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

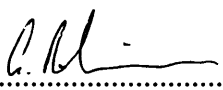My ever-supportive parents, Carla & Brian

&

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.

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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

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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.

following the River Caldew (i.e. sites 1, 3, 4, 8 and 11) and Roe Beck/Lower Caldew (i.e. sites 5-8 and 11) during base flows and high flows: (a) Phase 1; and (b) Phase 2.

List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
AF	Acid Fast
ANOVA	Analysis of Variance
ANTE	Abstracts in New Technologies & Engineering
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
ASFA	Aquatic Sciences & Fisheries Abstracts
BIDS	Bath Information and Data Services
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
°C	Degrees Celsius
CCC	Current Contents Connect
CC-PCR	Cell Culture PCR
CDSC	Communicable Disease Surveillance Centre
COGAP	Codes of Good Agricultural Practice
COWP	*Cryptosporidium* Oocyst Wall Protein
CREH	Centre for Research into Environment and Health
CRU	*Cryptosporidium* Reference Unit
CSA	Cambridge Scientific Abstracts
CSO	Combined Sewage Overflow
Ct	Threshold Cycle
CTAB	Cetyl Trimethylammonium Bromide
Da	Daltons
DABCO	1,4-diazabicyclo(2.2.2)octane
DAPI	4',6-diamidino-2-phenylindole

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 Immuosorbent 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
MgCl$_2$	Magnesium Chloride
MLFT	Multilocus Fragment Type
mZN	Modified Ziehl-Neelsen
NaCl	Sodium Chloride
NaHCO$_3$	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	Polythreonine 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

S-dsRNA	Small Double Stranded RNA
SDWA	Safe Drinking Water Act
SIGLE	System for Information of Grey Literature in Europe
spp.	Species
SSCP	Single Strand Conformation Polymorphism
SSU rRNA	Small Subunit Ribosomal RNA
TAMU	Texas A&M University
TE	Tris-EDTA
TRAP	Thrombospondin-Related Adhesive Protein
U	Units of Enzyme Activity
UCP	Ungar *Cryptosporidium parvum*
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volts
WHO	World Health Organisation

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*

2

1900

1907 – *Cryptosporidium muris*

1910

1912 – *Cryptosporidium parvum*

1920

1929 – Found in birds for 1st time

1930

1940

1950

1955 – *Cryptosporidium meleagridis* ——— 1955 – 1st found to be pathogenic

1960

1970

1971 – *Cryptosporidium wrairi* ——— 1971 – Disease in cattle 1st described

1976 – Disease in humans 1st described

1979 – *Cryptosporidium felis*
1980 – *Cryptosporidium serpentis* ——— **1980** — 1980 – Cattle disease found to be common

1982 – Common in AIDS patients
1983 – Common in all immune statuses
1986 – *Cryptosporidium baileyi* ——— 1983 – Widespread laboratory detection
1984 – Found to be waterborne pathogen

1990

1991 – Variation in *C. parvum* isolates
1993 – Milwaukee waterborne outbreak
1998 – *Cryptosporidium saurophilum* ——— 1997 – 2 transmission cycles in humans
1999 – *Cryptosporidium galli* ——— 1999 – UK drinking water regulations
2000 – *Cryptosporidium andersoni* ——— **2000** — 1999 – Non-*C. parvum* human infections
2001 – *Cryptosporidium canis*
2002 – *Cryptosporidium hominis*
2002 – *Cryptosporidium molnari*
2004 – *Cryptosporidium suis* ——— **2004**

Figure 1.1: A timeline summarising the discovery of individual species and important events involving *Cryptosporidium*

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wrairi, 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 serpentis* 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
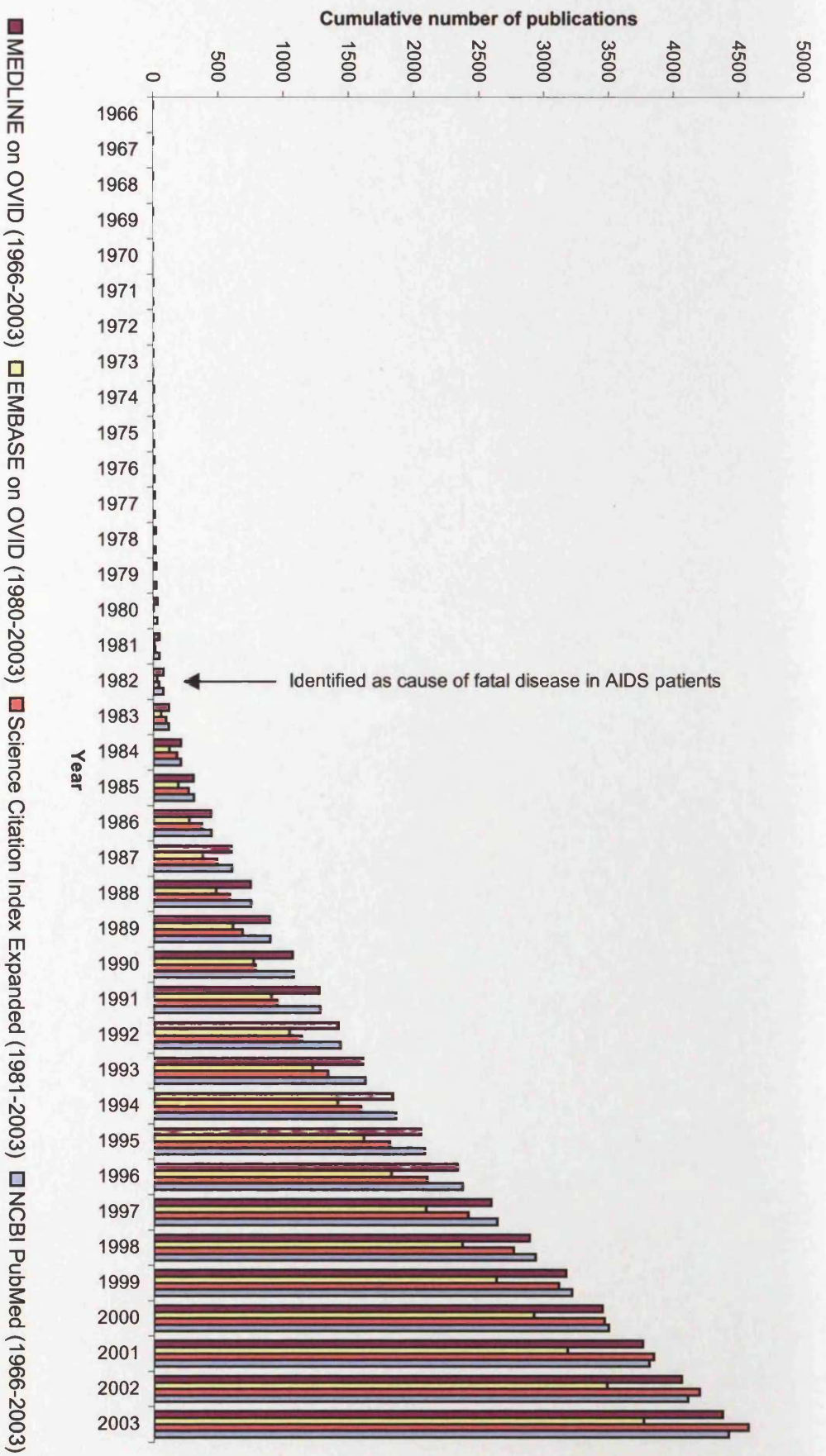
Figure 1.2: The cumulative increase of *Cryptosporidium* and cryptosporidiosis publications

MEDLINE on OVID (1966-2003) ■ EMBASE on OVID (1980-2003) ■ Science Citation Index Expanded (1981-2003) ■ NCBI PubMed (1966-2003)

Identified as cause of fatal disease in AIDS patients

Cumulative number of publications

Table 1.1: Proposed species of *Cryptosporidium* and their current status

Species	Current Status	Oocyst Size in μm (ranges)	GenBank Sequences SSU rRNA	GenBank Sequences hsp70	Type Host	M	B	R	F	Man
C. *muris* Tyzzer 1907	Valid	8.4 x 6.3 (7.5-9.8x5.5-7.0)	AF093498	AF221543	Mouse (*Mus musculus*)	Y	-	-	-	Yes
C. *parvum* Tyzzer 1912	Valid	5.0 x 4.5 (4.5-5.4x4.2-5.0)	L16996	AF221528	Mouse (*Mus musculus*)	Y	-	-	-	Yes
C. *crotali* Triffit 1925	Nomen nudum	-	-	-	Rattlesnake (*Crotalus* sp.)	-	-	-	-	-
C. *vulpis* Wetzel 1938	Nomen nudum	-	-	-	Fox (*Vulpes* sp.)	-	-	-	-	-
C. *baikalika* Matschoulsky 1947	Nomen nudum	-	-	-	Woodcock (*Scolopax rusticola*)	-	-	-	-	-
C. *meleagridis* Slavin 1955	Valid	5.2 x 4.6 (4.5-6.0x4.2-5.3)	AF112574	AF329189	Turkey (*Meleagris gallopavo*)	Y	Y	-	-	Yes
C. *tyzzeri* Levine 1961	Nomen nudum	-	-	-	Chicken (*Gallus gallus*)	-	-	-	-	-
C. *lampropeltis* Anderson et al. 1968	Nomen nudum	-	-	-	Kingsnake (*Lampropeltis calligaster*)	-	-	-	-	-
C. *ameivae* Arcay de Peraza & Bastardo de San Jose 1969	Nomen nudum	-	-	-	Lizard (*Ameiva ameiva*)	-	-	-	-	-
C. *ctenosauris* Duszynski 1969	Nomen nudum	-	-	-	Lizard (*Ctenosaura similis*)	-	-	-	-	-
C. *wrairi* Vetterling et al. 1971	Valid	5.4 x 4.6 (4.8-5.6x 4.0-5.0)	AF115378	AF221536	Guinea pig (*Cavia porcellus*)	Y	-	-	-	No
C. *agni* Barker & Carbonell 1974	syn. C. *parvum*	-	-	-	Sheep (*Ovis aries*)	-	-	-	-	-
C. *anserinum* Proctor & Kemp 1974	Nomen nudum	-	-	-	Goose (*Anser anser*)	-	-	-	-	-
C. *bovis* Barker & Carbonell 1974	syn. C. *parvum*	-	-	-	Cattle (*Bos taurus*)	-	-	-	-	-
C. *cuniculus* Inman & Takeuchi 1979	syn. C. *parvum*	-	-	-	Rabbit (*Oryctolagus cuniculus*)	-	-	-	-	-
C. *felis* Iseki 1979	Valid	4.6 x 4.0 (3.2-5.1x3.0-4.0)	AF112575	AF221538	Cat (*Felis catus*)	Y	-	-	-	Yes
C. *rhesi* Levine 1980	Nomen dubium	-	-	-	Rhesus monkey (*Macaca mulata*)	-	-	-	-	-
C. *serpentis* Levine 1980	Valid	6.2 x 5.3 (5.6-6.6x4.8-5.6)	AF093502	AF221541	Cork snake (*Elaphe guttata*)	-	-	Y	-	No
C. *garnhami* Bird 1981	Nomen nudum	-	-	-	Human (*Homo sapien*)	-	-	-	-	-
C. *nasoris* Hoover et al. 1981	Nomen nudum	-	-	-	Unicornfish (*Naso lituratus*)	-	-	-	-	-
C. *enteritidis* Payne et al. 1983	Nomen nudum	-	-	-	Human (*Homo sapien*)	-	-	-	-	-
C. *baileyi* Current et al. 1986	Valid	6.2 x 4.6 (5.6-6.3x4.5-4.8)	L19068	AF316634	Chicken (*Gallus gallus*)	-	Y	-	-	No
C. *curyi* Ogassawara et al. 1986	Nomen dubium	-	-	-	Cat (*Felis catus*)	-	-	-	-	-
C. *villithecum* Paperna et al. 1986	Nomen nudum	-	-	-	-	-	-	-	-	-
C. *varanii* Pavlasek et al. 1995	Nomen dubium	-	-	-	Emerald monitor (*Varanus prasinus*)	-	-	Y	-	-
C. *saurophilum* Koudela & Modry 1997	Valid	5.0 x 4.7 (4.4-5.6x4.2-5.2)	AY382170	AF221540	Skink (*Eumeces schneideri*)	-	-	Y	-	No
C. *galli* Pavlasek 1999	Valid	8.3 x 6.3 (8.0-8.5x6.2-6.4)	AY168847	AY168849	Chicken (*Gallus gallus*)	-	Y	-	-	No
C. *andersoni* Lindsay et al. 2000	Valid	7.4 x 5.5 (6.0-8.1x5.0-6.5)	L19069	AF221542	Cattle (*Bos taurus*)	Y	-	-	-	No

Table 1.1 cont.: Proposed species of *Cryptosporidium* and their current status

C. canis Fayer et al. 2001	Valid	5.0 x 4.7 (3.7-5.9x3.7-5.9)	AF112576	AF221529	Dog (*Canis familiaris*)	Y	-	Yes
C. blagburni Morgan et al. 2002	syn. *C. galli*	-	-		Gouldian finch (*Erythrura gouldiae*)	-	-	-
C. hominis Morgan-Ryan et al. 2002	Valid	4.9 x 5.2 (4.4-5.4x4.4-5.9)	L16997	AF401506	Human (*Homo sapien*)	Y	Y	Yes
C. molnari Alvarez-Pellitero & Sitja Bobadilla 2002	Valid*	4.7 x 4.5 (3.2-5.5x3.0-5.0)	N/A	N/A	Gilthead Seabream (*Sparus aurata*)	Y	Y	No
C. suis Ryan et al. 2004	Valid	4.6 x 4.2 (4.4-4.9x4.0-4.3)	AF108861	AF221533	Pig (*Sus scrofa*)	Y	Y	Yes
C. scophthalmi Alvarez-Pellitero et al. 2004	Valid*	4.4 x 3.9 (3.7-5.0x3.0-4.7)	N/A	N/A	Turbot (*Scophthalmus maximus*)	Y	Y	No
Piscicryptosporidium cichlids Paperna & Vilenkin 1996	*Nomen dubium**	4.3 x 3.3 (4.0-4.7x2.5-3.5)	N/A	N/A	Cichlid fish (*Oreochromis* hybrid sp.)	Y	Y	No
Piscicryptosporidium reichenbachklinkei Paperna & Vilenkin 1996	*Nomen dubium**	2.9 x 2.9 (2.4-3.2x2.4-3.0)	N/A	N/A	Gourami (*Trichogaster leeri*)	Y	Y	No

Valid – Based on biological and/or molecular biological differences that clearly distinguish between these species

syn. – The described species have since been identified as junior synonyms of a valid species

Nomen dubium – These species are of uncertain identity (*species inquirenda*) but with further description may be classed as valid species or invalidated

Nomen nudum – These species are not valid due to misidentification or inadequate description and could not be recognised with certainty in the future

N/A – None available

* – None of these species infecting fish have been characterised at the molecular level and whilst *C. molnari* and *C. scophthalmi* are valid as separate species based on biological differences, the other two species have been placed in a new genus (*Piscicryptosporidium*) based on the sporulation of oocysts deep within the epithelial cells. This is occasionally seen in other species of *Cryptosporidium* and further biological and molecular data are required to determine whether they warrant separate genus status, which is why they are *species inquirenda* and *nomen dubium*.

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. meleagridis*, *C. wrairi*, *C. felis*, *C. serpentis* 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 molnari* (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 'Syzygien' 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 *et al.*, 1980a), or that only four species (*C. muris*, *C. meleagridis*, *C. crotali* and *C. nasoris*) should be considered valid for those found in mammals, birds, reptiles and fish respectively (Levine, 1984). In 1985, obvious biological differences between *C. muris* and *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 *Cryptosporidium* species (Xiao *et al.*, 2004). However, some of the previously described species of *Cryptosporidium* were not adequately described and no molecular characterisation has yet been possible, resulting in them being designated *nomen dubium* (Table 1.1).

The definition of a new *Cryptosporidium* species must be carefully undertaken using both molecular and biological criteria (Egyed *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 *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 *et al.*, 2004).

Host specificity varies greatly in *Cryptosporidium* species with some (e.g. *C. parvum*) found to infect numerous host species and others (e.g. *C. wrairi*) that are currently thought to have a very narrow host range. In addition to the host specificity of a particular *Cryptosporidium* species there are also a number of "host-adapted" genotypes that were initially grouped with *C. parvum* or *C. muris* due to the similar morphology, but have displayed some genetic differences during the gradual development of some host specificity (Xiao *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 *C. parvum* genotype 1 to *C. hominis*, dog genotype to *C. canis* and pig genotype I to *C. suis*) (Ryan *et al.*, 2004; Fayer *et al.*, 2001; Morgan-Ryan *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
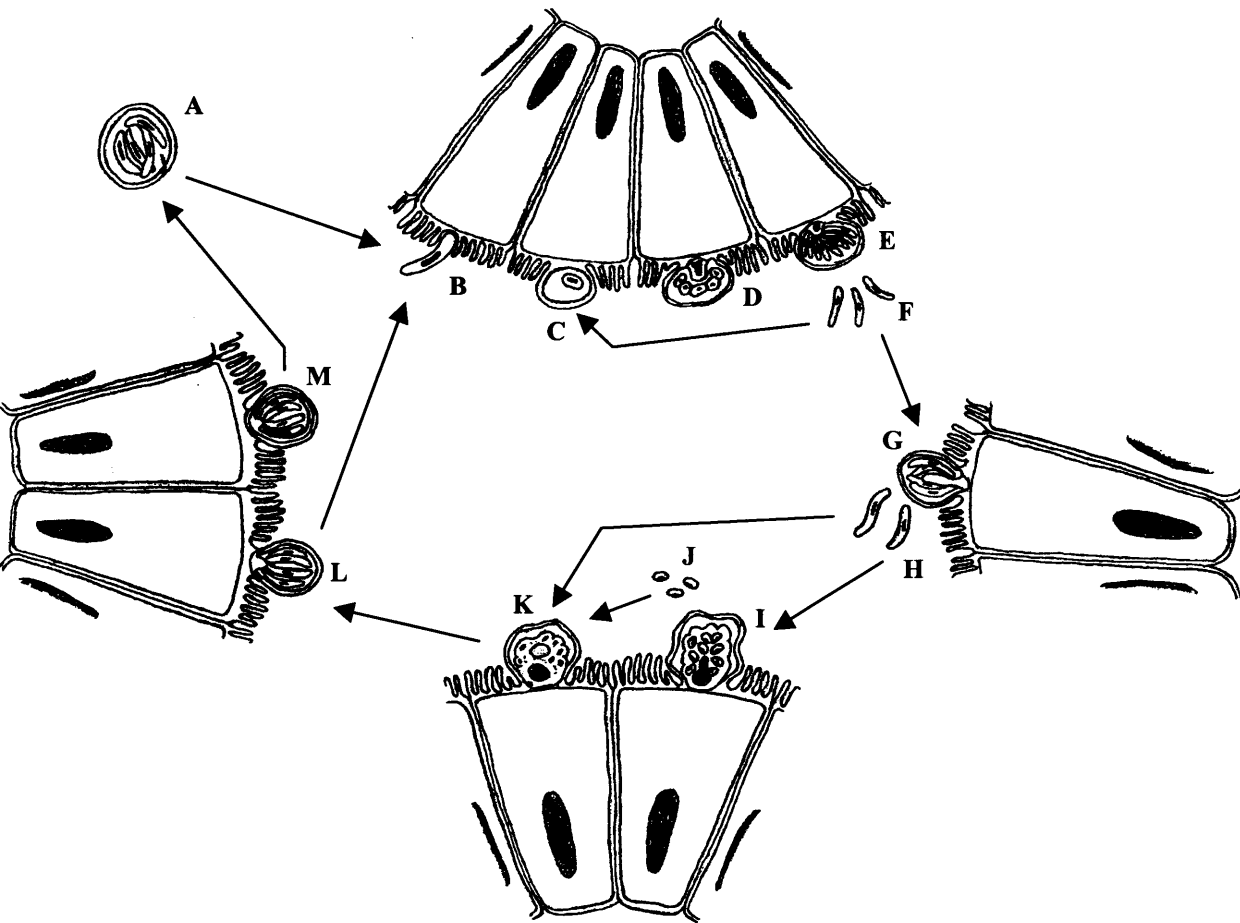
Figure 1.3: The life cycle of *Cryptosporidium*. Oocysts (A) are ingested by the host; Sporozoites (B) excyst and invade the brush border of epithelial cells; The sporozoite develops into a trophozoite (C); The trophozoite undergoes merogony to form a type I meront (D and E); Merozoites (F) are released 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 autoinfect 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. meleagridis* (Slavin, 1955). A type II meront contains four merozoites that when released attach again 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 microgamont 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; Jokipii and Jokipii, 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 anthroponotic 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 anthroponotic 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 anthroponotic 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.

C. hominis (%)

0   10   20   30   40   50   60   70   80   90   100

C. parvum (%)

100   90   80   70   60   50   40   30   20   10   0
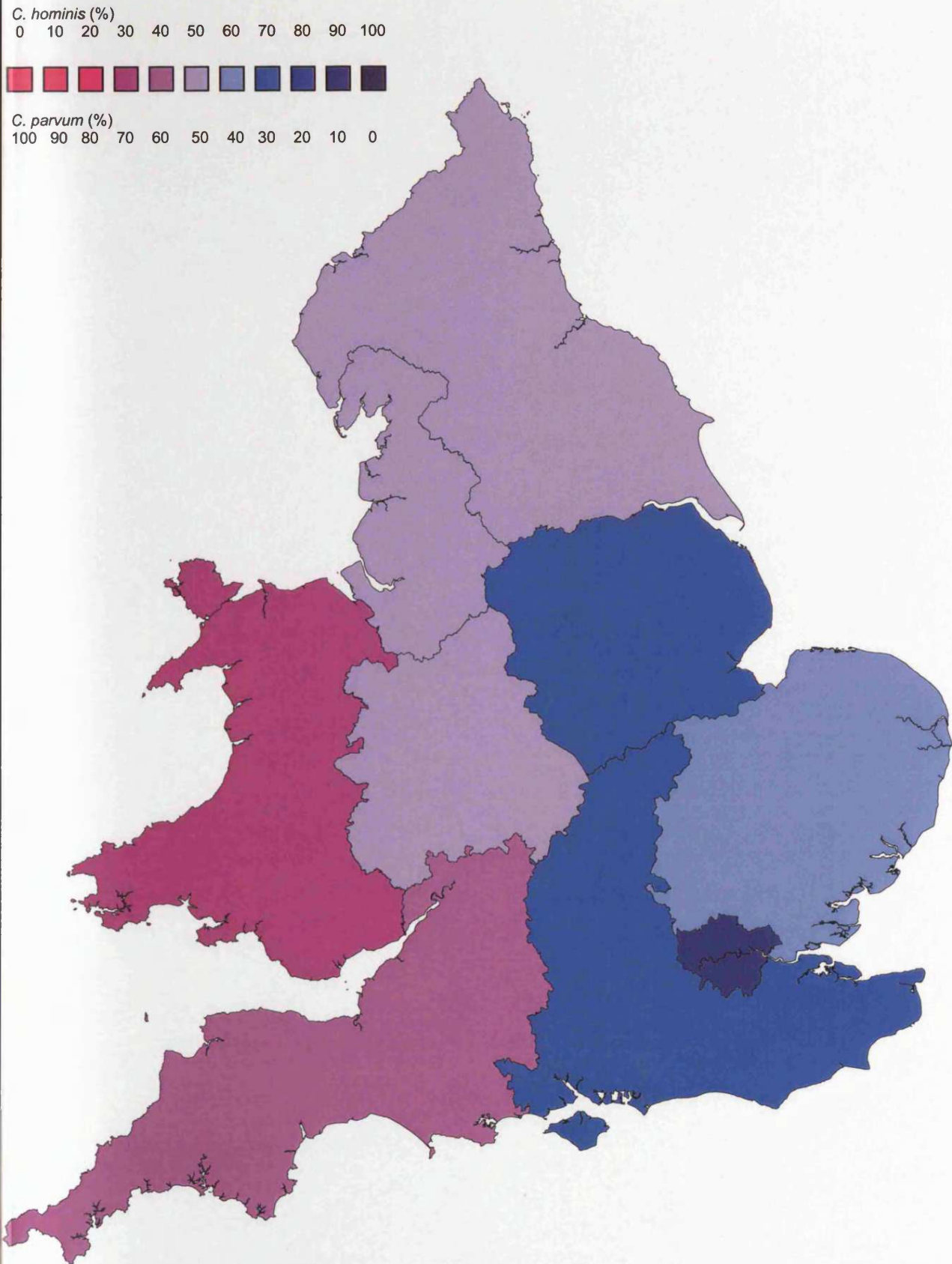
Figure 1.4: The geographical distribution of *C. hominis* and *C. parvum* cases reported in 2000 (used with permission from Dr. Rachel Chalmers)

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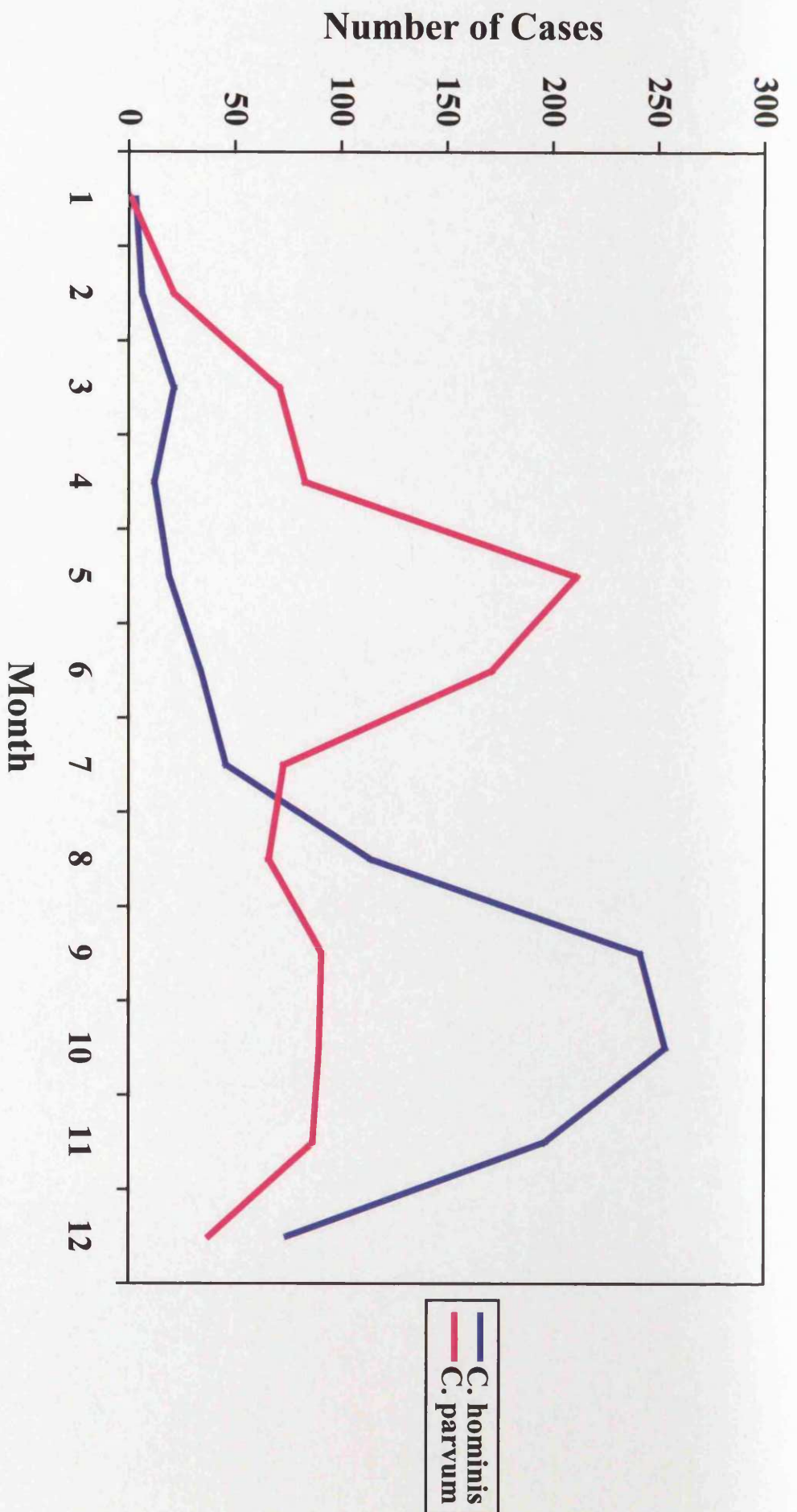
Figure 1.5: The seasonal distribution of disease caused by *C. hominis* and *C. parvum* in England and Wales during 2000 (used with permission from Dr Rachel Chalmers)

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 (Jokipii and Jokipii, 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 mucous 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% recieving 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 FE 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; Zarlenga 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 (Zarlenga 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

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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 fuchsin 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 upto 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
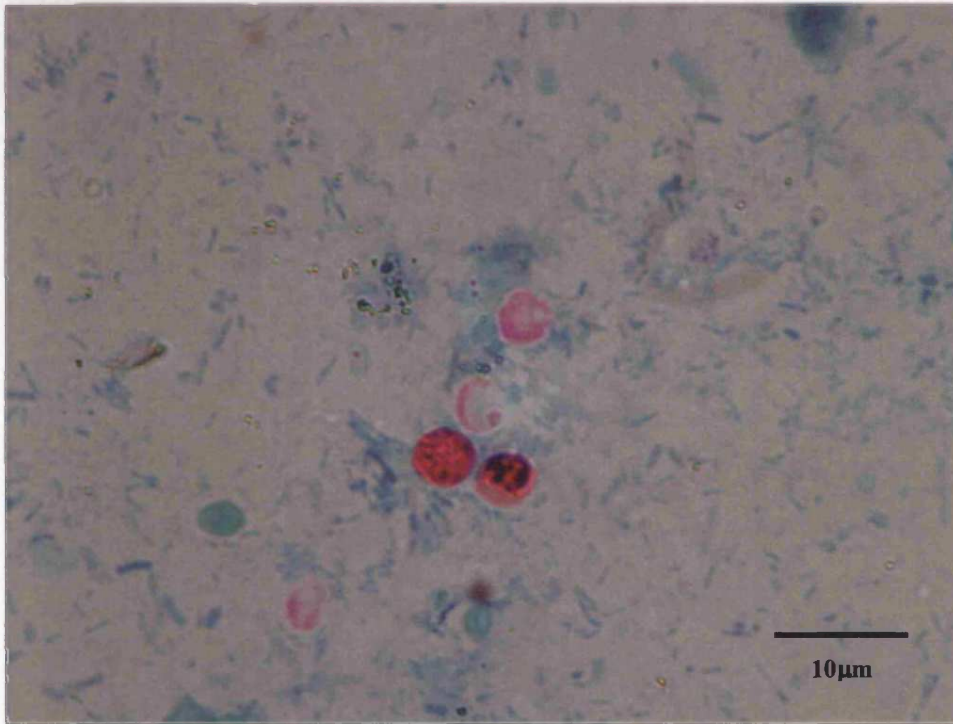
Figure 1.6: *Cryptosporidium parvum* oocysts stained by modified Ziehl-Neelsen using a x100 objective.
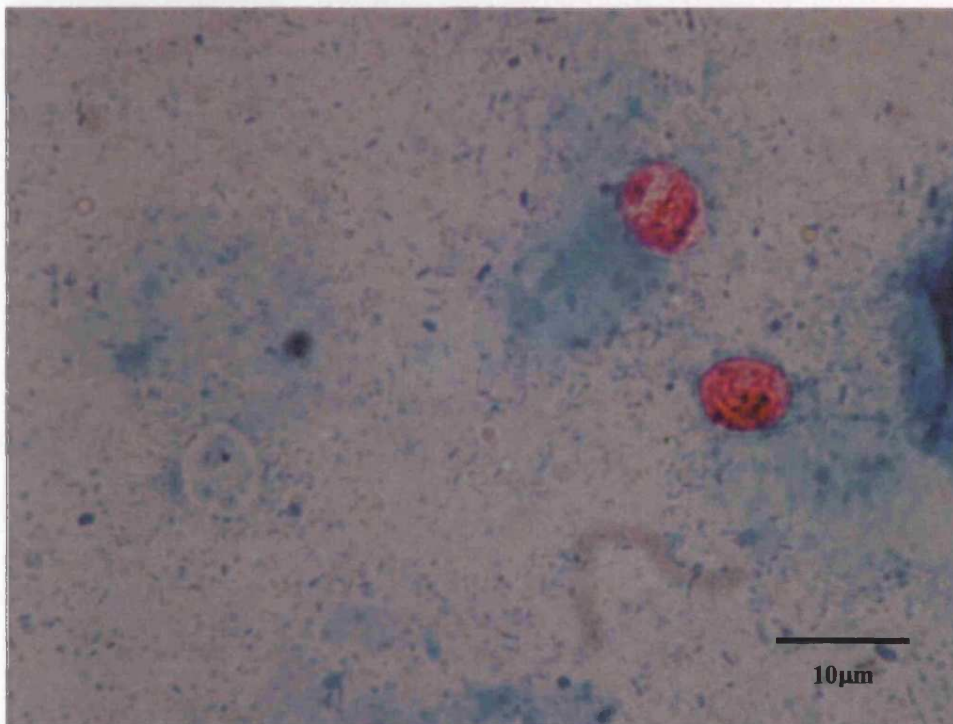
Figure 1.7: *Cryptosporidium andersoni* oocysts stained by modified Ziehl-Neelsen using a x100 objective.
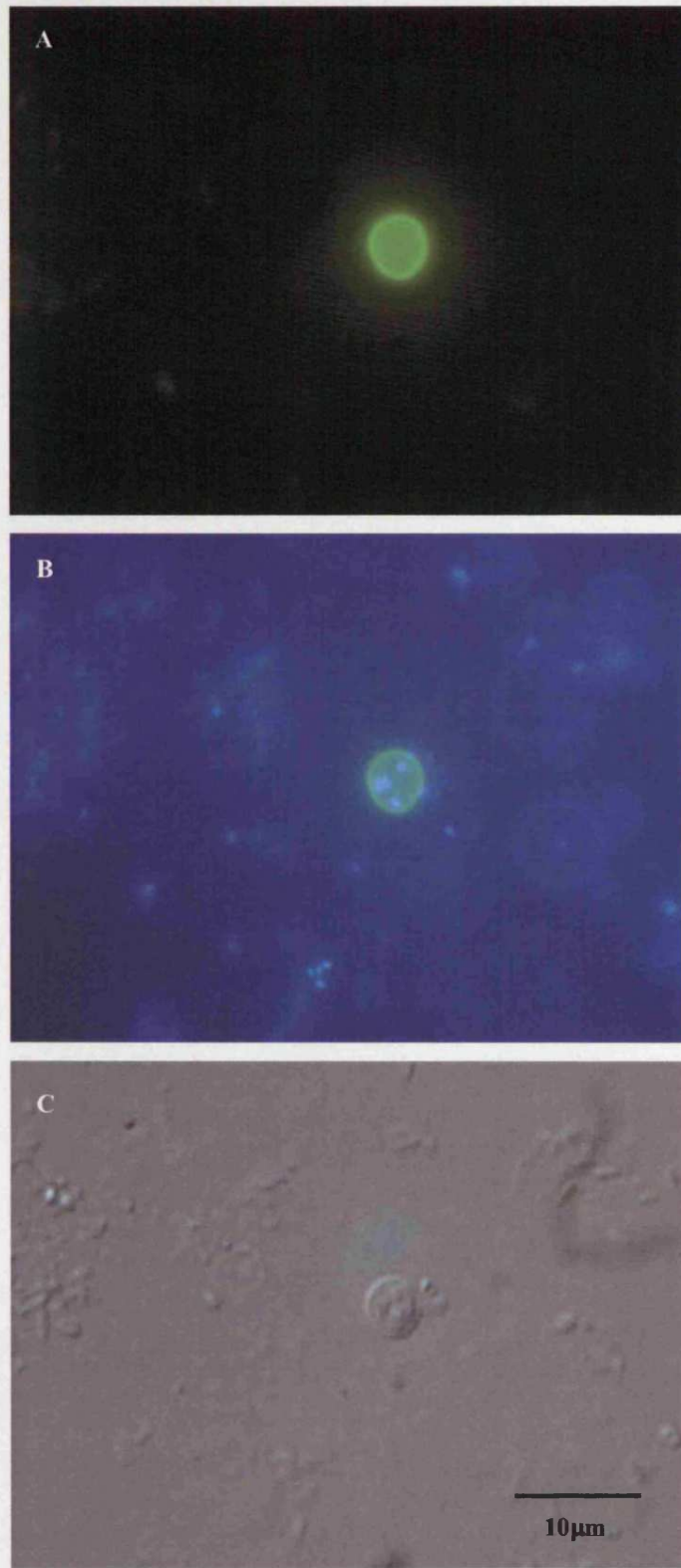
Figure 1.8: A *Cryptosporidium parvum* oocyst using a x100 objective: A) with the wall stained by a fluorescein-labelled antibody; B) with DAPI staining of four sporozoite nuclei; C) with sporozoites visible by DIC microscopy.
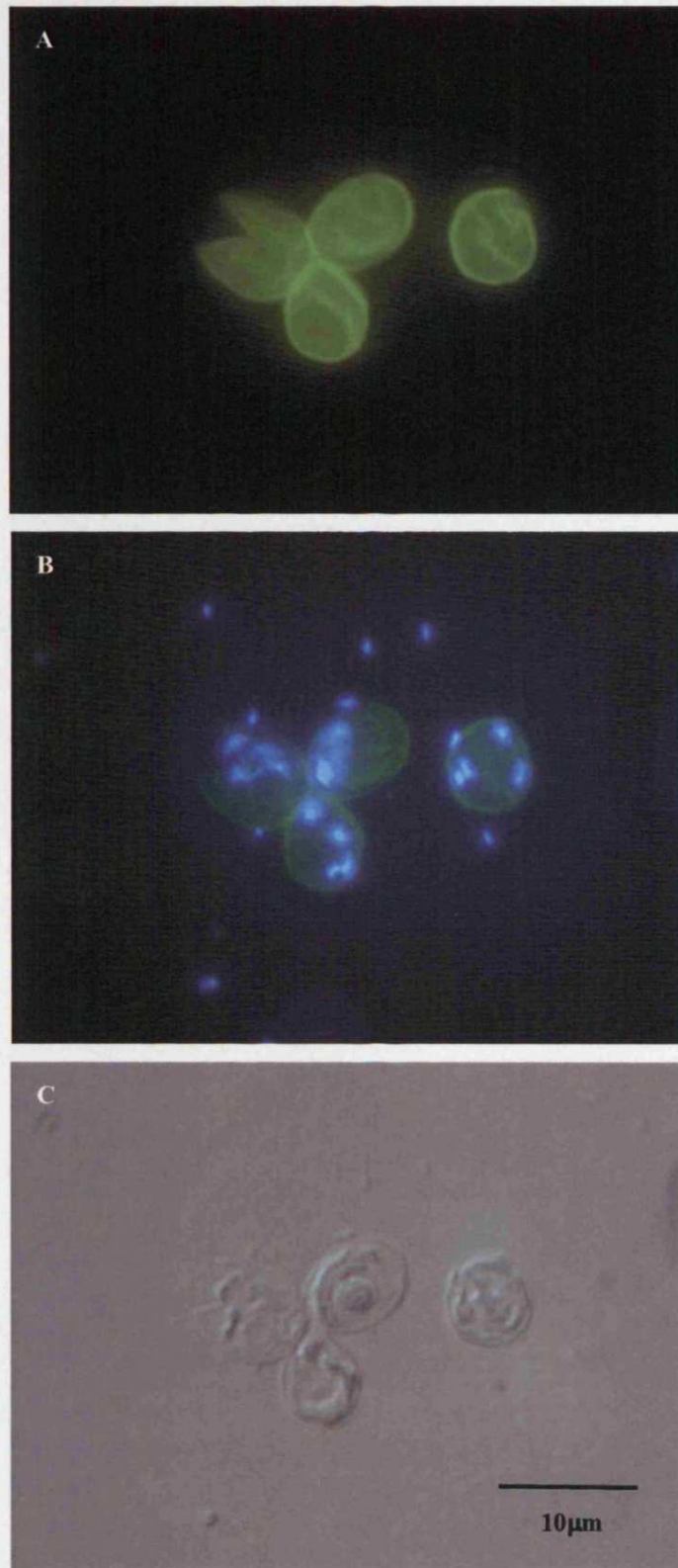
Figure 1.9: *Cryptosporidium andersoni* oocysts using a x100 objective: A) with the walls stained by a fluorescein-labelled antibody and showing one with the suture open; B) with DAPI staining of four sporozoite nuclei within each oocyst; C) with sporozoites and oocyst residual bodies visible by DIC 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 5×10^5 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}. Unger (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, Seradyn), 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, Merdian 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 unconcentrated 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 pubic 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 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 1.2: Target loci to distinguish between _C. parvum_ and _C. hominis_

Locus Product	References
Small-subunit (SSU) rRNA	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
Cryptosporidium oocyst wall protein (COWP)	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
Thrombospondin-related adhesive protein 1 (TRAP-C1)	Peng _et al._, 1997; Spano _et al._, 1998a; Spano _et al._, 1998b; Sulaiman _et al._, 1998; McLauchlin _et al._, 1999; Elwin _et al._, 2001
Thrombospondin-related adhesive protein 2 (TRAP-C2)	Peng _et al._, 1997; Sulaiman _et al._, 1998; Ong _et al._, 1999; Elwin _et al._, 2001
β-tubulin	Widmer _et al._, 1998a; Caccio _et al._, 1999; Rochelle _et al._, 1999b; Sulaiman _et al._, 1999a
Internal transcribed spacer 1 (ITS1)	Carraway _et al._, 1996; Spano _et al._, 1998a; Morgan _et al._, 1999; Ong _et al._, 1999
Polythreonine repeat (Poly-T)	Carraway _et al._, 1997; Spano _et al._, 1998a; Widmer _et al._, 1998b
Dihydrofolate reductase (DHFR)	Vasquez _et al._, 1996; Gibbons _et al._, 1998b; Ong _et al._, 1999
Ribonucleotide reductase (RNR)	Spano _et al._, 1998a; Widmer _et al._, 1998b
Acetyl-CoA synthetase	Morgan _et al._, 1998; Morgan _et al._, 2000
70kDa heat shock protein (hsp70)	Morgan _et al._, 2000; Gobet and Toze, 2001
Surface protein p23	Sturbaum _et al._, 2003
60 kDa glycoprotein (gp60)	Sturbaum _et al._, 2003
900 kDa glycoprotein (gp900)	Sturbaum _et al._, 2003
Actin	Sulaiman _et al._, 2002
Undefined genomic sequences	Bonnin _et al._, 1996; Morgan _et al._, 1997; Guyot _et al._, 2002

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. meleagridis_, _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. meleagridis* 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. meleagridis* 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/genotype 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. meleagridis* 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 shear 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 3-3.8×10^5 in wastewater, $0.5\text{-}1.36 \times 10^2$ in drinking water and $0.12\text{-}4.8 \times 10^4$ 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 use 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

Database	Gateway	Time Period Searched
Embase	Ovid	1980-2003
Medline	Ovid	1966-2003
Pre-Medline (live database)	Ovid	On 31/12/03
Abstracts in New Technologies & Engineering (ANTE)	CSA	1981-2003
AGRICOLA	CSA	1970-2003
Aqualine	CSA	1960-2003
Aquatic Sciences & Fisheries Abstracts (ASFA)	CSA	1978-2003
Biological Sciences	CSA	1982-2003
Biology Digest	CSA	1989-2003
Biotechnology & Bioengineering Abstracts	CSA	1982-2003
Conference Papers Index	CSA	1982-2003
Environmental Sciences & Pollution Management	CSA	1981-2003
Oceanic Research	CSA	1981-2003
TOXLINE	CSA	1994-2003
Water Resources Abstracts	CSA	1967-2003
CAB Abstracts	BIDS	1973-2003
Ingenta Online Articles	BIDS	1988-2003
Ingenta Uncover Plus	BIDS	1988-2003
Current Contents Connect (CCC) Databases: Agriculture, Biology & Environmental Sciences Clinical Medicine Engineering, Computing & Technology Life Sciences Physical, Chemical & Earth Sciences	ISI CCC	1997-2003
Science Citation Index Expanded	ISI Web of Science	1981-2003
System for Information on Grey Literature in Europe (SIGLE)	SilverPlatter	1980-2003

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 wild card 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

Search Term Reference	Search Terms
1	Cryptosporidi* AND molecular
2	Cryptosporidi* AND typing
3	Cryptosporidi* AND subtyp*
4	Cryptosporidi* AND sub-typ*
5	Cryptosporidi* AND subgenotyp*
6	Cryptosporidi* AND sub-genotype
7	Cryptosporidi* AND genotyp*
8	Cryptosporidi* AND geno-typ*
9	Cryptosporidi* AND PCR
10	Cryptosporidi* AND multiloc*
11	Cryptosporidi* AND multi-loc*
12	Cryptosporidi* AND fingerprint*
13	Cryptosporidi* AND finger-print*
14	Cryptosporidi* AND microsat*
15	Cryptosporidi* AND micro-sat*
16	Cryptosporidi* AND telomer*
17	Cryptosporidi* AND characteri*
18	Cryptosporidi* AND sequenc*
19	Cryptosporidi* AND DNA
20	Cryptosporidi* AND RNA

* - 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. meleagridis* 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

Table 2.3: The number of references retrieved from the databases with each search term

Database	Search Term Reference (see Table 2.2)																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Medline	209	16	12	2	7	0	217	0	232	14	3	10	0	15	0	6	414	303	382	191
Embase	199	15	9	2	7	1	172	1	212	9	2	9	0	11	0	5	347	234	199	67
Pre-Medline	5	0	1	0	1	0	2	0	4	0	0	0	0	0	0	0	3	6	3	0
AGRICOLA	89	3	1	1	0	0	62	0	44	5	1	6	0	8	0	2	65	84	84	31
ANTE	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Aqualine	12	1	0	1	0	0	12	0	36	1	0	0	0	1	0	1	59	20	34	7
ASFA	13	1	0	0	0	0	12	0	24	0	0	0	0	0	0	0	23	18	15	8
Biological Sciences	158	14	8	2	7	0	143	0	180	10	3	9	0	10	0	4	296	210	179	55
Biology Digest	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	1	0
Biotechnology & Bioengineering Abstracts	49	4	4	0	3	0	45	0	61	6	1	6	0	6	0	2	72	87	57	22
Conference Papers Index	9	0	0	0	2	0	8	0	3	2	1	0	0	1	0	1	8	3	2	1
Environmental Sciences & Pollution Management	36	2	0	1	1	0	23	0	64	0	1	1	0	1	0	1	111	39	55	21
Oceanic Abstracts	2	0	0	0	0	0	3	0	3	0	0	0	0	1	0	0	3	3	2	2
TOXLINE	14	2	0	0	0	0	13	0	20	0	0	0	0	1	0	0	12	14	21	7

Table 2.3 cont.: The number of references retrieved from the databases with each search term

Water Resources Abstracts	12	2	0	1	0	13	0	38	0	0	1	0	62	26	35	13
CAB Abstracts	354	17	12	11	9	12	236	248	21	8	9	1	405	285	273	137
Ingenta Online Articles	65	7	4	1	0	0	43	79	4	1	3	0	103	77	76	21
Ingenta Fax/Ariel Articles	54	3	1	0	2	0	66	80	6	0	1	4	76	23	33	10
Current Contents Connect Databases	222	13	9	2	8	0	194	291	15	3	11	19	286	252	218	126
Science Citation Index Expanded	274	17	8	2	8	0	200	334	16	3	12	18	420	314	295	156
SIGLE	4	1	0	0	0	1	0	0	0	0	1	0	1	1	1	

Table 2.4: Methods and loci used to subtype *Cryptosporidium* spp.

Method	Number of Papers (number that examined environmental isolates)	Number of papers that provided evidence (Typability, Reproducibility or Discriminatory power) for method evaluation	References (* examined environmental isolates) ([T,R,D] how methods were evaluated)
Sequence Analysis			
gp60 locus	13 (4)	0	Strong et al., 2000; Glaberman et al., 2001a*; Glaberman et al., 2001b; Peng et al., 2001; Sulaiman et al., 2001; Glaberman et al., 2002*; Leav et al., 2002; Alves et al., 2003b; Peng et al., 2003b*; Peng et al., 2003a; Stantic-Pavlinic et al., 2003; Wu et al., 2003; Zhou et al., 2003*
SSU rRNA locus in the rDNA unit	6	0	Carraway et al., 1994; Guyot et al., 2001; Glaberman et al., 2001b; Sulaiman et al., 2001; Gibbons-Matthews & Prescott, 2003; Navarro-i-Martinez et al., 2003
hsp70 locus	6 (3)	0	Di Giovanni et al., 1999*; LeChevallier et al., 2000*; Glaberman et al., 2001b; Sulaiman et al., 2001; Le Chevallier et al., 2003*; Peng et al., 2003a
Microsatellite loci			
ML1	6 (1)	0	Caccio et al., 2000; Caccio et al., 2001; Caccio & Pozio, 2001*; Huetink et al., 2001; Enemark et al., 2002; Mallon et al., 2003a
ML2	2 (1)	0	Caccio et al., 2001; Caccio & Pozio, 2001*
MS1	2	0	Aiello et al., 1999; Sulaiman et al., 2001
MS6	1	0	Aiello et al., 1999
5B12, 1FO7 & 12C07	1	0	Feng et al., 2000

Table 2.4 cont.: Methods and Loci used to Subtype *Cryptosporidium* spp.

Method / Locus			Reference
TP14, MS9 & GP15	1	0	Mallon et al., 2003a
Minisatellite regions			
MS1, MS5 & MS12	1	0	Mallon et al., 2003a
Extra-chromosomal S-dsRNA	4	1 (T, R &D), 1 (R)	Khramtsov et al., 2000; Xiao et al., 2001; Leoni et al., 2003b[R]; Leoni et al., 2003a[T,R,D]
Extra-chromosomal L-dsRNA	1	0	Khramtsov et al., 2000
ITS1-5.8S-ITS2 locus in the rDNA unit	1	0	Morgan et al., 1999b
TRAP-C2 locus	1	0	Sulaiman et al., 2001
Poly-T locus	1	0	Sulaiman et al., 2001
β-tubulin locus	2	1 (R)	Widmer et al., 1998; Rochelle et al., 2000[R]
RFLP Analysis			
β-tubulin locus	1	0	Widmer et al., 1998
Gp60 locus	1	0	Wu et al., 2003
Heteroduplex Mobility Assay			
Extra-chromosomal S-dsRNA	2	1 (T, R & D), 1 (R)	Leoni et al., 2003b[R]; Leoni et al., 2003a[T,R,D]
Mutation Scanning Electrophoresis			
Gp60 locus	1	0	Wu et al., 2003
SSU rRNA locus in the rDNA unit	1	1 (R – data not shown)	Gasser et al., 2003[R]
ITS2 locus in the rDNA unit	1	1 (R – data not shown)	Gasser et al., 2003[R]

Table 2.4 cont.: Methods and Loci used to Subtype *Cryptosporidium* spp.

Fragment size Analysis			
Microsatellite regions			
ML1	2	0	Enemark *et al.*, 2002; Mallon *et al.*, 2003b
ML2	1	0	Alves *et al.*, 2003a
5B12 & 1FO7	3	1 (R)	Feng *et al.*, 2000; Feng *et al.*, 2002[R]; Widmer *et al.*, 2000
7E1C, 4E12 & Cp273	2	1 (R)	Feng *et al.*, 2000; Feng *et al.*, 2002[R]
1G09, 12C07, 2G04 & 6B03	1	0	Feng *et al.*, 2000
Cp492 & Cp358	1	1 (R)	Feng *et al.*, 2002[R]
TP14, MS9 & GP15	1	0	Mallon *et al.*, 2003b
M1-telomere	1	1 (R)	Blasdall *et al.*, 2002[R]
Minisatellite regions			
MS1, MS5 & MS12	1	0	Mallon *et al.*, 2003b
AP-PCR/RAPD	3	1 (R)	Carraway *et al.*, 1994, Giles *et al.*, 2001[R], Shianna *et al.*, 1998
Electrophoretic Analysis of Proteins	3	1 (R)	Mead *et al.*, 1990; McLauchlin *et al.*, 1998[R]; El-Ghaysh & Barrett, 1999

Table 2.5: A description of the allele groups identified at the gp60 locus

Species	gp60 Allele	References
Cryptosporidium hominis	Ia	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
	Ia1	Wu *et al.*, 2003
	Ia2	Wu *et al.*, 2003
	Ib	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
	Ic*	Strong *et al.*, 2000
	Id	Strong *et al.*, 2000; Peng *et al.*, 2001; Sulaiman *et al.*, 2001; Leav *et al.*, 2002; Peng *et al.*, 2003a
	Ie	Peng *et al.*, 2001; Sulaiman *et al.*, 2001; Alves *et al.*, 2003b; Peng *et al.*, 2003a; Zhou *et al.*, 2003
	If**	Leav *et al.*, 2002; Alves *et al.*, 2003b; Wu *et al.*, 2003
Cryptosporidium parvum	IIa	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
	IIb	Peng *et al.*, 2001; Alves *et al.*, 2003b
	Ic*	Peng *et al.*, 2001; Leav *et al.*, 2002; Alves *et al.*, 2003b; Peng *et al.*, 2003a
	IId	Alves *et al.*, 2003b
	IIe	Peng *et al.*, 2003a
	IIa, IIb and IIc***	Wu *et al.*, 2003
	GP$_A$, GP$_B$ and GP$_C$****	Stantic-Pavlinic *et al.*, 2003
Cryptosporidium meleagridis	1	Glaberman *et al.*, 2001b
	2	Glaberman *et al.*, 2001b
	3	Glaberman *et al.*, 2001b
	4	Glaberman *et al.*, 2001b
	5	Glaberman *et al.*, 2001b
	6	Glaberman *et al.*, 2001b

* - 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 IIa, IIb and IIc. 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 *Rsa*I or *Alu*I 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 *Rsa*I or *Alu*I 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 polythreonine 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 2.6: A description of the alleles identified at various microsatellite and minisatellite loci

Locus	Reference	Number and description of samples	Alleles
Microsatellites			
ML1	Caccio et al., 2000	22 C. hominis isolates	H1 and H2
		72 C. parvum isolates from humans	C1, C2, C3 and C4
	Caccio et al., 2001	29 C. parvum isolates from calves	ML1-238 (=C1), ML1-226 (=C2) and ML1-220 (=C4)
		11 C. parvum isolates from kids	ML1-238 (=C1), ML1-226 (=C2) and ML1-220 (=C4)
		10 C. parvum isolates from lambs	ML1-238 (=C1), ML1-226 (=C2) and ML1-220 (=C4)
		9 C. parvum isolates from humans	ML1-238 (=C1), ML1-226 (=C2) and ML1-220 (=C4)
	Caccio and Pozio, 2001	Cryptosporidium spp. isolates from humans	H1, H2, C1, C2, C3 and C4
		samples	
	Huetink et al., 2001	64 Cryptosporidium spp. isolates from cattle (27 identified as C. parvum)	C1 and C3
	Enemark et al., 2002a	62 Cryptosporidium spp. isolates from humans (including at least 25 C. hominis, 18 C. parvum and 1 C. meleagridis isolates)	H1, C1, C2 and C3 (C. meleagridis ML1 not amplified)
		114 C. parvum isolates from cattle	C1, C2 and C3
		1 C. parvum isolate from a lamb	C2
		1 C. parvum isolate from a hedgehog	Novel subtype by sequence analysis, H1 by fragment analysis
		4 C. parvum 'porcine genotype' isolates	N.A.
	Mallon et al., 2003a	71 C. hominis isolates	4 (229bp) (=H1)
		64 C. parvum isolates from humans	2 (223bp) (=C3), 3 (226bp) (=C2) and 5 (238bp) (=C1)
		44 C. parvum isolates from cattle	5 (238bp) (=C1)
		1 C. meleagridis isolate	6 (N.A.)
	Mallon et al., 2003b	190 C. parvum isolates from cattle	5 (238bp) (=C1)
		39 C. parvum isolates from humans	2 (223bp) (=C3), 3 (226bp) (=C2) and 5 (238bp) (=C1)
		11 C. parvum isolates from sheep	5 (238bp) (=C1)
		2 C. parvum 'monkey genotype' isolates from humans	1 (220bp) (=C4)
ML2	Caccio et al., 2001	29 C. parvum isolates from calves	ML2-229, ML2-227, ML2-193, ML2-191, ML2-187
		11 C. parvum isolates from kids	ML2-227, ML2-191, ML2-187
		10 C. parvum isolates from lambs	ML2-231, ML2-227, ML2-187, ML2-191
		9 C. parvum isolates from humans	ML2-229, ML2-227, ML2-187, ML2-191, ML2-213
	Caccio and Pozio, 2001	Cryptosporidium spp. isolates from humans, animals and water samples	8 different alleles identified

Table 2.6 cont.: A description of the alleles identified at various microsatellite and minisatellite loci

Locus	Reference	Description	Alleles
5B12	Alves et al., 2003a	11 C. hominis isolates	ML2-179
		22 C. parvum isolates from humans	ML2-176, ML2-191
		31 C. parvum isolates from cattle	No alleles were identified due to stutter with amplicons >200bp.
	Feng et al., 2000	6 C. hominis isolates	2 and 5
		8 C. parvum isolates from calves	4, 5 and 6
		4 C. parvum isolates from humans	5 and 7
		1 Cryptosporidium spp. from a human	5
	Feng et al., 2002	2 parental C. parvum isolates from a human and a deer	Each isolate has unique allele
		Several generations of mixed isolates following GKO mouse passage	Alleles either mixed or same as one of the parental isolates
1F07	Widmer et al., 2000	10 C. parvum isolates	9 unique alleles
	Feng et al., 2000	6 C. hominis isolates	1, 2 and 3
		8 C. parvum isolates from calves	4 and 5
		4 C. parvum isolates from humans	5 and 7
		1 Cryptosporidium spp. from a human	4
	Feng et al., 2002	2 parental C. parvum isolates from a human and a deer	Each isolate has unique allele
		Several generations of mixed isolates following GKO mouse	Alleles either mixed or same as one of the parental isolates
4E12	Widmer et al., 2000	3 C. parvum isolates	2 unique alleles
	Feng et al., 2000	6 C. hominis isolates	2
		8 C. parvum isolates from calves	2
		4 C. parvum isolates from humans	1
		1 Cryptosporidium spp. from a human	2
	Feng et al., 2002	2 C. parvum parental isolates & 2 progeny of mixed infection	2 unique alleles
Cp273	Feng et al., 2000	6 C. hominis isolates	N.A.
		8 C. parvum isolates from calves	1 and 2
		4 C. parvum isolates from humans	1 and 3
		1 Cryptosporidium spp. from a human	N.A.
	Feng et al., 2002	2 C. parvum parental isolates & 2 progeny of mixed infection	2 unique alleles

Table 2.6 cont.: A description of the alleles identified at various microsatellite and minisatellite loci

Locus	Reference	Isolates	Alleles
7E1C	Feng et al., 2000	6 C. hominis isolates	2 and 3
		8 C. parvum isolates from calves	1 and 2
		4 C. parvum isolates from humans	1
1G09	Feng et al., 2002	1 Cryptosporidium spp. from a human	N.A.
		2 C. parvum parental isolates & 2 progeny of mixed infection	2 unique alleles
	Feng et al., 2000	6 C. hominis isolates	3 and 4
		8 C. parvum isolates from calves	1 and 2
		4 C. parvum isolates from humans	2
12C07	Feng et al., 2000	1 Cryptosporidium spp. from a human	2
		6 C. hominis isolates	2
		8 C. parvum isolates from calves	1, 2, 3 and 4
		4 C. parvum isolates from humans	2
2G04	Feng et al., 2000	1 Cryptosporidium spp. from a human	2
		6 C. hominis isolates	1
		8 C. parvum isolates from calves	2
		4 C. parvum isolates from humans	4
6B03	Feng et al., 2000	1 Cryptosporidium spp. from a human	3
		6 C. hominis isolates	2
		8 C. parvum isolates from calves	3
		4 C. parvum isolates from humans	N.A.
Cp492	Feng et al., 2002	1 Cryptosporidium spp. from a human	1
Cp358		2 C. parvum parental isolates & 2 progeny of mixed infection	2 unique alleles
	Feng et al., 2002	2 C. parvum parental isolates & 2 progeny of mixed infection	2 unique alleles
MS1	Aiello et al., 1999	67 C. hominis isolates	Hu A and Hu B
		9 C. parvum isolates from calves	Bov A and Bov B
MS6	Sulaiman et al., 2001	62 C. hominis isolates	A and B
	Aiello et al., 1999	8 C. hominis isolates	A and Bov B
		7 C. parvum isolates	N.A.
TP14	Mallon et al., 2003a	71 C. hominis isolates	Bov A and Bov B
		64 C. parvum isolates from humans	2 (279bp) and 3 (285bp)
		44 C. parvum isolates from cattle	4 (315bp), 5 (324bp) and 6 (333bp)
			5 (324bp) and 6 (333bp)
		1 C. meleagridis isolate	1 (258bp)

Table 2.6 cont.: A description of the alleles identified at various microsatellite and minisatellite loci

Locus	Reference	Isolates	Alleles
MS9	Mallon et al., 2003b	190 C. parvum isolates from cattle	5 (300bp) and 6 (309bp)
		39 C. parvum isolates from humans	4 (291bp), 5 (300bp) and 6 (309bp)
		11 C. parvum isolates from sheep	5 (300bp) and 6 (309bp)
		2 C. parvum 'monkey genotype' isolates from humans	3 (260bp)
	Mallon et al., 2003a	71 C. hominis isolates	2 (375bp)
		64 C. parvum isolates from humans	4 (432bp), 5 (450bp), 6 (456bp), 7 (462bp), 8 (522bp) and 9 (528bp)
GP15	Mallon et al., 2003b	44 C. parvum isolates from cattle	5 (450bp) and 7 (462bp)
		1 C. meleagridis isolate	1 (303bp)
	Mallon et al., 2003b	190 C. parvum isolates from cattle	5 (450bp) and 6 (456bp)
		39 C. parvum isolates from humans	4 (432bp), 5 (450bp), 6 (456bp), 7 (462bp) and 10 (433bp)
		11 C. parvum isolates from sheep	5 (450bp) and 10 (433bp)
		2 C. parvum 'monkey genotype' isolates from humans	3 (385bp)
	Mallon et al., 2003a	71 C. hominis isolates	10 (366bp), 12 (411bp), 13 (435bp), 14 (456bp) and 15 (N.A.)
		64 C. parvum isolates from humans	2 (312bp), 3 (327bp), 4 (330bp), 5 (333bp), 6 (336bp), 7 (339bp) and 8 (342bp)
Minisatellites MS1	Mallon et al., 2003b	44 C. parvum isolates from cattle	5 (333bp), 6 (336bp), 7 (339bp), 8 (342bp) and 9 (345bp)
		1 C. meleagridis isolate	1 (273bp)
	Mallon et al., 2003b	190 C. parvum isolates from cattle	4 (330bp), 5 (333bp), 6 (336bp), 7 (339bp), 8 (342bp), 9 (345bp) and 19 (414bp)
		39 C. parvum isolates from humans	3 (327bp), 5 (333bp), 6 (336bp), 7 (339bp), 8 (342bp), 9 (345bp), 16 (366bp), 17 (384bp), 18 (411bp), 19 (414bp) and 20 (417bp)
		11 C. parvum isolates from sheep	5 (333bp) and 6 (336bp)
		2 C. parvum 'monkey genotype' isolates from humans	10 and 11
	Mallon et al., 2003a	71 C. hominis isolates	4 (376bp)
		64 C. parvum isolates from humans	1 (352bp), 3 (364bp), 4 (376bp) and 5 (388bp)
		44 C. parvum isolates from cattle	3 (364bp), 5 (388bp) and 6 (400bp)
		1 C. meleagridis isolate	1 (352bp)
	Mallon et al., 2003b	190 C. parvum isolates from cattle	3 (364bp)
		39 C. parvum isolates from humans	1 (352bp) and 3 (364bp)
		11 C. parvum isolates from sheep	3 (364bp) and 7 (328bp)
		2 C. parvum 'monkey genotype' isolates from humans	2 (355bp)

Table 2.6 cont.: A description of the alleles identified at various microsatellite and minisatellite loci

MS5	Mallon *et al.*, 2003a	71 *C. hominis* isolates	3 (277bp), 5 (301bp), 8 (325bp) and 10 (349)
		64 *C. parvum* isolates from humans	2 (262bp), 4 (286bp), 7 (310bp), 9 (328bp) and 11 (352bp)
		44 *C. parvum* isolates from cattle	6 (304bp), 9 (328bp) and 11 (352bp)
		1 *C. meleagridis* isolate	12 (N.A.)
	Mallon *et al.*, 2003b	190 *C. parvum* isolates from cattle	9 (328bp)
		39 *C. parvum* isolates from humans	2 (262bp), 4 (286bp) and 9 (328bp)
		11 *C. parvum* isolates from sheep	9 (328bp)
		2 *C. parvum* 'monkey genotype' isolates from humans	1 (181bp)
MS12	Mallon *et al.*, 2003a	71 *C. hominis* isolates	3 (665bp)
		64 *C. parvum* isolates from humans	1 (544bp) and 2 (571bp)
		44 *C. parvum* isolates from cattle	1 (544bp) and 2 (571bp)
		1 *C. meleagridis* isolate	4 (N.A.)
	Mallon *et al.*, 2003b	190 *C. parvum* isolates from cattle	1 (544bp) and 2 (571bp)
		39 *C. parvum* isolates from humans	1 (544bp), 2 (571bp) and 5 (568bp)
		11 *C. parvum* isolates from sheep	1 (544bp) and 2 (571bp)
		2 *C. parvum* 'monkey genotype' isolates from humans	2 (571bp)

N.A. - no amplification

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. meleagridis*. 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 apposed 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 2×10^9 oocysts and McLaughlin *et al.* (1998) stated that 1×10^4 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*

al., 2002a; Alves *et al.*, 2003a; Mallon *et al.*, 2003a; Mallon *et al.*, 2003b). 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 DGPE 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 DGPE) 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 ($>1.2 \times 10^7$) (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. meleagridis*, *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

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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 $0.1\mu l$ 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.

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

Those found to contain $>2\times10^5$ 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

○ → Oocyst counted

● → Oocyst not counted
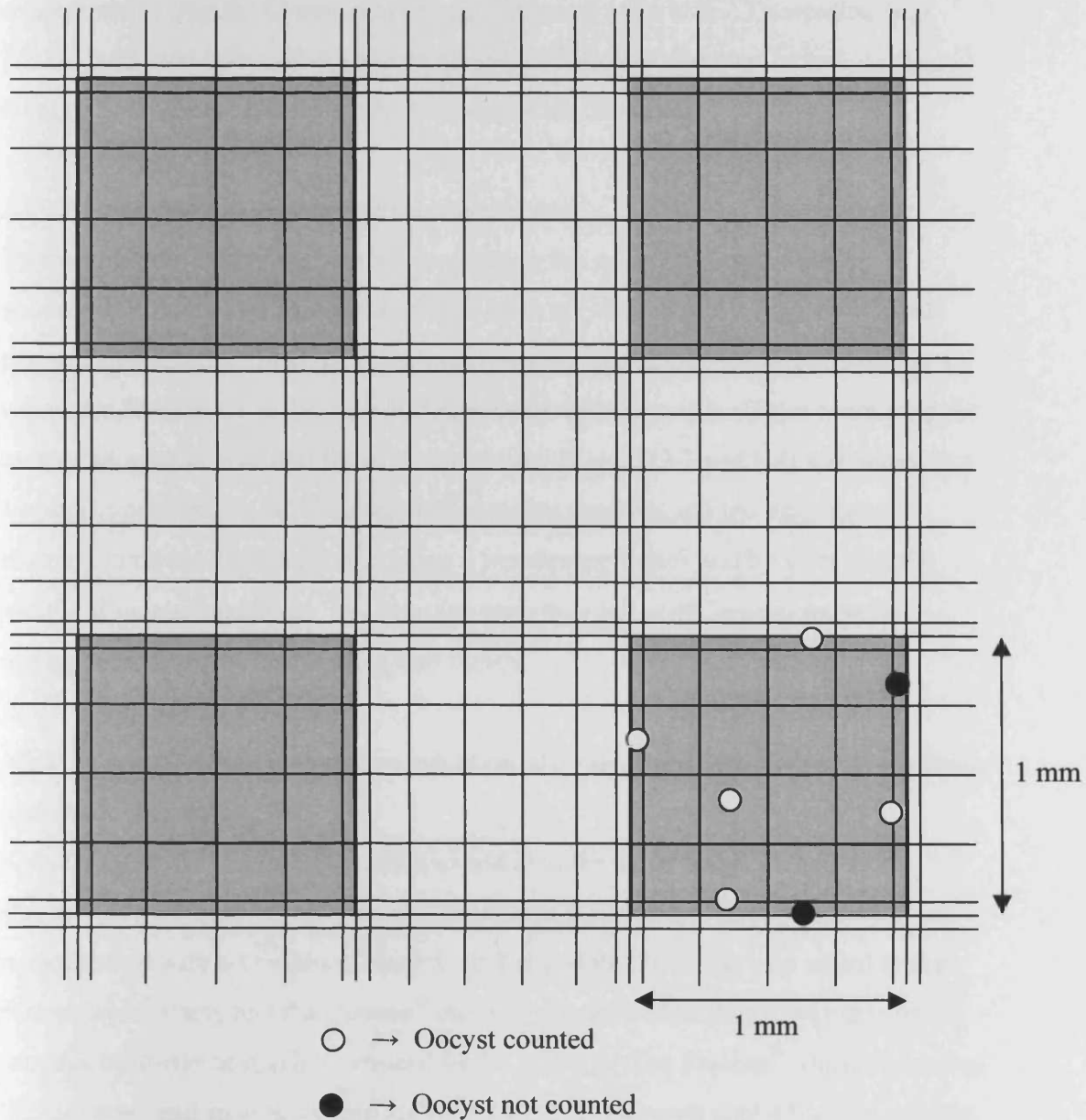
Figure 3.1: A diagrammatic representation of the improved Neubauer Haemocytometer grid. The four 0.1μl corner squares used for enumerating *Cryptosporidium* oocyst suspensions are shaded and an example of which oocysts are counted (oocysts touching the top or left central boundary are counted and those 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 ($O_{counted}$) was then adjusted for the dilution factor to give the total number of oocysts per well following concentration (O_{total}) using the formula:

$$O_{total} = \frac{O_{counted}}{D}$$

Mixing chamber

425μm mesh filter

Large debris

Sedimentation cone

Ethyl acetate and fatty plug

Formol solution containing oocysts removed and concentrated by centrifugation

Sediment debris
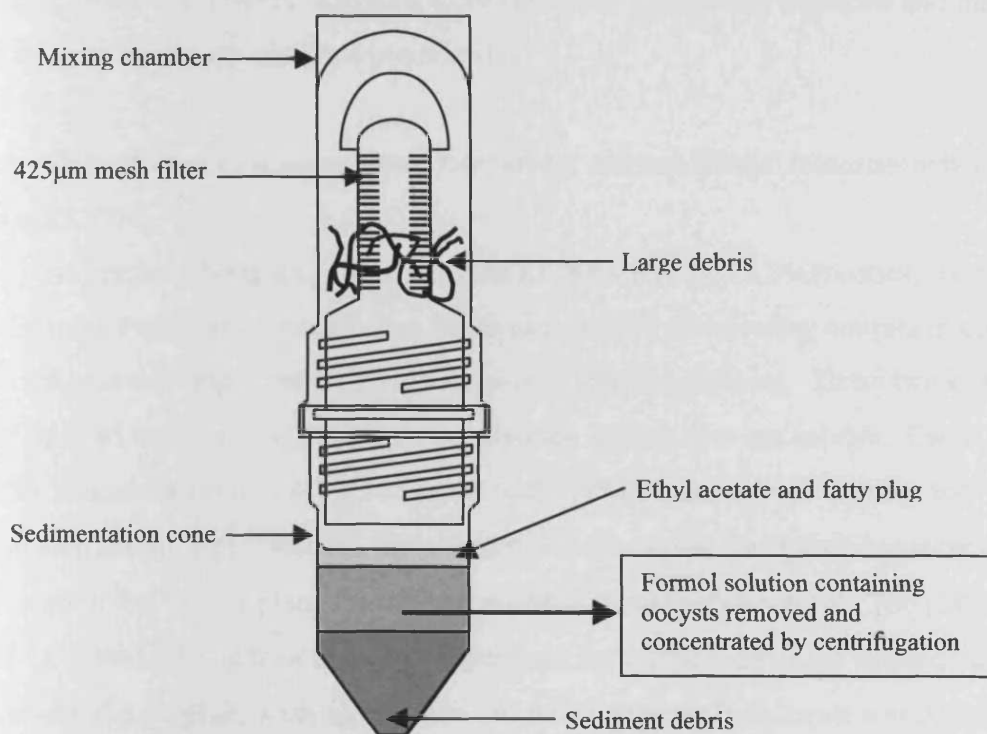
Figure 3.2: A diagrammatic representation of the Parasep® faecal concentrator, used in the modified formol-ethyl acetate method, showing the oocyst-containing layer which is removed for further concentration.

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 htII 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 1×10^5 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.

Sheep Faeces

↓

3 replicates spiked with 1×10^5 oocysts

↓

15 IFATs (5 from each replicate) for oocyst enumeration

↓

15 IFATs (5 from each replicate) for oocyst enumeration

Formol-ethyl acetate concentration

Cattle Faeces

↓

3 replicates spiked with 1×10^5 oocysts

↓

15 IFATs (5 from each replicate) for oocyst enumeration

↓

15 IFATs (5 from each replicate) for oocyst enumeration
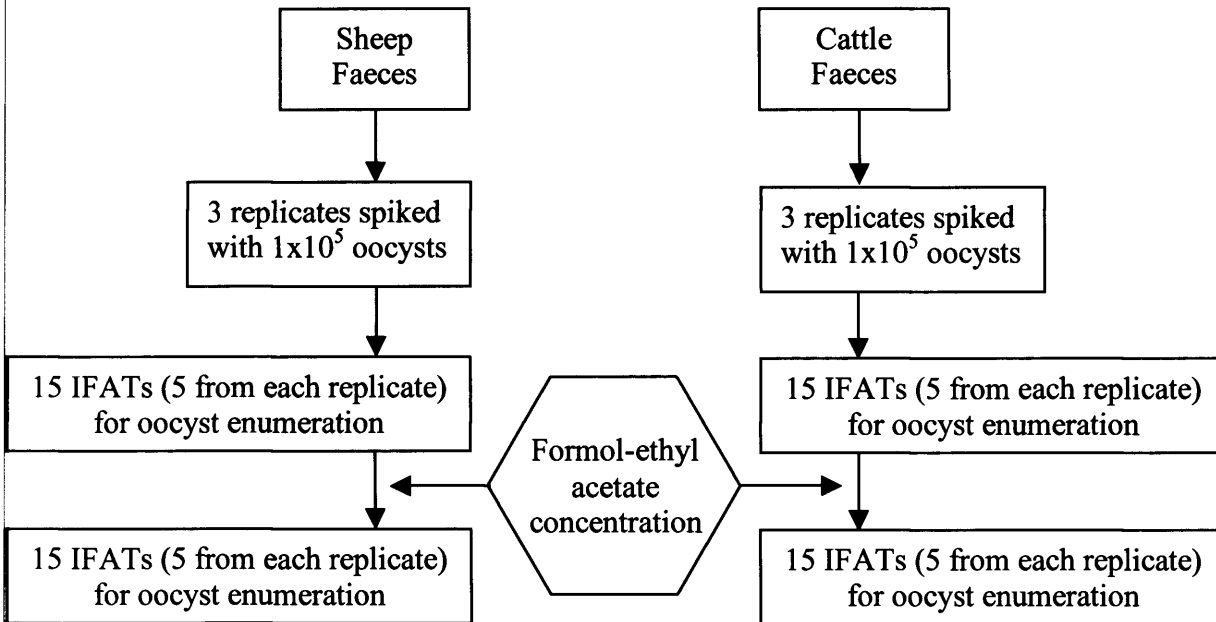
Figure 3.3: An algorithm of the experiment to determine the level of concentration achieved using the formol-ethyl acetate protocol with sheep and cattle faeces.

Cattle Faeces

2.5×10^3 oocysts	2×10^3 oocysts	1.5×10^3 oocysts	1×10^3 oocysts	0.5×10^3 oocysts

·········· Parasep

5 IFATs	5 IFATs	5 IFATs	5 IFATs	5 IFATs

Sheep Faeces

3.5×10^3 oocysts	3×10^3 oocysts	2.5×10^3 oocysts	2×10^3 oocysts	1.5×10^3 oocysts	1×10^3 oocysts	0.5×10^3 oocysts

·········· Parasep

5 IFATs	5 IFATs	5 IFATs	5 IFATs	5 IFATs	5 IFATs	5 IFATs
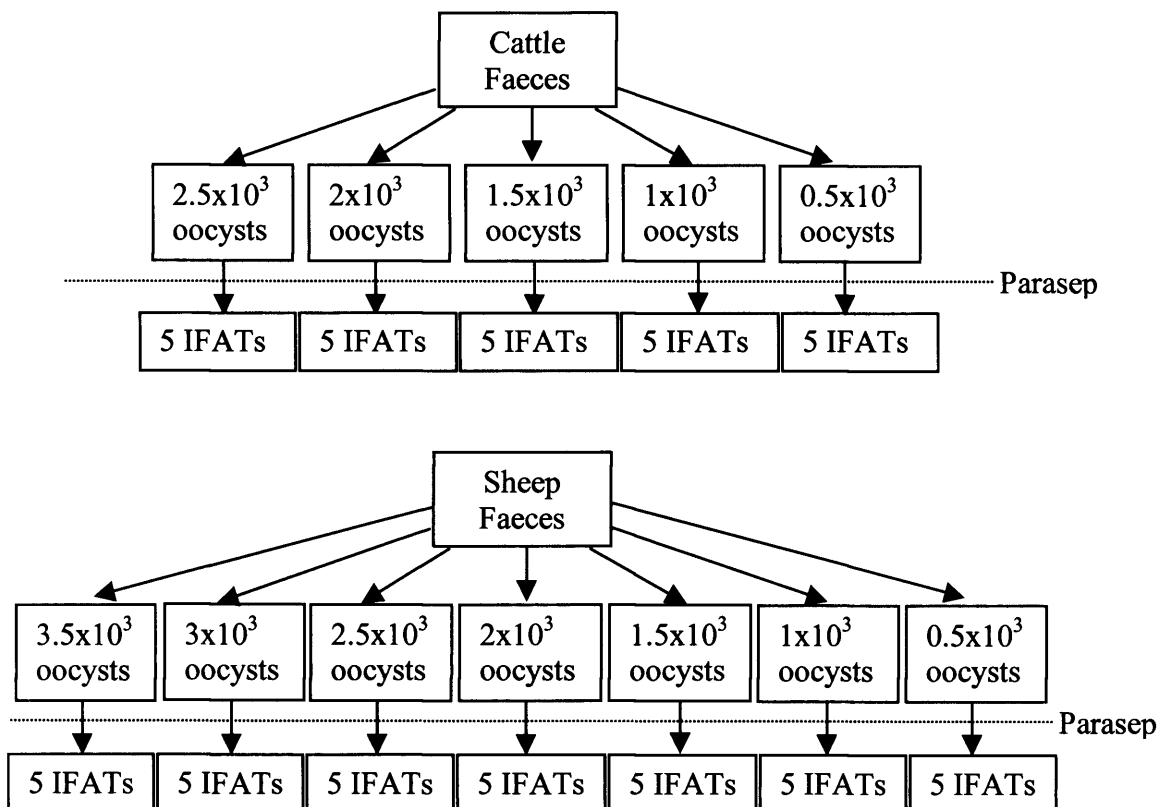
Figure 3.4: An algorithm of the experiment to determine the limit of oocyst detection in sheep and cattle faeces using formol-ethyl acetate concentration and immunofluorescence microscopy.

Table 3.1: Samples used to evaluate the use of ELISA following concentration by formol-ethyl acetate concentration

Sample	Number of oocysts seeded into sample	Replicates
Sheep faeces	2.5×10^3	5
Sheep faeces	2.0×10^3	5
Sheep faeces	1.5×10^3	5
Sheep faeces	1.0×10^3	5
Sheep faeces	0.5×10^3	5
Cattle faeces	2.5×10^3	5
Cattle faeces	2.0×10^3	5
Cattle faeces	1.5×10^3	5
Cattle faeces	1.0×10^3	5
Cattle faeces	0.5×10^3	5
Kit positive control	N/A	1 per plate
Non-concentrated positive faeces	N/A	1
Concentrated positive faeces	N/A	1
10^5 oocysts in suspension	N/A	1
10^4 oocysts in suspension	N/A	1
10^3 oocysts in suspension	N/A	1
Negative faeces	N/A	1
Deionised water blank	N/A	1 per plate

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

Table 3.2: Oocyst counts and means for the faecal samples prior to and following concentration using the formol-ethyl acetate protocol

Sample	Pre-Concentration			Post–Concentration			
	No. of oocysts	Mean per replicate	Mean per animal	No. of oocysts	Mean per replicate	Mean per animal	Without dilution
Sheep Faecal Replicate 1	16	13.4 ±6.1	13.1±4.0	91	86.4±21.8	69.9±19.4	210.1±78.9
	21			78			
	5			117			
	10			57			
	15			89			
Sheep Faecal Replicate 2	14	12.6±3.6		56	60.8±12.4		
	16			59			
	7			63			
	11			80			
	15			46			
Sheep Faecal Replicate 3	13	13.4±2.1		79	62.6±13.5		
	12			57			
	17			43			
	12			66			
	13			68			
Cattle Faecal Replicate 1	41	50.2±8.3	51.3±5.9	140	140.6±5.6	135.4±11.0	300.3±55.1
	43			140			
	61			132			
	55			144			
	51			147			
Cattle Faecal Replicate 2	46	53.2±5.2		139	138.4±10.2		
	50			138			
	54			131			
	57			129			
	59			155			
Cattle Faecal Replicate 3	54	50.6±4.1		123	127.2±12.5		
	44			116			
	51			116			
	50			140			
	54			141			
				116			

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 3×10^3 oocysts g^{-1} (Table 3.3). The limit of detection with the same sensitivity for the seeded typical cow faeces was 2×10^3 oocysts g^{-1} (Table 3.3). Oocysts were also detected in the faecal samples from both species at a level of 0.5×10^3 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 10^5, 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

| Sample | Oocysts | Replicates | | | | | Sensitivity |
		1	2	3	4	5	
Sheep Faeces	3.5×10^3	Positive	Positive	Positive	Positive	Positive	100%
	3.0×10^3	Positive	Positive	Positive	Positive	Positive	100%
	2.5×10^3	Positive	Positive	Positive	Negative	Positive	80%
	2.0×10^3	Negative	Positive	Positive	Positive	Positive	80%
	1.5×10^3	Positive	Negative	Negative	Negative	Positive	40%
	1.0×10^3	Negative	Negative	Positive	Positive	Positive	60%
	0.5×10^3	Negative	Negative	Negative	Positive	Negative	20%
Cattle Faeces	2.5×10^3	Positive	Positive	Positive	Positive	Positive	100%
	2.0×10^3	Positive	Positive	Positive	Positive	Positive	100%
	1.5×10^3	Positive	Positive	Positive	Negative	Positive	80%
	1.0×10^3	Positive	Negative	Negative	Positive	Positive	60%
	0.5×10^3	Positive	Positive	Negative	Negative	Positive	60%

Table 3.4: ELISA detection of *Cryptosporidium* following concentration with the formol-ethyl acetate protocol

| Sample | No. of Spiked Oocysts | Replicates | | | | | Sensitivity |
		1	2	3	4	5	
Sheep Faeces	2.5×10^3	Negative	Negative	Negative	Negative	Negative	0%
	2.0×10^3	Negative	Negative	Negative	Negative	Negative	0%
	1.5×10^3	Negative	Negative	Negative	Negative	Negative	0%
	1.0×10^3	Negative	Negative	Negative	Negative	Negative	0%
	0.5×10^3	Negative	Negative	Negative	Negative	Negative	0%
Cattle Faeces	2.5×10^3	Negative	Negative	Negative	Negative	Negative	0%
	2.0×10^3	Negative	Negative	Negative	Negative	Negative	0%
	1.5×10^3	Negative	Negative	Negative	Negative	Negative	0%
	1.0×10^3	Negative	Negative	Negative	Negative	Negative	0%
	0.5×10^3	Negative	Negative	Negative	Negative	Negative	0%
Kit positive	-	Positive	-	-	-	-	-
Non-Concentrated known positive	-	Positive	-	-	-	-	-
Concentrated known positive	-	Negative	-	-	-	-	-
10^5 suspension	-	Negative	-	-	-	-	-
10^4 suspension	-	Negative	-	-	-	-	-
10^3 suspension	-	Negative	-	-	-	-	-
Known Negative	-	Negative	-	-	-	-	-
Blank	-	Negative	-	-	-	-	-

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 *Cryptosporidium* oocyst disruption used by researchers prior to extraction of DNA for use in molecular techniques

Method	References
Proteinase K digestion in TE-SDS buffer at 60°C for 48 hours	Laxer *et al.*, 1991; Awad-El-Kariem *et al.*, 1994
Proteinase K digestion in TE-SDS buffer at 50°C for 3 hours	Ranucci *et al.*, 1993
Proteinase K digestion in TE-sarcosyl balathat buffer at 60°C for 2 hours	Balathat *et al.*, 1996
Proteinase K digestion in SDS buffer at 45°C overnight	Sluter *et al.*, 1997
Proteinase K digestion in TE-sarcosine buffer at 60°C for 4 hours	Deng *et al.*, 1997; Deng *et al.*, 2000
Proteinase K digestion in TE-SDS buffer at 56 °C for 2 hours	Ward and Wang, 2001
Proteinase K digestion in TE-sarcosyl buffer at 37°C for 1 hour then incubation in NaCl-CTAB at 65°C for 30 min followed by 1x Freeze-thaw cycle (-198°C ↔95°C, 2↔5 minutes)	Rochelle *et al.*, 1997a; Rochelle *et al.*, 1997b
Proteinase K digestion in NaCl-EDTA buffer at 65°C for 2 hours then 6x Freeze-thaw cycles in TE-SDS (-78°C ↔65°C, 2↔5 minutes)	Mayer and Palmer, 1996
Freeze-thaw cycles	Cai *et al.*, 1992; Johnson *et al.*, 1993
3x Freeze-thaw cycles (-78°C↔37°C)	Sluter *et al.*, 1997
3x Freeze-thaw cycles then Proteinase K digestion in SDS buffer at 37°C overnight	Carraway *et al.*, 1994; Carraway *et al.*, 1996
3x Freeze-thaw cycles in TE-Triton X-100 buffer then Proteinase K digestion at 56°C for 3 hours	Morgan *et al.*, 1995
3x Freeze-thaw cycles (-198°C ↔75°C, 2 minutes each) then Proteinase K digestion in TE-SDS buffer at 56°C for 2 hours	Ward and Wang, 2001
3x Freeze-thaw cycles in TE buffer then heating at 100°C for 5 minutes	Morgan *et al.*, 1996
3x Freeze-thaw cycles (-198°C ↔90°C) in TE-dithiothreitol buffer followed by heating at 90°C for 20 minutes	Webster, 1993
4x Freeze-thaw cycles in SDS buffer then heating at 100°C for 10 minutes	Spano *et al.*, 1997
4x Freeze-thaw cycles (-198°C ↔75°C, 5 minutes each) in Nonidet P 40-SDS-NaOH-Proteinase K buffer then Proteinase K digestion at 75°C for 3 hours	Leng *et al.*, 1996a ; Leng *et al.*, 1996b
5x Freeze-thaw cycles (-78°C↔37°C)	Sluter *et al.*, 1997
5x Freeze-thaw cycles (-198°C ↔90°C) in TE-dithiothreitol buffer	Webster *et al.*, 1996
5x Freeze-thaw cycles (-198°C ↔65°C, 1 minute each) in TE-LDS- dithiothreitol buffer	Stinear *et al.*, 1996
5x Freeze-thaw cycles (-198°C ↔65°C, 5 minutes each) in TE-sarcosyl-Proteinase K buffer then Proteinase K digestion at 55°C for 1 hour	Kim *et al.*, 1992
5-10x Freeze-thaw cycles (-198°C or -78↔42 to 50°C)	Higgins *et al.*, 2001
6x Freeze-thaw cycles (-78°C↔98°C, 1 to 2 minutes each) in 20% Chelex	Johnson *et al.*, 1995

Method	Reference
10x Freeze-thaw cycles (-198°C↔65°C, 5 minutes each) in TE-sarcosyl-Proteinase K buffer then Proteinase K digestion at 55°C for 1 hour	Laberge et al., 1996
10x Freeze-thaw cycles (-198°C↔65°C, 5 minutes each) in TE-sarcosyl-Proteinase K buffer then Proteinase K digestion at 55°C for 30 minutes	Chung et al., 1998
15x Freeze-thaw cycles in Tris-Tween 20 buffer then heating at 100°C for 15 minutes	Bonnin et al., 1996; Gobet et al., 1997
15x Freeze-thaw cycles (-198°C↔65°C, 1 minute each) in TE-SDS buffer then Proteinase K digestion at 55°C for 3 hours	Nichols et al., 2003
Incubation with TE-guanidinium thiocyanate-Triton X-100 lysis buffer at 20-25°C for 30 minutes	Spano et al., 1997
Bead disruption (2 minutes at maximum speed) then incubation with TE-guanidinium thiocyanate-Triton X-100 lysis buffer at 20-25°C for 5 minutes	Patel et al., 1998; McLauchlin et al., 1999
Bead Disruption (3 x 1 minute)	Higgins et al., 2001
Sonication (3 x 5 minutes)	Sluter et al., 1997
Electroporation (2.5kV, 129 Ω)	Sluter et al., 1997
Excystation in gall acid at 37°C for 4 hours then heating at 100°C for 15 minutes	Filkorn et al., 1994
Excystation in Trypsin and gall acid at 37°C for 1 hour then Proteinase K digestion in TE-SDS-dithiothreitol buffer at 37°C for 1 hour	Wagner-Wiening and Kimmig, 1995
Excystation and in vitro infection of cell culture then DNA extracted from infected cells	Rochelle et al., 1996; Rochelle et al., 1997b
Excystation in bile and NaHCO₃ at 37°C for 4 hours then incubation at 95°C for 10 minutes followed by 5 minutes at 4°C	Deng et al., 1997

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 1×10^5 oocysts were added to 1ml of deionised water to produce a working suspension of 1×10^5 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 1×10^5 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 manufacturers 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 ($MgCl_2$) 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 $MgCl_2$, 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 $MgCl_2$, forward primer and reverse primer in each sample

		MgCl₂ Concentration (mM)					
		1.5	2.0	2.5	3.0	3.5	4.0
Concentration of each primer (nM)	50	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
	100	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
	150	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18
	200	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
	250	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30

To test the limit of detection of this adapted PCR, DNA was extracted from 200µl of a 5.25×10^4 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

Dilution	Number of oocysts ml^{-1} equivalent	Number of oocysts equivalent per PCR (10µl)
1	5.25×10^4	1.05×10^3
1:10	5.25×10^3	1.05×10^2
1:100	5.25×10^2	1.05×10^1
1:1,000	5.25×10^1	1.05×10^0
1:10,000	5.25×10^0	1.05×10^{-1}
1:100,000	5.25×10^{-1}	1.05×10^{-2}
1:1,000,000	5.25×10^{-2}	1.05×10^{-3}

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 MgCl$_2$, 200 mM of each deoxynucleotide triphosphate, 200nM of forward primer (5'-GGAAGGGTTGTATTTATTAGATAAA

G-3'), 200nM of reverse primer (5'-AAGGAGTAAGGAACAACCTCCA-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

(Phorecus, Biogene Ltd.) gel at 110V for 60 minutes and recorded using a digital

imaging system (AlphaImager, 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 1.43×10^6 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 2.86×10^5 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

Dilution	Equivalent number of oocysts ml^{-1}	Equivalent number of oocysts 200μl^{-1}	Equivalent number of oocysts per PCR
1	1.43x10^6	2.86x10^5	2.86x10^4
1:5	2.86x10^5	5.72x10^4	5.72x10^3
1:10	1.43x10^5	2.86x10^4	2.86x10^3
1:50	2.86x10^4	5.72x10^3	5.72x10^2
1:100	1.43x10^4	2.86x10^3	2.86x10^2
1:500	2.86x10^3	5.72x10^2	5.72x10^1
1:1,000	1.43x10^3	2.86x10^2	2.86x10^1

AGTAAGGAACAACCTCCA-3'), 2.5U of HotStar Taq DNA polymerase (Qiagen Ltd., UK), 10μl of template DNA and 1:10 dilution of SYBR Green I 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 (C$_t$) 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 5x10^6 oocysts were added to 50ml of deionised water to give a working concentration of 1x10^5 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 4.5: Methods of oocyst disruption to evaluate for the maximum recovery of *Cryptosporidium* DNA

Method of Oocyst Disruption

1. Proteinase K and Buffer AL digestion at 56°C for 16 hours

2. Proteinase K and Buffer ATL digestion at 37°C for 10 minutes then Buffer AL at 70°C for 10 minutes

3. Proteinase K and Buffer ATL digestion at 37°C for 30 minutes then Buffer AL at 70°C for 10 minutes

4. Proteinase K and Buffer ATL digestion at 37°C for 60 minutes then Buffer AL at 70°C for 10 minutes

5. Proteinase K and Buffer ATL digestion at 37°C for 16 hours then Buffer AL at 70°C for 10 minutes

6. Proteinase K and Buffer ATL digestion at 56°C for 10 minutes then Buffer AL at 70°C for 10 minutes

7. Proteinase K and Buffer ATL digestion at 56°C for 30 minutes then Buffer AL at 70°C for 10 minutes

8. Proteinase K and Buffer ATL digestion at 56°C for 60 minutes then Buffer AL at 70°C for 10 minutes

9. Proteinase K and Buffer ATL digestion at 56°C for 16 hours then Buffer AL at 70°C for 10 minutes

10. 1 x Freeze-Thaw cycle (-78°C ↔ 37°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

11. 3 x Freeze-Thaw cycles (-78°C ↔ 37°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

12. 5 x Freeze-Thaw cycles (-78°C ↔ 37°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

13. 7 x Freeze-Thaw cycles (-78°C ↔ 37°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

14. 10 x Freeze-Thaw cycles (-78°C ↔ 37°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

15. 1 x Freeze-Thaw cycle (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

16. 3 x Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

17. 5 x Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

18. 7 x Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

19. 10 x Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

20. Heating at 100°C for 10 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

21. Heating at 100°C for 30 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

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 CaCl$_2$, 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

Method of Oocyst Disruption

1. Proteinase K and Buffer AL digestion at 56°C for 30 minutes

2. Proteinase K and Buffer AL digestion at 56°C for 16 hours

3. Proteinase K and Buffer ATL digestion at 56 °C for 10 minutes followed by Buffer AL at 70°C for 10 minutes

4. 3 Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 30 minutes

5. 3 Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer AL digestion at 56°C for 16 hours

6. 3 Freeze-Thaw cycles (-78°C ↔ 100°C) followed by Proteinase K and Buffer ATL digestion at 56 °C for 10 minutes then Buffer AL at 70°C for 10 minutes

7. Heating at 100°C for 60 minutes followed by Proteinase K and Buffer AL digestion at 56°C for 10 minutes

8. Heating at 100°C for 60 minutes followed by Proteinase K and Buffer ATL digestion at 56 °C for 10 minutes then Buffer AL at 70°C for 10 minutes

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 1×10^5 oocysts in 9ml of deionised water

and 1ml of 10x SL-Buffer A (Dynal Biotech) giving a working concentration of 1×10^4

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 1×10^3 oocysts

ml^{-1} and was repeated a further time to prepare a suspension of 1×10^2 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'-CCCATTTCCTTCGAAACAGGA-3'. The 50µl PCR reaction mixture contained 10x PCR buffer (Qiagen Ltd., UK), 6mM $MgCl_2$, 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'-GGAAGGGTTGTATTTATTAGATAAAG-3' and the reverse primer 5'-AAGGAGTAAGGAACAACCTCCA-3'. The PCR reaction mixture was identical to that in the primary PCR except that 3mM $MgCl_2$ 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

Day	Mean oocyst Concentration (ml⁻¹)	Standard Deviation	t-statistic	p-value
1	1.5×10^5	3.1×10^4	-	-
2	1.5×10^5	3.3×10^4	-0.92	0.365
3	1.4×10^5	3.0×10^4	0.65	0.517
4	1.4×10^5	3.0×10^4	0.39	0.698
5	1.3×10^5	3.3×10^4	2.86	0.007
8	1.0×10^5	2.8×10^4	6.69	0.000
15	1.2×10^5	2.9×10^4	3.09	0.004
22	1.1×10^5	2.6×10^4	4.42	0.000

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

	Before Concentration	After Concentration
No Disruption	1.0×10^5	4.4×10^5
Heat Disruption	0.6×10^5	2.2×10^5

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 $MgCl_2$ 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).
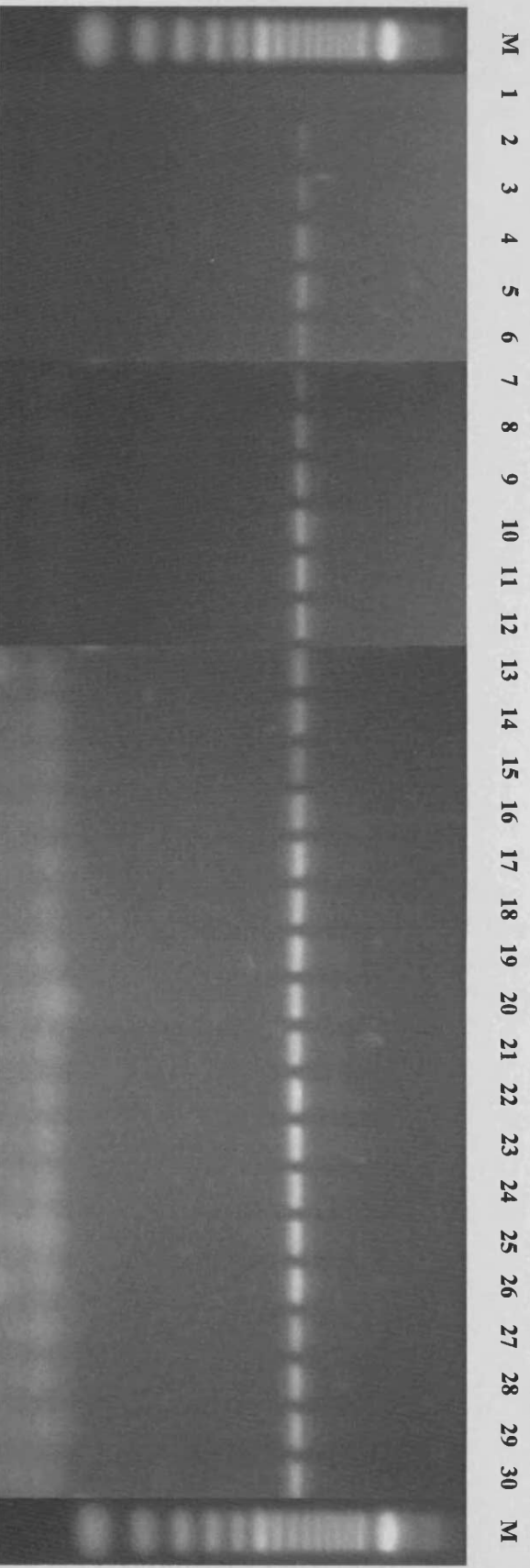
Figure 4.1: The ~830bp PCR products from the MgCl₂ and primer chequerboard (see Table 4.2 for concentrations).
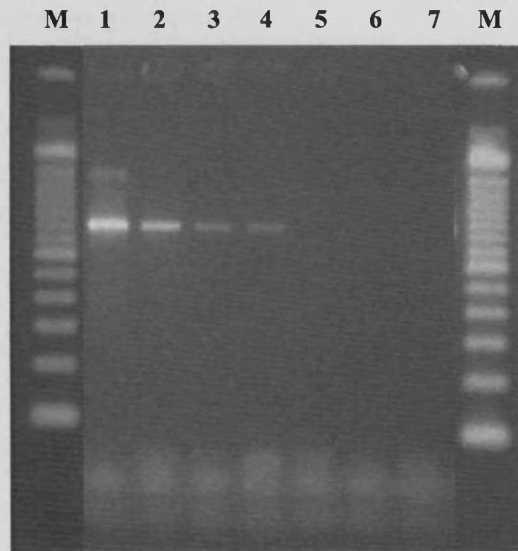
Figure 4.2: Products from the dilution series to test the limit of detection for the adapted SSU rDNA PCR. Lanes M = 100bp ladders; Lane 1 = 1,050 oocysts; Lane 2 = 105 oocysts; Lane 3 = 10.5 oocysts; Lane 4 = 1 oocyst; Lane 5 = 0.1 oocyst; Lane 6 = 0.01 oocyst; Lane 7 = 0.001 oocyst.
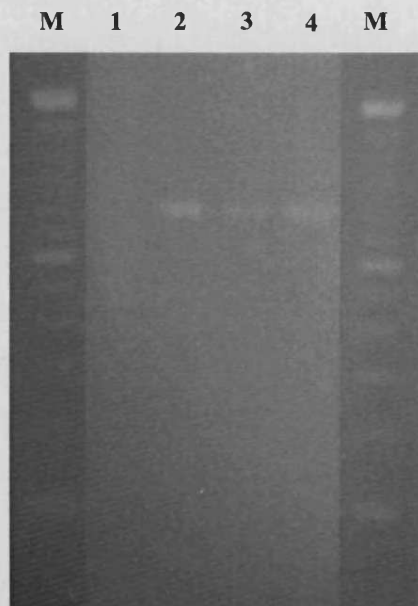
Figure 4.3: Amplification with the adapted SSU rDNA PRC with SYBR Green I present in the reaction mixture. Lanes M = 100bp ladders; Lane 1 = x1 working concentration of SYBR Green I; Lane 2 = 1:10 dilution; Lane 3 = 1:50 dilution; Lane 4 = 1:100 dilution.

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.b – 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
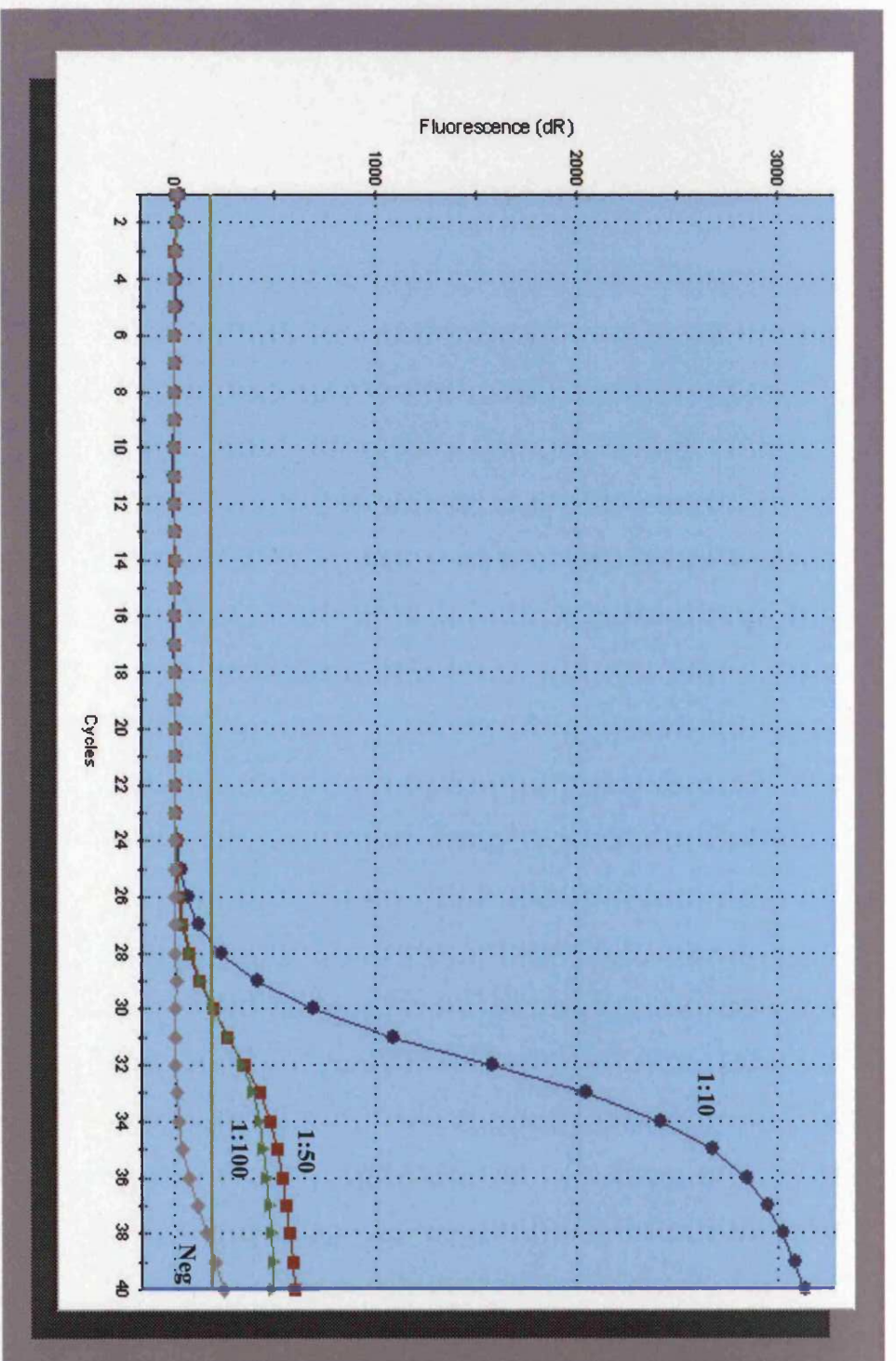
Figure 4.4: Amplification curves showing the level of fluorescence generated by the 1:10, 1:50 and 1:100 dilutions of SYBR Green I present in the PCR reaction mixtures.
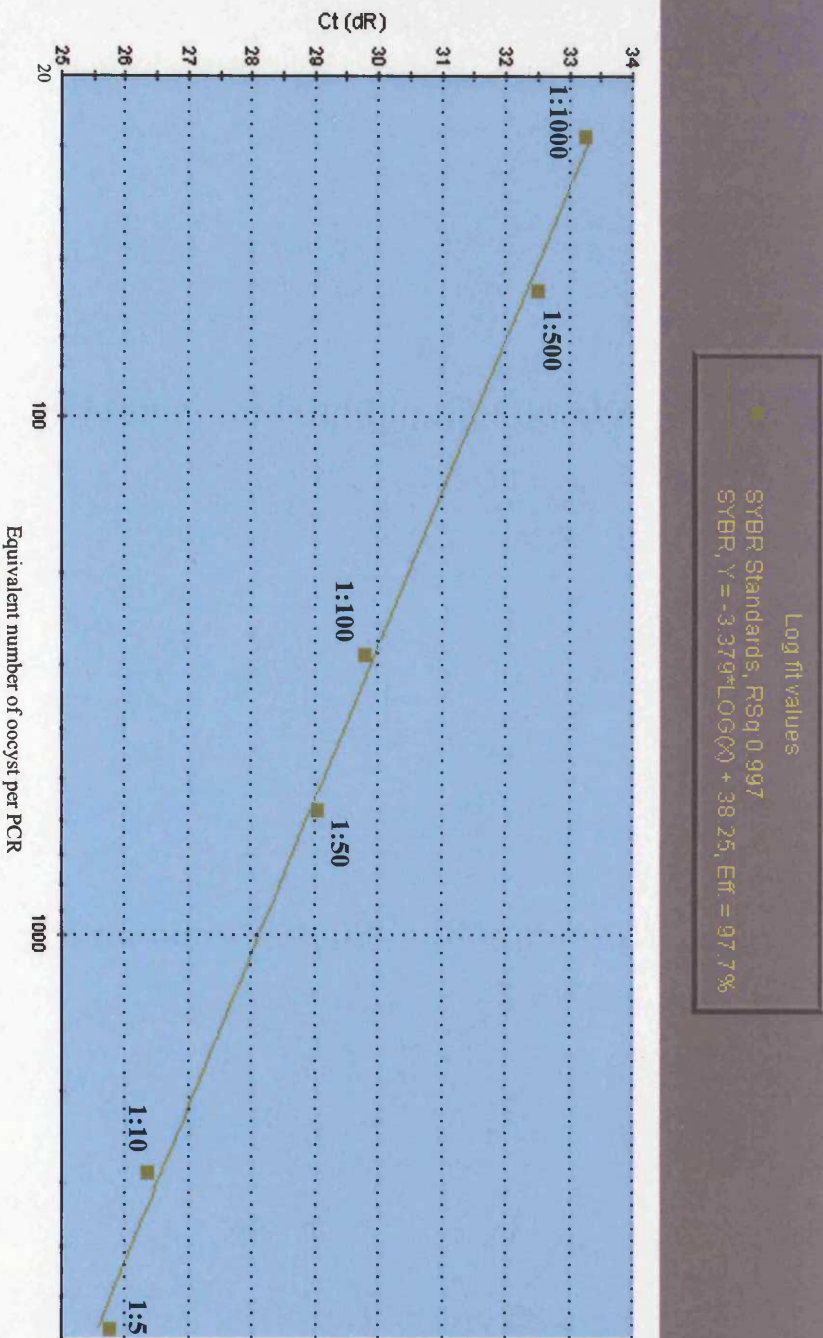
Figure 4.5: A standard curve for the SYBR Green I real-time PCR from a DNA dilution series, showing the result of the regression analysis (RSq) and amplification efficiency of the PCR (Eff).
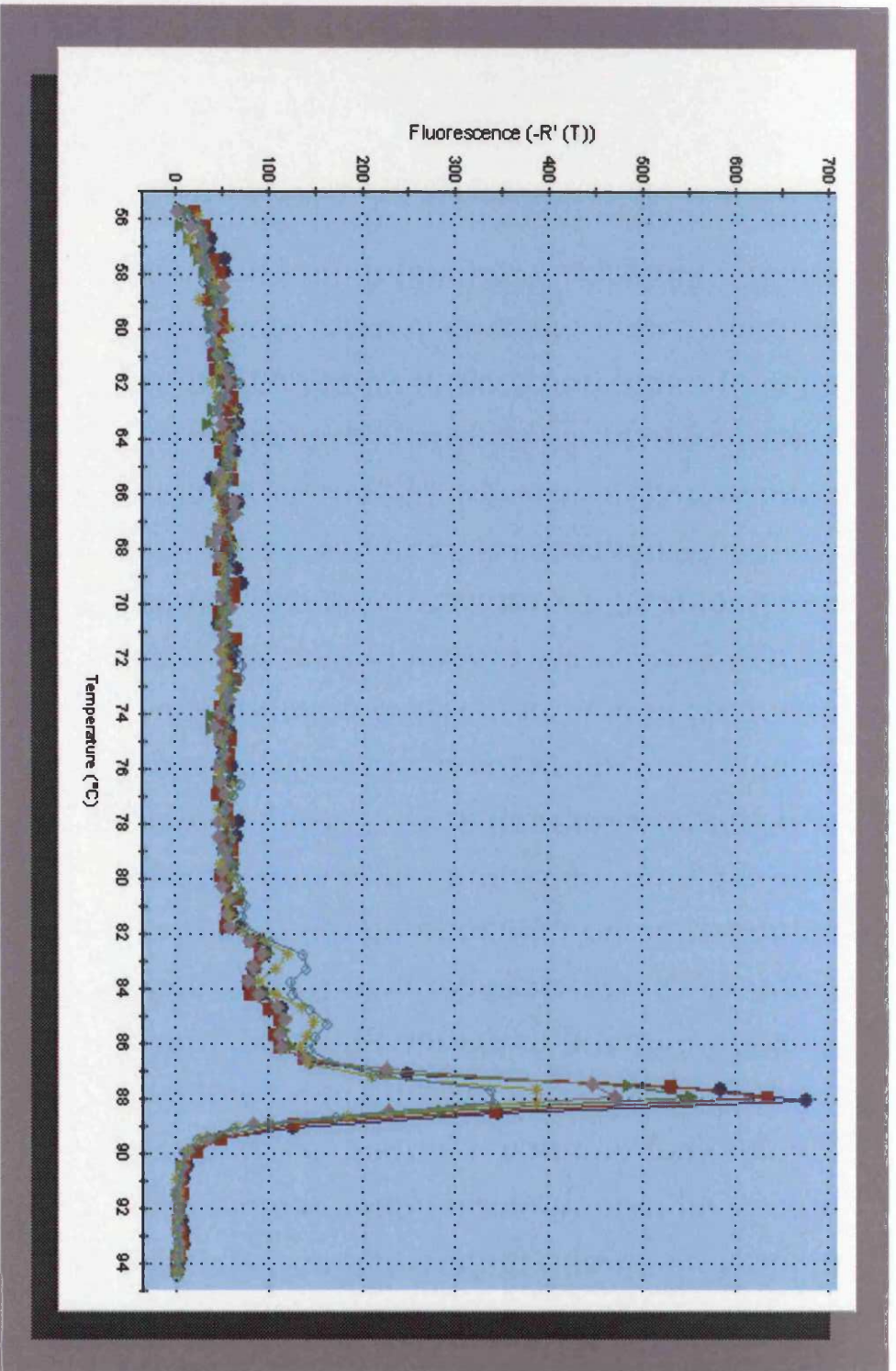
Figure 4.6: The dissociation curves for the DNA dilution series used to create the standard curves showing the typical 88°C peaks for these *Cryptosporidium* SSU rDNA fragments.

Table 4.9: The number of whole oocysts ml^{-1} when enumerated by improved Neubauer Haemocytometer following each disruption method

Method	Number of Whole Oocysts (ml^{-1})				
	Count 1	Count 2	Count 3	Mean	S.D.
1. Control (no treatment)	3.0×10^5	4.2×10^5	3.3×10^5	3.5×10^5	6.2×10^4
2. 10 min x Prot. K + Buffer AL (37°C)	1.2×10^5	1.2×10^5	1.3×10^5	1.2×10^5	5.8×10^3
3. 30 min x Prot. K + Buffer AL (37°C)	1.3×10^5	1.1×10^5	1.8×10^5	1.4×10^5	3.6×10^4
4. 60 min x Prot. K + Buffer AL (37°C)	1.7×10^5	1.7×10^5	1.2×10^5	1.5×10^5	2.9×10^4
5. 16 hrs x Prot. K + Buffer AL (37°C)	6.0×10^4	1.2×10^5	9.0×10^4	9.0×10^4	3.0×10^4
6. 10 min x Prot. K + Buffer AL (56°C)	1.2×10^5	1.4×10^5	1.5×10^5	1.4×10^5	1.5×10^4
7. 30 min x Prot. K + Buffer AL (56°C)	1.3×10^5	1.6×10^5	1.4×10^5	1.4×10^5	1.5×10^4
8. 60 min x Prot. K + Buffer AL (56°C)	1.1×10^5	1.1×10^5	6.5×10^4	9.5×10^4	2.6×10^4
9. 16 hrs x Prot. K + Buffer AL (56°C)	8.3×10^4	6.8×10^4	6.8×10^4	7.3×10^4	8.7×10^3
10. 10 min x Prot. K + Buffer ATL (37°C)	4.8×10^4	5.5×10^4	3.5×10^4	4.6×10^4	1.0×10^4
11. 30 min x Prot. K + Buffer ATL (37°C)	4.8×10^4	5.3×10^4	3.0×10^4	4.4×10^4	1.2×10^4
12. 60 min x Prot. K + Buffer ATL (37°C)	4.5×10^4	3.0×10^4	4.0×10^4	3.8×10^4	7.6×10^3
13. 16 hrs x Prot. K + Buffer ATL (37°C)	1.0×10^4	0	0	3.3×10^3	5.8×10^3
14. 10 min x Prot. K + Buffer ATL (56°C)	2.8×10^4	8.8×10^4	4.8×10^4	5.5×10^4	3.1×10^4
15. 30 min x Prot. K + Buffer ATL (56°C)	5.0×10^4	3.0×10^4	4.3×10^4	4.1×10^4	1.0×10^4
16. 60 min x Prot. K + Buffer ATL (56°C)	3.8×10^4	5.8×10^4	3.3×10^4	4.3×10^4	1.3×10^4
17. 16 hrs x Prot. K + Buffer ATL (56°C)	0	0	3.5×10^4	1.2×10^4	2.0×10^4
18. 1 x Freeze-Thaw (37°C)	4.8×10^5	4.1×10^5	4.4×10^5	4.4×10^5	3.5×10^4
19. 3 x Freeze-Thaw (37°C)	4.2×10^5	4.8×10^5	4.5×10^5	4.5×10^5	3.0×10^4
20. 5 x Freeze-Thaw (37°C)	5.0×10^5	2.8×10^5	3.6×10^5	3.8×10^5	1.1×10^5
21. 7 x Freeze-Thaw (37°C)	2.7×10^5	3.7×10^5	3.3×10^5	3.2×10^5	5.0×10^4
22. 10 x Freeze-Thaw (37°C)	2.0×10^5	2.0×10^5	2.5×10^5	2.2×10^5	2.9×10^4
23. 1 x Freeze-Thaw (100°C)	2.5×10^5	2.2×10^5	5.3×10^4	1.7×10^5	1.1×10^5
24. 3 x Freeze-Thaw (100°C)	4.8×10^4	9.8×10^4	2.5×10^4	5.7×10^4	3.7×10^4
25. 5 x Freeze-Thaw (100°C)	6.5×10^4	1.4×10^5	1.4×10^5	1.2×10^5	4.3×10^4
26. 7 x Freeze-Thaw (100°C)	9.5×10^4	7.5×10^4	8.0×10^4	8.3×10^4	1.0×10^4
27. 10 x Freeze-Thaw (100°C)	5.8×10^4	3.5×10^4	2.3×10^4	3.9×10^4	1.8×10^4
28. 10 min x Heat (100°C)	9.0×10^4	7.5×10^4	1.5×10^5	1.1×10^5	4.0×10^4
29. 30 min x Heat (100°C)	5.5×10^4	1.8×10^4	8.5×10^4	5.3×10^4	3.4×10^4
30. 60 min x Heat (100°C)	9.8×10^4	3.5×10^4	1.5×10^4	4.9×10^4	4.3×10^4
31. 10 min x Trypsin (37°C)	1.6×10^5	2.6×10^5	2.1×10^5	2.1×10^5	5.0×10^4
32. 30 min x Trypsin (37°C)	3.4×10^5	1.6×10^5	2.3×10^5	2.4×10^5	9.1×10^4
33. 60 min x Trypsin (37°C)	6.5×10^5	1.2×10^5	1.7×10^5	3.1×10^5	2.9×10^5
34. 16 hrs x Trypsin (37°C)	3.3×10^5	2.9×10^5	2.9×10^5	3.0×10^5	2.3×10^4
35. 1 min x Sonication (50/60 Hz, 80W)	2.1×10^5	2.8×10^5	2.6×10^5	2.5×10^5	3.6×10^4
36. 5 min x Sonication (50/60 Hz, 80W)	2.8×10^5	2.2×10^5	2.5×10^5	2.5×10^5	3.0×10^4
37. 10 min x Sonication (50/60 Hz, 80W)	3.1×10^5	3.4×10^5	4.6×10^5	3.7×10^5	7.9×10^4
38. 15 min x Sonication (50/60 Hz, 80W)	2.4×10^5	3.6×10^5	3.6×10^5	3.2×10^5	6.9×10^4
39. 20 min x Sonication (50/60 Hz, 80W)	4.0×10^5	4.5×10^5	4.1×10^5	4.2×10^5	2.6×10^4
40. 2 min x Vortexed Zirconia Beads	1.5×10^5	1.8×10^5	1.3×10^5	1.5×10^5	2.5×10^4
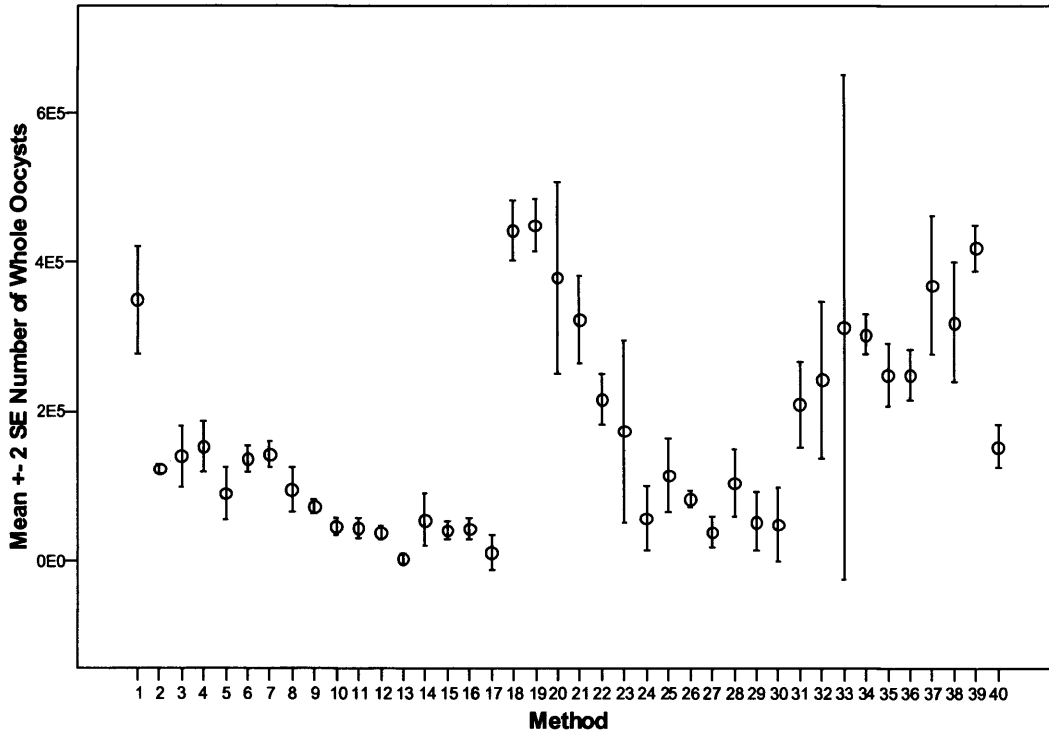
Figure 4.7: The mean oocyst counts for each method (see Table 4.5) with bars representing the standard error of the mean.
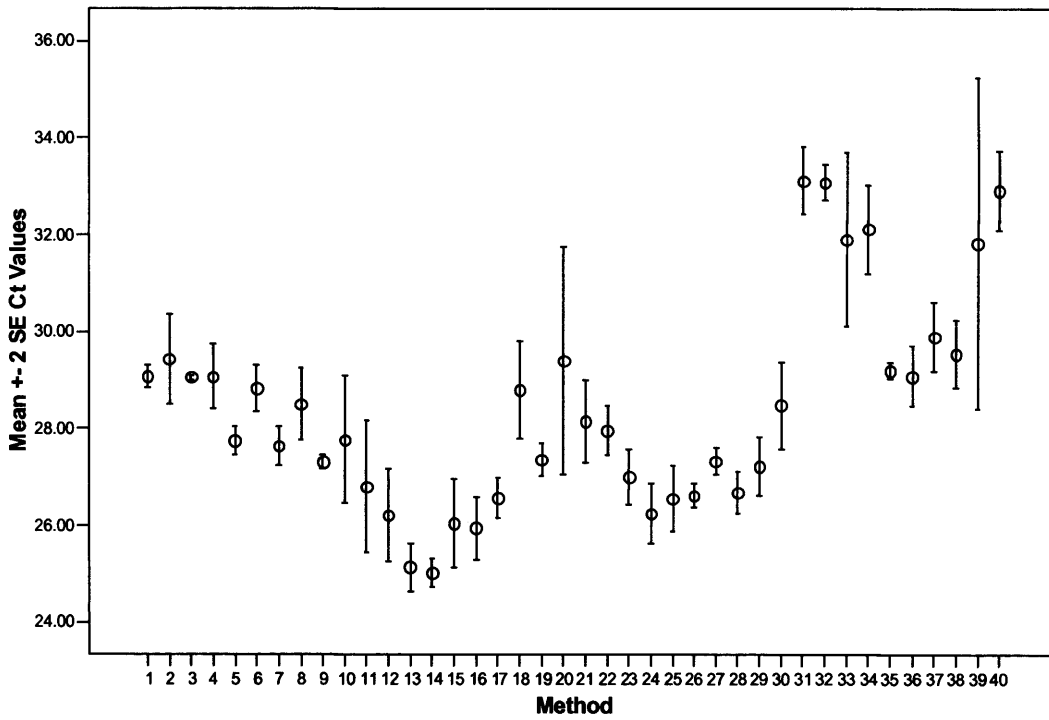
Figure 4.8: The mean Ct values for each method (see Table 4.5) with bars representing the standard error of the mean.

Table 4.10: The Ct values obtained for each replicate using the SYBR Green I PCR following each oocysts disruption treatment

	Method	SYBR Green I PCR Ct Values				
		1	2	3	Mean	S.D.
1.	Control (no treatment)	28.99	28.94	29.29	29.07	0.19
2.	10 min x Prot. K + Buffer AL (37°C)	NA	28.96	29.89	29.43	0.66
3.	30 min x Prot. K + Buffer AL (37°C)	29.05	29.02	29.10	29.06	0.04
4.	60 min x Prot. K + Buffer AL (37°C)	29.46	29.33	28.40	29.06	0.58
5.	16 hrs x Prot. K + Buffer AL (37°C)	27.67	27.53	28.02	27.74	0.25
6.	10 min x Prot. K + Buffer AL (56°C) (PKAL)	29.28	28.75	28.45	28.83	0.42
7.	30 min x Prot. K + Buffer AL (56°C)	27.60	27.30	28.00	27.63	0.35
8.	60 min x Prot. K + Buffer AL (56°C)	28.07	28.20	29.23	28.50	0.64
9.	16 hrs x Prot. K + Buffer AL (56°C)	27.35	27.19	27.42	27.32	0.12
10.	10 min x Prot. K + Buffer ATL (37°C)	26.83	27.43	29.03	27.76	1.14
11.	30 min x Prot. K + Buffer ATL (37°C)	26.83	25.61	27.96	26.80	1.18
12.	60 min x Prot. K + Buffer ATL (37°C)	26.12	27.09	25.44	26.22	0.83
13.	16 hrs x Prot. K + Buffer ATL (37°C)	25.58	25.11	24.72	25.14	0.43
14.	10 min x Prot. K + Buffer ATL (56°C)	24.73	25.20	25.15	25.03	0.26
15.	30 min x Prot. K + Buffer ATL (56°C)	26.57	25.15	26.42	26.05	0.78
16.	60 min x Prot. K + Buffer ATL (56°C)	26.23	25.29	26.31	25.94	0.57
17.	16 hrs x Prot. K + Buffer ATL (56°C)	26.20	26.93	26.55	26.56	0.37
18.	1 x Freeze-Thaw (37°C) + PKAL	28.22	29.81	28.36	28.80	0.88
19.	3 x Freeze-Thaw (37°C) + PKAL	27.59	27.46	27.01	27.35	0.30
20.	5 x Freeze-Thaw (37°C) + PKAL	28.35	28.11	31.74	29.40	2.03
21.	7 x Freeze-Thaw (37°C) + PKAL	27.89	28.99	27.56	28.15	0.75
22.	10 x Freeze-Thaw (37°C) + PKAL	27.71	27.71	28.46	27.96	0.43
23.	1 x Freeze-Thaw (100°C) + PKAL	26.53	27.51	26.95	27.00	0.49
24.	3 x Freeze-Thaw (100°C) + PKAL	25.81	26.08	26.84	26.24	0.53
25.	5 x Freeze-Thaw (100°C) + PKAL	26.19	26.23	27.24	26.55	0.60
26.	7 x Freeze-Thaw (100°C) + PKAL	26.74	26.73	26.37	26.61	0.21
27.	10 x Freeze-Thaw (100°C) + PKAL	27.14	27.59	27.24	27.32	0.24
28.	10 min x Heat (100°C) + PKAL	27.07	26.66	26.30	26.68	0.39
29.	30 min x Heat (100°C) + PKAL	27.16	26.74	27.77	27.22	0.52
30.	60 min x Heat (100°C) + PKAL	28.00	28.05	29.38	28.48	0.78
31.	10 min x Trypsin (37°C) + PKAL	32.82	33.00	33.43	33.08	0.31
32.	30 min x Trypsin (37°C) + PKAL	30.33	31.96	33.43	31.91	1.55
33.	60 min x Trypsin (37°C) + PKAL	31.62	31.70	33.03	32.12	0.79
34.	16 hrs x Trypsin (37°C) + PKAL	29.37	29.08	29.14	29.20	0.15
35.	1 min x Sonication (50/60 Hz, 80W) + PKAL	28.54	29.11	29.60	29.08	0.53
36.	5 min x Sonication (50/60 Hz, 80W) + PKAL	29.79	29.33	30.56	29.89	0.62
37.	10 min x Sonication (50/60 Hz, 80W) + PKAL	30.19	29.01	29.41	29.54	0.60
38.	15 min x Sonication (50/60 Hz, 80W) + PKAL	30.11	NA	33.53	31.82	2.42
39.	20 min x Sonication (50/60 Hz, 80W) + PKAL	33.09	32.13	33.52	32.91	0.71
40.	2 min x Vortexed Zirconia Beads + PKAL	28.99	28.94	29.29	29.07	0.19

NA – No Amplification

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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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Table 4.11: The amplification of *Cryptosporidium* DNA by SSU nested PCR for low numbers of oocysts following the use of selected disruption methods prior to DNA extraction

Method	SSU nested PCR Amplification											
	41 oocysts				5 oocysts				1 oocyst			
	1	2	3	Sensitivity	1	2	3	Sensitivity	1	2	3	Sensitivity
1. 30 min x Prot. K + Buffer AL (56°C)	+	+	+	100%	+	+	+	100%	+	+	-	67%
2. 16 hrs x Prot. K + Buffer AL (56°C)	+	+	+	100%	+	+	+	100%	+	-	-	33%
3. 10 min x Prot. K + Buffer ATL (56°C)	+	+	+	100%	+	+	+	100%	+	+	-	67%
4. 3 x Freeze-Thaw (100°C) + 30 min x Prot. K + Buffer AL (56°C)	+	+	+	100%	+	+	+	100%	+	+	+	100%
5. 3 x Freeze-Thaw (100°C) + 16 hrs x Prot. K + Buffer AL (56°C)	+	+	+	100%	+	+	+	100%	+	+	+	100%
6. 3 x Freeze-Thaw (100°C) + 10 min x Prot. K + Buffer ATL (56°C)	+	+	+	100%	+	+	+	100%	+	+	+	100%
7. 60 min x Heat (100°C) + 10 min x Prot. K + Buffer AL (56°C)	+	+	+	100%	+	-	+	67%	+	-	-	33%
8. 60 min x Heat (100°C) + 10 min x Prot. K + Buffer ATL (56°C)	+	+	+	100%	+	+	+	100%	+	+	-	67%

Table 4.12: The amplification of *Cryptosporidium* DNA by SYBR Green I real-time PCR for low numbers of oocysts following the use of selected disruption methods prior to DNA extraction

Method	SYBR Green I PCR Amplification											
	41 oocysts				5 oocysts				1 oocyst			
	1	2	3	Sensitivity	1	2	3	Sensitivity	1	2	3	Sensitivity
1. 30 min x Prot. K + Buffer AL (56°C)	+	+	+	100%	-	-	-	0%	-	-	-	0%
2. 16 hrs x Prot. K + Buffer AL (56°C)	+	+	+	100%	-	+	+	67%	-	-	-	0%
3. 10 min x Prot. K + Buffer ATL (56°C)	+	+	+	100%	+	-	-	33%	-	-	-	0%
4. 3 x Freeze-Thaw (100°C) + 30 min x Prot. K + Buffer AL (56°C)	+	+	+	100%	+	+	+	100%	+	+	+	100%
5. 3 x Freeze-Thaw (100°C) + 16 hrs x Prot. K + Buffer AL (56°C)	+	+	+	100%	+	+	+	100%	+	+	+	100%
6. 3 x Freeze-Thaw (100°C) + 10 min x Prot. K + Buffer ATL (56°C)	+	+	+	100%	+	+	+	100%	+	-	-	33%
7. 60 min x Heat (100°C) + 10 min x Prot. K + Buffer AL (56°C)	+	+	+	100%	+	+	+	100%	-	-	-	0%
8. 60 min x Heat (100°C) + 10 min x Prot. K + Buffer ATL (56°C)	+	+	+	100%	+	+	+	100%	-	-	-	0%

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

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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 1minute 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 identifed 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 idenification 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

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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 5.1: *Cryptosporidium* study surface water sites

Crypto. Study Site Number	EA Study site number	River	Location	Grid ref	Composite water type
Site 1	EA Site 1	Caldew	Grainsgill Beck	NY328326	Upland
Site 2	EA Site 5	Parkend Beck	Parkend	NY300388	Upland
Site 3	EA Site 3	Caldew	Hesket Newmarket	NY343886	Upland
Site 4	EA Site 10	Caldew	Sebergham	NY358419	Upland
Site 5	EA Site 6	Peel Gill	Skelton Wood End	NY407386	Lowland
Site 6	EA Site 9	Roe Beck	Roebank Bridge	NY393417	Lowland
Site 7	EA Site 11 (d/s of Farm A)	Roe Beck	Gaitsgill	NY387465	Lowland
Site 8	EA Site 12	Caldew	Green Dalston	NY370497	Lowland
Site 9	d/s of Farm C	Glenderamackin*	Mungrisdale	NY366290	Upland
Site 10	d/s of Farm B	Pow Beck	Pow Bank	NY386500	Lowland
Site 11	d/s of Farm D	Caldew	Cummersdale	NY393521	Lowland

d/s: downstream

* The River Glenderamackin is not within the Caldew catchment
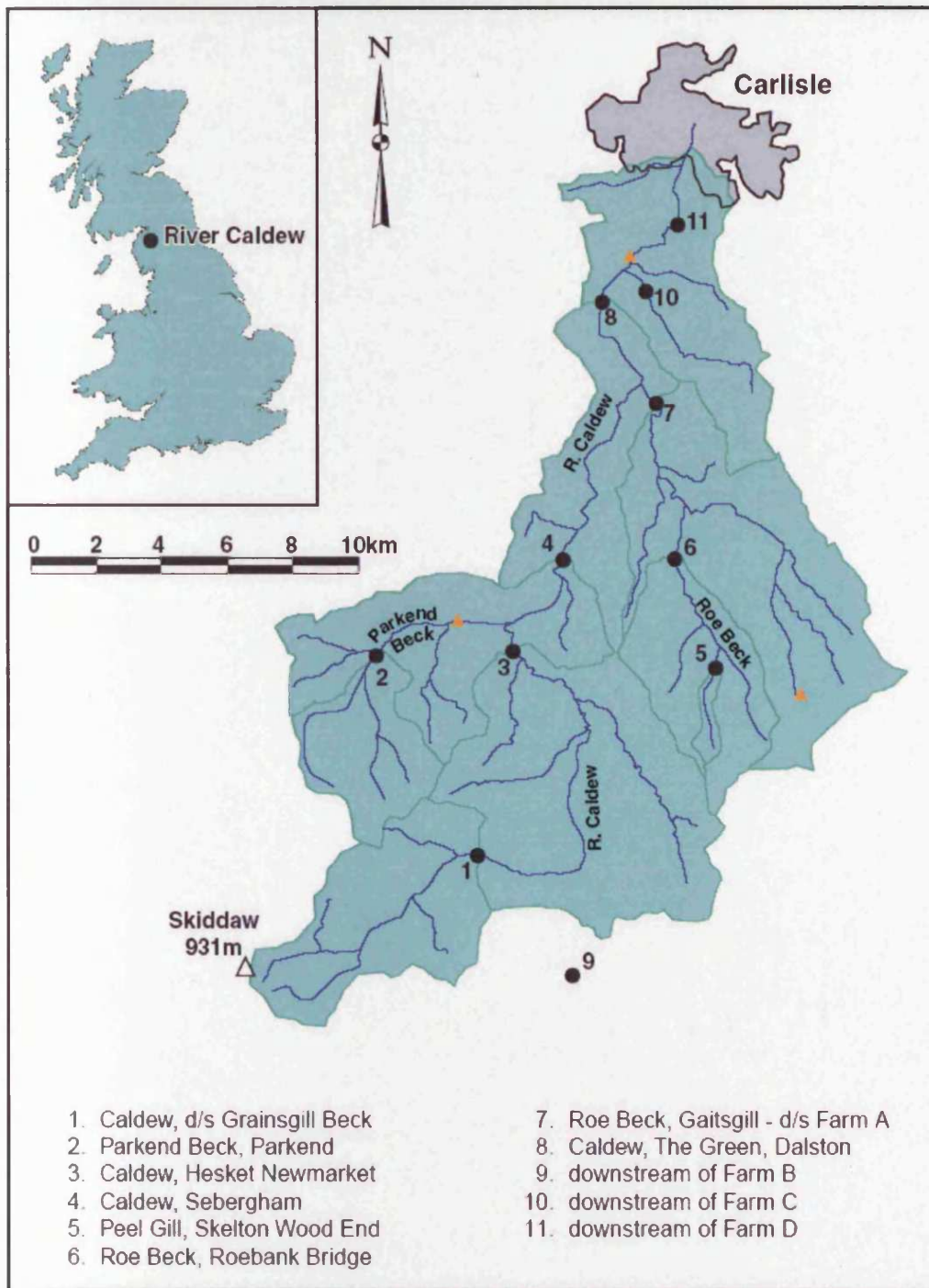
Figure 5.1: Location of the *Cryptosporidium* monitoring points in the River Caldew catchment (GIS map produced by Dr Carl Stapleton).

Legend (within figure):

1. Caldew, d/s Grainsgill Beck
2. Parkend Beck, Parkend
3. Caldew, Hesket Newmarket
4. Caldew, Sebergham
5. Peel Gill, Skelton Wood End
6. Roe Beck, Roebank Bridge
7. Roe Beck, Gaitsgill - d/s Farm A
8. Caldew, The Green, Dalston
9. downstream of Farm B
10. downstream of Farm C
11. downstream of Farm D

●9 *Cryptosporidium* sample site

— Sub-catchment Boundary

▲ Waste water treatment works

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

Farm	Stock status	Farm type	Farm size (ha)
A	Re-stocking	Sheep & dairy cattle	114.5
B	Re-stocking	Dairy cattle	137.0
C	Continually stocked	Sheep (Upland)	171.0 + fell
D	Continually stocked	Dairy cattle	81.0

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

Monthly Number of Cattle
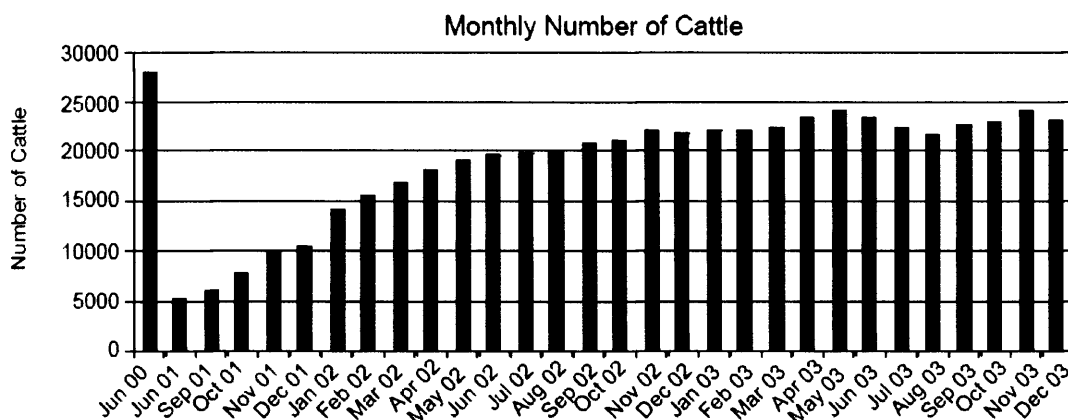
Figure 5.2: Calculated total number of cattle in the Caldew catchment, between June 2000 and December 2003 (Sanders *et al.*, 2004).

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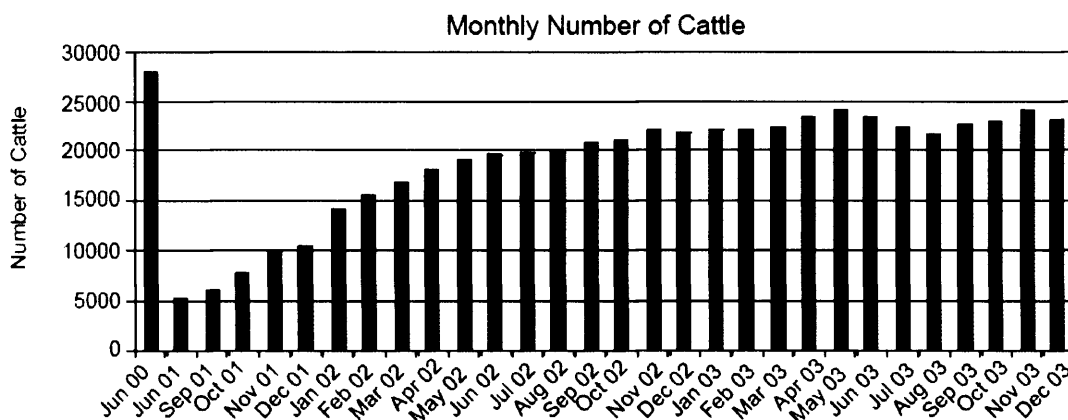
Figure 5.3: Calculated total number of cattle in the Caldew catchment, between June 2000 and December 2003 (Sanders *et al.*, 2004).

(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

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Table 5.3: Surface water sample sites and sample dates

Site Number	EA study site	Composite water type	Phase 1 Base flow	Phase 1 High flow	Phase 2 Base flow	Phase 2 High flow
Site 1	EA Site 1	Upland	16/01/02	17/01/02 23/01/02	26/03/03 02/04/03	01/04/03
Site 2	EA Site 5	Upland	16/01/02	17/01/02 23/01/02	27/03/03 02/04/03	01/04/03
Site 3	EA Site 3	Upland	16/01/02	17/01/02 23/01/02	27/03/03 02/04/03	01/04/03
Site 4	EA Site 10	Upland	19/01/02	17/01/02 23/01/02	27/03/03 02/04/03	01/04/03
Site 5	EA Site 6	Lowland	18/01/02	22/01/02 24/01/02	27/03/03 03/04/03	01/04/03
Site 6	EA Site 9	Lowland	18/01/02	22/01/02 24/01/02	27/03/03 03/04/03	01/04/03
Site 7	EA Site 11 Farm A	Lowland	18/01/02	22/01/02 24/01/02	28/02/03 27/03/03 03/04/03	01/04/03
Site 8	EA Site 12	Lowland	18/01/02	22/01/02 23/01/02	28/03/03 03/04/03	01/04/03
Site 9	Farm C	Upland	01/03/02	- -	26/03/03 02/04/03	01/04/03
Site 10	Farm B	Lowland	01/03/02	- -	28/03/03 03/04/03	01/04/03
Site 11	Farm D	Lowland	26/03/02	- -	28/02/03 28/03/03 03/04/03	01/04/03

Table 5.4: Farm animal sampling dates

Farm	Stock type	Date sampled Phase 1	Date sampled phase 2
A	Sheep	28/02/02	25-27/02/03
	Unweaned lambs	28/02/02	25-27/02/03
	Dairy cows	28/02/02	25/02/03
	Unweaned calves	28/02/02	25-27/02/03 & 27/03/03
B	Dairy cows	28/02/02	28/03/03
	Unweaned calves	28/02/02	28/03/03
C	Sheep	27/02/02	30/04/03
	Lambs	30/04/02 & 01/05/02	30/04/03
D	Dairy cows	26/03/02	25/02/03
	Calves	26/03/02 & 30/04/02	25-27/02/03 & 28/03/03

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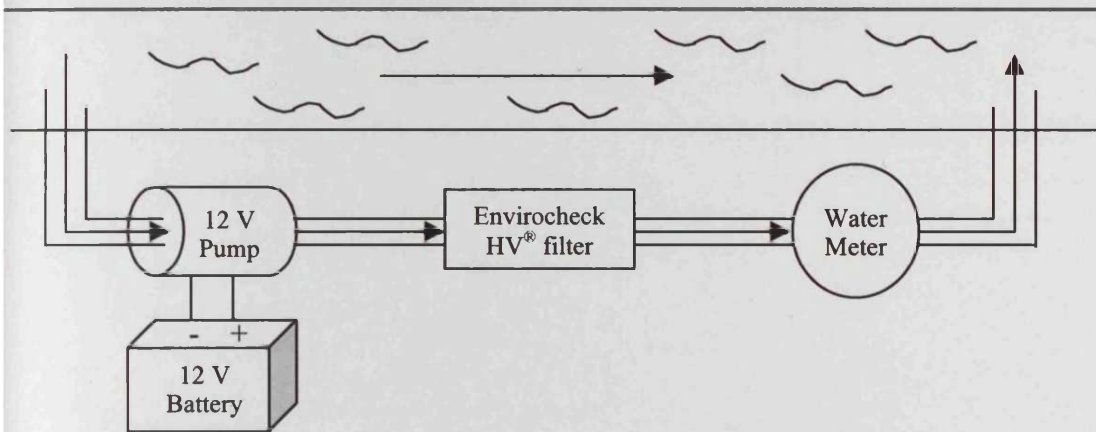
Figure 5.4: A diagrammatic representation of the sampling rig

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 animal to be tested) to estimate prevalence with 95% confidence

Number of animals in herd/flock	Predicted prevalence (worst acceptable prevalence of 0%)			
	40%	20%	10%	5%
10	10	10	9	9
30	28	27	25	21
60	52	48	42	33
80	66	60	51	38
100	79	71	58	42
150	107	93	72	49
200	130	110	82	53
300	165	135	95	59
400	192	152	103	62
500	212	165	108	64

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

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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 *Ssp*I and *Vsp*I to differentiate between the majority of *Cryptosporidium* species (Table 5.6). For samples containing *C. andersoni* or *C. muris* (both have similar *Ssp*I and *Vsp*I patterns), differentiation was undertaken by further digesting the PCR products with *Dde*I (Xiao *et al.*, 2001c). The 20μl digestion mixes contained 10U of restriction enzyme *Ssp*I, *Vsp*I or *Dde*I (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 (AlphaImager, Alpha Innotech).

Table 5.6: The different nested SSU rDNA PCR fragment sizes for each species of *Cryptosporidium* when digested by *Ssp*I, *Vsp*I and *Dde*I. Fragment sizes calculated using GenBank sequences trimmed to the primers and inserted into Restriction Mapper (http://www.restrictionmapper.org)

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

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'-CCTGCTTTAAGCAC TCTAATTTTC-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

CRU Reference	Host	Year Submitted	*Cryptosporidium* Species
W0144	Human	2000	*C. parvum*
W0240	Human	2000	*C. parvum*
W0456	Human	2000	*C. parvum*
W1871	Human	2000	*C. hominis*
W2360	Human	2000	*C. hominis*
W4878	Human	2002	*C. parvum*
W5018	Human	2002	*C. parvum*
W6132	Human	2002	*C. parvum*
W6325	Human	2002	*C. hominis*
W8265	Human	2003	*C. hominis*
W8609	Human	2003	*C. hominis*
A484	Bovine calf	2002	*C. parvum*
A486	Bovine calf	2002	*C. parvum*

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_

Cryptosporidium species	Microsatellite Marker (fragment size in bp)			*Multilocus Fragment Type (MLFT)
	ML1	ML2	gp60	
C. hominis	233	180	371	H1
	240	180	372	H2
	242	180	371	H3
	223	180	371	H4
	234	180	408	H5
	234	180	352	H6
	218	180	371	H7
	219	180	414	H8
	233	180	341	H9
C. parvum	242	230	341	P1
	242	230	338	P2
	228	193	329	P3
	227	195	338	P4
	242	232	341	P5
	242	234	338	P6
	242	232	338	P7
	242	234	341	P8
	242	225	341	P9
	242	228	338	P10
	242	230	332	P11
	242	230	359	P12
	242	230	347	P13
	242	232	356	P14
	242	232	338	P15
	242	232	344	P16
	242	232	347	P17
	242	234	347	P18
	242	236	338	P19
	242	238	341	P20
	243	232	349	P21
	227	193	320	P22
	227	194	327	P23
	227	224	333	P24
	227	197	310	P25
	227	232	341	P26
	227	195	353	P27
	227	193	327	P28
	227	193	330	P29
	227	195	333	P30
	241	238	338	P31
	242	215	344	P32
	242	230	350	P33
	242	207	338	P34
	242	203	338	P35

* 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'-CTAAAAATGGTGGAGAATATTC-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'-CAATG TAAGTTTACTTATGATTAT-3' and the reverse primer 5'-CGACTATAAAGATG AGAGAAG-3' (Caccio *et al.*, 2001). The gp60 fragment (synonymous with gp15) was amplified using the forward primer 5'-GCCGTTCCACTCAGAGGAAC-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

| Site | Stocking phase | Base flow samples (16/1/02, 18-19/4/02) | | High flow samples, Run 1 (17 & 22/1/02) | | High flow samples, Run 2 (23 & 24/1/02) | |
		Volume sampled (litres)	Number of oocysts per 10 litres	Volume sampled (litres)	Number of oocysts per 10 litres	Volume sampled (litres)	Number of oocysts per 10 litres
1	Phase 1	515	0.00	181	0.22	440	0.00
2	Phase 1	504	0.22	96	3.03	238	0.00
3	Phase 1	309	0.00	101	1.99	166	0.60
4	Phase 1	269	0.37	33	1.53	157	0.00
5	Phase 1	241	1.66	69	20.29	68	4.41
6	Phase 1	176	6.24	53	5.71	60	1.67
7	Phase 1	147	0.00	51	5.93	60	0.00
8	Phase 1	174	0.57	83	3.64	81	0.00
9	Phase 1	80	0.00	Not sampled		Not sampled	
10	Phase 1	70	0.00	Not sampled		Not sampled	
11	Phase 1	100	0.00	Not sampled		Not sampled	
Mean	Phase 1	235 (n=11)	0.82 (5 +ve)	Volume sampled (litres): 121 (n=16) Number of oocysts per 10 litres: 3.06 (11+ve)			
Supplementary samples during phase 1							
Downstream 6 (land drain)		Not sampled		20	0.00	10	0.00
Farm D (small beck)		10	0.00	Not sampled		Not sampled	

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

Site	Stocking phase	Base flow samples Run 1 (26-28/3/03)		Base flow samples, Run 2 (2-3/4/03)		High flow samples, (1/4/03)	
		Volume sampled (litres)	Number of oocysts per 10 litres	Volume sampled (litres)	Number of oocysts per 10 litres	Volume sampled (litres)	Number of oocysts per 10 litres
1	Phase 2	204	0.00	200	0.00	118	0.00
2	Phase 2	271	0.00	180	0.00	160	0.00
3	Phase 2	250	0.00	172	0.00	90	0.00
4	Phase 2	201	0.00	99	0.00	113	3.54
5	Phase 2	200	0.00	100	0.00	66	1.52
6	Phase 2	208	0.00	193	2.07	125	0.00
7	Phase 2	207	0.00	176	5.68	87	16.09
8	Phase 2	219	0.00	132	1.52	87	6.90
9	Phase 2	182	0.00	103	0.00	45	0.00
10	Phase 2	186	1.08	150	0.00	29	10.34
11	Phase 2	276	0.00	166	0.60	93	4.30
Mean	Phase 2	Volume sampled (litres): 185 (n=22) Number of oocysts per 10 litres:0.49 (5 +ve)				92 (n=11)	3.88 (6+ve)

Supplementary samples during phase 2						
7 (February)	100	49.00		Not sampled		Not sampled
11 (February)	100	9.00		Not sampled		Not sampled
Farm A land drain a (March)	Not sampled		1	4000.00		Not sampled
Farm A land drain b (March)	Not sampled		10	0.00		Not sampled
Farm A land drain c (March)	Not sampled		10	0.00		Not sampled

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%), χ^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%), χ^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%, χ^2=1.41, df=1, p=0.26; Phase 2 = 23%-55%, χ^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

Crypto Site No.	EA Site No.	Flow condition	Phase 1 discharge ($m^3 s^{-1}$)	Phase 2 discharge ($m^3 s^{-1}$)
Site 1	EA Site 1	Base flow	0.14	0.27
			-	0.30
		High flow	0.67	0.40
			1.10	-
Site 2	EA Site 5	Base flow	0.15	0.13
			-	0.13
		High flow	0.54	0.17
			0.99	-
Site 3	EA Site 3	Base flow	0.42	0.70
			-	0.69
		High flow	1.07	1.00
			2.54	-
Site 4	EA Site 10	Base flow	3.23	1.27
			-	1.18
		High flow	8.06	1.91
			7.98	-
Site 5	EA Site 6	Base flow	0.01	0.01
			-	0.01
		High flow	0.13	0.02
			0.09	-
Site 6	EA Site 9	Base flow	0.41	0.06
			-	0.05
		High flow	0.83	0.11
			0.94	-
Site 7	EA Site 11 Farm A	Base flow	1.33	0.19
			-	0.17
		High flow	2.62	0.38
			3.07	-
Site 8	EA Site 12	Base flow	7.16	1.48
			-	1.65
		High flow	19.07	3.14
			19.15	-

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,

(a) Phase 1

Cryptosporidium oocysts per 10 litre eq.

25

20

15

10

5

0

Base Flow (16 & 18-19/1/02)
High Flow (17 & 22/1/02)
High Flow (23-24/1/02)

Base flow only sampled 1/3/02
0 oocysts per 10 litres

Base flow only sampled 1/3/02
0 oocysts per 10 litres

Base flow only sampled 26/3/02
0 oocysts per 10 litres

1 2 3 4 9 5 6 7 8 10 11

Upland ← → ← Lowland →

(b) Phase 2

Cryptosporidium oocysts per 10 litre eq.

18
16
14
12
10
8
6
4
2
0

Base Flow (26-28/3/03)
Base Flow (2-3/4/03)
High Flow (1/4/03)

1 2 3 4 9 5 6 7 8 10 11

Upland ← → ← Lowland →

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.

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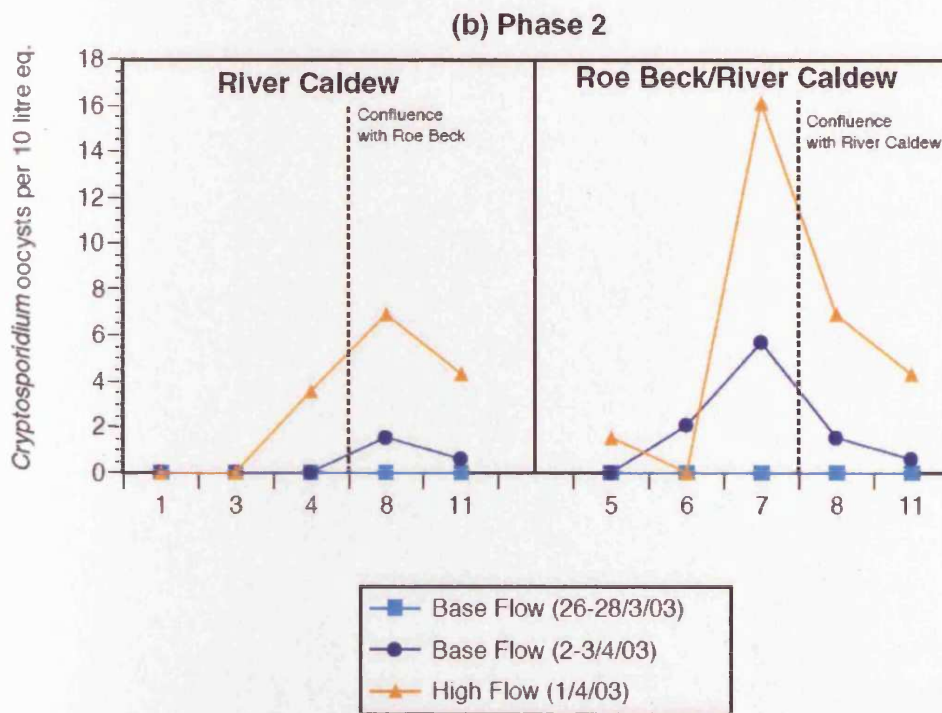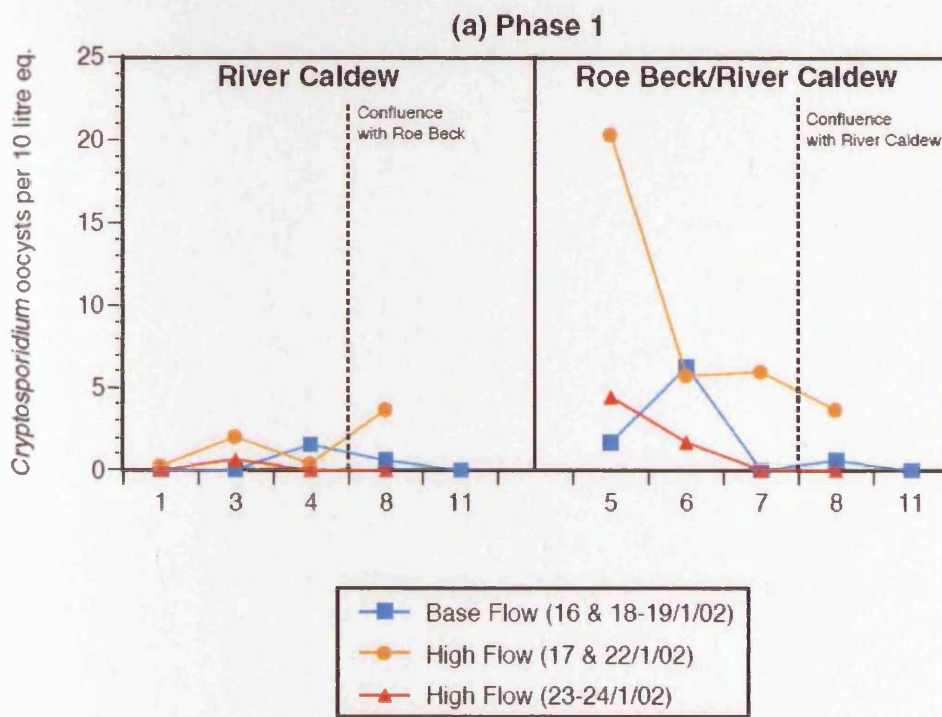
Figure 5.8: *Cryptosporidium* **oocyst concentration along transects following the River Caldew (i.e. sites 1, 3, 4, 8 and 11) and Roe Beck/Lower Caldew (i.e. sites 5-8 and 11) during base flows and high flows: (a) Phase 1; and (b) Phase 2.**

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 (χ^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 (χ^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 (χ^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 (χ^2=0.16, df=1, p=0.69).

Table 5.12: Sample prevalence of *Cryptosporidium* in farmed animals

Farm	Animal type	Phase 1		Phase 2	
		Population size	Number of positive samples / total number of samples	Population size	Number of positive samples / total number of samples
A Re-stocking	Dairy cow	120	0/58	180	0/57
	Calf	5	0/5	9	15/34
	Sheep	35	0/32	36	0/37
	Lamb	45	0/1	32	1/19
B Re-stocking	Dairy cow	70	1/43	90	0/62
	Calf	5	0/2	11	0/3
C Continually stocked	Sheep	574	0/180	600	3/131
	Lamb	320	1/135	334	0/197
D Continually stocked	Cattle	148	0/55	120	0/63
	Calf	46	19/59	31	22/99

Table 5.13: Sample prevalence of *Cryptosporidium* in wild animals

Farm type	Animal type	Phase 1 Number of positive samples / total number of samples	Phase 2 Number of positive samples / total number of samples
Re-stocking	Fox	2/5	0/5
	Pheasant	0/10	0/0
	Roe Deer	0/29	0/11
	Badger	0/3	0/6
Continually stocked	Fox	2/17	0/3
	Roe Deer	½	1/3
	Badger	0/5	0/5
	Rabbit	0/3	0/0
Close to water site 6	Fox	Not sampled	0/1
	Pheasant	Not sampled	0/2
	Roe Deer	Not sampled	1/1
	Badger	Not sampled	0/4

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

| Sample site | Number of oocysts per 10 litre equivalent of water sampled; species ID | | |
	Pre-event base flow	High flow samples, Run 1 (17 & 22/1/02)	High flow samples, Run 2 (23 & 24/1/02)
1	0.00; PCR neg	0.22; PCR neg	0.00; PCR neg
2	0.22; PCR neg	3.03; novel genotype	0.00; PCR neg
3	0.00; PCR neg	1.99; PCR neg	0.60; PCR neg
4	0.37; *C. andersoni*	1.53; *C. andersoni*	0.00; PCR neg
5	1.66; *C. andersoni*	20.29; *C. parvum*	4.41; *C. andersoni*
6	6.24; PCR neg	5.71; PCR neg	1.67; PCR neg
7	0.00; PCR neg	5.93; PCR neg	0.00; PCR neg
8	0.57; PCR neg	3.64; PCR. neg	0.00; *C. andersoni*
9	0.00; PCR neg	Not sampled	Not sampled
10	0.00; PCR neg	Not sampled	Not sampled
11	0.00; PCR neg	Not sampled	Not sampled
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
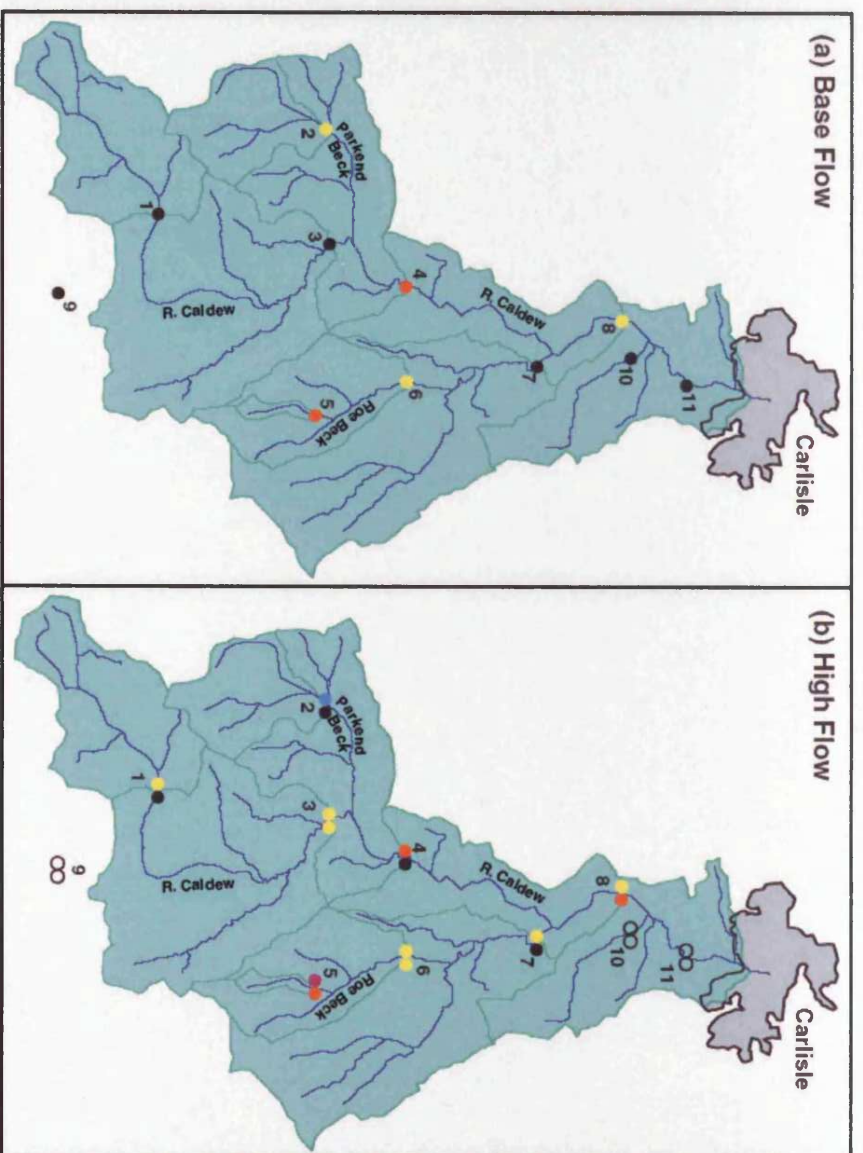
Figure 5.9: Results of *Cryptosporidium* species identification in surface waters during Phase 1: (a) Base flows; and (b) High flows.

NB each circle represents one sample (i.e. two high flow samples from different days analysed)
(GIS map produced by Dr Carl Stapleton)

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

| Sample site | Number of oocysts per 10 litre equivalent of water sampled; species ID | | |
	Pre-event base flow	High flow	Post-event base flow
1	0.00; PCR neg	0.00; PCR neg	0.00; PCR neg
2	0.00; PCR neg	0.00; PCR neg	0.00; PCR neg
3	0.00; PCR neg	0.00; PCR neg	0.00; PCR neg
4	0.00; PCR neg	3.54; *C. andersoni*	0.00; *C. andersoni*
5	0.00; PCR neg	1.52; PCR neg	0.00; PCR neg
6	0.00; PCR neg	0.00; PCR neg	2.07; PCR neg
7	0.00; PCR neg	16.09; *C. parvum*	5.68; PCR neg
8	0.00; PCR neg	6.90; *C. parvum*	1.52; PCR neg
9	0.00; PCR neg	0.00; PCR neg	0.00; PCR neg
10	1.08; *C. andersoni*	10.34; *C. andersoni*	0.00; *C. andersoni*
11	0.00; PCR neg	4.30; PCR neg	0.60; PCR neg
Supplementary samples phase 2			
Site 7 (February)	49.00; *C. parvum*	-	-
Site 11 (February)	9.00; PCR neg	-	-
Farm A land drain a (March)	-	-	4000.00; *C. parvum*
Farm A land drain b (March)	-	-	0.00; PCR neg
Farm A land drain c (March)	-	-	0.00; PCR neg
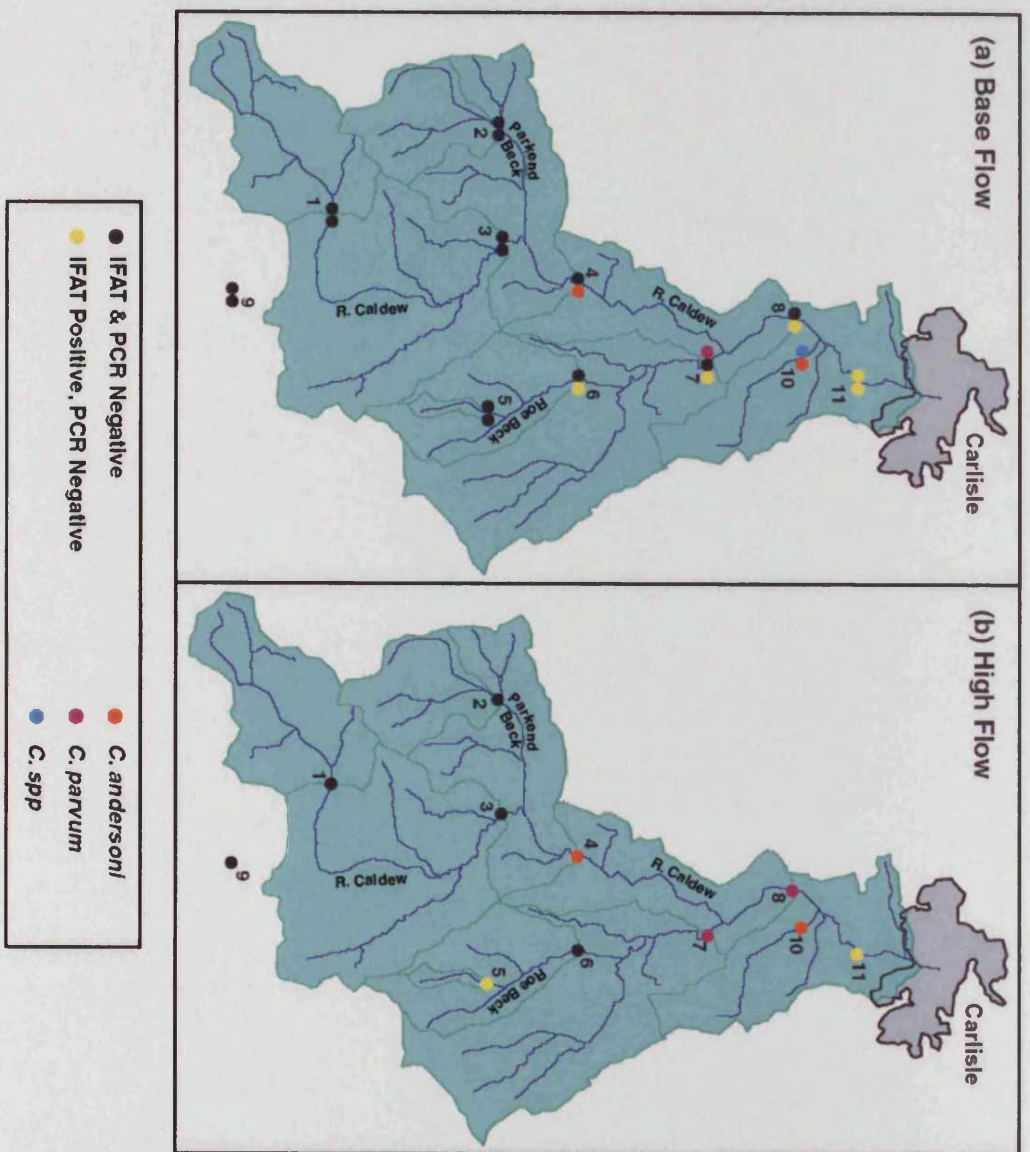
Figure 5.10: Results of *Cryptosporidium* species identification in surface waters during Phase 2: (a) Base flows; and (b) High flows.

NB each circle represents one sample (i.e. two base flow samples from different days analysed with the exception of site 7, which had an extra sample collected in February (positive for *C. parvum*)) (GIS map produced by Dr Carl Stapleton)
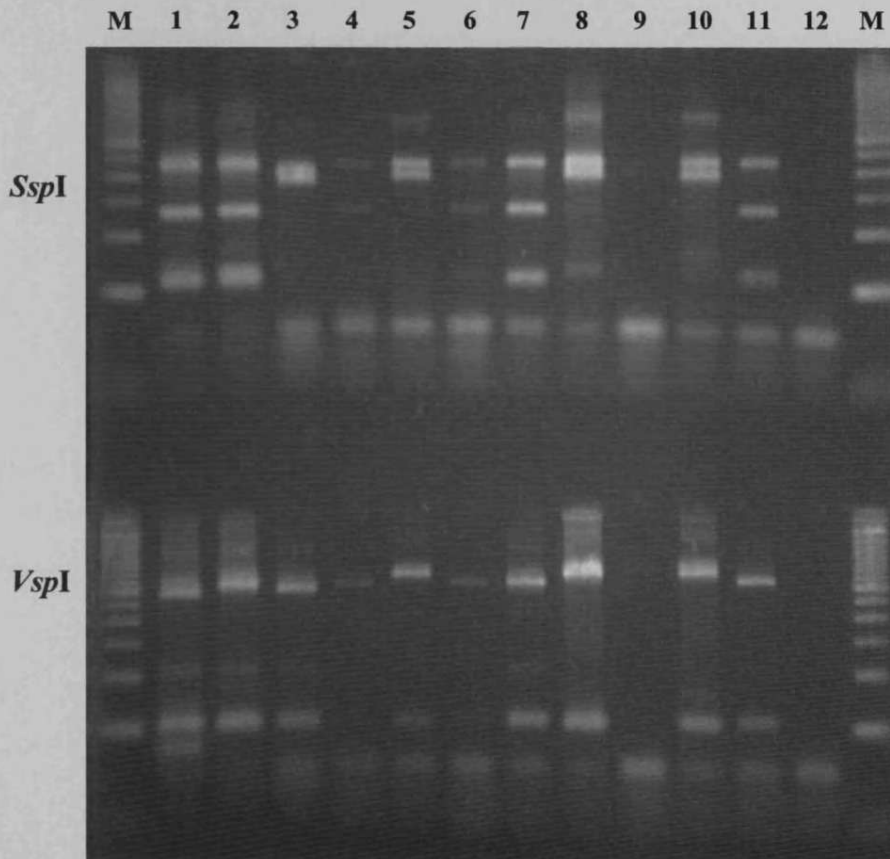
Figure 5.11: An agarose gel showing how the sample in lane 3 (which was later confirmed as _C. andersoni_ by sequence analysis) produced slightly smaller fragments than the other _C. andersoni_ isolates in lanes 5, 8, 9 and 10. Lane M = 100bp DNA ladder; Lane 1 = _C. hominis_; Lanes 2, 4, 6, 7, 11 = _C. parvum_; Lane 12 = negative control.

5.3.2.b – 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

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

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

Farm type	Animal type	Phase 1 Number of positive samples / total number of samples	Phase 2 Number of positive samples / total number of samples
Re-stocking	Fox	2/5 1x *C. parvum* 1x PCR neg	0/5
	Pheasant	0/10	0/0
	Roe Deer	0/29	0/11
	Badger	0/3	0/6
Continually stocked	Fox	2/17 1 x *C. parvum* 1 x PCR neg	0/3
	Roe Deer	1 /2 PCR neg	1/3 PCR neg
	Badger	0/5	0/5
	Rabbit	0/3	0/0
Close to water site 6	Fox	Not sampled	0/1
	Pheasant	Not sampled	0/2
	Roe Deer	Not sampled	1/1 *C. parvum*
	Badger	Not sampled	0/4

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

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

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 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

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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 5.19: *Cryptosporidium parvum* **multilocus fragment types in livestock**

Farm type	Animal type	Phase 1	Phase 2
Re-stocking	Calf	-	10 x *C. parvum* MLFT 5 4 x *C. parvum* NT
Continually stocked	Sheep	-	1 x *C. parvum* NT
	Lamb	1 x *C. parvum* MLFT 7	-
	Calf	16 x *C. parvum* NT 1 x novel genotype	15 x *C. parvum* MLFT 13 5 *C. parvum* NT

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 5.20: *Cryptosporidium parvum* multilocus fragment types in wildlife

Farm type	Animal type	Phase 1	Phase 2
Re-stocking	Fox	1x *C. parvum* MLFT 32	-
Continually stocked	Fox	1 x *C. parvum* NT	-
Close to water site 6	Roe Deer	-	*C. parvum* NT

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

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

* 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; Bouchier, 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*.

Legend (map):
- 1. Caldew, d/s Grainsgill Beck
- 2. Caldew, Mosedale Bridge
- 3. Caldew, Hesket Newmarket
- 4. Cald Beck, Caldbeck Village
- 5. Parkend Beck, Parkend
- 6. Peel Gill, Skelton Wood End
- 7. Roe Beck, Crown Point
- 8. River Ive, Low Braithwaite Bridge
- 9. Roe Beck, Roebank Bridge
- 10. Caldew, Sebergham
- 11. Roe Beck, Gaitsgill
- 12. Caldew, Green, Dalston
- 13. Caldew, Holmehead
- 14. R. Glenderamakin, Mungrisdale
- 15. Carrock Beck, Nr Calebreck

Legend (symbols):
- •9 Sample site
- ▪ Cattle and sheep (LFA)
- ▲ Cattle and sheep (Lowland)
- ● Cereal
- ○ Dairy
- ■ Horticulture
- △ Mixed
- ⬡ Pig and Poultry

Figure 5.12: Farm types of commercial sized enterprises in the River Caldew catchment (Sanders *et al.*, 2004)

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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, QIAamp® 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 5.22: Summary of manure applications made to agricultural land in the whole of the Caldew catchment in the period June 2000 to December 2003, including the effects of culling, re-stocking and the seasonality of animal numbers about the June census figures. Applications are expressed as tonnes (t), by livestock and manure type. (Sanders et al., 2004)

Stock:	Cattle				Sheep			Poultry		Pig			
Type:	Slurry		FYM		Voided			Litter		Slurry		FYM	
Land:	Grass	Arable	Grass	Arable	Grass	Grass	Rough	Grass	Arable	Grass	Arable	Grass	Arable
Jun 00	5092	26	1950	65	24424	4427	3745	70	1	371	3	76	1
Jun 01	981	5	376	12	4704	303	257	70	1	371	3	76	1
Sep 01	633	136	1628	90	5324	270	228	132	30	301	17	127	17
Oct 01	815	174	2096	115	4420	276	233	130	30	301	17	127	17
Nov 01	6280	41	1849	16	1548	319	270	41	3	301	4	122	2
Dec 01	6686	43	1968	17	65	689	583	41	3	301	4	122	2
Jan 02	8962	57	2613	22	86	1299	1099	41	3	301	4	124	2
Feb 02	17310	172	3135	46	78	1300	1100	168	5	301	8	183	1
Mar 02	18799	189	2404	50	102	1546	1308	168	5	301	8	183	1
Apr 02	19966	201	3616	53	3498	1515	1282	167	5	400	8	183	1
May 02	3477	18	1337	44	14770	1625	1375	70	1	375	3	76	1
Jun 02	3588	18	1374	45	17209	1561	1321	70	1	371	3	76	1
Jul 02	3634	18	1429	47	19557	1609	1362	70	1	371	3	76	1
Aug 02	2080	448	5349	294	19623	1640	1387	132	30	301	17	127	17
Sep 02	2149	462	5527	304	18076	1943	1644	132	30	301	17	127	17
Oct 02	2180	467	5605	309	11820	2385	2018	130	30	301	17	127	17
Nov 02	13804	89	4064	34	3402	2424	2051	41	3	301	4	122	2
Dec 02	13633	88	4013	34	132	2516	2129	41	3	301	4	122	2
Jan 03	13945	89	4066	34	133	2485	2103	41	3	301	4	124	2
Feb 03	24536	244	4443	65	111	2193	1856	168	5	400	8	183	1
Mar 03	24826	250	4496	66	135	2491	2108	168	5	400	8	183	1
Apr 03	25858	260	4683	68	4531	2644	2237	167	5	400	8	183	1
May 03	4392	22	1689	56	18656	2951	2497	70	1	375	3	76	1
Jun 03	4247	22	1626	54	20372	2739	2317	70	1	371	3	76	1
Jul 03	4072	21	1602	53	21916	2697	2282	70	1	371	3	76	1
Aug 03	2228	479	5729	315	21016	2537	2147	132	30	301	17	127	17
Sep 03	2334	502	6003	331	19636	2491	2107	132	30	301	17	127	17
Oct 03	2365	506	6082	335	12825	2541	2150	130	30	301	17	127	17
Nov 03	15024	97	4423	37	3703	2366	2002	41	3	301	4	122	2
Dec 03	14478	94	4262	36	140	2457	2079	41	3	301	4	122	2

Caldew Catchment Land Cover (ha): Arable = 1448.97; Grass = 12973.28; Rough = 8362.61

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 (χ^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 characterised, 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 Caldew 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, Mallon *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 possbile 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,

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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 Caldew 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 on-going, 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 6.1: Details of *Cryptosporidium* outbreaks identified to the Cryptosporidium Reference Unit between 2000 and 2003 (CRU data)

CDSC Outbreak Ref.	Region	Year	Month	Vehicle / route	Cases ill (Laboratory confirmed)	Faecal samples received by CRU (Typed)	Species by COWP	Further Sampling
00/219	North West	2000	March	††† Public drinking water	58 (58)	48 (45)	C. parvum	-
00/406	Trent	2000	May/June	††† Swimming pool	41 (41)	34 (34)	C. parvum	-
00/440	South West	2000	May/June	† Private water supply / Farmland	8 (3)	3 (3)	C. parvum	-
-	London	2000	July/Aug	Swimming pool	9 (?)	8 (7)	6 x C. hominis & 1 x C. parvum	-
00/723	London	2000	Aug/Sept	† Swimming pool	3 (3)	1 (1)	C. hominis	-
-	Yorkshire & Humberside	2000	Sept	Nursery	9 (6)	0	-	-
00/656	London	2000	Sept	†† Swimming pool	10 (10)	10 (10)	C. hominis	-
00/1022	Eastern	2000	Sept	†† Swimming pool	7 (7)	0	-	-
00/870	South West	2000	Sept/Oct	†† Swimming pool	12 (7)	1 (1)	C. parvum	-
00/1023	Trent	2000	Sept/Oct	†† Swimming pool	9 (9)	0	-	-
00/806	London	2000	Oct	Nursery	?	14 (14)	C. hominis	-
00/972	South West	2000	Oct/Nov	†† Swimming pool	5 (5)	6*	5 x C. hominis & 1 x C. parvum	-
01/136	South East	2001	Feb/March	††† Swimming pool	5 (4)	0	-	-
01/347	South East	2001	June	† Swimming pool	152** (8)	5 (5)	C. hominis	-
01/440	South West	2001	August	† Environmental contact	14 (5)	5 (5)	3 x C. parvum & 2 x C. hominis	-
01/442	South East	2001	Sept	Nursery	?	8 (8)	C. hominis	-
-	Wales	2001	Sept	Swimming pool	?	0	-	-
01/528	South West	2001	Oct/Nov	† Swimming pool	3	3 (3)	C. hominis	-
02/018	Yorkshire & Humberside	2002	March	† College PWS	50*** (1)	1 (1)	C. hominis	-
02/302	North West	2002	April	†† Leisure Centre	4 (2)	0	-	-
-	Wales	2002	June/July	PWS/Farm/childminder	4	4	C. parvum	-
02/399	London	2002	July	Unknown	6	0	-	-
02/1547	South East	2002	Nov	† Public drinking water	21 (21)	20 (20)	C. hominis	-
-	South West	2002	Nov	Unknown	?	6 (4)	C. hominis	-

EHO No.	Region	Year	Month	Setting	Cases (confirmed)	Confirmed	Species	Notes
-	South East	2002	Dec	Unknown	?	8 (7)	C. hominis	-
02/1701	South East	2002	Nov/Dec	††† Public drinking water	31 (31)	15 (15)	C. hominis	-
-	Yorkshire & Humberside	2002	Nov	Nursery	9 (9)	9 (8)	C. hominis	-
03/121	South East	2003	Feb	†† Swimming pool	20 (20)	4 (4) +1 contact	4 x C. hominis & 1 x C. parvum	-
-	Eastern	2003	March	Open farm general public	7	2 (2)	C. parvum	[1 & 2] C. parvum & cervine
03/197	North West	2003	March	Open farm school visit	17 (6)	6 (6)	C. parvum	[2] C. parvum
03/220	Yorkshire & Humberside	2003	Jan-April	††† Swimming pool	66 (48)	21 (21)****	C. hominis	[3] C. hominis
-	Wales	2003	April	Open farm school visit	36	11 (10)	C. parvum	[2] C. parvum
03/411	West	2003	August	†† Splash zone	122 (35)	32 (32)	31 x C. hominis & 1 x C. meleagridis	[2] C. parvum
-	Midlands	2003	August/September	Various	?	0	-	-
03/400	South West	2003	August	† Swimming pool	22 (1)	0	-	-
03/401	South West	2003	August/September	†† Splash zone / Petting zoo	52 (?)	31 (31)	29 x C. parvum & 2 x C. hominis	[2 & 3] C. parvum
03/409	South West	2003	August/September	††† Swimming pool	17 (17)	0	-	-
03/410	South West	2003	August	††† Fountain	4 (4)	0	-	-
-	South West	2003		Swimming pool	?	0	-	-
-	South West	2003		Swimming pool	?	0	-	-
-	South West	2003		Paddling pool	?	0	-	-
-	South West	2003		Open farm	?	0	-	-
03/438	South West	2003		Nursery	?	0	-	-

*EHO numbers do not reconcile with CDSC's.
**Concurrent community outbreak of Norovirus may account for a proportion of cases
***Diarrhoea and Vomiting outbreak with 1 Cryptosporidium and 1 Campylobactor confirmed
****21 samples from 19 patients
† - Possible; †† - Probable; ††† - Strong = Strength of association (Anon, 1996)

[1] Barn effluent samples
[2] Farm animal samples
[3] Water samples
PWS – Private water supply
? – Unknown

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 anthroponotic 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

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 occurence 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

Abu Al-Soud, W. and Radstrom, P. (1998) Capacity of nine thermostable DNA
polymerases to mediate DNA amplification in the presence of PCR-inhibiting
samples. *Applied and Environmental Microbiology* **64**: 3748-3753.

Aiello, A.E., Xiao, L., Limor, J.R., Liu, C., Abrahamsen, M.S. and Lal, A.A. (1999)
Microsatellite analysis of the human and bovine genotypes of
Cryptosporidium parvum. Journal of Eukaryotic Microbiology **46 Suppl:** 46S-
47S.

Alles, A.J., Waldron, M.A., Sierra, L.S. and Mattia, A.R. (1995) Prospective
comparison of direct immunofluorescence and conventional staining methods
for detection of *Giardia* and *Cryptosporidium* spp. in human fecal specimens.
Journal of Clinical Microbiology **33:** 1632-1634.

Alvarez-Pellitero, P. and Sitja-Bobadilla, A. (2002) *Cryptosporidium molnari* n. sp.
(Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus
aurata* L. and *Dicentrarchus labrax* L. *International Journal for Parasitology*
32: 1007-1021.

Alvarez-Pellitero, P., Quiroga, M.I., Sitja-Bobadilla, A., Redondo, M.J., Palenzuela,
O., Padros, F., Vazquez, S. and Nieto, J.M. (2004) *Cryptosporidium
scophthalmi* n. sp. (Apicomplexa: Cryptosporidiidae) from cultured turbot
Scophthalmus maximus. Light and electron microscope description and
histopathological study. *Diseases of Aquatic Organisms* **62:** 133-145.

Alves, M., Matos, O. and Antunes, F. (2001a) Multilocus PCR-RFLP analysis of
Cryptosporidium isolates from HIV-infected patients from Portugal. *Annals of
Tropical Medicine and Parasitology* **95:** 627-632.

Alves, M., Matos, O., Pereira, D.F., I, Delgado, E., Lourenco, A.M. and Antunes, F.
(2001b) Multilocus genotyping of *Cryptosporidium* isolates from human HIV-
infected and animal hosts. *Journal of Eukaryotic Microbiology* **48 Suppl:**
17S-18S.

Alves, M., Matos, O. and Antunes, F. (2003a) Microsatellite analysis of
Cryptosporidium hominis and C. parvum in Portugal: a preliminary study.
Journal of Eukaryotic Microbiology **50 Suppl:** S29-S30.

Alves, M., Xiao, L., Sulaiman, I., Lal, A.A., Matos, O. and Antunes, F. (2003b)
Subgenotype analysis of Cryptosporidium isolates from humans, cattle, and
zoo ruminants in Portugal. Journal of Clinical Microbiology **41:** 2744-2747.

Amadi, B., Mwiya, M., Musuku, J., Watuka, A., Sianongo, S., Ayoub, A. and Kelly,
P. (2002) Effect of nitazoxanide on morbidity and mortality in Zambian
children with cryptosporidiosis: a randomised controlled trial. Lancet **360:**
1375-1380.

Amar, C.F., Dear, P.H. and McLauchlin, J. (2004) Detection and identification by real
time PCR/RFLP analyses of Cryptosporidium species from human faeces.
Letters in Applied Microbiology **38:** 217-222.

Anderson, B.C. (1991) Prevalence of Cryptosporidium muris-like oocysts among
cattle populations of the United States: preliminary report. Journal of
Protozoology **38:** 14S-15S.

Anderson, B.C. (1998) Cryptosporidiosis in bovine and human health. Journal of
Dairy Science **81:** 3036-3041.

Anon (1982a) Cryptosporidiosis: assessment of chemotherapy of males with acquired
immune deficiency syndrome (AIDS). Morbidity and Mortality Weekly Report
31: 589-592.

Anon (1996) Strength of association between human illness and water: revised
definitions for use in outbreak investigations. Communicable Disease Report
Weekly **6:** 65.

Anon (2002a) From the Centers for Disease Control and Prevention. Manufacturer's
recall of rapid assay kits based on false positive Cryptosporidium antigen
tests--Wisconsin, 2001-2002. Journal of American Medical Association **287:**
1798

Anon (2002b) The development of a national collection for oocysts of
Cryptosporidium. Final report to DEFRA: Drinking Water Inspectorate,
Foundation for Water Research, Marlow, Bucks, UK.

Anon (2003) *Molecular fingerprinting of Cryptosporidium oocysts isolated during regulatory monitoring.* Final report to DEFRA: Drinking Water Inspectorate, Foundation for Water Research, Marlow, Bucks, UK.

Anon (2004) Manufacturer's recall of rapid cartridge assay kits on the basis of false-positive *Cryptosporidium* antigen tests--Colorado, 2004. *Morbidity and Mortality Weekly Report* **53:** 198

Anon (2005) *Investigation of Cryptosporidium clinical isolates and analysis with epidemiological data.* Final report to DEFRA: Drinking Water Inspectorate, Foundation for Water Research, Marlow, Bucks, UK.

Anusz, K.Z., Mason, P.H., Riggs, M.W. and Perryman, L.E. (1990) Detection of *Cryptosporidium parvum* oocysts in bovine feces by monoclonal antibody capture enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology* **28:** 2770-2774.

Arrowood, M.J. and Sterling, C.R. (1989) Comparison of conventional staining methods and monoclonal antibody-based methods for *Cryptosporidium* oocyst detection. *Journal of Clinical Microbiology* **27:** 1490-1495.

Arrowood, M.J. (1997) Diagnosis. In: *Cryptosporidium and Cryptosporidiosis*, (ed. Fayer, R.), CRC Press, Boca Raton, Florida. pp. 43-64.

Australian Government (2004) *National Water Quality Management Strategy: Australian Drinking Water Guidelines.* National Health and Medical Reseach Council & Natural Resource Management Ministerial Council, Australia.

Awad-El-Kariem, F.M., Warhurst, D.C. and McDonald, V. (1994) Detection and species identification of *Cryptosporidium* oocysts using a system based on PCR and endonuclease restriction. *Parasitology* **109:**19-22.

Awad-El-Kariem, F.M., Robinson, H.A., Dyson, D.A., Evans, D., Wright, S., Fox, M.T. and McDonald, V. (1995) Differentiation between human and animal strains of *Cryptosporidium parvum* using isoenzyme typing. *Parasitology* **110:** 129-132.

Badenoch, J. (1990) *Cryptosporidium in water supplies.* Report of the Group of Experts; Department of the Environment, Department of Health, London, UK. HMSO.

Badenoch, J. (1995) *Cryptosporidium in water supplies.* Second Report of the Group
of Experts; Department of the Environment, Department of Health, London,
UK. HMSO.

Balatbat, A.B., Jordan, G.W., Tang, Y.J. and Silva, J., Jr. (1996) Detection of
Cryptosporidium parvum DNA in human feces by nested PCR. *Journal of
Clinical Microbiology* **34:** 1769-1772.

Bang, P. and Dahlstrom, P. (1974) *Collins Guide to Animal Tracks and Signs,* Collins,
London.

Barta, J.R., Martin, D.S., Liberator, P.A., Dashkevicz, M., Anderson, J.W., Feighner,
S.D., Elbrecht, A., Perkins-Barrow, A., Jenkins, M.C., Danforth, H.D., Ruff,
M.D. and Profous-Juchelka, H. (1997) Phylogenetic relationships among eight
Eimeria species infecting domestic fowl inferred using complete small subunit
ribosomal DNA sequences. *Journal of Parasitology* **83:** 262-271.

Barta, J.R. (2001) Molecular approaches for inferring evolutionary relationships
among protistan parasites. *Veterinary Parasitology* **101:** 175-186.

Baxby, D., Blundell, N. and Hart, C.A. (1984) The development and performance of a
simple, sensitive method for the detection of *Cryptosporidium* oocysts in
faeces. *Journal of Hygiene* **93:** 317-323.

Bialek, R., Binder, N., Dietz, K., Joachim, A., Knobloch, J. and Zelck, U.E. (2002)
Comparison of fluorescence, antigen and PCR assays to detect
Cryptosporidium parvum in fecal specimens. *Diagnostic Microbiology and
Infectious Disease* **43:** 283-288.

Blasdall, S.A., Ongerth, J.E. and Ashbolt, N.J. (2002) Sub-species typing among
bovine *C. parvum* isolates by PCR-PAGE using a novel microsatellite +
telomere primer scheme. *Water Science & Technology: Water Supply* **2:** 81-
87.

Bodley-Tickell, A.T., Kitchen, S.E. and Sturdee, A.P. (2002) Occurrence of
Cryptosporidium in agricultural surface waters during an annual farming cycle
in lowland UK. *Water Research* **36:** 1880-1886.

Bonnin, A., Fourmaux, M.N., Dubremetz, J.F., Nelson, R.G., Gobet, P., Harly, G.,
Buisson, M., Puygauthier-Toubas, D., Gabriel-Pospisil, G., Naciri, M. and

Camerlynck, P. (1996) Genotyping human and bovine isolates of *Cryptosporidium parvum* by polymerase chain reaction-restriction fragment length polymorphism analysis of a repetitive DNA sequence. *FEMS Microbiology Letters* **137**: 207-211.

Bouchier, I. (1998) *Cryptosporidium in water supplies.* Third Report of the Group of Experts; Department of the Environment, Transport and the Regions, Department of Health, London, UK.

Bronsdon, M.A. (1984) Rapid dimethyl sulfoxide-modified acid-fast stain of *Cryptosporidium* oocysts in stool specimens. *Journal of Clinical Microbiology* **19**: 952-953.

Brownstein, D.G., Strandberg, J.D., Montali, R.J., Bush, M. and Fortner, J. (1977) *Cryptosporidium* in snakes with hypertrophic gastritis. *Veterinary Pathology* **14**: 606-617.

Bukhari, Z. and Smith, H.V. (1995) Effect of three concentration techniques on viability of *Cryptosporidium parvum* oocysts recovered from bovine feces. *Journal of Clinical Microbiology* **33**: 2592-2595.

Bukhari, Z. and Smith, H.V. (1996) Detection of *Cryptosporidium muris* oocysts in the faeces of adult dairy cattle in Scotland. *Veterinary Record* **138**: 207-208.

Bull, S., Chalmers, R., Sturdee, A.P., Curry, A. and Kennaugh, J. (1998) Cross-reaction of an anti-*Cryptosporidium* monoclonal antibody with sporocysts of *Monocystis* species. *Veterinary Parasitology* **77**: 195-197.

Caccio, S., Homan, W., van, D.K. and Pozio, E. (1999) Genetic polymorphism at the beta-tubulin locus among human and animal isolates of *Cryptosporidium parvum*. *FEMS Microbiology Letters* **170**: 173-179.

Caccio, S., Homan, W., Camilli, R., Traldi, G., Kortbeek, T. and Pozio, E. (2000) A microsatellite marker reveals population heterogeneity within human and animal genotypes of *Cryptosporidium parvum*. *Parasitology* **120**: 237-244.

Caccio, S., Spano, F. and Pozio, E. (2001) Large sequence variation at two microsatellite loci among zoonotic (genotype C) isolates of *Cryptosporidium parvum*. *International Journal for Parasitology* **31**: 1082-1086.

Caccio, S. and Pozio, E. (2001) Molecular identification of food-borne and water-borne protozoa. *Southeast Asian Journal of Tropical Medicine and Public Health* **32 Suppl 2:** 156-158.

Cai, J., Collins, M.D., McDonald, V. and Thompson, D.E. (1992) PCR cloning and nucleotide sequence determination of the 18S rRNA genes and internal transcribed spacer 1 of the protozoan parasites *Cryptosporidium parvum* and *Cryptosporidium muris*. *Biochimica et Biophysica Acta* **1131:** 317-320.

Cannon, R.M. and Roe, R.T. (1982) *Livestock surveys: a field manual for veterinarians,* Australian Bureau of Animal Health, Canberra.

Carraway, M., Widmer, G. and Tzipori, S. (1994) Genetic markers differentiate *C. parvum* isolates. *Journal of Eukaryotic Microbiology* **41:** 26S

Carraway, M., Tzipori, S. and Widmer, G. (1996) Identification of genetic heterogeneity in the *Cryptosporidium parvum* ribosomal repeat. *Applied and Environmental Microbiology* **62:** 712-716.

Carraway, M., Tzipori, S. and Widmer, G. (1997) A new restriction fragment length polymorphism from *Cryptosporidium parvum* identifies genetically heterogeneous parasite populations and genotypic changes following transmission from bovine to human hosts. *Infection and Immunity* **65:** 3958-3960.

Carreno, R.A., Schnitzler, B.E., Jeffries, A.C., Tenter, A.M., Johnson, A.M. and Barta, J.R. (1998) Phylogenetic analysis of coccidia based on 18S rDNA sequence comparison indicates that *Isospora* is most closely related to *Toxoplasma* and *Neospora*. *Journal of Eukaryotic Microbiology* **45:** 184-188.

Carreno, R.A., Martin, D.S. and Barta, J.R. (1999) *Cryptosporidium* is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. *Parasitology Research* **85:** 899-904.

Carreno, R.A., Pokorny, N.J., Lee, H., Trevors, J.T. and De Grandis, S.A. (2001) Phenotypic and genotypic characterization of *Cryptosporidium* species and isolates. *Journal of Industrial Microbiology and Biotechnology* **26:** 95-106.

Casemore, D.P., Armstrong, M. and Jackson, B. (1984) Screening for
Cryptosporidium in stools. *Lancet* **323:** 734-735.

Casemore, D.P., Armstrong, M. and Sands, R.L. (1985) Laboratory diagnosis of
cryptosporidiosis. *Journal of Clinical Pathology* **38:** 1337-1341.

Casemore, D.P. (1987) Gastroenteritis in a semi-rural population (The emergence of a
'new' pathogen, *Cryptosporidium*). PhD Thesis, School of Pharmacy,
Liverpool.

Casemore, D.P. (1990) Epidemiological aspects of human cryptosporidiosis.
Epidemiology and Infection **104:** 1-28.

Casemore, D.P. (1991) ACP Broadsheet 128: June 1991. Laboratory methods for
diagnosing cryptosporidiosis. *Journal of Clinical Pathology* **44:** 445-451.

Casemore, D.P., Wright, S.E. and Coop, R.L. (1997) Cryptosporidiosis - human and
animal epidemiology. In: *Cryptosporidium and cryptosporidiosis*, (ed. Fayer,
R.), CRC Press, Boca Raton, Florida. pp. 65-92.

Chalmers, R.M., Elwin, K., Reilly, W.J., Irvine, H., Thomas, A.L. and Hunter, P.R.
(2002a) *Cryptosporidium* in farmed animals: the detection of a novel isolate in
sheep. *International Journal for Parasitology* **32:** 21-26.

Chalmers, R.M., Elwin, K., Thomas, A.L. and Joynson, D.H. (2002b) Infection with
unusual types of *Cryptosporidium* is not restricted to immunocompromised
patients. *Journal of Infectious Diseases* **185:** 270-271.

Chalmers, R.M., Hughes, S., Thomas, A.L., Woodhouse, S., Thomas, P.D. and
Hunter, P. (2002c) Laboratory ascertainment of *Cryptosporidium* and local
authority policies for investigating sporadic cases of cryptosporidiosis in two
regions of the United Kingdom. *Communicable Disease and Public Health* **5:**
114-118.

Chalmers, R.M., Ferguson, C., Caccio, S., Gasser, R.B., Abs EL-Osta, Y.G., Heijnen,
L., Xiao, L., Elwin, K., Hadfield, S., Sinclair, M. and Stevens, M. (2005)
Direct comparison of selected methods for genetic characterisation of
Cryptosporidium parvum and *Cryptosporidium hominis* species. *International
Journal for Parasitology* **35:** 397-410.

Chan, R., Chen, J., York, M.K., Setijono, N., Kaplan, R.L., Graham, F. and Tanowitz,
H.B. (2000) Evaluation of a combination rapid immunoassay for detection of
Giardia and *Cryptosporidium* antigens. *Journal of Clinical Microbiology* **38**:
393-394.

Chappell, C.L., Okhuysen, P.C., Sterling, C.R., Wang, C., Jakubowski, W. and
DuPont, H.L. (1999) Infectivity of *Cryptosporidium parvum* in healthy adults
with pre-existing anti-*C. parvum* serum immunoglobulin G. *American Journal
of Tropical Medicine and Hygiene* **60**: 157-164.

Chung, E., Aldom, J.E., Chagla, A.H., Kostrzynska, M., Lee, H., Palmateer, G.,
Trevors, J.T., Unger, S. and De Grandis, S. (1998) Detection of
Cryptosporidium parvum oocysts in municipal water samples by the
polymerase chain reaction. *Journal of Microbiological Methods* **33**: 171-180.

Clavel, A., Arnal, A., Sanchez, E., Varea, M., Quilez, J., Ramirez, I. and Castillo, F.J.
(1996) Comparison of 2 centrifugation procedures in the formalin-ethyl
acetate stool concentration technique for the detection of *Cryptosporidium*
oocysts. *International Journal for Parasitology* **26**: 671-672.

Cole, D.J., Snowden, K., Cohen, N.D. and Smith, R. (1999) Detection of
Cryptosporidium parvum in horses: thresholds of acid-fast stain,
immunofluorescence assay, and flow cytometry. *Journal of Clinical
Microbiology* **37**: 457-460.

Current, W.L., Reese, N.C., Ernst, J.V., Bailey, W.S., Heyman, M.B. and Weinstein,
W.M. (1983) Human cryptosporidiosis in immunocompetent and
immunodeficient persons. Studies of an outbreak and experimental
transmission. *New England Journal of Medicine* **308**: 1252-1257.

Current, W.L., Upton, S.J. and Haynes, T.B. (1986) The life cycle of *Cryptosporidium
baileyi* n. sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. *Journal of
Protozoology* **33**: 289-296.

Current, W.L. and Reese, N.C. (1986) A comparison of endogenous development of
three isolates of *Cryptosporidium* in suckling mice. *Journal of Protozoology*
33: 98-108.

Current, W.L. and Garcia, L.S. (1991) Cryptosporidiosis. *Clinical Microbiology Reviews* **4**: 325-358.

D'Antonio, R.G., Winn, R.E., Taylor, J.P., Gustafson, T.L., Current, W.L., Rhodes, M.M., Gary, G.W., Jr. and Zajac, R.A. (1985) A waterborne outbreak of cryptosporidiosis in normal hosts. *Annals of Internal Medicine* **103**: 886-888.

Dagan, R., Fraser, D., el-On, J., Kassis, I., Deckelbaum, R. and Turner, S. (1995) Evaluation of an enzyme immunoassay for the detection of *Cryptosporidium* spp. in stool specimens from infants and young children in field studies. *American Journal of Tropical Medicine and Hygiene* **52**: 134-138.

Daniel, R.G., Staak, D., Robinson, G., Thomas, A.L., Hadfield, S.J., Elwin, K. and Chalmers, R.M. (2005) Detection of *Cryptosporidium andersoni* in a dairy cow with diarrhoea in the U.K. *Veterinary Record* **156**: 423

Davies, C.M., Kaucner, C., Deere, D. and Ashbolt, N.J. (2003) Recovery and enumeration of *Cryptosporidium parvum* from animal fecal matrices. *Applied and Environmental Microbiology* **69**: 2842-2847.

Deng, M.Q., Cliver, D.O. and Mariam, T.W. (1997) Immunomagnetic capture PCR to detect viable *Cryptosporidium parvum* oocysts from environmental samples. *Applied and Environmental Microbiology* **63**: 3134-3138.

Deng, M.Q. and Cliver, D.O. (1999) Improved immunofluorescence assay for detection of *Giardia* and *Cryptosporidium* from asymptomatic adult cervine animals. *Parasitology Research* **85**: 733-736.

Deng, M.Q., Lam, K.M. and Cliver, D.O. (2000) Immunomagnetic separation of *Cryptosporidium parvum* oocysts using MACS MicroBeads and high gradient separation columns. *Journal of Microbiological Methods* **40**: 11-17.

Di Giovanni, G.D., Hashemi, F.H., Shaw, N.J., Abrams, F.A., LeChevallier, M.W. and Abbaszadegan, M. (1999) Detection of infectious *Cryptosporidium parvum* oocysts in surface and filter backwash water samples by immunomagnetic separation and integrated cell culture-PCR. *Applied and Environmental Microbiology* **65**: 3427-3432.

Djuretic, T., Wall, P.G., Ryan, M.J., Evans, H.S., Adak, G.K. and Cowden, J.M. (1996) General outbreaks of infectious intestinal disease in England and Wales 1992 to 1994. *Communicable Disease Report Review* **6:** R57-R63.

Doing, K.M., Hamm, J.L., Jellison, J.A., Marquis, J.A. and Kingsbury, C. (1999) False-positive results obtained with the Alexon ProSpecT *Cryptosporidium* enzyme immunoassay. *Journal of Clinical Microbiology* **37:** 1582-1583.

DWI (1999) *Guidance on assessing risk from Cryptosporidium oocysts in treated water supplies to satisfy the Water Supply (Water Quality)(Amendment) Regulations 1999.* Department of the Environment, Transport and the Regions and the Welsh Office, London.

DWI (2005) *Standard operating protocol for the monitoring of Cryptosporidium oocysts in treated water supplies to satisfy the Water Supply (Water Quality) Regulations 2000, SI No. 3184 England and the Water Supply (Water Quality) Regulations 2001, SI No. 3911 (W.323) Wales.*

DuPont, H.L., Chappell, C.L., Sterling, C.R., Okhuysen, P.C., Rose, J.B. and Jakubowski, W. (1995) The infectivity of *Cryptosporidium parvum* in healthy volunteers. *New England Journal of Medicine* **332:** 855-859.

Egyed, Z., Sreter, T., Szell, Z. and Varga, I. (2003) Characterization of *Cryptosporidium* spp.--recent developments and future needs. *Veterinary Parasitology* **111:** 103-114.

El-Ghaysh, A. and Barrett, J. (1999) Isoenzyme activities of different strains of *Cryptosporidium parvum. Veterinary Parasitology* **81:** 195-200.

Elwin, K., Chalmers, R.M., Roberts, R., Guy, E.C. and Casemore, D.P. (2001) Modification of a rapid method for the identification of gene-specific polymorphisms in *Cryptosporidium parvum* and its application to clinical and epidemiological investigations. *Applied and Environmental Microbiology* **67:** 5581-5584.

Enemark, H.L., Ahrens, P., Juel, C.D., Petersen, E., Petersen, R.F., Andersen, J.S., Lind, P. and Thamsborg, S.M. (2002a) Molecular characterization of Danish *Cryptosporidium parvum* isolates. *Parasitology* **125:** 331-341.

Enemark, H.L., Ahrens, P., Lowery, C.J., Thamsborg, S.M., Enemark, J.M., Bille-Hansen, V. and Lind, P. (2002b) *Cryptosporidium andersoni* from a Danish cattle herd: identification and preliminary characterisation. *Veterinary Parasitology* **107**: 37-49.

European co-operation for Accreditation (2002) *Accreditation for Microbiological Laboratories*. EA-04/10.

Evans, H.S., Madden, P., Douglas, C., Adak, G.K., O'Brien, S.J., Djuretic, T., Wall, P.G. and Stanwell-Smith, R. (1998) General outbreaks of infectious intestinal disease in England and Wales: 1995 and 1996. *Communicable Disease and Public Health* **1**: 165-171.

Fairley, C.K., Sinclair, M.I. and Rizak, S. (1999a) Monitoring drinking water: the receding zero. *Medical Journal of Australia* **171**: 397-398.

Fairley, C.K., Sinclair, M.I. and Rizak, S. (1999b) Monitoring not the answer to *Cryptosporidium* in water. *Lancet* **354**: 967-969.

Fayer, R., Speer, C.A. and Dubey, J.P. (1990) General biology of *Cryptosporidium*. In: *Cryptosporidiosis of man and animals*, (eds. Dubey, J.P., Speer, C.A. and Fayer, R.), CRC Press, Boca Raton, Florida. pp. 1-29.

Fayer, R., Speer, C.A. and Dubey, J.P. (1997) The General Biology of *Cryptosporidium*. In: *Cryptosporidium and Cryptosporidiosis*, (ed. Fayer, R.), CRC Press, Boca Raton, Florida. pp. 1-41.

Fayer, R., Morgan, U. and Upton, S.J. (2000a) Epidemiology of *Cryptosporidium*: transmission, detection and identification. *International Journal for Parasitology* **30**: 1305-1322.

Fayer, R., Trout, J.M., Graczyk, T.K. and Lewis, E.J. (2000b) Prevalence of *Cryptosporidium*, *Giardia* and *Eimeria* infections in post-weaned and adult cattle on three Maryland farms. *Veterinary Parasitology* **93**: 103-112.

Fayer, R., Trout, J.M., Xiao, L., Morgan, U.M., Lai, A.A. and Dubey, J.P. (2001) *Cryptosporidium canis* n. sp. from domestic dogs. *Journal of Parasitology* **87**: 1415-1422.

Fayer, R. (2004) *Cryptosporidium*: a water-borne zoonotic parasite. *Veterinary Parasitology* **126**: 37-56.

Feldsine, P., Abeyta, C. and Andrews, W.H. (2002) AOAC International Methods
Committee Guidelines for Validation of Qualitative and Quantitative Food
Microbiological Official Methods of Analysis. *Journal of AOAC International*
85: 1187-1200.

Feng, X., Rich, S.M., Akiyoshi, D., Tumwine, J.K., Kekitiinwa, A., Nabukeera, N.,
Tzipori, S. and Widmer, G. (2000) Extensive polymorphism in
Cryptosporidium parvum identified by multilocus microsatellite analysis.
Applied and Environmental Microbiology **66:** 3344-3349.

Feng, X., Rich, S.M., Tzipori, S. and Widmer, G. (2002) Experimental evidence for
genetic recombination in the opportunistic pathogen *Cryptosporidium parvum*.
Molecular and Biochemical Parasitology **119:** 55-62.

Feng, Y.Y., Ong, S.L., Hu, J.Y., Song, L.F., Tan, X.L. and Ng, W.J. (2003) Effect of
particles on the recovery of *Cryptosporidium* oocysts from source water
samples of various turbidities. *Applied and Environmental Microbiology* **69:**
1898-1903.

Filkorn, R., Wiedenmann, A. and Botzenhart, K. (1994) Selective detection of viable
Cryptosporidium oocysts by PCR. *Zentralblatt fur Hygiene und
Umweltmedizin* **195:** 489-494.

Forgacs, P., Tarshis, A., Ma, P., Federman, M., Mele, L., Silverman, M.L. and Shea,
J.A. (1983) Intestinal and bronchial cryptosporidiosis in an immunodeficient
homosexual man. *Annals of Internal Medicine* **99:** 793-794.

Garcia, L.S., Bruckner, D.A., Brewer, T.C. and Shimizu, R.Y. (1983) Techniques for
the recovery and identification of *Cryptosporidium* oocysts from stool
specimens. *Journal of Clinical Microbiology* **18:** 185-190.

Garcia, L.S., Brewer, T.C. and Bruckner, D.A. (1987) Fluorescence detection of
Cryptosporidium oocysts in human fecal specimens by using monoclonal
antibodies. *Journal of Clinical Microbiology* **25:** 119-121.

Garcia, L.S., Shum, A.C. and Bruckner, D.A. (1992) Evaluation of a new monoclonal
antibody combination reagent for direct fluorescence detection of *Giardia*
cysts and *Cryptosporidium* oocysts in human fecal specimens. *Journal of
Clinical Microbiology* **30:** 3255-3257.

Garcia, L.S. and Shimizu, R.Y. (1997) Evaluation of nine immunoassay kits (enzyme immunoassay and direct fluorescence) for detection of *Giardia lamblia* and *Cryptosporidium parvum* in human fecal specimens. *Journal of Clinical Microbiology* **35**: 1526-1529.

Garcia, L.S. and Shimizu, R.Y. (2000) Detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens using the ColorPAC combination rapid solid-phase qualitative immunochromatographic assay. *Journal of Clinical Microbiology* **38**: 1267-1268.

Garcia, L.S., Shimizu, R.Y. and Bernard, C.N. (2000) Detection of *Giardia lamblia*, *Entamoeba histolytica/Entamoeba dispar*, and *Cryptosporidium parvum* antigens in human fecal specimens using the triage parasite panel enzyme immunoassay. *Journal of Clinical Microbiology* **38**: 3337-3340.

Garcia, L.S., Shimizu, R.Y., Novak, S., Carroll, M. and Chan, F. (2003) Commercial assay for detection of *Giardia lamblia* and *Cryptosporidium parvum* antigens in human fecal specimens by rapid solid-phase qualitative immunochromatography. *Journal of Clinical Microbiology* **41**: 209-212.

Gasser, R.B., El-Osta, Y.G. and Chalmers, R.M. (2003) Electrophoretic analysis of genetic variability within *Cryptosporidium parvum* from imported and autochthonous cases of human cryptosporidiosis in the United Kingdom. *Applied and Environmental Microbiology* **69**: 2719-2730.

Gatei, W., Ashford, R.W., Beeching, N.J., Kamwati, S.K., Greensill, J. and Hart, C.A. (2002a) *Cryptosporidium muris* infection in an HIV-infected adult, Kenya. *Emerging Infectious Diseases* **8**: 204-206.

Gatei, W., Suputtamongkol, Y., Waywa, D., Ashford, R.W., Bailey, J.W., Greensill, J., Beeching, N.J. and Hart, C.A. (2002b) Zoonotic species of *Cryptosporidium* are as prevalent as the anthroponotic in HIV-infected patients in Thailand. *Annals of Tropical Medicine and Parasitology* **96**: 797-802.

Gatei, W., Greensill, J., Ashford, R.W., Cuevas, L.E., Parry, C.M., Cunliffe, N.A., Beeching, N.J. and Hart, C.A. (2003) Molecular analysis of the 18S rRNA gene of *Cryptosporidium* parasites from patients with or without human

immunodeficiency virus infections living in Kenya, Malawi, Brazil, the United Kingdom, and Vietnam. *Journal of Clinical Microbiology* **41:** 1458-1462.

Gibbons, C.L., Rigi, F.M. and wad-El-Kariem, F.M. (1998a) Detection of *Cryptosporidium parvum* and *C. muris* oocysts in spiked backwash water using three PCR-based protocols. *Protist* **149:** 127-134.

Gibbons, C.L., Gazzard, B.G., Ibrahim, M.A.A., Morris-Jones, S., Ong, C.S.L. and wad-El-Kariem, F.M. (1998b) Correlation between markers of strain variation in *Cryptosporidium parvum*: Evidence of clonality. *Parasitology International* **47:** 139-147.

Gibbons-Matthews, C. and Prescott, A.M. (2003) Intra-isolate variation of *Cryptosporidium parvum* small subunit ribosomal RNA genes from human hosts in England. *Parasitology Research* **90:** 439-444.

Giles, M., Webster, K.A., Marshall, J.A., Catchpole, J. and Goddard, T.M. (2001) Experimental infection of a lamb with *Cryptosporidium parvum* genotype 1. *Veterinary Record* **149:** 523-525.

Glaberman, S., Moore, J.E., Lowery, C.J., Chalmers, R.M., Sulaiman, I., Elwin, K., Rooney, P.J., Millar, B.C., Dooley, J.S.G. and Lal, A.A. (2001a) Investigation of three drinking water associated outbreaks of cryptosporidiosis in Northern Ireland using genotyping and subgenotyping tools, 50th Annual Meeting of the American Society for Tropical Medicine, Atlanta, USA.

Glaberman, S., Sulaiman, I.M., Bern, C., Limor, J., Peng, M.M., Morgan, U., Gilman, R., Lal, A.A. and Xiao, L. (2001b) A multilocus genotypic analysis of *Cryptosporidium meleagridis*. *Journal of Eukaryotic Microbiology* **48 Suppl:** 19S-22S.

Glaberman, S., Moore, J.E., Lowery, C.J., Chalmers, R.M., Sulaiman, I., Elwin, K., Rooney, P.J., Millar, B.C., Dooley, J.S., Lal, A.A. and Xiao, L. (2002) Three drinking-water-associated cryptosporidiosis outbreaks, Northern Ireland. *Emerging Infectious Diseases* **8:** 631-633.

Gobel, E. and Brändler, U. (1982) Ultrastructure of microgametogenesis, microgametes and gametogamy of *Cryptosporidium* sp in the small-intestine of mice. *Protistologica* **18:** 331-344.

Gobet, P., Buisson, J.C., Vagner, O., Naciri, M., Grappin, M., Comparot, S., Harly, G., Aubert, D., Varga, I., Camerlynck, P. and Bonnin, A. (1997) Detection of *Cryptosporidium parvum* DNA in formed human feces by a sensitive PCR-based assay including uracil-N-glycosylase inactivation. *Journal of Clinical Microbiology* **35**: 254-256.

Gobet, P. and Toze, S. (2001) Sensitive genotyping of *Cryptosporidium parvum* by PCR-RFLP analysis of the 70-kilodalton heat shock protein (HSP70) gene. *FEMS Microbiology Letters* **200**: 37-41.

Goh, S., Reacher, M., Casemore, D.P., Verlander, N.Q., Chalmers, R., Knowles, M., Williams, J., Osborn, K. and Richards, S. (2004) Sporadic cryptosporidiosis, North Cumbria, England, 1996-2000. *Emerging Infectious Diseases* **10**: 1007-1015.

Goh, S., Reacher, M., Casemore, D.P., Verlander, N.Q., Charlett, A., Chalmers, R.M., Knowles, M., Pennington, A., Williams, J., Osborn, K. and Richards, S. (2005) Sporadic cryptosporidiosis decline after membrane filtration of public water supplies, England, 1996-2002. *Emerging Infectious Diseases* **11**: 251-259.

Graczyk, T.K., Cranfield, M.R. and Fayer, R. (1996) Evaluation of commercial enzyme immunoassay (EIA) and immunofluorescent antibody (FA) test kits for detection of *Cryptosporidium oocysts* of species other than *Cryptosporidium parvum*. *American Journal of Tropical Medicine and Hygiene* **54**: 274-279.

Griffin, K., Matthai, E., Hommel, M., Weitz, J.C., Baxby, D. and Hart, C.A. (1992) Antigenic diversity among oocysts of clinical isolates of *Cryptosporidium parvum*. *Journal of Protozoology Research* **2**: 97-101.

Guy, R.A., Payment, P., Krull, U.J. and Horgen, P.A. (2003) Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. *Applied and Environmental Microbiology* **69**: 5178-5185.

Guyot, K., Follet-Dumoulin, A., Lelievre, E., Sarfati, C., Rabodonirina, M., Nevez, G., Cailliez, J.C., Camus, D. and Dei-Cas, E. (2001) Molecular

characterization of *Cryptosporidium* isolates obtained from humans in France. *Journal of Clinical Microbiology* **39**: 3472-3480.

Guyot, K., Follet-Dumoulin, A., Recourt, C., Lelievre, E., Cailliez, J.C. and Dei-Cas, E. (2002) PCR-restriction fragment length polymorphism analysis of a diagnostic 452-base-pair DNA fragment discriminates between *Cryptosporidium parvum* and *C. meleagridis* and between *C. parvum* isolates of human and animal origin. *Applied and Environmental Microbiology* **68**: 2071-2076.

Hallier-Soulier, S. and Guillot, E. (1999) An immunomagnetic separation polymerase chain reaction assay for rapid and ultra-sensitive detection of *Cryptosporidium parvum* in drinking water. *FEMS Microbiology Letters* **176**: 285-289.

Hancock, C.M., Rose, J.B. and Callahan, M. (1998) Crypto and *Giardia* in US groundwater. *Journal American Water Works Association* **90**: 58-61.

Harris, J.R. and Petry, F. (1999) *Cryptosporidium parvum*: structural components of the oocyst wall. *Journal of Parasitology* **85**: 839-849.

Henriksen, S.A. and Pohlenz, J.F. (1981) Staining of cryptosporidia by a modified Ziehl-Neelsen technique. *Acta Veterinaria Scandinavica* **22**: 594-596.

Hewitt, R.G., Yiannoutsos, C.T., Higgs, E.S., Carey, J.T., Geiseler, P.J., Soave, R., Rosenberg, R., Vazquez, G.J., Wheat, L.J., Fass, R.J., Antoninievic, Z., Walawander, A.L., Flanigan, T.P. and Bender, J.F. (2000) Paromomycin: no more effective than placebo for treatment of cryptosporidiosis in patients with advanced human immunodeficiency virus infection. AIDS Clinical Trial Group. *Clinical Infectious Diseases* **31**: 1084-1092.

Higgins, J.A., Fayer, R., Trout, J.M., Xiao, L., Lal, A.A., Kerby, S. and Jenkins, M.C. (2001) Real-time PCR for the detection of *Cryptosporidium parvum*. *Journal of Microbiological Methods* **47**: 323-337.

Hijjawi, N.S., Meloni, B.P., Ryan, U.M., Olson, M.E. and Thompson, R.C. (2002) Successful in vitro cultivation of *Cryptosporidium andersoni*: evidence for the existence of novel extracellular stages in the life cycle and implications for the classification of *Cryptosporidium*. *International Journal for Parasitology* **32**: 1719-1726.

Hijjawi, N. (2003) In vitro cultivation and development of *Cryptosporidium* in cell culture. In: *Cryptosporidium: From Molecules to Disease*, (eds. Thompson, R.C.A., Armson, A. and Ryan, U.M.), Elsevier, Amsterdam. pp. 233-253.

Hijjawi, N.S., Meloni, B.P., Ng'anzo, M., Ryan, U.M., Olson, M.E., Cox, P.T., Monis, P.T. and Thompson, R.C. (2004) Complete development of *Cryptosporidium parvum* in host cell-free culture. *International Journal for Parasitology* **34**: 769-777.

Homan, W., van, G.T., Kan, Y.Y. and Hepener, J. (1999) Characterization of *Cryptosporidium parvum* in human and animal feces by single-tube nested polymerase chain reaction and restriction analysis. *Parasitology Research* **85**: 707-712.

Hommer, V., Eichholz, J. and Petry, F. (2003) Effect of antiretroviral protease inhibitors alone, and in combination with paromomycin, on the excystation, invasion and in vitro development of *Cryptosporidium parvum*. *Journal of Antimicrobial Chemotherapy* **52**: 359-364.

Huetink, R.E., van der Giessen, J.W., Noordhuizen, J.P. and Ploeger, H.W. (2001) Epidemiology of *Cryptosporidium* spp. and *Giardia duodenalis* on a dairy farm. *Veterinary Parasitology* **102**: 53-67.

Hunter, P.R. and Gaston, M.A. (1988) Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *Journal of Clinical Microbiology* **26**: 2465-2466.

Hunter, P.R. (1991) A critical review of typing methods for *Candida albicans* and their applications. *Crit Rev. Microbiol.* **17**: 417-434.

Hunter, P.R. and Syed, Q. (2001) Community surveys of self-reported diarrhoea can dramatically overestimate the size of outbreaks of waterborne cryptosporidiosis. *Water Sci. Technol.* **43**: 27-30.

Hunter, P.R. and Nichols, G. (2002) Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. *Clinical Microbiology Reviews* **15**: 145-154.

Hunter, P.R., Chalmers, R.M., Syed, Q., Hughes, L.S., Woodhouse, S. and Swift, L. (2003) Foot and mouth disease and cryptosporidiosis: possible interaction

between two emerging infectious diseases. *Emerging Infectious Diseases* **9:** 109-112.

Hunter, P.R., Hughes, S., Woodhouse, S., Raj, N., Syed, Q., Chalmers, R.M., Verlander, N.Q. and Goodacre, J. (2004a) Health sequelae of human cryptosporidiosis in immunocompetent patients. *Clinical Infectious Diseases* **39:** 504-510.

Hunter, P.R., Hughes, S., Woodhouse, S., Syed, Q., Verlander, N.Q., Chalmers, R.M., Morgan, K., Nichols, G., Beeching, N. and Osborn, K. (2004b) Sporadic cryptosporidiosis case-control study with genotyping. *Emerging Infectious Diseases* **10:**(7) 1241-1249.

Hutchison, M.L., Walters, L.D., Avery, S.M., Synge, B.A. and Moore, A. (2004) Levels of zoonotic agents in British livestock manures. *Letters in Applied Microbiology* **39:**(2) 207-214.

Ignatius, R., Eisenblatter, M., Regnath, T., Mansmann, U., Futh, U., Hahn, H. and Wagner, J. (1997) Efficacy of different methods for detection of low *Cryptosporidium parvum* oocyst numbers or antigen concentrations in stool specimens. *European Journal of Clinical Microbiology and Infectious Diseases* **16:** 732-736.

Iseki, M. (1979) *Cryptosporidium felis* sp.n. (Protozoa: Eimeriorina) from the domestic cat. *Japanese Journal of Parasitology* **28:** 285-307.

Jellison, K.L., Hemond, H.F. and Schauer, D.B. (2002) Sources and species of *Cryptosporidium* oocysts in the Wachusett Reservoir watershed. *Applied and Environmental Microbiology* **68:** 569-575.

Jenkins, M.B., Bowman, D.D. and Ghiorse, W.C. (1998) Inactivation of *Cryptosporidium parvum* oocysts by ammonia. *Applied and Environmental Microbiology* **64:** 784-788.

Jiang, J. and Xiao, L. (2003) An evaluation of molecular diagnostic tools for the detection and differentiation of human-pathogenic *Cryptosporidium* spp. *Journal of Eukaryotic Microbiology* **50 Suppl:** 542-547.

Johnson, D.W., Pieniazek, N.J. and Rose, J.B. (1993) Dna probe hybridization and PCR detection of *Cryptosporidium* compared to immunofluorescence assay. *Water Science and Technology* **27**: 77-84.

Johnson, D.W., Pieniazek, N.J., Griffin, D.W., Misener, L. and Rose, J.B. (1995) Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples. *Applied and Environmental Microbiology* **61**: 3849-3855.

Johnston, S.P., Ballard, M.M., Beach, M.J., Causer, L. and Wilkins, P.P. (2003) Evaluation of three commercial assays for detection of *Giardia* and *Cryptosporidium* organisms in fecal specimens. *Journal of Clinical Microbiology* **41**: 623-626.

Jokipii, L., Pohjola, S. and Jokipii, A.M. (1983) *Cryptosporidium*: a frequent finding in patients with gastrointestinal symptoms. *Lancet* **322**: 358-361.

Jokipii, L. and Jokipii, A.M. (1986) Timing of symptoms and oocyst excretion in human cryptosporidiosis. *New England Journal of Medicine* **315**: 1643-1647.

Kacena, K.A., Quinn, S.B., Hartman, S.C., Quinn, T.C. and Gaydos, C.A. (1998) Pooling of urine samples for screening for *Neisseria gonorrhoeae* by ligase chain reaction: accuracy and application. *Journal of Clinical Microbiology* **36**: 3624-3628.

Katanik, M.T., Schneider, S.K., Rosenblatt, J.E., Hall, G.S. and Procop, G.W. (2001) Evaluation of ColorPAC *Giardia/Cryptosporidium* rapid assay and ProSpecT *Giardia/Cryptosporidium* microplate assay for detection of *Giardia* and *Cryptosporidium* in fecal specimens. *Journal of Clinical Microbiology* **39**: 4523-4525.

Kay, D., Edwards, A.C., Ferrier, R.C., Francis, C., Kay, C., Rushby, L., Watkins, J., Mcdonald, A.T., Wyer, M., Crowther, J. and Wilkinson, J. (2003) Does farmyard runoff represent a significant source of faecal indicator organisms for the bathing waters of south west Scotland, *Proceedings Diffuse Pollution Conference*, Dublin, pp 6.22-6.28.

Kehl, K.S., Cicirello, H. and Havens, P.L. (1995) Comparison of four different methods for detection of *Cryptosporidium* species. *Journal of Clinical Microbiology* **33:** 416-418.

Kemp, J.S., Wright, S.E. and Bukhari, Z. (1995) On farm detection of *Cryptosporidium parvum* in cattle, calves and environmental samples. In: *Protozoan Parasites and Water*, Vol. 168 (eds. Betts, W.B., Casemore, D., Fricker, C., Smith, H. and Watkins, J.), Royal Society of Chemistry Special Publication, Cambridge. pp. 154-157.

Khalakdina, A., Vugia, D.J., Nadle, J., Rothrock, G.A. and Colford, J.M., Jr. (2003) Is drinking water a risk factor for endemic cryptosporidiosis? A case-control study in the immunocompetent general population of the San Francisco Bay Area. *BMC Public Health* **3:** 11

Khramtsov, N.V., Chung, P.A., Dykstra, C.C., Griffiths, J.K., Morgan, U.M., Arrowood, M.J. and Upton, S.J. (2000) Presence of double-stranded RNAs in human and calf isolates of *Cryptosporidium parvum*. *Journal of Parasitology* **86:** 275-282.

Kim, K., Gooze, L., Petersen, C., Gut, J. and Nelson, R.G. (1992) Isolation, sequence and molecular karyotype analysis of the actin gene of *Cryptosporidium parvum*. *Molecular and Biochemical Parasitology* **50:** 105-113.

Kimbell III, L.M., Miller, D.L., Chavez, W. and Altman, N. (1999) Molecular analysis of the 18S rRNA gene of *Cryptosporidium serpentis* in a wild-caught corn snake (*Elaphe guttata guttata*) and a five-species restriction fragment length polymorphism- based assay that can additionally discern *C. parvum* from *C. wrairi*. *Applied and Environmental Microbiology* **65:** 5345-5349.

Kistemann, T., Classen, T., Koch, C., Dangendorf, F., Fischeder, R., Gebel, J., Vacata, V. and Exner, M. (2002) Microbial load of drinking water reservoir tributaries during extreme rainfall and runoff. *Applied and Environmental Microbiology* **68:** 2188-2197.

Knipschild, P. (1994) Systematic Reviews: Some examples. *British Medical Journal* **309:** 721

Kotler, D.P., Francisco, A., Clayton, F., Scholes, J.V. and Orenstein, J.M. (1990) Small intestinal injury and parasitic diseases in AIDS. *Annals of Internal Medicine* **113**: 444-449.

Koudela, B., Modry, D. and Vitovec, J. (1998) Infectivity of *Cryptosporidium muris* isolated from cattle. *Veterinary Parasitology* **76**: 181-188.

Koudela, B. and Modry, D. (1998) New species of *Cryptosporidium* (Apicomplexa : Cryptosporidiidae) from lizards. *Folia Parasitologica* **45**: 93-100.

Kuczynska, E. and Shelton, D.R. (1999) Method for detection and enumeration of *Cryptosporidium parvum* oocysts in feces, manures, and soils. *Applied and Environmental Microbiology* **65**: 2820-2826.

Kvac, M., Kvetonova, D., Puzova, G. and Ditrich, O. (2003) Comparison of selected diagnostic methods for identification of *Cryptosporidium parvum* and *Cryptosporidium andersoni* in routine examination of faeces. *Journal of Veterinary Medicine B: Infectious Diseases, Veterinary and Public Health* **50**: 405-411.

Kvac, M. and Vitovec, J. (2003) Prevalence and pathogenicity of *Cryptosporidium andersoni* in one herd of beef cattle. *Journal of Veterinary Medicine B: Infectious Diseases, Veterinary and Public Health* **50**: 451-457.

Laberge, I., Ibrahim, A., Barta, J.R. and Griffiths, M.W. (1996) Detection of *Cryptosporidium parvum* in raw milk by PCR and oligonucleotide probe hybridization. *Applied and Environmental Microbiology* **62**: 3259-3264.

Lantz, P.G., Matsson, M., Wadstrom, T. and Radstrom, P. (1997) Removal of PCR inhibitors from human faecal samples through the use of an aqueous two-phase system for sample preparation prior to PCR. *Journal of Microbiological Methods* **28**: 159-167.

Last, J.M. (2001) *A Dictionary of Epidemiology,* 4th Oxford University Press, New York.

Laxer, M.A., Timblin, B.K. and Patel, R.J. (1991) DNA sequences for the specific detection of *Cryptosporidium parvum* by the polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene* **45**: 688-694.

Le Blancq, S.M., Khramtsov, N.V., Zamani, F., Upton, S.J. and Wu, T.W. (1997) Ribosomal RNA gene organization in *Cryptosporidium parvum*. *Molecular and Biochemical Parasitology* **90:** 463-478.

Leav, B.A., Mackay, M.R., Anyanwu, A., O' Connor, R.M., Cevallos, A.M., Kindra, G., Rollins, N.C., Bennish, M.L., Nelson, R.G. and Ward, H.D. (2002) Analysis of sequence diversity at the highly polymorphic Cpgp40/15 locus among *Cryptosporidium* isolates from human immunodeficiency virus-infected children in South Africa. *Infection and Immunity* **70:** 3881-3890.

LeChevallier, M.W., Abbaszadegan, M. and Di Giovanni, G.D. (2000) Detection of infectious *Cryptosporidium parvum* oocysts in environmental water samples using an integrated cell culture- PCR (CC-PCR) system. *Water, Air and Soil Pollution* **123:** 53-65.

LeChevallier, M.W., Di Giovanni, G.D., Clancy, J.L., Bukhari, Z., Bukhari, S., Rosen, J.S., Sobrinho, J. and Frey, M.M. (2003) Comparison of method 1623 and cell culture-PCR for detection of *Cryptosporidium* spp. in source waters. *Applied and Environmental Microbiology* **69:** 971-979.

Lee, J.J., Leedale, G.F. and Bradbury, P. (2000) *The Illustrated Guide to the Protozoa,* 2nd Society of Protozoologists, Lawrence, Kansas.

Leng, X., Mosier, D.A. and Oberst, R.D. (1996a) Simplified method for recovery and PCR detection of *Cryptosporidium* DNA from bovine feces. *Applied and Environmental Microbiology* **62:** 643-647.

Leng, X., Mosier, D.A. and Oberst, R.D. (1996b) Differentiation of *Cryptosporidium parvum, C. muris,* and *C. baileyi* by PCR-RFLP analysis of the 18S rRNA gene. *Veterinary Parasitology* **62:** 1-7.

Leoni, F., Gallimore, C.I., Green, J. and McLauchlin, J. (2003a) A rapid method for identifying diversity within PCR amplicons using a heteroduplex mobility assay and synthetic polynucleotides: application to characterisation of dsRNA elements associated with *Cryptosporidium*. *Journal of Microbiological Methods* **54:** 95-103.

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.

Levine, N.D. (1980) Some corrections of coccidian (Apicomplexa: Protozoa) nomenclature. *Journal of Parasitology* **66:** 830-834.

Levine, N.D. (1984) Taxonomy and review of the coccidian genus *Cryptosporidium* (protozoa, apicomplexa). *Journal of Protozoology* **31:** 94-98.

Lindsay, D.S., Upton, S.J., Owens, D.S., Morgan, U.M., Mead, J.R. and Blagburn, B.L. (2000) *Cryptosporidium andersoni* n. sp. (Apicomplexa: Cryptosporiidae) from cattle, *Bos taurus*. *Journal of Eukaryotic Microbiology* **47:** 91-95.

Lopez, F.A., Manglicmot, J., Schmidt, T.M., Yeh, C., Smith, H.V. and Relman, D.A. (1999) Molecular characterization of *Cyclospora*-like organisms from baboons. *Journal of Infectious Diseases* **179:** 670-676.

Lowery, C.J., Moore, J.E., Millar, B.C., Burke, D.P., McCorry, K.A., Crothers, E. and Dooley, J.S. (2000) Detection and speciation of *Cryptosporidium* spp. in environmental water samples by immunomagnetic separation, PCR and endonuclease restriction. *Journal of Medical Microbiology* **49:** 779-785.

Lowery, C.J., Moore, J.E., Millar, B.C., McCorry, K.A., Xu, J., Rooney, P.J. and Dooley, J.S. (2001) Occurrence and molecular genotyping of *Cryptosporidium* spp. in surface waters in Northern Ireland. *Journal of Applied Microbiology* **91:** 774-779.

Ma, P. and Soave, R. (1983) Three-step stool examination for cryptosporidiosis in 10 homosexual men with protracted watery diarrhea. *Journal of Infectious Diseases* **147:** 824-828.

Mackenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R. and Rose, J.B. (1994) A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. *New England Journal of Medicine* **331:** 161-167.

MacPherson, D.W. and McQueen, R. (1993) Cryptosporidiosis: multiattribute
   evaluation of six diagnostic methods. *Journal of Clinical Microbiology* **31:**
   198-202.

MAFF (1998) *Code of Good Agricultural Practice for the Protection of Water.*
   Ministry of Agriculture Fisheries and Food and Welsh Office Agricultural
   Department, London, UK.

Mallon, M., MacLeod, A., Wastling, J., Smith, H., Reilly, B. and Tait, A. (2003a)
   Population structures and the role of genetic exchange in the zoonotic
   pathogen *Cryptosporidium parvum*. *Journal of Molecular Evolution* **56:** 407-
   417.

Mallon, M.E., MacLeod, A., Wastling, J.M., Smith, H. and Tait, A. (2003b)
   Multilocus genotyping of *Cryptosporidium parvum* Type 2: population
   genetics and sub-structuring. *Infection, Genetics and Evolution* **3:** 207-218.

Matsubayashi, M., Kimata, I., Abe, N., Tani, H. and Sasai, K. (2004) The detection of
   a novel type of *Cryptosporidium andersoni* oocyst in cattle in Japan.
   Parasitology Research **93:** 504-506.

Mayer, C.L. and Palmer, C.J. (1996) Evaluation of PCR, nested PCR, and fluorescent
   antibodies for detection of *Giardia* and *Cryptosporidium* species in
   wastewater. *Applied and Environmental Microbiology* **62:** 2081-2085.

McDonald, A. and Kay, D. (1981) Enteric bacterial concentrations in reservoir feeder
   streams: baseflow characteristics and response to hydrograph events. *Water
   Research* **15:** 961-968.

McDonald, V., Deer, R.M., Nina, J.M., Wright, S., Chiodini, P.L. and McAdam, K.P.
   (1991) Characteristics and specificity of hybridoma antibodies against oocyst
   antigens of *Cryptosporidium parvum* from man. *Parasite Immunology* **13:**
   251-259.

McLauchlin, J., Casemore, D.P., Harrison, T.G., Gerson, P.J., Samuel, D. and Taylor,
   A.G. (1987) Identification of *Cryptosporidium* oocysts by monoclonal
   antibody. *Lancet* **329:** 51

McLauchlin, J., Casemore, D.P., Moran, S. and Patel, S. (1998) The epidemiology of
   cryptosporidiosis: application of experimental sub-typing and antibody

detection systems to the investigation of water-borne outbreaks. *Folia Parasitologica (Praha)* **45:** 83-92.

McLauchlin, J., Pedraza-Diaz, S., mar-Hoetzeneder, C. and Nichols, G.L. (1999) Genetic characterization of *Cryptosporidium* strains from 218 patients with diarrhea diagnosed as having sporadic cryptosporidiosis. *Journal of Clinical Microbiology* **37:** 3153-3158.

McLauchlin, J., Amar, C., Pedraza-Diaz, S. and Nichols, G.L. (2000) Molecular epidemiological analysis of *Cryptosporidium* spp. in the United Kingdom: results of genotyping *Cryptosporidium* spp. in 1,705 fecal samples from humans and 105 fecal samples from livestock animals. *Journal of Clinical Microbiology* **38:** 3984-3990.

McNabb, S.J., Hensel, D.M., Welch, D.F., Heijbel, H., McKee, G.L. and Istre, G.R. (1985) Comparison of sedimentation and flotation techniques for identification of *Cryptosporidium* sp. oocysts in a large outbreak of human diarrhea. *Journal of Clinical Microbiology* **22:** 587-589.

Mead, J.R., Humphreys, R.C., Sammons, D.W. and Sterling, C.R. (1990) Identification of isolate-specific sporozoite proteins of *Cryptosporidium parvum* by two-dimensional gel electrophoresis. *Infection and Immunity* **58:** 2071-2075.

Meinhardt, P.L., Casemore, D.P. and Miller, K.B. (1996) Epidemiologic aspects of human cryptosporidiosis and the role of waterborne transmission. *Epidemiologic Reviews* **18:** 118-136.

Meisel, J.L., Perera, D.R., Meligro, C. and Rubin, C.E. (1976) Overwhelming watery diarrhea associated with a *Cryptosporidium* in an immunosuppressed patient. *Gastroenterology* **70:** 1156-1160.

Mele, R., Gomez Morales, M.A., Tosini, F. and Pozio, E. (2003) Indinavir reduces *Cryptosporidium parvum* infection in both in vitro and in vivo models. *International Journal for Parasitology* **33:** 757-764.

Miller, J.R., Mojica, B., Nadle, J., Vugia, D.J., Waterman, S.H., Mamer, B., Hahn, C., Doing, K.M., Hamm, J.L., Buker, N., Beckett, G.A., Gensheimer, K.F., Kludt, P., DeMaria, A., Ennis, J., Keithly, J., Kondracki, S., Ackman, D., Smith, P.,

Warshauer, D., Proctor, M. and Davis, J. (1999) False-positive laboratory tests for *Cryptosporidium* involving an enzyme-linked immunosorbent assay - United States, November 1997 March 1998 (Reprinted from MMWR, vol 48, pg 4-8, 1999). *Journal of the American Medical Association* **281**: 411-412.

Monteiro, L., Bonnemaison, D., Vekris, A., Petry, K.G., Bonnet, J., Vidal, R., Cabrita, J. and Megraud, F. (1997) Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. *Journal of Clinical Microbiology* **35**: 995-998.

Morgan, U.M., Constantine, C.C., O'Donoghue, P., Meloni, B.P., O'Brien, P.A. and Thompson, R.C. (1995) Molecular characterization of *Cryptosporidium* isolates from humans and other animals using random amplified polymorphic DNA analysis. *American Journal of Tropical Medicine and Hygiene* **52**: 559-564.

Morgan, U.M., O'Brien, P.A. and Thompson, R.C. (1996) The development of diagnostic PCR primers for *Cryptosporidium* using RAPD-PCR. *Molecular and Biochemical Parasitology* **77**: 103-108.

Morgan, U.M., Constantine, C.C., Forbes, D.A. and Thompson, R.C. (1997) Differentiation between human and animal isolates of *Cryptosporidium parvum* using rDNA sequencing and direct PCR analysis. *Journal of Parasitology* **83**: 825-830.

Morgan, U.M., Sargent, K.D., Deplazes, P., Forbes, D.A., Spano, F., Hertzberg, H., Elliot, A. and Thompson, R.C. (1998) Molecular characterization of *Cryptosporidium* from various hosts. *Parasitology* **117**: 31-37.

Morgan, U.M., Deplazes, P., Forbes, D.A., Spano, F., Hertzberg, H., Sargent, K.D., Elliot, A. and Thompson, R.C. (1999) Sequence and PCR-RFLP analysis of the internal transcribed spacers of the rDNA repeat unit in isolates of *Cryptosporidium* from different hosts. *Parasitology* **118**: 49-58.

Morgan, U., Weber, R., Xiao, L., Sulaiman, I., Thompson, R.C., Ndiritu, W., Lal, A., Moore, A. and Deplazes, P. (2000) Molecular characterization of *Cryptosporidium* isolates obtained from human immunodeficiency virus-infected individuals living in Switzerland, Kenya, and the United States. *Journal of Clinical Microbiology* **38**: 1180-1183.

Morgan-Ryan, U.M., Fall, A., Ward, L.A., Hijjawi, N., Sulaiman, I., Fayer, R., Thompson, R.C., Olson, M., Lal, A. and Xiao, L. (2002) *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from Homo sapiens. *Journal of Eukaryotic Microbiology* **49:** 433-440.

Morrison, D.A. and Ellis, J.T. (1997) Effects of nucleotide sequence alignment on phylogeny estimation: a case study of 18S rDNAs of apicomplexa. *Molecular Biology and Evolution* **14:** 428-441.

Mtambo, M.M., Nash, A.S., Blewett, D.A. and Wright, S. (1992) Comparison of staining and concentration techniques for detection of *Cryptosporidium* oocysts in cat faecal specimens. *Veterinary Parasitology* **45:** 49-57.

Navarro-i-Martinez, L., Bornay-Llinares, F.J., Rueda, C., del, A.C., da Silva, A.J., Oleaga, A., Ramajo, V., Fenoy, S. and Pieniazek, N.J. (2003) Molecular characterization of *Cryptosporidium* sp. from animals in Spain. *Journal of Eukaryotic Microbiology* **50 Suppl:** 553-554.

Neumann, N.F., Ruecker, N., Bounsombath, N., Wallis, P., Ong, C. and Isaac-Renton, J. (2004) Assessing geospatial and geotemporal contamination of a watershed with *Cryptosporidium* and *Giardia* using molecular forensic profiling. *Proceedings of the International Giardia and Cryptosporidium Conference*, September 2004, Amsterdam.

Newman, R.D., Jaeger, K.L., Wuhib, T., Lima, A.A., Guerrant, R.L. and Sears, C.L. (1993) Evaluation of an antigen capture enzyme-linked immunosorbent assay for detection of *Cryptosporidium* oocysts. *Journal of Clinical Microbiology* **31:** 2080-2084.

Nichols, G.L., McLauchlin, J. and Samuel, D. (1991) A technique for typing *Cryptosporidium* isolates. *Journal of Protozoology* **38:** 237S-240S.

Nichols, R.A., Campbell, B.M. and Smith, H.V. (2003) Identification of *Cryptosporidium* spp. oocysts in United Kingdom noncarbonated natural mineral waters and drinking waters by using a modified nested PCR-restriction fragment length polymorphism assay. *Applied and Environmental Microbiology* **69:** 4183-4189.

245

Nichols, R.A. and Smith, H.V. (2004) Optimization of DNA extraction and molecular detection of *Cryptosporidium* oocysts in natural mineral water sources. *Journal of Food Protection* **67**: 524-532.

Nime, F.A., Burek, J.D., Page, D.L., Holscher, M.A. and Yardley, J.H. (1976) Acute enterocolitis in a human being infected with the protozoan *Cryptosporidium*. *Gastroenterology* **70**: 592-598.

Nina, J.M., McDonald, V., Deer, R.M., Wright, S.E., Dyson, D.A., Chiodini, P.L. and McAdam, K.P. (1992) Comparative study of the antigenic composition of oocyst isolates of *Cryptosporidium parvum* from different hosts. *Parasite Immunology* **14**: 227-232.

O'Donoghue, P.J. (1995) *Cryptosporidium* and cryptosporidiosis in man and animals. *International Journal for Parasitology* **25**: 139-195.

Ogunkolade, B.W., Robinson, H.A., McDonald, V., Webster, K. and Evans, D.A. (1993) Isoenzyme variation within the genus *Cryptosporidium*. *Parasitology Research* **79**: 385-388.

Okhuysen, P.C., Chappell, C.L., Sterling, C.R., Jakubowski, W. and DuPont, H.L. (1998) Susceptibility and serologic response of healthy adults to reinfection with *Cryptosporidium parvum*. *Infection and Immunity* **66**: 441-443.

Okhuysen, P.C., Chappell, C.L., Crabb, J.H., Sterling, C.R. and DuPont, H.L. (1999) Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. *Journal of Infectious Diseases* **180**: 1275-1281.

Ong, C.S., Eisler, D.L., Goh, S.H., Tomblin, J., wad-El-Kariem, F.M., Beard, C.B., Xiao, L., Sulaiman, I., Lal, A., Fyfe, M., King, A., Bowie, W.R. and Isaac-Renton, J.L. (1999) Molecular epidemiology of cryptosporidiosis outbreaks and transmission in British Columbia, Canada. *American Journal of Tropical Medicine and Hygiene* **61**: 63-69.

Ong, C.S., Eisler, D.L., Alikhani, A., Fung, V.W., Tomblin, J., Bowie, W.R. and Isaac-Renton, J.L. (2002) Novel *Cryptosporidium* genotypes in sporadic cryptosporidiosis cases: first report of human infections with a cervine genotype. *Emerging Infectious Diseases* **8**: 263-268.

Palmer, C.J., Xiao, L., Terashima, A., Guerra, H., Gotuzzo, E., Saldias, G., Bonilla, J.A., Zhou, L., Lindquist, A. and Upton, S.J. (2003) *Cryptosporidium muris*, a rodent pathogen, recovered from a human in Peru. *Emerging Infectious Diseases* **9:** 1174-1176.

Palmer, S.R. and Biffin, A.H. (1990) Cryptosporidiosis in England and Wales: prevalence and clinical and epidemiological features. Public Health Laboratory Service Study Group. *British Medical Journal* **300:** 774-777.

Panciera, R.J., Thomassen, R.W. and Garner, F.M. (1971) Cryptosporidial infection in a calf. *Veterinary Pathology* **8:**479-484.

Parisi, M.T. and Tierno, P.M., Jr. (1995) Evaluation of new rapid commercial enzyme immunoassay for detection of *Cryptosporidium* oocysts in untreated stool specimens. *Journal of Clinical Microbiology* **33:** 1963-1965.

Patel, S., Pedraza-Diaz, S., McLauchlin, J. and Casemore, D.P. (1998) Molecular characterisation of *Cryptosporidium parvum* from two large suspected waterborne outbreaks. Outbreak Control Team South and West Devon 1995, Incident Management Team and Further Epidemiological and Microbiological Studies Subgroup North Thames 1997. *Communicable Disease and Public Health* **1:** 231-233.

Pavlasek, I. (1995) [Cryptosporidia and other endoparasites in heifers imported into the Czech Republic]. *Veterinari Medicina (Praha)* **40:** 333-336.

Pavlasek, I. (1999) Cryptosporidia: biology, diagnosis, host spectrum, specificity, and the environment. *Remidia Klinicka Mikrobiologie* **3:** 290-301.

Pedraza-Diaz, S., Amar, C. and McLauchlin, J. (2000) The identification and characterisation of an unusual genotype of *Cryptosporidium* from human faeces as *Cryptosporidium meleagridis*. *FEMS Microbiology Letters* **189:** 189-194.

Pedraza-Diaz, S., Amar, C., Iversen, A.M., Stanley, P.J. and McLauchlin, J. (2001a) Unusual *Cryptosporidium* species recovered from human faeces: first description of *Cryptosporidium felis* and *Cryptosporidium* 'dog type' from patients in England. *Journal of Medical Microbiology* **50:** 293-296.

Pedraza-Diaz, S., Amar, C., Nichols, G.L. and McLauchlin, J. (2001b) Nested polymerase chain reaction for amplification of the *Cryptosporidium* oocyst wall protein gene. *Emerging Infectious Diseases* **7:** 49-56.

Pedraza-Diaz, S., Amar, C.F., McLauchlin, J., Nichols, G.L., Cotton, K.M., Godwin, P., Iversen, A.M., Milne, L., Mulla, J.R., Nye, K., Panigrahl, H., Venn, S.R., Wiggins, R., Williams, M. and Youngs, E.R. (2001c) *Cryptosporidium meleagridis* from humans: molecular analysis and description of affected patients. *Journal of Infection* **42:** 243-250.

Peng, M.M., Xiao, L., Freeman, A.R., Arrowood, M.J., Escalante, A.A., Weltman, A.C., Ong, C.S., Mackenzie, W.R., Lal, A.A. and Beard, C.B. (1997) Genetic polymorphism among *Cryptosporidium parvum* isolates: evidence of two distinct human transmission cycles. *Emerging Infectious Diseases* **3:** 567-573.

Peng, M.M., Matos, O., Gatei, W., Das, P., Stantic-Pavlinic, M., Bern, C., Sulaiman, I.M., Glaberman, S., Lal, A.A. and Xiao, L. (2001) A comparison of *Cryptosporidium* subgenotypes from several geographic regions. *Journal of Eukaryotic Microbiology* **48 Suppl:** 28S-31S.

Peng, M.M., Meshnick, S.R., Cunliffe, N.A., Thindwa, B.D., Hart, C.A., Broadhead, R.L. and Xiao, L. (2003a) Molecular epidemiology of cryptosporidiosis in children in Malawi. *Journal of Eukaryotic Microbiology* **50 Suppl:** S57-S59.

Peng, M.M., Wilson, M.L., Holland, R.E., Meshnick, S.R., Lal, A.A. and Xiao, L. (2003b) Genetic diversity of *Cryptosporidium* spp. in cattle in Michigan: implications for understanding the transmission dynamics. *Parasitology Research* **90:** 175-180.

Percival, S.L., Chalmers, R.M., Embrey, M., Hunter, P.R., Sellwood, J. and Wyn-Jones, P. (2004) *Cryptosporidium* spp. In: *Microbiology of Waterborne Diseases*, Elsevier Academic Press, London. pp. 237-266.

Pereira, M.D., Atwill, E.R. and Jones, T. (1999) Comparison of sensitivity of immunofluorescent microscopy to that of a combination of immunofluorescent microscopy and immunomagnetic separation for detection of *Cryptosporidium parvum* oocysts in adult bovine feces. *Applied and Environmental Microbiology* **65:** 3236-3239.

Perry, J.L., Matthews, J.S. and Miller, G.R. (1990) Parasite detection efficiencies of five stool concentration systems. *Journal of Clinical Microbiology* **28:** 1094-1097.

Pieniazek, N.J., Bornay-Llinares, F.J., Slemenda, S.B., da Silva, A.J., Moura, I.N., Arrowood, M.J., Ditrich, O. and Addiss, D.G. (1999) New *Cryptosporidium* genotypes in HIV-infected persons. *Emerging Infectious Diseases* **5:** 444-449.

Pohlenz, J., Moon, H.W., Cheville, N.F. and Bemrick, W.J. (1978) Cryptosporidiosis as a probable factor in neonatal diarrhea of calves. *Journal of the American Veterinary Medical Association* **172:** 452-457.

Power, M.L., Slade, M.B., Sangster, N.C. and Veal, D.A. (2004) Genetic characterisation of *Cryptosporidium* from a wild population of eastern grey kangaroos *Macropus giganteus* inhabiting a water catchment. *Infection, Genetics and Evolution* **4:** 59-67.

Quilez, J., Sanchez-Acedo, C., Clavel, A., del, C.E. and Lopez-Bernad, F. (1996) Comparison of an acid-fast stain and a monoclonal antibody-based immunofluorescence reagent for the detection of *Cryptosporidium* oocysts in faecal specimens from cattle and pigs. *Veterinary Parasitology* **67:** 75-81.

Quintero-Betancourt, W., Peele, E.R. and Rose, J.B. (2002) *Cryptosporidium parvum* and *Cyclospora cayetanensis*: a review of laboratory methods for detection of these waterborne parasites. *Journal of Microbiological Methods* **49:** 209-224.

Quiroz, E.S., Bern, C., MacArthur, J.R., Xiao, L., Fletcher, M., Arrowood, M.J., Shay, D.K., Levy, M.E., Glass, R.I. and Lal, A. (2000) An outbreak of cryptosporidiosis linked to a foodhandler. *Journal of Infectious Diseases* **181:**(2) 695-700.

Quy, R.J., Cowan, D.P., Haynes, P.J., Sturdee, A.P., Chalmers, R.M., Bodley-Tickell, A.T. and Bull, S.A. (1999) The Norway rat as a reservoir host of *Cryptosporidium parvum. Journal of Wildlife Diseases* **35:** 660-670.

Ralston, B.J., Cockwill, C.L., Guselle, N.J., Van Herk, F.H., McAllister, T.A. and Olson, M.E. (2003) Prevalence of *Giardia* and *Cryptosporidium andersoni* and their effects on performance in feedlot beef cattle. *Canadian Journal of Animal Science* **83:** 153-159.

Ranucci, L., Muller, H.M., La, R.G., Reckmann, I., Morales, M.A., Spano, F., Pozio, E. and Crisanti, A. (1993) Characterization and immunolocalization of a *Cryptosporidium* protein containing repeated amino acid motifs. *Infection and Immunity* **61:** 2347-2356.

Reduker, D.W., Speer, C.A. and Blixt, J.A. (1985a) Ultrastructural-changes in the oocyst wall during excystation of *Cryptosporidium parvum* (Apicomplexa, Eucoccidiorida). *Canadian Journal of Zoology* **63:** 1892-1896.

Reduker, D.W., Speer, C.A. and Blixt, J.A. (1985b) Ultrastructure of *Cryptosporidium parvum* oocysts and excysting sporozoites as revealed by high resolution scanning electron microscopy. *Journal of Protozoology* **32:** 708-711.

Relman, D.A., Schmidt, T.M., Gajadhar, A., Sogin, M., Cross, J., Yoder, K., Sethabutr, O. and Echeverria, P. (1996) Molecular phylogenetic analysis of *Cyclospora*, the human intestinal pathogen, suggests that it is closely related to *Eimeria* species. *Journal of Infectious Diseases* **173:** 440-445.

Ritchie, L.S. (1948) An ether sedimentation technique for routine stool examinations. *Bulletin of the US Army Medical Department* **8:** 326

Robert, B., Ginter, A., Antoine, H., Collard, A. and Coppe, P. (1990) Diagnosis of bovine cryptosporidiosis by an enzyme-linked immunosorbent assay. *Veterinary Parasitology* **37:** 1-8.

Robertson, B., Sinclair, M.I., Forbes, A.B., Veitch, M., Kirk, M., Cunliffe, D., Willis, J. and Fairley, C.K. (2002) Case-control studies of sporadic cryptosporidiosis in Melbourne and Adelaide, Australia. *Epidemiology and Infection* **128:** 419-431.

Robertson, L.J., Campbell, A.T. and Smith, H.V. (1992) Survival of *Cryptosporidium parvum* oocysts under various environmental pressures. *Applied and Environmental Microbiology* **58:** 3494-3500.

Robinson, G., Thomas, A.L., Daniel, R.G., Hadfield, S.J., Elwin, K. and Chalmers, R.M. (2005) *Cryptosporidium andersoni*: Review of the literature and prevalence within a dairy herd in the United Kingdom. *In preperation.*

Rochelle, P.A., Ferguson, D.M., Handojo, T.J., De, L.R., Stewart, M.H. and Wolfe, R.L. (1996) Development of a rapid detection procedure for *Cryptosporidium*, using in vitro cell culture combined with PCR. *Journal of Eukaryotic Microbiology* **43**: 72S

Rochelle, P.A., De, L.R., Stewart, M.H. and Wolfe, R.L. (1997a) Comparison of primers and optimization of PCR conditions for detection of *Cryptosporidium parvum* and *Giardia lamblia* in water. *Applied and Environmental Microbiology* **63**: 106-114.

Rochelle, P.A., Ferguson, D.M., Handojo, T.J., De, L.R., Stewart, M.H. and Wolfe, R.L. (1997b) An assay combining cell culture with reverse transcriptase PCR to detect and determine the infectivity of waterborne *Cryptosporidium parvum*. *Applied and Environmental Microbiology* **63**: 2029-2037.

Rochelle, P.A., De, L.R., Johnson, A., Stewart, M.H. and Wolfe, R.L. (1999a) Evaluation of immunomagnetic separation for recovery of infectious *Cryptosporidium parvum* oocysts from environmental samples. *Applied and Environmental Microbiology* **65**: 841-845.

Rochelle, P.A., Jutras, E.M., Atwill, E.R., De, L.R. and Stewart, M.H. (1999b) Polymorphisms in the beta-tubulin gene of *Cryptosporidium parvum* differentiate between isolates based on animal host but not geographic origin. *Journal of Parasitology* **85**: 986-989.

Rochelle, P.A., De Leon, R. and Atwill, E.R. (2000) Intra-isolate heterogeneity and reproducibility of PCR-based genotyping of *Cryptosporidium parvum* using the β-tubulin gene. *Quantitative Microbiology* **2**: 87-101.

Rose, J.B. (1997) Environmental ecology of *Cryptosporidium* and public health implications. *Annual Review of Public Health* **18**: 135-161.

Rose, J.B., Lisle, J.T. and LeChevallier, M. (1997) Waterborne Cryptosporidiosis: Incidence, Outbreaks, and Treatment Strategies. In: *Cryptosporidium and Cryptosporidiosis*, (ed. Fayer, R.), CRC Press, Boca Raton, Florida. pp. 93-109.

Rose, J.B., Huffman, D.E. and Gennaccaro, A. (2002) Risk and control of waterborne cryptosporidiosis. *FEMS Microbiology Reviews* **26**: 113-123.

Rosenblatt, J.E. and Sloan, L.M. (1993) Evaluation of an enzyme-linked immunosorbent assay for detection of *Cryptosporidium* spp. in stool specimens. *Journal of Clinical Microbiology* **31**: 1468-1471.

Rusnak, J., Hadfield, T.L., Rhodes, M.M. and Gaines, J.K. (1989) Detection of *Cryptosporidium* oocysts in human fecal specimens by an indirect immunofluorescence assay with monoclonal antibodies. *Journal of Clinical Microbiology* **27**: 1135-1136.

Ryan, U.M., Xiao, L., Read, C., Sulaiman, I.M., Monis, P., Lal, A.A., Fayer, R. and Pavlasek, I. (2003) A redescription of *Cryptosporidium galli* Pavlasek, 1999 (Apicomplexa: Cryptosporidiidae) from birds. *Journal of Parasitology* **89**: 809-813.

Ryan, U.M., Monis, P., Enemark, H.L., Sulaiman, I., Samarasinghe, B., Read, C., Buddle, R., Robertson, I., Zhou, L., Thompson, R.C. and Xiao, L. (2004) *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (Sus scrofa). *Journal of Parasitology* **90**: 769-773.

Sanders, B., Anthony, S., Stapleton, C.M., Kay, D. and Crowther, J. (2004) *The impact of destocking on the microbiological quality of rivers in the Caldew catchment*. Final report to the Environment Agency, North West Region. Ref: 12162.

Santin, M., Trout, J.M., Xiao, L., Zhou, L., Greiner, E. and Fayer, R. (2004) Prevalence and age-related variation of *Cryptosporidium* species and genotypes in dairy calves. *Veterinary Parasitology* **122**: 103-117.

Satoh, M., Hikosaka, K., Sasaki, T., Suyama, Y., Yanai, T., Ohta, M. and Nakai, Y. (2003) Characteristics of a novel type of bovine *Cryptosporidium andersoni*. *Applied and Environmental Microbiology* **69**: 691-692.

Schmidt, W., Wahnschaffe, U., Schafer, M., Zippel, T., Arvand, M., Meyerhans, A., Riecken, E.O. and Ullrich, R. (2001) Rapid increase of mucosal CD4 T cells followed by clearance of intestinal cryptosporidiosis in an AIDS patient receiving highly active antiretroviral therapy. *Gastroenterology* **120**: 984-987.

Scott, C.A., Smith, H.V., Mtambo, M.M. and Gibbs, H.A. (1995) An epidemiological study of *Cryptosporidium parvum* in two herds of adult beef cattle. *Veterinary Parasitology* **57**: 277-288.

Sharp, S.E., Suarez, C.A., Duran, Y. and Poppiti, R.J. (2001) Evaluation of the Triage Micro Parasite Panel for detection of *Giardia lamblia*, *Entamoeba histolytica/Entamoeba dispar* and *Cryptosporidium parvum* in patient stool specimens. *Journal of Clinical Microbiology* **39**: 332-334.

Sheather, A.L. (1923) The detection of intestinal protozoa and mange parasites by a flotation technique. *Journal of Comparative Pathology and Therapeutics* **36**: 266-275.

Shianna, K.V., Rytter, R. and Spanier, J.G. (1998) Randomly amplified polymorphic DNA PCR analysis of bovine *Cryptosporidium parvum* strains isolated from the watershed of the Red River of the North. *Applied and Environmental Microbiology* **64**: 2262-2265.

Siddons, C.A., Chapman, P.A. and Rush, B.A. (1992) Evaluation of an enzyme immunoassay kit for detecting *Cryptosporidium* in faeces and environmental samples. *Journal of Clinical Pathology* **45**: 479-482.

Silva, C.V., Ferreira, M.S., Goncalves-Pires, M.R. and Costa-Cruz, J.M. (2003) Detection of *Cryptosporidium*--specific coproantigen in human immunodeficiency virus/acquired immunodeficiency syndrome patients by using a commercially available immunoenzymatic assay. *Memorias do Instituto Oswaldo Cruz* **98**: 1097-1099.

Slapeta, J.R., Modry, D., Votypka, J., Jirku, M., Lukes, J. and Koudela, B. (2003) Evolutionary relationships among cyst-forming coccidia *Sarcocystis* spp. (Alveolata: Apicomplexa: Coccidea) in endemic African tree vipers and perspective for evolution of heteroxenous life cycle. *Molecular Phylogenetics and Evolution* **27**: 464-475.

Slavin, D. (1955) *Cryptosporidium meleagridis* (sp. nov.). *J. Comp Pathol.* **65**: 262-266.

Sluter, S.D., Tzipori, S. and Widmer, G. (1997) Parameters affecting polymerase chain reaction detection of waterborne *Cryptosporidium parvum* oocysts. *Applied Microbiology and Biotechnology* **48:** 325-330.

Smerdon, W.J., Nichols, T., Chalmers, R.M., Heine, H. and Reacher, M.H. (2003) Foot and mouth disease in livestock and reduced cryptosporidiosis in humans, England and Wales. *Emerging Infectious Diseases* **9:** 22-28.

Smith, H.V. (1998) Detection of parasites in the environment. *Parasitology* **117 Suppl:** S113-S141.

Smith, H.V. and Rose, J.B. (1998) Waterborne cryptosporidiosis: Current status. *Parasitology Today* **14:** 14-22.

Smith, H.V. (2004) New drugs and treatments for cryptosporidiosis. *Current Opinions in Infectious Diseases* **17:** 557-564.

Spano, F., Putignani, L., McLauchlin, J., Casemore, D.P. and Crisanti, A. (1997) PCR-RFLP analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene discriminates between *C. wrairi* and *C. parvum*, and between *C. parvum* isolates of human and animal origin. *FEMS Microbiology Letters* **150:** 209-217.

Spano, F., Putignani, L., Crisanti, A., Sallicandro, P., Morgan, U.M., Le Blancq, S.M., Tchack, L., Tzipori, S. and Widmer, G. (1998a) Multilocus genotypic analysis of *Cryptosporidium parvum* isolates from different hosts and geographical origins. *Journal of Clinical Microbiology* **36:** 3255-3259.

Spano, F., Putignani, L., Guida, S. and Crisanti, A. (1998b) *Cryptosporidium parvum:* PCR-RFLP analysis of the TRAP-C1 (thrombospondin-related adhesive protein of *Cryptosporidium*-1) gene discriminates between two alleles differentially associated with parasite isolates of animal and human origin. *Experimental Parasitology* **90:** 195-198.

Sreter, T., Egyed, Z., Szell, Z., Kovacs, G., Nikolausz, M., Marialigeti, K. and Varga, I. (2000) Morphologic, host specificity, and genetic characterization of a European *Cryptosporidium andersoni* isolate. *Journal of Parasitology* **86:** 1244-1249.

Stantic-Pavlinic, M., Xiao, L., Glaberman, S., Lal, A.A., Orazen, T., Rataj-Verglez, A., Logar, J. and Berce, I. (2003) Cryptosporidiosis associated with animal contacts. *Wiener Klinische Wochenschrift* **115:** 125-127.

Sterling, C.R. and Arrowood, M.J. (1986) Detection of *Cryptosporidium* sp. infections using a direct immunofluorescent assay. *Pediatric Infectious Disease* **5:** S139-S142.

Stibbs, H.H. and Ongerth, J.E. (1986) Immunofluorescence detection of *Cryptosporidium* oocysts in fecal smears. *Journal of Clinical Microbiology* **24:** 517-521.

Stinear, T., Matusan, A., Hines, K. and Sandery, M. (1996) Detection of a single viable *Cryptosporidium parvum* oocyst in environmental water concentrates by reverse transcription-PCR. *Applied and Environmental Microbiology* **62:** 3385-3390.

Strong, W.B., Gut, J. and Nelson, R.G. (2000) Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. *Infection and Immunity* **68:** 4117-4134.

Struelens, M.J., Bauernfeind, A., van Belkum, A., Blanc, D., Cookson, B.D., Dijkshoorn, L., El Solh, N., Etienne, J., Garaizar, J., Gerner-Smidt, P., Legakis, N., de Lencastre, H., Nicolas, M.H., Pitt, T.L., Römling, U., Rosdahl, V. and Witte, W. (1996) Consensus guidelines for appropriate use and evaluation of microbial epidemiologic typing systems. *Clinical Microbiology and Infection* **2:** 2-11.

Sturbaum, G.D., Reed, C., Hoover, P.J., Jost, B.H., Marshall, M.M. and Sterling, C.R. (2001) Species-specific, nested PCR-restriction fragment length polymorphism detection of single *Cryptosporidium parvum* oocysts. *Applied and Environmental Microbiology* **67:** 2665-2668.

Sturbaum, G.D., Klonicki, P.T., Marshall, M.M., Jost, B.H., Clay, B.L. and Sterling, C.R. (2002) Immunomagnetic separation (IMS)-fluorescent antibody detection and IMS-PCR detection of seeded *Cryptosporidium parvum* oocysts in natural waters and their limitations. *Applied and Environmental Microbiology* **68:** 2991-2996.

Sturbaum, G.D., Jost, B.H. and Sterling, C.R. (2003) Nucleotide changes within three Cryptosporidium parvum surface protein encoding genes differentiate genotype I from genotype II isolates. *Molecular and Biochemical Parasitology* **128:** 87-90.

Sturdee, A.P., Chalmers, R.M. and Bull, S.A. (1999) Detection of Cryptosporidium oocysts in wild mammals of mainland Britain. *Veterinary Parasitology* **80:** 273-280.

Sturdee, A.P., Bodley-Tickell, A.T., Archer, A. and Chalmers, R.M. (2003) Long-term study of Cryptosporidium prevalence on a lowland farm in the United Kingdom. *Veterinary Parasitology* **116:** 97-113.

Sulaiman, I.M., Xiao, L., Yang, C., Escalante, L., Moore, A., Beard, C.B., Arrowood, M.J. and Lal, A.A. (1998) Differentiating human from animal isolates of Cryptosporidium parvum. *Emerging Infectious Diseases* **4:** 681-685.

Sulaiman, I.M., Lal, A.A., Arrowood, M.J. and Xiao, L. (1999a) Biallelic polymorphism in the intron region of beta-tubulin gene of Cryptosporidium parasites. *Journal of Parasitology* **85:** 154-157.

Sulaiman, I.M., Xiao, L. and Lal, A.A. (1999b) Evaluation of Cryptosporidium parvum genotyping techniques. *Applied and Environmental Microbiology* **65:** 4431-4435.

Sulaiman, I.M., Lal, A.A. and Xiao, L. (2001) A population genetic study of the Cryptosporidium parvum human genotype parasites. *Journal of Eukaryotic Microbiology* **48 Suppl:** 24S-27S.

Sulaiman, I.M., Lal, A.A. and Xiao, L. (2002) Molecular phylogeny and evolutionary relationships of Cryptosporidium parasites at the actin locus. *Journal of Parasitology* **88:** 388-394.

Tebbe, C.C. and Vahjen, W. (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Applied and Environmental Microbiology* **59:** 2657-2665.

Tenter, A.M. and Johnson, A.M. (1997) Phylogeny of the tissue cyst-forming coccidia. *Advances in Parasitology* **39:** 69-139.

The Comptroller and Auditor General (2002) *The 2001 outbreak of Foot and Mouth Disease.* National Audit Office, The Stationery Office, London.

Thomas, A.L. and Chalmers, R.M. (2003) Investigation of the range of *Cryptosporidium* species detected by commercially available antibody-based tests, Health Protection Agency Inaugural Conference, Warwick.

Tiangtip, R. and Jongwutiwes, S. (2002) Molecular analysis of *Cryptosporidium* species isolated from HIV-infected patients in Thailand. *Tropical Medicine and International Health* **7:** 357-364.

Tsai, Y.L. and Olson, B.H. (1992) Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Applied and Environmental Microbiology* **58:** 754-757.

Tyrrel, S.F. and Quinton, J.N. (2003) Overland flow transport of pathogens from agricultural land receiving faecal wastes. *Journal of Applied Microbiology* **94:** 87S-93S.

Tyzzer, E.E. (1907) A sporozoan found in the peptic glands of the common mouse. *Proceedings of the Society for Experimental Biology and Medicine* **5:** 12-13.

Tyzzer, E.E. (1910) An extracellular coccidium, *Cryptosporidium muris* (gen. et sp. nov.), of the gastric glands of the common mouse. *Journal of Medical Research* **23:** 487-509.

Tyzzer, E.E. (1912) *Cryptosporidium parvum* (sp.nov.), a coccidium found in the small intestine of the common mouse. *Archiv fur Protistenkunde* **26:** 394-412.

Tyzzer, E.E. (1929) Coccidiosis in gallinaceous birds. *The American Journal of Hygiene* **10:** 269-383.

Tzipori, S., Angus, K.W., Campbell, I. and Gray, E.W. (1980a) *Cryptosporidium*: evidence for a single-species genus. *Infection and Immunity* **30:** 884-886.

Tzipori, S., Angus, K.W., Gray, E.W. and Campbell, I. (1980b) Vomiting and diarrhea associated with cryptosporidial infection. *New England Journal of Medicine* **303:** 818

Tzipori, S., Smith, M., Birch, C., Barnes, G. and Bishop, R. (1983) Cryptosporidiosis in hospital patients with gastroenteritis. *American Journal of Tropical Medicine and Hygiene* **32:** 931-934.

Tzipori, S. and Griffiths, J.K. (1998) Natural history and biology of *Cryptosporidium parvum. Advances in Parasitology* **40:** 5-36.

Ungar, B.L. (1990a) Enzyme-linked immunoassay for detection of *Cryptosporidium* antigens in fecal specimens. *Journal of Clinical Microbiology* **28:** 2491-2495.

Ungar, B.L.P. (1990b) Cryptosporidiosis in humans (*Homo sapiens*). In: *Cryptosporidiosis of Man and Animals*, (eds. Dubey, J.P., Speer, C.A. and Fayer, R.), CRC Press, Boca Raton, Florida. pp. 59-82.

Upton, S.J. and Current, W.L. (1985) The species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) infecting mammals. *Journal of Parasitology* **71:** 625-629.

USEPA (1998) *National Primary Drinking Water Regulations: Interim Enhanced Surface Water Treatment; Final Rule.* Part V. EPA, 40 CFR Parts 9, 141 and 142. Fed. Reg. **63:** 69478

USEPA (2001) *Method 1622: Cryptosporidium in Water by Filtration/IMS/FA, EPA-821-R-01-026. Office of Water, Engineering and Analysis Division, Washington, DC.*

USEPA (2002) *National Primary Drinking Water Regulations: Long Term 1 Enhanced Surface Water Treatment; Final Rule.* Part II. EPA, 40 CFR Parts 9, 141 and 142. Fed. Reg. **67:** 1812.

Vasquez, J.R., Gooze, L., Kim, K., Gut, J., Petersen, C. and Nelson, R.G. (1996) Potential antifolate resistance determinants and genotypic variation in the bifunctional dihydrofolate reductase-thymidylate synthase gene from human and bovine isolates of *Cryptosporidium parvum. Molecular and Biochemical Parasitology* **79:** 153-165.

Vetterling, J.M., Jervis, H.R., Merrill, T.G. and Sprinz, H. (1971) *Cryptosporidium wrairi* sp. n. from the guinea pig *Cavia porcellus*, with an emendation of the genus. *Journal of Protozoology* **18:** 243-247.

Wade, S.E., Mohammed, H.O. and Schaaf, S.L. (2000) Prevalence of *Giardia* sp, *Cryptosporidium parvum* and *Cryptosporidium muris* (*C. andersoni*) in 109

dairy herds in five counties of southeastern New York. *Veterinary Parasitology* **93**: 1-11.

Wagner-Wiening, C. and Kimmig, P. (1995) Detection of viable *Cryptosporidium parvum* oocysts by PCR. *Applied and Environmental Microbiology* **61**: 4514-4516.

Ward, L.A. and Wang, Y. (2001) Rapid methods to isolate *Cryptosporidium* DNA from frozen feces for PCR. *Diagnostic Microbiology and Infectious Disease* **41**: 37-42.

Ward, P.I., Deplazes, P., Regli, W., Rinder, H. and Mathis, A. (2002) Detection of eight *Cryptosporidium* genotypes in surface and waste waters in Europe. *Parasitology* **124**: 359-368.

Weber, R., Bryan, R.T., Bishop, H.S., Wahlquist, S.P., Sullivan, J.J. and Juranek, D.D. (1991) Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: evidence for low sensitivity of current diagnostic methods. *Journal of Clinical Microbiology* **29**: 1323-1327.

Weber, R., Bryan, R.T. and Juranek, D.D. (1992) Improved stool concentration procedure for detection of *Cryptosporidium* oocysts in fecal specimens. *Journal of Clinical Microbiology* **30**: 2869-2873.

Webster, K.A. (1993) Molecular methods for the detection and classification of *Cryptosporidium*. *Parasitology Today* **9**: 263-266.

Webster, J.P. and Macdonald, D.W. (1995) Cryptosporidiosis reservoir in wild brown rats (*Rattus norvegicus*) in the UK. *Epidemiology and Infection* **115**: 207-209.

Webster, K.A., Smith, H.V., Giles, M., Dawson, L. and Robertson, L.J. (1996) Detection of *Cryptosporidium parvum* oocysts in faeces: comparison of conventional coproscopical methods and the polymerase chain reaction. *Veterinary Parasitology* **61**: 5-13.

Widmer, G., Tchack, L., Chappell, C.L. and Tzipori, S. (1998a) Sequence polymorphism in the beta-tubulin gene reveals heterogeneous and variable population structures in *Cryptosporidium parvum*. *Applied and Environmental Microbiology* **64**: 4477-4481.

Widmer, G., Tzipori, S., Fichtenbaum, C.J. and Griffiths, J.K. (1998b) Genotypic and phenotypic characterization of *Cryptosporidium parvum* isolates from people with AIDS. *Journal of Infectious Diseases* **178:** 834-840.

Widmer, G., Feng, X. and Tzipori, S. (2000) Genetic fingerprinting and species characterisation of *Cryptosporidium* using microsatellite polymorphism, AWWA Water Quality Technology Conference Proceedings.

Wiedenmann, A., Kruger, P. and Botzenhart, K. (1998) PCR detection of *Cryptosporidium parvum* in environmental samples - a review of published protocols and current developments. *Journal of Industrial Microbiology & Biotechnology* **21:** 150-166.

Wilkinson, J., Jenkins, A., Wyer, M. and Kay, D. (1995) Modelling faecal coliform dynamics in streams and rivers. *Water Research* **29:** 847-855.

Wilson, I.G. (1997) Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology* **63:** 3741-3751.

World Health Organisation (2004) *Guidelines for Drinking-water Quality, Third Edition.* World Helath Organisation, Geneva.

Wu, Z., Nagano, I., Boonmars, T., Nakada, T. and Takahashi, Y. (2003) Intraspecies polymorphism of *Cryptosporidium parvum* revealed by PCR-restriction fragment length polymorphism (RFLP) and RFLP-single-strand conformational polymorphism analyses. *Applied and Environmental Microbiology* **69:** 4720-4726.

Wyer, M.D., Stapleton, C.M., Kay, D., Francis, C.A., Watkins, J. and Rushby, L. (2001) *Cryptosporidium, Giardia and faecal indicator organisms in surface water and sewage effluent from the Irvine catchment, Ayrshire.* Report to the Drinking Water Inspectorate, West of Scotland Water, the Scottish Environment Protection Agency, South Ayrshire Council and North Ayrshire Council. CREH, University of Wales, Aberystwyth.

Xiao, L., Herd, R.P. and McClure, K.E. (1994) Periparturient rise in the excretion of *Giardia* sp. cysts and *Cryptosporidium parvum* oocysts as a source of infection for lambs. *Journal of Parasitology* **80:** 55-59.

Xiao, L., Sulaiman, I., Fayer, R. and Lal, A.A. (1998) Species and strain-specific
typing of *Cryptosporidium* parasites in clinical and environmental samples.
Memorias do Instituto Oswaldo Cruz **93:** 687-691.

Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A.A., Montali, R.J., Fayer,
R. and Lal, A.A. (1999a) Phylogenetic analysis of *Cryptosporidium* parasites
based on the small-subunit rRNA gene locus. *Applied and Environmental
Microbiology* **65:** 1578-1583.

Xiao, L., Morgan, U.M., Limor, J., Escalante, A., Arrowood, M., Shulaw, W.,
Thompson, R.C., Fayer, R. and Lal, A.A. (1999b) Genetic diversity within
Cryptosporidium parvum and related *Cryptosporidium* species. *Applied and
Environmental Microbiology* **65:** 3386-3391.

Xiao, L., Alderisio, K., Limor, J., Royer, M. and Lal, A.A. (2000a) Identification of
species and sources of *Cryptosporidium* oocysts in storm waters with a small-
subunit rRNA-based diagnostic and genotyping tool. *Applied and
Environmental Microbiology* **66:** 5492-5498.

Xiao, L., Limor, J., Morgan, U.M., Sulaiman, I.M., Thompson, R.C. and Lal, A.A.
(2000b) Sequence differences in the diagnostic target region of the oocyst wall
protein gene of *Cryptosporidium* parasites. *Applied and Environmental
Microbiology* **66:** 5499-5502.

Xiao, L., Morgan, U.M., Fayer, R., Thompson, R.C. and Lal, A.A. (2000c)
Cryptosporidium systematics and implications for public health. *Parasitology
Today* **16:** 287-292.

Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L.,
Gilman, R.H. and Lal, A.A. (2001a) Identification of 5 types of
Cryptosporidium parasites in children in Lima, Peru. *Journal of Infectious
Diseases* **183:** 492-497.

Xiao, L., Limor, J., Bern, C. and Lal, A.A. (2001b) Tracking *Cryptosporidium
parvum* by sequence analysis of small double-stranded RNA. *Emerging
Infectious Diseases* **7:** 141-145.

Xiao, L., Singh, A., Limor, J., Graczyk, T.K., Gradus, S. and Lal, A. (2001c)
Molecular characterization of *Cryptosporidium* oocysts in samples of raw

surface water and wastewater. *Applied and Environmental Microbiology* **67**: 1097-1101.

Xiao, L., Bern, C., Arrowood, M., Sulaiman, I., Zhou, L., Kawai, V., Vivar, A., Lal, A.A. and Gilman, R.H. (2002a) Identification of the *Cryptosporidium* pig genotype in a human patient. *Journal of Infectious Diseases* **185**: 1846-1848.

Xiao, L., Sulaiman, I.M., Ryan, U.M., Zhou, L., Atwill, E.R., Tischler, M.L., Zhang, X., Fayer, R. and Lal, A.A. (2002b) Host adaptation and host-parasite co-evolution in *Cryptosporidium*: implications for taxonomy and public health. *International Journal for Parasitology* **32**: 1773-1785.

Xiao, L., Fayer, R., Ryan, U. and Upton, S.J. (2004) *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clinical Microbiology Reviews* **17**: 72-97.

Xiao, L. and Ryan, U.M. (2004) Cryptosporidiosis: an update in molecular epidemiology. *Current Opinions in Infectious Disease* **17**: 483-490.

Yagita, K., Izumiyama, S., Tachibana, H., Masuda, G., Iseki, M., Furuya, K., Kameoka, Y., Kuroki, T., Itagaki, T. and Endo, T. (2001) Molecular characterization of *Cryptosporidium* isolates obtained from human and bovine infections in Japan. *Parasitology Research* **87**: 950-955.

Young, K.H., Bullock, S.L., Melvin, D.M. and Spruill, C.L. (1979) Ethyl acetate as a substitute for diethyl ether in the formalin-ether sedimentation technique. *Journal of Clinical Microbiology* **10**: 852-853.

Zarlenga, D.S. and Trout, J.M. (2004) Concentrating, purifying and detecting waterborne parasites. *Veterinary Parasitology* **126**: 195-217.

Zhou, L., Singh, A., Jiang, J. and Xiao, L. (2003) Molecular surveillance of *Cryptosporidium* spp. in raw wastewater in Milwaukee: implications for understanding outbreak occurrence and transmission dynamics. *Journal of Clinical Microbiology* **41**: 5254-5257.

Zierdt, W.S. (1984) Concentration and identification of *Cryptosporidium* sp. by use of a parasite concentrator. *Journal of Clinical Microbiology* **20**: 860-861.

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-bye 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 feilds (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.

APPENDIX B: Caldew Catchment *Cryptosporidium* Results (IFAT and PCR positive samples)

Sample reference	Sampling phase	Stock status or event-based flow category	Sample site	Sample description	IFAT result*	Oocyst count per 10 l equivalent water	Microscopy comments	IMS	Nested SSU PCR amplicon	Nested SSU PCR-RFLP result	SSU rDNA sequencing result	ML1	ML2	gp15/60	Multilocus fragment type (MLFT)
P0097	1	Re-stocking	Farm A	Fox Faeces	+			Y	Y	C. parvum		242	215	344	P 32
P0099	1	Re-stocking	Farm A	Fox Faeces	+			Y	N	N					
P0156	1	Re-stocking	Farm B	Cow Faeces	+			Y	N	N					
P0245	1	Continually stocked	Farm C	Fox Faeces	+			Y	N	N					
P0447	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0448	1	Continually stocked	Farm D	Calf Faeces	++			Y	Y	C. parvum		neg	neg	neg	
P0450	1	Continually stocked	Farm D	Calf Faeces	+			Y	N	N					
P0451	1	Continually stocked	Farm D	Calf Faeces	+++			Y	Y	novel	novel	neg	neg	neg	
P0453	1	Continually stocked	Farm D	Roe Deer Faeces	+		Possibly 2 sizes of oocysts	Y	N	N					
P0455	1	Continually stocked	Farm D	Fox Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0466	1	Continually stocked	Farm D	Calf Faeces	+			Y	N	N					
P0470	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	215	neg	
P0471	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0481	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0483	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum (weak)		neg	neg	neg	
P0484	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	271	neg	
P0494	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0495	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0496	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0500	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0503	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0504	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0505	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum (weak)		neg	neg	neg	
P0507	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0509	1	Continually stocked	Farm D	Calf Faeces	+			Y	Y	C. parvum		neg	neg	neg	
P0542	1	Continually stocked	Farm C	Lamb Faeces	+++			Y	Y	C. parvum		242	232	338	P 7
P0703	2	Re-stocked	Farm A	Calf Faeces	+			Y	Y	C. parvum		242	232	341	P 5
P0706	2	Re-stocked	Farm A	Calf Faeces	+			Y	Y	C. parvum		242	232	341	P 5
P0707	2	Re-stocked	Farm A	Calf Faeces	++			N	Y	C. parvum		242	232	341	P 5
P0708	2	Re-stocked	Farm A	Calf Faeces	+			Y	N	N					

ID		Management	Site	Sample	Intensity	Small Crypto-like bodies also		Species				
P0711	2	Re-stocked	Farm A	Calf Faeces	++	Y	Y	C. parvum	neg	neg	neg	
P0712	2	Re-stocked	Farm A	Calf Faeces	+	Y	Y	C. parvum	232		341	P 5
P0715	2	Re-stocked	Farm A	Calf Faeces	+	Y	Y	C. parvum	232		341	P 5
P0721	2	Re-stocked	Farm A	Calf Faeces	+	Y	Y	C. parvum	232		341	P 5
P0722	2	Re-stocked	Farm A	Calf Faeces	+++	N	Y	C. parvum	232		341	P 5
P0723	2	Re-stocked	Farm A	Calf Faeces	++	N	Y	C. parvum	232		341	P 5
P0724	2	Re-stocked	Farm A	Calf Faeces	+	Y	Y	C. parvum	232		341	P 5
P0725	2	Re-stocked	Farm A	Calf Faeces	+	Y	Y	C. parvum	232		341	P 5
P0726	2	Re-stocked	Farm A	Calf Faeces	+	Y	Y	C. parvum	neg		341	P 5
P0727	2	Re-stocked	Farm A	Calf Faeces	+	Y	Y	C. parvum	232		neg	
P0728	2	Re-stocked	Farm A	Calf Faeces	+	Y	N	C. parvum	neg		neg	
P0766	2	Re-stocked	Farm A	Lamb Faeces	+	N	N	N	neg	neg	neg	
P0875	2	Continually stocked	Farm D	Calf Faeces	+	Y	Y	C. parvum	neg	neg	neg	
P0879	2	Continually stocked	Farm D	Calf Faeces	+	Y	N	N	neg	neg	neg	
P0886	2	Continually stocked	Farm D	Calf Faeces	++	N	Y	C. parvum	neg	230	347	P 13
P0889	2	Continually stocked	Farm D	Calf Faeces	++	N	Y	C. parvum	242	230	347	P 13
P0891	2	Continually stocked	Farm D	Calf Faeces	+	Y	Y	C. parvum	242	230	neg	P 13
P0894	2	Continually stocked	Farm D	Calf Faeces	+	Y	Y	C. parvum	242	230	347	P 13
P0898	2	Continually stocked	Farm D	Calf Faeces	+	Y	Y	C. parvum	neg	neg	neg	P 13
P0899	2	Continually stocked	Farm D	Calf Faeces	+	N	N	N	neg	neg	neg	
P0902	2	Continually stocked	Farm D	Calf Faeces	+	Y	Y	C. parvum	242	230	347	P 13
P0912	2	Continually stocked	Farm D	Calf Faeces	++	N	Y	C. parvum	242	230	347	P 13
P0913	2	Continually stocked	Farm D	Calf Faeces	+	Y	Y	C. parvum	242	230	347	P 13
P0914	2	Continually stocked	Farm D	Calf Faeces	++	N	Y	C. parvum	242	230	347	P 13
P0915	2	Continually stocked	Farm D	Calf Faeces	+++	N	Y	C. parvum	242	230	347	P 13
P0916	2	Continually stocked	Farm D	Calf Faeces	+++	N	Y	C. parvum	242	230	347	P 13
P0918	2	Continually stocked	Farm D	Calf Faeces	+	Y	Y	C. parvum	242	230	347	P 13
P0938	2	Continually stocked	Farm D	Calf Faeces	++	N	Y	C. parvum	242	230	347	P 13
P0939	2	Continually stocked	Farm D	Calf Faeces	++	N	Y	C. parvum	242	230	347	P 13
P0940	2	Continually stocked	Farm D	Calf Faeces	+++	N	Y	C. parvum	242	230	347	P 13
P0941	2	Continually stocked	Farm D	Calf Faeces	+++	N	Y	C. parvum	242	230	347	P 13
P0942	2	Continually stocked	Farm D	Calf Faeces	++	N	Y	C. parvum	242	230	347	P 13
P0943	2	Continually stocked	Farm D	Calf Faeces	++	Y	Y	C. parvum	242	230	347	P 13
P0944	2	Continually stocked	Farm D	Calf Faeces	+++	N	Y	C. parvum	242	230	347	P 13
P0949	2	Continually stocked	Farm D	Roe Deer Faeces	+	N	Y	N	neg	neg	neg	
P1048	2	Continually stocked	Site 6	Roe Deer Faeces	+	Y	Y	C. parvum Small Crypto-like bodies also	neg	neg	neg	

ID	Grp	Management	Site	Source	Result	Value	Notes	DAPI	DAPI	Species	Species	n1	n2	n3	P
P1243	2	Continually stocked	Farm C	Sheep Faeces	+		Plus 1 large Crypto-like body	Y	N	C. parvum		neg	neg	neg	
P1279	2	Continually stocked	Farm C	Sheep Faeces	+			Y	N	N		neg	neg	neg	
P1311	2	Continually stocked	Farm C	Sheep Faeces	+			Y	N	N					
H03	1	high flow	Site 4	Surface Water	Pos	1.53	Also CLBs but DAPI -ve	Y	Y	C. andersoni	C. andersoni	neg	neg	neg	
H04	1	pre-event base flow	Site 2	Surface Water	Pos	0.22		Y	N	N					
H05	1	high flow	Site 1	Surface Water	Pos	0.22		Y	N	N					
H06	1	high flow	Site 2	Surface Water	Pos	3.03		Y	Y	Novel	Novel	neg	neg	neg	
H07	1	post-event base flow	Site 5	Surface Water	Pos	20.29		Faint Y	Faint Y	C. parvum		242	232	338	
H08	1	high flow	Site 3	Surface Water	Pos	1.99	Bodies outside 5 micron size, 8.5 - 10 x 5 micron, 2 with sporozoites and 2 without	Y	N	N					
H09	1	pre-event base flow	Site 5	Surface Water	Pos	1.66		Y	Y	C. andersoni		neg	neg	neg	
H10	1	pre-event base flow	Site 6	Surface Water	Pos	6.24		Y	N	N					
H11	1	post-event base flow	Site 6	Surface Water	Pos	5.71	3 objects 7-8 micron 1 with sporozoites	Y	N	N					
H12	1	post-event base flow	Site 7	Surface Water	Pos	5.93		Y	N	N					
H13	1	pre-event base flow	Site 8	Surface Water	Pos	0.57		Y	N	N					
H15	1	pre-event base flow	Site 4	Surface Water	Pos	0.37		Y	Y	C. andersoni		neg	neg	neg	
H16	1	post-event base flow	Site 8	Surface Water	Pos	3.64	3 larger bodies, no sporozoites, 7-9 x 5-6 micron	Y	N	N					
H19	1	post-event base flow	Site 3	Surface Water	Pos	0.60		Y	N	N					
H21	1	high flow	Site 6	Surface Water	Pos	1.67		Y	N	N					
H23	1	high flow	Site 8	Surface Water	-	0.00		Y	Faint Y	C. andersoni		neg	neg	neg	
H25	1	high flow	Site 5	Surface Water	Pos	4.41		Y	Faint Y	C. andersoni		neg	neg	neg	
H40	2	pre-event base flow	Site 10	Surface Water	Pos	1.08	12 CLBs 5-6 micron, 1 possible C. andersoni	Y	Y	Novel	C. andersoni	neg	neg	neg	
H42	2	supplementary	Site 7	Surface Water	Pos	49.00		Y	Y	C. parvum		242	232	341	P 5
H43	2	supplementary	Site 11	Surface Water	Pos	9.00		Y	N	N					
H47	2	high flow	Site 4	Surface Water	Pos	3.54	2x 7 micron objects, sporozoites, 4 empty	Y	Y	C. andersoni		neg	neg	neg	
H48	2	high flow	Site 5	Surface Water	Pos	1.52		Y	N	N					
H50	2	high flow	Site 7	Surface Water	Pos	16.09		Y	Faint Y	C. parvum		242	230	350	P 33
H51	2	high flow	Site 8	Surface Water	Pos	6.90		Y	Y	C. parvum		242	232	338	P 7
H53	2	high flow	Site 10	Surface Water	Pos	10.34	Approx 70 objects 6x8 micron, contents no sporos	Y	Y	C. andersoni		242	232	338	P 7
H54	2	high flow	Site 11	Surface Water	Pos	4.30	2 objects 6 - 8 micron, contents no sporos	Y	N	N					
H58	2	post-event base flow	Site 4	Surface Water	-	0.00		Y	Faint Y	C. andersoni		neg	neg	neg	
H60	2	post-event base flow	Site 6	Surface Water	Pos	2.07		Y	N	N					
H61	2	post-event base flow	Site 7	Surface Water	Pos	5.68		Y	N	N					
H62	2	post-event base flow	Site 8	Surface Water	Pos	1.52	One oocyst 8x7 micron, 4 sporozoites	Y	N	N					
H64	2	post-event base flow	Site 10	Surface Water	-	0.00		Y	Y	C. andersoni	C. andersoni	neg	neg	neg	

| H65 | 2 | post-event base flow | Site 11 | Surface Water | Pos | 0.60 | Y | Y | N | N | 242 | 232 | 341 | P 5 |
| H66 | 2 | supplementary | Farm A | Land Drain | Pos | 4000.00 | Y | Y | N | C. parvum | 242 | 232 | 341 | P 5 |

Samples from National Collection of Cryptosporidium oocysts, resident within catchment

CRU ref.	Specimen date	Primary testing lab	Postcode	Origin	Age	Sex	Original diagnostic test	IMS	COWP PCR-RFLP	ML1	ML2	GP15/61	MLFT
W0144	29/03/2000	Carlisle PHL	CA7	Human clinical case	25	Female	modified Ziehl-Neelsen stain	N	C. parvum	242	207	338	P 34
W0240	28/04/2000	Carlisle PHL	CA2	Human clinical case	58	Male	modified Ziehl-Neelsen stain	N	C. parvum	neg	neg	neg	
W0456	14/05/2000	Carlisle PHL	CA2	Human clinical case	33	Female	modified Ziehl-Neelsen stain	N	C. parvum	242	234	338	P 6
W1871	11/09/2000	Carlisle PHL	CA2	Human clinical case	12	Female	modified Ziehl-Neelsen stain	N	C. hominis	233	180	371	H 1
W2360	25/10/2000	Carlisle PHL	CA2	Human clinical case	32	Female	modified Ziehl-Neelsen stain	N	C. hominis	233 + 227	180	329 + 371	H 1?+??
W4878	25/02/2002	Carlisle PHL	CA11	Human clinical case	1	Male	modified Ziehl-Neelsen stain	N	C. parvum	242	230	338	P 2
W5018	05/04/2002	Carlisle PHL	CA5	Human clinical case	1	Male	modified Ziehl-Neelsen stain	N	C. parvum	242	232	341	P 5
W6132	06/11/2002	Carlisle PHL	CA2	Human clinical case	NK	Male	modified Ziehl-Neelsen stain	N	C. parvum	242	203	338	P 35
W6325	02/12/2002	Carlisle PHL	CA2	Human clinical case	2	Female	modified Ziehl-Neelsen stain	N	C. hominis	233	180	371	H 1
W8265	13/10/2002	Carlisle PHL	CA2	Human clinical case	25	Female	modified Ziehl-Neelsen stain	N	C. hominis	233	180	371	H 1
W8609	11/12/2002	Carlisle PHL	CA2	Human clinical case	3	Male	modified Ziehl-Neelsen stain	N	C. hominis	233	180	371	H 1
A484	Sep-02	VLA Penrith		Bovine clinical case	calf		modified Ziehl-Neelsen stain	N	C. parvum	242	230	350	P 33
A486	Aug-02	VLA Penrith		Bovine clinical case	calf		modified Ziehl-Neelsen stain	N	C. parvum	242	230	338	P 2

* Semi-quantitative: + = 2 to 5 oocysts per field x400 mag; ++ = 6 to 10, +++ = 11-50, ++++ = >50