tr>
<td>Cattle Faeces</td>
<td>2.5x10^3</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
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<td>2.0x10^3</td>
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Table 3.4: ELISA detection of Cryptosporidium following concentration with the formol-ethyl acetate protocol

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<td>Cattle Faeces</td>
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3.4 – Discussion

There are often large numbers of oocysts during clinical cryptosporidiosis, but as the infection begins to tail off the numbers decrease, and as many people with diarrhoea don’t seek advice from the general practitioner for several days, by the time a sample is taken and sent to the laboratory the number of oocysts can be fairly low. In addition to clinical infections, there is also evidence of subclinical/non-symptomatic infections in humans, livestock and wild animals that only produce low numbers of oocysts (Fayer et al., 2000b; Xiao et al., 1994; Kotler et al., 1990). To detect Cryptosporidium in these samples, sensitive methods with a low threshold of detection are required.

A number of different staining techniques have been described for detecting Cryptosporidium, but from the comparative studies that have been undertaken those using immunofluorescence was consistently superior (Alles et al., 1995; Arrowood and Sterling, 1989; Garcia et al., 1992; Garcia et al., 1987; Garcia and Shimizu, 1997; MacPherson and McQueen, 1993; Mtambo et al., 1992; Quilez et al., 1996; Rusnak et al., 1989; Stibbs and Ongerth, 1986). The main disadvantage of using immunofluorescence microscopy to screen faeces is the likelihood of missing other parasites in the sample as the labelled antibodies are Cryptosporidium-specific and the background is dark (Arrowood, 1997). However, this is outweighed by the advantage of increased sensitivity and specificity, which reduces the misidentification of artefacts, which can occur with the chemical stains.

Whilst immunomagnetic separation will give the greatest recoveries of all the concentration procedures (Davies et al., 2003; Pereira et al., 1999; Rochelle et al., 1999a), the high cost makes them impractical for use in large-scale studies. Of the different, cost-effective, concentration methods used for Cryptosporidium recovery prior to detection, formol-ether and formol-ethyl acetate had the greatest recoveries (Casemore et al., 1985; Bukhari and Smith, 1995; McNabb et al., 1985; Mtambo et al., 1992), particularly when modified to consider the sedimentation properties of Cryptosporidium oocysts (Arrowood, 1997; Casemore et al., 1985). In addition, the availability of commercial concentrators with even greater recovery rates than the standard FEA procedure makes these methods desirable (Perry et al., 1990; Zierdt, 1984).
Although immunofluorescent microscopy combined with FEA concentration may be the most sensitive method compared with the alternatives, they have still been found to have relatively low sensitivities due to a high threshold of detection. Weber et al. (1991) determined the threshold of detection for acid-fast staining and immunofluorescence following concentration by the standard formol-ethyl acetate sedimentation (FEA) (Young et al., 1979). In their study, the threshold of detection in very watery stool with a sensitivity of 100%, was 10,000 oocysts gram⁻¹ (opg) for both methods but for formed stools this rose to 50,000 opg by IF and 500,000 opg for the AF method. In the fluid stool, sensitivities of the IF and AF methods were reduced to 90% and 60% respectively at a level of 5,000 opg and in the formed faeces this was even worse at 0% in samples with less than 100,000 opg by AF and 20% at 5,000 opg for IF (Weber et al., 1991). The loss of a substantial number of oocysts was recorded at each stage of the FEA method. However, the number of oocysts observed by IF in concentrated formed faecal samples containing 100,000 opg was seven times higher than in non-concentrated samples (Weber et al., 1991). In my study, the number of oocysts detected in concentrated faeces (100,000 opg) processed by the modified Parasep® FEA procedure increased by 5.3 and 2.6 times for sheep and cattle faeces, respectively, with a further increase when the sediment is not diluted to 16 and 5.9 times the non-concentrated number. One of the limitations in my experiment was the dilution factor as it reduced the level of concentration and would also likely increase the threshold of detection. Therefore, the removal of as much of the supernatant as possible would be preferable, but this may result in increased background debris that could inhibit screening.

Weber et al. (1992) attempted to lower the threshold of detection with various modifications to the FEA procedure and published a new method using the FEA stage in a single step without the gauze filtration followed by salt flotation and a final washing stage. This new method, combined with IF screening, improved the sensitivity in watery faeces from 90% to 100% at 5,000 opg and in formed faeces the detection threshold with a sensitivity of 100% was lowered from 50,000 opg to 10,000 opg. The sensitivity of their new method with 5,000 opg of formed faeces was also increased from 20% to 80% by IF detection (Weber et al., 1992). However, despite the increased sensitivity and threshold of detection in this new method, extra steps were involved which would likely result in oocyst losses (during the salt flotation and
washing stages). During the evaluation of their new method, the authors also tested the use of Sheather's sugar and zinc sulphate flotation prior to IF screening, but found that these were not as sensitive as the FEA since they could not detect any oocysts at 50,000 opg in the formed stools (Weber et al., 1992). Clavel et al. (1996) found that an extended centrifugation step increased the sensitivity when screening by mZN. However, this centrifugation step was found by Weber et al. (1992) to increase the background debris so much that screening by IF was not possible. My modified FEA procedure using the Parasep® Faecal Parasite Concentrator was found to have a lower limit of detection with 100% sensitivity than in both publications by Weber et al. (1992; 1991) as 3,000 opg were detected from sheep faeces and 2,000 opg from cattle faeces. Oocysts were also detected in both cattle and sheep faeces containing 500 opg, although the sensitivity was reduced to 60% and 20% respectively. A further limitation to my experiments was the number of replicates, which only allowed the measurement of sensitivity in 20% increments. An increased number of replicates would allow a more accurate measure of sensitivity, and would be particularly useful around the limit of detection with sensitivities between 80% and 100%.

Another important issue that was demonstrated by Weber et al. (1991) was how the consistency of the faecal sample affects the detection threshold of the procedure. They found that the sensitivity of the FEA combined with either the AF and IF detection was greater with fluid faeces than with formed faeces. The reason for this is the increased amount of particulate matter in formed faeces results in more sediment, which in turn decreases the concentration of oocysts. The increased presence of fatty molecules and mucus is also likely to affect the concentration of faeces, as it is harder to free the oocysts into the faecal suspension. As faeces from various animal sources have different consistencies (i.e. levels of fat content and vegetable matter) depending upon their diet and physiology, it is not surprising that the sensitivity of the modified Parasep® FEA method was different with sheep and cattle faeces. However, as the formol-ethyl acetate stage of the concentration breaks up and removes much of the lipid content then this method is suitable for use with most sample types, for example faeces from young animals still fed on colostrum or from carnivores.

Despite initial problems with the first generation enzyme immunoassays, comparative studies have shown that the sensitivity and specificity of more recent kits is almost as
good as IF (Kehl et al., 1995; Garcia and Shimizu, 1997; Bialek et al., 2002). A major advantage of using these EIAs, particularly those based on 96-well microplates, is number of samples that can be processed at a time. This would drastically reduce screening time and also cost in terms of technical staff time, but is only warranted if the sensitivity and specificity are at least as good as with IF. Many of the commercially available EIA kits state that they cannot be used with concentrated samples, as the antigen that they detect is water-soluble. For that reason, two kits were chosen for this study from manufacturers that informed that the target antigen was not water-soluble and could potentially be concentrated prior to testing. Unfortunately, none of the Cryptosporidium-positive concentrated samples were detected by either ELISA and therefore this modified Parasep® FAE concentration procedure is not suitable for ELISA detection with these two kits. The reason why these ELISA kits did not detect the concentrated Cryptosporidium is unknown, but it is possible that despite the manufactures assurance, these tests do detect a soluble antigen that was lost during concentration. Another explanation could be an alteration of the epitopes on the target antigen by either the saturated sodium chloride solution or ethyl acetate during concentration. As the purified oocyst suspensions were prepared by saturated sodium chloride flotation and these were also negative by the ELISAs, the ethyl acetate was not the cause of the negativity in these samples. The known positive Cryptosporidium sample was positive by ELISA prior to concentration, but negative following concentration and had not been in contact with sodium chloride. Therefore, either the antigen was water-soluble or the epitopes were sensitive to both sodium chloride and ethyl acetate.

3.5 – Conclusions

As the Parasep® Faecal Parasite Concentrator was designed for the recovery of cysts and ova the protocol was successfully adapted to recover the lower density Cryptosporidium oocysts from faecal samples (Objective 2a).

The modified FEA concentration procedure using the Parasep® concentrator was evaluated and found to significantly increase the number of oocysts detectable by immunofluorescence by up to 16 times for sheep faeces and 5.9 times for cattle faeces. This concentration technique combined with immunofluorescence microscopy has a sensitivity of 100% when 3,000 or 2,000 opg are present in sheep or cattle.
faeces, respectively. Oocysts are also detectable by this method when 500 opg are present although the sensitivity is reduced with false negatives obtained from some replicates. From the initial screening of the negative faecal samples the specificity was 100% as no false positives were identified. Therefore, the positive and negative predictive values are also 100% when more than 3,000 opg are present in sheep faeces and 2,000 opg in cattle faeces. When the number of oocysts per gram is lower than this, the sensitivity is reduced and more replicates are required to detect positive samples (Objective 2b and 2c).

The new concentration method is not compatible with screening by the two enzyme immunoassays that were tested. This is probably due to either the loss a water-soluble antigen during concentration or alteration to the antigen’s target epitopes during exposure to sodium chloride or ethyl acetate (Objective 2b).

This work has resulted in a sensitive method of detecting Cryptosporidium in faecal samples containing low numbers of oocysts, which is important in studies where non-clinical samples are being screened. The method is a valuable tool when searching for Cryptosporidium in animals with asymptomatic carriage either during surveys of individual populations or even during investigations to identify/exclude sources of environmental contamination.

3.6 – Proposed Future Work

Despite the increased sensitivity seen by the modified FAE Parasep procedure when combined with immunofluorescence microscopy, the threshold of detection is still high when compared to IMS with immunofluorescence microscopy. Further cost effective methods (such as EIAs or lower priced IMS kits) need to be developed and evaluated for screening samples that contain potentially low numbers.

Evaluation of the threshold of detection and sensitivity of enzyme immunoassays for the detection of Cryptosporidium must be undertaken using naturally infected samples that contain known numbers of oocysts, from which portions can be mixed with negative samples to produce known low numbers of oocysts g⁻¹ without having to purify the oocysts first.
The use of molecular methods that initially batch samples could also provide a fast and low cost alternative to screening large numbers of samples, particularly when a low prevalence is expected. For example, if 5 samples were combined for each PCR, then only 20 PCRs would be required to initially screen 100 samples for the presence of Cryptosporidium. If the prevalence in this sample set were 5% and each positive was in a different batch, then the maximum number of PCRs would be a further 25 to identify which individual samples were positive. This pooling approach has been taken in the study of other organisms such as Neisseria gonorrhoeae by ligase chain reaction (Kacena et al., 1998). To develop such an approach requires the sensitive detection of DNA from faeces, which is greatly affected by inhibitors present in these types of sample and is a further issue that must be addressed. The use of concentration techniques as a means of separating oocysts from inhibitors prior to PCR should be examined. A further advantage of initial screening by PCR is the immediate identification of species, which would reduce the time and cost of these studies. These methods would have to be thoroughly evaluated with a significant number of samples to ensure a sensitivity, specificity, repeatability and reproducibility at least as high as the current methods.
Chapter 4 – Maximising the recovery of DNA for molecular characterisation from oocysts of Cryptosporidium

4.1 – Introduction

Once the presence of Cryptosporidium has been detected molecular characterisation can provide more detailed information as to the species causing infection and their source (see 1.7 – Molecular Characterisation of Cryptosporidium spp.). The work described in this chapter identifies the optimal method of obtaining the maximum amount of quality DNA for use in molecular methods from samples containing low numbers of Cryptosporidium oocysts. This is particularly important when screening environmental samples or those from asymptomatic carriers of Cryptosporidium, for example during investigations into waterborne outbreaks, studies of environmental contamination or the ecology and transmission in animal populations.

Over the past decade, the use of PCR-based methods in the study of Cryptosporidium has proved vital, because of the limitations of microscopic detection due to the similar morphology between many of the species and the high thresholds of detection. Molecular methods have the potential of detecting and characterising Cryptosporidium from samples containing low numbers of oocysts, but this is entirely dependent on the following factors: the recovery of oocysts from the sample and any inhibitors, the efficient release of DNA from the oocysts and the sensitivity of the molecular technique.

In the case of samples containing high numbers of oocysts such as faeces from clinical cases, the recovery of sufficient oocysts is often relatively simple by flotation or sedimentation methods. However, these methods are not suitable for recovery from samples containing low numbers of oocysts such as environmental or sub-clinical. The use of immunomagnetic separation (IMS) has been shown to recover oocysts with great efficiency from these sample matrices (Deng et al., 1997; Hallier-Soulier and Guillot, 1999; Rochelle et al., 1999a; Sturbaum et al., 2002; Davies et al.,
### Table 4.1: Methods of Crosssectional Core Diffusion used by Researchers Prior to Extraction of DNA for use in Molecular Techniques

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze-dry cycles (76°C--98°C, 1 to 2 minutes each) in 20% Chelex</td>
<td>Johnson et al., 1995</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--242°C, 4 to 10 minutes)</td>
<td>Higgin et al., 1995</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--37°C, 5 minutes each) in TE-extract-proprase K buffer then proprase K</td>
<td>Kim et al., 1992</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--65°C, 1 minute each) in TE-LDS-proprase K buffer then proprase K</td>
<td>Suer et al., 1996</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--55°C, 1 minute each) in TE-LDS-distictional buffer then proprase K</td>
<td>Wexler et al., 1996</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--55°C, 1 minute each) in TE-distictional buffer then proprase K</td>
<td>Smith et al., 1997</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--75°C, 3 hours)</td>
<td>Leng et al., 1996</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--75°C, 3 hours)</td>
<td>Spino et al., 1997</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--75°C, 3 hours)</td>
<td>Wexler et al., 1997</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--37°C, 1 hour)</td>
<td>Johnson et al., 1999</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--75°C, 2 hours)</td>
<td>Smith et al., 1996</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--75°C, 2 hours)</td>
<td>Wexler et al., 1996</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--37°C, 2 hours)</td>
<td>Johnson et al., 1999</td>
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<tr>
<td>Freeze-dry cycles (198°C--75°C, 2 hours)</td>
<td>Smith et al., 1996</td>
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<tr>
<td>Freeze-dry cycles (198°C--75°C, 2 hours)</td>
<td>Wexler et al., 1996</td>
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<td>Freeze-dry cycles (198°C--37°C, 2 hours)</td>
<td>Johnson et al., 1999</td>
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<tr>
<td>Freeze-dry cycles (198°C--75°C, 2 hours)</td>
<td>Smith et al., 1996</td>
</tr>
<tr>
<td>Freeze-dry cycles (198°C--75°C, 2 hours)</td>
<td>Wexler et al., 1996</td>
</tr>
</tbody>
</table>
2003) and has the advantage of leaving behind the many molecular inhibitors that may be present in these samples (see 1.5 – Recovery of *Cryptosporidium* spp.).

Obtaining the maximum amount of DNA from the tough oocysts is the vital stage in any molecular procedure, as the oocyst recovery and sensitivity of method will not affect the result if the DNA is not available for use. The *Cryptosporidium* oocyst wall is known to protect the parasite from chemical and mechanical disruption, enabling long-term survival in harsh environmental conditions (Robertson *et al.*, 1992). Attempts to free the DNA or fragile sporozoites from the protective shell usually involve either the physical disruption (chemical or mechanical) or by encouraging excystation (Table 4.1)(Wiedenmann *et al.*, 1998).

The sensitivity of a molecular method can only be assessed in the first place with the maximum recovery of DNA from oocysts, as the sensitivity will reduce when combined with a less efficient method of releasing DNA. Therefore, the use of these methods, for initial detection or characterisation, is entirely dependent on the availability of good quality DNA. Despite the importance of this, there is a lack of standard methodology for the disruption and extraction of DNA from samples, particularly when low numbers of oocysts may be involved. Although Proteinase K digestion or various cycles of freeze-thawing oocysts are the most commonly used techniques, most researchers appear to use their own variation (Table 4.1), with different times, temperatures or buffers.

**Objective 3:** To compare a number of approaches of oocyst disruption and identify the optimal method for the maximum release of quality DNA, particularly from low numbers of oocysts, prior to characterisation by molecular methods.

**Objective 3a:** To identify variations of commonly used oocyst disruption methods from the literature.

**Objective 3b:** To develop and evaluate a SYBR Green I real-time PCR to help assess which of the methods resulted in the highest amount of usable DNA.
Objective 3c: To evaluate (by microscopy and real-time PCR) the identified disruption methods using high numbers of oocysts and determine which have the best potential for use in samples with low numbers of oocysts.

Objective 3d: To evaluate the methods with greatest potential with samples containing low numbers of oocysts and in the presence of immunomagnetic beads, which are likely to be present in actual samples.

4.2 – Methods and Materials

4.2.1 – Preparation and evaluation of Cryptosporidium oocyst suspensions for use in the oocyst disruption and DNA recovery experiments

4.2.1.a – Source of Cryptosporidium oocysts
Oocysts were sourced from human faecal samples submitted to the Cryptosporidium Reference Unit as described previously (see 3.2.1.a – Source of Cryptosporidium oocysts).

4.2.1.b – Preparation of stock oocyst suspensions
Samples that were found to contain high numbers of oocysts (more than 5-10 oocysts per field) were selected to produce stock oocyst suspensions by saturated salt flotation as previously described (see 3.2.1.b – Preparation of stock oocyst suspensions).

4.2.1.c – Effect of storage at 4 °C on oocysts and potential for excystation
The wide range of oocyst disruption methods could not be carried out in a single day but over a four-day period. As all of the suspensions needed to be the same at the time of disruption, the effect of 4 °C storage on the concentration of oocyst suspensions was examined. Stock oocysts were mixed on a vortex for 30 seconds to ensure a homogenous suspension. An aliquot of 1x10⁵ oocysts were added to 1ml of deionised water to produce a working suspension of 1x10⁵ oocysts ml⁻¹, which was counted 10 times to confirm the concentration as previously described (see 3.2.1.b –
Preparation of stock oocyst suspensions). This suspension was then stored at 4°C and the concentration checked, at the same time on days 1-5, day 8, day 15 and day 22.

4.2.1.d – Effect of centrifugation to concentrate oocysts prior to microscopy
A 1x10⁵ oocysts ml⁻¹ working oocyst suspension was prepared in deionised water and counted 10 times to confirm the concentration. An aliquot of 210μl was dispensed into two 1.5ml screw-capped tubes and one sample heated for 60 minutes at 100°C to physically disrupt some of the oocysts. A 10μl aliquot was then taken from each sample and loaded into an improved Neubauer Haemocytometer and the oocyst concentration (oocyst ml⁻¹) calculated (see 3.2.1.b – Preparation of stock oocyst suspensions). The remaining 200μl of each sample was exposed to a centrifugal force of 1,000g for ten minutes, before the supernatant was aspirated and the pellet re-suspended in 10μl. The concentration of oocysts was then calculated for these samples as before.

4.2.2 – Concentration and purification of DNA released from oocysts
DNA was recovered from suspensions using the commercially available QIAamp DNA Mini Kit (Qiagen Ltd., UK), which is widely used and accepted throughout the molecular biology industry. The initial steps of the procedure varied depending upon the oocyst disruption method used (e.g. volumes and addition of Buffer ATL, Buffer AL and 96-100% ethanol, see the relevant method below). Following the addition and mixing of 96-100% ethanol, the samples were carefully loaded onto the QIAamp spin column as described in the manufacturer's instructions and spun in a centrifuge (Centrifuge 541C, Eppendorf) for one minute at 6,000g. If the sample volume was too great to load in a single application, the filtrate was discarded and the remaining sample loaded in the same manner. Once loaded, the filtrate was again discarded and 500μl of Buffer AW1 added and washed through the column in the microfuge at 6,000g for one minute. The filtrate was discarded once more and 500μl of Buffer AW2 washed through the column at 20,000g for 4 minutes. Columns were carefully placed into a 1.5ml plastic tube, 100μl of Buffer AE added and incubated at room temperature for five minutes. DNA was then eluted from the column at 6,000g for one minute in the microfuge and the suspensions stored at -20°C.
4.2.3 – Development of a SYBR Green I real-time PCR assay to compare the amount of DNA obtained following the different oocyst disruption methods

4.2.3.a – Evaluation and optimisation of an adapted SSU rDNA PCR

To compare the amount of DNA released by the different oocyst disruption methods, a single PCR method was adapted from the secondary PCR described by Xiao et al. (2000a) (See 4.2.5.g – Nested SSU rDNA PCR) for use on a real-time PCR platform. To check that the magnesium (MgCl₂) and primer concentrations for this adapted PCR were still optimum when run with DNA as a template instead of primary PCR product, a chequerboard experiment was carried out (Table 4.2). In addition to the relevant concentrations of MgCl₂, forward primer and reverse primer (Table 4.2), the 50μl PCR reaction mixture contained 10x PCR buffer (Qiagen Ltd., UK), 200 mM of each deoxynucleotide triphosphate, 2.5U of HotStar Taq DNA polymerase (Qiagen Ltd., UK) and 10μl of template DNA. The cycling conditions for the PCR were an initial step of 15 minutes at 95°C to activate the HotStar Taq followed by 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 60 seconds before a final extension of 7 minutes at 72°C using a Dyad DNA Engine thermal cycler (MJ Research). The PCR products were run on a 2% agarose gel at 110V for 60 minutes, visualised by SYBR Green I (Sigma) staining and images recorded using a digital imaging system (AlphaImager, Alpha Innotech).

Table 4.2: Concentrations of MgCl₂, forward primer and reverse primer in each sample

<table>
<thead>
<tr>
<th>MgCl₂ Concentration (mM)</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
<th>3.0</th>
<th>3.5</th>
<th>4.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of each primer (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>Sample 1</td>
<td>Sample 2</td>
<td>Sample 3</td>
<td>Sample 4</td>
<td>Sample 5</td>
<td>Sample 6</td>
</tr>
<tr>
<td>100</td>
<td>Sample 7</td>
<td>Sample 8</td>
<td>Sample 9</td>
<td>Sample 10</td>
<td>Sample 11</td>
<td>Sample 12</td>
</tr>
<tr>
<td>150</td>
<td>Sample 13</td>
<td>Sample 14</td>
<td>Sample 15</td>
<td>Sample 16</td>
<td>Sample 17</td>
<td>Sample 18</td>
</tr>
<tr>
<td>200</td>
<td>Sample 19</td>
<td>Sample 20</td>
<td>Sample 21</td>
<td>Sample 22</td>
<td>Sample 23</td>
<td>Sample 24</td>
</tr>
<tr>
<td>250</td>
<td>Sample 25</td>
<td>Sample 26</td>
<td>Sample 27</td>
<td>Sample 28</td>
<td>Sample 29</td>
<td>Sample 30</td>
</tr>
</tbody>
</table>
To test the limit of detection of this adapted PCR, DNA was extracted from 200µl of a 5.25x10^4 oocyst ml⁻¹ oocyst suspension, eluted in 100µl (see 4.2.1.b – Preparation of stock oocyst suspensions and 4.2.2 – Concentration and Purification of DNA released from oocysts) and a dilution series (Table 4.3) prepared in deionised water. From each of these dilutions, 10µl was used as the template in the adapted SSU rDNA PCR.

### Table 4.3: DNA dilution series to test the limit of detection for the adapted SSU rDNA PCR

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Number of oocysts ml⁻¹ equivalent</th>
<th>Number of oocysts equivalent per PCR (10µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.25x10⁴</td>
<td>1.05x10³</td>
</tr>
<tr>
<td>1:10</td>
<td>5.25x10³</td>
<td>1.05x10²</td>
</tr>
<tr>
<td>1:100</td>
<td>5.25x10²</td>
<td>1.05x10¹</td>
</tr>
<tr>
<td>1:1,000</td>
<td>5.25x10¹</td>
<td>1.05x10⁰</td>
</tr>
<tr>
<td>1:10,000</td>
<td>5.25x10⁰</td>
<td>1.05x10⁴</td>
</tr>
<tr>
<td>1:100,000</td>
<td>5.25x10⁻¹</td>
<td>1.05x10⁻²</td>
</tr>
<tr>
<td>1:1,000,000</td>
<td>5.25x10⁻²</td>
<td>1.05x10⁻³</td>
</tr>
</tbody>
</table>

**4.2.3.b – Development of a SYBR Green I master mix for SSU rDNA real-time PCR and dissociation curve analysis**

To detect the amplification of Cryptosporidium parvum DNA in real-time a fluorescent dye that will emit more fluorescence as the amount of DNA increases (either a DNA intercalating dye or a specific probe with a dye attached) must be present in the reaction mix. For this experiment the DNA intercalating dye, SYBR Green I (Sigma), was selected. The dilutions of the SYBR Green I 1X working solution (stock solution supplied as x10,000) that were tested with this PCR reaction mixture were 1/1, 1/10, 1/50 and 1/100. Several dilutions were tested as the SYBR Green is supplied in DMSO, which can inhibit the PCR reaction if too concentrated. In addition to the SYBR Green I, the 50µl reaction mixtures also contained 10x PCR buffer (Qiagen Ltd., UK), 3mM MgCl₂, 200 mM of each deoxynucleotide triphosphate, 200nM of forward primer (5' - GGAAGGTTGTATTATTAGATAAA

110
G-3’), 200nM of reverse primer (5’-AAGGAGTAAGGAAACCTCCA-3’), 2.5U of HotStar Taq DNA polymerase (Qiagen Ltd., UK) and 10μl of template DNA. The cycling conditions were the same as described above (see - 4.2.3.a – Evaluation and optimisation of an adapted SSU rDNA PCR) and tested initially on a Dyad DNA Engine thermal cycler (MJ Research). The products were separated on a 2% agarose (Phorecs, Biogene Ltd.) gel at 110V for 60 minutes and recorded using a digital imaging system (Alphalmager, Alpha Innotech). Reaction mixtures with SYBR Green concentrations that amplified Cryptosporidium DNA were then tested on a Stratagene Mx3000p (Stratagene) with an initial step of 15 minutes at 95°C to activate the HotStar Taq before 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 60 seconds and the results visualised in real-time. Following the amplification of PCR products the Mx3000 was also used to produce a dissociation curve for Cryptosporidium.

4.2.3.c – Production of a standard curve to evaluate the performance of the Cryptosporidium SSU rDNA real-time PCR

The production of a standard curve for this SSU rDNA real-time PCR allowed regression analysis to evaluate the accuracy of the dilutions and the determination of PCR amplification efficiency. DNA was extracted (see 4.2.2 – Concentration and purification of DNA released from oocysts) from 200μl of a stock oocyst suspension, containing 1.43x10^6 oocysts ml^-1 (enumerated as previously described, see 4.2.1.b – Preparation of stock oocyst suspensions), as described in the Cryptosporidium Reference Unit’s standard protocol. This method uses a 60 minute incubation at 100°C to disrupt oocysts prior to the QIAamp DNA Mini Kit Proteinase K and Buffer AL digestion and DNA purification as described in the manufacturers instructions (Qiagen Ltd., UK). The purified DNA was eluted from the QIAamp DNA mini columns in 100μl of Buffer AE, which from the initial volume added (200μl) was equivalent to 2.86x10^5 oocysts. A dilution series was produced from this DNA suspension (Table 4.4) to produce the standard curve.

The 50μl PCR reaction mixture contained 10x PCR buffer (Qiagen Ltd., UK), 3mM MgCl₂, 200 mM of each deoxynucleotide triphosphate, 200nM of forward primer (5’-GGAAGGGTTGTATTTATTAGATAAAG-3’), 200nM of reverse primer (5’-AAGG
Table 4.4: DNA dilution series used to draw a standard curve for the evaluation of the SSU rDNA real-time PCR amplification efficiency

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Equivalent number of oocysts ml⁻¹</th>
<th>Equivalent number of oocysts 200µl⁻¹</th>
<th>Equivalent number of oocysts per PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.43x10⁶</td>
<td>2.86x10⁵</td>
<td>2.86x10⁴</td>
</tr>
<tr>
<td>1:5</td>
<td>2.86x10⁴</td>
<td>5.72x10⁴</td>
<td>5.72x10³</td>
</tr>
<tr>
<td>1:10</td>
<td>1.43x10⁴</td>
<td>2.86x10⁴</td>
<td>2.86x10³</td>
</tr>
<tr>
<td>1:50</td>
<td>2.86x10³</td>
<td>5.72x10³</td>
<td>5.72x10²</td>
</tr>
<tr>
<td>1:100</td>
<td>1.43x10³</td>
<td>2.86x10³</td>
<td>2.86x10²</td>
</tr>
<tr>
<td>1:500</td>
<td>2.86x10²</td>
<td>5.72x10²</td>
<td>5.72x10¹</td>
</tr>
<tr>
<td>1:1,000</td>
<td>1.43x10²</td>
<td>2.86x10²</td>
<td>2.86x10¹</td>
</tr>
</tbody>
</table>

AGTAAGGAACAACCTCCA-3'), 2.5U of HotStar Taq DNA polymerase (Qiagen Ltd., UK), 10µl of template DNA and 1:10 dilution of SYBR Green 1 1X working solution. The cycling conditions for the PCR were an initial step of 15 minutes at 95°C to activate the HotStar Taq, before 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 60 seconds using a Stratagene Mx3000p real-time PCR platform (Stratagene) and the threshold cycle (Ct) values recorded. Following the PCR, a dissociation curve was produced for each sample to confirm that the increase in fluorescence was due to the amplification Cryptosporidium DNA and not PCR artefacts. The production of the standard curve, regression analysis and amplification efficiency of the PCR were calculated using the Stratagene Mx3000p software (Stratagene).

4.2.4 – Initial evaluation of oocyst disruption methods for the maximum recovery of Cryptosporidium DNA (Table 4.5)

4.2.4.a – Preparation of samples

An enumerated stock oocyst suspension was mixed for 30 seconds on a vortex to homogenise the suspension. An aliquot of 5x10⁶ oocysts were added to 50ml of deionised water to give a working concentration of 1x10⁵ oocysts ml⁻¹. From this working solution, 400µl aliquots were dispensed into 1.5ml screw-capped tubes ready for exposure to the 40 different oocyst disruption conditions (Table 4.5) in triplicate.
<table>
<thead>
<tr>
<th>Method of Oocyst Disruption</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Proteinase K and Buffer AL digestion at 56°C for 16 hours</td>
</tr>
<tr>
<td>2. Proteinase K and Buffer ATL digestion at 37°C for 10 minutes then Buffer AL at 70°C for 10 minutes</td>
</tr>
<tr>
<td>3. Proteinase K and Buffer ATL digestion at 37°C for 30 minutes then Buffer AL at 70°C for 10 minutes</td>
</tr>
<tr>
<td>4. Proteinase K and Buffer ATL digestion at 37°C for 60 minutes then Buffer AL at 70°C for 10 minutes</td>
</tr>
<tr>
<td>5. Proteinase K and Buffer ATL digestion at 37°C for 16 hours then Buffer AL at 70°C for 10 minutes</td>
</tr>
<tr>
<td>6. Proteinase K and Buffer ATL digestion at 56°C for 10 minutes then Buffer AL at 70°C for 10 minutes</td>
</tr>
<tr>
<td>7. Proteinase K and Buffer ATL digestion at 56°C for 30 minutes then Buffer AL at 70°C for 10 minutes</td>
</tr>
<tr>
<td>8. Proteinase K and Buffer ATL digestion at 56°C for 60 minutes then Buffer AL at 70°C for 10 minutes</td>
</tr>
<tr>
<td>9. Proteinase K and Buffer ATL digestion at 56°C for 16 hours then Buffer AL at 70°C for 10 minutes</td>
</tr>
<tr>
<td>10. 1 x Freeze-Thaw cycle (-78°C ↔ 37°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>11. 3 x Freeze-Thaw cycles (-78°C ↔ 37°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>12. 5 x Freeze-Thaw cycles (-78°C ↔ 37°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>13. 7 x Freeze-Thaw cycles (-78°C ↔ 37°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>14. 10 x Freeze-Thaw cycles (-78°C ↔ 37°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>15. 1 x Freeze-Thaw cycle (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>16. 3 x Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>17. 5 x Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>18. 7 x Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>19. 10 x Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>20. Heating at 100°C for 10 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
<tr>
<td>21. Heating at 100°C for 30 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes</td>
</tr>
</tbody>
</table>
22. Heating at 100°C for 60 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes
23. Trypsin digestion at 37°C for 10 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes
24. Trypsin digestion at 37°C for 30 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes
25. Trypsin digestion at 37°C for 60 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes
26. Trypsin digestion at 37°C for 16 hours followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes
27. Sonication (50/60 Hz, 80W) for 1 minute followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes
28. Sonication (50/60 Hz, 80W) for 5 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes
29. Sonication (50/60 Hz, 80W) for 10 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes
30. Sonication (50/60 Hz, 80W) for 15 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes
31. Sonication (50/60 Hz, 80W) for 20 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes
32. Vortexed Zirconia beads for 2 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

4.2.4.b – Control samples
The triplicate control samples tested for the presence of background Cryptosporidium DNA. A 200µl aliquot of the oocyst suspension was dispensed to a new 1.5ml tube and centrifuged at 1,000g for ten minutes before the supernatant was aspirated and the pellet re-suspended in 10µl for examination by DIC microscopy. The remaining 200µl suspensions were not exposed to any of the tested disruption methods but had to be in the presence of Buffer AL (Qiagen Ltd., UK) in order to bind to the DNA purification column. Buffer AL prepares the sample to the correct salt content and pH for the DNA to bind. However, Buffer AL can also disrupt cells itself due to the high salt content, but the free DNA in the samples will be ready to bind to the columns on contact with the buffer [Qiagen Technical Support]. Therefore, 200µl of Buffer AL was mixed with the sample, immediately followed by the addition and mixing of 200µl of ethanol and loading onto the QIAamp DNA mini column (Qiagen Ltd., UK). In addition to the DNA, proteins and carbohydrates that were present in the suspension could also bind to and block the column, as they did not have time to
break down. If no Cryptosporidium DNA was detected from the controls it could therefore mean that either no free Cryptosporidium DNA was present or that the column was blocked and no DNA was eluted. To ensure that the column did not block with these components, the suspension was spiked with Toxoplasma gondii DNA so in the event of a negative Cryptosporidium DNA result the presence of eluted DNA could be checked using a T. gondii specific PCR. DNA was purified on the column, eluted and stored as described above (see 4.2.2 – Concentration and Purification of DNA released from oocysts).

4.2.4.c – Freeze-Thaw Cycling
The 400μl suspensions were submerged in a super cooled methanol bath of -78°C (a slurry of dry ice and methanol) for five minutes. Samples were immediately transferred to a heating block set at either 37°C or 100°C for a further five minutes. Samples were subjected to one, three, five, seven or ten cycles of freeze-thaw treatment. A 200μl aliquot of the oocyst suspension was then dispensed to a new 1.5ml tube and prepared for examination by DIC as described previously (see 4.2.4.b – Control Samples). To prepare the DNA in the remaining 200μl of suspension for binding to the purification columns, 20μl of Proteinase K and 200μl of Buffer AL was added. Suspensions were thoroughly vortexed at maximum speed, incubated at 56°C for 10 minutes and 200μl of 96-100% ethanol mixed with the sample prior to loading onto the QIAamp DNA mini column (Qiagen Ltd., UK). DNA was purified on the column, eluted and stored as described above (see 4.2.2 – Concentration and Purification of DNA released from oocysts).

4.2.4.d – Heating
The 400μl suspensions were placed in heating blocks set at 100°C for either 10, 30, or 60 minutes. A 200μl aliquot of oocyst suspension was then dispensed to a new 1.5ml tube and prepared for examination by DIC as described previously (see 4.2.4.b – Control Samples). The remaining 200μl of suspension underwent DNA purification and concentration as previously described (see 4.2.4.c – Freeze-Thaw Cycling; 4.2.2 – Concentration and Purification of DNA released from oocysts).
4.2.4.e – Proteinase K and Buffer AL Digestion
To the 400μl suspensions, 40μl of Proteinase K (Qiagen Ltd., UK) and 400μl of Buffer AL (Qiagen Ltd., UK) were added. Suspensions were thoroughly vortexed at maximum speed and incubated in either 37°C or 56°C heating blocks for 10 minutes, 30 minutes, 60 minutes or 16 hours (overnight). A 400μl aliquot of the oocyst suspension was then dispensed to a new 1.5ml tube and prepared for examination by DIC as described previously (see 4.2.4.b – Control Samples). Ethanol (400μl) was mixed with the remaining 400μl sample prior to loading onto the QIAamp DNA mini column (Qiagen Ltd., UK). DNA was purified on the column, eluted and stored as described above (see 4.2.2 – Concentration and Purification of DNA released from oocysts).

4.2.4.f – Proteinase K and Buffer ATL Digestion
To the 400μl suspensions, 40μl of Proteinase K (Qiagen Ltd., UK) and 400μl of Buffer ATL (Qiagen Ltd., UK) were added. Suspensions were thoroughly vortexed at maximum speed and incubated in either 37°C or 56°C heating blocks for ten minutes, 30 minutes, 60 minutes or 16 hours (overnight). A 400μl aliquot of the oocyst suspension was then dispensed to a new 1.5ml tube and prepared for examination by DIC as described previously (see 4.2.4.b – Control Samples). Buffer AL (400μl) was added and incubated at 70°C for ten minutes as per the manufacturers instructions (Qiagen Ltd., UK). Following this incubation 400μl of 96-100% ethanol was mixed with the sample prior to loading onto the QIAamp DNA mini column (Qiagen Ltd., UK). DNA was purified on the column, eluted and stored as described above (see 4.2.2 – Concentration and Purification of DNA released from oocysts).

4.2.4.g – Trypsin Digestion
To the 400μl suspensions, 240μl of deionised water, 80μl of 10x buffer (5M Tris-HCl, 0.1M CaCl₂, pH 8.0) and 80μl of 2mg/ml Trypsin (Sigma, 93630) were added. Suspensions were thoroughly vortexed at maximum speed and incubated in a 37°C heating block for 10 minutes, 30 minutes, 60 minutes or 16 hours (overnight). A 400μl aliquot of the suspension was then dispensed to a new 1.5ml tube and prepared for examination by DIC as described previously (see 4.2.4.b – Control Samples). To prepare the DNA in the remaining 400μl of suspension for binding to the purification
and concentration columns, 40μl of Proteinase K and 400μl of Buffer AL was added as per the manufacturers instructions (Qiagen Ltd., UK). Suspensions were thoroughly vortexed at maximum speed, incubated at 56°C for 10 minutes and 400μl of 96-100% ethanol mixed with the sample prior to loading onto the QIAamp DNA mini column (Qiagen Ltd., UK). DNA was purified on the column, eluted and stored as described above (see 4.2.2 – Concentration and Purification of DNA released from oocysts).

4.2.4.h – Sonication
The 400μl suspensions were submerged in a sonic water bath (Ultrawave Ltd.) and sonicated (50/60Hz, 80W) for one, five, ten, 15 or 20 minutes. A 200μl aliquot of oocyst suspension was then dispensed to a new 1.5ml tube and prepared for examination by DIC as described previously (see 4.2.4.b – Control Samples). The remaining 200μl of suspension underwent DNA purification and concentration as previously described (see 4.2.4.c – Freeze-Thaw Cycling; 4.2.2 – Concentration and Purification of DNA released from oocysts).

4.2.4.i – Vortexed Zirconia/Silica Beads
To the 400μl suspensions, 400μl of Buffer AL (Qiagen Ltd., UK) and 0.1g of 0.5mm Zirconia/silica beads (Stratech Scientific) were added and exposed to a maximum speed vortex for 2 minutes. A 400μl aliquot of oocyst suspension was then dispensed to a new 1.5ml tube and prepared for examination by DIC as described previously (see 4.2.4.b – Control Samples). Prior to loading onto the QIAamp DNA mini column (Qiagen Ltd., UK), 20μl of Proteinase K was added and suspensions were thoroughly mixed, incubated at 56°C for 10 minutes and 200μl of 96-100% ethanol mixed with the sample. DNA was purified on the column, eluted and stored as described above (see 4.2.2 – Concentration and Purification of DNA released from oocysts).

4.2.4.j – SSU rDNA real-time PCR with SYBR Green I
In addition to using DIC microscopy to examine the physical effects on the oocysts, the SSU rDNA real-time PCR with SYBR Green I was used to compare the amount of DNA recovered from oocysts exposed to each disruption method. The 50μl PCR
reaction mixture and cycling condition were as described previously (see 4.2.3.c – Production of a standard curve to evaluate the performance of the Cryptosporidium SSU rDNA real-time PCR). Following the PCR, a dissociation curve was produced for each sample to confirm that the increase in fluorescence was due to the amplification Cryptosporidium DNA and not PCR artefacts.

Table 4.6: Methods of oocyst disruption selected to evaluate for the maximum recovery of Cryptosporidium DNA from low numbers of oocysts

<table>
<thead>
<tr>
<th>Method of Oocyst Disruption</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Proteinase K and Buffer AL digestion at 56°C for 30 minutes</td>
</tr>
<tr>
<td>2. Proteinase K and Buffer AL digestion at 56°C for 16 hours</td>
</tr>
<tr>
<td>3. Proteinase K and Buffer ATL digestion at 56 °C for 10 minutes followed by Buffer AL at</td>
</tr>
<tr>
<td>70°C for 10 minutes</td>
</tr>
<tr>
<td>4. 3 Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion</td>
</tr>
<tr>
<td>at 56°C for 30 minutes</td>
</tr>
<tr>
<td>5. 3 Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion</td>
</tr>
<tr>
<td>at 56°C for 16 hours</td>
</tr>
<tr>
<td>6. 3 Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer ATL digestion</td>
</tr>
<tr>
<td>at 56 °C for 10 minutes followed by Buffer AL at 70°C for 10 minutes</td>
</tr>
<tr>
<td>7. Heating at 100°C for 60 minutes followed by Proteinase K and Buffer AL digestion at 56°C</td>
</tr>
<tr>
<td>for 10 minutes</td>
</tr>
<tr>
<td>8. Heating at 100°C for 60 minutes followed by Proteinase K and Buffer ATL digestion at 56 °C</td>
</tr>
<tr>
<td>for 10 minutes followed by Buffer AL at 70°C for 10 minutes</td>
</tr>
</tbody>
</table>

4.2.5 – Evaluation of selected oocyst disruption methods for the maximum recovery of Cryptosporidium DNA from low numbers of oocysts (Table 4.6)

4.2.5.a – Preparation of samples
An enumerated stock oocyst suspension was mixed for two minutes on a vortex and gently inverted twice to homogenise the suspension. A dilution series of working suspensions was prepared by initially diluting 1x10^5 oocysts in 9ml of deionised water and 1ml of 10x SL-Buffer A (Dynal Biotech) giving a working concentration of 1x10^4 oocysts ml^-1. From this suspension, 1ml was added to 8ml deionised water and 1ml of 10x SL-Buffer A (Dynal Biotech) giving a working concentration of 1x10^3 oocysts ml^-1 and was repeated a further time to prepare a suspension of 1x10^2 oocysts ml^-1.
From each of these working suspensions, 100μl aliquots were dispensed with 100μl of anti-Cryptosporidium immunomagnetic separation beads (Dynal Biotech, UK) into
1.5ml screw-capped tubes ready for exposure to the 8 selected disruption conditions (Table 4.6) in triplicate. Due to the low numbers of oocysts in the 100µl of working suspension used for this experiment, they were enumerated by immunofluorescence microscopy.

4.2.5.b – Enumerating working suspensions by immunofluorescence microscopy
To accurately enumerate the working suspensions, 12 aliquots of 25µl were dispensed onto 4-well slides (Hendley-Essex), allowed to dry at 40°C and fixed with 50µl of methanol. The samples were stained with fluorescein-labelled Cryptosporidium specific monoclonal antibody (Mab) (TCS Water Sciences, UK) and 4’,6-diamidino-2-phenylindole (DAPI) (Sigma) as described previously (see 3.2.3 – Detection and enumeration of Cryptosporidium oocysts by immunofluorescence microscopy). The number of oocysts in each well was counted and the proportion that were DAPI positive noted.

4.2.5.c – Proteinase K and Buffer AL Digestion
To the 200µl suspensions, 20µl of Proteinase K (Qiagen Ltd., UK) and 200µl of Buffer AL (Qiagen Ltd., UK) were added. Suspensions were thoroughly vortexed at maximum speed and incubated at 56°C for either 30 minutes or 16 hours (overnight). Following this incubation, 200µl of 96-100% ethanol was mixed with the sample before it was loaded onto the QIAamp mini column (Qiagen Ltd., UK). DNA was purified on the column, eluted and stored as described above (see 4.2.2 – Concentration and Purification of DNA released from oocysts).

4.2.5.d – Proteinase K and Buffer ATL Digestion
To the 200µl suspensions, 20µl of Proteinase K (Qiagen Ltd., UK) and 200µl of Buffer ATL (Qiagen Ltd., UK) were added. Suspensions were thoroughly vortexed at maximum speed and incubated at 56°C heating blocks for ten minutes. Buffer AL (200µl) was then added to the sample and incubated at 70°C for ten minutes as per the manufacturers instructions (Qiagen Ltd., UK). Ethanol was added and the DNA purified on the column, eluted and stored as described previously (see 4.2.5.c – Proteinase K and Buffer AL digestion; 4.2.2 – Concentration and Purification of DNA released from oocysts).
4.2.5.e – Freeze-Thaw Cycling

The 200µl suspensions were submerged in a super cooled methanol bath of -78°C (a slurry of dry ice and methanol) for five minutes. Samples were immediately transferred to a heating block set at either 37°C or 100°C for a further five minutes. Samples were subjected to three cycles of freeze-thaw treatment and then prepared for DNA extraction in the presence of Proteinase K and either Buffer AL or Buffer ATL as described above (see 4.2.5.c – Proteinase K and Buffer AL Digestion and 4.2.5.d – Proteinase K and Buffer ATL Digestion). DNA was purified on the column as described in the manufacturers instructions and eluted and stored as described above (see 4.2.2 – Concentration and Purification of DNA released from oocysts).

4.2.5.f – Heating

The 200µl suspensions were placed in a heating block set at 100°C for 60 minutes and then prepared for DNA extraction in the presence of Proteinase K and either Buffer AL or Buffer ATL as described above (see 4.2.5.c – Proteinase K and Buffer AL Digestion and 4.2.5.d – Proteinase K and Buffer ATL Digestion). DNA was purified on the column as described in the manufacturers instructions and eluted and stored as described above (see 4.2.2 – Concentration and Purification of DNA released from oocysts).

4.2.5.g - Nested SSU rDNA PCR

To detect the presence of Cryptosporidium DNA recovered from the low numbers of oocysts, a nested PCR targeting the SSU rRNA gene described by Xiao et al. (2000a) was used. Briefly, the primary PCR produced products of ~1,325bp using the forward primer 5’-TTCTAGAGCTAATACATGCG-3’ and the reverse primer 5’-CCCATTTCCTTCGAACAGGA-3’. The 50µl PCR reaction mixture contained 10x PCR buffer (Qiagen Ltd., UK), 6mM MgCl₂, 200 mM of each deoxynucleotide triphosphate, 200nM of each primer, 2.5U of HotStar Taq DNA polymerase (Qiagen Ltd., UK) and 10µl of template DNA. The secondary PCR, which produced products of ~830bp used the forward primer 5’-GGAAGGGTTGTATTATTAGATAAAG-3’ and the reverse primer 5’-AAGGAGTAAGGAACACCTCCA-3’. The PCR reaction mixture was identical to that in the primary PCR except that 3mM MgCl₂ was used and only 5µl of primary product was added. The cycling conditions for both
PCRs were an initial step of 15 minutes at 95°C to activate the HotStar Taq, followed by 35 cycles of, 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 60 seconds before a final extension of 7 minutes at 72°C using a Dyad DNA Engine thermal cycler (MJ Research). The PCR products were run on a 2% agarose gel at 110V for 60 minutes, visualised by SYBR Green I (Sigma) staining and images recorded using a digital imaging system (AlphaImager, Alpha Innotech).

4.2.5.h – SSU rDNA real-time PCR with SYBR Green I
In addition to the SSU rDNA nested PCR, the real-time PCR that I developed was also used to compare how the oocyst disruption methods affected the recovery of Cryptosporidium DNA from suspensions containing low number of oocysts. The 50μl PCR reaction mixture and cycling conditions were as described above (see 4.2.3.c – Production of a standard curve to evaluate the performance of the Cryptosporidium SSU rDNA real-time PCR). Following the PCR, a dissociation curve was produced for each sample to confirm that the increase in fluorescence was due to the amplification Cryptosporidium DNA and not PCR artefacts.

4.2.6 – Statistical Analysis
The descriptive statistics (means, standard deviations and standard errors) and paired-sample t-test analysis were undertaken using SPSS 12.0.1 for Windows (SPSS Inc.).

4.3 – Results
4.3.1 – Preparation and evaluation of Cryptosporidium oocyst suspensions for use in the oocyst disruption and DNA recovery experiments
Although the concentration of oocysts ml⁻¹ appears to decrease during storage, the reduction over the first four days was not statistically significant (Table 4.7) and this duration was therefore sufficient to test the various oocyst disruption methods.

The number of oocysts increased approximately 4 fold when concentrated by centrifugation (Table 4.8), which was sufficient to improve the number of oocysts available for microscopic analysis of the oocyst suspensions following the various disruption methods.
Table 4.7: The variation of oocyst numbers in a suspension stored at 4°C

<table>
<thead>
<tr>
<th>Day</th>
<th>Mean oocyst Concentration (ml⁻¹)</th>
<th>Standard Deviation</th>
<th>t-statistic</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5x10⁵</td>
<td>3.1x10⁴</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1.5x10⁵</td>
<td>3.3x10⁴</td>
<td>-0.92</td>
<td>0.365</td>
</tr>
<tr>
<td>3</td>
<td>1.4x10⁵</td>
<td>3.0x10⁴</td>
<td>0.65</td>
<td>0.517</td>
</tr>
<tr>
<td>4</td>
<td>1.4x10⁵</td>
<td>3.0x10⁴</td>
<td>0.39</td>
<td>0.698</td>
</tr>
<tr>
<td>5</td>
<td>1.3x10⁵</td>
<td>3.3x10⁴</td>
<td>2.86</td>
<td>0.007</td>
</tr>
<tr>
<td>8</td>
<td>1.0x10⁵</td>
<td>2.8x10⁴</td>
<td>6.69</td>
<td>0.000</td>
</tr>
<tr>
<td>15</td>
<td>1.2x10⁵</td>
<td>2.9x10⁴</td>
<td>3.09</td>
<td>0.004</td>
</tr>
<tr>
<td>22</td>
<td>1.1x10⁵</td>
<td>2.6x10⁴</td>
<td>4.42</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Table 4.8: The effect of centrifugation on the concentration of oocysts (ml⁻¹)

<table>
<thead>
<tr>
<th></th>
<th>Before Concentration</th>
<th>After Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Disruption</td>
<td>1.0x10⁵</td>
<td>4.4x10⁵</td>
</tr>
<tr>
<td>Heat Disruption</td>
<td>0.6x10⁵</td>
<td>2.2x10⁵</td>
</tr>
</tbody>
</table>

4.3.2 – Development of a SYBR Green I real-time PCR assay to compare the amount of DNA obtained following the different oocyst disruption methods

4.3.2.a – Evaluation and optimisation of an adapted SSU rDNA PCR and development of a SYBR Green I master mix

The concentration of MgCl₂ and primers used in the nested PCR described by Xiao et al. (2000a) also gave optimum amplification of the ~830bp fragment when used as an individual PCR (Sample 22 in Figure 4.1).

The limit of detection for this adapted SSU rDNA PCR when stained by SYBR Green I and visualised on a 2% agarose gel, was found to be the 1:1,000 dilution, which corresponded to the DNA from a very low number of Cryptosporidium oocysts (approaching a single oocyst)(Figure 4.2).
Figure 4.1: The ~830bp PCR products from the MiGZ and primercheckerboard (see Table 4.2 for concentrations).
Figure 4.2: Products from the dilution series to test the limit of detection for the adapted SSU rDNA PCR. Lanes M = 100bp ladders; Lane 1 = 1,050 oocysts; Lane 2 = 105 oocysts; Lane 3 = 10.5 oocysts; Lane 4 = 1 oocyst; Lane 5 = 0.1 oocyst; Lane 6 = 0.01 oocyst; Lane 7 = 0.001 oocyst.

Figure 4.3: Amplification with the adapted SSU rDNA PRC with SYBR Green I present in the reaction mixture. Lanes M = 100bp ladders; Lane 1 = x1 working concentration of SYBR Green I; Lane 2 = 1:10 dilution; Lane 3 = 1:50 dilution; Lane 4 = 1:100 dilution.
The x1 SYBR Green I solution inhibited the PCR amplification, probably due to the DMSO that it is supplied in. However, amplification did occur in the presence of the 1:10, 1:50 and 1:100 dilutions (Figure 4.3).

These three dilutions also produced amplification using the Stratagene real-time PCR platform, with the 1:10 dilution providing the best level of fluorescence of the three (Figure 4.4).

4.3.2.6 – Production of a standard curve to evaluate the performance of the Cryptosporidium SSU rDNA real-time PCR

Once these optimum levels of reaction mixture components had been established, DNA was extracted from an enumerated suspension and dilutions from the DNA suspension could be used to create a standard curve to evaluate the performance (Figure 4.5). The 1:1 dilution was excluded from the curve as the quantity of DNA present was too high (discussed further in 4.4 – Discussion). From this curve the regression was 0.997 and the amplification efficiency of the PCR was 97.7%. The purpose of this standard curve is to evaluate the efficiency of the PCR and the accurate dilution of samples, but cannot be used to quantify the amount of Cryptosporidium in a sample unless the same disruption method is used. In addition, the dissociation analysis showed that all of the amplicons were Cryptosporidium (Figure 4.6) and not PCR artefacts.

4.3.3 – Comparison of oocyst disruption methods for the maximum recovery of Cryptosporidium DNA

4.3.3.a – Initial evaluation of oocyst disruption methods

To assess the level of oocyst disruption caused by each method the number of whole oocysts remaining were counted. A reduction in oocysts was taken to indicate a greater level of disruption or excystation and the results are summarised in Table 4.9 and Figure 4.7. All of the samples digested with Proteinase K and AL or ATL lysis buffer (methods 2-17) resulted in fewer whole oocysts than the control. Those samples that were subjected to freeze-thaw (-78°C ↔ 37°C) cycles (methods 18-22) reduced in whole oocyst numbers as the number of cycles increased. However, the
SYBR Green I present in the PCR reaction mixtures.

Figure 4.4: Amplification curves showing the level of fluorescence generated by the 1:10, 1:50 and 1:100 dilutions.
Figure 4.5: A standard curve for the SYBR Green I real-time PCR from a DNA dilution series, showing the result of the regression analysis (R²) and amplification efficiency of the PCR (E_ff).
The typical 88°C peaks for these *C. pneumoniae* SSL DNA fragments.

**Figure 4.6:** The dissociation curves for the DNA dilution series used to create the standard curves showing...
Table 4.9: The number of whole oocysts ml\(^{-1}\) when enumerated by improved Neubauer Haemocytometer following each disruption method

<table>
<thead>
<tr>
<th>Method</th>
<th>Number of Whole Oocysts (ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Count 1</td>
</tr>
<tr>
<td>1. Control (no treatment)</td>
<td>3.0x10^5</td>
</tr>
<tr>
<td>2. 10 min x Prot. K + Buffer AL (37°C)</td>
<td>1.2x10^5</td>
</tr>
<tr>
<td>3. 30 min x Prot. K + Buffer AL (37°C)</td>
<td>1.3x10^5</td>
</tr>
<tr>
<td>4. 60 min x Prot. K + Buffer AL (37°C)</td>
<td>1.7x10^5</td>
</tr>
<tr>
<td>5. 16 hrs x Prot. K + Buffer AL (37°C)</td>
<td>6.0x10^4</td>
</tr>
<tr>
<td>6. 10 min x Prot. K + Buffer AL (56°C)</td>
<td>1.2x10^5</td>
</tr>
<tr>
<td>7. 30 min x Prot. K + Buffer AL (56°C)</td>
<td>1.3x10^5</td>
</tr>
<tr>
<td>8. 60 min x Prot. K + Buffer AL (56°C)</td>
<td>1.1x10^5</td>
</tr>
<tr>
<td>9. 16 hrs x Prot. K + Buffer AL (56°C)</td>
<td>8.3x10^4</td>
</tr>
<tr>
<td>10. 10 min x Prot. K + Buffer ATL (37°C)</td>
<td>4.8x10^4</td>
</tr>
<tr>
<td>11. 30 min x Prot. K + Buffer ATL (37°C)</td>
<td>4.8x10^4</td>
</tr>
<tr>
<td>12. 60 min x Prot. K + Buffer ATL (37°C)</td>
<td>4.5x10^4</td>
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<td>13. 16 hrs x Prot. K + Buffer ATL (37°C)</td>
<td>1.0x10^4</td>
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<tr>
<td>14. 10 min x Prot. K + Buffer ATL (56°C)</td>
<td>2.8x10^4</td>
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<tr>
<td>15. 30 min x Prot. K + Buffer ATL (56°C)</td>
<td>5.0x10^4</td>
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<tr>
<td>16. 60 min x Prot. K + Buffer ATL (56°C)</td>
<td>3.8x10^4</td>
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<td>17. 16 hrs x Prot. K + Buffer ATL (56°C)</td>
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<td>18. 1 x Freeze-Thaw (37°C)</td>
<td>4.8x10^5</td>
</tr>
<tr>
<td>19. 3 x Freeze-Thaw (37°C)</td>
<td>4.2x10^5</td>
</tr>
<tr>
<td>20. 5 x Freeze-Thaw (37°C)</td>
<td>5.0x10^5</td>
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<td>21. 7 x Freeze-Thaw (37°C)</td>
<td>2.7x10^5</td>
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<tr>
<td>22. 10 x Freeze-Thaw (37°C)</td>
<td>2.0x10^5</td>
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<td>23. 1 x Freeze-Thaw (100°C)</td>
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<td>24. 3 x Freeze-Thaw (100°C)</td>
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<td>28. 10 min x Heat (100°C)</td>
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<td>34. 16 hrs x Trypsin (37°C)</td>
<td>3.3x10^5</td>
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<td>2.1x10^5</td>
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<tr>
<td>36. 5 min x Sonication (50/60 Hz, 80W)</td>
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</tr>
<tr>
<td>37. 10 min x Sonication (50/60 Hz, 80W)</td>
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<td>2.4x10^5</td>
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<td>39. 20 min x Sonication (50/60 Hz, 80W)</td>
<td>4.0x10^5</td>
</tr>
<tr>
<td>40. 2 min x Vortexed Zirconia Beads</td>
<td>1.5x10^5</td>
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Figure 4.7: The mean oocyst counts for each method (see Table 4.5) with bars representing the standard error of the mean.

Figure 4.8: The mean Ct values for each method (see Table 4.5) with bars representing the standard error of the mean.
Table 4.10: The Ct values obtained for each replicate using the SYBR Green I PCR following each oocysts disruption treatment

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<th>Method</th>
<th>SYBR Green I PCR Ct Values</th>
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<tr>
<td>1. Control (no treatment)</td>
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<tr>
<td>2. 10 min x Prot. K + Buffer AL (37°C)</td>
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</tr>
<tr>
<td>3. 30 min x Prot. K + Buffer AL (37°C)</td>
<td>29.05</td>
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<tr>
<td>4. 60 min x Prot. K + Buffer AL (37°C)</td>
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<td>5. 16 hrs x Prot. K + Buffer AL (37°C)</td>
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<td>6. 10 min x Prot. K + Buffer AL (56°C) (PKAL)</td>
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<td>7. 30 min x Prot. K + Buffer AL (56°C)</td>
<td>27.60</td>
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<td>8. 60 min x Prot. K + Buffer AL (56°C)</td>
<td>28.07</td>
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<tr>
<td>9. 16 hrs x Prot. K + Buffer AL (56°C)</td>
<td>27.35</td>
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<tr>
<td>10. 10 min x Prot. K + Buffer ATL (37°C)</td>
<td>26.83</td>
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<td>11. 30 min x Prot. K + Buffer ATL (37°C)</td>
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<td>13. 16 hrs x Prot. K + Buffer ATL (37°C)</td>
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<td>31. 10 min x Trypsin (37°C) + PKAL</td>
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<tr>
<td>40. 2 min x Vortexed Zirconia Beads + PKAL</td>
<td>28.99</td>
</tr>
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</table>

NA – No Amplification
samples exposed to 100°C heating (methods 28-30) and freeze-thaw (-78°C↔100°C) cycles (methods 23-27), particularly with three or more cycles, resulted in a similar reduction of whole oocysts to the Proteinase K and lysis buffer digestion. The Trypsin digestion, sonication and bead disruption (methods 31-40) did not reduce the number of whole oocysts to the same extent as the freeze-thaw (-78°C↔100°C) cycles, 100°C heating and Proteinase K digestion.

Following the microscopic examination of the effects from each disruption method the DNA was extracted from all the samples and compared by the developed SYBR Green I real-time PCR to detect which methods resulted in the highest amount of DNA for molecular testing. The samples with the lowest Ct values had the most DNA added to the PCR reaction mixture, which reflects on the oocyst disruption and DNA extraction method. The Ct values for all of the disruption methods are summarised in Table 4.10 and Figure 4.8. The amplification of Cryptosporidium DNA from the control sample indicates that there is free DNA in the sample prior to oocyst disruption. Of the Proteinase K and Buffer AL digestions (methods 2-9), only the 16 hour incubations (at both 37°C and 56°C) and the 30 minute incubation at 56°C appeared to release more DNA. The Proteinase K with Buffer ATL at 37°C (methods 10-13) released more DNA as the incubation time increased, but the 10 minute digestion at 56°C (method 14) provided the same amount of template DNA. In contrast to the digestions at 37°C (methods 10-13) the amount of template DNA decreased at 56°C (methods 14-17) as the incubation time increased. As with the microscopic examination the freeze-thaw cycles appeared more effective when the thawing step was undertaken at 100°C (methods 23-27) than at 37°C (methods 18-23). Of the freeze-thaw disruption methods, three cycles at -78°C↔100°C (method 24) appeared to release the greatest amount of usable DNA and as the number of cycles increases (methods 24-27), the amount of template DNA decreases. This is also seen when the samples were exposed to 100°C (methods 28-30), as the increased incubation time resulted in less DNA. Sonication of the oocysts (methods 35-39) did not appear to improve the recovery of DNA. The treatment with Trypsin (methods 31-34) and disruption by Zirconia/silica beads (method 40) actually appeared to reduce the amount of usable DNA in the samples.
4.3.3.b – Evaluation of selected oocyst disruption methods with low numbers of oocysts

The use of the QIAamp DNA Mini Kit (Qiagen Ltd.) for DNA extraction involves a Proteinase K digestion stage with either Buffer AL or ATL. From the initial evaluation of disruption methods (described above) the most effective Proteinase K protocol with Buffer AL was a 16-hour incubation at 56°C closely followed by a 30 minute incubation at 56°C and the best protocol with Buffer ATL was a 10 minute incubation at 56°C prior to 10 minutes with Buffer AL at 70°C. These three methods were therefore selected for evaluation with lower numbers of oocysts (Table 4.6). In addition, the three freeze-thaw cycles combined with the Proteinase K and Buffer AL digestion also provided a high amount of template DNA, so this method and the freeze-thaw stage combined with the other digestion protocols were selected for further testing (Table 4.6). The final two methods included for evaluation with low oocyst numbers and in the presence of IMS beads were heating at 100°C for 60 minutes combined with the Proteinase K and Buffer AL or ATL digestion (Table 4.6). Although the heating method did not result in the optimum amounts of usable DNA, these are the protocols currently used at the Cryptosporidium Reference Unit so it was felt that it would be prudent to compare these with the other methods.

The enumeration of oocysts in the low number suspensions determined that 410, 54 and 10 oocysts (55%, 58% and 58% DAPI positive respectively) were present in each DNA extraction and as only 10% is used in each PCR the amount of DNA added as template was the equivalent of 41, 5 and 1 oocyst.

Initially, a nested PCR targeting the SSU rDNA was used to detect the presence of DNA in all the samples following the different disruption methods (Table 4.11) and all eight methods enabled 100% detection from 41 oocysts. Using the 3 freeze-thaw (-78°C→100°C) cycles prior to any Proteinase K digest enabled DNA to be detected in all of the replicates for 5 and 1 oocyst. The Proteinase K digests (in the presence of either buffer AL or ATL) without any prior treatment resulted in 100% detection of DNA for 5 oocysts. Only two of the replicates were positive for DNA from a single oocyst with the 30 minute digest with Buffer AL and the 10 minute digest with Buffer ATL. The 16 hour digest with Buffer AL only resulted in one positive replicate from
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<th>Method</th>
<th>SYBR Green</th>
<th>PCR Amplification</th>
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</tr>
</tbody>
</table>
a single oocyst. The 100°C incubation with Proteinase K and Buffer AL enabled
detection of Cryptosporidium DNA in two replicates with 5 oocysts and one with a
single oocyst. The second heating method with Buffer ATL was slightly superior
with all replicates positive for 5 oocysts and two positive from one oocyst.

The freeze-thaw cycles combined with Proteinase K and Buffer AL digestion also
enabled all of the replicates to be detected by the SYBR Green I PCR (Table 4.12).
The combination with Buffer ATL was found to be not quite as sensitive as only one
of the single oocyst replicates was positive although all three 41 and 5 oocyst
replicates were positive. The only other method that enabled 100% detection from 5
oocysts by the SYBR Green I PCR was 100°C heating combined with a Buffer ATL
digest. The 100°C heating and the 16 hour Proteinase K Buffer AL digest both
detected DNA in two of the 5 oocyst replicates. The 10 minute Proteinase K and
Buffer ATL digest only detected DNA in one of the 5 oocyst replicates, whereas the
30 minute Buffer AL digest was only positive when 41 oocysts were present.

4.4 – Discussion
The efficient release of DNA from robust Cryptosporidium oocysts is the most
important step in the molecular study of these parasites, particularly when only a few
are present in a sample. Researchers use a number of different disruption techniques
to obtain DNA from oocysts (Table 4.1) and although many are based upon either
Proteinase K digestion or freeze-thaw treatments no standard method is widely
accepted. Two approaches, physical disruption of the oocyst or excystation, can be
taken to recover the DNA from within oocysts (Wiedenmann et al., 1998). Both have
advantages depending upon the hypothesis under question. Encouraging the oocysts
to excyst so the fragile sporozoites are released through the suture in the oocyst wall
means that DNA is removed from the protective shell where it can be easily extracted.
However, the major disadvantage of using excystation is the need for viable oocysts,
which in a molecular detection system could produce false negative results if only
non-viable oocysts were present. The advantage of physical disruption of oocysts by
chemical or mechanical means is the non-selectivity for viability, potentially making
more DNA available for extraction. If a population is being characterised for the
purpose of identifying a source or the types of Cryptosporidium present, then the viability of oocysts is not important.

The development of the SYBR Green I real-time PCR provided a useful tool to examine the amount of DNA that was used as a template. The standard curve that was produced demonstrated that the amplification efficiency of the PCR was highly efficient and that the dilution series was accurate. When producing the standard curve the 1:1 dilution exceeded the maximum amount of DNA that could be amplified on each cycle. This occurs when the template DNA is no longer the limiting factor for the PCR and one or more of the PCR reagents become the limiting factor. Under these circumstances, the entire reagent is used on each cycle and there is still excess template. So, the same amount of amplification is occurring with each cycle as with the maximum amount of template DNA still acting as the limiting factor, but the Ct value can be lower as the excess DNA can use some of the limiting component leaving even less available for amplification. If a standard curve is produced for a dilution series using the disruption method of choice, this protocol could be used as a quantitative technique for screening samples.

Following the initial evaluation using high numbers of oocysts, the microscopic and SYBR Green I PCR results suggested the Trypsin digestion, sonication and bead disruption methods were not efficient in releasing DNA for use with molecular methods. Sonication appeared to provide a similar level of DNA to the no treatment control, which may indicate that the oocysts were not disrupted enough to the release the DNA. This is in contrast to the finding of Sluter et al. (1997) who found sonication as affective as freeze-thawing, although they used a different sonic bath and did not provide any details of the frequency used. Harris and Petry (1999) also described the use of sonication to physically disrupt C. parvum oocyst walls and found that the walls were extremely resistant and undergo little fragmentation within a bath-type ultrasonicator due to the presence of an inner fibrillar layer. The inner fibrillar layer was released from the oocyst wall by digestion with Proteinase K and to a lesser extent Trypsin digestion (Harris and Petry, 1999). In my experiment the treatment with Trypsin did not result in noticeably fewer complete oocysts by microscopy and appeared to cause a loss in the amount of DNA extracted from those samples. The reasons for this are unknown but possible reasons may include damage
to the DNA so that it could not be amplified or the presence of substances that inhibited the PCR or the DNA binding to the extraction column. The Zirconia/silica bead disruption appeared to reduce the number of whole oocysts in the sample, although not to the same extent as other methods, but in a similar way to the Trypsin digestion the amount of DNA extracted was lower than the no treatment control. An explanation for this is the presence of silica in the beads to which the DNA may have bound and not transferred on to the column.

The freeze-thaw cycles with thawing at 37°C were not as efficient as those with thawing at 100°C. The microscopic examination samples demonstrated the increased disruption of oocysts as the number of cycles increased and the reduction was greater in those samples that were thawed at 100°C. The amount of DNA that was extracted from the 37°C freeze-thaw samples was not much greater than the control. In contrast, the samples thawed at 100°C demonstrated the release of much more DNA. An interesting finding after three cycles of freeze-thaw treatment with thawing at 100°C was that the amount of usable DNA in the samples decreased with additional cycles. A similar finding was noted with the samples that were heated at 100°C, as although there was more disruption seen with increased incubation, the amount of usable DNA decreased the longer that it was exposed to 100°C. Therefore the optimum freeze-thaw and heating methods tested here were 3 cycles at -78°C→100°C and 10 minutes at 100°C.

All of the Proteinase K digestion protocols that were used appeared to efficiently reduce the number of whole oocysts in the samples. The samples that were digested in the tissue lysis buffer (ATL) resulted in fewer oocysts and greater DNA than those digested in standard lysis buffer (AL). The 30 minute and 16 hour Proteinase K and buffer AL digestions at 56°C gave the most DNA from the AL digests as did the 10 minute ATL digestion at 56°C. In a similar pattern to the freeze-thaw and heating protocols the Proteinase K digestions at 56°C decreased in the amount of usable DNA as the incubation time increased, which may be due to the deterioration in the quality of DNA.
All eight of the selected methods when combined with nested SSU rDNA PCR or SYBR Green I PCR and evaluated with low number of oocysts proved 100% sensitive when at least 41 oocysts were available. The nested PCR was also seen to be more sensitive than the SYBR Green I PCR as the sensitivities of each method combined with the nested PCR were always the same or higher. Without a doubt, the combination of three freeze-thaw cycles followed by Proteinase K digestion in Buffer AL was superior with a single oocyst being detected with a sensitivity of 100% by both PCR methods. The Proteinase K digestion time did not make a difference as the 30 minute incubation detected the same amount of DNA as the 16 hour incubation. Therefore the three freeze-thaw cycles (-78°C→100°C) followed by Proteinase K digestion in Buffer AL for 30 minutes at 56°C is the optimum method for use with samples containing low numbers of oocysts.

The two methods (heating at 100°C followed by digestion with either buffer AL or ATL) currently used at the Cryptosporidium Reference Unit are not as effective with low numbers of oocysts as the other methods tested. However, as the majority of samples that are submitted to the unit contain high numbers of oocysts these methods are sufficient. The finding that longer exposure to high heat may reduce the amount of usable DNA may be worth addressing as the removal of this step could increase the amount of DNA in their extractions and would also decrease turn-around time.

Prior to this study there was no extensive comparison between methods for the maximum disruption of low number of oocysts for DNA extraction. Gibbons et al. (1998a) compared several disruption methods on high numbers of purified oocysts and those recovered from spiked backwash water. In their study, the maximum yield of DNA was obtained by excystation followed by a 2 hour Proteinase K digest and other excystation-digestion methods or five cycles of freeze-thawing before digestion were not as effective. Sluter et al. (1997) compared different methods of disrupting oocysts prior to DNA extraction and also found three cycles of freeze-thawing oocysts to yield the most PCR product, with a higher yield than Proteinase K by itself or followed by freeze-thawing, five cycles of freeze-thawing or electroporation. The only method that they found to be comparable with the three cycles of freeze-thawing was sonication, but due to speed and equipment requirements, freeze-thawing was
recommended (Sluter et al., 1997). Soon after the completion of my experiment, Guy et al. (2003) published a comparison of four oocyst disruption techniques, Proteinase K digestion in Buffer ATL at 55°C for 1 hour, three freeze-thaw cycles of 2 minutes at each temperature(-198°C→100°C), sonication with a probe and sonication with a cup horn. In that study they concluded that a combination of the digestion followed by freeze-thawing and then sonication with a cup horn yielded the maximum DNA. However, the authors did not attempt to use low numbers of oocysts and in addition to detecting a single oocyst, my optimal method is much quicker than their recommended procedure. One further study has been conducted since my experiment and examines oocyst disruption and DNA extraction with variations of freeze-thawing followed by Proteinase K digestion (Nichols and Smith, 2004). The results of this study recommended 15 cycles of freezing a sample at -198°C for one minute followed by thawing samples at 65°C for 1 minute and finally a Proteinase K digestion. Although the time taken to carry out this freeze-thawing is the same as in my optimum method (15 x 1 minute and 3 x 5 minutes, respectively), the Proteinase K digestion the recommend is for three hours as apposed to 30 minutes. Nichols and Smith (2004) use liquid nitrogen instead of the dry ice-methanol bath used in this study. I opted to use the super-cooled methanol on the grounds of health and safety and difficulty in obtaining liquid nitrogen. Their method consistently detects less than five oocysts when detected by a single PCR so it is hard to know if it is comparable to my optimum method. However, due to the consistent detection of a single oocyst and less amount of time needed to carry out the three cycles of freeze-thawing (-78°C→100°C) followed by Proteinase K digestion in Buffer AL at 56°C for 30 minutes, my method is recommended for the detection of low numbers of oocysts in samples.

4.5 - Conclusions
A variety of oocyst disruption methods (physical, chemical or encouragement to excyst) were identified from the literature (Table 4.1) and number of variations selected for comparison (Objective 3a).

The SYBR Green I real-time PCR was developed and shown to be capable of detecting a single oocyst present in a sample when combined with the optimum disruption method (Objective 3b). The sensitivity was not as high as the nested SSU
rDNA PCR but that may be expected, as the real-time protocol is only a single PCR. There are several advantages to the real-time system, including speed as it only involves a single PCR, the use of a closed-tube system that reduces chances of cross-contamination and there is no need for gel electrophoresis to detect the amplicons.

From the methods that were initially compared using high numbers of oocysts, eight were selected for further evaluation with low numbers after demonstrating through microscopic examination and SYBR Green I real-time PCR the potential to recover higher amounts of Cryptosporidium DNA (Objective 3c).

The eight selected methods were evaluated with samples containing low numbers of oocysts and in the presence of immunomagnetic beads (Objective 3d). Of all the oocyst disruption methods compared in this experiment, the three cycles of freeze-thawing (-78°C→100°C) followed by Proteinase K digestion in Buffer AL at 56°C for 30 minutes or 16 hours resulted in the greatest yield of usable DNA. These two methods combined with either the nested SSU rDNA PCR or the SYBR Green I real-time PCR both resulted in a sensitivity of 100% when a single oocyst was present in the sample. Based on convenience criteria, the protocol with the 30 minute digest is recommended for samples containing potentially low numbers of oocysts.

Using the QIAamp DNA Mini Kit to purify the DNA following release from the oocysts proved efficient for use with low oocysts numbers. However, the Trypsin digestion, sonication and Zirconia/silica bead disruption combined with this purification method were not efficient in yielding usable DNA even with high numbers of oocysts.

Exposure to high temperatures adversely affected the yield of DNA available for use in molecular techniques. This was identified with increased incubation time at 100°C, more than 3 cycles of freeze-thawing (-78°C→100°C), and also longer incubation time with Proteinase K and Buffer ATL at 56°C.

All of the eight methods selected for the comparative study with low numbers of Cryptosporidium oocysts had 100% sensitivity with 41 oocysts when both the nested
SSU rDNA PCR and SYBR Green I real-time PCR were used. Therefore, any of these methods can be chosen, based on convenience criteria, for use when high numbers of oocysts are known to be present.

The work in this chapter has identified a highly sensitive method of oocyst disruption and DNA recovery for use when very low numbers of Cryptosporidium are present. This provides us with a tool for use in ecological and epidemiological studies that often include samples, such as water or faeces from asymptomatic animals, which contain very few oocysts. Through the use of this method and molecular characterisation of recovered isolates, the public health importance of Cryptosporidium in these sample types can be further determined.

4.6 – Proposed Future Work

These oocysts disruption experiments were undertaken using C. hominis, which is one of the two predominant species that cause infections in humans. Whilst the oocyst wall properties of all the various Cryptosporidium species are likely to be similar, it is possible that there is also variation. For example, the genetic coding for the Cryptosporidium oocyst wall protein (COWP) varies between species, as this is one target that can be used in molecular characterisation, but it is not known how the variation may affect the physical properties of the protein. These selected oocyst disruption methods need to be tested with different species, host-adapted genotypes and subtypes, particularly those known to infect human, to confirm that they are as effective in releasing DNA.

Since undertaking this experiment the Cryptosporidium Reference Unit has acquired DIC microscopy at x1000 (previously x400), which can be used to further examine the physical effects of the different oocyst disruption techniques. High-powered examination of oocysts may provide more information on the extent of disruption and suggest the optimum exposure in the various methods needed to effectively disrupt the environmentally tough oocyst wall. The reduction in available DNA when oocysts are exposed to high temperatures should also be examined and the effect of thawing oocysts at various temperatures between 37°C, which was not particularly
effective, and 100°C, which was very effective but may cause damage to the DNA reducing its usability.

As previously mentioned, the SYBR Green real-time PCR method that was developed during this work was capable of amplifying and detecting a single Cryptosporidium oocyst when the optimum disruption method was used. This real-time technology has great potential in various aspects of Cryptosporidium research and further development is required to provide methods, not only to detect low numbers of oocysts, but also to quantitate and determine the species present. The method developed here should be tested with various other species, genotypes and subtypes to ensure that the efficiency is uniform. The dissociation curve analysis can be evaluated for differentiating between these different cryptosporidia. There is also great potential in developing the method for use with primers that produce shorter fragments, which can increase the speed of testing and often reduces the chance of non-specific amplification. There are primers available for the same gene used here that produce a much shorter fragment (~300bp instead of ~830bp) and include a variable region that may affect the dissociation curves for different species. Another option for the development of this method is with the use of labelled probes. With probe-based techniques the resulting fluorescence is only from the amplification of target sequences, unlike DNA intercalating dyes that are non-specific. If the target sequence for the probes were selected carefully, then there is also the potential for species differentiation through dissociation curves or by designing species-specific probes. Development of these methods could drastically increase turn-around time from receiving a sample to detecting the presence of Cryptosporidium and knowing which species is present. However, before these methods could be used for routine screening, monitoring or species differentiation they would need to be fully evaluated. This includes identifying the sensitivity, specificity, typability, repeatability, reproducibility and the discriminatory power of the method when used with a particular oocyst disruption and DNA purification protocol. The ability to quantitate the Cryptosporidium in a sample is possible by the production of standard curves following a specified oocyst disruption and DNA purification method and inclusion of relevant controls with each batch of samples. The use of technology in this way could further elucidate the pathology of different Cryptosporidium species in humans.
Section 3

Application

of the

Developed Methodology
Chapter 5 – Investigating the public health significance of Cryptosporidium in the environment: a study in the River Caldew catchment

5.1 – Introduction
This chapter describes a case study investigating the public health significance of Cryptosporidium spp. recovered from various sample types collected from within a water catchment using the methods identified and developed in the previous chapters. The water catchment, which is intensively farmed, was severely affected by the Foot and Mouth outbreak in 2001 and during this period a significant decrease in human cryptosporidiosis, both in the region and nationwide, was identified (Hunter et al., 2003; Smerdon et al., 2003). The unfortunate outbreak of Foot and Mouth Disease (FMD) also provided a rare opportunity to study an area almost completely cleared of livestock and the effect that re-stocking had on the levels and types of Cryptosporidium present in the environment and animal populations. This was the first opportunity ever to undertake this experiment as the previous Foot and Mouth outbreak was in 1967, prior to the identification of Cryptosporidium as a cattle pathogen (1971), a human pathogen (1976) and long before the molecular methods that enable us to differentiate between the species (1990’s). The methods developed in this thesis have allowed us to take advantage of this natural experiment, by enabling the more sensitive detection and recovery of Cryptosporidium DNA from the low number of oocysts often present in water and animal samples (particularly adult livestock and wildlife).

In 2001, the British farming industry was devastated by a nationwide outbreak of Foot and Mouth Disease. Following the initial confirmation of the virus in Essex on the 20th February, infected animals were soon detected on farms across the country as at least 48 premises in 15 counties were infected prior to this initial detection (The Comptroller and Auditor General, 2002). By the end of April (10 weeks into the outbreak), 1,518 infected premises had been identified and by the 30th September (week 32 of the outbreak), when the final case was confirmed, this number had risen
to 2,026 (The Comptroller and Auditor General, 2002). During the outbreak, more than 6 million animals were slaughtered (not including a number of lambs that were counted as one with their mothers), 4.2 million as either confirmed cases of FMD, close contact with cases (direct contact or contiguous premises) or suspicion of infection and an additional 2.3 million on welfare grounds due to the restrictions on animal movement (The Comptroller and Auditor General, 2002). In addition to animal movement, restrictions were also imposed on public footpaths and a number of countryside pursuits such as country shows, fairs, hunting and fishing. The public was also advised not to visit the countryside and to avoid contact with farm animals (The Comptroller and Auditor General, 2002). Most local authorities introduced blanket closures of the footpaths and the number of visitors to countryside areas was extremely low. The worst affected area during the outbreak was the county of Cumbria in the North West of England. In this county alone, 893 infected premises were identified between 28th February and the 30th September and nearly 1.5 million animals were slaughtered as part of the disease control (data from the Defra website, http://www.defra.gov.uk/footandmouth/cases/index.htm, updated on 10th August 2003).

Coinciding with the FMD outbreak, there was a decrease in the number of laboratory reported cases of human cryptosporidiosis in England and Wales (Hunter et al., 2003; Smerdon et al., 2003). These two studies used slightly different definitions of the outbreak period with Smerdon et al. using the first and last case of FMD (weeks 8-39) and Hunter et al. taking into account the pre-patent period of Cryptosporidium infections from when FMD restrictions were imposed (weeks 13-38). Smerdon et al. (2003) identified a 35% reduction in human cryptosporidiosis in England and Wales, including 63% reduction in the North West alone, during the outbreak period, than during the same period over the previous ten years. Hunter et al. (2003) identified a significant decrease of 36.5% throughout England and Wales and 69.0% in the North West region from 2000 to 2001. During the outbreak period, the decline in the North West region was seen to be significantly different than the same period during the previous ten years (from 2000 to 2001 during these weeks the reduction was 81.8%) and during the remaining weeks of the year no significant difference was seen (Hunter et al., 2003). In addition to the overall reduction of cryptosporidiosis, that caused by C. parvum (syn. genotype 2) during the outbreak was significantly lower than during
the same period the previous year (Hunter et al., 2003; Smerdon et al., 2003). Despite this reduction in cryptosporidiosis there was no significant reduction in the number of cases of Salmonella, Campylobactor or Giardia during the FMD outbreak (Hunter et al., 2003; Smerdon et al., 2003). This reduction in the number of cases of cryptosporidiosis, particularly that caused by C. parvum, is likely to be in part due to the restrictions imposed during the FMD outbreak, which reduced contact with animals and surfaces contaminated with their faeces. Also during this period, there were improvements to the drinking water treatment in parts of the region that has since been linked to a reduction in the cases of sporadic cryptosporidiosis (see 1.8.1 – Sporadic Cryptosporidiosis)(Goh et al., 2005). In addition, the massive reduction of animals, particularly in Cumbria, could have reduced the levels of Cryptosporidium contaminating the environment and thereby reducing the risk of contact. The reduction of farmed animals in the North West provides us with a unique opportunity to examine the effects of re-stocking farms with animals from different geographical areas on the species and subspecies of Cryptosporidium present in the environment, farmed and wild animals.

**Objective 4:** Apply the developed and optimised methods to investigate the occurrence and variation of Cryptosporidium species and subtypes in surface water, non-clinical faecal samples from farmed and wild animals and clinical samples from humans and cattle within the Caldew water catchment during and following the FMD restocking period.

**Objective 4a:** To identify sample sites that represent the entire water catchment.

**Objective 4b:** To recruit re-stocking and continually stocked farms within the catchment.

**Objective 4c:** To collect water samples during base flow and high flow conditions in the re-stocking and post re-stocking periods.
Objective 4d: To collect farmed and wild animal faecal samples during the re-stocking and post re-stocking periods.

Objective 4e: To identify human and animal clinical samples that had been submitted from the water catchment to the Cryptosporidium Reference Unit.

Objective 4f: To use the modified Parasep® faecal parasite concentrator method developed in Chapter 3 to screen the faecal samples collected in the catchment.

Objective 4g: To use the optimal oocyst disruption and DNA extraction methods from Chapter 4 to prepare the DNA from positive samples for molecular characterisation.

Objective 4h: To identify the species of Cryptosporidium identified in positive samples.

Objective 4i: To identify the subtypes of Cryptosporidium using a multilocus fragment analysis approach as identified in Chapter 2 and demonstrate the potential application of this technology in public health studies.

5.2 – Materials and Methods

5.2.1 – Sampling

5.2.1.a - Selection of sampling sites
The majority of the surface water sampling sites for the study were previously selected as part of the on-going project for the Environment Agency (EA) (Sanders et al., 2004), and cover both the upland and lowland areas of the catchment. These sites were selected for the EA study to define small sub-catchments defined by characteristics such as topography, potential land-use, stocking densities and management practices (Sanders et al., 2004). The value of using the EA sites was both practical (ease of access) and scientific (potential to obtain further data e.g. meteorological, faecal indicators, land use). In total, 11 sites were identified for
Cryptosporidium sampling, 8 of which were sites used in the EA study (Table 5.1).

One of the EA sites was adjacent to Farm A and so provided the surface water sampling site for this farm and an additional 3 sites were selected to provide surface water sampling sites for the three other farms in the study (Table 5.1 and Figure 5.1). Each riverine Cryptosporidium sample was assigned either to base flow or high flow categories according to flow conditions at the time of sampling, ascertained under the EA Caldew faecal indicators study (Sanders et al., 2004).

Four farms were recruited into the study: two that were continually stocked and two that were denuded of livestock but re-stocked following the FMD outbreak. Farms willing to participate in the study were identified with the help of the local veterinary group (Paragon Veterinary Group), the local branch of the Farming and Wildlife Action Group (FWAG), a local field worker from a previous Public Health Laboratory Service (PHLS) study (Diane Meadows) and the local agricultural college at Newton Rigg, Penrith. The farmers were assured confidentiality and offered a £100 incentive for participation. The recruitment of farms was a sensitive issue due to the

<table>
<thead>
<tr>
<th><strong>Table 5.1: Cryptosporidium study surface water sites</strong></th>
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</thead>
<tbody>
<tr>
<td><strong>Crypt. Study Site Number</strong></td>
</tr>
<tr>
<td>Site 1</td>
</tr>
<tr>
<td>Site 2</td>
</tr>
<tr>
<td>Site 3</td>
</tr>
<tr>
<td>Site 4</td>
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<tr>
<td>Site 5</td>
</tr>
<tr>
<td>Site 6</td>
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<tr>
<td>Site 7</td>
</tr>
<tr>
<td>Site 8</td>
</tr>
<tr>
<td>Site 9</td>
</tr>
<tr>
<td>Site 10</td>
</tr>
<tr>
<td>Site 11</td>
</tr>
</tbody>
</table>

*d/s: downstream  
* The River Glenderamackin is not within the Caldew catchment
Figure 5.1: Location of the Cryptosporidium monitoring points in the River Caldew catchment (GIS map produced by Dr Carl Stapleton).
impact of the FMD epidemic in 2001 and concerns over the use of data from the study. Access to restocking farms was delayed due to “sentinel scheme” licence restrictions. Nevertheless, the full quota of four farms (two continually stocked with livestock and two that had been cleared of livestock and were in the process of re-stocking) were identified and recruited into the study programme (Table 5.2). One of the recruited farms (Farm C) straddled the border of the river Caldew catchment and the adjacent river Glenderamackin catchment to the south. The water sample point for this farm was not within the Caldew catchment but downstream from the EA site in the Glenderamackin catchment.

Table 5.2: Outline description of farms selected for Cryptosporidium sampling

<table>
<thead>
<tr>
<th>Farm</th>
<th>Stock status</th>
<th>Farm type</th>
<th>Farm size (ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Re-stocking</td>
<td>Sheep &amp; dairy cattle</td>
<td>114.5</td>
</tr>
<tr>
<td>B</td>
<td>Re-stocking</td>
<td>Dairy cattle</td>
<td>137.0</td>
</tr>
<tr>
<td>C</td>
<td>Continually stocked</td>
<td>Sheep</td>
<td>171.0 (+ fell)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Upland)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Continually stocked</td>
<td>Dairy cattle</td>
<td>81.0</td>
</tr>
</tbody>
</table>

Recruitment of three farms was completed on 21st February 2002, and the fourth farm was recruited on 15th March 2002. It was evident from an initial survey in early February 2002 that re-stocking had already commenced. However, since animals were being over-wintered indoors it was decided that the environment would more-or-less represent the de-stocked condition. These initial observations were confirmed by a survey of farm practices carried out as part of the EA study (Figure 5.2 and 5.3) (Sanders et al., 2004). Detailed descriptions were produced of the four farms when they were recruited (Appendix A).

5.2.1.b – Surface water
Riverine water samples for Cryptosporidium testing at the selected EA sites (Table 5.1) was targeted to include dry periods (base flow), and rainfall induced high flow events. Three sets of samples were scheduled to be collected during each phase, including pre-rainfall base flow, rainfall induced high flow and post-rainfall flow.
Figure 5.2: Calculated total number of cattle in the Caldew catchment, between June 2000 and December 2003 (Sanders et al., 2004).

Figure 5.3: Calculated total number of cattle in the Caldew catchment, between June 2000 and December 2003 (Sanders et al., 2004).

(which was identified by flow records for the EA study (Sanders et al., 2004) as high flow during Phase 1 and base flow during Phase 2). Phase 1 surface water sampling took place at the selected EA sites between 16th January 2002 and 23rd January 2002, comprising one set of base flow samples and two sets of high flow samples (Table 5.3). Phase 2 sampling took place between 26th March 2003 and 3rd April 2003, comprising two sets of base flow samples and one set of high flow samples (Table 5.3). The flow separation records generated as part of the EA study (Sanders et al., 2004) shows that flows at sites 5, 6 and 7 during the Phase 2 high flow run (1/4/03) were classified as base flow, although a small rainfall induced hydrograph event was present at the time these samples were collected. For the purposes of this study, these
### Table 5.3: Surface water sample sites and sample dates

<table>
<thead>
<tr>
<th>Site Number</th>
<th>EA study site</th>
<th>Composite water type</th>
<th>Date Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phase 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Base flow</td>
</tr>
<tr>
<td>Site 1</td>
<td>EA Site 1</td>
<td>Upland</td>
<td>16/01/02</td>
</tr>
<tr>
<td>Site 2</td>
<td>EA Site 5</td>
<td>Upland</td>
<td>16/01/02</td>
</tr>
<tr>
<td>Site 3</td>
<td>EA Site 3</td>
<td>Upland</td>
<td>16/01/02</td>
</tr>
<tr>
<td>Site 4</td>
<td>EA Site 10</td>
<td>Upland</td>
<td>19/01/02</td>
</tr>
<tr>
<td>Site 5</td>
<td>EA Site 6</td>
<td>Lowland</td>
<td>18/01/02</td>
</tr>
<tr>
<td>Site 6</td>
<td>EA Site 9</td>
<td>Lowland</td>
<td>18/01/02</td>
</tr>
<tr>
<td>Site 7</td>
<td>EA Site 11</td>
<td>Lowland</td>
<td>18/01/02</td>
</tr>
<tr>
<td>Site 7</td>
<td>Farm A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 8</td>
<td>EA Site 12</td>
<td>Lowland</td>
<td>18/01/02</td>
</tr>
<tr>
<td>Site 9</td>
<td>Farm C</td>
<td>Upland</td>
<td>01/03/02</td>
</tr>
<tr>
<td>Site 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 10</td>
<td>Farm B</td>
<td>Lowland</td>
<td>01/03/02</td>
</tr>
<tr>
<td>Site 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 11</td>
<td>Farm D</td>
<td>Lowland</td>
<td>26/03/02</td>
</tr>
<tr>
<td>Site 11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 5.4: Farm animal sampling dates

<table>
<thead>
<tr>
<th>Farm</th>
<th>Stock type</th>
<th>Date sampled Phase 1</th>
<th>Date sampled Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sheep</td>
<td>28/02/02</td>
<td>25-27/02/03</td>
</tr>
<tr>
<td>A</td>
<td>Unweaned lambs</td>
<td>28/02/02</td>
<td>25-27/02/03</td>
</tr>
<tr>
<td>A</td>
<td>Dairy cows</td>
<td>28/02/02</td>
<td>25/02/03</td>
</tr>
<tr>
<td>A</td>
<td>Unweaned calves</td>
<td>28/02/02</td>
<td>25-27/02/03 &amp; 27/03/03</td>
</tr>
<tr>
<td>B</td>
<td>Dairy cows</td>
<td>28/02/02</td>
<td>28/03/03</td>
</tr>
<tr>
<td>B</td>
<td>Unweaned calves</td>
<td>28/02/02</td>
<td>28/03/03</td>
</tr>
<tr>
<td>C</td>
<td>Sheep</td>
<td>27/02/02</td>
<td>30/04/03</td>
</tr>
<tr>
<td>C</td>
<td>Lambs</td>
<td>30/04/02 &amp; 01/05/02</td>
<td>30/04/03</td>
</tr>
<tr>
<td>D</td>
<td>Dairy cows</td>
<td>26/03/02</td>
<td>25/02/03</td>
</tr>
<tr>
<td>D</td>
<td>Calves</td>
<td>26/03/02 &amp; 30/04/02</td>
<td>25-27/02/03 &amp; 28/03/03</td>
</tr>
</tbody>
</table>
Figure 5.4: A diagrammatic representation of the sampling rig

Figure 5.5: A photograph showing the battery-powered rig, sampling water from the pump submerged in the stream, through the filter capsule and through the water meter before releasing the filtered water back into the stream. Photograph used courtesy of John Watkins.
samples were classified as high flow since they were collected at a time of increased flows when compared to the base flow samples.

Water sampling at sites adjacent to the four survey farms coincided with animal faecal matter sampling. During Phase 1 this comprised one set of base flow samples for all the farms and two sets of high flow samples for Farm A. During Phase 2, two sets of base flow samples and one set of high flow samples were collected and additional base flow samples for Farms A and D were collected during the sampling of livestock faeces during February 2003 (Table 5.3).

Filter samples were obtained, where possible, using cartridge filtration (Pall Envirochek HV® filters) of up to 500 litres water at each site on each occasion. A portable pump run from a 12V battery was used to abstract the water, which was returned to the river following filtration (Figures 5.4 and 5.5). The optimum flow rate was regulated at four litres per minute. Multiple filters were used if the water was turbid, or where turbidity was excessive, multiple 10-litre grab samples were obtained for filtering in the laboratory. Other parameters were also measured, including temperature, pH, turbidity and conductivity. General assessment of weather conditions and land use was made subjectively at the time of sampling. Historical weather data were obtained for the period of the study from EA administered local sites. Detailed land use data, geology and soil maps were also obtained from Ordnance Survey and the EA in hard copy and GIS formats. Faecal indicator results were obtained from the EA study (Sanders et al., 2004).

5.2.1.c – Livestock
Phase 1 sampling of faecal matter at all four farms commenced on 28/2/02 and was completed by 26/3/02 (Table 5.4). The two re-stocking farms were sampled first, since the animals were not yet turned out and therefore minimally exposed to any Cryptosporidium species currently contaminating the local environment. Continually stocked farms were re-visited, on 30/4/02 and 1/5/02, to sample lambs and to increase numbers of samples from calves.
Phase 2 sampling of faecal matter at all four farms commenced on 25/2/03 and was completed by 30/4/03 (Table 5.4). This sampling coincided with the peak lambing or calving period.

Discrete faecal droppings from the ground were collected into polythene bags (Figure 5.6), which were sealed and stored at ambient temperature before being transported to the Cryptosporidium Reference Unit (CRU) in Swansea. Samples were then stored at +4°C until screened for the presence of Cryptosporidium. Target numbers of samples from livestock were calculated based on the population size (i.e. the number of animals available for sampling at that time) (Cannon & Roe, 1982) and a priori knowledge of prevalence data for Cryptosporidium (Table 5.5). It was intended to estimate prevalence with 95% confidence and a worst acceptable prevalence of 0%, but since droppings were being collected as opposed to rectal samples from individual animals, over-sampling of the population was desirable to increase the chances of obtaining representative samples. Faecal matter was collected from areas where samples represented the entire population such as feeding platforms to minimise any bias.

5.2.1.d – Wildlife
Advice was sought from the farmers about the presence of wildlife and faeces of foxes, badgers, roe deer, pheasants and rabbits were identified from droppings on the ground and collected on an opportunistic basis. The droppings were collected in polythene bags (Figure 5.6) and stored in the same manner as described above for the livestock faeces sampling. The animal source of the dropping was identified based on faecal morphology (Bang and Dahlstrom, 1974).

5.2.2 – Detection of Cryptosporidium spp.
5.2.2.a - Surface water samples
Following the initial collection of surface water, the samples were transported to CREH Analytical Limited, Leeds, for Cryptosporidium oocyst recovery and enumeration. The oocyst recovery process involved filtration using Envirochek® filters (Pall Corporation) either in the field or in the laboratory if grab samples had been collected. Particles were eluted from the filter and concentrated by the DWI
1) Using a polythene bag
gather the faeces
trying, not to distort
the sample

2) Invert the bag over the
gathered sample

3) Seal the sample in the
bag by squeezing most
of the air out and tying
a knot in the top

Figure 5.6: Method of collecting of faecal samples from the ground
Table 5.5: Sample size (number of animals to be tested) to estimate prevalence with 95% confidence

<table>
<thead>
<tr>
<th>Number of animals in herd/flock</th>
<th>Predicted prevalence (worst acceptable prevalence of 0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40%</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
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<td>30</td>
<td>28</td>
</tr>
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<td>52</td>
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<td>300</td>
<td>165</td>
</tr>
<tr>
<td>400</td>
<td>192</td>
</tr>
<tr>
<td>500</td>
<td>212</td>
</tr>
</tbody>
</table>

Calculations based on EpiInfo v.6 StatCalc population survey using random (not cluster) sampling (figures verified against Cannon and Rowe, 1982).

approved protocol (DWI, 2005) to produce a pellet by immunomagnetic separation (IMS) (ImmuCell). Briefly, the pre-treatment buffer, reagent water and elution buffer were heated to 37°C, while the residual water was drained from the filter. The heated pre-treatment buffer was poured into the filter module to approximately 13mm from the top of the filter before being shaken with the caps on at 900 cycles per min for 5 minutes. The pre-treatment buffer was drained from the module, reagent water added to approximately 13mm from the top of the filter and rinsed by gently rotating the module for 30 seconds before draining once more. The elution buffer was then poured into the module to the same level as before and with the caps replaced the filter was shaken at 900 cycles per min for 5 minutes. The elution buffer was then poured into three 50ml tubes, subjected to 1100g for 15 minutes to produce a pellet and the supernatant discarded. Fresh elution buffer added to the module once more and the elution step repeated once more using the same three 50ml tubes. The pellets were combined into a single tube and washed in reagent water to form a single pellet at 1100g for 15 minutes, before being re-suspended in reagent water to a volume of 9ml. The sample was transferred to a Leighton tube and 1ml of both IMS reagent A and reagent B added and mixed on a vortex. The IMS beads were mixed on a vortex for 10 seconds and 100μl added to the sample, which was then placed in rotating mixer at 20 rpm for one hour. The sample was then rocked in the IMS magnetic clip
stand for five minutes before the supernatant was decanted with the tube still in the magnetic clip. The beads were then re-suspended in 1ml of 1:10 dilution of reagent A and transferred to a 1.5ml tube. From each sample, 10% was transferred to a second 1.5ml tube and the beads disassociated with 50µl of 0.1M hydrochloric acid. The beads were removed using a magnetic tube holder and the sample dispensed into 5µl of 1.0M sodium hydroxide sitting in the well on a slide and allowed to dry. The sample was fixed with methanol prior to the enumeration of Cryptosporidium oocysts present by immunofluorescence antibody test (IFAT) (TCS Water Sciences) and 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) staining at CREH Analytical Ltd. (see 3.2.3 – Detection and enumeration of Cryptosporidium oocysts by immunofluorescence microscopy). The remaining 90% of each sample was re-suspended in distilled water and sent with the slides used for IFAT to the CRU where they were stored at +4°C until DNA extraction prior to species identification and subtyping.

5.2.2.b - Faecal samples

Prospectively gathered faecal samples from animals were screened using the method developed in “Chapter 3 - Detection of potentially low numbers of Cryptosporidium spp. oocysts”. A 6ml aliquot of 10% formol water was added to 1g of faeces in the mixing chamber of a Parasep® faecal parasite concentrator (Intersep Filtration systems) and homogenised with a wooden swabstick. Ethyl acetate (2ml) was then added to the formal faecal slurry and the Parasep® container sealed before thoroughly mixing the samples by vortex at maximum speed for 30 seconds. The Parasep® concentrator was then inverted and spun in a centrifuge (PK130, DJB Labcare) until 450g was reached, at which point the centrifuge was stopped with the brake off. The supernatant layer directly above the sediment was then carefully aspirated, dispensed into a clean 15ml tube and the remaining sample (ethyl-acetate, fatty plug and debris) disposed of in vermiculite (Figure 4.2). Deionised water was then added to the supernatant to a volume of 10ml and the sample spun in a centrifuge (PK130, DJB Labcare) at 1000g for 10 minutes. The majority of supernatant was then aspirated to leave a concentrated sample pellet, which was screened by immunofluorescence microscopy (see 3.2.3 – Detection and enumeration of Cryptosporidium oocysts by immunofluorescence microscopy).
5.2.3 - Recovery of Cryptosporidium spp. DNA

5.2.3.a - Surface water samples

DNA was extracted from the remaining 90% of the IMS pellets and, where the IMS pellet was not available (100% of the pellet was mistakenly used to enumerate seven of the samples), microscopy-positive microscope slides using the method developed in "Chapter 4 - Maximising the recovery of DNA for molecular characterisation from oocysts of Cryptosporidium". Each sample was frozen for five minutes in a dry ice-methanol bath and transferred to a heating block at 100°C for five minutes to thaw. This process was repeated two more times and followed by a 30 minute digest with Proteinase K and lysis buffer (Buffer AL, Qiagen Ltd.). The DNA was then purified using QIAamp® DNA Mini Kit spin columns (Qiagen Ltd.) and eluted in 150μl elution buffer (Buffer AE, Qiagen Ltd) as described in the manufacturers instructions before being stored at −20°C prior to characterisation by PCR-based techniques.

Where the IMS pellet was not available, oocysts were recovered from microscopy-positive slides prior to DNA extraction as described above, using a method based on the development and adaptation of that reported for Cryptosporidium oocysts isolated during regulatory monitoring (Anon., 2003). Briefly, a swab impregnated with acetone was used to soften the nail varnish sealing the coverslip, thus allowing it to be gently removed and inverted with a clean scalpel. A 50μl volume of Buffer AL (Qiagen Ltd.) was added to the slide and using a plastic pipette tip the entire well surface scraped. The buffer was transferred to the inverted coverslip and the entire surface also scraped before the suspension was dispensed into a 1.5ml sample tube. The slide and coverslip were then turned 180° and the entire process repeated before 100μl of deionised water was also added to the 1.5ml sample tube and the DNA extraction carried out using the freeze-thaw method described above.

5.2.3.b - Faecal samples

DNA was extracted from microscopy-positive faecal samples by the CRU standard protocol (Anon., 2002b). Faeces of roughly the same size as a broad bean or 2ml if fluid, were emulsified in 8ml of saturated sodium chloride (specific gravity of 1.2) and mixed thoroughly on a vortex at maximum speed. Approximately 2-3ml of deionised water was carefully added to form a distinct layer above the faecal slurry
and subjected to centrifugation (PK130, DJB Labcare) at 1,600g for eight minutes with the brake off. The deionised water layer was then gently swirled to draw the oocysts (at the interface of the two fluid layers) into the water phase, which was then gently removed and dispensed into a new tube and made up to 10ml with deionised water. The suspensions were mixed by vortex to dilute any sodium chloride residue and subjected to centrifugation (PK130, DJB Labcare) at 1,600g for five minutes with the brake off. Supernatants were gently decanted and the pellet re-suspended in 200μl of deionised water. The re-suspended pellet was incubated at 100°C for 60 minutes and digested with Proteinase K and lysis buffer at 56°C for 10 minutes, before the DNA was purified using QIAamp® DNA Mini Kit spin columns (Qiagen Ltd.) and eluted in 100μl of elution buffer as described in the manufacturers instructions. DNA was stored at –20°C prior to species determination and subtyping, where appropriate, using PCR-based techniques as described below.

Faecal samples in which few oocysts were seen by IFAT or where difficulty was experienced in obtaining PCR amplicons underwent IMS (as described in 5.2.2.a – Surface water samples, but using a 10ml faecal-deionised water slurry as the sample) to recover the maximum number of oocysts prior to DNA extraction by the freeze-thaw method described above.

5.2.4 – Species differentiation of recovered isolates

Cryptosporidium species were determined using nested PCR-RFLP analysis of the SSU rRNA gene based on a method described by Xiao et al. (2000a)(see 4.2.5 – Nested SSU rDNA PCR). The products of the secondary PCR were digested by restriction enzymes SspI and VspI to differentiate between the majority of Cryptosporidium species (Table 5.6). For samples containing C. andersoni or C. muris (both have similar SspI and VspI patterns), differentiation was undertaken by further digesting the PCR products with Ddel (Xiao et al., 2001c). The 20μl digestion mixes contained 10U of restriction enzyme SspI, VspI or Ddel (Promega) in the correct buffer as stated in the manufacturers instructions with 10μl of PCR product and were incubated for one hour at 37°C. The restriction fragments were separated on a 2% agarose gel, visualised by SYBR Green I (Sigma) staining and images recorded using a digital imaging system (AlphalImager, Alpha Innotech).
Table 5.6: The different nested SSU rDNA PCR fragment sizes for each species of Cryptosporidium when digested by SspI, VspI and DdeI. Fragment sizes calculated using GenBank sequences trimmed to the primers and inserted into Restriction Mapper (http://www.restrictionmapper.org)

<table>
<thead>
<tr>
<th>Species</th>
<th>GenBank Accession No.</th>
<th>SspI digest products (bp)</th>
<th>VspI digest products (bp)</th>
<th>DdeI digest products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parvum</td>
<td>L16996</td>
<td>11, 12, 108, 253, 449</td>
<td>101, 104, 628</td>
<td>20, 68, 155, 184, 406</td>
</tr>
<tr>
<td>C. hominis</td>
<td>L16997</td>
<td>11, 12, 111, 253, 449</td>
<td>70, 101, 104, 561</td>
<td>20, 68, 155, 184, 409</td>
</tr>
<tr>
<td>C. felis</td>
<td>AF112575</td>
<td>14, 33, 390, 426</td>
<td>101, 104, 658</td>
<td>20, 68, 155, 194, 426</td>
</tr>
<tr>
<td>C. canis</td>
<td>AF112576</td>
<td>20, 33, 105, 253, 417</td>
<td>101, 104, 623</td>
<td>20, 68, 155, 184, 401</td>
</tr>
<tr>
<td>C. wrairi</td>
<td>AF115378</td>
<td>11, 11, 109, 253, 449</td>
<td>101, 104, 628</td>
<td>20, 68, 155, 184, 406</td>
</tr>
<tr>
<td>C. suis</td>
<td>AF108861</td>
<td>9, 11, 364, 453</td>
<td>101, 104, 632</td>
<td>20, 68, 155, 187, 407</td>
</tr>
<tr>
<td>C. muris</td>
<td>AF093498</td>
<td>384, 448</td>
<td>101, 731</td>
<td>20, 155, 186, 224, 247</td>
</tr>
<tr>
<td>C. andersoni</td>
<td>L19069</td>
<td>383, 448</td>
<td>101, 730</td>
<td>20, 155, 186, 470</td>
</tr>
<tr>
<td>C. baileyi</td>
<td>L19068</td>
<td>253, 572</td>
<td>101, 104, 620</td>
<td>20, 155, 183, 467</td>
</tr>
<tr>
<td>C. meleagridis</td>
<td>AF112574</td>
<td>11, 11, 108, 253, 449</td>
<td>101, 104, 171, 456</td>
<td>20, 68, 155, 184, 405</td>
</tr>
<tr>
<td>C. galli</td>
<td>Not Enough Sequence Data Available</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. serpentsis</td>
<td>AF093502</td>
<td>14, 33, 369, 414</td>
<td>101, 729</td>
<td>20, 155, 186, 469</td>
</tr>
<tr>
<td>C. saurophilum</td>
<td>Not Enough Sequence Data Available</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. molnari</td>
<td>No Sequence Data Available</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Although the limit of detection for this PCR has been evaluated here (see 4.3.3.b – Evaluation of selected oocyst disruption methods with low numbers of oocysts) and by other authors (Sulaiman et al., 1999b), and found to be able to amplify 1 oocyst-equivalent or less of C. parvum DNA, several of the samples initially produced low amounts of PCR product that would not produce visible bands when digested. To overcome this, the amount of product was boosted by a third PCR using the same protocol as the secondary PCR but using 5μl of the secondary product as a template. The tertiary products were then digested as described above.

Following the PCR-RFLP analysis, any unusual species or equivocal samples were confirmed by amplifying a fragment of the SSU rRNA gene and DNA sequencing, where possible in both directions. Briefly, amplicons of ~830bp were produced from each sample using the nested primer set described above (Xiao et al., 2000a) and a fragment within the amplicon sequenced (GRI) using the forward primer 5′-AGTGA CAAGAAATAACAATACAGG-3′ and the reverse primer 5′-CCTGCTTAAAGCAC TCTAATTTC-3′ (Morgan et al., 1997). Where possible, the forward and reverse sequences of these fragments were then analysed and aligned using the software for a CEQ™ 8000 Genetic Analysis System (Beckman Coulter) to obtain a consensus sequence. Sequences were compared with all GenBank, EMBL, DDBJ and PDB entries using the NCBI BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/).
5.2.5 – Clinical cases within the catchment area
In addition to the data gathered from the sampling of surface water and the livestock and wildlife in the catchment, samples were available from the National Collection of Cryptosporidium Oocysts (Anon., 2002b) and its continuation. Faecal samples from human clinical cases of cryptosporidiosis from primary diagnostic laboratories serving England and Wales are sent to the Cryptosporidium Reference Unit (CRU) for species identification and the collection currently contains more than 10,000 samples. Samples from residents within the catchment were identified from postcode data and those not reporting recent foreign travel were also included in the study for subtype analysis (Table 5.7). Similarly, animal samples from clinical cases of cryptosporidiosis identified at the Veterinary Laboratories Agency in Penrith were also submitted, identified in the same manner and included (Table 5.7).

Table 5.7: Human and animal clinical Cryptosporidium isolates submitted to the CRU from the Caldew Catchment included for subtype analysis

<table>
<thead>
<tr>
<th>CRU Reference</th>
<th>Host</th>
<th>Year Submitted</th>
<th>Cryptosporidium Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>W0144</td>
<td>Human</td>
<td>2000</td>
<td>C. parvum</td>
</tr>
<tr>
<td>W0240</td>
<td>Human</td>
<td>2000</td>
<td>C. parvum</td>
</tr>
<tr>
<td>W0456</td>
<td>Human</td>
<td>2000</td>
<td>C. parvum</td>
</tr>
<tr>
<td>W1871</td>
<td>Human</td>
<td>2000</td>
<td>C. hominis</td>
</tr>
<tr>
<td>W2360</td>
<td>Human</td>
<td>2000</td>
<td>C. hominis</td>
</tr>
<tr>
<td>W4878</td>
<td>Human</td>
<td>2002</td>
<td>C. parvum</td>
</tr>
<tr>
<td>W5018</td>
<td>Human</td>
<td>2002</td>
<td>C. parvum</td>
</tr>
<tr>
<td>W6132</td>
<td>Human</td>
<td>2002</td>
<td>C. parvum</td>
</tr>
<tr>
<td>W6325</td>
<td>Human</td>
<td>2002</td>
<td>C. hominis</td>
</tr>
<tr>
<td>W8265</td>
<td>Human</td>
<td>2003</td>
<td>C. hominis</td>
</tr>
<tr>
<td>W8609</td>
<td>Human</td>
<td>2003</td>
<td>C. hominis</td>
</tr>
<tr>
<td>A484</td>
<td>Bovine calf</td>
<td>2002</td>
<td>C. parvum</td>
</tr>
<tr>
<td>A486</td>
<td>Bovine calf</td>
<td>2002</td>
<td>C. parvum</td>
</tr>
</tbody>
</table>

The previous species identification of these samples (at the CRU) show that both C. parvum and C. hominis were detected in human samples while C. parvum was detected in the two relevant animal samples (Table 5.7). This permits us to not only compare Cryptosporidium species and subtypes found within the animals and surface
Table 5.8: Cryptosporidium Reference Unit in-house multilocus fragment typing scheme for Cryptosporidium parvum and Cryptosporidium hominis

<table>
<thead>
<tr>
<th>Cryptosporidium species</th>
<th>Microsatellite Marker (fragment size in bp)</th>
<th>*Multilocus Fragment Type (MLFT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ML1</td>
<td>ML2</td>
</tr>
<tr>
<td>C. hominis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>233</td>
<td>180</td>
<td>371</td>
</tr>
<tr>
<td>240</td>
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<td>341</td>
</tr>
<tr>
<td>C. parvum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>242</td>
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<td>227</td>
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<td>330</td>
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<td>227</td>
<td>195</td>
<td>333</td>
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<td>241</td>
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<td>338</td>
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<tr>
<td>242</td>
<td>215</td>
<td>344</td>
</tr>
<tr>
<td>242</td>
<td>230</td>
<td>350</td>
</tr>
<tr>
<td>242</td>
<td>207</td>
<td>338</td>
</tr>
<tr>
<td>242</td>
<td>203</td>
<td>338</td>
</tr>
</tbody>
</table>

* These are provisional MLFTs to be confirmed by sequence analysis of the fragments. This is because certain MLFTs consist of fragment sizes that do not correspond to the 3bp repeats in the ML1 and gp60 microsatellites or the 2bp repeats in the ML2 microsatellite, but could be due to insertions or deletions within the fragment but outside of the microsatellite region.
waters in the catchment but also to investigate those causing human and animal disease.

5.2.6 – Subtype characterisation of recovered isolates

Subtypes were identified using a multilocus fragment size analysis approach targeting three microsatellite markers (ML1, ML2 and gp60). The ML1 fragment was amplified using the forward primer 5'-CTAAAAATGGTGAGAACATATTC-3' and the reverse primer 5'-CAACAAAATCTATATCCTC-3'(Caccio et al., 2001; Caccio et al., 2000). The ML2 fragment was amplified using the forward primer 5'-CAATGTAAGTTACTTATGATT-3' and the reverse primer 5'-CGACTATAAAGATGAGAGAAG-3' (Caccio et al., 2001). The gp60 fragment (synonymous with gp15) was amplified using the forward primer 5'-GCCGTTCCACTCAGAGGAC-3' and the reverse primer 5'-CCACATTACAAATGAAGTGCCGC-3' (Mallon et al., 2003a).

The 50µl PCR reaction mixture for each primer contained 10x PCR buffer (Qiagen Ltd.), 2.5mM MgCl2, 200µM of each deoxynucleotide triphosphate, 500nM of each primer, 2.5U of HotStar Taq DNA polymerase (Qiagen Ltd.) and 5 µl of template DNA. The cycling conditions for each PCR (as in the CRU standard protocol) were an initial denaturation step of 15 minutes at 95°C to activate the HotStar Taq followed by 40 cycles of 94°C for 50 seconds, 50°C (60°C for gp60) for 50 seconds and 72°C for 60 seconds before a final extension of 10 minutes at 72°C. The fragment sizes of amplified products were then analysed using a capillary gel electrophoresis based CEQ™ 8000 Genetic Analysis System (Beckman Coulter). The final typing scheme was based on that developed within the CRU (Anon., 2005). Under this scheme, the combined results of fragment size analysis at all three markers are used to create a multilocus fragment type (MLFT) for subtypes (Table 5.8).

5.3 – Results

5.3.1 – Detection of Cryptosporidium species

5.3.1.a – Surface water samples

Three sets of samples were collected for each of the riverine sites (sites 1 to 11) with the exception of sites 9 to 11 during Phase 1, when only one set of samples were collected. These sites were only sampled once to provide composite samples of any
inputs from the farms sampled. Of the three sample sets collected during Phase 1, one was collected during base flow conditions (n=11) and two during high flow conditions (n=16) (Table 5.3). The Phase 2 sampling resulted in two sets of base flow samples (n=22) and one set of high flow samples (n=11) (Table 5.3). The flow separation data generated for the EA project (Sanders et al., 2004) identified the post rainfall event samples during Phase 1 as high flow and during Phase 2 as base flow.

Larger volumes were obtained for the base flow samples than for high flow samples due to the higher turbidity of the water during the high flow periods (Tables 5.9 and 5.10). Generally, discharges were greater during Phase 1, and at some sites the Phase 2 high flow discharge was lower than that during Phase 1 base flow collection (Table 5.11).

### Table 5.9: The number of Cryptosporidium oocysts detected per 10 litre equivalent of surface water sampled during Phase 1

<table>
<thead>
<tr>
<th>Site</th>
<th>Stocking phase</th>
<th>Base flow samples (16/1/02, 18-19/4/02)</th>
<th>High flow samples, Run 1 (17 &amp; 22/1/02)</th>
<th>High flow samples, Run 2 (23 &amp; 24/1/02)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Volume sampled (litres)</td>
<td>Number of oocysts per 10 litres</td>
<td>Volume sampled (litres)</td>
</tr>
<tr>
<td>1</td>
<td>Phase 1</td>
<td>515</td>
<td>0.00</td>
<td>181</td>
</tr>
<tr>
<td>2</td>
<td>Phase 1</td>
<td>504</td>
<td>0.22</td>
<td>96</td>
</tr>
<tr>
<td>3</td>
<td>Phase 1</td>
<td>309</td>
<td>0.00</td>
<td>101</td>
</tr>
<tr>
<td>4</td>
<td>Phase 1</td>
<td>269</td>
<td>0.37</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>Phase 1</td>
<td>241</td>
<td>1.66</td>
<td>69</td>
</tr>
<tr>
<td>6</td>
<td>Phase 1</td>
<td>176</td>
<td>6.24</td>
<td>53</td>
</tr>
<tr>
<td>7</td>
<td>Phase 1</td>
<td>147</td>
<td>0.00</td>
<td>51</td>
</tr>
<tr>
<td>8</td>
<td>Phase 1</td>
<td>174</td>
<td>0.57</td>
<td>83</td>
</tr>
<tr>
<td>9</td>
<td>Phase 1</td>
<td>80</td>
<td>0.00</td>
<td>Not sampled</td>
</tr>
<tr>
<td>10</td>
<td>Phase 1</td>
<td>70</td>
<td>0.00</td>
<td>Not sampled</td>
</tr>
<tr>
<td>11</td>
<td>Phase 1</td>
<td>100</td>
<td>0.00</td>
<td>Not sampled</td>
</tr>
<tr>
<td>Mean</td>
<td>Phase 1</td>
<td>235</td>
<td>0.82</td>
<td>(n=11)</td>
</tr>
</tbody>
</table>

**Supplementary samples during phase 1**

- **Downstream 6 (land drain)**: Not sampled
- **Farm D (small beck)**: Not sampled

164
Table 5.10: The number of Cryptosporidium oocysts detected per 10 litre equivalent of surface water sampled during Phase 2

<table>
<thead>
<tr>
<th>Site</th>
<th>Stocking phase</th>
<th>Base flow samples Run 1 (26-28/3/03)</th>
<th>Base flow samples, Run 2 (2-3/4/03)</th>
<th>High flow samples, (1/4/03)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Volume sampled (litres)</td>
<td>Number of oocysts per 10 litres</td>
<td>Volume sampled (litres)</td>
</tr>
<tr>
<td>1</td>
<td>Phase 2</td>
<td>204</td>
<td>0.00</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>Phase 2</td>
<td>271</td>
<td>0.00</td>
<td>180</td>
</tr>
<tr>
<td>3</td>
<td>Phase 2</td>
<td>250</td>
<td>0.00</td>
<td>172</td>
</tr>
<tr>
<td>4</td>
<td>Phase 2</td>
<td>201</td>
<td>0.00</td>
<td>99</td>
</tr>
<tr>
<td>5</td>
<td>Phase 2</td>
<td>200</td>
<td>0.00</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>Phase 2</td>
<td>208</td>
<td>0.00</td>
<td>193</td>
</tr>
<tr>
<td>7</td>
<td>Phase 2</td>
<td>207</td>
<td>0.00</td>
<td>176</td>
</tr>
<tr>
<td>8</td>
<td>Phase 2</td>
<td>219</td>
<td>0.00</td>
<td>132</td>
</tr>
<tr>
<td>9</td>
<td>Phase 2</td>
<td>182</td>
<td>0.00</td>
<td>103</td>
</tr>
<tr>
<td>10</td>
<td>Phase 2</td>
<td>186</td>
<td>1.08</td>
<td>150</td>
</tr>
<tr>
<td>11</td>
<td>Phase 2</td>
<td>276</td>
<td>0.00</td>
<td>166</td>
</tr>
</tbody>
</table>

Mean Phase 2
Volume sampled (litres): 185 (n=22)
Number of oocysts per 10 litres: 0.49 (5 +ve)

Supplementary samples during phase 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume sampled (litres)</th>
<th>Number of oocysts per 10 litres</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 (February)</td>
<td>100</td>
<td>49.00</td>
</tr>
<tr>
<td>11 (February)</td>
<td>100</td>
<td>9.00</td>
</tr>
<tr>
<td>Farm A land drain a (March)</td>
<td>Not sampled</td>
<td>Not sampled</td>
</tr>
<tr>
<td>Farm A land drain b (March)</td>
<td>Not sampled</td>
<td>1</td>
</tr>
<tr>
<td>Farm A land drain c (March)</td>
<td>Not sampled</td>
<td>10</td>
</tr>
</tbody>
</table>

The range in the numbers of oocysts detected in scheduled surface water samples was greater during Phase 1 (Phase 1 = 0 to 20.29 oocysts per 10 litres equivalent; Phase 2 = 0 to 16.09 oocysts per 10 litres equivalent)(Table 5.9 and 5.10). The percentage of base flow samples containing Cryptosporidium oocysts was greater during Phase 1 than Phase 2, but this was not statistically significant (Phase 1 = 5/11 (45%), Phase 2 = 5/22 (23%), $\chi^2=1.74$, df=1, p=0.24). The percentage of high flow samples containing Cryptosporidium oocysts was also greater during Phase 1 than Phase 2, but again this was not statistically significant (Phase 1 = 11/16 (69%), Phase 2 = 6/11 (55%), $\chi^2=0.54$, df=1, p=0.69). The proportion of samples containing
Cryptosporidium oocysts increased from base flow to high flow conditions in both phases, but this was also not statistically significant (Phase 1 = 45%-69%, $\chi^2=1.41$, df=1, $p=0.26$; Phase 2 = 23%-55%, $\chi^2=3.24$, df=1, $p=0.12$). Additional base flow samples taken during Phase 2 from Farms A and D, whilst animal faecal matter sampling in February 2003, had counts of 49 and 9 oocysts per 10 litres equivalent respectively and an opportunistic land drain sample collected during Phase 2 water sampling contained 4000 oocysts per 10 litres equivalent.

Table 5.11: Discharge (m$^3$ s$^{-1}$) at the time of collection of Cryptosporidium samples. Data generated by and printed with permission from the Centre for Research into Environment and Health

<table>
<thead>
<tr>
<th>Crypto Site No.</th>
<th>EA Site No.</th>
<th>Flow condition</th>
<th>Phase 1 discharge (m$^3$ s$^{-1}$)</th>
<th>Phase 2 discharge (m$^3$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td>EA Site 1</td>
<td>Base flow</td>
<td>0.14</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High flow</td>
<td>-</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.67</td>
<td>0.40</td>
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<td></td>
<td></td>
<td></td>
<td>1.10</td>
<td>-</td>
</tr>
<tr>
<td>Site 2</td>
<td>EA Site 5</td>
<td>Base flow</td>
<td>0.15</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High flow</td>
<td>-</td>
<td>0.13</td>
</tr>
<tr>
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<td></td>
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<td>0.54</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.99</td>
<td>-</td>
</tr>
<tr>
<td>Site 3</td>
<td>EA Site 3</td>
<td>Base flow</td>
<td>0.42</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High flow</td>
<td>-</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.07</td>
<td>1.00</td>
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<td></td>
<td></td>
<td>2.54</td>
<td>-</td>
</tr>
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<td>Site 4</td>
<td>EA Site 10</td>
<td>Base flow</td>
<td>3.23</td>
<td>1.27</td>
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<tr>
<td></td>
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<td>High flow</td>
<td>-</td>
<td>1.18</td>
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<td></td>
<td></td>
<td></td>
<td>8.06</td>
<td>1.91</td>
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<td></td>
<td></td>
<td>7.98</td>
<td>-</td>
</tr>
<tr>
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<td>EA Site 6</td>
<td>Base flow</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High flow</td>
<td>-</td>
<td>0.01</td>
</tr>
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<td></td>
<td></td>
<td>0.13</td>
<td>0.02</td>
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<td></td>
<td></td>
<td></td>
<td>0.09</td>
<td>-</td>
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<tr>
<td>Site 6</td>
<td>EA Site 9</td>
<td>Base flow</td>
<td>0.41</td>
<td>0.06</td>
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<tr>
<td></td>
<td></td>
<td>High flow</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.83</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.94</td>
<td>-</td>
</tr>
<tr>
<td>Site 7</td>
<td>EA Site 11</td>
<td>Base flow</td>
<td>1.33</td>
<td>0.19</td>
</tr>
<tr>
<td>Farm A</td>
<td></td>
<td>High flow</td>
<td>-</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.62</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td>3.07</td>
<td>-</td>
</tr>
<tr>
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<td>EA Site 12</td>
<td>Base flow</td>
<td>7.16</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High flow</td>
<td>-</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19.07</td>
<td>3.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19.15</td>
<td>-</td>
</tr>
</tbody>
</table>
Differences were observed in the presence and numbers of oocysts detected depending on whether the sample site was influenced by upland or lowland terrain (Figure 5.7). *Cryptosporidium* oocysts were found more often and in greater numbers at sites influenced by lowland terrain, particularly during rain-induced high flow, in both Phases 1 and 2. This is also apparent in the concentration of oocysts at each site along the upland-influenced River Caldew (sites 1, 3 and 4) and lowland-influenced Roe Beck (sites 5, 6 and 7) prior to their convergence (Figure 5.8). During both phases, only low numbers of oocysts were isolated from the River Caldew prior to its convergence with Roe Beck. In contrast to this, higher levels of oocysts were seen in Roe Beck, particularly during high flow. Below the confluence of the two streams the concentration appears to decrease (site 8), suggesting that the higher concentration in Roe Beck is diluted by the lower concentration in the upland influenced waters of the River Caldew (Figure 5.8). The only exception to this is with the base flow during Phase 1 where, although the concentrations of oocysts are low in both, the number in the upper River Caldew is higher just prior to the confluence (site 4). However, the dilution effect is still seen during this sampling period as the concentration in the upper River Caldew is reduced when the combined with the waters from Roe Beck (site 8)(Figure 5.8).

All the surface water samples were also tested for the presence of *Cryptosporidium* DNA by nested PCR of the SSU rDNA. Of the 30 samples positive by IFAT, 13 were also positive by PCR while 17 were PCR negative. In addition to these IFAT and PCR positives, three samples were IFAT negative but PCR positive (described in greater detail in 5.3.2 – *Cryptosporidium* species identification, Table 5.14 and 5.16).

5.3.1.b – Livestock faecal samples
A total of 570 samples were obtained from farmed animals during Phase 1 (Table 5.12). The numbers collected achieved over-sampling (see 5.2.1.c – Livestock) of the populations of dairy cows and sheep at all farms, lambs at Farm C and calves at Farm D. At Farm A only one lamb sample was obtained because lamb droppings were difficult to find amongst the bedding. At Farm C the lambs were at pasture and droppings were visible in the short grass. The calf population at Farm A was very small since most calves had been sold, so the entire population of 5 calves was sampled. A total of 21/570 (4%) faecal samples contained *Cryptosporidium* oocysts,
Figure 5.7: Comparison of numbers of Cryptosporidium oocysts detected in surface waters during base flow and rainfall-induced high flow event conditions: (a) Phase 1; and (b) Phase 2.
Figure 5.8: Cryptosporidium oocyst concentration along transects following the River Caldw (i.e. sites 1, 3, 4, 8 and 11) and Roe Beck/Lower Caldw (i.e. sites 5-8 and 11) during base flows and high flows: (a) Phase 1; and (b) Phase 2.
detected by IFAT: 19/66 (29%) calves, 1/156 dairy cows, 1/136 lambs and 0/212 sheep.

A total of 702 faecal samples were obtained from farmed animals during Phase 2 (Table 5.12). The numbers collected achieved over-sampling of the populations of dairy cows and sheep at all farms, calves at Farm A and D and lambs at Farm C. Once again lamb faeces at Farm A were difficult to find amongst the bedding and over-sampling was not quite achieved with only 19 samples being collected. Cryptosporidium spp. oocysts were detected by IFAT in 41/702 (6%) samples: 37/136 (27%) calves, 0/182 dairy cows, 1/216 lambs and 3/168 (2%) sheep.

During Phase 1, fewer farmed animals in total were positive for Cryptosporidium but the difference was not statistically significant ($\chi^2=3.15$, df=1, $p=0.08$). On the continually stocked farms there were also slightly fewer animal positive for Cryptosporidium during Phase 1 (20/429, 4.7%) than in Phase 2 (25/490, 5.1%), but the difference was also not significant ($\chi^2=0.10$, df=1, $p=0.76$). However, the increase in the number of Cryptosporidium positive farmed animals on the re-stocking farms from Phase 1 (1/141, 0.7%) to Phase 2 (16/212, 7.5%) was highly statistically significant ($\chi^2=8.61$, df=1, $p=0.003$).

5.3.1.c – Wildlife faecal samples
A total of 74 wild animal droppings were collected during Phase 1 and Cryptosporidium oocysts were detected by IFAT in 5 (7%): 4/22 (18%) fox, 1/31 (3%) roe deer, with no positive results from 10 pheasant, 8 badger and 3 rabbit samples (Table 5.13).

A total of 41 droppings were collected from wild animals during Phase 2 and Cryptosporidium oocysts were detected in 2 (5%) samples, both (2/15, 13%) from roe deer. Negative results were obtained from 9 fox, 2 pheasant and 15 badger samples (Table 5.13). Although the prevalence was slightly higher in wild animals during Phase 1 the difference was not statistically significant ($\chi^2=0.16$, df=1, $p=0.69$).
Table 5.12: Sample prevalence of Cryptosporidium in farmed animals

<table>
<thead>
<tr>
<th>Farm</th>
<th>Animal type</th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Population size</td>
<td>Number of positive samples / total number of samples</td>
<td>Population size</td>
</tr>
<tr>
<td>A Re-stocking</td>
<td>Dairy cow</td>
<td>120</td>
<td>0/58</td>
</tr>
<tr>
<td></td>
<td>Calf</td>
<td>5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>35</td>
<td>0/32</td>
</tr>
<tr>
<td></td>
<td>Lamb</td>
<td>45</td>
<td>0/1</td>
</tr>
<tr>
<td>B Re-stocking</td>
<td>Dairy cow</td>
<td>70</td>
<td>1/43</td>
</tr>
<tr>
<td></td>
<td>Calf</td>
<td>5</td>
<td>0/2</td>
</tr>
<tr>
<td>C Continually stocked</td>
<td>Sheep</td>
<td>574</td>
<td>0/180</td>
</tr>
<tr>
<td></td>
<td>Lamb</td>
<td>320</td>
<td>1/135</td>
</tr>
<tr>
<td>D Continually stocked</td>
<td>Cattle</td>
<td>148</td>
<td>0/55</td>
</tr>
<tr>
<td></td>
<td>Calf</td>
<td>46</td>
<td>19/59</td>
</tr>
</tbody>
</table>

Table 5.13: Sample prevalence of Cryptosporidium in wild animals

<table>
<thead>
<tr>
<th>Farm type</th>
<th>Animal type</th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of positive samples / total number of samples</td>
<td>Number of positive samples / total number of samples</td>
<td></td>
</tr>
<tr>
<td>Re-stocking</td>
<td>Fox</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Pheasant</td>
<td>0/10</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>Roe Deer</td>
<td>0/29</td>
<td>0/11</td>
</tr>
<tr>
<td></td>
<td>Badger</td>
<td>0/3</td>
<td>0/6</td>
</tr>
<tr>
<td>Continually stocked</td>
<td>Fox</td>
<td>2/17</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>Roe Deer</td>
<td>½</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>Badger</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>0/3</td>
<td>0/0</td>
</tr>
<tr>
<td>Close to water site 6</td>
<td>Fox</td>
<td>Not sampled</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>Pheasant</td>
<td>Not sampled</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>Roe Deer</td>
<td>Not sampled</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td>Badger</td>
<td>Not sampled</td>
<td>0/4</td>
</tr>
</tbody>
</table>
5.3.2 – Species differentiation of recovered isolates

5.3.2.a – Surface water samples

From the surface water samples collected during Phase 1, Cryptosporidium spp. were detected by PCR in six of the IFAT positive samples from three of the sites (Sites 2, 4 and 5)(Table 5.14 and Figure 5.9). In addition to these, Cryptosporidium DNA was also detected by PCR in a single IFAT negative sample from site 8. C. andersoni was the most commonly identified species during Phase 1 being found in five samples from three sites (Sites 4, 5 and 8), whereas C. parvum was only identified in one sample (at Site 5). The Cryptosporidium detected at the other PCR-positive site during Phase 1 (Site 2) was identified as a novel genotype. Sequence analysis of this novel genotype revealed that it had 95% sequence identity with a Cryptosporidium spp. isolated from a ground squirrel, over a 382bp fragment of the SSU rRNA gene.

Table 5.14: Species identification from surface waters by PCR-RFLP in Phase 1

<table>
<thead>
<tr>
<th>Sample site</th>
<th>Number of oocysts per 10 litre equivalent of water sampled; species ID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-event base flow</td>
</tr>
<tr>
<td>1</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>2</td>
<td>0.22; PCR neg</td>
</tr>
<tr>
<td>3</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>4</td>
<td>0.37; C. andersoni</td>
</tr>
<tr>
<td>5</td>
<td>1.66; C. andersoni</td>
</tr>
<tr>
<td>6</td>
<td>6.24; PCR neg</td>
</tr>
<tr>
<td>7</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>8</td>
<td>0.57; PCR neg</td>
</tr>
<tr>
<td>9</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>10</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>11</td>
<td>0.00; PCR neg</td>
</tr>
</tbody>
</table>

Supplementary samples phase 1

| Land Drain d/s Site 6 | Not sampled | 0.00; PCR neg | 0.00; PCR neg |
| Farm D Small Beck    | 0.00; PCR neg | Not sampled | Not sampled |

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CIS map produced by Dr. Carl Shapleton

Two high flow samples from different days.

NB: Each circle represents one sample (i.e. one high flow).

Figure 5.9: Results of C. propinquum

(a) Base Flow
(b) High Flow
From the surface water samples collected during Phase 2, Cryptosporidium spp. were detected by PCR in five of the IFAT positive samples from four of the sites (Sites 4, 7, 8 and 10) and from two IFAT positive supplementary samples from Site 7 and a land drain at Farm A (Table 5.15 and Figure 5.10). In addition to these, Cryptosporidium DNA was also detected in two IFAT negative samples from sites 4 and 10, during base flow conditions. C. andersoni was again the most commonly identified species during Phase 2, in five samples from two sites (Sites 4 and 10).

One of these isolates found at site 10 produced an RFLP pattern that was different from the others (Figure 5.11), but when a 300bp fragment of the SSU rDNA was sequenced, it showed 100% homology with C. andersoni isolates in the BLAST database. C. parvum was identified more often during Phase 2, in two scheduled samples at sites 7 and 8 and in the supplementary samples from site 7 and the land drain on Farm A.

Table 5.15: Species identification results in surface waters by PCR-RFLP during Phase 2

<table>
<thead>
<tr>
<th>Sample site</th>
<th>Number of oocysts per 10 litre equivalent of water sampled; species ID</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-event base flow</td>
</tr>
<tr>
<td>1</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>2</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>3</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>4</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>5</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>6</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>7</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>8</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>9</td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td>10</td>
<td>1.08; C. andersoni</td>
</tr>
<tr>
<td>11</td>
<td>0.00; PCR neg</td>
</tr>
</tbody>
</table>

Supplementary samples phase 2

<table>
<thead>
<tr>
<th>Sample site</th>
<th>Number of oocysts per 10 litre equivalent of water sampled; species ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 7 (February)</td>
<td>49.00; C. parvum</td>
</tr>
<tr>
<td>Site 11 (February)</td>
<td>9.00; PCR neg</td>
</tr>
<tr>
<td>Farm A land drain a (March)</td>
<td>-</td>
</tr>
<tr>
<td>Farm A land drain b (March)</td>
<td>-</td>
</tr>
<tr>
<td>Farm A land drain c (March)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4000.00; C. parvum</td>
</tr>
<tr>
<td></td>
<td>0.00; PCR neg</td>
</tr>
<tr>
<td></td>
<td>0.00; PCR neg</td>
</tr>
</tbody>
</table>
GIS map produced by Dr. Carl Stappleton (positive for C. parvum) had an extra sample collected in February which was analyzed with the exception of site 7, which two base flows samples from different days.

NB each circle represents one sample (i.e. high flows during Phase 2). 

Figure 5.10: Results of Ciprofloxacin
Figure 5.11: An agarose gel showing how the sample in lane 3 (which was later confirmed as *C. andersoni* by sequence analysis) produced slightly smaller fragments than the other *C. andersoni* isolates in lanes 5, 8, 9 and 10.

Lane M = 100bp DNA ladder; Lane 1 = *C. hominis*;
Lanes 2, 4, 6, 7, 11 = *C. parvum*; Lane 12 = negative control.
5.3.2.5 - Livestock faecal samples

With the exception of one novel genotype all of the Cryptosporidium isolates from farmed animals that could be characterised by PCR-RFLP were found to be C. parvum (Table 5.16). Of the 20 IFAT-positive samples from farmed animals on continually stocked farms in Phase 1, 17 were found to contain C. parvum (16 from calves and one from a lamb), one was a novel genotype and two isolates were negative by PCR. The novel genotype, which was isolated from a calf on Farm D, could not be identified from its RFLP pattern and sequence analysis showed no homology with any currently recognised species or genotypes. The closest identity to this novel genotype in the BLAST database was 94% similarity to an unknown Cryptosporidium species recovered from a fox over 483bp. Cryptosporidium was only identified in one faecal sample from the animals being used to re-stock farms, which was in a dairy cow at farm B, but unfortunately the species of this isolate could not be determined as it was PCR negative. The oocyst morphology of all PCR-negative isolates was similar to C. parvum.

Table 5.16: Cryptosporidium spp. in livestock by PCR-RFLP

<table>
<thead>
<tr>
<th>Farm</th>
<th>Animal type</th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Dairy cow</td>
<td>0/58</td>
<td>0/57</td>
</tr>
<tr>
<td>Re-stocking</td>
<td>Calf</td>
<td>0/5</td>
<td>15/34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14 x C. parvum</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 PCR neg</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>0/32</td>
<td>0/37</td>
</tr>
<tr>
<td></td>
<td>Lamb</td>
<td>0/1</td>
<td>1/19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 x PCR neg</td>
</tr>
<tr>
<td>B</td>
<td>Dairy cow</td>
<td>1/43</td>
<td>0/62</td>
</tr>
<tr>
<td>Re-stocking</td>
<td>Calf</td>
<td>0/2</td>
<td>0/3</td>
</tr>
<tr>
<td>C</td>
<td>Sheep</td>
<td>0/180</td>
<td>3/131</td>
</tr>
<tr>
<td>Continually</td>
<td></td>
<td></td>
<td>1 x C. parvum</td>
</tr>
<tr>
<td>stocked</td>
<td></td>
<td></td>
<td>2 x PCR neg</td>
</tr>
<tr>
<td></td>
<td>Lamb</td>
<td>1/135</td>
<td>0/197</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 x C. parvum</td>
</tr>
<tr>
<td>D</td>
<td>Cattle</td>
<td>0/55</td>
<td>0/63</td>
</tr>
<tr>
<td>Continually</td>
<td>Calf</td>
<td>19/59</td>
<td>22/99</td>
</tr>
<tr>
<td>stocked</td>
<td></td>
<td></td>
<td>16 x C. parvum</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 x C. parvum</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 x novel genotype</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 x PCR neg</td>
</tr>
</tbody>
</table>

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During Phase 2, 35 isolates of *C. parvum* were confirmed while six isolates were negative by PCR (Table 5.16). Of the 25 IFAT-positive samples from continually-stocked farms, 21 were found to contain isolates of *C. parvum* (20 from calves and one from a sheep) and 4 were PCR-negative. From the 16 re-stocking samples positive by IFAT in Phase 2, 14 isolates from calves were also confirmed as *C. parvum*, while 2 (one from a calf and one from a lamb) were negative by PCR. The oocyst morphology of all PCR-negative isolates was similar to *C. parvum*.

<table>
<thead>
<tr>
<th>Farm type</th>
<th>Animal type</th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re-stocking</td>
<td>Fox</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1x <em>C. parvum</em></td>
<td>1x PCR neg</td>
</tr>
<tr>
<td></td>
<td>Pheasant</td>
<td>0/10</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>Roe Deer</td>
<td>0/29</td>
<td>0/11</td>
</tr>
<tr>
<td></td>
<td>Badger</td>
<td>0/3</td>
<td>0/6</td>
</tr>
<tr>
<td>Continuously stocked</td>
<td>Fox</td>
<td>2/17</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x <em>C. parvum</em></td>
<td>1 x PCR neg</td>
</tr>
<tr>
<td></td>
<td>Roe Deer</td>
<td>1/2</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCR neg</td>
<td>PCR neg</td>
</tr>
<tr>
<td></td>
<td>Badger</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td>0/3</td>
<td>0/0</td>
</tr>
<tr>
<td>Close to water site 6</td>
<td>Fox</td>
<td>Not sampled</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>Pheasant</td>
<td>Not sampled</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>Roe Deer</td>
<td>Not sampled</td>
<td>1/1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>C. parvum</em></td>
</tr>
<tr>
<td></td>
<td>Badger</td>
<td>Not sampled</td>
<td>0/4</td>
</tr>
</tbody>
</table>

### Table 5.17: *Cryptosporidium* spp. in wild animals by PCR-RFLP

#### 5.3.2.c - Wildlife faecal samples

All of the *Cryptosporidium* isolates from IFAT-positive wildlife faeces that could be characterised were found to be *C. parvum* (Table 5.17). Of the five positive samples collected during Phase 1, 2 isolates (from foxes on a continually-stocked and a re-stocking farm) were confirmed as *C. parvum*, while 3 samples (one from a fox on a re-stocking farm and two from a roe deer and a fox on a continually-stocked farm)
were PCR-negative. During Phase 2, the microscopy-positive isolate from a roe deer sample collected close to water site 6 was confirmed as *C. parvum* and other roe deer *Cryptosporidium* isolate (from a continually stocked farm) was negative by PCR. The oocyst morphology of all PCR-negative isolates was similar to *C. parvum*.

### Table 5.18: *Cryptosporidium* species and *C. parvum* multilocus fragment types in surface waters

<table>
<thead>
<tr>
<th>Sample site and stocking phase</th>
<th>Pre-event base flow</th>
<th>High flow samples, Run 1 (17 &amp; 22/1/02)</th>
<th>High flow samples, Run 2 (23 &amp; 24/1/02)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>novel genotype</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td><em>C. andersoni</em></td>
<td><em>C. andersoni</em></td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td><em>C. andersoni</em></td>
<td><em>C. andersoni</em></td>
<td><em>C. parvum NT</em></td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td><em>C. andersoni</em></td>
<td>-</td>
</tr>
<tr>
<td>Phase 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td><em>C. andersoni</em></td>
<td><em>C. andersoni</em></td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td><em>C. parvum MLFT 33</em></td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td><em>C. parvum NT</em></td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td><em>C. andersoni</em></td>
<td><em>C. andersoni</em> and <em>C. parvum MLFT 7</em></td>
<td><em>C. andersoni</em></td>
</tr>
<tr>
<td>Phase 2 Supplementary samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 7 (February)</td>
<td><em>C. parvum MLFT 5</em></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Farm A land drain a (March)</td>
<td>-</td>
<td>-</td>
<td><em>C. parvum MLFT 5</em></td>
</tr>
</tbody>
</table>

NT: not typable

### 5.3.3 – Subspecies characterisation of recovered isolates

#### 5.3.3.a – Surface water samples

Of the isolates recovered from the water samples, the multilocus fragment typing scheme could only be applied to those of *C. parvum* as neither *C. andersoni* or the novel genotype amplified at any of the three microsatellite loci. The proportion of *C. parvum* isolates from the water samples that could be characterised to the subspecies level using this typing scheme was 67% (4/6), and 3 different multilocus fragment types (MLFTs) were identified (MLFT 5, 7 and 33) (Table 5.18). The single *C. parvum* isolate detected in the surface water samples during Phase 1 (site 5) was only positive at the ML1 locus and therefore could not be assigned a multilocus fragment type.
type (MLFT). During Phase 2, three of the four *C. parvum* isolates recovered from water samples amplified at all three loci and could be assigned MLFTs. Two of these were from site 7 (MLFT 5 and the novel MLFT 33) and one (MLFT 5) from the land drain on Farm A, which is situated slightly upstream from water site 7. The *C. parvum* isolate found at site 8 was PCR-negative at all three loci and could not be assigned an MLFT. In addition to this, one of the samples containing *C. andersoni* produced a *C. parvum* MLFT (MLFT 7), which suggests that this sample was mixed and initially seen as negative for *C. parvum* by PCR-RFLP due to PCR bias towards the higher numbers of *C. andersoni* in the sample.

5.3.3.b – Livestock faecal samples

The proportion of *C. parvum* isolates recovered from the faeces of farmed animals that could be characterised to the subspecies level was 50% (26/52), and 3 different MLFTs were identified (MLFT 5, 7 and 13)(Table 5.19). During Phase 1, the only *C. parvum* isolates recovered were from continually stocked farms. However, only the isolate recovered from a lamb on Farm C amplified at all three loci and was found to be MLFT 7 (the same as that found at site 10) and the remaining 16 *C. parvum* isolates, which were recovered from calves on Farm D could not be assigned a type as they did not amplify. As with the novel genotype recovered from the water samples, the novel genotype recovered from a calf sample did not amplify at any of the microsatellite loci either.

<table>
<thead>
<tr>
<th>Farm type</th>
<th>Animal type</th>
<th>Phase 1</th>
<th>Phase 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re-stocking</td>
<td>Calf</td>
<td>-</td>
<td>10 x <em>C. parvum</em> MLFT 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 x <em>C. parvum</em> NT</td>
</tr>
<tr>
<td>Continuously</td>
<td>Sheep</td>
<td>-</td>
<td>1 x <em>C. parvum</em> NT</td>
</tr>
<tr>
<td>stocked</td>
<td>Lamb</td>
<td>1 x <em>C. parvum</em> MLFT 7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Calf</td>
<td>16 x <em>C. parvum</em> NT</td>
<td>15 x <em>C. parvum</em> MLFT 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x novel genotype</td>
<td>5 <em>C. parvum</em> NT</td>
</tr>
</tbody>
</table>

NT: not typable
Of the 14 *C. parvum* isolates recovered from Farm A (re-stocking) during Phase 2, 10 were found to be MLFT 5 (the same MLFT as found in the water samples from site 7 and the land drain on Farm A) while four could not be assigned an MLFT. Of these four, two did not amplify at any locus, one amplified at the ML2 locus only and one amplified at the gp60 locus only. The two isolates that amplified at only one locus had the same fragment type (232bp for ML2 and 341bp for gp60) at that locus as the 10 MLFT 5 isolates from the same farm (Appendix B). Of the *C. parvum* recovered from the continually stocked farms, the single sheep isolate failed to amplify at any locus, but 15/20 isolates from the calves on Farm D were identified as MLFT 13.

Two of the five calf isolates from Farm D that could not be assigned a subtype did not amplify at any of the loci, where as the other three amplified at two of the three loci and corresponded to the respective fragments that make up MLFT 13 (Appendix B).

5.3.3.c – *Wildlife faecal samples*

Of the three *C. parvum* isolates identified from wild animal faeces only 1 could be assigned an MLFT (Table 5.20). The isolate recovered from a fox sample collected on re-stocking Farm C during Phase 1 was found to be a novel MLFT (MLFT 32) while the other two (one from a fox on Farm D during Phase 1 and one from a roe deer close to site 6 in Phase 2), were both PCR-negative at all three loci. The *C. parvum* isolate from the fox that did not amplify at any loci was collected at the same time (during Phase 1) as the 16 calf isolates from the same farm (Farm D), which also failed to amplify.

<table>
<thead>
<tr>
<th>Table 5.20: <em>Cryptosporidium parvum</em> multilocus fragment types in wildlife</th>
</tr>
</thead>
<tbody>
<tr>
<td>Farm type</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Re-stocking</td>
</tr>
<tr>
<td>Continuously stocked</td>
</tr>
<tr>
<td>Close to water site 6</td>
</tr>
</tbody>
</table>

NT: not typable
5.3.3.d – Human and animal clinical samples

The proportion of *C. parvum* isolates from clinical samples submitted to the CRU, originating from residents or animals within the Caldew catchment, which could be characterised to the subspecies level was 88% (7/8). Six different MLFTs were identified (MLFT 2, 5, 6, 33, 34 and 35), of which MLFT 34 and 35 were novel (Table 5.21). Of the three *C. parvum* isolates from human samples submitted in 2000, one was MLFT 6, one was MLFT 34 and one was not typable. Two *C. hominis* samples were also submitted in 2000, one was *C. hominis* MLFT 1 (Table 5.21) and one was a mixed infection (Appendix B). The presence of a 233bp ML1 fragment indicates that at least one *C. hominis* subtype is present, but the other could be either a *C. hominis* or a *C. parvum* subtype. The three *C. parvum* and one *C. hominis* isolates that were submitted to the CRU in 2002 were found to be three different *C. parvum* MLFTs (2, 5 and 35) and *C. hominis* MLFT 1. In addition to the human samples in 2002, two clinical calf samples were submitted and when subtyped they were found to be *C. parvum* MLFT 2 and *C. parvum* MLFT 33. The two human isolates, submitted to the CRU in 2003, were also both *C. hominis* MLFT 1.

Table 5.21: Species and multilocus fragment type of human and animal clinical Cryptosporidium isolates submitted to the CRU from the Caldew Catchment

<table>
<thead>
<tr>
<th>CRU Reference</th>
<th>Host</th>
<th>Year</th>
<th>Cryptosporidium Species</th>
<th>MLFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>W0144</td>
<td>Human</td>
<td>2000</td>
<td><em>C. parvum</em></td>
<td>P 34</td>
</tr>
<tr>
<td>W0240</td>
<td>Human</td>
<td>2000</td>
<td><em>C. parvum</em></td>
<td>NT</td>
</tr>
<tr>
<td>W0456</td>
<td>Human</td>
<td>2000</td>
<td><em>C. parvum</em></td>
<td>P 6</td>
</tr>
<tr>
<td>W1871</td>
<td>Human</td>
<td>2000</td>
<td><em>C. hominis</em></td>
<td>H 1</td>
</tr>
<tr>
<td>W2360</td>
<td>Human</td>
<td>2000</td>
<td><em>C. hominis</em></td>
<td>*</td>
</tr>
<tr>
<td>W4878</td>
<td>Human</td>
<td>2002</td>
<td><em>C. parvum</em></td>
<td>P 2</td>
</tr>
<tr>
<td>W5018</td>
<td>Human</td>
<td>2002</td>
<td><em>C. parvum</em></td>
<td>P 5</td>
</tr>
<tr>
<td>W6132</td>
<td>Human</td>
<td>2002</td>
<td><em>C. parvum</em></td>
<td>P 35</td>
</tr>
<tr>
<td>W6325</td>
<td>Human</td>
<td>2002</td>
<td><em>C. hominis</em></td>
<td>H 1</td>
</tr>
<tr>
<td>W8265</td>
<td>Human</td>
<td>2003</td>
<td><em>C. hominis</em></td>
<td>H 1</td>
</tr>
<tr>
<td>W8609</td>
<td>Human</td>
<td>2003</td>
<td><em>C. hominis</em></td>
<td>H 1</td>
</tr>
<tr>
<td>A484</td>
<td>Bovine calf</td>
<td>2002</td>
<td><em>C. parvum</em></td>
<td>P 33</td>
</tr>
<tr>
<td>A486</td>
<td>Bovine calf</td>
<td>2002</td>
<td><em>C. parvum</em></td>
<td>P 2</td>
</tr>
</tbody>
</table>

* Mixed infection with either 2 subtypes of *C. hominis* or *C. parvum* and *C. hominis*
NT: Not typable
5.4 – Discussion

Although water utilities regularly sample and test raw waters for the presence of *Cryptosporidium* oocysts for operational monitoring purposes, there have been a limited number of published studies into the occurrence of *Cryptosporidium* in surface waters in the UK. Several studies have been reviewed in the series of reports *Cryptosporidium in water supplies: Report of the Group of Experts* (Badenoch, 1990; Badenoch, 1995; Boucher, 1998), although these rarely included species identification. Of those that have been published, the characterisation of the recovered *Cryptosporidium* isolates to the species or subspecies levels is extremely rare as the detection and identification is generally based solely on microscopic observation.

At an individual farm level Kemp *et al.* (1995) reported the presence of ‘*C. parvum*-like’ oocysts in drainage waters from a farm that had a history of cryptosporidiosis in calves and noted a rise in levels during periods of peak calving and spreading of slurry and manure. More recently, Bodley-Tickell *et al.* (2002) also sampled agricultural surface water for the presence of *Cryptosporidium* oocysts and found that 66% of the samples from a ditch that drained a farmyard and the stream into which it flowed contained oocysts morphologically similar to *C. parvum*. Although cryptosporidiosis was not a major cause of morbidity among the livestock on this farm, long-term study demonstrated that both the farmed and wild animal populations were at times infected with ‘*C. parvum*-like’ organisms and *C. muris* was also detected in the rodent populations (Sturdee *et al.*, 2003). The high prevalence of *Cryptosporidium* in the surface water on the farm in that study may therefore have been due to the close proximity of oocyst-shedding animals. As well as a stream passing through this farm estate, Bodley-Tickell *et al.* (2002) also sampled a pond on a neighbouring wholly arable farm and found that 65% of these samples were also *Cryptosporidium* positive. Since the pond was at the top of the watershed, it was speculated that contamination might have been due to faecal matter of wild animals being washed into the standing water during wet weather. To support this, the authors observed extensive rat activity around the pond including individuals swimming and when sampled, 25% were positive for ‘*C. parvum*-like’ oocysts (Bodley-Tickell *et al.*, 2002; Quy *et al.*, 1999).
On a larger geographical scale, a study of upland surface waters in the Pennines and Lake District reported 32% samples contained *C. parvum*-like oocysts ("Cryptosporidium levels in upland valleys and the implications for water quality management”. Swindale Project Final Report to North West Water, quoted with permission of Keith Osborn). In addition to the lower occurrence in upland waters, the report also noted a much lower mean concentration of oocysts per litre in the upland study compared with the Warwickshire (lowland) study.

The same relationship in the occurrence of *Cryptosporidium* oocysts, when detected by IFAT, was seen with the scheduled surface water samples for the Caldew study (64% of lowland samples and 54% of upland samples during Phase 1, and 56% of lowland samples and 7% of upland samples during Phase 2). The concentration of oocysts during both phases of this study displayed a marked difference between the upland and lowland sites. The mean concentration of oocysts per 10 litre equivalent of *Cryptosporidium* positive samples was lower in the upland waters (5.57 in lowland samples and 1.14 in upland samples during Phase 1, and 5.01 in lowland samples and 3.54 in upland samples), reflecting the differences noted between the Swindale and Warwickshire projects. The lower occurrence and concentrations of oocysts at the upland sites (sites 1-4 and 9)(Figure 5.7) in this study could be a result of the differences in landscape and land usage. The upland areas are dominated by beef cattle and upland sheep systems with much lower stocking densities than the dairy systems which predominate in the lower Caldew and Roe Beck/River Ive subcatchments (Figure 5.12)(Sanders *et al.*, 2004). The highest concentrations of *Cryptosporidium* oocysts were found in the Roe Beck/River Ive subcatchments (sites 5, 6 and 7) as were the highest faecal indicator organism concentrations found by Sanders *et al.* (2004). The pattern of low concentrations in the River Caldew upstream of the confluence with Roe Beck, but higher concentrations downstream, coupled with a decrease in concentrations between the lowest site on Roe Beck and the next site downstream on the River Caldew, was also replicated in the faecal indicator organism study (Sanders *et al.*, 2004). All of these data indicate that the more intensively stocked dairy farms in these lowland areas of the catchment are likely to be the main source of environmental *Cryptosporidium*. 

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Figure 5.12: Farm types of commercial sized enterprises in the River Caldew catchment (Sanders et al., 2004)
During studies of raw waters in Northern Ireland far lower sample prevalence of *Cryptosporidium* was detected. Lowery *et al.* (2000) identified *Cryptosporidium parvum* oocysts in 3 of 160 water samples (1.9%) from different sources by IMS-PCR. Lowery *et al.* (2001) also published the results of a 4-year study of the occurrence of *Cryptosporidium* in surface waters in Northern Ireland. Between 1996 and 1998 the occurrence of *Cryptosporidium* declined from 5.1% samples positive to 0%, using IFAT to detect oocysts. In 1999, the sample prevalence rose to 7.4% due to the inclusion of reservoir water from which *C. parvum* had been detected and combining IMS recovery with the IFAT detection. During the final two years (1998 and 1999) of this study, the occurrence was also measured using PCR and IMS-PCR, respectively, to detect *Cryptosporidium* in the samples and these methods of detection found the prevalence, compared to IFAT and IMS-IFAT detection, increased from 0% to 2.5% in 1998 and from 7.4% to 8.5% in 1999 (Lowery *et al.*, 2001). However, a direct comparison cannot be made between studies at different locations due to differences including geography, local animals, animal husbandry and water quality. In addition, oocyst recovery and detection methods often differ between studies, which highlights the need for standardised protocols and the full evaluation of recovery, detection and characterisation techniques to be agreed within the scientific community.

The *Cryptosporidium* prevalence in Caldew water samples was higher by IFAT microscopy (53% and 37% for Phases 1 and 2 respectively) than when tested by PCR (23% for Phase 1 and 24% for Phase 2). Overall, of the 30 water samples found to be positive by IFAT only 13 were PCR positive. There are a several possible reasons for this:

i. It has been demonstrated that the composition of water from different local sources has different inhibitory properties that can directly affect PCR (Sluter *et al.*, 1997), although this should have been minimised by the use of IMS to separate the oocysts from inhibitory components in the sample matrix and by the use of spin columns during DNA extraction.

ii. The degradation of DNA before sampling and during the storage prior to PCR could potentially occur in the presence of nucleases. If the
Cryptosporidium oocysts are damaged the DNA could come into contact with any nucleases present in the environment or sample, which in turn may have an adverse affect on the PCR amplification.

iii. Although the nested PCR method used in this study has been shown to have excellent sensitivity (Sulaiman et al., 1999b; see 5.3.3.b - Evaluation of selected oocyst disruption methods with low numbers of oocysts), the method ultimately relies upon the presence of DNA. However, the fluorescent labelled monoclonal antibody that is used in the IFAT does not depend on the presence of DNA and can detect both intact and excysted oocysts. The use of DAPI staining allows the detection of DNA within oocysts but with low numbers of oocysts present some samples may include only DAPI-negative (excysted) oocysts.

iv. As far as is known, the nested PCR used in this study amplifies SSU rDNA from all currently known species of Cryptosporidium. However, it is possible that these primers may not amplify some currently undefined species. However, the same is also true for the specificity of the IMS and IFAT used in this study. While this has been tested for all Cryptosporidium species infecting mammals, and it is likely that the antibody is indeed genus-specific, there may be differences in avidity for different Cryptosporidium species (Thomas and Chalmers, 2003).

In the Caldew study, three surface water samples that were IFAT negative were PCR positive (one from Phase 1 and two from Phase 2). The three isolates that were PCR positive/IFAT negative were identified as *C. andersoni*, which is larger than the oocyst morphology guidelines (centred around *C. parvum*-sized oocysts) in the DWI drinking water regulations. Of the 16 PCR positive samples, 13 were characterised to the species level by PCR-RFLP of the SSU rDNA while 3 could not be assigned a species by this method due to a novel RFLP-pattern or none-digestion of the secondary PCR product. During Phase 1 *C. andersoni* was the most prevalent species (71% of positive samples) while *C. parvum* was confirmed by molecular methods in just one sample. In Phase 2, the most prevalent species was *C. andersoni* once again (56% of positive samples), which included one isolate that could not be characterised...
by PCR-RFLP, but had a similarity of 100% to *C. andersoni* when part of its SSU rDNA was aligned with those retrieved using the NCBI BLAST database. This isolate was amplified by the nested PCR but following the RFLP digest the fragments produce the same pattern as other *C. andersoni* isolates, but appeared smaller (Figure 5.11). It is possible that this could be a different subtype of *C. andersoni* and the smaller fragments be due to deletions in the ~830bp fragment amplified by the nested PCR, and if these deletions were outside of the ~300bp fragment used for sequence analysis then the alignment could still produce 100% similarity to other *C. andersoni* isolates. The prevalence of *C. parvum* during Phase 2 increased with 4 isolates being identified by PCR-RFLP and a further isolate detected from a sample containing *C. andersoni* when amplified with the primers for the multilocus fragment analysis.

Xiao et al. (2001c) used very similar methodology to that used in the Caldew study (including the use of Envirochek® filters, IMS, QIAGen® DNA mini columns and the nested SSU rDNA PCR) to study the *Cryptosporidium* species in raw surface water and wastewater in the USA. From the surface water samples that they collected, 46% (25/55) were *Cryptosporidium* positive and from the wastewater samples, 25% (12/49) were positive. In the surface waters they detected *C. andersoni* (most frequently), *C. hominis*, *C. parvum*, *C. canis*, *C. muris*, *C. felis* and the cervine genotype. However, the frequent finding of *C. andersoni* in the surface waters in the Caldew catchment is unprecedented in the UK. It is interesting that it was not detected in any of the animal samples tested within the catchment and the source(s) of this species in these waters remains unknown.

The two restocking farms included within this study both sourced stock from within England and at the time of this study there had never been any reports of *C. andersoni* isolated from animal sources in this country. However, in 1996 there was a report of *C. muris* being detected in the faeces of dairy cattle in Scotland prior to the discrimination between *C. andersoni* and *C. muris* (Bukhari and Smith, 1996), and it is likely that this was in fact *C. andersoni*, although it was never confirmed. Since the completion of the Caldew study the first confirmed *C. andersoni* infections in the UK were identified from dairy cattle on a farm in south-west Wales (Daniel et al., 2005). The prevalence within the herd on this farm was 16/101 (16%) and slurry was collected from these animals for application to stubble fields, possibly identifying a
route of environmental contamination (Robinson et al., in preparation). In addition to England, anecdotal evidence from the study carried out by Sanders et al. (2004) indicated that farmers also restocked from outside the UK, mainly from Ireland and the Low Countries, which could also provide a source of *C. andersoni*. The provenance of stock may be traced through the Animal Movements Licensing System (AMLS) and it may be possible to confirm this potential route of transmission through sampling of animals sourced from outside the UK. A study in the Czech Republic from 1993-1995, identified *C. muris*-like oocysts in 4.5% and 7.9% of imported cattle from France and Germany respectively (Pavlasek, 1995). However, it must be remembered that the *C. andersoni* isolates were also detected during Phase 1 when restocking was just beginning and the new animals were still being housed, so it is likely that these isolates were also present in the catchment prior to restocking. In addition, all of the livestock samples collected from the restocking farms during this period (with the exception of a single dairy cow sample) appeared to be negative for *Cryptosporidium*. During the earlier study in the Lake District and Pennines, which took place during the late 1990s, no *C. andersoni*-size oocysts were detected in farmed animal or surface water samples ("Cryptosporidium levels in upland valleys and the implications for water quality management". Swindale Project Final Report to North West Water, quoted with permission of Keith Osborn).

Study of the natural history of *C. andersoni* is at an early stage. So far, the major natural host for *C. andersoni* has been identified as adult or post-weaned cattle (Santin et al., 2004; Wade et al., 2000), but has also been identified in bactrian camels and a sheep (Xiao et al., 2004). Host specificity appears to be high as cattle-derived oocysts are not infectious to most rodent species, chickens or goats (Koudela et al., 1998; Lindsay et al., 2000), and differences have even been reported in the susceptibility of cattle to experimental infection (Enemark et al., 2002a). Following their transmission experiments and the data from other studies, Koudela et al. (1998) suggest that wild rodents are not likely to be the source of environmental contamination. However, as the Mongolian gerbils (*Meriones unguiculatus*) in their study appeared readily susceptible to infection with cattle-derived *C. muris* oocysts, the possibility that some wild rodent or other animal species could be a natural host cannot be ruled out. Infections with *C. andersoni* in cattle have been reported to be chronic but cause no overt illness, although reduced milk production has been seen from those animals.
(Anderson, 1998). The majority of studies examining the prevalence of *C. andersoni* infections among cattle have been carried out in the USA. Anderson et al. (1991) carried out a nationwide survey of bovine *C. muris* and found a low prevalence of 1.4% within US cattle populations, although certain pens had higher prevalences, particularly in dairy herds (e.g. 31% in a Connecticut dairy). One study in Michigan examined diarrhoeic faecal samples from human infections, cows and calves on 12 farms and water samples from 7 of the farms for the presence of *Cryptosporidium* (Peng et al., 2003b). Whereas *C. parvum* was identified on all but one farm (and sampling on that farm only consisted of two samples), *C. andersoni* was only identified on two of the 12 farms and only in four samples out of the 98 samples collected on these farms. Another survey in the state of New York identified *C. andersoni* oocysts in cattle faeces from 20 of 109 farms all of which were from animals older than 50 days whereas *C. parvum* was only recovered from animals less than 30 days old (Wade et al., 2000). The susceptibility of older animals to infection in this study could explain some of the difficulty in experimentally infecting cattle, as transmission studies have tended to use calves (Enemark et al., 2002a). *C. andersoni* has also been reported from cattle in Hungary, Denmark, the Czech Republic, Japan and Canada (Enemark et al., 2002b; Kvac and Vitovec, 2003; Matsubayashi et al., 2004; Satoh et al., 2003; Sreter et al., 2000; Ralston et al., 2003) and has only recently been molecularly confirmed in the UK in the peer-reviewed scientific literature (Daniel et al., 2005; Robinson et al., in preparation).

Jellison et al. (2002) identified *C. muris*, which may well have been *C. andersoni*, in a cattle manure pit and also in a brook that is though to be contaminated by agricultural runoff. This could highlight an important source of environmental contamination as adult cattle have been seen to chronically shed *C. andersoni* oocysts at a rate of $10^6$ oocysts per gram (Anderson, 1998) and spreading of slurry or solid waste on agricultural land is common practice. Manure management in the Caldew catchment was studied by Sanders et al. (2004) and the majority of manure applied to agricultural land is from adult cattle and spreading is mainly carried out over the winter months due to limited storage capacity at most farms (Table 5.22), which is just prior to our sampling periods. This could be an explanation for the higher prevalence in the Caldew water samples of *C. andersoni* than *C. parvum*, which may be more likely stem from the faeces of younger animals and therefore contamination
### Table 5.2: Summary of manure applications made to agricultural land in the whole of the Calder catchment in the period June 2000 to December 2003

<table>
<thead>
<tr>
<th>Land Use</th>
<th>Area of Land (ha)</th>
<th>Arable</th>
<th>Grass</th>
<th>Pasture</th>
<th>Shurry</th>
<th>Volcanic</th>
<th>Total</th>
</tr>
</thead>
</table>

**Applications are expressed as tonnes (t) by livestock and manure type (cubards or 1, 2004).**

December 2003, including the effects of cattle re-stocking and the seasonality of animal numbers on the June census figures.

<table>
<thead>
<tr>
<th>Land Use</th>
<th>Area of Land (ha)</th>
<th>Arable</th>
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<th>Volcanic</th>
<th>Total</th>
</tr>
</thead>
</table>

**Table 5.2: Summary of manure applications made to agricultural land in the whole of the Calder catchment in the period June 2000 to December 2003, including the effects of cattle re-stocking and the seasonality of animal numbers on the June census figures.**
Figure 5.13: An example of increased risk to water contamination through directly voided faeces from sheep grazing unrestricted next to the fast flowing upland surface water.

Figure 5.14: A photograph taken in the Caldew water catchment of increased risk to surface water contamination by slurry applied to a field by broadcast method over the top of a drainage ditch.
from farmyards rather than fields. It should also be noted that the samples for this study were taken whilst the majority of cattle were housed, thus the direct input of oocysts via directly voided excreta is limited to grazing sheep and wild animals, that often have unrestricted access to surface water (Figure 5.13). The slurries within the catchment are almost solely spread by broadcast methods to the land surface (Sanders et al., 2004) rather than injected into the soil matrix, which exposes the manure to processes such as saturated overland flow. The risk of environmental contamination from slurry applied by broadcast methods also increases through poor management and non-compliance with the Code of Good Agricultural Practice (COGAP)(Figure 5.14)(MAFF, 1998).

Although water utilities regularly monitor raw waters for Cryptosporidium, this is undertaken under the same sample screening criteria as DWI regulations and, thus, only C. parvum-sized oocysts are recorded. It is therefore not clear whether C. andersoni is a recent introduction or not, although from the Warwickshire and Swindale studies (Bodley-Tickell et al., 2002; Swindale Project Final Report) in which all oocysts sizes were searched for and in which larger oocysts were not detected, may suggest its recent introduction or presence in localised areas.

The prevalence of Cryptosporidium in animals has been more widely published than in surface waters around the UK. Scott et al. (1995) reported 61% and 66% in adult Scottish beef cattle from two farms (one with a history of cryptosporidiosis and the other without) and when the samples were concentrated prior to microscopy the prevalence increased to 92%. Chalmers et al. (2002a) reported a prevalence of 53% in apparently healthy lambs, also in Scotland, which also increased to 74% when concentrated prior to screening. However, in other studies much lower figures have been reported. Sturdee et al. (2003) reported the results of a 6-year survey (1992-1997) of the prevalence of Cryptosporidium in farmed and wild animals on a lowland Warwickshire farm which did not have a history of a problem with cryptosporidiosis. The livestock animals that were sampled as part of this study were bull beef, dairy cows, ewes, horses, homebred calves, bought-in calves and lambs. Only C. parvum-like oocysts were detected in the livestock, with lower cumulative 6-year prevalences in the adult animals (bull beef, 3.6%; dairy cows, 3.5%; ewes, 6.4%; horses, 8.9%) than in the young (homebred calves, 52%; bought-in calves, 23.2%; lambs, 12.9%).
In the upland study in the Pennines and Lake District, low prevalence of *Cryptosporidium* was reported in extensively farmed animals but was related to husbandry site (Swindale Project Final Report). Hutchison *et al.* (2004) sampled fresh and stored waste from cattle, pigs, poultry and sheep around Great Britain between 2000 and 2002, to identify the prevalence of zoonotic agents. From the fresh faecal samples that they collected, the prevalence of *Cryptosporidium* was 5.4% in cattle, 13.5% in pigs and 29.2% in sheep. The total prevalence of *Cryptosporidium* in farmed animals from the Caldew water catchment was 4% during Phase 1 and 6% during Phase 2, but the difference was not statistically significant. Although the difference in prevalence between farmed animals positive for *Cryptosporidium* in Phase 1 and 2 was not significant, the difference between positive farmed animals on re-stocking farms in phases 1 (1/141) and 2 (16/212) was statistically significant ($\chi^2=8.61$, df=1, p=0.003). The increase may be due to the greater number of calves that were sampled in Phase 2, but as the calf prevalence in this phase was 44%, the same prevalence during the previous year would have meant that at least two of the five calves (of which all were sampled) would have been positive. This significant increase of *Cryptosporidium* positive animals could also imply that the adult animals being used to re-stock these farms were healthy and free of *Cryptosporidium*. The highest prevalence during both sampling phases was in the calves (29% n=66 and 27% n=136 from phases 1 and 2 respectively), which is supported by the study on a Warwickshire farm as in both cases the farms did not have a history of problems with cryptosporidiosis. The prevalence of *Cryptosporidium* in adult livestock and lambs appears lower in the Caldew catchment than reported in these other surveys as only one cow in phase 1, three sheep in phase 2 and 1 lamb in each phase were shedding oocysts.

A number of wild animals in the UK have been identified as potential reservoirs for *Cryptosporidium* spp. Webster and MacDonald (1995) identified ‘C. parvum-like’ oocysts in 63% of the brown rats that they sampled. Sturdee *et al.* (1999) also studied the prevalence of *Cryptosporidium* in British wild animals. They described ‘C. parvum-like’ oocysts from foxes (9%, n=23), badgers (15%, n=26), muntjac deer (10%, n=42), fallow deer (6%, n=16), rabbits (7%, n=28), pygmy shrews (10%, n=10), common shrews (35%, n=20) and one of four hedgehogs sampled. These figures appear higher than those found in the Caldew wildlife, although the numbers
of samples were low. The difference in Cryptosporidium sample prevalence for wild animals in the Caldew catchment from each phase was not statistically significant (7% and 5% for phase 1 and 2 respectively). Although a relatively low number of wild animal samples were collected and it is therefore difficult to draw conclusions from these data, the prevalence in both wild and farmed animals could demonstrate a low baseline level of Cryptosporidium in the catchment, particularly on the farms included in the study. It is also difficult to compare the results of this study with those previously published as prevalence data could be expected to differ between geographical regions, due to the intensity and type of farming as well as the levels of wildlife, use of communal grazing and even the type of landscape and geology.

Of the 69 animal samples found to be Cryptosporidium positive by IFAT, 56 (81%) were amplified by the PCR targeting the SSU rDNA. With the exception of a single isolate that was recovered from a calf, all of the Cryptosporidium isolates were characterised to the species level by SSU rDNA PCR-RFLP and found to be C. parvum. This may have been expected as the majority of oocysts were isolated from calf faeces and C. parvum is a common parasite in such animals, although it is perhaps surprising when C. andersoni was the predominant species found in the surface waters. The C. andersoni isolates that were detected in the water samples are likely to have originated from other farms in the area that were not sampled in this study and via the spreading of slurry and manure of the adult animals onto local fields. Despite the limited host range currently known for C. andersoni, a wild animal reservoir for this species cannot be ruled out and may be identified in future studies.

The characterisation of isolates from wildlife as C. parvum is also not surprising as C. parvum-like oocysts have been found in several species of wild animal (Sturdee et al., 1999) and a wide host range, including humans, is well known. To my knowledge this is the first molecular confirmation of C. parvum oocysts from fox and roe deer faeces. However, the apparently asymptomatic presence of Cryptosporidium in animals such as foxes, since the faeces appeared normal, could also indicate spurious infection as their diet often includes other hosts of Cryptosporidium such as rabbits, rodents and livestock carcasses in fields and fells and the oocysts may be solely passing through. Nonetheless, these animals could act as vehicles of infection for other host species.
Of the isolates identified from the National Collection of *Cryptosporidium* Oocysts at
the Cryptosporidium Reference Unit (CRU) in Swansea only *C. hominis* and *C. parvum* were identified as causing human disease and *C. parvum* in the two animal
samples. *C. andersoni* has never been found in any human clinical isolates submitted
to the collection, which now exceeds 10,000 isolates and is not currently considered a
human pathogen. The multilocus fragment typing identified the subtypes *C. parvum*
MLFT 2, 5, 6, 34, and 35, and *C. hominis* MLFT 1 in the human clinical cases and *C. parvum* MLFT 2 and 33 in the animal clinical cases.

Of the *C. parvum* multilocus fragment types found in the surface water samples
(MLFT 5, 7 and 33), all were found in animals in the Caldew catchment. MLFT 7
was recovered from the faeces of a single lamb in Phase 1 (2002), MLFT 5 was
identified in 10 calves on Farm A in Phase 2 (2003) and a case of human
cryptosporidiosis in 2002 and MLFT 33 was found in a clinical case of calf
cryptosporidiosis submitted to the CRU in 2002. One of the human samples was
found to contain the same *C. parvum* MLFT (2) as an isolate that caused calf
cryptosporidiosis, both of which were submitted during the same year (2002). Of the
fragment types that were identified from the human clinical cases submitted to the
CRU, only *C. parvum* MLFT 5 was also found in animals sampled as part of this
study. While the source of the human infection has not been established, this MLFT
is fairly common (Anon., 2005). However, this fragment type is obviously infectious
to humans and as well as the isolates from the calves on Farm A and those recovered
from the water in at site 7 (downstream of Farm A), this subtype was also isolated
from farmyard waste water running out of a land drain on Farm A. The land drain
runs directly into a brook that connects with the Roe Beck slightly upstream of site 7.
With the evidence from these samples a source and route of environmental
contamination with an isolate of *C. parvum* that causes human and animal infection
has been identified and preventative measures can be implemented to reduce the risk.
These results demonstrate the usefulness of the MLFT scheme in tracing and
identifying the source or point of contamination. Although the isolates causing
clinical disease in humans and animals that were submitted to the CRU cannot in this
instance be linked to the source of their infection with further epidemiological data it
may be possible to establish these relationships.
All of the isolates that were recovered from different farms, which could be
classified, had different fragment types. This coupled with six different *C.
parvum* fragment types identified in 8 epidemiologically unrelated clinical samples
demonstrates the high level of discrimination that the typing scheme provides with the
use of only 3 molecular markers. The use of these three markers as a typing scheme
has recently been published since the end of this case study (Chalmers et al., 2005).
Chalmers et al. (2005) described the trial of five subtyping methods including the
multilocus sequence analysis of the three loci used for the Caldwel study. They found
that the multilocus approach was comparable with the other highly discriminatory
methods in their trial (gp60 sequencing and SSCP of the ITS-2 region), but stated that
the applicability of the method to species and genotypes other than *C. parvum* and *C.
hominis* and to environmental samples needs to be evaluated. I demonstrated that the
multilocus approach using these markers is applicable to environmental samples and
that *C. andersoni* as well as certain *Cryptosporidium* genotypes may not be amplified
using these primers. However, a full evaluation of the method, particularly using the
fragment analysis approach, with epidemiological unrelated samples is required.

There have been several publications of these microsatellite loci used individually. At
the ML1 locus Caccio et al. (2000) described four *C. parvum* subtypes by sequence
analysis and Mallon et al. (2003b) identified the same four types by fragment
analysis. At the ML2 locus Caccio et al. (2001) described seven different *C. parvum*
subtypes by sequence analysis and Alves et al. (2003a) used fragment analysis but
could not detect all of the subtypes due to fragment stutter on traces. Mallon et al.
(2003a; 2003b) identified 15 different *C. parvum* subtypes by sequence and fragment
analysis of the gp60 microsatellite. Although the three markers were not all used,
million et al. (2003a; 2003b) used ML1 and gp60 along with two other microsatellites
and three minisatellites to generate multilocus types. By increasing the number of
loci examined in a multilocus scheme, the discriminatory power can be increased, but
the number of loci chosen has to be balanced with the level of discrimination that is
actually required and the practicalities (such as time and cost) of carrying out the
work. The combined fragment analysis of ML1, ML2 and gp60 has to date identified
35 different *C. parvum* and 9 different *C. hominis* fragment types (Table 5.8). This
three-locus typing scheme is more practical for epidemiological purposes than those
employing greater numbers of loci for the study of population genetics, as it is less expensive and time consuming while still providing good discrimination.

The fact that only one of the clinical samples could not be assigned a fragment type also indicates that the method may have a high level of typability, but this needs to be assessed with a larger number of samples. It is unfortunate that only 60% of the *C. parvum* positive water samples and 50% of the prospectively gathered non-clinical *C. parvum* isolates within the catchment could be typed. However, the reduction is most probably due in part to the sensitivity of the PCR methods as the initial species identification was carried out with a nested PCR and the subtyping used single PCRs. Nested PCRs for each microsatellite were investigated but due to generation of non-specific amplicon further work needs to be undertaken in order to optimise these protocols. In addition to the sensitivity of the PCR, the SSU rDNA used to differentiate species has five copies per genome, where as the loci used for subtyping only have a single copy per genome. However, the gene coding the SSU rDNA is not a good target for subtyping since few intra-species polymorphisms have been identified and intra-isolate heterogeneity exists (see Chapter 2 - Review of subtyping methods and implications for the investigation of environmental transmission). The microsatellite MLFT scheme currently appears to be only applicable to *C. parvum* or *C. hominis*, since the other species and genotypes identified in the Caldew samples (*C. andersoni* and the two novel genotypes), did not amplify by the primers used. It is possible that the *C. parvum* isolate(s) infecting the calves at Farm D during Phase 1 were of an unusual strain for which PCR primer specificity was lacking in the subtyping scheme, as none of them produced any amplicons by these methods but some could be easily amplified by the species differentiation PCR. The finding of such a high number of *C. andersoni*-positive samples was unprecedented and identification of this species, regardless of its subtypes, is novel. The usefulness of the subtyping scheme for public health purposes is not compromised by its specificity since *C. parvum* and *C. hominis* predominate in human disease in the UK.

One mixed sample was identified in this study, containing both *C. parvum* and *C. andersoni*. This water sample had been found to contain *C. andersoni* by PCR-RFLP but gave a *C. parvum* fragment type when subtyped indicating that it is likely to be a mixed sample. The reason that the sample was not originally identified as being
mixed is probably due to a competition effect within the PCR between the higher numbers and therefore rate of amplification of C. andersoni than that of C. parvum. If the amplification of C. andersoni reached a plateau before the detectable limit of C. parvum had been produced then only C. andersoni would be visible. However, as the fragment typing appears to be specific to C. parvum and C. hominis this competition effect was removed and only C. parvum amplified.

The elevation of Cryptosporidium concentrations in surface waters during rainfall induced high flow events has previously been observed by Wyer et al. (2001) in samples collected from three rivers in Ayrshire. Elevations in high flow geometric mean concentrations were observed at all three sites, two of which were statistically significant. The proportion of positive samples also increased from 55.9% during base flow conditions to 81.3% during high flow conditions (Wyer et al., 2001). Whilst statistical tests on the low number of samples from each site from this project are not prudent, an elevation in concentrations is evident between base flow and high flow samples, particularly at lowland sites (Figure 5.7, Table 5.9 and 5.11). In addition, where species identification was possible, the higher concentrations of oocysts in water samples appears to be related to C. parvum, which could indicate a specific point of contamination (i.e. drainage from a farmyard) rather than a more diluted source of contamination (i.e. runoff from fields). Although not statistically significant, the proportion of positive samples during high flow events (Phase 1: 69%; Phase 2: 55%) was also greater than the proportion of positive base flow samples (Phase 1: 45%; Phase 2: 23%). Flows at the time of sampling during the high flow event of Phase 2 were much lower than those during Phase 1 for any given site, which may explain the lower proportion of positives during Phase 2 (Table 5.11).

The increased concentrations of Cryptosporidium during rainfall induced high flow events can be attributed to similar processes to those inducing increases in faecal indicator organism concentrations, i.e. saturated overland flow (Tyrrel and Quinton, 2003), runoff from farm hard-standing areas (Kay et al., 2003) and stream stage rise over bank areas provides a pathway for microorganism delivery to the rivers, whilst increased velocities may re-entrain organisms from settled sediments (McDonald and Kay, 1981; Wilkinson et al., 1995). Furthermore, increased discharges from the
sewerage infrastructure, most noticeably combined sewage overflows (CSOs) may provide additional sources.

It is hard to assess whether the flow state has any effect on the species distribution due to the low number of samples containing Cryptosporidium that could be assigned a species. However, of the scheduled samples collected, C. parvum was only found in high flow samples and C. andersoni in both base flow and high flow samples (Table 5.14 and 5.16). This pattern was seen during both phases of sampling.

The housing of cattle in winter and grazing in summer, seasonal patterns of manure management and the breeding cycles of the livestock (Cryptosporidium was mainly found in the faecal samples of calves and lambs) all suggest that Cryptosporidium oocyst concentrations in surface waters are likely to vary over the year. The results of the EA study of faecal indicator organism concentrations in the Caldew catchment demonstrated lower concentrations during the winter periods, even during high flow conditions (Sanders et al., 2004).

Through this study it was possible to take advantage of the rare opportunity created by the FMD outbreak through the near complete culling of livestock in the area. The methods developed in the earlier chapters enabled the characterisation of Cryptosporidium from environmental and animal samples containing low numbers of oocysts prior to and following the re-stocking of the catchment. It is interesting to note the higher proportion of surface water samples positive for Cryptosporidium during Phase 1 (Table 5.9 and 5.11). Despite the drastic reduction of livestock levels as a result of the FMD cull, the hypothesis that Cryptosporidium was less prevalent in the environment therefore appears to be erroneous. However, species identification suggested that C. andersoni dominated in surface waters (Table 5.14 and 5.16), which to date, has not been associated with human illness. Nevertheless, the spatial distribution of positive samples, which covers the majority of the sample sites across catchment, suggests a relatively ubiquitous source. It is possible that livestock-derived oocysts from before the FMD cull remained in the environment (in fields or in the silt on river beds, which is disturbed more during rain-induced high flow). The wild animals in the catchment could also contribute to the environmental contamination during the period when livestock was at a minimum, although the
results of this study do not provide any evidence of this with only a low proportion of wildlife found positive. A more likely source of environmental contamination is through the spreading of ‘disinfected’ slurries during the restricted period. Adherence to the COGAP for handling and disposal of farm waste (MAFF, 1998) should help minimise contamination of watercourses, although it is impossible to completely eradicate it (Bouchier, 1998). Slurries were treated with lime to increase the pH (or alternatively, treated to reduced the pH) to destroy FMD. While survival of Cryptosporidium oocysts in terms of viability and therefore onward transmission is affected during storage of manure and slurry due to pH, temperature and ammonia concentrations (Jenkins et al., 1998), their detection and identification if non-viable but still intact, by IMS, IFAT or PCR would not necessarily be affected.

5.5 – Conclusions
Water sampling sites were successfully identified and samples collected during base flow and high flow conditions in both Phases 1 (2002) and 2 (2003)(Objectives 4a and 4c). Two continually stocked farms and two re-stocking farms, with animals that are located within the catchment, were recruited into the study and representative samples were successfully collected during both Phases 1 and 2 (Objectives 4b and 4d). All of the human and animal clinical cases that have been submitted to the CRU from within the catchment and reported no foreign travel were identified through postcode data for inclusion in the study (Objective 4e). The modified Parasep® method developed for faecal screening in Chapter 3 was used to screen all 1,387 faecal samples from farmed and wild animals in Phases 1 and 2 (Objective 4f). The optimised oocyst disruption methods were used to prepare the DNA from positive faecal and water samples prior to molecular characterisation (Objective 4g). The species of Cryptosporidium in positive samples was identified using nested SSU rDNA PCR-RFLP analysis (Objective 4h). Subtyping was carried out on PCR positive samples using multilocus (ML1, ML2 and gp60) fragment analysis (Objective 4i).

There was no significant difference in the prevalence of Cryptosporidium in the surface water samples collected during Phase 1 and those during Phase 2 and the increase from base flow to high flow was also not significant during either phase. This suggests that the restrictions and denuding of livestock in the area may not have had any significant effect on the levels of environmental Cryptosporidium. However,
the influence of upland or lowland terrain did affect the presence and numbers of Cryptosporidium in the surface water, with greater environmental contamination occurring in lowland influenced water.

The prevalence of Cryptosporidium infections in livestock was 4% during Phase 1 and 6% during Phase 2 but this difference was not significant. Whilst there was not a significant difference between the prevalence on continually stocked farms from Phase 1 to Phase 2, the difference on re-stocking farms was highly significant (p=0.003). This could be due to the age of imported animals, as C. parvum is predominantly a major infection in young animals, or restocking with healthy animals. In the wild animal samples that were collected there was no significant difference seen between the two phases (7% and 5% respectively), but the numbers of samples were very low.

The Cryptosporidium IFAT positive samples (species by PCR-RFLP) from during the whole study were, 56/202 (28%) calf samples (50 C. parvum, one novel genotype and five PCR negative), 1/338 (0.3%) adult dairy cow samples (one PCR negative), 2/352 (0.6%) lamb samples (one C. parvum, one PCR negative), 3/380 (0.8%) adult sheep samples (one C. parvum, two PCR negative), 4/31 (13%) fox samples (two C. parvum, two PCR negative) and 3/46 (7%) roe deer samples (one C. parvum, two PCR negative). Only C. parvum and a single novel genotype were identified in the faecal samples collected from animals in the catchment. C. parvum was identified on both the re-stocking and continually stocked farms during both phases although on the re-stocking farms the only species confirmation in Phase 1 was from a fox.

As only 13 of the 30 IFAT positive water samples were PCR positive, molecular methods cannot yet be used instead of microscopic detection methods, but molecular characterisation of Cryptosporidium in positive samples is still very important. The reasons for the low sensitivity with these environmental samples are unknown. The predominant species of Cryptosporidium identified in the water samples during both phases was C. andersoni, which is unprecedented in the UK. The source of this environmental contamination is unknown and this species was not identified in any of the animal samples that were collected.
C. parvum subtypes seen to be pathogenic to humans by typing those recovered from clinical cases in the catchment, were identified in both livestock and water samples. A point source of environmental contamination with one such subtype (C. parvum MLFT 5) was also identified, demonstrating the potential use of these techniques in enhancing source tracking during incident investigations (Objective 4i). The subtypes in animals on re-stocking or continually stocked farms were seen to be different and on each farm type the subtypes were also different during Phase 1 and Phase 2.

5.6 – Proposed Future Work

The unprecedented finding of the predominance of C. andersoni in surface water samples raises questions as to the source. The presence in so many samples from different areas around the catchment suggests a fairly ubiquitous source. However, none of the animals sampled in this study were positive for C. andersoni. Future studies are needed to identify the sources of this environmental contamination and could be based on using water sampling to trace it to a certain area and then target potential wild and farmed animal sources around that area.

Future studies should also examine the seasonal variation of environmental Cryptosporidium. Much of the livestock in the catchment is housed overwinter and due to the limited storage capacity, slurry spreading increased. The effects of less directly voided faeces on fields and the application of manure and slurry, in addition to other seasonal agricultural activity may affect the levels and types of Cryptosporidium present in the environment.

Only animals from 4 farms were included in this study, partially due to the proximity to the FMD outbreak and farmers were cautious, but further studies should be carried out to gain a representative sample of the area. In order to screen the larger number of samples from more farms, a method involving the pooling of samples for PCR detection (as described in 4.6 – Future Work) could be developed.

During the Caldew study, the farmyard of one of the recruited farms was found to be a point source for environmental contamination, with a human pathogenic C. parvum subtype contaminating a watercourse via a land-drain. The opportunistic sample that was collected from this drain contained a concentration of Cryptosporidium oocysts
of 4000 oocysts per 10 litres. Future studies, should attempt to assess the environmental impact of Cryptosporidium from farmyard hard-standings as the Caldew EA study found most drain into directly into a ditch or stream.

At the outset of this study, the methodology for recovering DNA from samples on slides had not been developed, however the successful amplification of Cryptosporidium DNA was achieved from a slide in this study. Therefore in future studies, 100% of the IMS sample can be dissociated and examined by microscopy to accurately know how many oocysts are present in the sample and the proportion of them that are DAPI positive, prior to recovery from the slide for molecular characterisation.

For the purposes of this study, various samples were collected in an attempt to take an holistic approach to the study of Cryptosporidium within the catchment. However, certain samples were not included this time, including slurry, farmyard manure and waste from septic tanks, which should be examined in future studies for the holistic approach to be complete.

Recent work in Canada has used repetitive dilution PCR to identify different species that are present within the same water sample (Neumann et al., 2004). The principle is to use less template DNA and repeat the PCR several times in an attempt to remove the PCR bias from the most dominant species in the sample. This approach could be used in future studies and may also be useful in identifying samples with mixed subtypes present.

Whilst the use of the multilocus fragment typing appears to be fairly discriminatory from this and other studies, a formal evaluation of the method with epidemiologically unrelated samples is required to determine the typability, reproducibility, discriminatory power and limit of detection.
Section 4

General Discussion

and

Future Research
Chapter 6 – General Discussion

In this chapter I discuss the public health significance of *Cryptosporidium* and how molecular characterisation of these species and genotypes is important in studying the epidemiology of these ubiquitous parasites and in making informed health-based decisions. The main conclusions from the work described in the thesis are discussed and how they can benefit the investigation of environmental *Cryptosporidium* and their impact on human populations.

6.1 – The public health significance of environmental *Cryptosporidium*

Although *Cryptosporidium* was first recognised as a human pathogen in 1976 (Nime *et al.*, 1976; Meisel *et al.*, 1976), it wasn’t until 1982 and 1983 that it became of major concern to public health as the cause of fatal disease in individuals with AIDS (Ma and Soave, 1983; Current *et al.*, 1983; Forgacs *et al.*, 1983; Anon., 1982a). During 1983 it also became apparent that cryptosporidiosis was a frequent cause of self-limiting profuse, watery diarrhoea in otherwise healthy people (Jokipii *et al.*, 1983; Current *et al.*, 1983; Tzipori *et al.*, 1983), and the laboratory diagnosis of *Cryptosporidium* infections in humans soon became common practice (Casemore *et al.*, 1985).

The public health significance of *Cryptosporidium* in the environment is dependent on the likelihood of infection and the extent of human disease that may be caused by these isolates. Therefore, some of the key questions are:

- Are these species, genotypes or subtypes infectious to humans?
- If infectious, how pathogenic and virulent are they?
- What is the likely outcome of a human infection?
- Are they viable?
- Is it likely that humans will come into contact with them?

The first three of these key questions are entirely dependent on the accurate characterisation of the *Cryptosporidium* detected, although their answers are also
dependent on various parasite and host factors that influence the risk of infection and disease. There is some level of evidence of these host and parasite factors from surveillance data and epidemiological studies (see 1.4 – Epidemiology, Clinical Features and Treatment). To determine this significance to the health of communities or populations at risk, there is a need to revisit these factors influencing the host-parasite interactions.

The parasite factors that determine their significance to public health include the pathogenicity, virulence and viability. The pathogenicity to humans is dependent on the host specificity of the species, genotype and even subtype of Cryptosporidium that is detected. In environmental samples there is the potential to find a wide number of different species, genotypes and subtypes, many of which have not been found to cause disease in humans. In the Caldwew study (see Chapter 5), the predominant Cryptosporidium species characterised from surface water was C. andersoni, currently thought to be non-pathogenic to humans. The detection of Cryptosporidium oocysts without identifying the species is not always sufficient information upon which to base public health intervention. For example, in drinking water it could lead to a boil water advisory notice that could actually carry greater risk (scald injuries; loss of public confidence in the water; costs to industry and health care) than that posed by the oocysts themselves.

The virulence of different Cryptosporidium species and subtypes varies, as demonstrated in infectivity studies in healthy human volunteers (see 1.4.3 – Infective Dose and Duration of Infection). However, the differences between species and particularly within species are not yet fully understood and specific virulence markers have not yet been identified for Cryptosporidium.

The viability of oocysts is vital to their public health significance and has important implications particularly in environmental samples. The hardy nature of Cryptosporidium oocysts means that they can remain viable for long periods even following exposure to different environmental conditions and disinfectants, explaining their success in waterborne transmission. Whilst oocysts are environmentally tough, the proportion of viable organisms recovered in these samples is hard to assess. The microscopic detection of Cryptosporidium used by the water industries in both the UK
and the US does not determine the viability or the species and can therefore not be used to assess their public health significance. Assessing the viability of Cryptosporidium in the environment is beyond the scope of the work described in this thesis, and further studies are required in this area since there are inherent problems in determining viability. Two approaches are currently used, infectivity in an animal model or cell culture, or by using a surrogate. The former relies on availability of an appropriate susceptible model, which poses some difficulty for C. hominis. While experimental infection has been established in neonatal animals, the infective dose used in these studies was particularly high (in the order of >$10^5$ oocysts)(Giles et al., 2001). Surrogates for infectivity are the exclusion and inclusion of two fluorogenic vital dyes (DAPI and Propidium Iodide), the induction of sporozoite excystation, fluorescence in situ hybridisation (FISH) and reverse transcription-PCR (RT-PCR). However, all of these approaches have their own drawbacks and lack both sensitivity and specificity, particularly when low numbers are present, such as in environmental samples. The potential advantage of the FISH and RT-PCR is the simultaneous differentiation of species/genotype.

In addition to the parasite, various host factors affect the public health significance of Cryptosporidium acquired directly from the source or via an environmental route. The primary host factors that determine the populations at risk are the immune system and risk factors for exposure, including the frequency and amount thereof. For example, activities that result in exposure to faecal material such as crawling on the ground, poor personal hygiene, visiting farms, changing nappies or travelling to areas of the world where hygiene and water treatments are not as controlled can increase the chance of infection. Surveillance data shows that most cases occur in the 1 to 4 year olds, and an epidemiological study incorporating genotyping demonstrated significant risks were specific for C. parvum (contact with farmed animals) and C. hominis (changing nappies and recent foreign travel) (Hunter et al., 2004b). This important study, and the long term enhanced molecular surveillance (Anon 2002b; CRU ongoing, unpublished data) demonstrate that, since the epidemiology of the two species and risk factors are different, species identification is desirable for Cryptosporidium.

Individuals with immature or weakened immune systems, such as young children, those with immunodeficient diseases or suppressed immunity, are more likely to
develop severe and prolonged cryptosporidiosis. In contrast, individuals repeatedly exposed probably develop immunity over time and populations with a high level of exposure to Cryptosporidium can develop herd immunity, providing a certain level of protection against disease (Casemore et al., 1997). Circulating antibodies to C. parvum have been detected in patients with C. hominis infection and it is likely that immunity may be cross protective (CRU unpublished data).

It is the interaction between these host-parasite factors that actually determines the public health significance of Cryptosporidium, making it extremely difficult to predict without prior knowledge of the population and history of, for example, the watershed. However, decisions can be made to protect public health by considering these factors. For example, a protected watershed that provides the source water for Sydney, Australia, has a population of wild kangaroos. A study of the Cryptosporidium species/genotypes in the kangaroos revealed types not known to be infectious to humans and this information has been included in the risk assessment for the water supply (Power et al. 2004). Molecular characterisation of isolates paired with comprehensive source (for environmental isolates) or epidemiological and pathological (for clinical isolates) data will help identify additional reservoirs, pathogenicity, virulence and risk factors for the transmission of human pathogenic species and genotypes, as well as those thought to have no aetiological importance to human cryptosporidiosis, leading to greater understanding of the public health impact of these different taxa.

Whilst waterborne transmission of Cryptosporidium was identified in the 1984 (D'Antonio et al., 1985), the importance to public health of this route was highlighted in 1993 when a massive waterborne outbreak occurred in Milwaukee (Mackenzie et al., 1994). In England and Wales, several unconfirmed waterborne outbreaks were suspected during the 1980's, but in 1989 an outbreak in the Oxford and Swindon regions was identified and attributed to the consumption of mains water from a polluted reservoir (Casemore, 1990). During this outbreak 516 cases were confirmed but it is likely that there were further undiagnosed cases. During the 1990's a number of outbreaks were linked to the consumption of water from the public supplies, which led to the drinking water regulations that came into effect in 1999 (see 1.8.3 – Drinking Water Regulations). Due to these regulations, continual monitoring,
improvements to water treatment, catchment and source water protection have helped reduce the number of outbreaks associated with public drinking water with only three (including one that only had a possible association with public drinking water) reported in England and Wales since 2000 compared with 10 between 1995 and 2000 (data from twice yearly reports on the surveillance of waterborne disease and water quality in CDR Weekly). Neither viability nor species/genotype are identified in regulatory monitoring in England and Wales, while in Scotland a project is in place to identify species in all incidents involving detection of oocysts in monitored water supplies, thus providing further data to inform the risk assessment.

Although the waterborne outbreaks via public drinking water have become less common, other waterborne routes of transmission such as private water supplies and recreational water have remained a threat (Table 6.1). Recreational waters, including swimming or paddling pools, interactive or ornamental fountains and rivers have all been linked to outbreaks in the UK, with 16 recreational waterborne outbreaks reported to the CDSC since 2000 and 14 between 1995 and 2000 (data from twice yearly reports on the Surveillance of waterborne disease and water quality in CDR Weekly). Improved education and awareness may result in the reduction of recreational waterborne outbreaks, as the contamination of these waters is often from human sources, such as children having faecal accidents.

The application of species/genotyping to outbreak samples has demonstrated that *C. parvum* causes human outbreaks both via environmental routes (e.g. the North West drinking water outbreak in 2000) and from a human source (swimming pool outbreaks). More discriminatory molecular characterisation has also been shown to add support to descriptive and analytical epidemiology. Following an outbreak at a splash zone and petting zoo in the South West of England during 2003, 31 *Cryptosporidium* positive samples were sent the CRU for typing. Of these 31, 29 were *C. parvum* and linked to the outbreak but 2 were *C. hominis* and therefore probably not linked. Using the splash zone was the significant risk factor and *C. parvum* oocysts were detected in water samples. The main suspected source was cross contamination from the farm animals to the splash zone via children’s dirty footwear. Although none of the animals present prior to the outbreak were sampled, a
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Count</th>
<th>Species of CRPV identified in</th>
<th>Sampled Plant</th>
<th>Region</th>
<th>Year</th>
<th>Month</th>
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<td>6 (9)</td>
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<td>2000</td>
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<td></td>
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<td>London</td>
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<td>Jan</td>
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<tr>
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<td></td>
<td></td>
<td>Public drinking water</td>
<td>Hambrook</td>
<td>2000</td>
<td>Jan</td>
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<td></td>
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<td>South West</td>
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<td>Feb</td>
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<td></td>
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<td>North West</td>
<td>2000</td>
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<td>South East</td>
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<td>Public drinking water</td>
<td>South East</td>
<td>2000</td>
<td>Apr</td>
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</table>

Table 6.1: Details of CRPV outbreaks identified to the CRPV Resistance Index between 2000 and 2003 (CRPV dam)
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Date</th>
<th>Location</th>
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</tr>
<tr>
<td>Example 3</td>
<td>2005</td>
<td>South East</td>
</tr>
</tbody>
</table>

*Note: Table data is illustrative and not based on actual content.*
goat that arrived as a replacement during the outbreak was found to be shedding *C. parvum* oocysts, as was its handler. Subtyping analysis using gp60 DNA sequencing demonstrated that the *C. parvum* recovered from cases who were visitors to the park were all of the same subtype (IIaA16G2). However, the *C. parvum* recovered from the goat and its handler were found to be a different subtype (IIaA19G1). Unfortunately, *C. parvum* subtyping was not possible from the DNA recovered from the diagnostic slide from the water sample (which only contained 1 oocyst). However, the work in this thesis provides the tools for a more efficient recovery of DNA from slides and will be of use in similar studies in the future.

Prior to the molecular characterisation of *Cryptosporidium*, our analysis and interpretation of surveillance data and epidemiological information was based on the genus as a whole. Since the development of these tools, much more data has become available and the epidemiological understanding has developed. The geographical distribution of the genus *Cryptosporidium* is worldwide and human cryptosporidiosis has been reported from all continents. However, although cryptosporidiosis was known to occur nationwide in England and Wales, molecular characterisation identified differences in the epidemiology, as the predominant species causing human infection in each geographical area varies greatly (see 1.4 – Epidemiology, Clinical Features & Treatment). In the urban areas of the UK, greater levels of *C. hominis* were identified, inferring an anthropontic source and in the rural areas *C. parvum* was predominant, which could suggest either source. By using greater resolution methods these *C. parvum* isolates may be separated and allow a specific source to be identified. There is some evidence from recent studies that human-only isolates and markers of zoonotic potential may exist within certain subtypes of the species *C. parvum* (Xiao and Ryan, 2004; Mallon *et al.*, 2003b; Anon, 2005). Studying the seasonal distribution of *Cryptosporidium* has also benefited from molecular methods as the two typical, annual peaks of human cryptosporidiosis found in England and Wales could be separated (see 1.4 – Epidemiology, Clinical Features & Treatment). The spring peak is predominantly caused by *C. parvum* whereas the autumn peak more commonly by *C. hominis*. This *C. parvum* spring peak coincides with major lambing and calving times as well as increased rainfall, which could be a major risk factor in the transmission via environmental routes. In addition, national surveillance between 1992-2004 identified 11 *Cryptosporidium* outbreaks associated with animal
contact on farms, 10 of which occurred during the spring (HPA data). The *C. hominis* autumn peak coincides with foreign travel to areas where the risk of anthroponotic infection may be greater and it also coincides with the increased use of recreational water, which as mentioned above, could also result in *Cryptosporidium* from anthroponotic sources.

From a public health point of view it is preferable to identify *Cryptosporidium* before they have caused human infection, including those circulating in animal reservoirs and those contaminating the environment. Whilst using genus-specific methods will detect all the *Cryptosporidium*, the role of molecular characterisation in epidemiological studies is vital to understand the public health impact of these *Cryptosporidium* species, such as the routes of transmission, risk factors, the virulence and pathology, age distribution, seasonality and the geographical distribution of individual species or subtypes. In addition, the use of genus specific methods does not enable the tracking of contamination back to the source, whereas molecular characterisation of isolates recovered from environmental samples could be used for this purpose, allowing interventions to protect the environment from contamination in the future. Although, the use of molecular epidemiology has increased over the past couple of years to demonstrate the differences between species (see 1.4 – Epidemiology, Clinical Features and Treatment), this line of *Cryptosporidium* research is still relatively new.

At present water companies cannot make public health decisions regarding *Cryptosporidium* in their source water, as they rely solely on genus-specific detection, which does not determine whether the detected oocysts are pathogenic, virulent or viable. The species and subtypes of *Cryptosporidium* found in water samples can vary greatly between different geographic locations and reasons for this include:

- The type and amount of agriculture and animal husbandry in the area
- Geological features
- The weather and climate
- Local wild animal populations
- The location of sewage treatment works and septic tanks
- The location of abattoirs

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To gain further understanding on environmental *Cryptosporidium*, molecular characterisation data needs to be coupled with detailed information from the area of interest. Understanding and identifying the source of pathogenic *Cryptosporidium* is important in any control strategies that may be implemented and is possible using high-resolution molecular methods, such as sequence analysis or multilocus typing. This approach can identify points of environmental contamination and link them to incidents and outbreaks of human disease as the strain involved can be distinguished from others that are not of public health importance. Monitoring the finished water is important, but with spatial sampling across a water catchment, key source areas can be identified and improved management and protection strategies implemented.

**6.2 – Conclusions of the work described in this thesis and how it will assist in investigating the public health significance of *Cryptosporidium* in the environment**

The detection and characterisation of environmental *Cryptosporidium* involves several stages: the detection of what are often very few oocysts; the maximal recovery and purification of DNA from those oocysts; and the use of highly discriminatory molecular methods that can not only distinguish between but also within different species. The work in this thesis has addressed these different stages and demonstrated their potential use in the Caldew catchment case study.

The choice of molecular method depends upon the balance of resolution required and logistics of using such methods (see Chapter 2 – Review of subtyping methods and implications for the investigation of environmental transmission). Whilst the use of DNA sequence analysis can provide the highest level of discrimination, there are drawbacks to using such an approach. The convenience criteria are important, as time, cost and skills required to carry out DNA sequence analysis can be excessive when a large number of samples must be processed. Environmental samples also have the potential to contain mixed isolates, which can be difficult to identify with sequence analysis. However, fragment size analysis can be used to detect these mixed population samples, as was seen during the Caldew study. In addition to sequence and fragment analysis, the literature review identified the mutation scanning technique, SSCP, to provide a comparable level of discrimination. However, specialist equipment that is not readily available in many laboratories is required to
use this method. As well as the method of analysis, the choice of locus is important for the molecular characterisation of Cryptosporidium isolates as the loci contain different amounts of polymorphism. From the literature review, the gp60 locus and several microsatellite loci were identified as promising for the high-resolution characterisation of Cryptosporidium. This level of discrimination was increased when multiple loci were used.

However, before these methods can be used to investigate the public health significance of Cryptosporidium in environmental samples the oocysts must be detected and recovered. This is often made more difficult in environmental samples as the numbers of oocysts can be low. Whilst the use of IMS enables the detection and recovery of these low numbers, its use is particularly expensive and not practical for studies with large sample numbers, such as surveys screening non-symptomatic faecal samples from herds of farmed animals and wildlife. The development of the modified FEA method using the Parasep® faecal parasite concentrator (see Chapter 3 – Detection of potentially low numbers of Cryptosporidium spp. oocysts) enabled a 16 and 5.9 fold increase in the numbers of oocysts detectable from sheep and cattle faecal samples, respectively. Oocysts were still detectable in both types of faeces at a concentration of 500 opg when detected by immunofluorescence microscopy. The use of this method in the Caldew catchment study enabled the detection of Cryptosporidium oocysts in non-symptomatic farmed and wild animals, which will assist in elucidating the potential reservoirs of human-pathogenic Cryptosporidium.

Once the oocysts have been detected, the optimal method of DNA extraction and purification is required prior to the molecular characterisation these isolates. The optimal methods that were identified during the evaluation of the various oocyst disruption and DNA purification experiments (see Chapter 4 – Maximising the recovery of DNA for molecular characterisation from oocysts of Cryptosporidium) demonstrated that the DNA from a single oocyst can be recovered and amplified by molecular techniques. In addition, the recommended method of three freeze-thaw cycles followed by Proteinase K digestion for 30 minutes at 56°C was used successfully in the recovery of Cryptosporidium DNA from environmental surface water samples, including positive results from isolates recovered from slides. This
will facilitate, the characterisation of Cryptosporidium isolates found in water samples during routine monitoring to determine whether they are infectious to humans. This information could be used reactively during incidents or proactively to provide historical data about the catchment. In previous studies, such as in the splash zone and petting zoo outbreak described above, Cryptosporidium DNA was not successfully recovered from slides for molecular characterisation beyond species. However, the work in this thesis advances these studies by providing an evaluated and efficient method of DNA recovery and extraction from samples including slides for discriminatory typing.

All of these methods were successfully combined and used in the Caldew water catchment case study to investigate the presence of Cryptosporidium spp. and subtypes in surface water, farmed and wild animals and clinical samples from both cattle and humans. The unprecedented finding of C. andersoni as the most prevalent species in the surface waters is important, as prior to this study there were no data of the variation in species present in UK waters. This study demonstrated the potential for these methods to be used in epidemiological investigations, which will help to determine whether the species and subtypes in these samples are of public health significance. With these tools specific transmission cycles can be identified within and between various host types and reservoirs, such as human specific C. parvum subtypes. As seen in the Caldew catchment study there is the potential for tracing sources of environmental contamination and it will be possible to link environmental isolates with incidents and outbreaks of human cryptosporidiosis. By tracking subtypes from the source of contamination it is also possible to investigate how these isolates spread throughout the environment in surface water and via slurry/manure spreading on land. With greater knowledge in these areas, better strategies can be developed to protect the environment and the human populations who may be exposed to the pollution through consumption or recreational use of contaminated waters and the countryside.

The knowledge and methods generated by the work in this thesis have now been adopted by the Cryptosporidium Reference Unit as routine and are being used to study the species and subtypes of Cryptosporidium recovered as part of various investigations. For example, using these methods the unit has been able to
characterise *Cryptosporidium* recovered from swimming pools, surface waters, septic tanks and animal faecal samples to provide data that influences the direction of investigations into outbreaks, water quality incidents and studies into the occurrence of *Cryptosporidium* in various animal species.

**6.3 – Future Research**

Armed with the new tools that are being developed for detection and characterisation, the future of *Cryptosporidium* research will involve the investigation of individual species and strains. There are a number of important gaps in our knowledge regarding *Cryptosporidium* that these tools can assist with. For environmental *Cryptosporidium* this includes:

- What are the sources of environmental contamination with individual species and strains?
- What is the distribution of them?
- Are they infectious to humans?
- What is the infectious dose of each strain?
- What is the clinical outcome of an infection with each species or strain?
- How can this environmental contamination be controlled at the source?

In order to answer some of these questions it is important to continue environmental surveillance using molecular tools and to link the data generated from these studies to those from sporadic and outbreak disease surveillance.

The effect of improved drinking water treatment on public health through epidemiological studies must also be further investigated and involve both disease surveillance and serological studies. Serological studies will provide data on the background exposure of populations to *Cryptosporidium* and can be compared to the amount of disease that is reported in those communities. The approach to providing safe drinking water can then be re-assessed based on health related data and adjusted accordingly: whether this involves continual monitoring, identifying and removing risks from the catchment prior to treatment or monitoring the efficiency of treatment processes.
The risk factors associated with different species and strains can also be further evaluated using molecular epidemiology. This will enable improved practices and interventions to be implemented to reduce the potential for infection as well as increasing our knowledge of the nature and severity of Cryptosporidium infections with individual species and strains. These studies may identify genetic markers that could indicate the source of transmission, such as zoonotic, anthroponotic or even individual reservoir species.

Whether oocysts are found in water, soil, wild or farmed animals, the future of investigating the public health significance of these Cryptosporidium species and strains is dependent on their identification based on molecular characterisation.
Section 5

References

and

Appendices
References


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Leoni, F., Gallimore, C.I., Green, J. and McLauchlin, J. (2003b) Molecular epidemiological analysis of Cryptosporidium isolates from humans and
animals by using a heteroduplex mobility assay and nucleic acid sequencing based on a small double-stranded RNA element. *Journal of Clinical Microbiology* **41**: 981-992.


APPENDIX A: Detailed farm descriptions at the time of sampling

Farm A.

A 114.5 hectare Dairy farm with a small flock of Texel sheep

Completely culled out of dairy cattle, beef cattle and sheep due to FMD on premises. Re-stocking has taken place under a sentinel scheme with 140 heifers and 35 Texel sheep. The beef cattle will not be replaced.

The replacement cows came from Gloucestershire and are mostly in milk but some are still to calve. There are 20 calves at present. Calving should be over by mid-April 2002. The cows are housed on slatted flooring in a spacious shed and the dry cows are over the road in separate housing (in same area as lambing shed). The cows will be housed until 1st April 2002.

The sheep arrived in-lamb; all but 2 had lambed by 28th February 2002. The sheep are housed in a shed in the same area as the dry cows and will be kept in until the weather improves when they will go outside initially during the day. Sampling the adult sheep is not a problem but samples from the lambs is near impossible, as there is complete lack of lamb faeces on the ground.

Slurry tower contains a mixture of old and new slurry (contains urine and rainwater); underground slurry store contains “new” slurry.

The land belonging to this farm is spread over a large area and includes a few wooded areas, providing plenty of wildlife habitats; therefore plenty of time is needed for walking the areas (woodland and borders). There are Badger sets (although these could not be accessed) in the woodland that borders field 671 and Roe Deer as well as evidence of fox activity (faeces and bird kills) were seen close to this woodland. There was also evidence of game shooting by this field and woodland. Roe deer were also seen along the border of another woodland area in field 407. Fox, pheasant, hare and rabbit activity have also been seen in this field.
Sampling of water that runs off from this farm can be carried out at one of the sites selected for the original water survey (EA Site 11, OS National Grid Reference: NY 387465 (338760,546580)), as this site is just downstream from a beck that drains the water from the farm.

More time is needed for sampling this site as the land is spread over a vast area.

**Farm B.**

**A 137 hectare dairy farm**

Completely culled out due to FMD on the premises. Now re-stocking with 91 Holstein Friesian (Hfr) from Staffordshire and Wiltshire, which arrived on 7th February 2002. 36 were bought in-milk and the rest were bought dry but in-calf (due to calve by the end of April). The female Hfr calves will be kept for herd replacement and the others sold after weaning. The unweaned calves are kept in dual pens and the farmer is happy for us to sample them (shed with calf pens is adjacent to milk storage room).

The cows are currently in 2 sheds, separating the milking and dry cows. The sheds are quite cramped but could be sampled, particularly when the cows leave for milking. The floors are slatted to collect the slurry into underground storage but slurry also includes urine etc. Some spreading has been done (old slurry).

The farm traditionally over wintered sheep on tack but had none over the past 12 months. They will probably over winter sheep this year and these will arrive in November 2002.

Wildlife has increased over the past year. There are a lot of mice in the long tatty ungrazed grass, which would not normally happen. There is a big badger set on the other side of the stream. The set can be sampled when it is possible to cross the stream, i.e. when water level is low or via tree that bridges river depending upon conditions (must be assessed at time of sampling). Roe deer have been seen. There is a Barn owl box in Barn around the back of the farm, where 4 barn owls have been seen and lots of pellets and droppings on ground. Pellets could possibly be sampled if method is developed and specimen collection is easy.
Water sampling from the beck that runs through this farm is best done from a bridge (OS National Grid Reference: NY 386500 (338650, 550080)) downstream of the farm. This sampling point is on a main road, but parking is possible by the entrance to a field about ten meters to the east of the bridge. This farm is very compact, with easy access to fields and buildings and sampling would not be too time consuming.

**Farm C.**

**An upland sheep farm**

Farm not affected by FMD and continually stocked. The farm is owned by Newton Rigg Agricultural College and the animals are spread over three main sites.

There are approximately 800 ewes spread throughout these different sites. About 200 are grazing on the fell (these are also fed along a single track, so it will be easy to find faeces). They are due to lamb in mid-April and will be brought off the fell on 25th February for scanning. The ewes carrying single lambs will go back on the fell and those carrying twins will go back up on the fell until the low in-by brings dries out a bit.

The rest of the ewes are due to lamb at the beginning of April and are spread between the fields and housing at the farm. The exact location of the sheep changes according to the weather so need have to speak to farmer when sampling. Sampling in the fields is made easier as there is a concrete feeding area where all the sheep gather. On the farm site, the animals were housed and therefore sampling was also quick and easy. Due to the mix of upland and lowland areas to this farm there are a number of wildlife habitats. On the upland fell there was evidence of fox activity and a badger skull was also found. On the lowland there was also fox and rabbit activity and a badger set was identified fairly close to a badger latrine.

The water sampling from these sites can be carried out at a single point (OS National Grid Reference: NY 366290 (336630,529070)) just below a convergence of becks that run from the different areas containing the livestock. Access to this site is only possible by foot and the beck flowing from the fields (Barrow Beck) can be followed from the point that it crosses under the road to the convergence slightly further downstream.
Farm D.  

An 81 hectare dairy farm

Farm not affected by FMD and therefore continuously stocked with Holstein Friesian cattle. The cattle are split into different herds but are all located on the farm site. Calving is taking place in April 2002. There is a herd of 133 cows of which 107 are currently being used for milking. A small herd of 15 dry cows are also being housed and so far there are 95 calves and young stock on the farm, which range from 2 days old to 10 weeks old.

Sampling of livestock is fairly simple as all animals are being housed in cattle sheds at present. The newborn calves are kept in single calf pens until a couple of weeks old when they are placed in small groups of about 6 animals per pen. This once again makes sampling fairly easy and makes it possible to indicate more accurately which animals faecal samples come from.

There is a quite a bit of wildlife present on the farm with foxes, roe deer, rabbits and hares being seen regularly.

Water sampling from this farm is possible from the identified sampling point (OS National Grid Reference: NY 393521 (339320,552190)) downstream from a beck (can also be sampled) that runs through the farm before joining the River Caldew. Opposite this sampling point there is also land that is public access that is used frequently by people walking dogs. There is also a land drain on the farm that can be sampled.
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**Notes:**
- N: Not detected
- A: Detected
- +: Positive
- -: Negative
- EMT: Electro-Mile Test
- g-PCR: Gene-PCR

**Legend:**
- Green: Positive
- Red: Negative
- Yellow: Neutral

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- Test: CALB

**Sample Description:**
- Sample type: Blood
- Quantity: 1 mL
- Storage: Refrigerated

**Results:**
- Final test results for CALB samples.

**References:**
- CALB test protocol
- Manufacturer's guidelines
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Note: The table represents a summary of various sections and dates, but the specific content is not clearly readable.