# Sub-species molecular typing of Cryptosporidium parvum

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# Sub-Species Molecular Typing of

# *Cryptosporidium parvum*

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Of the requirements for the degree of

**Doctor of Philosophy**

by

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CRC - Water Quality and Treatment

School of Civil and Environmental Engineering

## Certificate of Originality

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## Abstract

*Cryptosporidium parvum* is a protozoan parasite that infects virtually all mammalian hosts, including neonates of common livestock (cattle, sheep, horses, pigs). The organism has a sexual reproductive cycle occurring in the gastrointestinal tract of the host. An auto infective component of the cycle produces large numbers of environmentally resistant oocysts that are shed in the faeces. The majority of significant cryptosporidiosis outbreaks have been attributed to public water systems and 1998 *Cryptosporidium* was detected in Sydney's filtered drinking water.

Genotyping of *Cryptosporidium* has developed over the past 10 years and serval different primer sets provide reliable differentiators of *Cryptosporidium's* host of origin *ie*. TRAP-C1, TRAP-C2, COWP, β-tubulin, *Hsp*70 and various rRNA primer sets. Not all the primer sets which were used are *C. parvum* specific, cross-reacting with *C. hominis*, *C. meleagridis* and sometimes other *Cryptosporidium* species.

Conventional diagnostic methods for cryptosporidiosis based on morphological features have limitations and restrict clear species identification (Morgan *et al.* 1998; Morgan *et al.* 1998). Application of sensitive molecular approaches have indicated genetic heterogeneity among isolates of *Cryptosporidium* from different species of vertebrates. The data gathered suggests that a series of host-adapted genotypes-strains-species of the parasite may exist. The predictive value of achieving a sound understanding of the host *Cryptosporidium* interactions requires sub-species typing. An effective molecular based tying strategy would increase our understanding of the epidemiology, transmission and controlling outbreaks of the disease. Furthermore, environmental managers, swimming pool operators and water utilities would have an increased knowledge and understanding of *Cryptosporidium* interactions within their controlled areas. This understanding would also aid in sound management by treatments plant operators and swimming pool operators, so as to effectively minimise outbreaks associated with their water service.

The PCR typing protocol which has been developed is effective in sub-species typing of *C. parvum, C. hominis*. The T primer, is a 2 times teleomere repeat based on the

most frequent repeat unit found in the telomeric region of *C. parvum* (Liu *et al.* 1998). When this primer is used alone in a PCR, it does not produce products. The *Cryptosporidium* specificity is reliant upon the M1 being in the 3' area of a chromosome and in close proximity to the T primers. When combined they are able to be used to differentiate *Cryptosporidium* from different sampling locations rather thank just the host origin.

The method has successfully be used to produce DNA fingerprints for *Cryptosporidium* from both animal (Chapter 3) and human (Chapter 4) origin genotypes. Effective fingerprints were produced from samples of three types including widely spaced geographical sources. These included multiple hosts at the same dairy herd location, both in samples at the same date and sample dates extending over a four year period, as well as samples of a defined and passage-propagated isolate. It distinguishes between unrelated sources within a narrow geographical area (*eg.* 20 km radius). Reproducible fingerprints were achieved for each isolate (up to 10) taken from the same location on the same sampling date. The fingerprints observed in samples from Syd-SW appeared to change over the full four-year period of observation. Comparison to samples of the defined "Iowa" strain over a comparable period (2 years) revealed no change. Examination of individual isolates over a longer period may shed light on the long-term stability of the target region of the *Cryptosporidium* genome. The PCR method is applicable to DNA extracted from faeces without oocyst isolation. That is stable for a single homogenous source over time. With the human isolates from outbreaks the M1 + T PCR distinguishes between related and unrelated cases among an epidemiologic contact group.

Based on results reported here, the differentiation provided by the primer set in combination with apparent gene region stability establishes a useful procedure for epidemiologic investigation of outbreaks and related infections. Current applications of the protocol is useful for the water industry in the determination of clinical human isolates which form part of outbreaks ie swimming pool, veterinary industry tracking at dairy farms and outbreaks within domestic animals as well as handlers. When the additional further developments are carried out as described in section 5.4 are completed, additional applications may then be added. Such as being able to apply this

method for the detection of *Cryptosporidium* fingerprints from environmental isolates. Being able to apply this PCR method to samples would give a tracking and tracing tool That could be applied to the identification of an outbreak source. Furthermore, the application could also be used for the tracing of *Cryptosporidium* isolate stability in environmental sources *eg.* longitudinal studies at farms. An environmental water application could be to use the technique as a method for identification of sources of *Cryptosporidium* within a catchment or during water associated outbreaks of *Cryptosporidium*. The later application would be used in conjunction with widely used genotyping methods to fully characterise isolates which are responsible for the contamination event. A further use for the M1 + T PCR method would be to identify the strain or strains within an outbreak group or to monitor the stability of an isolate at an environmental location.

**References:**

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Ms. Marilyn Marshall, University of Arizona, Tucson USA,
Dr. Martha Sinclair, Monash University Melbourne, Victoria, Australia and
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## Abbreviations:

The abbreviations used within this thesis are those found in the "Instructions to Authors" (2004) *Biochem J.* **http://www.biochemj.org/bj/bji2a.htm#table4**

| | |
|---|---|
| COWP | *Cryptosporidium* Oocyst Wall Protein |
| DAPI | 4',6-diamidino-2-phenylindole, dihydrochloride |
| DHFR | Dihydrofolate reductase |
| FITC | Fluorescein isothiocyanate |
| HACCP | Hazard Analysis and Critical Control Point |
| *hTERT* | Human telomerase reverse transcriptase gene |
| Hsp 70 | Heat Shock Protein 70 |
| IFA | Immuno-florescent Antibody |
| IMS | Immuno-Magnetic Separation |
| ITS 1 | Intergenic spacer region of rRNA |
| PCR-RFLP | Polymerase Chain Reaction – Restriction Length Polymorphism |
| PI | Propidium iodide |
| Poly(T) | Open reading frame with several series of T repeats |
| TRAP-C1 | Thrombospondin-Related Adhesive Protein of *Cryptosporidium* – C1 |
| TRAP C2 | Thrombospondin-Related Adhesive Protein of *Cryptosporidium* – C2 |

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## 1.1 *Cryptosporidium* Introduction

The first *Cryptosporidium* species to be described was by Tyzer. Tyzer provided a brief but adequate description of what was most likely *Cryptosporidium muris*, from *Mus musculus* and established the genus *Cryptosporidium*. A second member of the genus, *Cryptosporidium parvum*, again from a mouse was identified by Tyzer in 1912 (Fayer *et al.* 1990). *C. parvum* was described as a new species based on differences in the morphology of both the oocyst and developmental stages compared to the previously identified *C. muris*.

*Cryptosporidium* spp. were classified as unicellular protozoa within the phylum *Apicomplexa*, order *Eucoccidiorida*, suborder *Eimeriorina* and family *Cryptosporidiidae* (DuPont *et al.* 1995). However, recent discussion places the genus in another family and molecular biology techniques have been utilized in re-defining the taxonomy. Molecular tools have revealed the extent of genetic diversity in *C. parvum* and this information has made a significant contribution to studies on its population genetics, phylogeny and taxonomy (Thompson and Chalmers 2002). Epidemiology studies have benefited from the application of molecular biology techniques especially when studying parasite life cycles and transmission. In 1990 there were only six species of *Cryptosporidium* recognised, whereas today, 14-15 species are now accepted as a result of molecular characterisation (Ryan *et al.* 2004; Xiao *et al.* 2004). Additionally there are at least eight distinct genotypes which appear to be host-adapted and are currently being debated as to warrant species status (Thompson and Chalmers 2002; Xiao *et al.* 2000). Nonetheless, multiple species of *Cryptosporidium* can occur in each vertebrate host. This has resulted in several new species of *Cryptosporidium* being named (Xiao *et al.* 2004).

Presently, the identification and naming of genotypes is based largely on host origin (Table 1.1). The genotype designation has generally reflected the significant genetic differences among *Cryptosporidium* isolates, yet not all genotypes differ from each other to the same extent. The term "subgenotype" is used to describe relatively minor intragenotypic variations. The use of genotypes and subgenotypes has become very common yet there are no clear guidelines to make the determinations (Xiao *et al.* 2004).

Nevertheless, the different species largely infect different hosts (Casemore *et al.* 1994; Flanigan and Soave 1992; Goodgame 1996; Juranek 1995; Keusch *et al.* 1995; Ryan *et al.* 2004) and include *C. andersoni* (cattle), *C. baileyi* (chicken and some other birds), *C. canis* (dogs), *C. felis* (cats), *C. galli* (birds), *C. hominis* (humans), *C. meleagridis* (birds and humans), *C. molnari* (fish), *C. muris* (rodents and some other mammals), *C. parvum* (ruminants, marsupials and humans), *C. wrairi* (guinea pigs), *C. suis* (pigs), *C. saurophilum* (lizards and snakes), and *C. serpentis* (snakes and lizards) (Table 1.1). Other morphologically distinct *Cryptosporidium* spp. have been found in fish, reptiles, birds and mammals and are yet to be named (Xiao *et al.* 2004).

The well recognised *C. parvum* genotypes which are host adapted to predominately infect 'human' and 'cattle' were often referred to as genotypes I and II respectively (section 1.4). In recent years many of the previously host-adapted genotypes of *C. parvum* have been well characterised. Several have been redescribed as different species, hence the increase in *Cryptosporidium* spp. number in the last couple of years (Thompson and Chalmers 2002; Xiao *et al.* 2004). A confusing situation is arising in the literature due to an increasing number of reports describing novel genotypes, which are being increasingly isolated from both environmental samples and different species of vertebrate hosts. One example was reported by McLauchlin *et al.* (2000) where a numerical system was used as a reference to genetically distinct forms of *C. parvum* isolated from a range of clinical samples. The report referred to a "genotype III" which corresponded to *C. meleagridis.* Another recent publication by Perz and Le Blancq (2001), molecular biology techniques were used to characterise *C. parvum* isolated from wildlife, two novel genotypes were referred to as genotype III and IV. From the information which was provided in both journal articles there seems to be no evidence that the genotype III described was identical (Thompson and Chalmers 2002).

Table 1.1: *Cryptosporidium* species and host of origin

(Fayer R *et al.* 2005; Ryan *et al.* 2004; Xiao *et al.* 2004)

| **Species** | **Major host** | **Minor host** |
|---|---|---|
| *C. andersoni* | Cattle, bactrian camels | Sheep |
| *C. baileyi* | Chickens, turkeys | Cockatiels, quails, ostriches, ducks |
| *C. bovis* | Cattle | |
| *C. canis* | Dogs | Humans |
| *C. felis* | Cats | Humans, cattle |
| *C. galli* | Finches, chicken, capercalles, grosbeaks | |
| *C. hominis* | Humans, monkeys | Dugongs, sheep |
| *C. meleagridis* | Turkeys, humans | Parrots |
| *C. molnari* | Fish | |
| *C. muris* | Rodents, bactrian camels | Humans, rock hyrax, mountain goats |
| *C. parvum* | Cattle, sheep, goats, Humans | Deer, mice, pigs |
| *C. saurophilum* | Lizards | Snakes |
| *C. serpentis* | Snakes, lizards | |
| *C. suis* | Pigs | |
| *C. wrairi* | Guinea pigs | |

Host-parasite co-evolution is also common in *Cryptosporidium*, as closely related hosts usually had related *Cryptosporidium* parasites. Results of phylogenetic analyses suggest that the *Cryptosporidium* bovine genotype and *C. meleagridis* were originally parasites of rodents and mammals, respectively, but have subsequently expanded their host ranges to include humans (Xiao *et al.* 2002). Understanding the evolution of *Cryptosporidium* species is important not only for clarification of the taxonomy of the parasites but also for assessment of the public health significance of *Cryptosporidium* parasites from animals (Xiao *et al.* 2002).

Of the *Cryptosporidium* species, genotype I (*C. hominis)* is the recognised causative agent of several waterborne outbreaks (Dalle *et al.* 2003; Leoni *et al.* 2003; Rose *et al.* 2002) and poses the greatest risk to humans. *C. hominis* is the primary species, known to infect humans, however genotype II is found in several mammalian species that act as carriers (humans, cattle pig, sheep, goat, *etc*), which leads to broad environmental distribution of potentially human infectious oocyst (Juranek 1995).

It was not until 1976 that two independent journal reports revealed *C. parvum* was able to infect humans, with Nime *et al.* (1976) describing *C. parvum* in the stool of a 3-year-old girl from Tennessee, USA. In the same year Meisel *et al.* (1976) reported a

39-year-old man with diarrhoea, duodenal and ileal biopsies revealing severe mucosal injury. The organisms described were 2-4 μm spherical structures on the epithelial surfaces, which were determined to be *C. parvum* (Fayer *et al.* 1997).

Little is known about the causes of sporadic cryptosporidiosis in humans. Low numbers of *C. parvum* oocysts are commonly detected in surface water supplies (Barwick *et al.* 2000; CDC 1984; Chung *et al.* 1999; French *et al.* 2000; Hancock *et al.* 1998; Lerman de Abramovich *et al.* 1999; Morris and Foster 2000; Skerrett and Holland 2000; Stein 2000; Zanelli *et al.* 2000). It is not known how few oocysts can cause disease, but the minimum dose used by DuPont *et al.* (1995) was 30 oocysts. Part of the uncertainty relates to current detection methods being insensitive and not distinguishing between viable and non-viable oocysts. Furthermore, standard methods, which rely on fluorescently-labelled monoclonal antibodies, do not discriminate between different species of *Cryptosporidium(US-EPA 1999)*.

### 1.1.1 Life Cycle of *C. parvum*

The life cycle of *C. parvum* is depicted in Figure 1.1 and begins with ingestion of the sporulated oocyst. The oocyst is the resistant stage found in the environment. Each oocyst contains four infective sporozoites (A), which exit from the oocyst. The preferred site of infection is the mammalian ileum and sporozoites penetrate individual epithelial cells in this region (B) Figure 1.2. Parasites reside on the lumenal surface of the cells (C) and were once thought to occur extracellularly. However, ultrastructural observations have clearly shown these parasites to be intracellular, enclosed by a thin layer of host cell cytoplasm (Fayer *et al.* 1997). A desmosome-like attachment organelle, plus accessory folding of the parasite membranes, develop at the interface between the parasite proper and the host cell cytoplasm, which results in the formation of eight merozoites within a meront. These meronts are termed Type I (D, E) meronts and rupture, releasing free merozoites. Once these merozoites penetrate new cells, they undergo merogony to form additional meronts. Type I merozoites are thought to be capable of recycling indefinitely, hence the potential exists for new Type I meronts to arise continuously via asexual reproduction (Fayer *et al.* 1997).

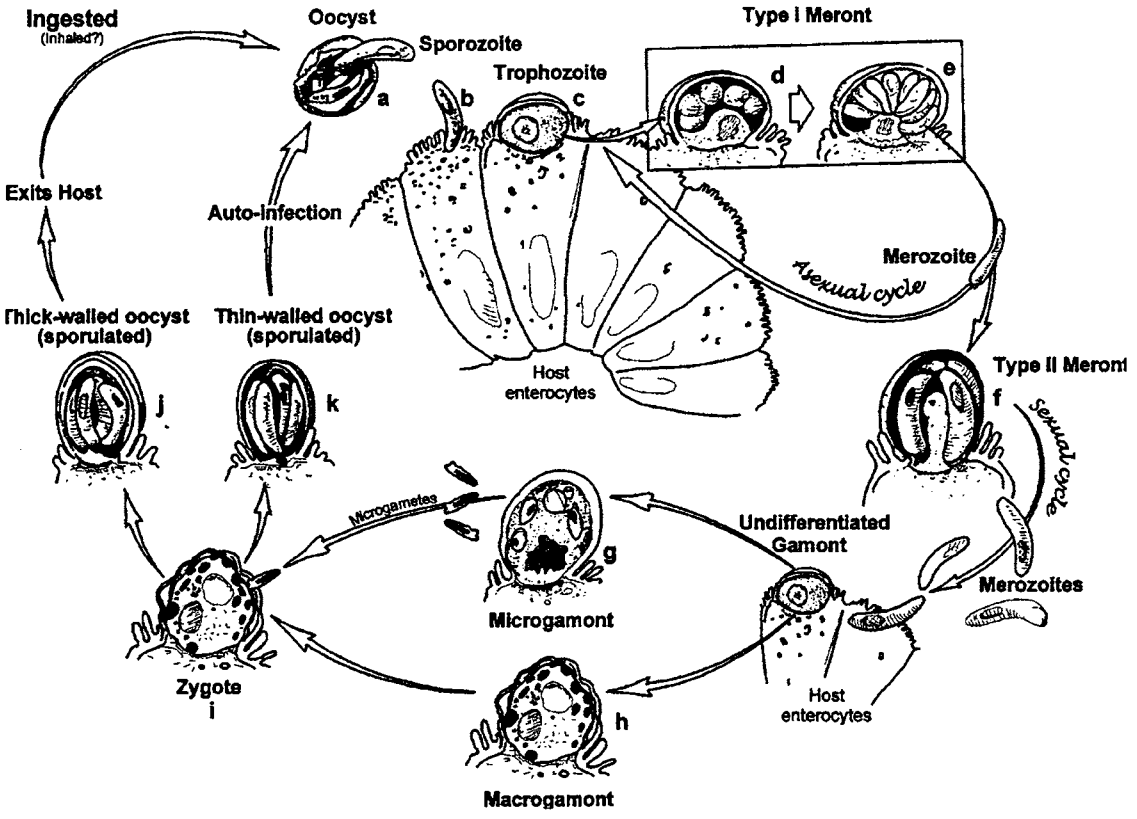


Figure 1.1: Life cycle of *C. parvum*
(Juranek 2000)

It is thought that some Type I merozoites are somehow triggered into forming a second type of meront, the Type II meront (F, Figure 1.1), which contains only four merozoites. Once liberated, the Type II merozoites form the sexual stages. Some Type II merozoites enter cells, enlarge and form macrogametes (H). Others undergo multiple fission once inside cells, forming microgametocytes containing sixteen non-flagellated microgametes (G). A zygote (I) is formed when the microgametes rupture from the microgametocyte and penetrate macrogametes. A resistant oocyst wall is then formed around the zygote (the only diploid stage in the life cycle), meiosis occurs and four sporozoites are formed in the process (J). Formation of sporozoites is termed sporogony. These oocysts form in the epithelial infected cells (Figure 1.2) are passed in the faeces and into the environment, but only the thick-walled oocytes (J) persist in the environment (Juranek 2000).

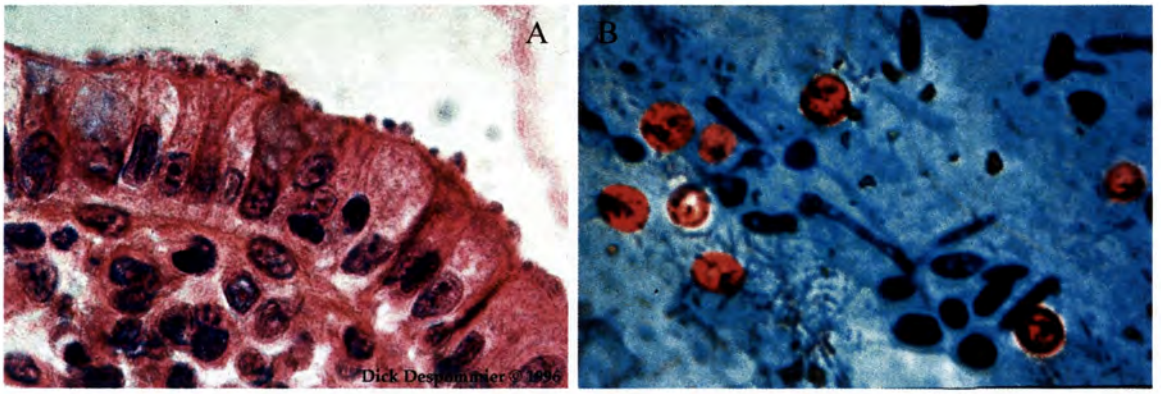


Figure 1.2: *C. parvum* in the gastrointestinal tract and stool sample

A: *C. parvum* - Trophozoite ring forms in red blood cells

B: *C. parvum* - Cysts in stool (Acid fast staining )

(http://www-medlib.med.utah.edu/parasitology/Cryptospor.html)

It has been estimated that approximately 20 % of the oocysts produced in the gut fail to form an oocyst wall (Fayer *et al.* 1997). A series of membranes surround the developing sporozoites. These "oocysts", devoid of a wall, are sometimes termed "thin-walled oocysts" (K, Figure 1.1). It is believed that the resulting sporozoites produced from thin-walled oocysts can excyst while still within the gut and infect new cells. Thus, *C. parvum* appears to have two autoinfective cycles one which is a continuous recycling of Type I meronts and the second where the sporozoites rupture from thin-walled oocysts.

Shedding experiments under laboratory conditions have been carried out. The prepatent period is the shortest time after ingestion of the infective oocysts to complete the endogenous life cycle and excrete newly developed oocysts (Fayer 1997). This time is dependent on two factors:

- the host being infected and
- the species of *Cryptosporidium*.

Patency is the length of time oocysts are in the host before they are shed in the faeces (Fayer 1997). The patency period of *C. parvum* in calves has been determined to be 2 - 7 days (Lorenzo *et al.* 1998) and 4 - 22 days in humans (Anonymous 1984; Anonymous 1991; Moore *et al.* 1993; Moore *et al.* 1994).

### 1.1.2 Life Cycle Research

Figure 1.3 illustrates the estimated waterborne acute gastrointestinal illness attributed to various microrganisms within the USA. In recent years *C. parvum* has been associated with consumption of (filtered or unfiltered) contaminated public drinking water (*e.g.* in the Milwaukee, USA, 1994, approximately 403 000 people were affected) (MacKenzie *et al.* 1994; Rose 1997). Although researchers are able to recover small numbers of oocysts from treated drinking water, current routine laboratory methods do not enable them to determine if these oocysts are viable or infectious.

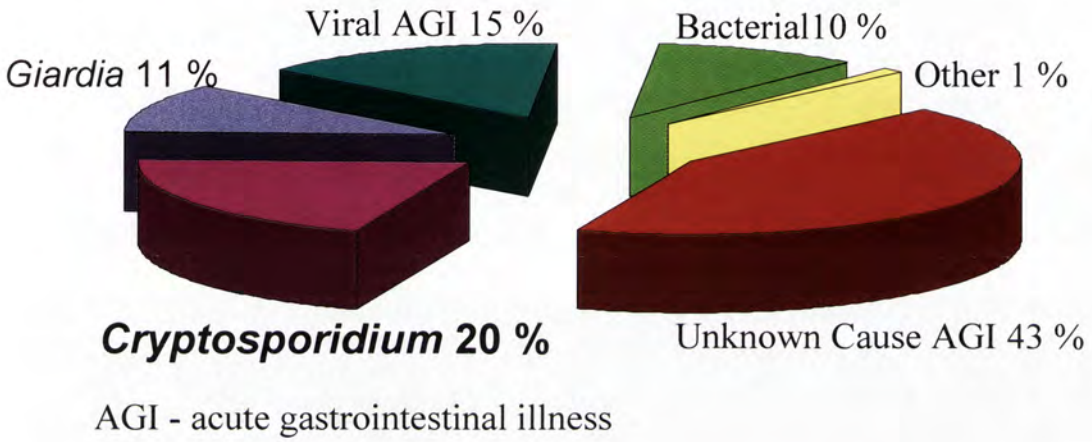


Figure 1.3: Waterborne outbreaks of acute gastrointestinal illness attributed to various microorgasisms.
(Anonymous 1984; Barwick *et al.* 2000; Kramer *et al.* 1996; Levy *et al.* 1998)

The usefulness of the nuclear fluorochrome 4', 6-diamidino-2-phenylindole (DAPI) as an adjunct in the identification of *Cryptosporidium* spp. oocysts by immunofluorescence has been studied. Because DAPI binds to the nuclei of sporozoites contained within oocysts it can be used to confirm the presence of sporulated oocysts by epifluorescence microscopy (Grimason *et al.* 1994). This increased number of observable internal features enhances the ability to identify sporulated oocysts derived from water samples. This method should facilitate confirmation of putative *Cryptosporidium* spp. oocysts detected in water and water-related samples also reducing the likelihood of either false-positives or false-negative results (Smith *et al.* 2002).

Until recently the use of cell culture was not available to *C. parvum* researchers (Hijjawi *et al.* 2001; Hijjawi *et al.* 2004; Rochelle *et al.* 2002; Slifko *et al.* 1997). A complete *C. parvum* life cycle within an *in vitro* cell culture system was described for the for the first time by Hijjawi *et al*, 2001 (Hijjawi *et al.* 2001; Hijjawi *et al.* 2004; Rochelle *et al.* 2002; Slifko *et al.* 1997). Hijjawi *et al*, 2001 described the complete development and long-term maintenance of *C. parvum* of both cattle and human genotypes, yet genotype I, could not be propagated or maintained in culture for a long period *i.e.* >25 days. Prior to Meloni and Thompson (1996) investigating the *in vitro* culturing there had only been two studies describing the complete development of *C. parvum* (not genotyped) originating from immunocompromised human patients (Current and Haynes, 1984; Burand *et al.* 1991). It would be most advantageous to genotype different isolates of *C. parvum* to allow interpretation of differences in their development, pathogenesis, host cell interactions and susceptibility to chemotherapeutic agents. A current limitation with cell culture is that it is labour-intensive and expensive. However, it has the potential to replace the current systems which purify oocysts from large animals such as pigs and calves. Hence, *in vitro* culturing systems should provide a model for the maintenance and propagation of human-infective *Cryptosporidium* genotypes.

### 1.1.3 Clinical Outcome and Treatment

Infection by *C. parvum* and C. *hominis* has been reported in six continents and identified in patients aged 3 days to 95 years (Flanigan and Soave 1992). Transmission is usually faecal-oral, often through water contaminated by mammalian faeces. In some individuals, specifically young children, the elderly and immunosuppressed patients, cryptosporidiosis can become chronic and life-threatening. At the present time it is nearly impossible to determine the origin of many individual cases of cryptosporidiosis.

Persons most likely to be infected by *Cryptosporidium* include (Casemore *et al.* 1994; Flanigan and Soave 1992; Goodgame 1996; Hunter *et al.* 2004; Juranek 1995; Keusch *et al.* 1995).

- infants and younger children in day-care centres,
- those whose drinking water that is unfiltered and not disinfected,
- involved in farming practices such as lambing, calving and muck-spreading,
- engaging in sexual practices that brings a person into oral contact with faeces of an infected individual,

- patients in a nosocomial setting with other infected patients or health-care employees,
- veterinarians who come in contact with farm animals,
- travellers to areas with untreated water,
- living in densely populated urban areas and
- owners of infected household pets (rare).

Hunter *et al.* (2004) identified the main risk factors as being travel abroad, contact with a patient, and handling cattle. Travel outside the United Kingdom was found to be significant for *C. hominis* infection but not illness due to *C. parvum.* The relationship between *C. hominis* infection and overseas travel was also identified as a risk factor by McLauchlin *et al.* (2000) and Nichols *et al.* (2002). The risk for *C. parvum* infection increased significantly upon contact with cattle when all patients were compared to controls. Previous research has associated farm animal contact with outbreaks of *Cryptosporidium* (Hunter *et al.* 2004). Direct calf contact and lamb contact was identified as risk factors for sporadic infection (Robertson *et al.* 2002). Several outbreaks of *C. parvum* have been associated with farm visits within the United Kingdom. The risk for contact with other farm animals was not significant. The association was found to be with *C. parvum* but not *C. hominis* (Hunter *et al.* 2004). The various symptoms of cryptosporidiosis differ greatly between immunocompetent and immunocompromised individuals. In immunocompetent patients, cryptosporidiosis is an acute, yet self-limiting diarrhoeal illness (1 - 2 week duration). Symptoms include (Juranek 1995):

- frequent, watery diarrhoea,
- nausea,
- vomiting,
- abdominal cramps and
- low-grade fever

For immunocompromised persons, the illness is much more severe (Connolly 1990; Juranek 1995) including:

- debilitating, cholera-like diarrhoea (up to 20 litres/day),
- severe abdominal cramps,
- malaise,
- low-grade fever,
- weight loss and
- anorexia.

*C. parvum* is predominantly a parasite of neonate mammals. Although exceptions occur, older animals generally develop minor infections, even when unexposed

previously to this parasite. Experimental laboratory infections in immunosuppressed adult animals have shown that *C. parvum* build up slowly and only occasionally progress to the level found in neonates. In contrast, humans, are the one host that can be infected at any time in their lives (Fayer *et al.* 1997). However cryptosporidiosis can therefore be transmitted (via the oocyst) from person-to-person, ingestion of contaminated water (Figure 1.3) or food, from animal to person, or by oral contact with faecally contaminated environmental surfaces.

### 1.1.4 The Health Risk from *C. parvum*

Dose-response data are currently available for only a few isolates of *C. parvum* that were evaluated in healthy volunteers (Chappell *et al.* 2001), all of which were of the same sub-genotype GP60 group (Xiao, per com). In one study the 50 % Infectious Dose was ($ID_{50}$) estimated to be 132 oocysts. One volunteer was infected with as few as 30 oocysts (DuPont *et al.* 1995). Another study using a more virulent horse isolate (TAMU) suggests that $ID_{50}$ = 10 oocysts (Chappell *et al.* 1999). Teunis *et al.* (2002) analysed data from volunteer dose-response studies which compared three *C. parvum* isolates (Iowa, UCP and TAMU). Analysis of $ID_{50}$'s from these data ranged from a high of 1 042 oocysts for the UCP isolate to a low of nine oocysts for the TAMU. The Iowa fell between these extremes and had an $ID_{50}$ of 87 oocysts (Tuenis *et al.* 2002). Humans, like animals, appear to have various degrees of susceptibility to different strains of this parasite. The infective dose is likely to vary between individuals and amongst isolates and again different sub-genotypes should be examined.

Several surveys have been conducted to gain some idea about prevalence of the parasite in humans. In industrialised nations, around 0.4 % of the population appears to be passing oocysts in their faeces at any one time (Fayer *et al.* 1997). For patients who were admitted to hospitals for diarrhoea, 2 - 2.5 % were passing oocysts, yet sero-prevalence appeared to be much higher, 30 - 35 % of the US population have antibodies to *C. parvum* (Anonymous 1984; Anonymous 1991; Moore *et al.* 1994). In developing countries, the sero-prevalence is even higher and up to 60 - 70 % of people in these countries may have circulating antibodies to this pathogen. In AIDS patients, the numbers of individuals suffering from chronic cryptosporidiosis has been estimated to 10 % in industrialised nations and up to 40 % in other regions. In the last couple of

years, however, the use of proteinase inhibitors plus nucleoside analogs in AIDS patients has fortuitously lowered the actual numbers of AIDS patients suffering from chronic cryptosporidiosis in the US (Fayer *et al.* 1997). The very young, the elderly, pregnant women and the immunocompromised are at the greatest risk of serious illness and mortality from water and food borne enteric microorganisms (Gerba *et al.* 1996). This segment of the population currently represents almost 20 % of the population of the US and is expected to increase (Gerba *et al.* 1996). Pregnant mothers are some ten times more likely to die from waterborne disease than the general pop during outbreaks and immunocompromised and transplant patients greater risk of dying (Gerba *et al.* 1996).

### 1.1.5 Source of Infection and Risk Factors

Oocysts of *C. parvum* are widespread in the environment and can be found in surface lakes and streams and groundwater and are included within the outbreaks reported in Figure 1.4 A. Ruminants, cervids, swine, cats, dogs and other mammals as well as sewerage may all contribute to numbers of *Cryptosporidium* oocysts in the environment both in rural and urban areas. Hence it is no surprise that breakdowns in public water supply systems result in community outbreaks of cryptosporidiosis and other waterborne disease (Figure 1.4 B) (Anonymous 1984; Barwick *et al.* 2000; Kramer *et al.* 1996; Levy *et al.* 1998).

There is considerable circumstantial evidence that low level (non-epidemic) transmission of *C. parvum* species through drinking water may be occurring throughout the United States (Frost *et al.* 1995). Furthermore, studies indicate that *C. parvum* oocysts are present in 65 % - 97 % of surface waters (rivers, lakes *etc.*) tested throughout the USA (LeChevallier and Moser 1995; LeChevallier *et al.* 1991).

*C. parvum* species are highly resistant to chlorine disinfectants used in the treatment of drinking water (Korich *et al.* 1990). Therefore physical removal of the parasite from contaminated water by filtration is a key component in the supply of safe drinking water. However, a filtration system, especially one that is not well maintained and operated, does not afford absolute protection. Most waterborne outbreaks of cryptosporidiosis within the USA have occurred in communities where water utilities

met state and federal standards for acceptable quality of drinking water (Kaminski *et al.* 1994). Surveys of the occurrence of *C. parvum* oocysts in fully treated (disinfected and filtered) municipal water demonstrate that small numbers of oocysts breach filters and were present in tap water in 27 %-54 % of the communities evaluated (LeChevallier and Moser 1995; LeChevallier *et al.* 1991). The health significance of these oocysts is unknown, due to lack of data on human infective genotypes and the viability of the oocysts.

Nonetheless, there have been many outbreaks of human disease due to the consumption of contaminated water and in recent years outbreaks due to *C. parvum* have been the most common in the USA where the agent was identified. The largest reported outbreak involved over 400 000 people in the city of Milwaukee in 1993 (Mackenzie 1994). This outbreak was associated with some 100 deaths among persons with immune deficiencies. In general, during outbreaks of waterborne cryptosporidiosis, the levels of organisms seen in the drinking water have been low and in some cases have not been detected (MacKenzie *et al.* 1994; Mackenzie *et al.* 1995; Proctor *et al.* 1998). This is due in part to not recognising the outbreak until well after the event.

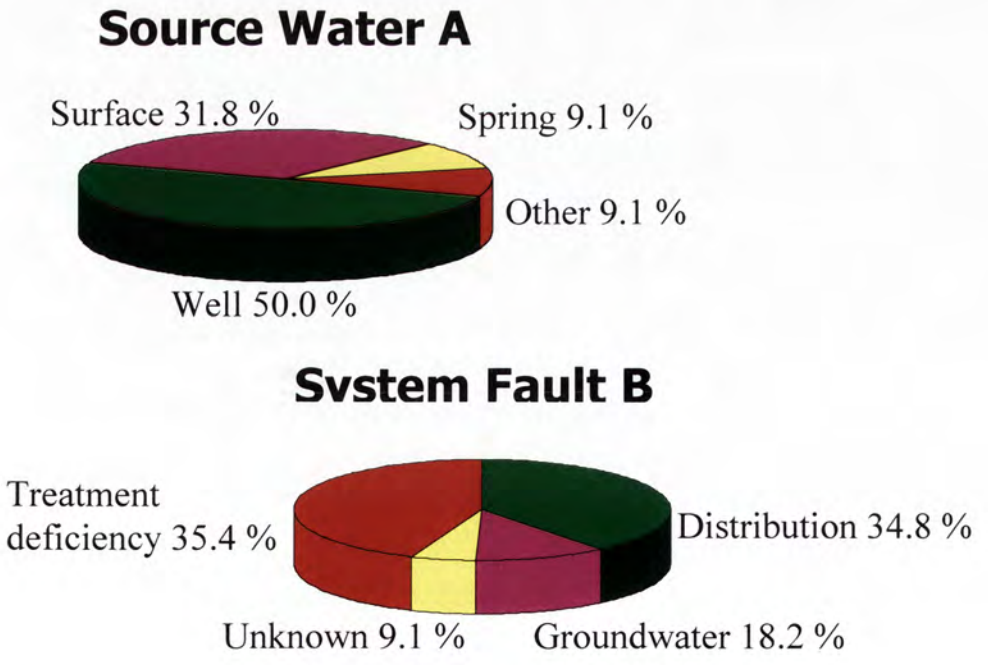


Figure 1.4: Waterborne disease in USA by: (A) water source, (B) system failure (Anonymous 1984; Barwick *et al.* 2000; Kramer *et al.* 1996; Levy *et al.* 1998)

Cryptosporidiosis outbreaks via water and food are well documented in the United States (D'Antonio *et al.* 1985; Herwaldt *et al.* 1992; Kramer *et al.* 1996; Kramer *et al.* 1996; Levy *et al.* 1998; Moore *et al.* 1994). The source of drinking water used by utilities in these outbreaks included surface water (lakes, rivers, streams), well water and spring water (Figure 1.5). Several outbreaks have also been associated with swimming pools and amusement park wave pools or water slides where accidental faecal release associated with children were the likely sources (Joce *et al.* 1991; Sorvillo *et al.* 1992).

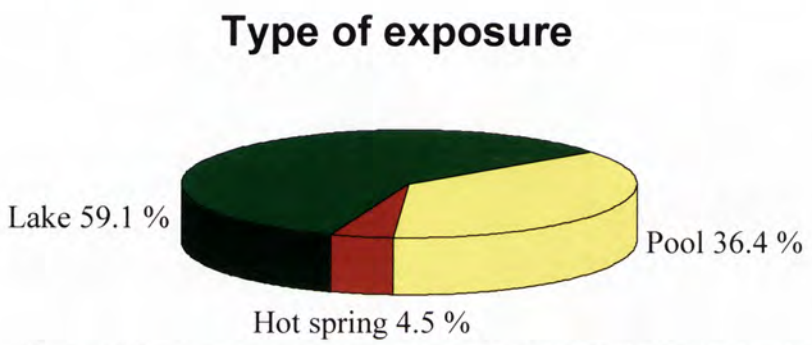


Figure 1.5: Type of *C. parvum* exposure resulting in USA waterborne outbreaks (Anonymous 1984; Barwick *et al.* 2000; Kramer *et al.* 1996; Levy *et al.* 1998)

## 1.2 The Sydney Incident

The levels of oocyst contamination in the water supply system during the 1998 Sydney events were sufficiently high to cause concern and may have endangered the health of the public. If the organisms identified were of the specific types known to infect humans and were infective, then significant disease would have been expected in the population of Sydney. The concentrations estimated in Sydney drinking water were at a level which would have resulted in an average consumption of up to 10 *C. parvum* per person per day (McClellan 1998).

The Health Department intensively investigated the level of disease within the community. No increase in infection with *C. parvum* was identified. Possible explanations to explain the lack of increase in infection were that the oocysts were not viable, were of a non-human infective type or were mis-identified as *C. parvum*.

## 1.3 Water Treatment and *C. parvum*

Municipal water utilities provide relatively good protection against water-borne *C. parvum* infection. There were only six major documented cases of cryptosporidiosis outbreaks via drinking water between 1984 – 1994 within the USA, even though the 1993 outbreak in Milwaukee caused over 400 000 cases (Jakubowski 1995). Since this massive outbreak, a huge amount of research continued to be undertaken to eliminate the possibility of further outbreaks via public drinking water.

Municipal drinking water is generally purified by chemical coagulation followed by granular medium filtration (conventional treatment). Chlorination is used most frequently to disinfect drinking water by effectively killing most pathogens (viruses, bacteria and some protozoa). Nonetheless, studies have shown that *C. parvum* is 240 000 times more resistant to chlorination than *Giardia* (Jakubowski 1995) and *C. parvum* oocyst viability was not affected by exposure to 1.05 and 3 % chlorine for up to 18 hours (Korich *et al.* 1990). Also, Korich, *et al.* (1990), demonstrated that chlorine dioxide and monochloramine were also ineffective in inactivating *C. parvum* oocysts in drinking water. Excystation and mouse infectivity were evaluated to assess oocyst viability. Korich *et al.* (1990) study also found that treating oocysts with 1mg/L of ozone for five minutes inactivated over 90 % of the oocysts. Thus, although ozone may be a potential disinfectant for inactivating *C. parvum* oocysts, one should not assume that traditional chemical disinfectants (even if they inactivate coliform bacteria and *Giardia*) effectively eliminate the risk of infection by *Cryptosporidium.*

Hence filtration is preformed for removing *C. parvum* oocysts from municipal drinking water. In recent years, ultra-fine membranes (ultra filtration, nanofiltration and reverse osmosis) have been developed to remove various contaminants from drinking water whereas microfiltration suffice for bacterial and protozoan pathogens (Jakubowski 1995). Table 1.2 lists the different membrance filters and their removal capabilities.

Table 1.2 The removal size ranges of membrane.
(McClellan 1998)

| Filter Type | Removal Capability |
|---|---|
| conventional filtration | 5 - 100μm, removes human hair, the smallest particles visible to the naked eye and red blood cells |
| microfiltration | 0.1 - 5μm, removes the smallest yeast cells, protozoa and most bacteria |
| ultrafiltration | removes carbon black and viruses |
| reverse osmosis | removes particles in the ionic range, such as aqueous salts and metal ions |

Thus, since *C. parvum* oocysts are about 2 - 5 μm (Flanigan and Soave 1992), at least microfiltration is needed to reliably remove oocysts from the water supply. Given that *C. parvum* oocysts are so resistant to chlorine disinfectants, ultrafiltration or nanofiltration should provide ideal protection against all waterborne outbreaks via drinking water (*i.e.* including enteric viruses) (Jakubowski 1995).

### 1.3.1 Sydney's Drinking Water Treatment

Since late 1996, all of Sydney's water supply has been filtered. Eleven water treatment plants are used to filter drinking water supplied to the greater Sydney, regional population of 4 million people. Seven of these facilities are owned and operated by Sydney Water Corporation, being are located at Orchard Hills, Cascade, North Richmond, Nepean, Warragamba, Linden and Greaves Creek (McClellan 1998a). The remaining four privately-owned and operated plants are at Prospect, Macarthur, Illawarra and Woronora all provide filtered water under contract to Sydney Water. These latter four plants provide more than 90 % of Sydney's drinking water.

Sydney Water Corporation (SWC) provides approximately 1 500 ML/day to more than 3.8 million people in the Sydney, Blue Mountains and the Illawarra regions. A network of nine major dams plus several minor storage reservoirs is used to collect and store water which, in turn, is delivered to a network of over 20 000 km of water mains, 165 pumping stations and 261 service reservoirs. The water supply is drawn from catchments on four main river systems - the Upper Nepean, the Warragamba, the Shoalhaven and the Woronora - with minor supplies drawn from the Hawkesbury River and tributaries of the Grose, Fish and Duckmaloi Rivers (McClellan 1998b).

Water treatment plants in Sydney generally use alternatives to conventional treatment because the water quality is deemed to contain low levels of pathogens and to be of consistently high quality. A process of contact or direct filtration treats nearly all of Sydney's water. There are two exceptions, North Richmond and Nepean plants. North Richmond, which has a run of river water supply uses dissolved air flotation and a clarification process followed by filtration using granular activated carbon contactors. Nepean, has high levels of colour and turbidity from time to time, uses a process of absorption, clarification and filtration. Disinfection for all plants is achieved after filtration by a chlorination process and fluoride is added for the protection of dental health (McClellan 1998b).

### 1.3.2 Role of Coagulation and Flocculation

The majority of particles that are being removed from drinking water range in size from one tenth to one hundred thousandth of a millimetre (100 - 0.01 μm). The larger the particles the easier they are to remove. Removal is achieved with the smaller particles by making them adhere to form flocs by a process that starts with chemically assisted coagulation. The small particles (colloids) usually have a net negative charge. By adding a suitable compound with a large positive charge, the negatively charged particles are attracted and will clump together. These compounds are called coagulants and are usually trivalent iron or aluminium salts that dissolve in water to form positive ions which attract the negatively charged particles. The process is sometimes enhanced by the addition of positively charged (cationic) polymers (Kawabata 1993). After the coagulant is added and mixed in the water, small groups of colloids start forming structures called flocs. Flocculation is the next stage where the size of the floc is increased, making it easier to remove by the settling or filtration process. The flocculation process is frequently aided by slow stirring of the water using large paddles, which causes the floc particles to gently collide and stick together. If the water is agitated too hard the larger floc will be sheared apart. Addition of a polymer during the flocculation stage is frequently used to strengthen the large floc. The surface charge of *C. parvum* oocysts is also negative (pI ~3.1, [Ongerth *et al.* 1996]), hence they are expected to adhere to flocs.

### 1.3.3 Clarification/Settling and Filtration of Water Particulates

Treated water flocs can be removed by use of a clarifier or large sedimentation chamber (Standen *et al.* 1997). Hence clarification may take the form of horizontal flow chambers where the water travels slowly through the chamber with several hours detention time. Alternatively, they may be more compact structures called clarifiers where the water is allowed to slowly rise or fall through a vertical chamber and the clarified water is decanted from the surface into troughs. Absorption clarifiers are alternatives to conventional clarifiers or roughing filters that consist of either a coarse artificial or granular media, such clarifiers and roughing filters remove large floc prior to polishing filtration (Standen *et al.* 1997). For large plants rapid gravity dual media filters are most frequently used for this latter purpose. The filters are regularly backwashed to remove accumulated floc.

Parasitic protozoa occur in water as dormant infectious cysts, which have natural mortality rates largely determined by temperature and incident UV light. In principle, removal or disinfection at the water source should be sufficient to prevent contamination of drinking water by enteric protozoa, provided that adequate measures are in place to prevent later re-contamination. Nonetheless, oocyst filter breakthrough has been reported and protozoan cysts are generally more resistant to chlorine disinfectants than most bacteria and viruses (Bouchier 1998; Goldstein *et al.* 1996). *Cryptosporidium* and *Giardia* species are likely to be the most important enteric protozoa in water in Australia, although infection by *Entamoeba histolytica* is also endemic in some communities. All these organisms cause moderate to severe enteritis in susceptible people and they appear, in Australia, to be transmitted mostly by direct contact with a carrier (McClellan 1998b). *Cryptosporidium* oocysts can be acquired from pasture animals and possibly from pets (Anonymous 1994; McClellan 1998a; McClellan 1998b).

### 1.3.4 Direct or Contact Filtration

In the process of contact filtration (also known as in-line filtration) a coagulant is added to the water immediately ahead of the filters in order to form a floc which is then trapped directly by the filter. Flocculation tanks are frequently added prior to the filters to provide a longer detention times thus allowing more time for the floc to increase in

size. The latter process is known as direct filtration. Direct and contact filtration processes differ from conventional filtration by eliminating the settling/clarification stage. Although more cost effective than the conventional settling/filtration method, these processes require optimal performance at all times and requires careful control in systems where the source water quality varies greatly. Modern instrumentation and control technology provide for reliable operation and management of filtration plants using the direct or contact filtration process (Nieminski and Ongerth 1995).

### 1.3.5 Dissolved Air Flotation (DAF)

Dissolved air flotation relies on flotation of particles instead of settling as described in the settling/filtration process. Prior to flotation, the water must first be treated with coagulant to allow the flocculation process to take place. Air is dissolved in water to super-saturation so microscopic bubbles form. The air/water solution flows up through the flotation chamber carrying flocs to the top of the tank where they can be skimmed off. The floc moves upward as the air bubbles attach to the floc, which makes them lighter than water. Flotation is frequently used where algae and colour are of more concern rather than silt or clay particles and is often used for treatment of running river water. DAF can be effective for the removal of some 90 - 99 % of *Cryptosporidium* and *Giardia* (Plummer *et al.* 1995).

## 1.4 Genotyping of *C. parvum*

Polymerisation chain reaction (PCR) based technologies have taken over from phenotypic characterisations of microorganisms. The early phenotypic characterisations included methods such as two-dimensional gel electrophoresis, antigenic analysis and restrictions fragment length polymorphism (RFLP) (Mead *et al.* 1990; Nina *et al.* 1992; Ortega *et al.* 1991). PCR has promoted the rise of a new generation of diagnostics. Nonetheless, sensitive "genetic fingerprinting" methods are required to determine the origin of *C. parvum* oocysts. Molecular methods such as random amplified polymorphic DNA (RAPD) analysis can be used to determine species as well as to differentiate the host of origin for *C. parvum* (Morgan *et al.* 1995; Widmer *et al.* 1998). Nonetheless the use of RAPD analysis to detect *C. parvum* has been limited because the methods used to purify the oocysts result in the presence of

contaminating microorganisms and may alter the fingerprinting pattern (Deng and Cliver 1998).

Higher resolution "typing" of *Cryptosporidium* has been explored with several gene targets using PCR, PCR-RFLP and RT-PCR for example to discriminate between human and bovine origin oocyst populations (Table 1.4). The majority of typing studies carried out to date employing PCR-based technologies have analysed a single genetic locus and *C. parvum* has been shown to be dimorphic (Spano *et al.* 1998). Overlapping transmission cycles may result in these genotyping methods providing insufficient resolution. Furthermore, poor inter-laboratory reproducibility is a problem (Davies *et al.* 2004). Hence, a genotyping methodology which was reproducible and host specific was required, the 18S rRNA PCR typing developed by Xiao *et al.* (2004), has met this criteria and has become standard for species description. However, for further differentiation at the sub-species level no such standard exists. The suitable marker would need to be stable to ensure consistent sub-species fingerprinting and with sufficient variation to be able to differentiate isolates from different geographical locations.

PCR-RFLP when used in tandem has the potential to determine species and to differentiate between the host of origin (Awad-El-Kariem *et al.* 1994; Bonnin *et al.* 1996; Peng *et al.* 1997; Spano *et al.* 1997). The application of PCR-RFLP markers have been used to designate two genotypes namely, genotype I (human) and genotype II (predominantly from domestic animals), which were consistent between several laboratories (Awad-El-Kariem *et al.* 1994; Bonnin *et al.* 1996; Carraway *et al.* 1996; Carraway *et al.* 1997; Morgan *et al.* 1995; Peng *et al.* 1997; Penner *et al.* 1993; Spano *et al.* 1997; Widmer *et al.* 1998).

New *Cryptosporidium* species until recently have been named based predominately on morphologic descriptions or developmental studies and to a lesser extent host of origin. Most animals can be naturally infected with multiple *Cryptosporidium* spp. (Table 1.1) (Xiao *et al.* 2004). Further differentiation has been achieved since the development of DNA sequencing methods. Common sequences have been used to differentiate *C. andersoni* from *C. muris*, *C. canis* from *C. parvum*. The newest proposed species is *C. hominis* and is distinct from *C. parvum* (Xiao *et al.* 2004). Nucleotide sequences are

playing a pivotal role in differentiating species of *Cryptosporidium* and as a consequence there are now 15 recognised species as detailed in Table 1.1 (Fayer R *et al.* 2005; Ryan *et al.* 2004; Xiao *et al.* 2004).

Coding genes have predominantly been used to differentiate genotypes with a few studies targeting non-coding regions (Aiello *et al.* 1999; Caccio *et al.* 2000; Feng *et al.* 2000; Mallon *et al.* 2003):

- 18S rRNA (Kilani and Wenman 1994; Rochelle *et al.* 1997);
- *C. parvum* oocysts wall protein (COWP) (Spano *et al.* 1997; Xiao *et al.* 2000);
- dihydrofolate reductase (DHFR) (Brophy *et al.* 2000);
- GP60 (Zhou *et al.* 2003)
- heat shock proteins (Khramtsov *et al.* 1995; Sulaiman *et al.* 2000);
- intergenic spacer region (ITS 1) of rRNA (Morgan-Ryan *et al.* 2001);
- microsattelites (Aiello *et al.* 1999; Caccio *et al.* 2000; Feng *et al.* 2000; Mallon *et al.* 2003);
- poly(T) (open reading frame with several series of T repeats) (Carraway *et al.* 1997),
- small sub-unit rRNA (Xiao *et al.* 1999);
- thrombospondin-related adhesive protein of *Cryptosporidium* (TRAP - C2) (Spano *et al.* 1998);
- thrombospondin-related adhesive protein of *Cryptosporidium* (TRAP - C2) (Peng *et al.* 1997) and
- tubulin (Caccio *et al.* 1999; Rochelle *et al.* 1999; Sulaiman *et al.* 1999; Widmer *et al.* 1998).

The PCR genotyping targets listed above have produced a wide range of typing protocols which target different genes or markers. All evolve at different rates and may contribute to the different genotyping results which have been reported and observed. However, several methods have shown to be of more use when investigating sub-species genotyping and include, gp60, sequencing of the 18S rRNA loci, the dsDNA virus present in *Cryptosporidium* and microsatelites. A universal typing strategy is yet to be agreed to and even if a method was agreed to, one protocol would not be sufficient to address all genotyping needs. A consensus protocol for distinguishing between *Cryptosporidium* has been described, which compares the sequence homologies of the 18S rRNA (Xiao *et al.* 2004). Thus, the clarification of *Cryptosporidium* taxonomy has been useful for better understanding the biology of *Cryptosporidium*, as well as assessing the public health significance of *Cryptosporidium* in animals, the environment, characterising transmission dynamics, and tracking infection and contamination sources (Xiao *et al.* 2004).

Sequence analysis of the gp60 divides *C. hominis* and *C. parvum* into several allelic groups, each of which consists of multiple subtypes (Tautz and Schloeterer 1994). One of the allele families, Ib, was responsible for the waterborne outbreaks of cryptosporidiosis in Milwaukee, Wis., which caused illness in over 400,000 people (Zhou *et al.* 2003). An important feature of this gene is its high degree of sequence polymorphism, particularly among *C. hominis* isolates, which is far greater than any other *Cryptosporidium* genetic loci examined to date. Within each allele, there are different sub-genotypes based on the number of a trinucleotide repeat. These results highlight the usefulness of the sub-genotype analysis for fingerprinting *Cryptosporidium* isolates (Alves *et al.* 2003).

A high number of two extrachromosomal virus-like double-stranded dsRNAs were detected in oocysts of several calf isolates of *C. parvum* (Khramtsov *et al.* 1997). The function of the putative protein encoded by the small dsRNA (S-dsRNA) remains unknown (Khramtsov and Upton 1998). The importance of these molecules for the parasite and its presence in human isolates with different genotypes are as yet unknown. However, dsRNAs are common in many parasitic protozoa (Patterson, 1990; Wang and Wang, 1991), and some are known to modulate virulence of pathogenic fungi (Nuss and Koltin 1990). Continued investigation into the biology and occurrence of these dsRNAs in both human and animal isolates of *Cryptosporidium* (Khramstov *et al.* 2000) is required.

Microsatelites, or simple sequence repeats, constitute a rich source of polymorphisms and have been used extensively for high-resolution genotyping and mapping. Aiello *et al.* (1999) analysed six microsatelites in *C. parvum*. Five loci showed sequence differences between human and bovine isolates but no polymorphism was observed within isolates from the same host species. Cacciò *et al.* (2000) identified a polymorphic *C. parvum* microsatelite located within what appears to be a protein coding sequence. The availability of polymorphic microsatelite markers allows significant improvement over the older PCR-RFLP methods. A practical advantage of the microsatelite polymorphisms is the reliance on conventional PCR methods and the potential for automation when using labelled primers. The use of multiple markers will

generate multilocus fingerprints capable of discriminating between individual isolates (Feng *et al.* 2000).

Using a series of microsatelite markers, variation within the *C. hominis* and *C. parvum* genotypes have been reported, thus demonstrates polymorphism within the two major lineages (*Aiello et al. 1999*; *Feng et al. 2000*; Caccio *et al.* 2001). The majority of the microsatelites which appear in the literature flank coding regions within the genome of *Cryptosporidium*. For example, a tri-nucleotide repeat has been described by Caccio *et al.* (2001), Strong *et al.* (2000) described repeats flanking an antigen (GP15/45/60), while Khramtsov *et al.* 1995 described flanking repeats in the hsp 70 gene sequence. These microsatelites formed part of the multilocus genotyping study conducted by Mallon *et al.* (2003). Mallon *et al.* (2003) concluded that multilocus genotyping will have substantial applications for defining the origins of outbreaks, although markers with higher levels of polymorphism may be required to provide the necessary resolution. Furthermore, the identification of more highly polymorphic markers are required to provide the necessary resolution as a disease-tracking tool over larger geographical distances. This genotyping system could be used to define the geographical origin of the outbreak. As a number of outbreaks are water- or food-borne, the diversity of the population could be exploited to define whether such outbreaks are from a single or multiple sources.

Given the wide diversity of possible *C. parvum* oocysts in source waters, water quality managers need to know if the oocyst sources under their control (*e.g.* domestic animals/sewage) are significant. Furthermore, epidemiologists also need to identify the source of human infective oocysts in the community. Therefore a form of sub-species typing is required. The successful application of molecular biology offers tools which are able to provide further insight into the relative relatedness of oocysts identified within the outbreak group. The agriculture industry would also be able to take advantage of the same methodology to identify the source of an outbreak within a dairy herd for example.

*Cryptosporidium* parasites infecting humans, have been previously designated *C. parvum* human genotype, genotype I. There is general consensus for this genotype to be recognised as a separate species, *C. hominis*, based on molecular and biological differences (Morgan-Ryan *et al.* 2002). *C. hominis* is morphologically identical to *C. parvum*, 4.6 to 5.4 by 3.8 to 4.7 μm (mean, 4.2 μm) with a length/width ratio of 1.21 to 1.15 (mean, 1.19) (Xiao *et al.* 2004). Yet unlike *C. parvum*, *C. hominis* is considered non-infective for mice, rats, cats, dogs, and cattle. Further, several studies conducted over the past several years have shown genetic and biological differences between *C. hominis* and *C. parvum* (Nichols *et al.* 2002, Xiao *et al.* 2004). There is also a lack of genetic exchange between *C. hominis* and *C. parvum* (McLauchlin *et al.* 2000, Xiao *et al.* 2004). There also appears to be distinct differences in oocyst shedding patterns between *C. hominis* and *C. parvum* in humans (McLauchlin *et al.* 2000, Nichols *et al.* 2002, Xiao *et al.* 2004).

Considering the frequency and prevalence of *Cryptosporidium* infections, surprisingly little is known about its molecular genetics. Abrahamsen *et al.* (2004) completed the genome sequence of *C. parvum* (Iowa "type II" isolate) and its predicted protein complement. Its genome consists of 8 chromosomes varying in size between ~1 and 1.5Mb, giving a total genome size of approximately 9.1 Mb (Abrahamsen *et al.* 2004). This is approximately the same size as the genome of *Sacharomyces cerevisiae*. The *C. parvum* genome is thus quite compact relative to the genome of *Plasmodium falciparum* (23-Mb, 14-chromosomes) (Gardner *et al.* 2002). This size difference is predominantly the result of shorter intergenic regions, fewer introns, and a smaller number of genes (Abrahamsen *et al.* 2004). Each haploid nucleus of the sporozoite contains eight chromosomes (Table 1.3), 1.04 - 1.54 million base pairs of DNA with very few introns *e.g.* β–Tubulin and a 35 kilobase extrachromosomal circular DNA molecule (Blunt *et al.* 1997; Piper *et al.* 1998; Spano and Crisanti 2000). Piper *et al.* (1998) developed a limited codon usage table (Table 1.3) from the 15 - 20 completely sequenced genes of *C. parvum*, which are listed in Genbank (Piper *et al.* 1998). Such a table is useful for researchers trying to locate more complete genes. An interesting observation about the genome of *C. parvum* is that the third position of the codon is dominated by U. Normally the third position has the most variability (Lewin 2000).

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The map and library are a considerable aid in gene-hunting studies of *Cryptosporidium*. The genome map is particularly useful in comparison with related species to try and identify specific or common genes that are conserved in both species *e.g. Toxoplasma* spp. In addition, evolutionary and phylogenetic studies should be aided by a better understanding of the genomic structure of *Cryptosporidium*'s genome and aid in its classification. It would also be useful in any studies which involve gene regulation, allowing isolation of both gene and regulatory elements on the same clone. However for this dissertation, genomic structure is most useful to identify regions suited to sub-species typing.

### 1.4.1 Problems with Current Typing Strategies

Research on *C. parvum* has been hampered by the lack of sufficient supplies of oocysts from well characterised isolates to allow parallel testing by different laboratories. The establishment of oocyst banks with a diverse range of well documented isolates and propagation of selected isolates by animal culture would greatly facilitate comparative work (Johnson 1998). Cooperation is also needed to optimise standard methods for purification and storage of viable oocysts and for extraction of nucleic acids and proteins for preservation.

Table 1.3: General features of the *C. parvum* genome. (Abrahamsen *et al.* 2004)

| | |
|---|---|
| Size (Mbp) | 9.1 |
| (G_C) content (%) | 30 |
| No. of genes | 3807 |
| Mean gene length (bp) excluding introns | 1795 |
| Gene density (bp per gene) | 2382 |
| Percent coding | 75.3 |
| Genes with introns (%) | 5 53. |
| Intergenic regions | |
| (G_C) content % | 23.9 |
| Mean length (bp) | 566 |
| RNAs | |
| No. of tRNA genes | 45 |
| No. of 5*S* rRNA genes | 6 |
| No. of 5.8*S*, 18*S*, and 28*S* rRNA units | 5 |

Availability of standardised material will allow the application of a range of molecular and biochemical techniques to identify markers that can distinguish between individual isolates. Once such markers are defined they will permit the tracing of individual strains and some may ultimately provide an indicator for human infectivity (Johnson 1998).

Recently multi-locus approaches have become the more common for genotyping *C. parvum* (Spano *et al.* 1998). Such an approach has been utilised in human paternity testing. From the environmental point of view high-resolution fingerprinting could assist in identifying the source of oocysts isolated from surface water, which would facilitate the implementation of measures to reduce the access of oocysts to drinking water. The potential virulence of each genotype is unknown and differences in symptoms may also be eluded to. A significant limitation with most typing methods is that they rely upon some 100 ng of DNA, which equates to about $10^6$ oocysts. The genome of *C. parvum* is about 10.4 Mb or 10.6 fg of DNA. One femtogram of DNA corresponds to less than 1 sporozoite (Zhu *et al.* 1998).

Hence, typing of environmentally collected oocysts by conventional PCR methods is very difficult. To effectively genotype samples with few oocyst numbers very sensitive PCR methods are required. One example is the 18S rRNA nested PCR which can detect femtogram levels of DNA (Xiao *et al.* 2004).

Table 1.4: *C. parvum*: codon usage table

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| UUU | 26.6 | (861) | UCU | 26.5 | (857) | UAU | 24.2 | (784) | UGU | 15.3 | (496) |
| UUC | 13.3 | (432) | UCC | 6.2 | (200) | UAC | 8.1 | (261) | UGC | 8.9 | (287) |
| UUA | 28.8 | (933) | UCA | 26.3 | (851) | UAA | 0.8 | (25) | UGA | 0.3 | (9) |
| UUG | 17.1 | (554) | UCG | 4.3 | (139) | UAG | 0.2 | (8) | UGG | 8.6 | (279) |
| CUU | 16.6 | (539) | CCU | 13.3 | (432) | CAU | 12.9 | (417) | CGU | 4.9 | (159) |
| CUC | 5.9 | (192) | CCC | 2.9 | (95) | CAC | 5.2 | (168) | CGC | 1.2 | (39) |
| CUA | 7.4 | (241) | CCA | 28.7 | (930) | CAA | 26.7 | (864) | CGA | 1.6 | (51) |
| CUG | 3.0 | (97) | CCG | 2.3 | (73) | CAG | 7.9 | (256) | CGG | 0.3 | (9) |
| AUU | 47.5 | (1538) | ACU | 27.4 | (889) | AAU | 44.0 | (1424) | AGU | 14.2 | (461) |
| AUC | 11.8 | (381) | ACC | 7.0 | (227) | AAC | 16.9 | (547) | AGC | 7.7 | (250) |
| AUA | 19.5 | (631) | ACA | 26.3 | (851) | AAA | 38.7 | (1254) | AGA | 24.1 | (781) |
| AUG | 22.2 | (720) | ACG | 3.5 | (113) | AAG | 28.0 | (906) | AGG | 5.9 | (192) |
| GUU | 32.4 | (1049) | GCU | 22.4 | (724) | GAU | 41.0 | (1329) | GGU | 28.8 | (932) |
| GUC | 6.1 | (197) | GCC | 5.1 | (165) | GAC | 12.1 | (392) | GGC | 6.5 | (209) |
| GUA | 18.4 | (597) | GCA | 21.8 | (707) | GAA | 44.4 | (1439) | GGA | 25.3 | (821) |
| GUG | 4.8 | (154) | GCG | 2.7 | (88) | GAG | 21.6 | (700) | GGG | 5.7 | (185) |

**Notes:**
fields: triplet, frequency: per thousand, (number) **42 CDS's (32391 codons)**

| | | | | | |
|---|---|---|---|---|---|
| Coding GC | 36.29 % | | | | |
| 1st letter GC | 43.99 % | 2nd letter GC | 38.59 % | 3rd letter GC | 26.29 % |
| Reference | http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=*Cryptosporidium+parvum*+[gbinv] | | | | |

Chalmers *et al.* (2005) conducted a comparative *Cryptosporidium* typing which included six methods. Three of the methods showed superior variation within *C. parvum* and *C. hominis* typing resolution (SSCP analysis of ITS-2, gp60 and microsatelite multi-locus genotyping (MS MLG)). However, it is clear from the estimates in this trial that typability was essentially 'driven' by the sensitivity of the PCR. SSCP analysis of ITS-2 was able to determine 'subgenotypes' in samples which contained DNA from both species and is cost effective both in terms of time and consumables. Sequence analysis provided a good comparison to the results produced by SSCP using the ITS-2 method. The markers which were used for sequencing were gp60 and microsatelite markers (ML1, ML2) (Mallon *et al.* 2003). MS MLG depends on the combined sensitivity of the multiple markers used: this also reflects the typability of the method, thus the results reported here are for this particular combination of markers alone. Where the information from only one marker was available, that result is reported, but no MLG was assigned. MS markers offer good discrimination but require improved sensitivity and careful selection of markers depending on sample type or source (Chalmers *et al.* 2005).

The method with the highest typability was SSCP of ITS-2 region. However, it is clear from the estimate of typability was essentially driven by the sensitivity of the PCR. The sensitivity achieved by the MS MLG depended on choice of markers used in combination of the multiple markers used (Chalmers *et al.* 2005).

The majority of published *C. parvum* molecular methods (as described in Section 1.4) lie within protein-coding regions which are known to be more homogenous than non-coding sequences. Due to overlapping transmission cycles current genotyping methods are insufficient. The commonly utilised typing methods target conserved regions of the genome (*i.e.* rRNA, functional genes). The utilisation of conserved regions of the genome results in a limited differentiation between isolates. Hence, there is a need to develop a genotyping methodology which is both stable, reproducible and able to differentiate source types while covering both the functional and non-functional regions of the *C. parvum* genome. Furthermore a stable marker for viability is required for assessing oocysts from water supply systems.

Previous studies investigating *Cryptosporidium* are difficult to compare directly due to:

- some report oocysts numbers for sensitivity;
- some report DNA concentrations for sensitivity;
- some use purified oocysts;
- some use seeded water and
- some use clinical samples and bovine faeces.

Few report recovery efficiency for DNA extraction though the number of oocysts identified by microscopy are often mentioned. The total oocysts which an IFA can detect include proportions of ruptured ('ghosts': dead), PI-positive (PI+: permeable and dead), DAPI-positive PI-negative (DAPI+PI−: semipermeable and viable) and DAPI-negative PI-negative (DAPI−PI−: impermeable and potentially viable after further trigger). The conventional method for determination of viable oocyst recovery is to quantify by enumerating more than 100 oocysts in each sample with the sum of impermeable (DAPI−PI−) and semipermeable (DAPI+PI−) oocysts considered to be the number of total viable oocysts (Campbell *et al.* 1992). Typing generally requires large numbers of oocysts and disproportionate mixed strain isolates, which is not always likely. Both of which are is a major hindrance when applying current techniques to the routine tracing of source of outbreaks of human cryptosporidiosis (Awad-El-Kariem *et al.* 1998).

### 1.4.2 Requirements for Molecular Based Typing and Identification

Genotyping beyond the species level will improve the strength of public health surveillance, incident management systems and proactive catchment management. The protocols which should be selected need to be relatively straight forward and able to determine one or more of the following including genotypic information, genetic linage, relationship of isolates and outbreaks and viability.

The qualities of the ideal detection system need to be applicable to clinical and environmental samples with appropriate sensitivity. In a diagnostic laboratory the protocol needs to be simple enough in order to allow a high sampling number.

Furthermore the typing markers need to be stable, robust, accurate, quick, cheap and reproducible. The viability and potentially infectivity are important attributes, given that catchment practices are designed to hold up pathogens, so when they finally reach the treatment plans they are largely expected to be non-viable. The achievement of

these aims would then allow further studies to develop rigorous genetic typing able to generate knowledge on the viability of *C. parvum* and how it could be accurately determined.

### 1.4.3 Rationale for Potential Targets within the Genome

The simultaneous analysis of several genetic markers would have the potential to better define the population structure of *C. parvum* and assess the degree of genetic isolation of the "clonal" strains. The selection of target genome areas will also play an influential role in finding unlinked polymorphic loci. On this basis the genome areas which could be targeted will not all be of the "same" gene type and some may not be from coding regions of the *C. parvum* genome. Selecting from a diverse background of genome types maximises the potential for detecting polymorphic differences between *C. parvum* populations. This is due to selective pressures not occurring evenly across the genome.

## 1.5 Conclusions

Public health and epidemiology experts agreed that the development of a typing system to distinguish individual strains would provide greater understanding of:

- the relative importance of different sources and
- routes of disease transmission within the community.

International cooperation is needed to improve and standardise surveillance methods, provide advice and information to the medical profession and environmental laboratory services.

Cryptosporidiosis in developing regions emphasises the need to understand the epidemiology of the disease and to identify the transmission routes. It will thereby facilitate the implementation of successful prevention management measures. However, epidemiological as well as taxonomy studies have been hindered by the lack of stable biochemical, immunological or genetic markers for isolate determination. Several laboratories have identified microsatellites in the genome of *C. parvum* and have investigated the level of polymorphism at these loci (Mallon *et al.* 2003; Widmer *et al.* 2004). Multilocus haplotypes based on such markers are suitable for

discriminating individual isolates of *C. parvum* (Mallon *et al.* 2003; Widmer *et al.* 2004) .

## 1.6 Objectives and Aims

The main objective of this thesis was to explore a novel method of PCR typing of *C. parvum*. Hence, the specific aims were:-

1. Compare banding patterns achieved with Microsatellites to those published by Morgan, Peng and Widmer (Morgan *et al.* 1995; Peng *et al.* 1997; Widmer 1998; Widmer *et al.* 2000);
2. Design primer sets that provide a higher resolution of genotyping information, in order to be able to distinguish sample origin, focusing on microsatelite and telomere primers, which are suitable for sub-species genotyping *Cryptosporidium*;
3. Optimise Microsatellite and Telomere Primer Set:
    - PCR conditions, Mg and Formamide titrations;
    - reproducibility of PCR using the same sample;
    - reproducibility of PCR at each sampling location and
    - determine stability of marker using two different *C. parvum* isolate sources.
4. Use the microsatellite and telomere primer set for "sub-species" typing of *C. parvum* animal isolates from within Australia an International Sources and human isolates from outbreak grouping or sporadic cases.

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## 2.1 Introduction

The hypothesis being tested in this method development chapter is whether the telomere region of the chromosome can be used to aid sub-species fingerprinting of *C. parvum* genotypes.

Telomeres are protein–DNA complexes at the termini of linear chromosomes. They constitute unique chromosome domains that perform at least two critical functions. First, they allow for the complete replication of genes at chromosome ends, which in most organisms is accomplished with the help of a specialised cellular reverse transcriptase called telomerase. In addition, telomeres mask chromosome ends, which would otherwise resemble accidental DNA breaks and initiate DNA damage response mechanisms that promote cell-cycle arrest and DNA repair (Blackburn *et al.* 1995; Brenner 1997; Zakian 1995).

Hence, telomeres are the very ends of linear eukaryotic chromosomes and are necessary for normal chromosomal activity. Telomeres act to prevent aberrant recombination and degradation of the chromosomal ends. It is thought that they might also be important for anchoring the chromosomes to the inner nuclear membrane (Zakian 1995). Telomeric DNA consists of a G-rich strand (5' - 3') and a C-rich strand (3' - 5') and is made up of short, simple repeats (Zakian 1995).

Telomere nucleotide sequences are found in all eukaryotic organisms. A telomere repeat sequence is different between species, hence combining a telomere repeat primer (species specific) with a non-specific PCR primer (*ie. a* microsatellite), differential genotyping which is also species specific should be able to be achieved. The telomeric repeat sequence appears critical for telomere function in eukaryotes (Blackburn *et al.* 1995). Telomere DNA of at least human somatic cells shortens at each cell division. Such shortening could be described as a mitotic clock which provides a finite proliferative capacity of human somatic cells (Zakian 1995). Telomerase is required for cells to proliferate indefinitely, maintaining telomere length, as in germ lines and most cancer cells. Several studies have established a causal relationship between telomere shortening and cellular senescence (Aakra *et al.* 1999). These investigations

show that transfection of the human telomerase reverse transcriptase gene (*hTERT*) into various human mortal somatic cells leads to elongation of telomere length and extension of the *in vitro* replicative life span (Kawaharasaki *et al.* 1999).

Advances in telomere biology have pointed to telomeres as important elements for cell survival, yet they cannot be fully replicated by the conventional DNA polymerase complex, but require an RNA primer to initiate DNA synthesis. In normal human cells, the average loss of human somatic telomere DNA has been estimated to be 30 to 200 bp/cell doubling *in vitro* (Delange 1998). Telomere shortening is especially a problem for rapidly dividing cells and shortening can lead to cellular senescence and death after a limited number of cell divisions, as has been demonstrated for the yeasts *Kluyveromyces lactis*, *S. cerevisiae* and *S. pombe* (Bottius *et al.* 1998; Patel *et al.* 1999). Sequence loss is usually balanced by the *de novo* addition of telomere repeats onto chromosome ends by a ribonucleoprotein enzyme called telomerase. The telomerase complex is a specialised reverse transcriptase which uses its RNA moiety to template the addition of new telomeric repeats to chromosomal DNA ends. In a wide phylogenetic range of eukaryotic cells, telomerase compensates for potentially fatal telomere shortening and probably contributes to cell immortalisation (Blackburn *et al.* 1995; Brenner 1997; Zakian 1995).

## 2.2 Telomere Structure, Role and Function

Typically, eukaryotic telomeric sequences are composed of largely invariant 5 – 8 bp repeats (Table 2.1). However, variable repeat sequences in telomeres have been shown to exist in several eukaryotes, including budding yeast (Patel *et al.* 1999), *Plasmodium* (Scherf 1996), *Giardia lamblia* (Le Blancq *et al.* 1998) and *C. parvum* (Liu *et al.* 1998). Telomere maintenance in most eukaryotic organisms is mediated by telomerase, which has known reverse transcriptase activity (Cooke 1996; Zakian 1995).

DNA polymerases can only synthesise a new DNA strand in the 5' - 3' direction and needs a primer that provides a free 3' OH end. The cellular replication machinery is unable to duplicate the 3' ends of linear chromosomes unless special mechanisms are operative (Delange 1998). Telomeres seem to shorten continuously in human somatic

cells because of the "end replication" problem. Yet it appears that telomere length is maintained in cancer cells, cell line and unicellular organisms like yeast (Cooper *et al.* 1997; Lue 1999). The mechanism involves the enzyme telomerase, which elongates the 3' ends of telomeres, however, a more complicated mechanism is necessary to ensure that there is no net gain or loss of telomeric ends (Blackburn *et al.* 1995; Brenner 1997; Zakian 1995).

### 2.2.1 Coordinating DNA Replication and Telomerase Action

In most eukaryotic organisms examined to date, the chromosome ends have multiple tandem repeats of a simple sequence $T_2AG_3$ in mammals and $T_{1\text{-}3}AG_{2\text{-}3}$ in most other eukaryotes (Table 2.1).

Table 2.1: Comparison of telomeric sequences from different organisms

| | |
|---|---|
| *Cryptosporidium parvum* | TTTAGG [a] |
| *Giardia* spp. | TAGGG |
| *Plasmodium falciparum* | TT(T/C)AGGG |
| *Saccharomyces cerevisiae* | $T(G)_{2\text{–}3}\,(TG)_{1\text{–}6}$ |
| *Caenorhabditis elegans* | TTAGGC |
| *Homo sapiens* | TTAGGG |
| *Mus* spp. | TTAGGG |

a Most common repeat unit identified (Liu *et al.* 1998).

Telomere repeats can vary considerably in length between different species (*e.g.* ~50 kb in the mouse *M. musculus*, ~10 kb in humans and ~300 bp in *S. cerevisiae*) but each species maintains a fixed average telomere length in its cell line (Liu *et al.* 1998). There are three interrelated questions regarding telomerase action and regulation which are still being researched:

- is telomerase action coordinated with normal DNA replication and if so, how is this brought about;
- how is telomerase recruited to the chromosome ends and
- how is telomerase regulated such that telomere length is maintained about a fixed average value.

Pioneering studies by Zakian *et al.* (1995) demonstrated that a telomeric G-rich single-strand overhang is presumed to be a necessary substrate for telomerase action. Either it is generated or extended during late stationary phase in *S. cerevisiae*, suggesting that

telomerase addition occurs at or near the time when the conventional replication machinery copies the chromosome ends. Marcand *et al.* (2000), provided the first direct evidence that telomerase elongation and normal DNA replication are in fact coincidental. This conclusion is consistent with work reported by Diede and Gottschling (1999), who showed HO endonuclease generated double-strand break. occur only if cells are allowed to pass through the stationary phase of the cell growth cycle. Cells held in G1 (the period during interphase in the cell cycle between mitosis and the S phase (Anonymous 2004), although they contain an active telomerase enzyme, are unable to extend the break (Diede *et al.* 1999). The temporal coincidence of telomeric DNA replication and telomerase action suggests that the two processes might be mechanistically coupled. In strong support for this idea, telomerase addition at a double strand break requires DNA polymerase α and δ function, as well as primase activity (Diede *et al.* 1999; Zakian 1995). Likewise, elongation of a critically shortened telomere after removal of internal repeats is significantly diminished if a replication fork is prevented from reaching the telomere (Marcand *et al.* 2000). Molecular mechanisms underlying the recruitment of telomerase to the chromosome end and the coordination of DNA replication with telomerase action, are also beginning to come into focus. Recruitment and regulation of telomerase thus appears to be a complex, multi-component process. Understanding the precise mechanisms involved are an issue for current research (DuBois *et al.* 2000).

### 2.2.2 Telomere Binding Proteins and Telomere Length Regulation

An additional key to understanding telomerase recruitment and telomere-length regulation is the telomere repeat tract itself. It acts as a platform for the assembly of a large complex of proteins. For example budding yeast, telomeric $TG_{1-3}$ repeats generate high-affinity binding sites for regulatory protein *Rap*1 which has additional non-telomeric roles in both activation and repression of transcription (Blackburn *et al.* 1995; Brenner 1997; Zakian 1995). The carboxyl terminus of *Rap*1p negatively regulates telomere elongation (Krauskopf *et al.* 1996) and appears to function as part of a negative feedback mechanism to achieve telomere-length homeostasis. According to one telomere–chromosome model called the protein "counting" model, *Rap*1p is part of a system that measures telomere length (Blackburn *et al.* 1995; Brenner 1997; Cooke 1996; Dokudovskaya *et al.* 1997; Katayama *et al.* 1999; Zakian 1995).

The mammalian telomere repeat binding protein *TRF*1 also regulates telomere elongation through a proposed negative-feedback mechanism (Steensel *et al.* 1997). Shortened telomeres initially elongate at a relatively rapid rate (~15 bp per generation) that decreases in a roughly linear fashion with increasing telomere length. There is evidence that the telomere-length-sensing mechanism does not regulate a finely-tuned "on/off" switch (Steensel *et al.* 1997). Controlling telomerase addition, instead works like a rheostat. Interestingly, newly formed telomeres in *Trypanosoma brucei* also display initial rapid elongation, which trails off as the eventual equilibrium length (~15 kb) is approached (Carducci *et al.* 2000). In summary, the connection between mechanisms that sense telomere length and those that ultimately control the action of telomerase remains largely obscure. Undoubtedly, this will be an area of intensive investigation in the coming years.

### 2.2.3 An Emerging DNA-Damage Checkpoint Connection

Despite the apparent re-direction of end-joining proteins towards an end protection or "capping" function at telomeres, a number of studies suggest a surprisingly intimate connection between telomere function and the cellular response to DNA damage. Three reports (Martin 1999; McAinsh *et al.* 1999) have shown that Sir and Ku proteins are released from telomeres in response to agents causing DSBs. Sir proteins released from telomeres appear to relocate to the sites of damage in a *MEC1*-dependent manner where they might promote repair. On balance, the evidence suggests that the primary effect of Sir proteins on repair is through their influence on mating-type gene expression. Cells that express both a and α mating-type genes (typical of diploid cells, also true of *SIR* mutant haploids) down regulate the Ku-dependent repair pathways. Nevertheless, additional studies have revealed connections between telomeric heterochromatin and DNA damage checkpoint systems (Brenner 1997; Cooke 1996; Katayama *et al.* 1999). These preliminary observations hint at a complex interplay between the DNA damage checkpoint and telomeric chromatin. Additional studies are clearly required to understand such mechanisms and their biological significance. These mysterious connection between the DNA-damage checkpoint and telomere function in yeast is unlikely to be limited to fungal studies. For example, a recent study of the nematode *Caenorhabditis elegans*. (Gartner *et al.* 2000) has identified a

homologue of the yeast DNA damage checkpoint gene *RAD17*, called *mrt-2*. Remarkably, *mrt-2* (mortal cell line) mutant worms exhibit progressive telomere shortening and accumulate end-to-end chromosome fusions. The cell lines of these animals are also hypersensitive to both X-rays and transposon activity (Gartner *et al.* 2000). This may indicate that some form of DNA-damage checkpoint activation is required for proper telomere replication.

Telomeres in both budding and fission yeast and probably many other organisms (Blackburn *et al.* 1995; Zakian 1995) exist in a dynamic heterochromatic state. In budding yeast, telomeric heterochromatin is influenced both by the process of telomere replication and by DNA damage response systems, either directly or indirectly. Recently considerable progress towards an understanding of the relationship between normal chromosomal replication and the telomerase-based mechanism for preserving telomere repeat tract sequences has emerged. Molecular mechanisms underlying gene silencing at telomeres has been enriched by the discovery that a key factor in this process, the Sir2 protein. It is a novel type of NAD-dependent deacetylase (Blackburn *et al.* 1995; Brenner 1997; Cooke 1996; Dokudovskaya *et al.* 1997; Katayama *et al.* 1999; Zakian 1995). Finally, there has been a growing awareness, through studies in both yeast and mammalian cells, that a number of DNA repair and recombination proteins lead a 'double life'. Nonetheless, maintaining telomere structure and preventing the telomere acting upon as a double strand break are poorly understood roles (Cooke 1996; Dokudovskaya *et al.* 1997; Zakian 1995).

## 2.3 Telomeres in Protozoan Parasites

Telomerase, a specialised cellular reverse transcriptase, compensates for chromosome shortening during the proliferation of most eukaryotic cells and contributes to cellular immortalisation. The mechanism used by single-celled protozoa to complete theirs replication of linear chromosomes, such as in the malaria parasite *Plasmodium falciparum* are currently unknown. Yet telomerase activity has been identified in cell extracts of *P. falciparum* and other protozoa (Bottius *et al.* 1998). The *de novo* synthesis of highly variable telomere repeats to the 3' end of DNA occurs with oligonucleotide primers and plasmodial telomerase. In addition to elongating

pre-existing telomere sequences, protozoan telomerase can also add telomere repeats onto non-telomeric 3' ends (Bottius *et al.* 1998). The efficiency of non-telomeric primer elongation has been shown to be dependent on the presence of a G-rich cassette upstream of the 3' terminus. Furthermore, oligonucleotide primers based on natural *P. falciparum* chromosome breakpoints are efficiently used as telomerase substrates. These results imply that *P. falciparum* telomerase contributes to chromosome maintenance and to *de novo* telomere formation on broken chromosomes. Reverse transcriptase inhibitors such as dideoxy GTP efficiently inhibit *P. falciparum* telomerase activity *in vitro* and may be a target for the development of drugs that could induce parasite cell senescence (Bottius *et al.* 1998).

Unicellular protozoan parasites such as *Plasmodium* spp., *Giardia* spp., *C. parvum* and trypanosomes represent a large group of human and animal pathogens with significant impact on the health and economies of many countries. Protozoan parasites are generally capable of very rapid replicative divisions and in many cases the severity of the disease correlates with the high parasite load found in the vertebrate host. Given that protozoan cells can undergo an unlimited number of divisions, they must have a mechanism for overcoming the problem of incomplete chromosome replication. Thus, interfering with parasite telomere maintenance might limit growth of these parasites (Bottius *et al.* 1998; Lanzer *et al.* 1995).

There have only been a limited numbers of studies which investigated the telomereic region of protozoan parasites' chromosomes. The majority of our knowledge of the area is based on yeast studies associated enzyme functions or the structure of the 3' chromosomal region. Focusing on the molecular analysis of *P. falciparum* a number of randomly broken chromosomes occur naturally and suggest that a plasmodial telomerase might be implicated in the reformation of a functional telomere by the addition of new telomere repeats to broken chromosomes (Debruin *et al.* 1994). The 14 linear chromosomes of *P. falciparum* are bounded by closely related G-rich repeats and the most frequent type, of telomere repeat motifs consists of GGGTTT/CA (Table 2.1). The average telomere length has been estimated to be approximately 1.3 kb (Bottius *et al.* 1998; Dore *et al.* 1994). The *G. lamblia* genome is known for its plasticity and its hypervariable sub-telomeric regions of homologous chromosomes

provide the structural basis of the chromosome size heterogeneity that is characteristic of *G. lamblia* (Le Blancq *et al.* 1998). Its chromosomes are compartmentalised into conserved central domains and polymorphic chromosome ends. The genes which reside towards the telomere-proximal domain of the chromosomes are frequently antigen-encoding genes. It has been speculated that the genetic flexibility of the chromosome ends is useful as a tool for the evasion of the hosts immune system (Lanzer *et al.* 1995).

## 2.4 The Telomere in *Cryptosporidium*

The *Cryptosporidium* genome contains several identified repetitive elements. Liu *et al.* (1998) identified a clone, CpGR 254, which contained 48 copies of a repeat sequence $T_{(2\text{-}12)}AG_{(2\text{-}5)}$. In addition, four copies of the repeat TTTAGA were present in the 5 % sequences flanking the imperfect direct repeats. The variability of the identified repeat units suggests that replication of the *C. parvum* telomeric sequence is imprecise (Liu *et al.* 1998). Comparison of the repeat units with sequences present in GenBank demonstrated that the basic repeat structure was similar in composition and structure to telomeric sequences characterised from other lower and higher eukaryotes (Table 2.1) (Aiello *et al.* 1999; Binder *et al.* 1998). To characterise the variations of the repeat sequence motif, each of the repeat units were grouped based on the length and composition of the repetitive element. Liu *et al.* (1998) reported that a total of 28 of the 48 repeat units (58 %) were 6 bp in length and contain the sequence TTTAGG (Liu *et al.* 1998). In general, the most common *C. parvum* repeat motif differed to telomeric sequences of other eukaryotic organisms by only a single T or G nucleotide (Table 2.1). As there are very few nucleotide substitutions present in the *C. parvum* telomeric sequences, generally only deletion and addition of nucleotides, the most likely mechanism for generating the variability in the repeat units is a 'stuttering' mechanism first identified in yeast (DuBois *et al.* 2000; Lue 1999). In this mechanism, the telomerase prematurely dissociates from the template and 'stutters' in the middle of synthesising a telomeric repeat, generating variation in the telomeric sequence. The high variability in the putative *C. parvum* telomeric sequences suggest that the *C. parvum* telomerase is error-prone and that the high error rate in telomeric sequence

synthesis may reflect one of the unique aspects of *C. parvum* biology (Liu *et al.* 1999; Liu *et al.* 1998; Liu *et al.* 1999).

## 2.5 Use of Telomere as a Molecular Marker

Telomere repeat sequences are often used to indicate the period of persistent viral infections (Flint *et al.* 1997). The repeat region lengthens with persistent infections due to the activity of telomerase. Virulent strains of *C. parvum* may have a longer repeat sequence than non-virulent strains. Various repeat lengths have been reported (Binder *et al.* 1998). Hence the telomerase seems to be very error prone in *C. parvum* and therefore may provide an opportunity to investigate strain variability in *C. parvum* by genotyping of the telomere. *C. parvum* has been reported to have 48 copies of $T_{(2\text{-}12)}AG_{(2\text{-}5)}$, with the most common repeat unit being TTTAGG (Liu *et al.* 1998). This is the basic repeat unit which will be trialled as the 3' primer for the PCR sub-species typing. The variable repeat lengths of isolates from different location should be variable due to different environmental pressures, as well as the repeat length should become shorter as the number of infection and re-infection cycles increases.

Pathogen subspecies typing is an essential tool of modern epidemiology. It also provides a powerful tool for environmental management of pathogen sources (Chalmers *et al.* 2002). *C. parvum* is an important environmentally transmitted pathogen, yet despite broad efforts, features of its life cycle and biology have contributed to severely limited success in developing a typing scheme with sufficient resolution at the host level (Chapter 1.4).

Previous genotyping methods have been directed to coding regions of the genome. Furthermore they yield predominantly only two genotypes and have not revealed regional host-isolate differences. Higher resolution using microsatellites (Caccio *et al.* 2000; Feng *et al.* 2000; Mallon *et al.* 2003) provide varying success and as with previous approaches, generally up to four PCR derived bands provide the basis for differentiation.

Accordingly, to better manage the source(s) of human infective parasites there is an apparent need for a reliable and sensitive method for identifying and distinguishing *C. parvum* subspecies as well as subspecies of other eukaryotic organisms.

## 2.6 Objectives

The objectives of this chapter were to:

- Regenerate banding patterns with previously used and reported Microsatellites (Morgan *et al.* 1995);
- Design Primer sets which provide high resolution sub-species genotyping information, in order to be able to distinguish sample origin, using a Microsatellite and Telomere primer set.
- Optimise the Microsatellite and Telomere Primer Set and PCR condition (Mg and Formamide titrations);
- Assess the reproducibility of the dual PCR target using the same sample and between sample location; and
- Investigate the relationship between a range of DNA archived from faeces collected from dairies, piggeries and humans.

## 2.7 Materials

### 2.7.1 Chemicals and Reagents

All chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich, Castle Hill NSW, Australia) except were noted. All chemical reagents were AR grade unless otherwise stated.

## 2.8 Isolation of *Cryptosporidium* Oocysts

Three different faecal oocysts separation methods were used. The method used was dependent on the final application. For oocysts which were being used for PCR, salt-flotation was used as the primary oocyst isolation (Chalmers *et al.* 2002). For all oocysts which were being used for microscopy or microscopy followed by PCR, Sheathers solution flotation followed by percoll gradients were used (Ongerth *et al.* 1987).

### 2.8.1 *Cryptosporidium* Isolation Using Salt Flotation

The method used was described by Elwin *et al.* (2001). This method is a modification of that first described by Rylet *et al.* (1976). Ryley *et al.* (1976) measured the specific gravity of the saturated salt solution to be 1.06 (Rylet *et al.* 1976). The method as described by Elwin *et al.* (2001) was to use two 15 mL polypropylene centrifuge tubes which were labelled per faecal sample. A saturated NaCl (8 mL) solution was added to one of the tubes. Approximately 2 mL of calf faeces was emulsified with the aid of a swab stick and added to the salt solution tube. The samples were then vortexed for 15 s to obtain a faeces/salt suspension. Deionised water (2 - 3 mL) was carefully placed onto the surface of the salt solution/faecal pellet suspension. The tubes were centrifuged for 10 min at 1 500 x g. Using a tip of a Pasteur pipette, the liquid in the water layer was swirled until cloudy (drawing oocysts from the salt solution/water interface into the water). A Pasteur pipette was used to remove the entire interphase layer and was transferred into the second labelled 15 mL centrifuge tube and 10 mL of $dH_2O$ was added. The tube's contents were mixed by inversion and centrifuge for 5 min at 1 500 x g and the supernatant then decanted off. The pellet was resuspended in $dH_2O$ (1 mL) and pipetted into a 1.5 mL microfuge tube and oocysts stored at 4 °C until analysis (Elwin *et al.* 2001).

### 2.8.2 *Cryptosporidium* Isolation Using Sheathers Solution

*Cryptosporidium* oocysts were prepared from naturally infected dairy calf faecal samples as previously described (Ongerth *et al.* 1987). In brief, faeces (1g) was resuspended in 40 mL of Sheathers and the tubes centrifuged for 10 min at 1 500g. Deionised water (5 mL) was used as an overlay and using the tip of a pipette oocysts were lifted into the aqueous layer by gently swirling. The 5 mL $dH_2O$ layer was removed to a clean 50 mL centrifuge tube. Using $dH_2O$ the volume was made up to 30 mL. The tubes were centrifuged for 10 min at 1 500g. The $dH_2O$ wash was repeated a further two times. The pelleted oocysts were resuspended in 40 mL of $dH_2O$ and oocysts preparations were stored at 4°C.

### 2.8.3 *Cryptosporidium* isolation using percoll

Each *C. parvum* sample was split across three to four 15 mL centrifuge tubes for purification (Grenier, Interpath Sydney, NSW Australia) were used per sample. A 4

mL of 1.05 g/ mL percoll overlayed was carefully layered over the bottom layer of 4 mL of 1.09 g/ mL percoll. A 4 - 6 mL faecal suspension was then introduced over the percoll layers. The tubes were centrifuged for 10 min at 1 500 x g and 4 °C. Using a Pasteur pipette the interphase between the 1.09 g/ mL and 1.05 g/ mL layers was harvested and placed into a fresh 15 mL centrifuge tube. To each tube 10 mL of $dH_2O$ was added and mixed by inversion. All tubes were centrifuged for 10 min at 1 500 x g and 4 °C. The $dH_2O$ wash was repeated a further 2 times. The pelleted oocysts were resuspended in 10 mL of $dH_2O$ then stored at 4 °C.

## 2.9 Immunofluorescence Staining

Immunofluorescent staining with a FITC-labelled monoclonal antibody (Crypto-Glo, *Giardia*-Glo from Waterborne Inc. New Orleans, LA USA) was preformed as described by Stibbs and Ongerth (1986) using 13 mm filters, 1 μm pore size (Millipore North Ryde, NSW, Australia). Figure 2.1 B illustrates a typical IFA staining of an oocysts suspension and in Figure 2.2 typical IFA stained oocysts from a concentrate made from 10 L of raw water.

### 2.9.1 Staining of Oocysts with PI

The stock solution of propidium iodide (PI) (MW = 668.4) was made by dissolving 1 mg/ mL (1.5 mM) $dH_2O$ and stored at 4 °C, protected from light for up to 4 weeks. PI was used with the IFA in a single staining incubation as described by Stibbs and Ongerth (1986) (Figure 2.1 D).

### 2.9.2 Staining Oocysts with DAPI

A stock solution of 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (MW = 350.3) was prepared by using $dH_2O$ to give a final concentration of 100 μM, which was store at 4 °C, protected from light for up to one week.

The working solution of DAPI was prepared by diluting the DAPI stock solution to 300 nM with PBS. The working solution of DAPI (240 μL) was added to the IFA solution (described in section 2.9) and a single staining incubation as described by Stibbs and Ongerth (1986) (Figure 2.1 B).

## 2.10 Microscopy

Epifluorescent microscopy was undertaken with a Leitz, Laborlux S, (Leica Microsystems, Gladesville, NSW, Australia), using a 50 W lamp and fitted with the following filters, for fluorescein isothiocyanate (FITC) (excitation 490 nm, emission 525 nm), PI (excitation 5363 nm, emission 617 nm) and DAPI (excitation 350 nm, emission 470 nm). All photography of oocysts were taken using wild MPS 46 photoautomat which was attached to a Leitz, Laborlux S, (Leica Microsystems, Gladesville, NSW, Australia) using 400 ASA Fuji film (colour negative).

## 2.11 Sterilisation of Biological Materials

Solutions that were heat stable, disposable plastic microfuge tubes (Integrated Sciences, Willoughby NSW Australia) and micropipette tips were autoclaved for 20 min at 121 °C, 125 kPa. For solutions which were heat labile, filter sterilisation through disposable sterile syringe filters (32 mm, 0.1 μm pore size) were used (Pal, Gelman Sciences, Lane Cove NSW, Australia).

All biological contaminated material was autoclaved at 121 °C, 125 kPa for 30 min prior to disposal.

## 2.12 DNA Extraction and Concentration

Qiagen QIAamp DNA extraction kit (Qiagen, Castle Hill NSW, Australia) was used for isolating DNA as per manufacturers instructions, typically starting with 1 000 oocysts prepared as described in section 2.8.3.

DNA was precipitated using 0.1 volume of 3M sodium acetate (pH 5) and an equal volume of spectral grade isopropanol. The mixture was keep at –20 °C for 20–30 min, then the precipitated DNA was collected by centrifugation at 14 000 x g for 15 min. The resultant pellet was washed with aqueous 70 % (v/v) ethanol and the washing solution removed. The samples were air dried for 5 min and redissolved in an appropriate volume of Tris EDTA buffer (TE: 100 mM Tris base, 10 mM EDTA, pH 8.2 ).

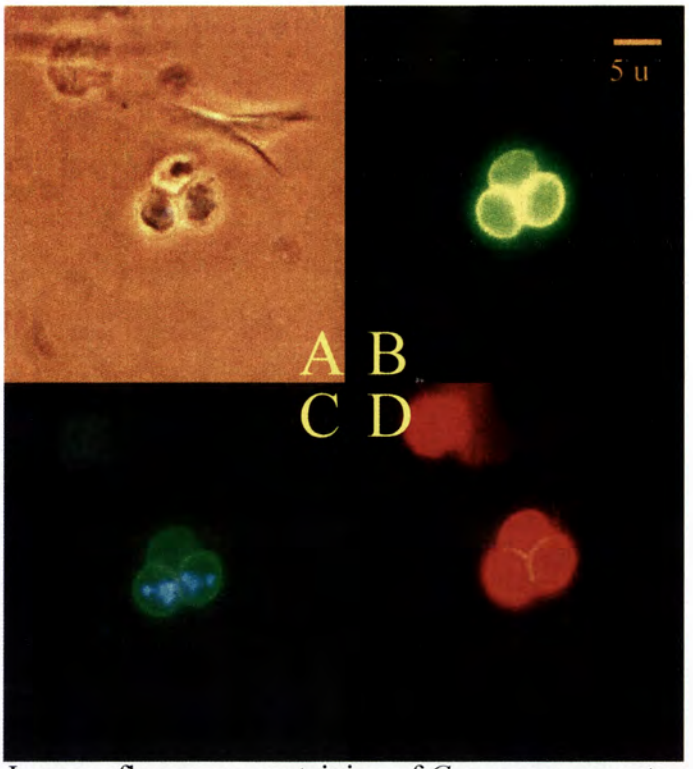


Figure 2.1: Immunofluorescence staining of *C. parvum* oocysts

A: Bright field
B: Immunofluorescence Staining with FITC mAb
C: Stained Oocysts with DAPI
D: Stained Oocysts with PI

DNA concentrations were deter mined by using a spectrophotometer at a wavelength of 260 nm (Cary UV Vis, 1E, Varian, Inc. Mulgrave Victoria, Australia). It was assumed that at a wavelength of 260 nm with a light path of 1 cm, 50 μm/ mL of double stranded DNA give and absorbance of 1.0 (Sambrook *et al.* 1989).

## 2.13 Gel Electrophoresis

### 2.13.1 Agarose

DNA preparations and aliquot's from PCRs were routinely analysed by electrophoresis through 1 % (w/v) agarose gels (Agarose Molecular Grade, Promega, Annandale NSW, Australia). Samples were mixed with gel loading dye (40 % [v/v] sucrose, 0.25 % [v/v] bromophenol blue, 0.25 % [v/v] xylene cynol) resolved by electrophoresis at 80-100 mA (EneMate, Mini vertical gel system, Gradipore, Frenchs Forest NSW,

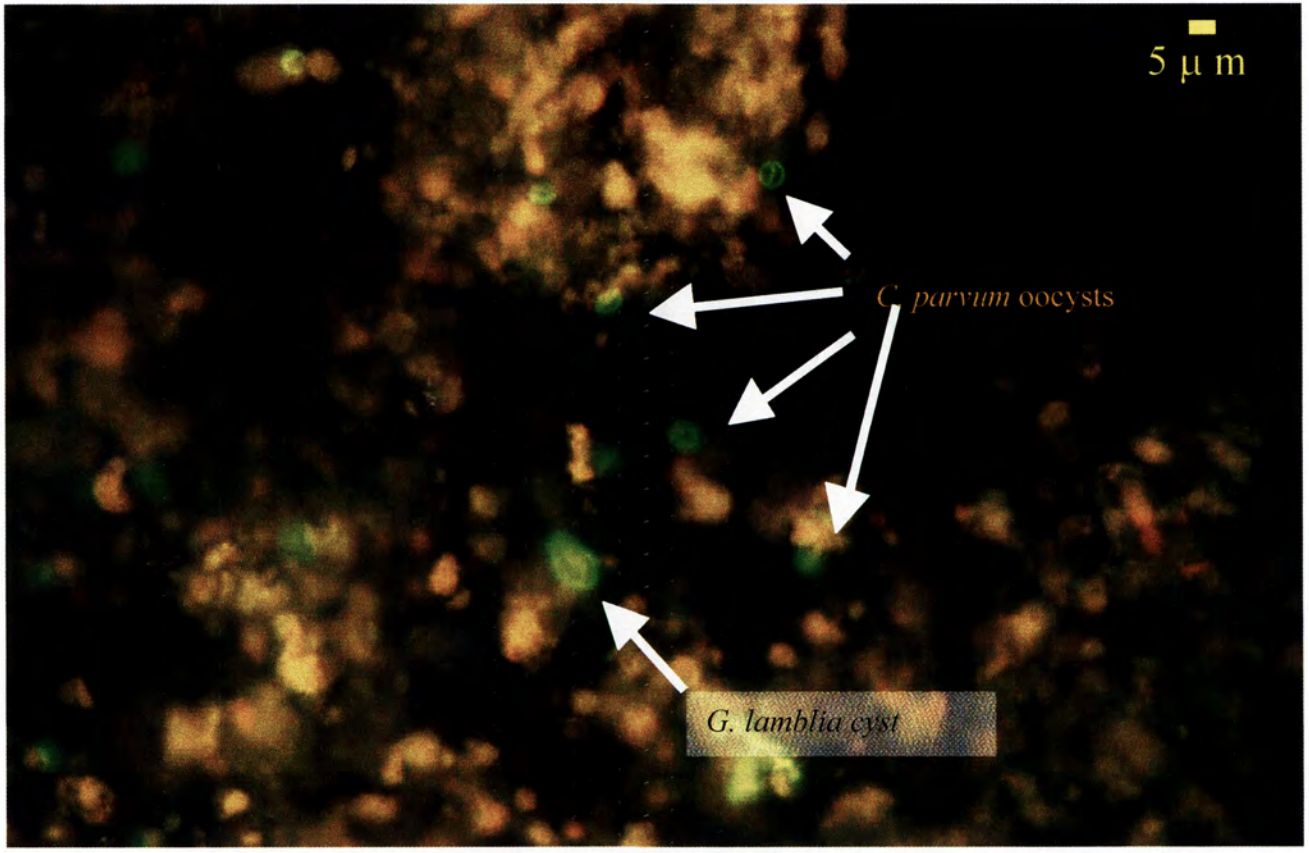


Figure 2.2: Immunofluorescence staining of *C. parvum and G. lamblia* within a $10^4$-fold concentrate from a water sample

Australia) in TAE buffer for 20 - 30 min in a submarine gel tank at room temperature. The DNA was stained with ethidium bromide (10 mg/ mL by adding 20 µL was added to the TAE running buffer prior to electrophoresis. Agarose gels were used for PCR-RFLP studies. Agarose gels were initially used for the first few PCRs, however the switch to PAGE occurred so that all the all the PCR bands easily be identified.

### 2.13.2 PAGE

Polyacrylamide gels (8 % igels, Gradipore, Frenchs Forest NSW, Australia) were used for analysis of all PCR amplicons. Each gel was run in accordance to the manufacturers recommendations, which were 10 min pre electrophoresis with TG running Buffer (Tris 2.9 g, Glycine 14.4 g in one litre of $dH_2O$) running buffer. The pre-electrophoresis buffer was removed and replaced by the running buffer TBE running Buffer (Tris 1.08 g, Borate 5.5 g, EDTA 0.75 g) for 60 - 90 min at 200 volts. PAGE gels were used for all microsatellite and telomere subtyping studies. The switch to PAGE was because the agarose gels were not providing sufficient resolution for the identification of all the PCR bands.

### 2.13.3 Photography of Gels

Agarose and PAGE gels were examined with a transilluminator (Pathtech, Box Hill Victoria, Australia) at a wavelength of 254 nm. They were photographed using a Polaroid CU-5 camera (Sigma-Aldrich, Castle Hill NSW, Australia) and Polaroid type 667 film (Sigma-Aldrich, Castle Hill NSW, Australia). A second digital photograph was also taken using a Kodak DC-290 camera (Integrated Sciences, Willoughby NSW, Australia) which was used for banding analysis (section 2.14).

## 2.14 PCR Banding Analysis

Digital photographs of gels were examined by EDAS-290 1D analysis software (Integrated Sciences, Willoughby NSW, Australia) according to manufacture's instructions. The software was used to deter mine the band sizes for all samples.

## 2.15 *Cryptosporidium* Isolates

The *Cryptosporidium* isolates which were used for the development of the typing scheme are shown in Table 2.2. A total of 67 isolates from calves were examined.

All the isolates which we collected were identified using fluro-labelled Ab and manually identified using a fluro microscope. The cattle samples were identified as *Cryptosporidium*. A *C. andersoni* Ab was also used and no sample was identified as positive for *C. andersoni*. When the experimental assays for this thesis were preformed *C. suis*, had not been identified. The pig isolates which were used were positive for *C. parvum* by immuno assay.

### 2.15.1 Non *Cryptosporidium* Isolates

Table 2.3 lists the non-*C. parvum* isolates which were used during the establishment and development of the sub-species typing scheme. The *Giardia* from Sydney South West (Syd SW) and Sydney North West (Syd NW) (Sydney) were detected at the time of *C. parvum* identification. All other non-*C. parvum* isolates were Laboratory cultured strains (Table 2.2).

## 2.16 Development of PCR Protocols

The microsatellites used were first described by Morgan *et al.* (1995), being $(GAA)_5$ (M1) and $(GACA)_4$ (M2). Both of these primers were reported to give discrimination between genotypes I and II.

During the development of the PCR protocols, each isolate underwent four PCRs, which consisted of two separate DNA extractions and then two PCRs was preformed for each DNA extraction.

Table 2.2: Isolates used during the comparison of previous "fingerprinting" protocols *C. parvum* isolates

| **Sample type** | **Location of Collection** | **Date Collected** | **No of Isolates** | **Host Age Range at Collection** |
|---|---|---|---|---|
| Bovine | Syd SW (Sydney) | September 1988 | 12 | 6-50 days |
| | Syd NW (Sydney) | January 1999 | 6 | 7-47 days |
| | NSW C (New South Wales) | February 1999 | 4 | 14-18 days |
| | NSW C (New South Wales) II | February 1999 | 3 | unknown |
| | Bel[+] (Europe) | November 1999 –February 2000 | 2 | unknown |
| | Iowa (Arizona)* | September 1998 | 2 | unknown |
| | Iowa (Wales)* | | 2 | unknown |
| Porcine | Sth NSW | February 1999 | 6 | 6–7 weeks |
| Ovine | NSW W (Fowler's Gap NSW) | April 2001 | 6 | unknown |
| | Victoria A | September 2000 | 6 | unknown |
| | Victoria B | September 2000 | 6 | unknown |
| Red Kangaroo | NSW W (Fowler's Gap NSW) | April 2001 | 6 | unknown |
| Grey Kangaroo | NSW W (Fowler's Gap NSW) | April 2001 | 6 | unknown |

\+ Samples provided by Ms. Cisha Schets from RIVM, Bilthoven The Netherlands.
* Samples provided by Ms. Marilyn Marshall from University of Arizona, Tucson USA

Table 2.3: Isolates used during the comparison of previous "fingerprinting" protocols: Non-*C. parvum* isolates

| Sample type | Organism | Date Collected | Location of Collection | No. of Isolates | Host Age Range at Collection |
|---|---|---|---|---|---|
| Bovine | *Giardia* sp. | September 1988 | SydSW (Sydney) | 4 | 28-36 days |
| | *Giardia* sp. | January 1999 | SydNW (Sydney) | 4 | 25-32 days |
| Laboratory culture | *Giardia*[a] sp. | - | - | 4 | - |
| | *Plasmodium vivax*[b] | - | - | 2 | - |
| | *Plasmodium falciparum*[b] | - | - | 2 | - |
| | *B. fragilis* HSP40 | - | - | 4 | - |
| | *E. coli* ATCC 10798D | - | - | 4 | - |
| | *Cryptosporidium meleagridis*[c] | - | - | 2 | - |

a: From Assoc. Prof. Mike Edwards, School of Biotechnology and Molecular Sciences, UNSW
b: From Dr. John Watkins, Westmead Hospital, Sydney NSW, Australia
c: From Dr. Rachael Chalmers, PHLS *Cryptosporidium* Reference Unit, Swansea Wales

### 2.16.1 PCR Fingerprinting Using Microsatellite 1

Reaction mix used for microsatellite 1 (M1) consisted of:

| | | |
|---|---|---|
| DNA | 2 μL | (DNA concentration was 100 ng/μL) |
| dNTP | 1 μL | (initial concentration was 10 mM) |
| Microsatellite 1 primer | 2 μL | (10 pM $(GAA)_5$) |
| 10x Buffer for Red Hot *Taq* | 4 μL | (Integrated Sciences, Willoughby NSW, Australia) |
| $Mg^{2+}$ | 4 μL | (initial concentration was 25 mM) |
| Red Hot *Taq* | 0.25 U | (Integrated Sciences, Willoughby NSW, Australia) |

PCR contained 40 μL total reaction vol

The optimised PCR cycling conditions were preformed on a FTS-1 Thermal Cycler (Corbett Research, Mortlake NSW, Australia) and consisted of:-
94 °C for 30 seconds, followed by 30 cycles of 92 °C (20 seconds), 45 °C (20 seconds), 72 °C (30 seconds). A final anneal of primers was preformed for 45 seconds at 45 °C followed by 5 min at 72 °C .

As expected, the M1 primer provided substantial resolution between bovine (Figure 2.3 A lanes 3 - 7 and C 3,4, 6,7), ovine (C lane 5) and porcine (B lanes 3 - 7) hosts. For the one host (samples of bovine origin), however, there was no resolution (Figure 2.3 C) seen within the one source from different geographical regions. The other two sample were in lane 2, *B. fragilis* and lane 5, Ovine from Western NSW.

When the PCR using the M1 primer alone was used on the *C. parvum* isolates from geographically diverse sources only one banding pattern was consistently observed, with the bovine isolates gave banding patterns on the PAGE in the 250 to 3 000 bp range (Figure 2.3).

### 2.16.2 PCR Fingerprinting Using Microsatellite 2

As for M1, conditions were optimised for the M2 primers, which turned out to be the same as described for M1 (section 2.16.1).

Figure 2.4 shows that for the PCR using M2 primers alone *C. parvum* isolates from geographically diverse sources consistently yielded only one banding

pattern. Therefore, like M1, when M2 is used alone no geographic discrimination was seen within the bovine host.

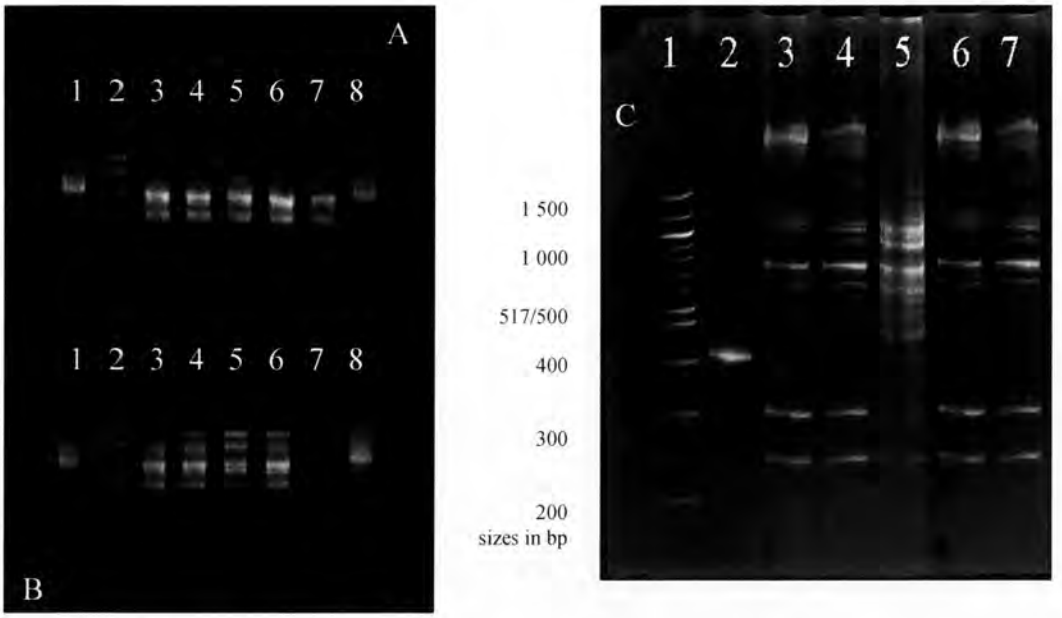


Figure 2.3: M1 PCR PAGE on isolated *C. parvum* DNA from zoonotic sources

Panel A: Lane 1 100 bp Size Standard, Lane 2 *B. fragilis*, Lane 3 Bovine 1 Syd SW, Lane 4 Bovine 2 Syd NW, Lane 5 Bovine 3 NSW CW 1, Lane 6 Bovine 4 NSW CW 2, Lane 7 Bovine 5 Syd SW, Lane 8 100 bp Size Standard (New England Biolabs, Brisbane AU).

Panel B: Lane 1 100 bp Size Standard, Lane 2 *B. fragilis,* Lane 3 Pig 6E, Lane 4 Pig 7A, Lane 5 Pig 7D, Lane 6 Pig 7E, Lane 7 Bovine 1 SydSW, Lane 8 100 bp Size Standard.

Panel C: Lanes 1 100 bp ladder (New England Biolabs, Brisbane AU), Lane 2 Bacterial sample (*B. fragilis*), lane 3 Isolate from SydSW, lane 4 Isolate from SydNW, lane 5 Isolate from Western NSW, lane 6 Isolate from NSWC, lane 7 Isolate from Bel.

### 2.16.3 PCR Development Using M1 + M2

To investigate whether M1 + M2 primers could provide geographical host discrimination, PCR conditions were optimised for M1 + M2 primers, though the same conditions as used for M1 and M2 primers alone (section 2.16.1) were found to be satasfactory. The $Mg^{2+}$ concentration was optimised by performing a PCR with increasing $Mg^{2+}$ concentrations, ranging from 0.5 mM to 10 mM final concentration. Also the primer concentration was also varied in order to obtain the optimal PCR conditions. The results of the PCR with the optimised $Mg^{2+}$ and primer concentrations shown in Figure 2.5.


Figure 2.4: M2 PCR PAGE on isolated *C. parvum* DNA from zoonotic sources
Lane 1, 100 bp Size Standard, Lane 2 Syd SW, Lane 3 Syd SW, Lane 4 Syd SW, Lane 5 Syd SW, Lane 6 Bovine 3 Syd NW, Lane 7 NSW CW 1, Lane 8 NSW CW 2 Lane 9 Syd SW, Lane 10 1 KB Size Standard (New England Biolabs, Brisbane AU).

In contrast to M1 or M2 alone, when combined, both microsatelite primers provided little information due to inconsistent amplification of products (Figure 2.5). Furthermore, the bacterial control gave a very similar banding pattern to the *C. parvum* isolates. The results shown in Figure 2.5 is of one PCR run, however the experiment was repeated a further three times and the same results as those shown were achieved.

### 2.16.4 PCR Development Using Telomere Primers With M1

A total of 67 samples (Tables 2.2 and 2.3) were used during the establishment of the dual M1 + T PCR protocol. Each sample was amplified at least twice and the same fingerprint was achieved (Figure 2.6) with the preferred protocol described below. When the T only primer was used alone within a PCR no bands were visible. This was the case for all PCRs trialled. DNA from bacterial spp or protozoan spp gave the same no band PCR result.

The PCR reagent mixture (per 40 μL) was:

| | | |
|---|---|---|
| DNA | 2 μL | (DNA concentration was 100ng/μL) |
| dNTP | 1 μL | (initial concentration was 10mM) |
| Froward M1 primer | 3 μL | (10 pM $(GAA)_5$) |

| | | |
|---|---|---|
| Reverse T1 primer | 3 µL | (10 pM CCTAAA CCTAAA or |
| Reverse T2 primer | 3 µL | (10 pM G CCTAAA CCTAAA) |
| 10x Buffer for Red Hot *Taq* | 4 µL | (Integrated Sciences, Willoughby NSW, Australia) |
| $Mg^{2+}$ | 4 µL | (initial concentration was 25mM) (see section 2.16.4.1) |
| Red Hot *Taq* | 0.25 U | (Integrated Sciences, Willoughby NSW, Australia) |

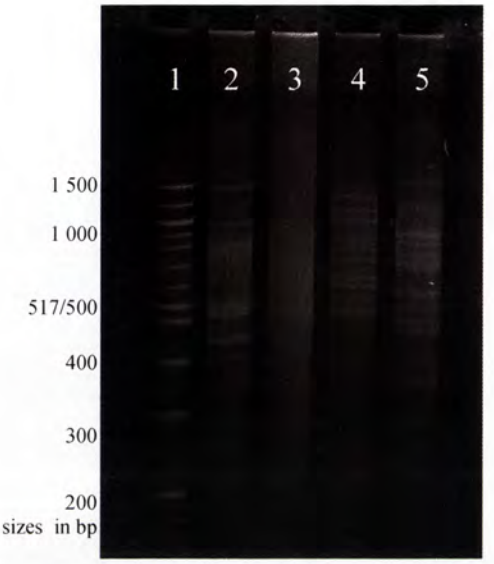


Figure 2.5: M1 and M2 PCR PAGE on isolated *C. parvum* DNA from zoonotic sources

Lane 1. 100 bp Size Standard, Lane 2 Bovine 1 Syd SW, Lane 3 Bovine 2 Syd SW, Lane 4 Bovine 3 Syd NW, Lane 5 Bovine 4 NSW CW 1.

The optimised PCR cycling conditions were performed on a FTS-1 Thermal Cycler (Corbett Research, Mortlake NSW, Australia) and consisted of:-
94 °C for 30 seconds, followed by 30 cycles of 92 °C (20 seconds), 40 °C (20 seconds), 60 °C (120 seconds). A final anneal of primers was preformed for 45 seconds at 40 °C followed by 5 min at 72 °C .

### 2.16.4.1 Method Optimisation: $Mg^{2+}$ Titre And Formamide Concentration Titration For M1 + T Primers

The magnesium concentration titration ranged from 0.5 mM to 10 mM final concentration (Figure 2.6). It was deter mined that 4 mM was optimal and was therefore used in all following PCR setups. A formamide concentration was also trailed (Figure 2.7) to determine if the banding patterns observed were due to non-specific binding. The titration covered the range of 0.001–0.1 % formamide in the final PCR tube. All bands observed with no formamide added to the PCR were still observed with most concentrations of formamide. The only two

exceptions were the 0.05 and 0.1 % formamide tubes, were no PCR product was observed, presumably due to inhibition of the DNA polymerase.

Due to the low annealing temp – had to be used due to the shortness of the primers. The temperature used was 2°C below their melting points. In order to increase the specificity of the PCR formamide was added to the PCR to increase the binding stringency of the primers. By doing this the specificity of the reaction was increase and the chance of non specific binding of the primers is reduced. This technique is commonly utilised in DNA/RNA hybridisations. Section 2.16.4.1 explained the reasoning for this experiment set – add a section in the conclusions of chapter 2 mentioning the specificity of the primers due to the stringency being increased caused by the addition of formamide

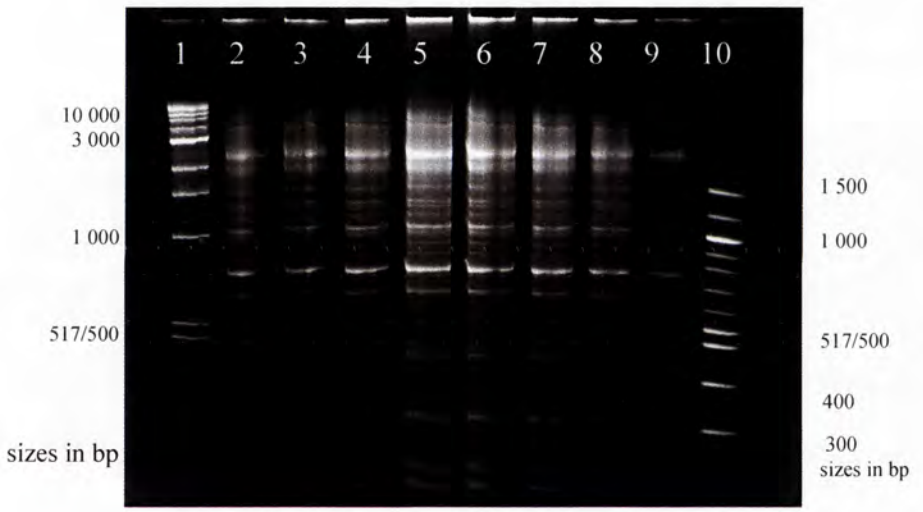


Figure 2.6: M1 + T PCR PAGE magnesium titration using and isolate from Syd SW 1

Lanes 1 1Kp ladder (New England Biolabs), lane 2: 0.5mM of Mg, lane 3: 1mM of Mg, lane 4: Isolate 2mM of Mg, lane 5: 4mM of Mg, lane 6: 5mM of Mg, lane7: 6mM of Mg, lane 8: Isolate 8mM of Mg, lane 9: 10mM of Mg, lane 10: 100 bp ladder (New England Biolabs).

### 2.16.4.2 Host-Specific Fingerprints With Dual M1 + T Primers

M1 + T PCR PAGE was applied to DNA from non *C. parvum* spp. For the determination of host specificity. Figure 2.8 illustrates the fingerprint profiles achieved when M1 was the sole primer and compared to the PCR fingerprint produced by M1 + T primers. For DNA samples from bacteria, *Giardia* and *P. vivax* there was no difference between the M1 alone and M1 + T primer PCR.

The only organism where the two PCRs differed was when DNA from *C. parvum* was amplified. The results from this PCR experiment illustrates that the M1 + T primer PCR was *Cryptosporidium* specific. Expand and make clearer – mention that the crypto isolate was as pure as possible – isolated from percol gradient – specific gravity.

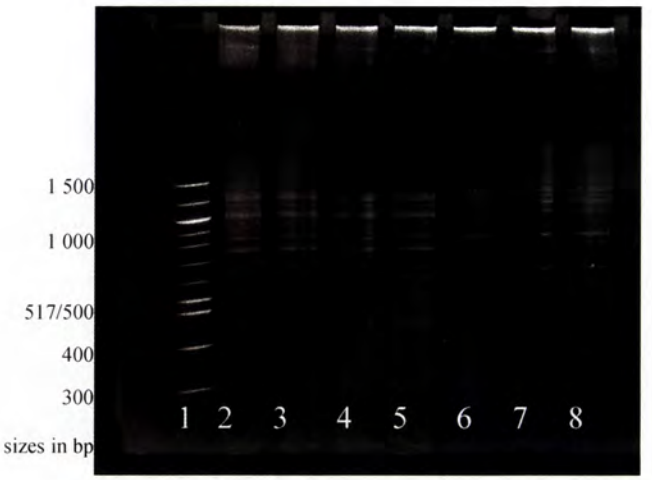


Figure 2.7: M1 + T primers PCR PAGE formamide titration

Lanes 1 100bp ladder (New England Biolabs), lane 2: Syd SW 1, with 0.001 % formamide, lane 3: Syd SW 1, with 0.0025 % formamide, lane 4: Isolate Syd SW 1, with 0.05 % formamide, lane 5: Syd SW 1, with 0.01 % formamide, lane 6: Syd SW 1, with 0.025 % formamide, lane 7: Syd SW 1, 0.05 % formamide, lane 8: Isolate Syd SW 1, with 0.1 % formamide


Figure 2.8: M1 + T PCR PAGE for genotyping

Lanes 1 100bp ladder (New England Biolabs), lane 2 Bacterial sample with M1 only, lane 3 Bacterial sample with M1 + T primers, lane 4 Isolate from Syd SW 1 with M1 only, lane 5 Isolate from Syd SW 1 with M1 + T primers, lane 6 *Giardia* Isolate with M1 only, lane7 *Giardia* Isolate with M1 + T primers, lane 8 *P. vivax* with M1 only, lane 9 *P. vivax* with M1 + T primers, lane 10 1Kb ladder (New England Biolabs).

### 2.16.5 PCR Development Using M2 + Telomere Primers

The telomere primer was first used with M2 (section 2.16.2). The initial samples used were the same isolates examined with the initial Microsatellite PCRs (Table 2.2). An example of the results achieved is shown in Figure 2.9. The primers did not give any useful information regarding sub-species genotyping for *C. parvum*. The bacterial samples appear identical and give the same banding pattern as if the telomere primer was not there. All *C. parvum* and *Giardia* isolates smeared and no sub-species information obtained.

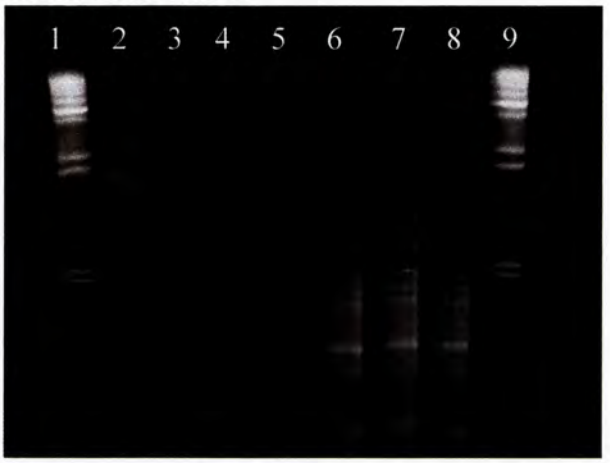


Figure 2.9: M2 + T PCR PAGE with DNA from *C. parvum*

Lane 1. 100 bp Size Standard; Lane 2 Bovine 1 Syd SW; Lane 3 Bovine 2 Syd SW; Lane 4 Bovine 3 Syd NW; Lane 5 Bovine 4 NSW CW 1; Lane 6 Bovine 5 NSW CW 2 Lane 7 Bovine 6 Syd SW; Lane 8. 1 KB Size Standard (New England Biolabs, Brisbane AU).

### 2.16.6 M1 + T PCR PAGE With Degrading DNA

A concern with the M1 + T PCR PAGE was the production of bands of 500 bp and lower (results not shown). It was believed that the telomere repeat region was shorting. To confirm this hypothesis isolates from Syd SW had their DNA sheared by passing it several times through a syringe with a 30 gauge needle, DNA samples from other isolates also from Syd SW were subjected to six round of freeze thaw cycling in an attempt to induce partial chromosomal fracturing. Figure 2.10 shows the results of this investigation. Lanes 3-5 are three isolates where freeze thaw was used and lanes 7 and 8 the DNA was passed through the syringe prior to the PCR PAGE. Both sets of results reproduced similar banding patterns which would be possible if the 3' ends of the chromosome were shorting either by loss of repeat number or chromosomal end damage.

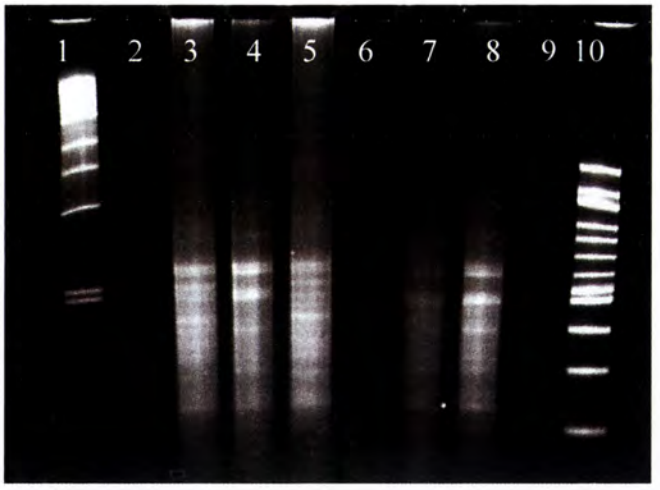


Figure 2.10: M1 + T PCR PAGE from isolates with degraded DNA

Lane 1. 100 bp Size Standard, Lane 2 Bovine 1 Syd SW, Lane 3 Bovine 2 Syd SW, Lane 4 Bovine 3 Syd NW, Lane 5 Bovine 4 NSW CW 1, Lane 6 Bovine 5 NSW CW 2 Lane 7 Bovine 6 Syd SW, Lane 8. 1 Kb Size Standard (New England Biolabs, Brisbane AU).

### 2.16.7 M1 + T PCR PAGE With DNA Mixed From Two or More Isolates

Isolates from several sampling locations were mixed together in different ratios to assess the ability of the M1 + T PCR PAGE to identify samples from mixed sources. It was shown that when three or more isolates were combined no individual PCR were able to be identified and only a smear was observed. Figure 2.11 illustrates some of the possible outcomes when two samples are mixed in differing ratios. Lane 2 is an isolate from Syd SW and lane 3 is from Syd NW. All the other lanes are different ratios of the two isolates. Lane 4 is a 1:1 ratio of the two isolates. Lane 5 is 60 % Syd SW with 40 % Syd NW, while lane 6 is the reverse of this. Lane 7 is 80 % % Syd SW with 20 % Syd NW.


Figure 2.11: M1 + T PCR PAGE with DNA mixed from two or more isolates

Lane 1, 100 bp Size Standard, Lane 2 Bovine 1 Syd SW, Lane 3 Bovine 2 Syd NW, Lane 4 Bovine 3 Syd NW & Syd NW 1:1, Lane 5 60 % Syd SW with 40 % Syd NW, Lane 6 40 % Syd SW with 60 % Syd NW, Lane 7 80 % Syd SW with 20 % Syd NW, Lane 8, 1 Kb Size Standard (New England Biolabs, Brisbane AU).

## 2.17 Dendogram Construction

Phenotypic analysis of the profiles identified by EDAS 1D Gel Analysis (section 2.14) was performed by calculating pairwise genetic distances from a binary matrix. Each isolate was identified by the presence or absence of each band. The band sizes which were included in the matrix ranged from 3 000 bp to 200 bp, giving 114 possible unique identifiers. The resulting tree (Figure 2.12) was constructed by using the neighbour-joining method and the NJTREE program by the Australian National Genome Information Service, BioManager (http://www.angis.org.au) (Sydney, Australia) (Neilan *et al.* 1997).

The fingerprint profiles were also used for cladistic analysis in which character changes along branches of the derived tree were minimized. The rules of parsimony were applied to the profile data by using the Dollo parsimony method supplied by the DOLLOP program in the PHYLIP package (version 3.57c) (Felsenstein 1993).

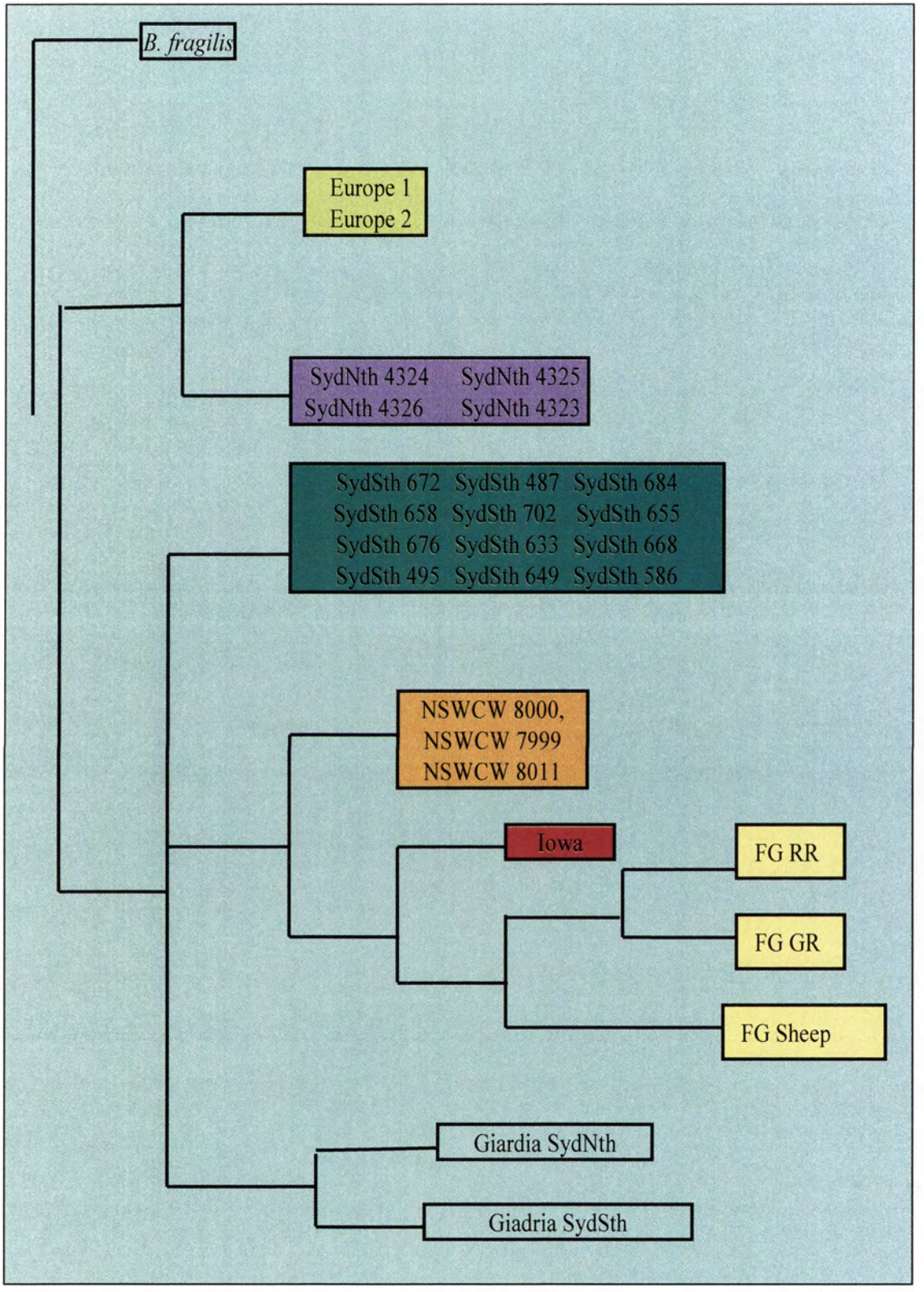


Figure 2.12: M1 + T PCR PAGE relative banding dendrogram

The phylogeny programs were made available by the Australian National Genome Information Service, BioManager (http://www.angis.org.au) (Sydney, Australia).

## 2.18 Conclusions

Microsatellites alone or in combination can provide valuable information as to the possible host species of *Cryptosporidium* oocysts (Nichols *et al.* 2003; Smith 1998; Webster *et al.* 1996). Nonetheless, best management practices are controlling animal faecal run-off at the contributing farm level. Hence there is a real need to further sub-type at the host-farm/zone level. The dual M1 + T PCR protocol developed appears to exploit an apparent fortuitous microsatellite-telomere region in the genome of *Cryptosporidium.*

Accordingly, the present protocol provides a method for the sub-species identification of *C. parvum* with higher resolution than previously reported. The method exploited differences from the 3' non-coding telomeric region of the genome. Whilst there are 8 chromosomes in *C. parvum* (Piper *et al.* 1998), it is unclear whether one, some or all of *C. parvum* chromosomes are involved in the banding patterns produced. Therefore, each chromosome could result in several bands

The PCR using the M1 + T2 primers provide sub-species information which appears specific for *Cryptosporidium* (Figure 2.8). Furthermore, the PCR banding patterns achieved were consistent between PCR runs and exactions. Each sample underwent two independent DNA isolations and each isolation was run twice by PCR. The DNA fingerprints which were observed was the same and consistent for each of the four PCRs per sample. All isolates from the same farm had the same fingerprint. In contrast, but as expected all *Giardia* isolates produced different banding patterns regardless of their source location and all the bacterial (*B. fragilis*) samples appear to be identical with the M1 or with M1 and T2 primers. Furthermore, all pigs isolates had the same banding pattern, which

differed from the patterns produced by bovine isolates of *C. parvum.* PCRs using the *B. fragilis* was an attempt to see what any bacterial comtamination could pose to the PCR

Due to the low annealing temp – had to be used due to the shortness of the primers. The temperature used was 2°C below their melting points. In order to increase the specificity of the PCR formamide was added to the PCR to increase the binding stringency of the primers. By doing this the specificity of the reaction was increase and the chance of non specific binding of the primers is reduced. This technique is commonly utilised in DNA/RNA hybridisations. Section 2.16.4.1 explained the reasoning for this experiment set – add a section in the conclusions of chapter 2 mentioning the specificity of the primers due to the stringency being increased caused by the addition of formamide.

### 2.18.1 Microsatelite Primer PCR

The M1 or M2 PCR set up was able to amplify all DNA isolates regardless of source, but only provided species level information, although different between M1 or M2 primers. For example, all bovine isolates gave the same fingerprint which was different from the pig isolates, but neither resolved between geographical location. There was no advantage in combining M1 and M2 as the PCR banding pattern achieved by this protocol was weak and inconsistent. Indeed, the of isolates gave a smearing pattern. Previous reports using these two primers alone was shown to be useful for the descrimination between genotype I and genotype II (Morgan *et al.* 1995)

### 2.18.2 M1 + T primer PCR

The only primer set examined that provided sub-species "finger printing" information was the M1 + T. Each geographic location investigated with the M1 + T PCR produced their own consistent fingerprint profile. Every isolate from the one location produce the same PCR banding pattern. Therefore, the PCR

appears to be specific for *Cryptosporidium* and when the banding patterns are compared to the M1 PCRs only *C. parvum* isolates provide a different fingerprint profiles. The bacterial and *Giardia* isolates have their own unique profiles, which did not change when M1 alone or M1 + T primers were used, suggesting genus specificity of the telomere primers.

### 2.18.3 M1 + T2 Primer PCR Condition for Sub-Species Typing

The final PCR protocol which was used for all subsequence work in this thesis used a 40 μL reaction mixture which contained:

| | | |
|---|---|---|
| Froward M1 primer | 3 μL | (10 pM $(GAA)_5$) |
| Reverse T2 primer | 3 μL | (10 pM G $(CCTAAA)_2$) |
| dNTP | 1 μL | (initial concentration was 10mM) |
| 10x Buffer for Red Hot *Taq* | 4 μL | (Integrated Sciences, Willoughby NSW, Australia) |
| $Mg^{2+}$ | 8 μL | (initial concentration was 25mM) |
| Red Hot *Taq* | 0.25 U | (Integrated Sciences, Willoughby NSW, Australia) |

The optimised PCR cycling conditions were preformed on a FTS-1 Thermal Cycler (Corbett Research, Mortlake NSW, Australia); using 94 °C for 30 seconds, followed by 30 cycles of 92 °C (20 seconds), 40 °C (20 seconds), 60 °C (120 seconds). A final anneal of primers was preformed for 45 seconds at 40 °C followed by 5 min at 72 °C .

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# 3 Chapter 3: Fingerprinting of *Cryptosporidium* from Animal Sources


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## 3.1 Introduction

*Cryptosporidium* species have been found in over 79 different mammalian species (O'Donoghue 1995). Infections, have been describes in domestic, wild and captive animals. However, young animals are more susceptible to clinical disease and mortalities have been recorded in neonates of many species, especially amongst ruminants. Outbreaks of disease within animal populations have predominantly been associated with intense husbandry (Olson *et al.* 1997), seasonal breeding and mixed grazing practises, with specific contamination events including fodder, water and holding facilities (O'Donoghue *et al.* 1987).

### 3.1.1 Epidemiology of *Cryptosporidium* in Animals

Epidemiology data for *Cryptosporidium* infection in animals is largely confined to economically important livestock and in particular ruminants such as cattle and sheep (Casemore *et al.* 1997; Fayer *et al.* 2000)

Infections detected in adult animals are mainly asymptomatic or with mild clinical signs and symptoms. There have been several reports of severe infection in immunosuppressed or immumocompromised hosts including dogs, cats, horses as well as monkeys (Baskerville *et al.* 1991; Snyder *et al.* 1978; Turnwald *et al.* 1988).

The most common symptom of cryptosporidiosis in animals is watery diarrhoea. Excreta may also appear pale yellow in colour and have an offensive smell. Other observed clinical signs include dehydration, fever, anorexia, weight loss, weakness and progressive loss of condition with rough coat and occasionally bloating (Fayer 1997). The prepatent period can vary considerable in *Cryptosporidium* infections and reflects the:-

- host species;
- strain of the parasite;
- age of host at infection and
- size and condition of the inoculum.

*Cryptosporidium* in commercial farm animals is well studied, with a strong focus on cattle. The world-wide incidence of *C. parvum* in cattle is most commonly reported for neonates. The typical *C. parvum* infection characteristics reported are a prepatent period of 5 - 12 days, followed by oocyst shedding, often with the onset of diarrhoea. The shedding typically lasts 3 - 12 days with a peak of $10^{6-7}$ oocysts per gram of faeces (Casemore *et al.* 1997).

Age-related resistance to infection has been demonstrated in several species. The age of the animal when it is exposed to the parasite can radically alter the severity of the infection and extent of the oocyst shedding (Harp *et al.* 1990; Ortega-Mora *et al.* 1994). The number of oocysts ingested can also have considerable influence on the length of the prepatent period (Blewett *et al.* 1993).

One reason for the lack of data on infection prevalence is that is can be difficult to identify oocysts in the fibrous faeces of many animals (Casemore *et al.* 1997; Davies *et al.* 2003). Furthermore, infections appear to be asymptomatic, with only small numbers of oocysts being excreted, possibly over a protracted period.

Studies to date investigating prevalence have been limited. A broad study was undertaken in Canadian farms at different geographical locations to investigate the prevalence of *Cryptosporidium* from 15 different Canadian geographical locations (Olson *et al.* 1997). The *Cryptosporidium* prevalence detected were

- 20 % in 104 cattle;
- 23 % in 89 sheep;
- 11 % in 236 pigs and
- 17 % in 35 horses.

*Cryptosporidium* was present in cattle, horse and sheep in all sites sampled, in four out of six pig operations. The prevalence of *Cryptosporidium* was greater in calves and lambs compared to adults (Davies *et al.* 2003; Olson *et al.* 1997).

A second relevant prevalence study was carried out in the UK (Sturdee *et al.* 1999). The isolates were from both domesticated and wild animal populations. The study compared detected prevalence in the UK with other surveys reported in literature

reviews. Within the UK, *Cryptosporidium* was been reported from 11 wild mammals. Overall, 12 % of the faecal samples tested positive for *Cryptosporidium* via IFA which detected oocysts. Their findings also emphasised a widespread distribution of *Cryptosporidium* amongst wild mammals in the UK (Chalmers *et al.* 2002; Sturdee *et al.* 1999). Webster *et al.* (1996) reported that the recovery of oocysts for various commonly used techniques was significantly less than expected. Another study conducted by Davies *et al.* (2003) also investigated the variation within recovery of oocysts from a range of faecal sources and reported that the percent recoveries of oocysts from cattle, kangaroo, pig, and sheep faeces (juveniles and adults) collected in a subsequent watershed animal faecal survey were far more wide ranging than predicted by the validation data (Davies *et al.* 2003; Webster *et al.* 1996).

A longitudinal sample survey testing for *Cryptosporidium* in livestock and small wild mammals conducted over six years (1992 – 1997, 3721 isolates) in England, showed that *Cryptosporidium* was endemic and persistently present in all mammalian categories tested with IFA. *C. muris* was rarely found in wild mammals and *C. andersoni* oocysts were not detected. Olsen (2001) reported that *C. andersoni* oocysts can be identified in up to 25 % of feedlot calves (Olsen 2001). Studree *et al.* (2003) observed prevalence of *Cryptosporidium* in several lowland farm in the United Kingdom:-

- bull beef, 3.6 %;
- dairy cows, 3.5 %;
- ewes, 6.4 %;
- horses, 8.9 %;
- calves (home bred), 52 %;
- calves (bought-in) 23.2 %;
- lambs, 12.9 %;
- rodents (living in and around farm buildings), 32.8 % and
- small wild mammals (mainly rodents) living in areas of pasture, 29.9 %.

Analysis of annual and seasonal data for each animal category revealed that patterns of infection were variable and sporadic; this means that short-term sampling was never likely to provide a true or representative picture. Seasonally combined data for adult livestock, young livestock and small wild mammals showed all three categories tended to have the highest *Cryptosporidium* prevalence in the autumn (Sturdee *et al.* 1999).

There are only a few studies which have investigated *C. parvum* from household domestic animals and these are mainly limited to cats. Two studies have been carried out in Glasgow which compared *Cryptosporidium* incidence in feral, domestic and farm cats (Mtambo *et al.* 1991; Nash *et al.* 1993). The *Cryptosporidium* occurrence were 12 % of feral cats, 8.8 % farm cats and 5 % domestic cats based on *Cryptosporidium* excreted at sampling.

### 3.1.2 *Cryptosporidium* in Wild Animals

Very little prevalence data exists for wild animal populations. *Cryptosporidium* has been detected in a variety of wild and feral animal populations such as

- cottontail rabbits (Ryan *et al.* 1986);
- grey squirrel (Sundberg *et al.* 1982);
- grey foxes (Davidson *et al.* 1992);
- juvenile raccoons (Snyder 1988);
- both wild and domestic deer (Heuschele *et al.* 1986; Simpson 1992);
- Canadian geese (Graczyk *et al.* 1998) and
- kangaroos (Power *et al.* 2001; Power *et al.* 2004).

Epidemiologic data for feral and wild animals is sparse and mainly confined to observations in zoological parks. As a result the populations which are studied are limited to small groups. A possible reservoir of infection within the agriculture setting are rodents (Chalmers *et al.* 1995). They are wide spread in all agriculture settings and *Cryptosporidium* have been shown to be capable of transmission between mice and cattle (Klesius *et al.* 1986).

### 3.1.3 Transmission Experiments and Animal Models for *C. parvum*

Early transmission experiments revealed that various animals were readily susceptible to infection from other domestic and laboratory animal hosts (Tzipori 1983). Experimental cross-transmission studies have now been preformed on a range of clinical isolates from mammals, birds and reptiles. The majority of attempts to transmit infections between hosts belonging to the same vertebrate class (mammal-to-mammal or bird-to-bird) have been successful whereas attempts between different vertebrate

hosts have failed (O'Donoghue 1995). *C. parvum* cross-transmission experiments have been preformed on mammals and it is readily transmitted between a wide range of hosts. Nonetheless different species can also infect mammals such *as C. muris, C. andersoni etc.* (Sturdee *et al.* 1999).

There are various limitations to animal transmission studies, however largely due to the limited amount of material available (Fayer *et al.* 1985; Heine *et al.* 1984; Olson *et al.* 1997; Tzipori *et al.* 1983). Recently better techniques have now been developed for parasite detentions and harvest (Davies *et al.* 2003; Power *et al.* 2003). Many studies are hampered by low parasite numbers, especially when using clinical isolates. One approach used to over come this problem is the development of reproducible animals models. Different models have been established by different laboratories to investigate infection and disease, pathology, immunology, molecular biology as well as drug evaluation studies (O'Donoghue 1995). Various experimental infections have been successfully established in a range of domestic and laboratory animals. The main animal types which have been used include neonates, immunosuppressed or immunodeficient animals. Domestic animal neonates, particularly calves (Fayer *et al.* 1985; Heine *et al.* 1984; Tzipori *et al.* 1983) have been used extensively for clinical investigations. The resources needed to maintain large animal models are considerable and beyond the scope of most projects. Currently the models represent the only available methods for producing large numbers of oocysts. Small animal models have difficulties due to the problem of establishing a continual infection. Infections in immunocompetent mice are self - limiting in 2 - 4 weeks post infection (Klesius *et al.* 1986). Hijjawi *et al.* (2001) described the complete life cycle *C. parvum* successfully being cultured in the HCT-8 cell line. However, further developments by Hijjawi *et al.* (2004) demonstrated a cell-free culture method for *Cryptosporidium* research (Hijjawi *et al.* 2004). Together these two developments could provide a stable supply of oocysts and be useful aids for many aspects of *Cryptosporidium* research (Hijjawi *et al.* 2001; Hijjawi *et al.* 2004).

### 3.1.4 Genotyping of Animal Isolates

Over recent years several difference genotyping methods have been developed as described in Chapter 1 (Section 1.4). Genetic polymorphisms within *Cryptosporidium spp.* have being detected at a continuously growing rate, owing to the widespread use of modern molecular techniques (Egyed *et al.* 2003). The most common typing methods are able to differentiate at the host of origin level *i.e.* animal and human. Typical typing strategies utilise polymorphisms which can be visualised by RFLP patterns.

The most common genes investigated are:-

- *Cryptosporidium* oocyst wall protein (COWP) gene (Spano *et al.* 1997),
- thrombospondin related adhesive protein (TRAP-C2) gene (Sulaiman *et al.* 1998) and
- 70 kDa heat shock protein (HSP70) gene (Rochelle *et al.* 1997).

More recently multilocus genotype *Cryptosporidium* have been utilised. Several studies have used combinations of the above described PCR-RFLP targets. The failure of the abovementioned techniques to address questions concerning population genetics in relation to host, temporal and geographical sub-structuring has lead researchers to investigate the non-coding regions of the *Cryptosporidium* genome. The PCR based methods which were developed utilise micro- and minisatellite markers. To date the micro- and minisatellite marker PCRs have not found evidence to support geographic or temporal sub-structuring of the populations (Mallon *et al.* 2003).

## 3.2 Objectives

The overall objective of this chapter was to determine the range of fingerprints from different animal types and locations using the M1 + T PCR as described in Chapter 2.19.3. Concerned with this was an evaluation of the reproducibility of the assay and the stability of the DNA fingerprint in a herd over time.

## 3.3 Materials and Methods

### 3.3.1 Sample Information

A total of 202 isolates (Table 2.2) were investigated from ten location within Australia and international sources. Of the source locations used, eight were within Australia (Figure 3.1) and two international (one from Belgium and the other being the Iowa isolate propagated at the University of Arizona, Tucson USA). The Australian locations were: - Sydney (Sth West & Nth West), two locations in Central Western NSW, Western NSW and two locations within northern Victoria. Bovine isolates were the predominant host of origin with 150 samples being used. The other host animals included pigs (6), sheep (30) and kangaroos (*Macropus rufus* 12, *Macropus giganteus* 4).

All bovine origin samples were collected directly from the calf by digital stimulation. Each sample was taken from an individual calf and no samples were pooled. For the sheep and kangaroo samples, each sample was collected post the defecation event. The sample was only collected from defecation events which were observed to ensure that each sample was from an individual animal. The piglet samples were a composite collection. Each sample was taken from an individual pen, therefore, each sample represented an entire litter.

### 3.3.2 Sample Preparation

Two independent DNA extractions (Chapter 2.12) were undertaken for each sample (Table 3.1). All DNA extracts were PCRed at least twice and all produced the same DNA fingerprint after independent PCRs.


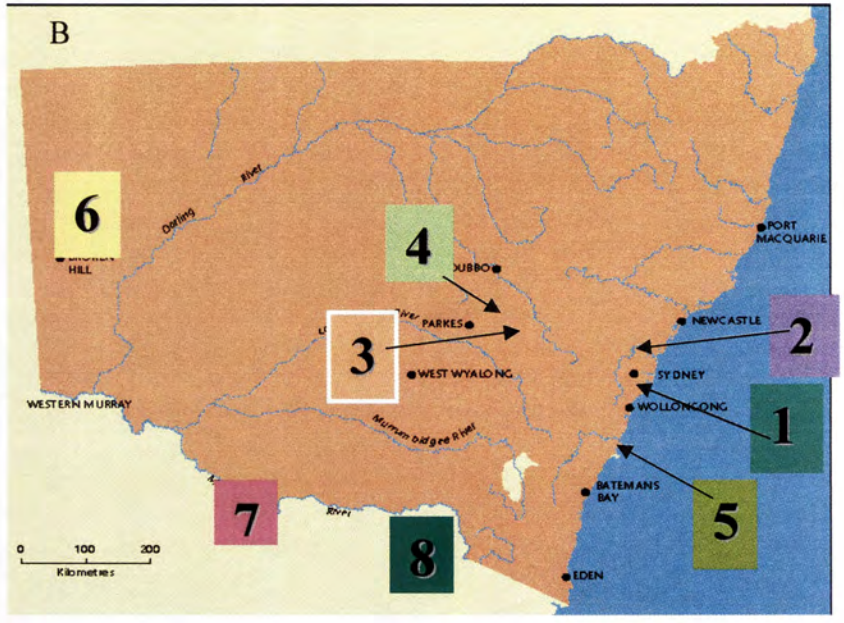


Figure 3 1: Location of sampling sites within Australia

Panel A: Map of Australia
Panel B: Map of NSW and Nth Victoria showing the approximate sampling locations

### 3.3.3 PCR-RFLP – TRAP C2

Peng *et al.* 1997 described a set of PCR primers which amplified a single specific band of 369 bp, of the *C. parvum* TRAP-C2 gene and sequence alignments of the PCR bands showed two primary genotypes (Peng *et al.* 1997). Elwin *et al.* 2001 described a modified PCR-RFLP for TRAP-C2 which generated a nested PCR product of the expected size (266 bp). The PCR amplicon contained a recognition sequence for either *Hae*III (*C. hominis*) or *Bst*EII (*C. parvum*) (Elwin *et al.* 2001).

The PCR-RFLP which was used to confirm *C. hominis* or *C. parvum* was in accordance to Elwin *et al.* (2001).

## 3.4 Results

### 3.4.1 Sydney South West Isolates

Samples from Sydney SW were collected at regular intervals from November 1997 to August 2001 (Appendix 1 Table A1.1). A total of 90 *C. parvum* positive faecal samples, all from calves, were used. The positive 1998 samples were described in Chapter 2.8.2.16. During the collection times no *C. parvum* nor *C. andersoni* were detected in any adult cattle at this location.

Several calf samples were positive for *Giardia* and were used for the genus specificity testing, Chapter 2.17.4.2. From 1998 onwards there were several samples from each collection point which were not *C. parvum* positive. The range of *C. parvum* detected was 0 - $10^7$ oocysts / g of wet weight faeces.

The PCR fingerprints which were produced from isolates collected during 1997 (4 isolates) and 1998 isolates were all identical. Figure 3.2 shows all the 1997 isolates and eight of the thirty samples from 1998. The bands which were produced range from approx 2 Kb down to 250 bp. The eight 1998 isolates shown in Figure 3.2 were not used establishing the protocol as detailed in Chapter 2.17.

Table 3 1: List of isolates locations and sample numbers

| Sample Host | Location of Collection | Date Collected | Number of Isolates | Age Range at Collection |
|---|---|---|---|---|
| **Bovine** | Syd SW (Sydney, South West) | November 1997 | 4 | unknown |
| | | October 1988 | 30 | 6-16 days |
| | | June 1999 – September 1999 | 20 | 11-49 days |
| | | May 2000 – August 2000 | 20 | 22-50 days |
| | | Feb 2001 – Aug 2001 | 16 | 7-34 days |
| | Syd NW (Sydney, North West) | January 1999 | 6 | 9-47 |
| | | Jan 2000 – August 2000 | 12 | 12-28 |
| | | Feb 2001 – Aug 2001 | 12 | 12-37 |
| | NSW C (New South. Wales) 1 | February 1999 | 4 | 14-18 |
| | NSW C (New South. Wales) 2 | February 1999 | 4 | unknown |
| | Belgium[+] (Europe) | Nov 1999 + Feb 2000 | 2 | unknown |
| | Uni of Arizona - Iowa strain* | September 1998 | 2 | unknown |
| | | Feb 1999 – Nov 1999 | 6 | unknown |
| | | Jan 2000 – Oct 2000 | 6 | unknown n |
| | | Feb 2001 – Aug 2001 | 6 | unknown |
| **Porcine** | South Eastern NSW | Feb 2001 | 6 | 6 – 7 weeks |
| **Ovine** | NSW W (Western New Sth. Wales ) | April 2001 | 6 | unknown |
| | Vic-A (Northern Victoria) | August 2000 | 12 | unknown |
| | Vic-B (Northern Victoria) | August 2000 | 12 | unknown |
| **Kangaroo- Red** | NSW W (Western New Sth. Wales ) | April 2001 | 12 | unknown |
| **Kangaroo- Grey** | NSW W (Western New Sth. Wales ) | April 2001 | 6 | unknown |

202 Isolates

\+ Samples provided by Ms. Cisha Schets from RIVM, The Netherlands.

* Samples provided by Ms. Marilyn Marshall from University of Arizona, Tucson USA.

During 1999 the consistent fingerprint profile for isolates from Syd SW site changed (Figure 3.3), compared to the profile observed in 1998 and 1997 (Figure 3.2). The banding pattern shifted down to lower molecular weight bands. During 1999 a mycoplasma infection was detected in the calf population and associate with approximately 60 calve deaths (personal communication with the Veterinarian of the dairy farm). The banding profile produced during the mycoplasma infection period shows faint bands between 500 – 1000bp which were present in the stronger profile established in 2000.

The third DNA fingerprint was found at the Syd SW location. A total of 36 isolates were collected and PCRed during 2000 (20 isolates) and 2001 (16 isolates). The banding profile was consistent over the two years. The major bands were a higher molecular weight from 450 bp to around 1 500 bp, four isolates from 2000 and four isolates from 2001 (Figure 3.4). Therefore, over a five year period at the Sydney South West location a total of three fingerprints were detected (Figure 3.5).

In 2000 – 2001 (Figure 3.4) a single new profile was detected. The new profile which was constant over the two year period had a banding pattern which ranged from 1 500 – 450 bp. Several of the stronger bands of this profile can be seen as very faint bands in the 1999 profile. Therefore, the profile which establishes itself as the sole fingerprint from 2000 on was able to be seen as early as 1999 (Figure 3.5). The 1999 samples are most likely a mixed fingerprint from 2 different isolates. During the setup stages of the M1 + T PCR PAGE (section 2.18.3) method an investigation into mixtures was carried out. The results of that study showed that discrete PCR bands were able to be seen with 1 or 2 isolates, but when 3 or more isolates were mixed only smears were detected.

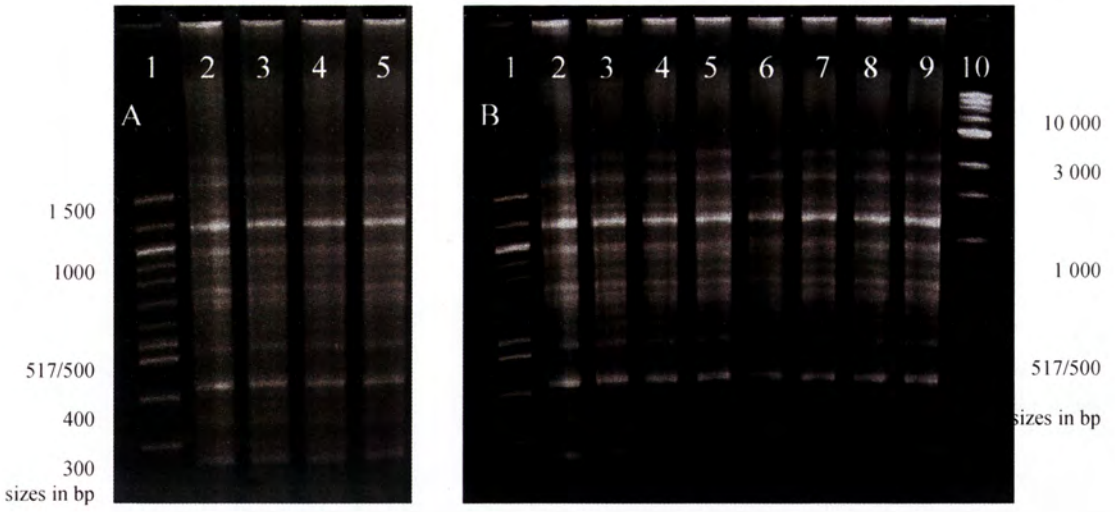


Figure 3 2: M1 + T PCR PAGE of isolates from Syd SW location, 1997 and 1998

Panel A: Lane 1 100 bp size standard (New England BioLabs, Brisbane, AU), Lane 2 April 1997 Lane 3 Syd SW April 1997, Lane 4 Syd SW Oct 1997, Lane 5 Syd SW Oct 1997.

Panel B: Lane 1 100 bp size standard (New England BioLabs, Brisbane, AU), Lane 2 Syd SW April 1998, Lane 3 Syd SW April 1998, Lane 4 Syd SW Oct 1998, Lane 5 Syd SW Oct 1998, Lane 6 Syd SW Oct 1998, Lane 7 Syd SW Oct 1998, Lane 8 Syd SW Oct 1998, Lane 9 Syd SW Oct 1998, Lane 10 1Kb size standard (New England BioLabs, Brisbane, AU).

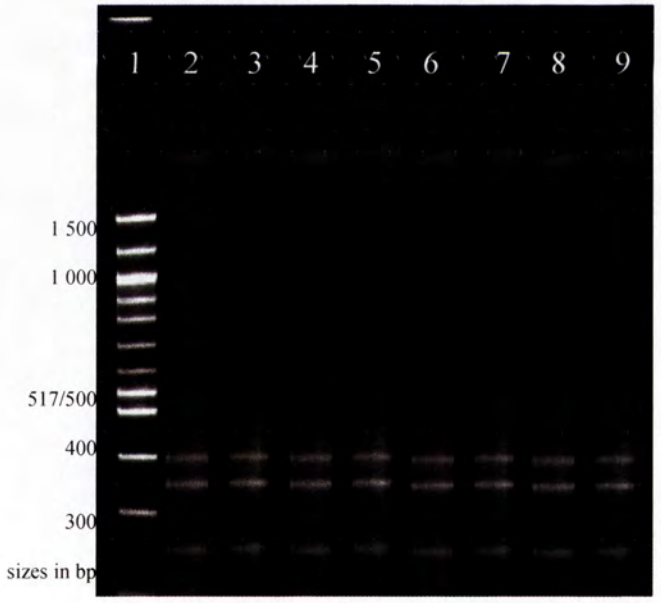


Figure 3 3: M1 + T PCR PAGE of isolates from Syd SW 1999 Isolates

Lane 1 100 bp size standard (New England BioLabs, Brisbane, AU), Lane 2 Syd SW Oct 1998, Lane 3 Syd SW Oct 1998, Lane 4, Syd SW 1999, Lane 5 Syd SW 1999, Lane 6 Syd SW 2000, Lane 7 Syd SW 2000, Lane 8 Syd SW 2001, Lane 9 Syd SW 2001.

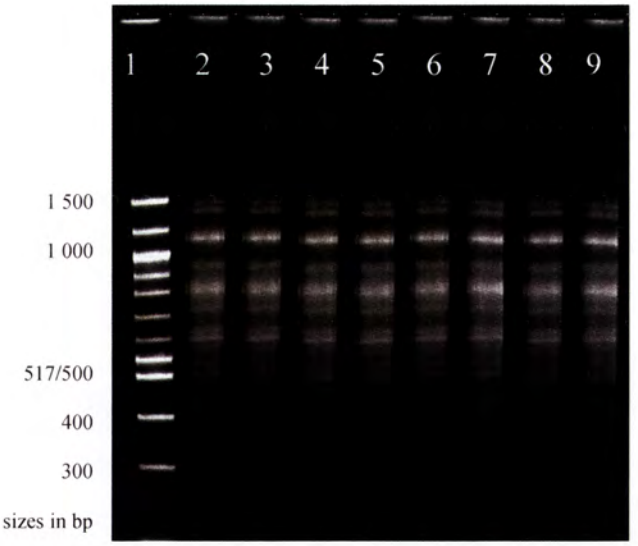


Figure 3 4: M1 + T PCR PAGE of isolates from Syd SW 2000 - 2001

Lane 1 100 bp size standard (New England BioLabs, Brisbane, AU), Lane 2 Syd SW Oct 1998, Lane 3 Syd SW Oct 1998, Lane 4 Syd SW 1999, Lane 5 Syd SW 1999, Lane 6 Syd SW 2000, Lane 7 Syd SW 2000, Lane 8 Syd SW 2001, Lane 9 Syd SW 2001.

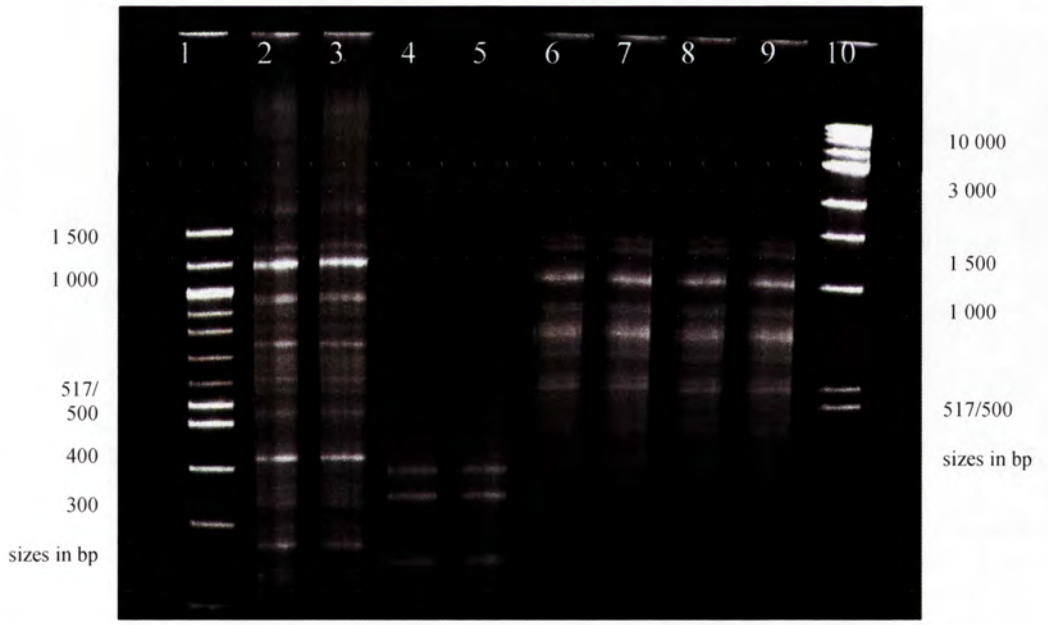


Figure 3 5: M1 + T PCR PAGE of Sydney South isolates 1998 - 2001

Lane 1 100 bp size standard (New England BioLabs, Brisbane, AU), Lane 2 Syd SW Oct 1998, Lane 3 Syd SW Oct 1998, Lane 4 Syd SW 1999, Lane 5 Syd SW 1999, Lane 6 Syd SW 2000, Lane 7 Syd SW 2000, Lane 8 Syd SW 2001, Lane 9 Syd SW 2001, Lane 10 1 Kb size standard (New England BioLabs, Brisbane, AU).

### 3.4.2 Sydney North West Isolates

A total of 30 isolates were collected from the Syd NW site from 1999 – 2001 (Appendix 1 Table A1.2).

At the location both the calves and several adult cattle samples were obtained for *C. parvum* or *C. andersoni* and *G. lamblia*. No *C. andersoni* was detected and no adult cow was detected positive for *C. parvum*. Several calf samples were positive for *Giardia* which were used for the genus-specific testing Chapter 2.17.4.2. At each sampling period there were between 12 – 20 calves present. The range of *C. parvum* detected was >0 – $10^6$ oocysts / g ww faeces.

In the years 2000 and 2001 all *C. parvum* positive samples were PCRed. A single consistent fingerprint was detected at this location over the whole of the sampling period (Figure 3.7).

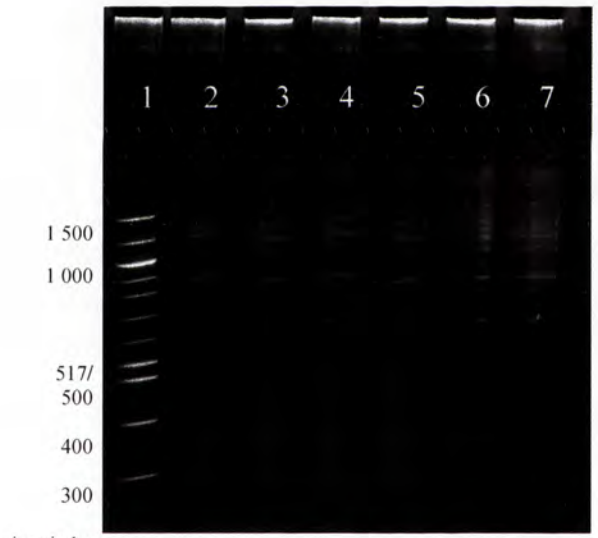


Figure 3 6: M1 + T PCR PAGE of Sydney North West isolates

Lane 1 100 bp size standard (New England BioLabs, Brisbane, AU), Lane 2 Syd NW Jan 1999, Lane 3 Syd NW Jan 1999, Lane 4 Syd NW Jan 2000, Lane 5 Syd NW Aug 2000, Lane 6 Syd NW Feb 2001, Lane 7 Syd NW Aug 2001.

### 3.4.3 New South Wales Central West Isolates

Two dairy farms (Appendix 1 Table A1.3) were surveyed for the NSW CW isolate in February 1999. The two farms were owned by the same family and shared breeding

stock, they were located only 10 km apart. From the one sampling trip a total of eight *C. parvum* isolates were detected from individual calves, four from each farm. All eight isolates gave the same fingerprint (Figure 3.7). The two dairy farms were the only two locations to have the same DNA fingerprint profiles, across the ten sites examined for this thesis. No calf sample was positive for *Giardia.*

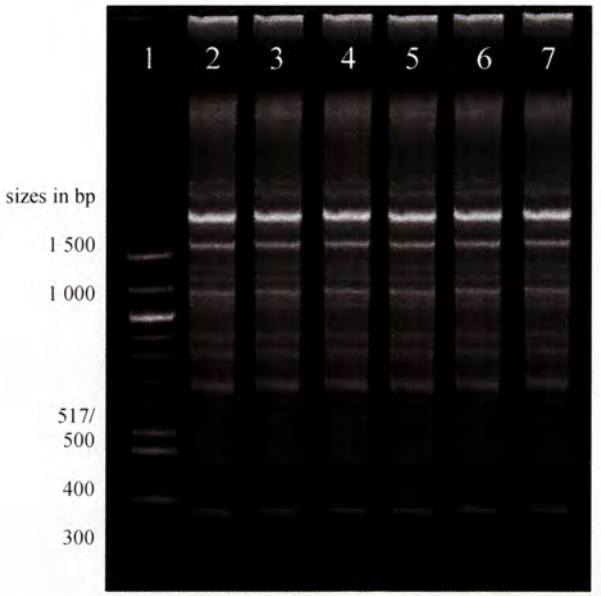


Figure 3 7: M1 + T PCR PAGE of New South Wales Central West isolates

Lane 1 100 bp size standard (New England BioLabs, Brisbane, AU), Lane 2 NSW CW 1 Feb 1999, Lane 3 NSW CW 1 Feb 1999, Lane 4 NSW CW 1 Feb1999, Lane 5 NSW CW 2 Feb1999, Lane 6 NSW CW 2 Feb 1999, Lane 7 NSW CW 2 Feb 1999.

### 3.4.4 New South Wales Western Isolates

The NSW W site was the only location examined where different mammalian species were grazing together and only one sampling occurred. It was during September 2000. The animals from which *Cryptosporidium* was detected were sheep (N = 6), red kangaroos (*M. rufus* N = 12) and grey kangaroos (*M. giganteus*, N = 4).

The oocyst numbers from the sheep ranged from $10^2$ - $10^6$ oocysts/g ww faeces. All the kangaroo isolates came from adults and the oocyst numbers were significantly lower than from sheep from the same area ranging from $10^1$ - $10^4$ oocysts/g ww faeces with M1 + T pattern shown in Figure 3.8. Faecal samples represent both juvenile and adult

kangaroos; these animals also appear to have asymptomatic infections (no diarrhoea at time of testing). A similar infection pattern was observed in post-weaned and adult cattle where *Cryptosporidium* prevalence was 20.7 % (Fayer *et al.*, 2000).

Figure 3.8 shows the sub-species fingerprint for all of the sheep origin isolates. The banding patterns were identical and ranged approximately 3 Kb – 500 bp. Figure 3.9 illustrates the same kangaroo and sheep fingerprints. These results suggested the same strain was present regardless of the animal host.

Furthermore, a single genotype was successfully obtained for these isolates using TRAP-C2 PCR-RFLP (section 3.3.3). All the isolates from NSW W are *Cryptosporidium* (Figure 3.10).

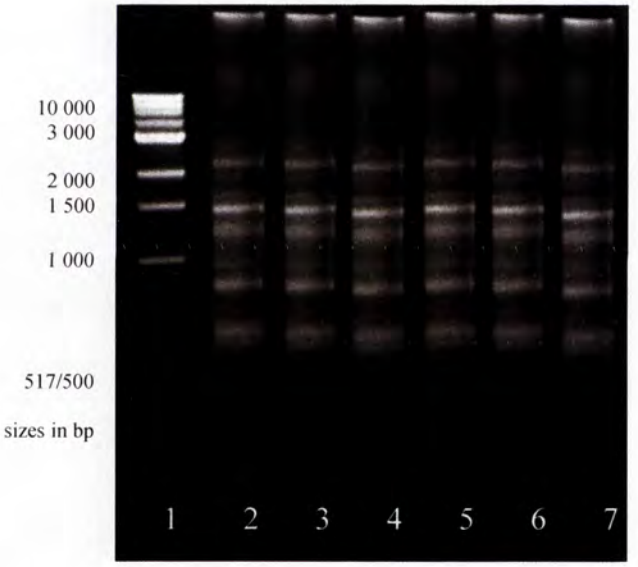


Figure 3 8: M1 + T PCR PAGE of Western New South Wales sheep isolates

Lane 1 100 bp size standard (New England BioLabs, Brisbane, AU), Lane NSW W Sheep 1 Sept 2000, Lane 3 NSW W Sheep 2 Sept 2000, Lane 4 NSW W Sheep 3 Sept 2000, Lane 5 NSW W Sheep 4 Sept 2000, Lane 6 NSW W Sheep 5 Sept 2000, Lane 7 NSW W Sheep 6 Sept 2000.

### 3.4.5 Victorian Sheep Isolates

Two Victorian locations provided sheep isolates which were collected during October 2000 (Appendix 1 Table A1.5). Each location (Vic A and B) gave a separate and unique DNA fingerprint as shown in Figure 3.11. At location Vic A twelve lamb

isolates were positive for *C. parvum*. The second location Vic B, four sheep and eight lambs were found to be shedding *C. parvum* at the time of collection. No *Giardia* was detected in any of the sheep isolates. The oocyst numbers ranged from $10^4$ - $10^7$ oocysts / g ww faeces.

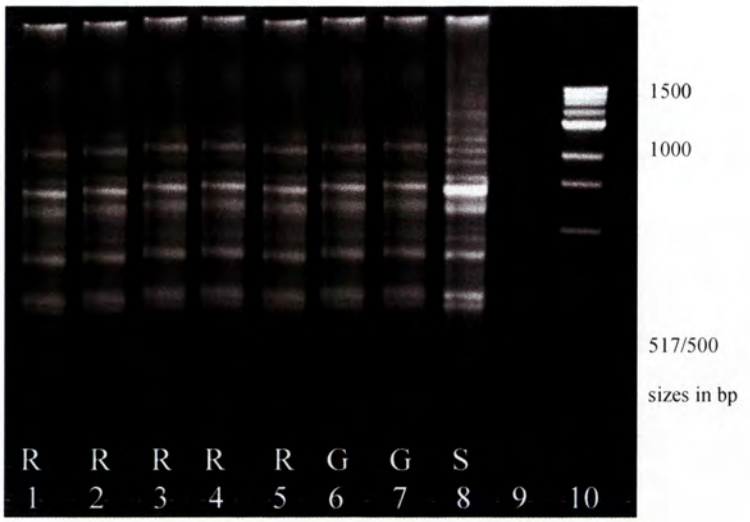


Figure 3 9: M1 + T PCR PAGE of Western New South Wales sheep and kangaroo isolates

Lane 1 NSW W Red Roo 1 Sept 2000, Lane 2 NSW W Red Roo 1 Sept 2000, Lane 3 NSW W Red Roo 2 Sept 2000, Lane 4 NSW W Red Roo 3 Sept 2000, Lane 5 NSW W Red Roo 4 Sept 2000, Lane 6 NSW W Grey Roo 1 Sept 2000, Lane 7 NSW W Grey Roo 2 Sept 2000, Lane 8 NSW W Sheep 1 Sept 2000, Lane 9 Blank, Lane 10 100 bp size standard (New England BioLabs, Brisbane, AU).

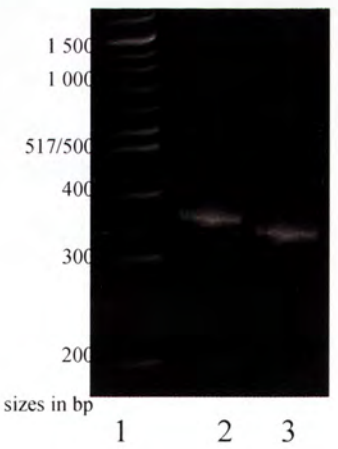


Figure 3 10: TRAP-C2 PCR-RFLP primer of Western New South Wales sheep and kangaroo isolates

Lane 1 100 bp size standard (New England BioLabs, Brisbane, AU), Lane 2 NSW W Sheep 1, Lane 3 NSW W Kangaroo 1

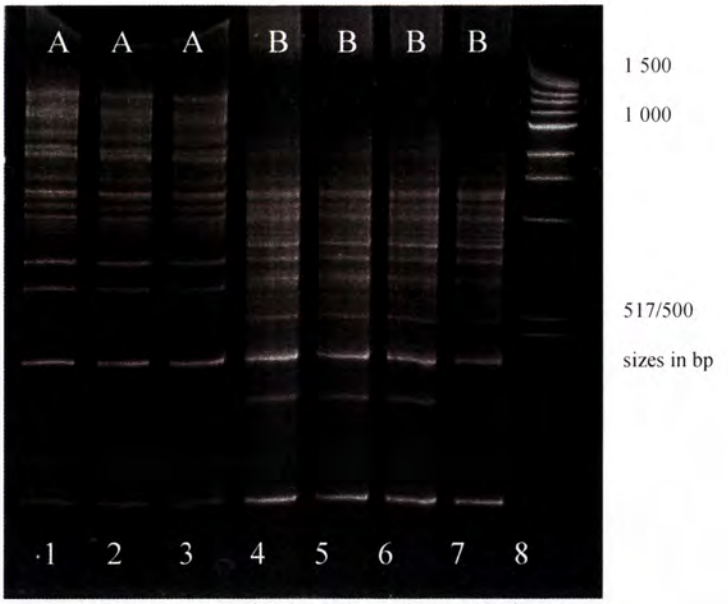


Figure 3 11: M1 + T PCR PAGE of Victorian sheep isolates

Lanes 1 Isolate from Vic A, Lane 2 Isolate from Vic A, lane 3 Isolate from Vic A, lane 4 Isolate from Vic B, lane 5 Isolate from Vic B, lane 6 Isolate from Vic B, lane 7 Isolate from Vic B, Lane 8 100 bp ladder (New England Biolabs, Brisbane AU).

Three of the Vic A lambs isolates PCR results are shown in Lanes 1 - 3. The remaining four sample lanes are two sheep (lanes 4 - 5) and two lambs (lanes 6 - 7) from the second Victorian location.

### 3.4.6 South Eastern NSW, Porcine Isolates

A total of 20 pig faecal samples were collected from the Sth Eastern NSW location (Appendix 1 Table A1.6 and Figure 3.12). When the experimental assays for this thesis were preformed *C. suis*, had not been identified. The pig isolates which were used were positive for *Cryptosporidium* by IFA, and *C. parvum* was identified by using TRAP-C2 PCR-RFLP. Further experimental analysis utilising Ryan's 18S rDNA method would be required to confirm the *C. suis* genotype (Ryan *et al.* 2004).

Only one collection occurred during February 1999. All samples were collected from several piglets of the one sow which were kept in the same pen. Of the twenty samples only four (Appendix 1 Table A1.6) were found to be positive for *Cryptosporidium*, in piglets aged 6-7 weeks old, no *Giardia* was detected. The age range of the piglets was 3 - 12 weeks at the time of sampling. The oocyst numbers in the positive samples were

$10^2$ - $10^4$ oocysts / g ww faeces. The piglet isolates were used in conjunction with Syd SW bovine origin *Cryptosporidium* samples during the establishment of the DNA fingerprint strategy (Chapter 2.16). Each of the 5 piglet samples had the same DNA profile which differed to all other sampling locations.

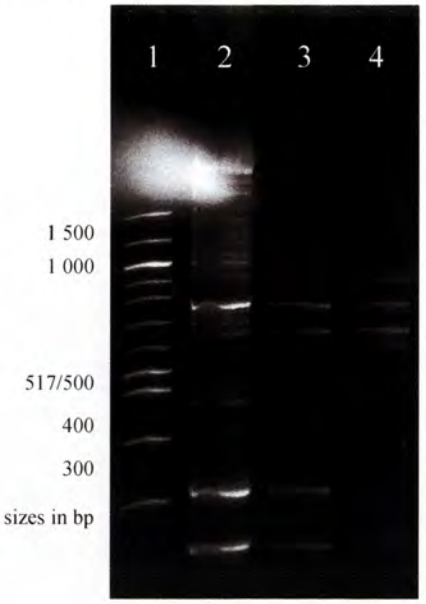


Figure 3 12: M1 + T PCR PAGE of South Eastern NSW, porcine isolate

Lanes 1 100 bp ladder (New England Biolabs, Brisbane AU), Lane 2: South Eastern NSW 6c, lane 3: South Eastern NSW 6e, lane 4: South Eastern NSW 7d.

### 3.4.7 *C. parvum* Isolates from Outside Australia

Two of the ten animal faecal sampling sites were from outside of Australia. Table 3.8 details the isolates which were provided from two sources. The "Belgian" isolate samples were provided by Ciska Scheis from RIVM, Bilthoven The Netherlands. The isolates provided were post passage through a *C. parvum*-naive calf. The initial inoculum came from the same type of source, a *C. parvum* positive calf from a dairy farm. Figure 3.13 shows the PCR results for the two isolates Each isolate under went two separate DNA extractions and Figure 3.12 shows identical fingerprints were obtained. The bands for this isolate were from approximately 2 kb to just below 400 bp. The second non-Australian source of *C. parvum* isolates were kindly provided by Ms. Marilyn Marshall from The University of Arizona, Tucson Arizona USA. This isolate was originally an "Iowa" isolate and has been serially passaged through *C. parvum* naive calves.

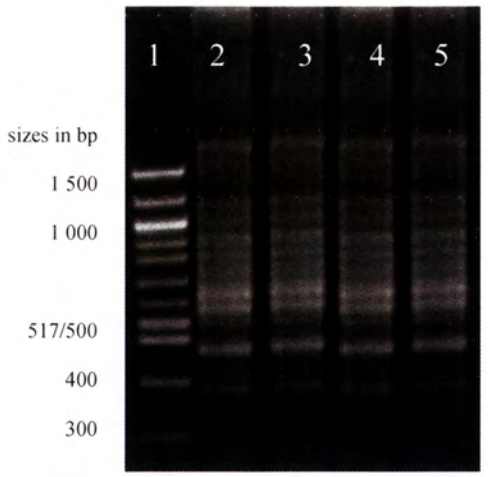


Figure 3 13: M1 + T PCR PAGE primer of Belgium calve isolates

Lane 1 100 bp size standard (New England BioLabs, Brisbane, AU), Lane 2 661/99.1, Lane 3 661/99.2, Lane 4 679/00.1, Lane 5 679/00.2.

A total of twenty isolates were PCRed (Appendix 1 Table A1.7) from 1998 to 2001. All twenty samples produced the same sub-species fingerprint and eight samples are shown in Figure 3.14. The profile was constant at each time point over the four year time course.

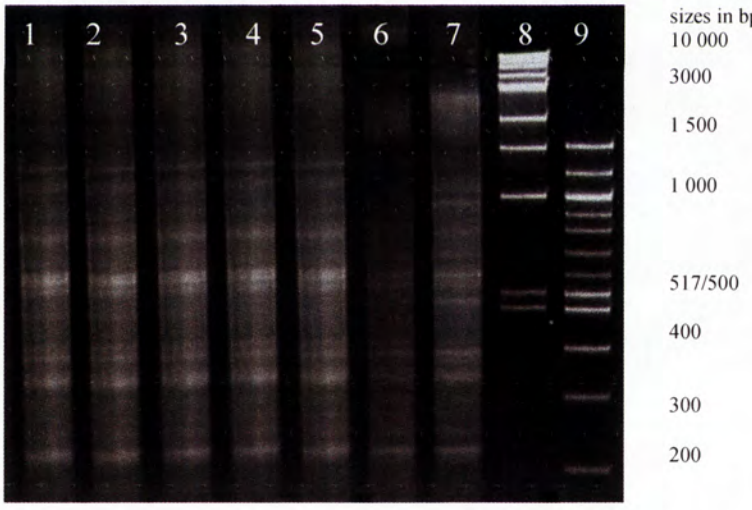


Figure 3 14: M1 + T PCR PAGE of Iowa strain isolates

Lane 1 UAI 1, Lane 2 UAI 3, Lane 3 UAI 8, Lane 4 UAI 12, Lane 5 UAI 16, Lane 6 UAI 22, Lane 7 UAI 25, Lane 8 1 Kb size standard (New England BioLabs, Brisbane, AU), Lane 9 100 bp size standard (New England BioLabs, Brisbane, AU).

## 3.5 Discussion

A total of 202 isolates were fingerprinted using the M1 + T PCR protocol as described in Chapter 2.19.3. Ten different locations (Figure 3.1, Australian locations), eight from within Australia and two International sources were investigated. The animal hosts from which *C. parvum* was detected and fingerprinted included calves, sheep, piglets and kangaroos.

Sub-species identification of *Cryptosporidium* animal isolates was demonstrated by application of our novel PCR-PAGE procedure that uses primers consisting of a microsatellite along with a 3' telomere anchor. Reproducible fingerprints were produced by all the isolates, which were unique for each geographic location. Other commonly used PCR typing methods which target one of the following 18S ribosomal RNA, COWP gene, TRAP-C1 and TRAP-C2 genes, are not able to provide the same level of sub-species resolution (McLauchlin *et al.* 2000; Pedraza-Diaz *et al.* 2001; Xiao *et al.* 2001). The only information which is able to be attributed to these typing methods is the determination of genotype. The genotype is assigned based on the amplified DNA amplicons being characterised by restriction enzyme digestion and/or DNA sequencing. As such identification is limited to *C. hominis* (which nearly only infects humans), *C. parvum* (animal and human hosts) and host specific species as described in Table 1.1. The only geographic information usually attributed to isolates which are typed by the common fingerprinting methods is described as the location of where the sample was collected. Using the M1 + T PCR PAGE described in this dissertation, however, different collection sites can also be compared to determine if there is a common sub-type at the different sample location, within and across host species.

Cacciò *et al.* (2001) utilised microsatellite primers for a PCR sequencing study. They showed that some alleles were associated exclusively with an animal (calf, kid, lamb) or human host. A few alleles were associated with a single host and a final category of alleles were demonstrated to be found within or were geographically restricted host location. The outcome of this study was the usefulness of microsatellite markers for the molecular identification of *Cryptosporidium,* that provide useful information regarding the source of infection of outbreaks and single cases, as well as for genetic studies

(Cacciò *et al.* 2001). The M1 + T PCR system described provided a greater level of resolution when applied to different geographic location compared to the Caccio *et al.* method. The new method is not host specific as were the markers described by Cacciò *et al* 2001, rather discrete sub-species were identified to the hosts encounters within a discrete geographical location. Such an approach we would be able to match clinical outbreaks to the source providing the correct environmental samples were obtained.

### 3.5.1 Fingerprinting *Cryptosporidium* Isolates from Geographically Diverse Sources

The application of the M1 + T typing procedure was applied to isolates from 10 distinct geographically locations. The majority of isolates were from bovine hosts (150) from five different sampling sites (Syd SW, Syd NW, NSW CW, University of Arizona, Europe). All bovine samples were from *C. parvum* positive calves, including two locations outside of Australia. Three of the bovine source locations (Syd SW, Syd NW, University of Arizona isolates) were sampled over a number of years (Section 3.5.3).

The sheep isolates were collected from two Victorian and one NSW (NSW W) locations. Both adult and neonates were sampled. No difference in PCR banding pattern was found between sheep and lambs when sampled at the same location. Piglet *Cryptosporidium* isolates were only collected from one location and the incidence of *Cryptosporidium* at this location was low when compared to the calf and lamb samples. The kangaroo isolates were collected from only one location (NSW W). Despite the few *Cryptosporidium* isolates, most interestingly kangaroos at the NSW W location had the same fingerprint profile as the nearby sheep (further discussion in Section 3.5.2). No identical patterns have yet been found in oocysts from logically unrelated samples (*i.e.* geographically diverse). Each representative from the same location had the same fingerprint and demonstrated the reproducibility – consistency of the PCR-PAGE method.

### 3.5.2 Common Fingerprint from Sheep and Kangaroo Isolates from NSW West Site

At the NSW W sampling site three different animal hosts (Sheep, Red and Grey Kangaroos) were shown to be positive for *Cryptosporidium*. The *Cryptosporidium* genotype from this location appeared to infect the domestic animals as well as two wild kangaroo populations. Other *Cryptosporidium* typing methods have shown that marsupials have a different genotype to those of domesticated animals. The most common genotyping method for marsupials uses 18S rRNA internal transcribed spacer 1 specific primers. Each marsupial previously examined appear to have their own genotype, which is refereed to as the "marsupial" genotype (Morgan *et al.* 1997). Some variation amongst this genotype has been identified (Power *et al.* 2004). Nonetheless, "marsupial" genotype had not been previously identified in any non-marsupial *Cryptosporidium* sample. Furthermore *Cryptosporidium*, which was found in a range of mammalian hosts, had not been identified in any marsupial.

The finding of the same sub-species fingerprint at NSW W in *Cryptosporidium* isolates from different hosts makes this location unique. A genotyping PCR (Chapter 3.4.4) was preformed on several isolates from NSW W using TRAP-C1 primers (Spano *et al.* 1998). A single genotype was successfully obtained for these isolates using TRAP-C2 PCR-RFLP (Chapter 3.3.3) (Elwin *et al.* 2001). All the isolates from NSW W were *C. parvum* (Figure 3.10). No other isolate has been reported to do so. Other marsupials which have been investigated to date all belong to the same genotype which has become known at the "marsupial" genotype (Xiao *et al.* 1999). Hence, sheep and kangaroo isolates demonstrate it is possible for the same *Cryptosporidium* biotype to successful infect domesticated farming and wild animals that are sharing water and grazing resources.

### 3.5.3 Fingerprint Stability Over Time

Three of the bovine origin *C. parvum* sites were sampled over a number of years. Each location was sampled at least twice a year for 3 - 5 years. The sample locations were Syd SW (5 years), Syd NW (3 years) and University of Arizona, Iowa strain (4 years). The Syd NW (Figure 3.6) and Uni of Arizona, Iowa strain (Figure 3.13) fingerprints did

not change over the sampling period, except for the short change at Syd SW as discussed before. *Mycoplasma bovis* is found worldwide and is a major cause of respiratory disease, mastitis, and arthritis in cattle. In Europe, it is estimated to be responsible for at least a quarter to a third of all calf pneumonia (Nicholas *et al.* 2003). As only few laboratories regularly monitor for mycoplasmas the numbers of infections attributed to *M. bovis* are an underestimation. Currently there are no vaccines available for the control of *M. bovis* infections. The treatment for *M. bovis* is by antibiotics, including tetracycline, tilmicosin and spectinomycin. These antibiotics work on *M. bovis* because it does not possess a cell wall; however evidence is accumulating that strains of *M. bovis* are becoming resistant (Nicholas *et al.* 2003).

Hence, the 1999 isolates could be explained by a mixture of two competing isolates with the 2001 isolate becoming the sole detected fingerprint over the last two years of the study. The low molecular weight bands detected in the 1999 isolates are presumably due to the shortening of the telomere repeats. While the shortening of the telomere would not complete with the replication of the chromosome, it could lead to this isolate not having all of the functions of the former genotype. Hence, the isolate was reduced in infectivity or was out competed by a "stronger" strain. In this location it is believed that during the time of collection, the profiles produced during 1999 showed a state of competition between two strains.

The fingerprints derived in samples from the Syd SW samples appeared to change over the full five-year period of observation (Figure 3.5). During the first two years, the fingerprint produced by all isolates from this location were identical. A change in fingerprint occurred in 1999 associated with an outbreak of mycoplasma infection amongst the calves. Approximately 60 calves died due to the infection (personal communication with the veterinarian of the dairy farm). The major bands of the 1999 isolates (Figure 3.3) were all lower molecular weight indicating that the telomere repeat length has shortened (Figures 3.3 and 3.5). Examination of individual isolates over a longer period may shed light on the long-term stability of the target region of the *C. parvum* genome.

## 3.6 Conclusions

*C. parvum* have recently been studied using multilocus (Section 3.1.4) PCR - RFLP targets. The failure of the abovementioned techniques to address questions concerning population genetics in relation to host, temporal and geographical sub-structuring has lead researchers to investigate the non-coding regions of the *C. parvum* genome.

Further PCR based methods were developed which utilised micro- and minisatellite markers. Nonetheless, current micro- and minisatellite markers described have not support geographic or temporal sub-structuring of the populations (Mallon *et al.* 2003). The differentiation between geographical isolates samples provided by M1 + T primer set demonstrates a procedure useful for epidemiological investigation of outbreaks and related infections. The PCR targeted area appears to have region stability over the period which were investigated, which at several locations extends over four year. With the use of microsatellite markers, the source of infection leading to cryptosporidiosis can be traced back by comparing fingerprints of oocysts shed by infected individuals with those present in the suspected source(s) of infection (water, food, animals, or humans). Moreover, the severity of human infection has been found to vary depending on the specific *Cryptosporidium* isolate (Okhuysen *et al.* 1999). Therefore, it will be of particular interest to genotype such isolates using microsatellite markers, or to perform experimental infections of healthy volunteers using oocysts having different genetic backgrounds defined by microsatellite analysis. Finally, the availability of microsatellite markers will allow the structure of the parasite's population to be critically evaluated and it will be possible to confirm or reject the hypothesis of clonality, as discussed for other protozoan parasites (Cacciò *et al.* 2001; Tibayrenc 1998).

## 3.7 References


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# 4 Chapter 4: Fingerprinting of Human Samples


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## 4.1 Introduction

*Cryptosporidium* was first detected and diagnosed in human patients in 1976 (Meisel *et al.* 1976; Nime *et al.* 1976). In that year two immunocompromised patients were diagnosed with cryptosporidiosis and over the following years *Cryptosporidium* was isolated from "healthy" adults (Ungar 1990). Hence cryptosporidiosis was and still is considered to be a disease predominantly found in patients who are immunocompromised and it was thought to be a rare opportunistic pathogen. Furthermore, *Cryptosporidium* was associated with a significant mortality rate within AIDS patients but this too is now decreasing (Hunter *et al.* 2001). Overall infections have now been detected and recorded in many patients groups from over fifty countries and the endemic incidence in western countries is thought to be 0.1 – 1 case per 100 000 (O'Donoghue 1995).

### 4.1.1 Epidemiologic Studies

Young children, typically under five years of age, appear to be the most susceptible group to cryptosporidiosis within the human population. Factors which contribute to their higher susceptibility may include an immature immune system as well as unhygienic behaviour (O'Donoghue 1995).

Epidemiologic studies investigating cryptosporidiosis have demonstrated that it is widespread and more common that had been previously thought. The prevalence of the infection within a given human population is difficult to determine with any degree of accuracy but estimates range from <0.1 % in adults to over 20 % in day care children (Wheeler *et al.* 1999). Instances of human-to-human transmission have been recorded between household and family members, sexual partners, hospital patients as well as staff and children attending day care centres. Surveys investigating asymptomatic individuals in an attempt to estimate the prevalence of infection have used two main detection methods. The first approach looks directly for oocysts being shed and underestimates the prevalence due to failure in detection of oocysts as they are excreted sporadically or in low numbers (Smith 1998). The second approach used serological assays and provides an indication of the exposure level within the population (Frost *et*

*al.* 1998). The serological assays do not discriminate between past and current infection (O'Donoghue 1995), hence rising titres or significant changes are all that can be indicated.

Human-to-human transmission has been recorded between household and family members, sexual partners, in hospitals both patients and staff, children attending daycare centres (Casemore 1990; Ungar 1990) and in recent years from contaminated food (Fayer *et al.* 2000) and water (*i.e.* drinking water and swimming pools) (Fayer *et al.* 2000; Hellard *et al.* 2000; MacKenzie *et al.* 1994). Zoonotic transmission is also another source where humans can become infected with *C. parvum* (Chalmers *et al.* 1995; Chalmers *et al.* 1997). The most common reports of humans being infected from an animal source come from those who handle animals as a profession, such as animal attendants, research students and workers and handling veterinary isolates (Enriquez *et al.* 2001). The most common animal sources of infection include pets and farm animals, particularly calves and lambs (Anderson 1998; Robertson *et al.* 2000).

### 4.1.2 Genotyping Human Isolates

Gene sequencing of *Cryptosporidium* has shed light on the genetic diversity and evolution of *Cryptosporidium* parasites. The main genes which have been of partial interest included the partial ssrRNA, actin, and 70 kDa heat shock protein (HSP70) genes. The results from several studies indicate that there are 14-15 *Cryptosporidium* spp. and eight *Cryptosporidium* genotypes (Ryan *et al.* 2005; Xiao *et al.* 2002) Results of multi-locus genetic characterisation indicate that host adaptation is a general phenomenon in the genus *Cryptosporidium*, because specific genotypes were usually associated with specific groups of animals (Xiao *et al.* 1999; Xiao *et al.* 2002).

Based on biological and molecular data, the '*C. parvum*' genotype I infecting the intestine of humans was proposed as being a new species *Cryptosporidium hominis* (Morgan-Ryan *et al.* 2002). The supporting evidence included a preliminary sequence scan of the entire genome demonstrating that *C. hominis* is distinct from *C. parvum.* This finding also demonstrates a lack of recombination, providing further support for its species status (Morgan-Ryan *et al.* 2002; Xiao *et al.* 2002).

## 4.2 Objectives

The objective of this chapter were to apply the M1 + T PCR fingerprinting to differentiate:

- *C. hominis* outbreak and sporadic cases from Australian sources,
- *C. hominis* outbreak and sporadic cases from England/Wales sources,
- human *C. parvum* outbreak cases from two England/Wales sources and
- *C. hominis* outbreak and human *C. parvum* from Scotland and Northern Ireland.

## 4.3 Sample Information

A total of 90 (Table 4.1) *Cryptosporidium* human isolates were fingerprinted using M1 + T primers as described in Chapter 2.19.3. All isolates were provided as purified DNA and the initial genotyping was preformed prior to this study by the institution proving the sample. The only exception was the South Australian samples which were provided as faecal samples. For these samples *Cryptosporidium* oocysts and DNA were isolated and extracted as described in Chapter 2.8 and 2.10. The South Australian samples were genotyped using a TRAP-C2 PCR as described in Chapter 3.3.3.

*C. hominis* isolates consisted of three outbreak groups (Western Australia, England/Wales, Scotland and Northern Ireland) and two sporadic groups (South Australia, England/Wales) giving a total of 61 samples. The 29 *C. parvum* samples consisted of three outbreak groupings (England/Wales 1 & 2, Scotland and Northern Ireland). All the Scotland and Northern Ireland isolates were provided as cell lysates. Before PCR occurred, all of the samples were ethanol precipitated and resuspended in 30 µL of TE.

Table 4.1: *Cryptosporidium* human isolates

| Location of Collection | Genotype | Isolate Number |
|---|---|---|
| Western Australian[1] | C. *hominis* | 10 |
| South Australian[2] | C. *hominis* | 12 |
| England / Wales Isolates[3] | C. *hominis* | 14 |
| | C. *hominis* | 16 |
| | *C. parvum* | 18 |
| | *C. parvum* | 3 |
| Scotland and Northern Ireland Isolates [4] | C. *hominis* | 9 |
| | *C. parvum*I | 8 |

1. Isolates provided by Associate Professor Una Ryan from Murdoch University Fremantle, Western Australia, Australia
2. Isolates provided by Dr Martha Sinclair from Monash University Melbourne, Victoria, Australia
3. Isolates provided by Dr Rachel Chalmers from PHLS *Cryptosporidium* Reference Unit, Swansea, Wales
4. Isolates provided by Professor. Huw Smith from Scottish Parasite Diagnostic Laboratory, Scotland

## 4.4 Results

### 4.4.1 Western Australian Human *C. hominis* Isolates

The M1 + T typing procedure was applied to ten human *C. hominis* isolates (Figure 4.1 panel A lanes 6 - 9 and lanes 3 - 9 Panel B) from a homogeneous source (children from a day care facility). The results showed a predominant consistent pattern for five of the isolates. The five isolates with the common banding pattern was WA 5, 7 - 10. The remaining five samples had unique fingerprints, suggesting different sources.

### 4.4.2 South Australian Human *C. hominis* Isolates, Sporadic Cases

The twelve South Australia (SA) human isolates (Table 4.3) were all sporadic cases and thought not to be related (Sinclair, per comm.). Nonetheless, there were two isolates which gave the same fingerprint profile (Figure 4.2 panel B lanes 6 and 7), which were subsequently discovered to have come from the same person on two different collection dates. All of the other isolates gave unique fingerprints (Figure 4.2) and panel A lane 6 was a blank.

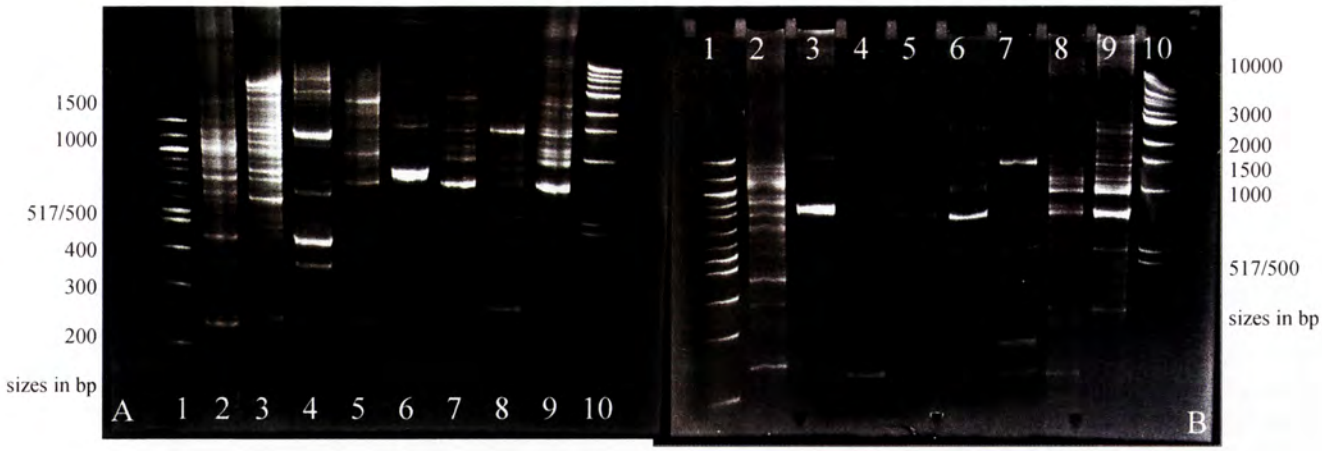


Figure 4.1: M1 + T PCR-PAGE, of bovine and Western Australia human isolates

Panel A: Lane 1 100 bp size standard (New England BioLabs Brisbane), Lane 2 Syd SW, Lane 3 Syd NW April 1998, Lane 4 NSWCW, Lane 5 Europe, Lane 6 WA 1 Human, Lane 7 WA 5 Human, Lane 8 WA 4 Human, Lane 9 WA 6 Human, Lane 10 1 Kb size standard (New England BioLabs Brisbane).

Panel B: Lane 1 100 bp size standard (New England BioLabs Brisbane), Lane 2 Syd SW, Lane 3 WA 1 Human, Lane 4 WA 2 Human, Lane 5 WA 3 Human, Lane 6 WA 5 Human, Lane 7 WA 6 Human, Lane 8 WA 9 Human, Lane 9 WA 10 Human, Lane 10 1 Kb size standard (New England BioLabs Brisbane).

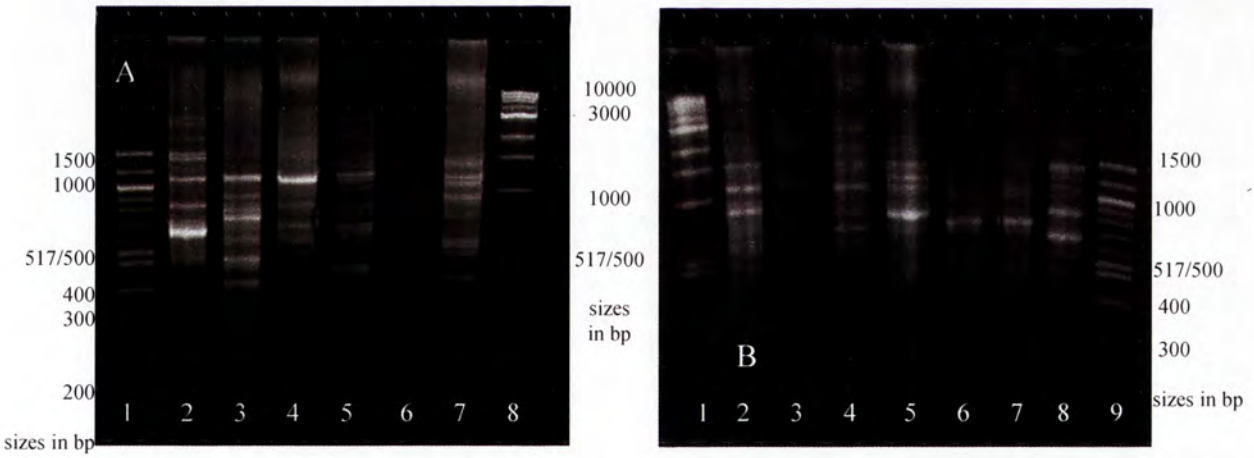


Figure 4.2: M1 + T PCR PAGE of South Australian human isolates

Panel A: Lane 1 100 bp size standard (New England BioLabs Brisbane), Lane 2 SA 1, Lane 3 SA 2, Lane 4 SA 3, Lane 5 SA 4, Lane 6 SA 5, Lane 7 SA 6, Lane 8 1 Kb size standard (New England BioLabs Brisbane).

Panel B: Lane 1 100 bp size standard (New England BioLabs Brisbane), Lane 2 SA 7, Lane 3 SA 8, Lane 4 SA 9, Lane 5 SA 10, Lane 6 SA 11, Lane 7 SA 11, Lane 8 SA 12, Lane 9 1 Kb size standard (New England BioLabs Brisbane).

### 4.4.3 England/Wales Human *C. hominis* and *C. parvum* Isolates

The M1 + T typing procedure was applied to a second homogeneous source of human *Cryptosporidium* samples (from children with diarrhoea at a day care facility) (Table 4.4). It resulted in a predominantly consistent pattern (Figure 4.3). The first 6 isolates where collected on the same day and the remaining eight were all collected three weeks later. Ten (DC 1-5, 10-14) of the fourteen samples had the same fingerprint with one other sample appearing very similar (DC 8). Another two isolates (DC 6 & 7) had a second fingerprint and appeared to be a mixture of two fingerprints. Further investigation of mixed isolates occurred (Chapter 2.17.7). In general, isolates which were mixtures usually had a lower intensity banding pattern with many bands all being close together.

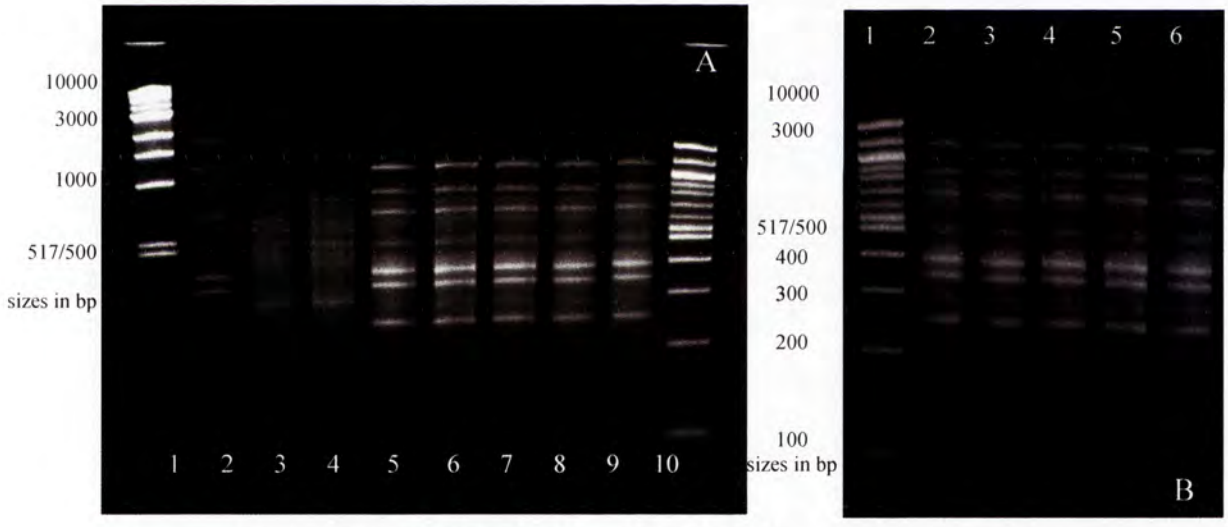


Figure 4.3: M1 + T PCR PAGE of South UK Daycare Centre isolates

Panel A: Lane 1, 100 bp size standard (New England BioLabs, Brisbane), Lane 2, DC **8**, Lane 3, DC **7**, Lane 4, DC 6, Lane 5, DC 5, Lane 6, DC **4**, Lane 7, DC **3**, Lane 8, DC 2, Lane 9, DC 1, Lane 10, 1 Kb size standard (New England BioLabs, Brisbane).

Panel B: Lane 1, 100 bp size standard (New England BioLabs, Brisbane), Lane 2, DC 10, Lane 3, DC 11, Lane 4, DC 12, Lane 5, DC 13, Lane 6, DC 14.

The ten UK human sporadic isolates (Figure 4.4) were collected at a similar time and region to the England/Wales Daycare Centre isolates. Nonetheless, all of the former sporadic isolates gave unique fingerprints and there are no common profile with the England/Wales Daycare Centre isolates.

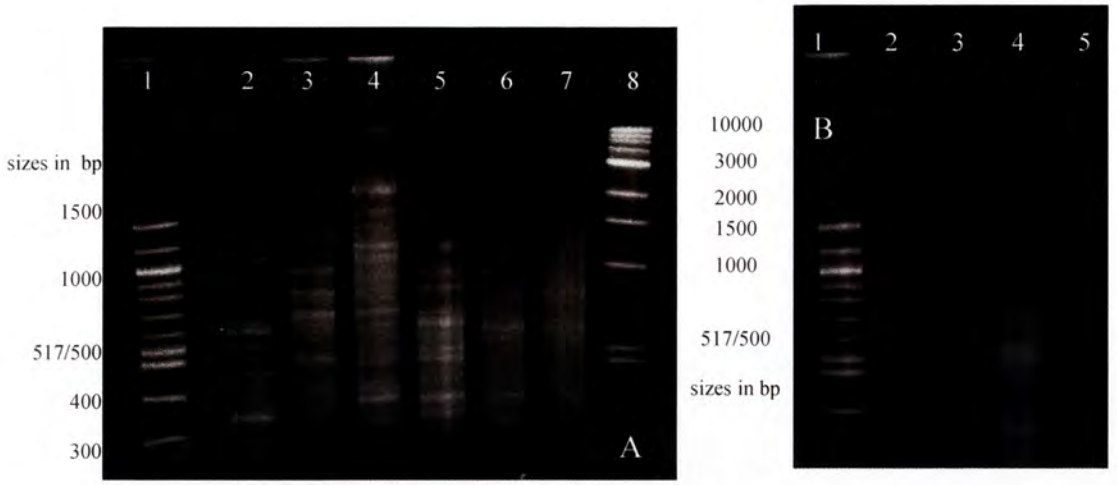


Figure 4.4: M1 + T PCR PAGE of England/Wales human *C. hominis* sporadic isolates

Panel A: Lane 1 100 bp size standard (New England BioLabs Brisbane); Lane 2 UK Sp 1; Lane 3 UK Sp 2; Lane 4 UK Sp 3; Lane 5 UK Sp 4; Lane 6 UK Sp 5; Lane 7 UK Sp 6; Lane 8 1 Kb size standard (New England BioLabs Brisbane).

Panel B: Lane 1 100 bp size standard (New England BioLabs Brisbane); Lane 2 UK Sp 7; Lane 3 UK Sp 8; Lane 4 UK Sp 9; Lane 5 UK Sp 10.

Two distinct *C. parvum* sets were examined from England/Wales. The larger group consisted of 18 human *C. parvum* isolates. Two of the samples did not amplify (UK T2 7 & UK T2 14). Figure 4.5 shows the fingerprints obtained for 16 of the samples. The most common banding pattern was found in six (UK T2 1 - 2, UK T2 10 - 13) isolates. Three isolates (UK T2 5, UK T2 16-17) had the same fingerprint and it was different to the other profiles found within this outbreak grouping. Another three isolates (UK T2 4, UK T2 15, UK T2 18) also has some common bands while the remaining isolates had unique fingerprints. Sample UK T2 8 did not amplify satisfactory as the DNA in the telomeric region appeared to have degraded. When sample UK T2 8 was examined by PCR-RFLP using the TRAP-C2 protocol, as described in Chapter 3.3.3, a *C. parvum* profile was produced.

The second *C. parvum* set from England/Wales consisted of three isolates (UK T2 B 1 - 3) (Table 4.7 and Figure 4.6) and all showed the same DNA fingerprint profile.

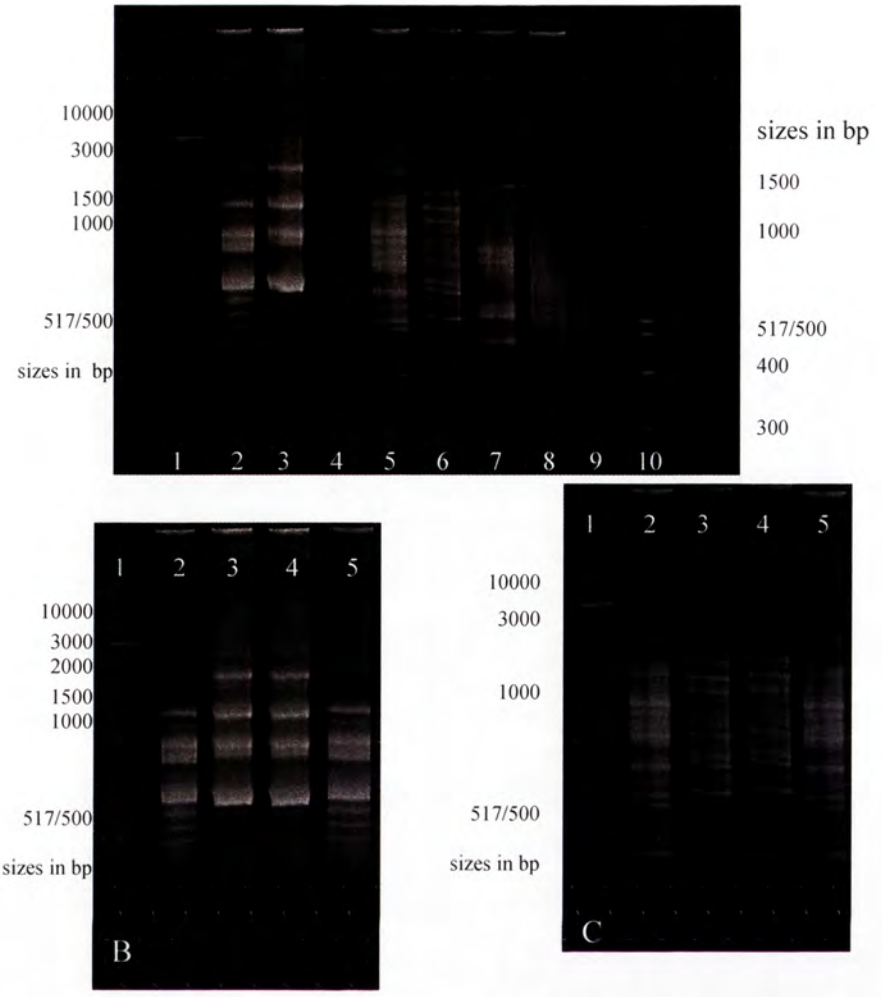


Figure 4.5: M1 + T PCR PAGE of England/Wales human *C. parvum* isolates group 1

Panel A: Lane 1 1 Kb size standard (New England BioLabs Brisbane), Lane 2 Sth UK T2 1, Lane 3 Sth UK T2 2, Lane 4 Sth UK T2 3, Lane 5 Sth UK T2 4, Lane 6 Sth UK T2 5, Lane 7 Sth UK T2 6, Lane 8 Sth UK T2 8, Lane 9 Sth UK T2 9, Lane 10 100 bp size standard (New England BioLabs Brisbane).

Panel B: Lane 1 1 Kb size standard (New England BioLabs Brisbane), Lane 2 Sth UK T2 10, Lane 3 Sth UK T2 11, Lane 4 Sth UK T2 12, Lane 5 Sth UK T2 13.

Panel C: Lane 1 1 Kb size standard (New England BioLabs Brisbane), Lane 2 Sth UK T2 15, Lane 3 Sth UK T2 16, Lane 4 Sth UK T2 17, Lane 5 Sth UK T2 18.

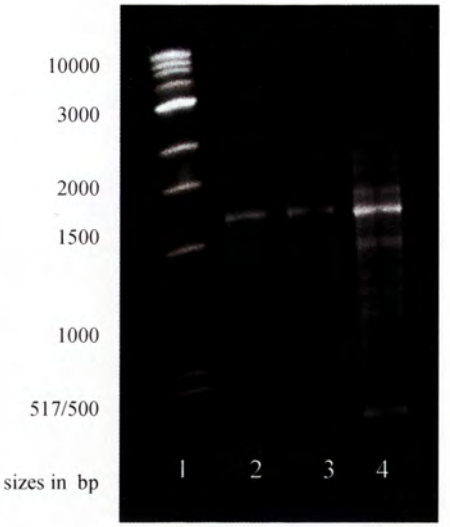


Figure 4.6: M1 + T PCR PAGE of England/Wales human *C. parvum* isolates group 2

Lane 1, 1 Kb size standard (New England BioLabs, Brisbane), Lane 2, Sth UK T2 B 1, Lane 3, Sth UK T2 B 2, Lane 4, Sth UK T2 B 3.

### 4.4.4 North UK Human *C. hominis* and *C. parvum* Isolates

Nine *C. hominis* and eight *C. parvum* isolates were investigated from Scotland (Figure 4.7) and Northern Ireland (Table 4.8).

From the nine Scotland *C. hominis* isolates, five unique M1 + T fingerprints were detected: UK Nth T1 1 and 2; UK Nth T1 4 and 6, UK Nth T1 5 and 10; each of UK Nth T1 7 - 9.

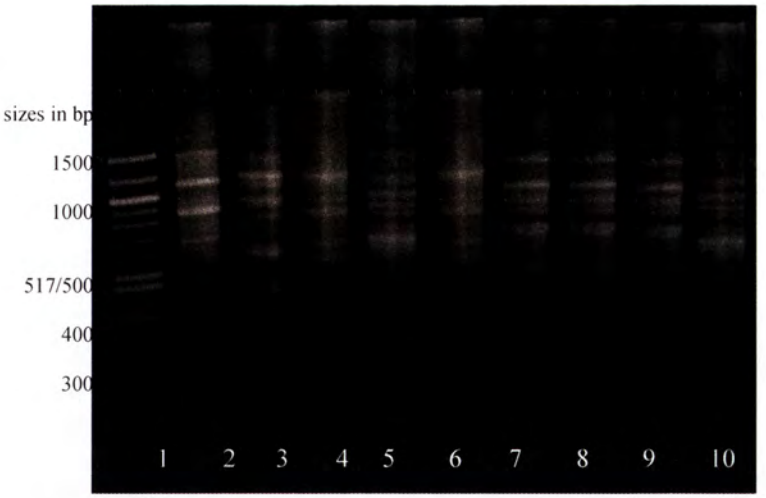


Figure 4.7: M1+ T PCR PAGE of North UK human *C. hominis* isolates

Lane 1 100 bp size standard (New England BioLabs Brisbane), Lane 2 UK Nth T1 1, Lane 3 UK Nth T1 2, Lane 4 UK Nth T1 3, Lane 5 UK Nth T1 4, Lane 6 UK Nth T1 5, Lane 7 UK Nth T1 6, Lane 8 UK Nth T1 7, Lane 9 UK Nth T1 8, Lane 10 1 Kb size standard (New England BioLabs Brisbane).

Of the eight Northern Ireland *C. parvum* isolates, a total of four fingerprints were produced after PCR using M1 + T primers. The dominant profile was found in five (UK Nth T2 2, 3, 5, 7 and 8) of the eight isolates. The remaining three isolates had unique profiles.

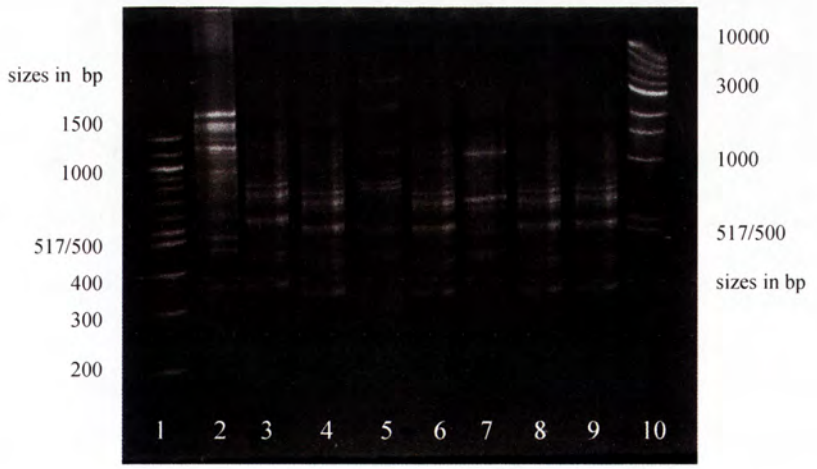


Figure 4.8: M1 + T PCR PAGE of North UK human *C. parvum* isolates

Lane 1 100 bp size standard (New England BioLabs Brisbane), Lane 2 UK Nth T2 1, Lane 3 UK Nth T2 2, Lane 4 UK Nth T2 3, Lane 5 UK Nth T2 4, Lane 6 UK Nth T2 5, Lane 7 UK Nth T2 6, Lane 8 UK Nth T2 7 Lane 9 UK Nth T2 8 Lane 10 1 Kb size standard (New England BioLabs Brisbane).

## 4.5 Discussion

The novel M1 + T subspecies typing methodology (Chapter 2) was shown to be applicable to *Cryptosporidium* isolates from a range of human hosts. With each of the outbreak related samples examined, a dominate fingerprint was produced. Yet, there was more diversity amongst the members of each outbreak group when compared to the relative homogeneity of animal *C. parvum* from the one geographic location (Chapter 3). Factors which could contribute to the greater diversity of the human origin isolates include:- different source origins reflecting less common exposure to faecal matter containing the same oocysts, the majority of groups were a one off collection and the dominate profile may not have established itself and the greater mobility of the human population may have also contributed to a greater diversity of infecting isolates being detected. The very nature of human day to day activity would make it harder for a dominate *C. hominis* isolate to establish itself, unless it was highly infectious or virulence. One possible source which has not been investigated during this study was an outbreak of *C. parvum* within a family group. A family group could possible be similar to the animal group isolates *i.e.* less diversity in the *C. hominis* fingerprint profile, similar to what was detected in the day care outbreak. With the *C. parvum* isolates, unfortunately, the associated animal isolates did not amplify so no direct comparison between fingerprints within the animals host and human outbreak group was undertaken.

The England/Wales *C. hominis* isolates were the only outbreak group investigated that had isolates collected over three weeks. The dominate profile which was found in this group comprised of ten out of the fourteen isolates. Each of the isolates was collected from a different individual. Five (1-5) of the common profile isolates were collected in week one and the other five (10-14) were collected three weeks later.

Currently outbreak groupings are determined by epidemiology methods and genotyping of the *Cryptosporidium* isolates. The standard genotyping methods are not able to determine any further information about the source of the *Cryptosporidium* or if the members of the outbreak group all have the same infecting isolate. By using M1 + T primer PCR it was established that within each outbreak group investigated there were several strains of *Cryptosporidium.* Other questions which cannot be addressed with

define genotyping is whether or not a dominate strain is able to establish within the outbreak group. Some evidence of this occurring has been shown within the England/Wales daycare centre group. Also with the M1 + T typing, individuals with more than one *C. hominis* strain can be identified which the usually genotyping methods cannot identify.

Micro- and minisatellites have been used for genotyping other parasites such as *Plasmodium* spp. and *Trypanosoma* spp. The information they provide has increased understanding of the epidemiology and population genetic structure of these parasites. However, there have only been a few studies which have utilised microsatelite markers (Cacciò *et al.* 2001; Mallon *et al.* 2003; Morgan *et al.* 1995). Mallon *et al.* (2003) used seven micro- and minisatellite markers to determine the multilocus genotype of bovine and human isolates from a discrete area in the north-east of Scotland. The results showed that the genotype I population was not panmictic and probably clonal or epidemic, but that the majority of human genotype II isolates were from the same randomly mating population as the local bovine isolates (Mallon *et al.* 2003). Caccio *et al.* (2001) used two microsatellite markers to study variation from different hosts and different geographical locations in Italy and identified possible host and geographical specific alleles.

Oocysts isolated from the faeces of infected animals or humans sometimes contained a mixture of different *Cryptosporidium* "genotypes"/species (McLauchlin *et al.* 1999). Characterising non-homogenous isolates can result in misleading conclusions (Egyed *et al.* 2003). The identification of different "genotypes" of various *Cryptosporidium* species may have important epidemiologic consequences in terms of understanding transmission cycles and sources of infection in outbreak situations (Morgan *et al.* 1999). Without genetic analysis, it is impossible to accurately predict host specificity or infectious potential of a specific isolate of *Cryptosporidium.*

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# 5 Chapter 5: Discussion and Conclusions

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## 5.1 Genotyping of *Cryptosporidium*

Genotyping of *Cryptosporidium* has developed over the past 10 years and serval different primer sets provide reliable differentiators of *C. parvum*'s host of origin *i.e.* TRAP-C1, TRAP-C2, COWP, β-tubulin, *Hsp*70 and various rRNA primer sets. Most primer sets which were used for *C. parvum* specific, cross-react with *C. hominis*, *C. meleagridis* and sometimes other *Cryptosporidium* species.

Conventional diagnostic methods for cryptosporidiosis are based on morphological features of oocysts, which have limitations and restrict clear species identification (Morgan *et al.* 1998; Morgan *et al.* 1998). Application of sensitive molecular approaches have indicated genetic heterogeneity among isolates of *Cryptosporidium* from different species of vertebrates. The data gathered suggests that a series of host-adapted genotypes-strains-species of the parasite may exist. The predictive value of achieving a sound understanding of the host *C. parvum* interactions requires sub-species typing. An effective molecular based typing strategy would increase our understanding of the epidemiology, transmission and control of outbreaks of the disease. Furthermore, environmental managers, swimming pool operators and water utilities would have an increased knowledge and understanding of *C. parvum* interactions within their controlled areas. This understanding would also aid in sound management by treatments plant operators and swimming pool operators, so as to effectively minimise outbreaks associated with their water service.

Clinically, patients with AIDS or otherwise immunocompromised are those most at risk from cryptosporidiosis. The development of appropriate molecular characterisation procedure would assist in the identification of sources of transmission and strain differences. Furthermore, such methods need to correlate with clinically important parameters such as severity of infection. Hence, there is an urgent need to determine the extent of genetic diversity within *Cryptosporidium* isolates affecting humans

### 5.1.1 Phenotypic variability within *Cryptosporidium*

Few differences have been found in the morphological characteristics and developmental cycles of parasites currently classified as *Cryptosporidium.* Increasing evidence of behavioural differences have been reported between isolates over the last few years, reinforcing the belief that *Cryptosporidium* is uniform species. Differences include virulence, pathogenesis, infectivity and drug sensitivity (Current *et al.* 1986; Fayer *et al.* 1986; Griffin *et al.* 1992; Mead *et al.* 1990; Nichols *et al.* 1991). Western-blot studies have shown antigenic differences among mammalian isolates (Griffin *et al.* 1992; McDonald *et al.* 1991; Nichols *et al.* 1991; Nina *et al.* 1992). More recently, analysis of oocyst wall antigens have been used to recognise multiple types within *Cryptosporidium* (McLauchlin *et al.* 1998). Such heterogeneity may explain differences in clinical incidence and response to drugs. All these factors contribute to complicate immunological approaches to diagnosis (Frost *et al.* 2003). Variable responses to treatment are also considered to be a factor in the clinical management of cryptosporidiosis (Morgan *et al.* 1999).

The genetic basis of such behavioural variance has still to be demonstrated, but it has been suggested that variability in virulence could be correlated to specific strains, whether or not the source of infection was of zoonotic origin (Thompson *et al.* 1996). Early results on the molecular characterisation of *C. parvum* isolates supported the presence of zoonotic transmission (Awad-El-Kariem *et al.* 1995) and subsequently have been confirmed by a wealth of data on the genetic variability of *C. parvum* (Hunter *et al.* 2002). Results from different laboratories examining isolates of human origin often produced variable results, which is now known as a reflection of the host specificity of the parasite. Cross-transmission studies of genotyped isolates indicate that the *C. parvum* readily infects mice and cattle, but the *C. hominis* does not (Meloni *et al.* 1996; Peng *et al.* 1997; Widmer *et al.* 1998). These results therefore support earlier workers who reported differences in infectivity between isolates of human and cattle origin (Current *et al.* 1986; Current *et al.* 1983; Pozio *et al.* 1992). Teunis *et al.* (2002) showed that three isolates of *C. parvum* (Iowa, TAMU, and UCP) have different ID50s, indicating substantial variation in their infectivity for humans and thus important for quantitative risk assessment. The evidence supports heterogeneity in infectivity among isolates of the same species (Teunis *et al.* 2002).

### 5.1.2 *Cryptosporidium* in humans

Less than 20 years ago, it was proposed that *Cryptosporidium* might be a `single species genus' (Tzipori *et al.* 1980) and a rare human infection (Bird 1981). There is now strong evidence from genetic and biological characterisation studies to indicate that there are at least two distinct genotypes of *Cryptosporidium*. A `human' (*C. homonis*), which to date has been found only in humans (Xiao *et al.* 2004) and possibly in a captive monkey (Xiao *et al.* 1999), and a `cattle' (*C. parvum*), which can be found in domestic livestock such as cattle, sheep, goats, *etc.*, as well as humans (Bonnin *et al.* 1996; Morgan *et al.* 1998; Ortega *et al.* 1991; Peng *et al.* 1997; Spano *et al.* 1997; Xiao *et al.* 1999) and is infectious for other animals such as laboratory rodents (Peng *et al.* 1997; Widmer *et al.* 1998) and Canada geese (Graczyk *et al.* 1997).

Genetic diversity in *C. parvum* has been identified by several molecular methods. Initial procedures utilised isoenzyme analysis and differentiated between human as well as animal isolates. Some human isolates exhibiting animal profiles due to zoonotic transmission (Awad-El-Kariem *et al.* 1995; Awad-El-Kariem *et al.* 1998; Ogunkolade *et al.* 1993). Initially RAPD analysis and the development of other PCR based methods were the initial molecular based protocols used to detect genotypic differences between human and animal *C. parvum* (Carraway *et al.* 1996; Deng *et al.* 1998; Morgan *et al.* 1995; Shianna *et al.* 1998). Major limitations such as the low number of oocysts recovered from many environmental and some faecal samples and the presence of contaminants, have hampered the successes of many PCR methods. The majority of genetic characterisation studies utilise parasite specific PCR primers in an attempt to overcome these problems with varying success. Specific PCR primer sets utilised to date are directed at either 18S rDNA genes or functional genes. Targeting the conserved regions of the genome has the limitation that these areas are highly regulated to maintain sequence integrity. The amount of variability between isolates would therefore be limited. Targeting of the non-coding regions within the genome may result in further differentiation, as is the case when using M1 + T primer set, as it is able to differentiate between similar samples collected from different locations (Chapter 3-4).

Sequence and/or PCR-RFLP analysis of the 18S rDNA gene (Carraway *et al.* 1996; Morgan *et al.* 1997; Xiao *et al.* 1998) and the more variable internal transcribed rDNA spacers (ITS1 and ITS2) (Carraway *et al.* 1996; Morgan *et al.* 1999) are commonly utilised. Indeed, this has become a defacto standard for comparison between isolate groupings, as well as being the main PCR used for describing different genotypes. Other genes of *C. parvum* which have been targeted include:-

- acetyl-CoA synthetase gene (Morgan *et al.* 1998);
- COWP gene (Spano *et al.* 1997);
- dihydrofolate reductase-thymidylate synthase (DHFR) gene (Gibbons *et al.* 1998; Vasquez *et al.* 1996) ;
- 70 kDa *Hsp*70 (Stinear *et al.* 1996) and
- thrombospondin related adhesion protein (TRAP-C1 and TRAP-C2) genes (Peng *et al.* 1997; Spano *et al.* 1998; Sulaiman *et al.* 1998).

All the gene specific PCRs confirmed the genetic distinctness of the *C. hominis* and *C. parvum*, but produce no additional information regarding the *C. parvum* oocyst such as; virulence, regional differentiation, infectivity and persistence within the environment.

Multilocus PCR approaches have become more common over the last few years. One example of such a typing approach investigated 28 isolates of *Cryptosporidium* originating from Europe, North and South America and Australia (Spano *et al.* 1998). PCR-RFLP which were used included the polythreonine (poly(T)) and COWP gene, TRAP-C1 gene and ribonucleotide reductase gene (RNR), and genotype-specific PCR of the rDNA ITS 1 region. All the isolates clustered into two groups. One comprised both human and animal isolates, whilst the other only human origin isolates (Spano *et al.* 1998). In this study, neither recombinant genotypes nor mixed infections were detected. Furthermore the separation of isolates into phylogenetic clades was purely based on host adapted specificity. No other genetic adaptations were detected by these PCRs. These results suggests that there is very little differentiation between *Cryptosporidium* isolates from a common host across all continents. In contrast, the benefits of also using a 'sub-species' typing methodology would be the further differentiation of the major phylogenetic clades identified.

Studies to date have indicated substantial genetic differences between the human and cattle genotypes, but very little variation within these genotypes. Only minor differences within the human genotype isolates have been identified in the 18S rRNA (Xiao *et al.* 1998), TRAP-C2 (Peng *et al.* 1997), (Sulaiman *et al.* 1998) and poly (T) genes (Widmer *et al.* 1998). However, the significance and prevalence of these intragenotype differences, are not clear.

Waterborne, day-care and food borne outbreaks of *cryptosporidiosis* of both the human and bovine genotypes of *Cryptosporidium* have been identified. The human genotype (*C. hominis*) is more frequently identified (Morgan *et al.* 1999). Several outbreaks caused by the bovine genotype have all been linked to contamination from or direct contact with animals (Chalmers *et al.* 2002; Howe *et al.* 2002; Preiser *et al.* 2003, Roy *et al.* 2004, Slifko *et al.* 2000). Examples include, the Maine apple cider outbreak in 1995, the British Colombia outbreak in 1996 and the Minnesota zoo outbreak in 1997 (Sulaiman *et al.* 1998). Results of these studies were also very useful in clarifying the source of contamination in outbreaks, such as the massive one in Milwaukee during 1993, were the probably cause was *Cryptosporidium* of human origin contaminating the water supply (Peng *et al.* 1997; Sulaiman *et al.* 1998). To date it is still assumed that isolates are all of the same genotype within an outbreak. All of the above outbreaks did not utilitise PCR methods which could distinguish sub-species by different DNA fingerprints. Therefore it is assumed that all isolates from the outbreak were the "same". PCR methods which are able to distinguish at a 'sub-species' level would in theory be able to identify common isolates within an outbreak group. Indeed, in the Milwalkee outbreak based in epidemiology data (Hunter per comm.) suggested that possibly only some 12 000 of the estimated 400 000 cases may have come via the water pollution incident.

Phylogenetic analysis of *C. hominis* and *C. parvum* genotypes at the 18S rRNA locus has revealed that they exhibit a similarity of 99.7%. This is the main focus that lead to the two genotypes being split into two different species (Morgan *et al.* 1999). The main thrust of the argument in other related species such as *T. gondii* and *N. caninum* is that there is 99.8% similarity within a species at 18S rRNA locus. For the ITS locus, the similarity between *C. hominis* and *C. parvum* genotypes of *Cryptosporidium* is

much less, 82%. The similarity between *T. gondii* and *N. caninum* at this locus is 89% (Morgan *et al.* 1999). There are also biological differences between the *C. hominis* and *C. parvum* genotypes the major being the host range which they infect naturally. The *C. hominis* genotype does not readily infect neonatal mice nor cattle (Peng *et al.* 1997; Widmer *et al.* 1998). The absence of recombinant genotypes using isoenzyme and multilocus analysis at unlinked loci (Peng *et al.* 1997; Widmer *et al.* 1998) indicates reproductive isolation between the `human' and `cattle' genotypes. This forms the basis for the two genotypes being classified as discrete species (Morgan *et al.* 1999).

Geographic and temporal sub-structuring is increasingly being utilised to track isolates in the environment and may identify major infective strains within an outbreak (Tait *et al.* 2004). The most commonly use PCR methods do not provide sufficient differentiation between isolates to be useful in answering such questions. The use of microsatellite markers as PCR primers are showing promise. The M1 + T2 typing method is able to provide the information necessary for the identification of sub-populations within outbreak groups as well as being a useful tool for the identification of environmental sources (Chapter 3-4).

Human cryptosporidiosis is mainly caused by *C. parvum* and *C. hominis* (previously known as the *C. parvum* human genotype I) (Morgan-Ryan *et al.* 2002). As described above, *C. hominis* is found almost exclusively in humans, whereas *C. parvum* is found in domestic livestock, wild animals, and humans (Morgan *et al.* 2000; Morgan *et al.* 1999). The occurrence in humans of both *Cryptosporidium* parasites has provided evidence that both anthroponotic and zoonotic cycles can occur in human infections (Alves *et al.* 2003). Naming and identification of genotypes is largely based on the host origin. The most common method adopted for the assignment of new genotypes utilises sequence differences from the existing genetic data. A new genotype is named after a host from which it is isolated and the latest species to be assigned is *C. hominis*. This is done so to minimise controversy which was occurring with the identification of new genotypes during the late 1990s. This genotype designation scheme is designed to reflect significant genetic differences among *Cryptosporidium* parasites and taking into account that not all genotypes differ from each other genetically to the same extent (Morgan *et al.* 2000; Morgan *et al.* 1999; Morgan-Ryan *et al.* 2002).

### 5.1.3 *Cryptosporidium* in domestic livestock

Little evidence for geographic or temporal sub-structuring within *C. parvum* has been reported in Scotland. Several distinct areas have now been analysed using a typing system with a high level of resolution (Caccio *et al.* 2000). Previous genotyping did not indicate geographic or temporal sub-structuring of Scottish isolates (Mallon *et al.* 2003). The markers which were used however, lacked the necessary level of resolution to distinguish different transmission routes and sources of infection or there may be other factors yet to be determined. However, with appropriate primers, such as M1 + T, significant discrimination was evident and able to identify and assign different fingerprints to samples collected from testing locations as close as 40 km (Syd SW and Syd NW, chapter 3.4.1 – 3.4.2).

*Cryptosporidium* was reported in pigs (*Sus scrofa*) in 1977 (Kennedy *et al.* 1977). Since that date *C. parvum* has been detected within porcine populations of Australia, Europe, Japan, the US and Viet Nam (Morgan *et al.* 1999). Genetic characterisation has revealed that pigs are infected with a genetically distinct specific form of *C. parvum* (Morgan *et al.* 1999, Ryan *et al.* 2004). Other investigations have detected pigs infected with the `cattle' strain. Therefore pigs have the potential to act as a reservoirs of infection for humans and other animals (Morgan *et al.* 1999). Phylogenetic analysis has provided support for the classification of the `pig' genotype as a distinct and valid species (Morgan *et al.* 1999).

#### 5.1.3.1 *Cryptosporidium* in marsupials the `marsupial' genotype

Little is known about the prevalence of *Cryptosporidium* in marsupials. *Cryptosporidium* infections have been reported in southern brown bandicoots (*Isoodon obesulus*), a hand-reared juvenile red kangaroo (*M. rufus*) from South Australia and a Tasmanian wallaby (*Thylogale billardierii*) (O'Donoghue 1995). To date, only three marsupial isolates of marsupial origin *Cryptosporidium* have been genotyped and published (Morgan *et al.* 1999; Power *et al.* 2004; Xiao *et al.* 1999). The isolates are

from a South Australian koala (*Phascolarctos cincereus*), a second isolate was a Western Australian red kangaroo and more recently, another isolate from a Western Australian koala (Morgan *et al.* 1997; Morgan *et al.* 1999; Xiao *et al.* 1999). However, 18S rDNA, ITS, HSP70, COWP, DHFR sequence analysis and RAPD analysis have all confirmed their genetic identity and distinctness from all other genotypes of *Cryptosporidium*. Indeed, at the ITS locus, the marsupial genotype exhibited only 64 % and 58 % similarity to the *C. hominis* and *C. parvum* genotypes, respectively (Morgan *et al.* 1999). Hence, marsupial genogroup is likely to be names as a new species.

The sheep and kangaroo isolates which were collected at Fowlers Gap all showed the same genotype when TRAP-C2 PCR typing was applied. *C. parvum* was found in all samples studies as described in Table 3.5. Figure 3.10 illustrates the genotyping by M1 + T PCR-RFLP. *C. parvum* had not been shown in any previous marsupial isolate. M1+T2 typing showed all samples to have the same fingerprint profile, therefore the strain at this location is sufficiently host adapted to be able to be infective to both kangaroo and sheep populations. Although few samples were investigated, the fingerprint of the *C. parvum* at Fowlers Gap was unique to that location.

### 5.1.4 Population genetics and mode of reproduction

Understanding the extent and nature of genetic variation in *Cryptosporidium* is an essential prerequisite in determining the epidemiology and transmission of this pathogen. It is especially important to know whether genotypes of *Cryptosporidium* are stable (indicative of clonal propagation) or unstable (due to frequent genetic recombination). Although the population structure of *Cryptosporidium* has not been conclusively determined, it appears likely to be clonal, based on several criteria that have been used as a basis for a "clonal" population structure in the related parasite *Toxoplasma* (Morgan *et al.* 1999).

The widespread occurrence of identical genotypes is an important criterion supporting this concept of a "clonal" population structure in *Cryptosporidium* (Morgan *et al.* 1997). The broad distribution of the cattle, human, pig and mouse genotypes supports

this concept, as does the correlation between phenotypic and genotypic markers (Awad-El-Kariem 1996; Awad-El-Kariem *et al.* 1998) and more recently, evidence of parity between unlinked genetic loci (Morgan *et al.* 1999; Spano *et al.* 1998; Widmer *et al.* 1998). Parity between two sets of independent genetic markers suggests that recombination might be biologically restricted. This is one of a number of criteria claimed to provide evidence of clonal population structure (Morgan *et al.* 1999). Conversely, it has been argued that for regions under selection, linkage disequilibrium can be maintained despite sufficient recombination (Morgan *et al.* 1999).

To test adequately the "clonal" model theory for *C. parvum* and determine population structure, a population genetic study is required. This would require the data to be analysed in terms of allelic and genotypic frequencies, rather than simply assessing distances. An examination of a much larger number of isolates from localised endemic foci would be required. There are a couple of studies which are now in progress, both are collaboration between colleagues in Australia, North America and Europe. One is headed by the CDC-USA (Morgan *et al.* 1998) and the other by PHLS *Cryptosporidium* Reference Laboratory, Wales, UK. The evaluation of intragenotype diversity will also be helpful for the elucidation of *Cryptosporidium* population structure in view of the proposed species status of some of the current genotypes. Future research should provide a clearer understanding of the population structure and transmission characteristics of this ubiquitous parasite (Morgan *et al.* 1998).

## 5.2 Using M1+T Primer PCR in Conjunction with Genotyping Methods

The cytoplasmic ribosomal RNA (rRNA) genes of *Cryptosporidium* have been analysed with respect to size, copy number, organisation and structure. The small and large subunit rRNAs are 1.7 and 3.6 kb, respectively. A 151 bp putative 5.8S rRNA gene has been identified. There are five copies of the rDNA unit per haploid genome and they are not organised in a conventional head to tail tandem array with a conserved external transcribed spacer. The rDNA units are dispersed through the genome to at least three chromosomes. At least two of the rDNA units are single unlinked copies on different chromosomes. There are two structurally distinct types of rDNA unit, Type A

and B, with marked differences in the internal transcribed spacer regions. There are four copies of the Type A rDNA unit and one copy of the Type B rDNA unit (Le Blancq *et al.* 1997).

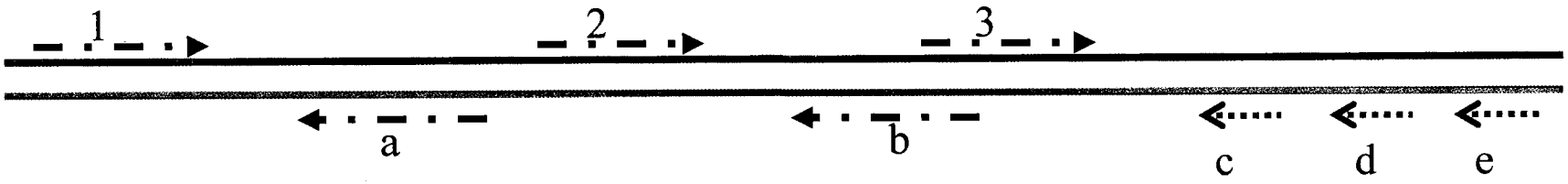
Microsatellite based methods, which only over recent years have become a common PCR method for differentiating *Cryptosporidium* oocysts have shown that there are mixed isolates. Microsatellites have recently been identified in the genome of *Cryptosporidium* (Aiello *et al.* 1999; Cacciò *et al.* 2000; Cacciò *et al.* 2001; Feng *et al.* 2000) and are becoming another PCR tool for genotyping and further sub-typing of *C. parvum* isolates. Mallon *et al.* (2003) has revealed that there is little evidence for geographical or temporal sub-structuring within *C. parvum* isolates in Scotland from several distinct areas encompassing 348 samples which included both human and animal origin isolates. The distribution of the human *C. parvum* cases was more varied in each of the three regions than the animal origin samples. No temporal sub-structuring of *C. parvum* was found (Mallon *et al.* 2003). The markers which were utilised, however may lack the necessary level of resolution to distinguish different transmission routes and sources of infection.

The current *Cryptosporidium* genotype designation system is also not in line with the genotype naming systems widely used for other microorgasisms, which are usually based on single nucleotide polymorphism in rapid-evolving genes such as ITS, immunodominant antigens, or therapeutic targets. Thus, species designation for some of the well-characterised *Cryptosporidium* genotypes would relieve the confusion. The naming of *Cryptosporidium* genotypes or species after the host of initial isolation can be problematic with *Cryptosporidium* parasites that have broad host range (Xiao *et al.* 2000).

The novel M1+T subspecies typing methodology (Chapter 2), was able to amplify *C. parvum* and *C. hominis* from a range of human hosts. With each of the outbreak related samples examined, a dominate fingerprint was produced. Yet, there was more diversity amongst the members of each outbreak group when compared to the relative homogeneity of animal *Cryptosporidium* from the one geographic location (Chapter 3).

Factors which could contribute to the greater diversity of the human origin isolates include:- different source origins reflecting less common exposure to faecal matter containing the same oocyst; the majority of groups were a one off collection and the dominate profile may not have established itself and the greater mobility of the human population may also contribute to a greater diversity of infecting isolates being detected. The very nature of human day to day activity would make it harder for a dominate *Cryptosporidium* isolate to establish itself, unless it was highly infectious or virulence. One possible source which has not been investigated during this study was an outbreak of *Cryptosporidium* within a family group. A family group could possible be similar to the animal group isolates *ie.* less diversity in the *Cryptosporidium* fingerprint profile. With the *C. parvum* isolates, unfortunately, the associated animal isolates did not amplify so no direct comparison between fingerprints within the animals host and human outbreak group was undertaken.

Application of M1 + T primers achieves a higher level of resolution compared to PCRs utilising the M1 primer alone. This is achieved by directing the PCR to the 3' ends of the chromosome. The T primer can bind in multiple positions (Figure 5.1) and ensures that only M1 priming position sufficiently close to the 3' end of the chromosome are utilised in the PCR. The T primer which has been utilised was the most common telomere repeat as identified by Lui *et al.* (1998).




**Gel information**

Lane 1: Size Standard in bp

Lane 2: Bands obtained from the above priming locations (bands labelled by the primer identification number and letter)

Microsatellite 1 Primer (1,2, 3 – acting as a forward primer)

Microsatellite 1 Primer (a, b – acting as a reverse primer)

Telomere Primer

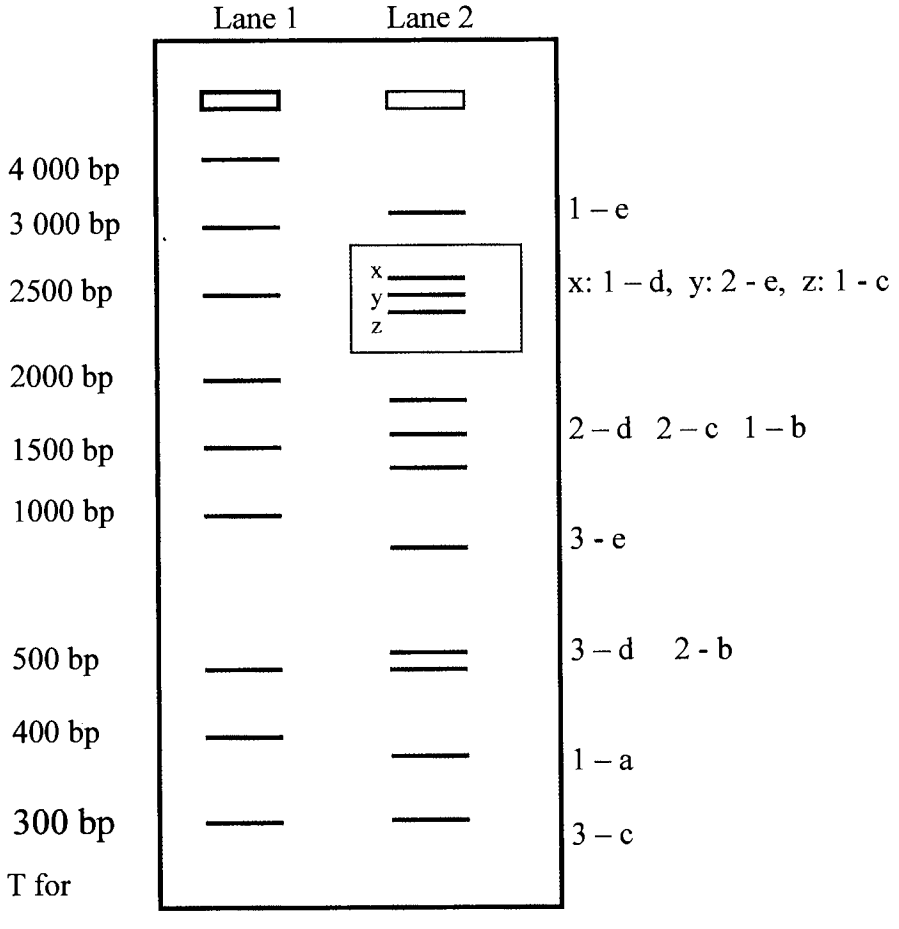



Figure 5.1: Theoretical schematic for potential primer binding of M1 + T for each *Cryptosporidium* chromosome

The M1 primer is able to be a forward and reverse primer as indicated in Figure 5.1. The *Cryptosporidium* specificity is achieved by comparing the M1 primer PCR to the M1 + T PCR as shown in Figure 2.8. The multiple PCR bands per isolate can be attributed to the amplicons being located on more than one of *Cryptosporidium*'s chromosomes or as detailed in Figure 5.1 the amplicons are from the one chromosome. The differentiation achieved between different isolates can be attributed to the above outlined possibilities of potential primer binding locations. It is possible for each different isolate to have several fingerprinting bands from the same chromosome, while other fingerprints could be the due to several bands being located on different chromosomes. For a full analysis of the PCR banding per chromosome further studies are required to be carried out. Within this type of study each chromosome from several isolates (ideally 2-3 from each of the ten sampling locations investigated within chapter 3) would need to be PCRed using the M1 + T PCR PAGE. The results of these PCRs would be able to confirm the relative M1 priming domains on the eight chromosomes of *Cryptosporidium.* Furthermore, the number of PCR bands from each individual chromosome would able to be determined for each of the fingerprints determined within Chapter 3 .

Three of the bovine origin *C. parvum* sites were sampled over a number of years. Each location was sampled at least twice a year for 3 - 5 years. The sample locations were Syd SW (5 years), Syd NW (3 years) and University of Arizona, Iowa strain (4 years). The Syd NW (Figure 3.6) and Uni of Arizona, Iowa strain (Figure 3.13) fingerprints did not change over the sampling period, except for the short change at Syd SW as discussed before.

Multilocus genotype *C. parvum* has recently been utilised by several studies using combinations of previously described (Section 3.1.4) PCR-RFLP targets. The failure of the abovementioned techniques to address questions concerning population genetics in relation to host, temporal and geographic sub-structuring has lead researchers to investigate the non-coding regions of the *C. parvum* genome.

Further PCR based methods have been developed which utilised micro- and minisatellite markers. The current micro- and minisatellite markers described,

however, do not support geographic or temporal sub-structuring of the populations (Mallon *et al.* 2003). The differentiation between geographic isolates samples provided by M1 + T2 primer set demonstrates a procedure useful for epidemiologic investigation of outbreaks and related infections. The PCR targeted area appears to have regional stability over the period which were investigated, which at several locations extends over four year. With the use of microsatellite markers, the source of infection of cryptosporidiosis can be traced back by comparing fingerprints of oocysts shed by infected individuals with those present in the suspected source(s) of infection (water, food, animals, or humans). Moreover, the severity of human infection has been found to vary depending on the specific *Cryptosporidium* isolate (Okhuysen *et al.* 1999). Therefore, it will be of particular interest to sub-type such isolates using microsatellite markers, or to perform experimental infections of healthy volunteers using oocysts having different genetic backgrounds defined by microsatellite analysis. Finally, the availability of microsatellite markers will allow the structure of the parasite's population to be critically evaluated and it will be possible to confirm or reject the hypothesis of clonality, as discussed for other protozoan parasites (Cacciò *et al.* 2001; Tibayrenc 1998). The branch orders of individual species or genotypes within the gastric or intestinal *Cryptosporidium* parasites differed somewhat among the three gene trees. These differences were probably due to differences in the rate and nature of genetic variations among the three genes. The ssrRNA gene of *Cryptosporidium* parasites evolves slowly, with sequence variations limited to several regions of the gene (Xiao *et al.* 2002).

Presently, the identification and naming of genotypes is largely based on the host origin. When significant or consistent sequence differences from the existing genetic data are identified, a new genotype is named after a host from which it is isolated. New species are generally not named right away because of a lack of biological characterisations and to avoid controversy. Although this genotype designation scheme generally reflects significant genetic differences among *Cryptospridium* parasites, not all genotypes differ from each other genetically to the same extent. Thus, some genotypes differ from each extensively whereas others are closely related to each other. One reason responsible for this is the need to differentiate related *Cryptosporidium* parasites when there is no species designation for the group of parasites, such is the

case for the *Cryptosporidium* marsupial, monkey and rabbit genotypes. The current *Cryptosporidium* genotype designation system is also not in line with the genotype naming systems widely used in other microorganisms, which are usually based on single nucleotide polymorphism in rapid-evolving genes such as ITS, immunodominant antigens, or therapeutic targets. Thus, species designation for some of the well-characterised *Cryptosporidium* genotypes would relieve the confusion. The naming of *Cryptosporidium* genotypes or species after the host of initial isolation can be problematic with *Cryptosporidium* parasites that have broad host range.

The host-adapted nature of most *Cryptosporidium* parasites indicates that the majority of *Cryptosporidium* parasites probably do not have a high infectivity to humans (Teunis *et al.* 2002). On the other hand, the presence of more than one genotype of *Cryptosporidium* parasites in a host species such as opossums, foxes, or cattle reaffirms that humans are not unique in being susceptible to more than one *Cryptosporidium* species.

The host expansion in the *C. parvum* bovine genotype certainly is not only limited to cattle, because this parasite is now responsible for more infections in humans in Europe than the *C. hominis* (Alves *et al.*, 2001; Guyot *et al.*, 2001; McLauchlin *et al.*, 2000; Morgan *et al.*, 2000a,b). Because the latter is still responsible for most human cryptosporidiosis in the rest of the world (Sulaiman *et al.*, 1998; Morgan *et al.*, 2000a,b; Xiao *et al.*, 2001; Yagita *et al.*, 2001; Leav *et al.*, 2002; Ong *et al.*, 2002; Tiangtip and Jongwutiwes, 2002), perhaps the expansion of the *C. parvum* bovine into humans may be due in part to the historic development of intensive husbandry practice for various livestock species and the associated high concentrations of young animals at these feeding operations. High concentrations of susceptible hosts such as young calves and lambs can lead to high rates of endemic cryptosporidiosis and the potential for high rates of environmental loading of, as in this case, the bovine origin *C. parvum*.

## 5.3 Further Work and Refinements to M1 +T PCR PAGE Method

The PCR methodology which has been developed still requires several refinements. Currently the number of oocysts which are needed for each PCR is fairly high (approx.

100 – 200). The number of oocysts which are needed for each PCR means that the current assay is only effective to be utilised for clinical isolates. For this method to become a true molecular epidemiology tool, isolates from water samples need to be PCRed as well as low oocyst numbers from other environmental samples. To achieve this, a >10 fold increase in sensitivity is required. Each PCR fingerprint needs to be produced from the DNA of 1-10 oocysts. The most effective way to increase sensitivity is to modify the detection methods. Currently PCR amplicons are visualised by ethidium bromide (EtBr) staining post electrophoresis. SYBR Gold is one stain which is more sensitive than EtBr. SYBR Gold is a non-specific nucleic acid gel stain and is a sensitive fluorescent stain which detects double or single-stranded DNA or RNA in electrophoretic gels, using standard ultraviolet transilluminators. Furthermore, SYBR Gold stain is a proprietary unsymmetrical cyanine dye. It exhibits >1000-fold fluorescence enhancement upon binding to nucleic acids and has a high quantum yield (~0.6) upon binding to double or single-stranded DNA as well as RNA (Molecular Probes 1999). Excitation maxima for dye–nucleic acid complexes are at ~495 nm in the visible spectrum and ~300 nm, in the ultraviolet. The emission maximum is ~537 nm. SYBR Gold stain is able to penetrate thick and high percentage agarose and PAGE gels rapidly. Agarose gels containing formaldehyde do not require distaining, due to the low intrinsic fluorescence of the unbound dye. Dye is readily removed from nucleic acids by ethanol precipitation, resulting in pure templates available for subsequent manipulation or analysis (Molecular Probes 1999). SYBR Gold can be used with the same transilluminator at used for EtBr staining as stained gels may be viewed with 300 nm ultraviolet transillumination or 254 nm epior transillumination. The SYBR Gold stain can be efficiently removed from nucleic acids by precipitating the DNA or RNA with ethanol. More than 97 % of the dye is removed by a single precipitation step. More than 99 % of the dye is removed when ammonium acetate is used as the salt in the precipitation procedure. Unlike EtBr, however no data is available regarding the mutagenicity or toxicity of SYBR Gold nucleic acid gel stain. Because this reagent binds to nucleic acids, it should be treated as a potential mutagen and handled with appropriate care (Molecular Probes 1999). As with all nucleic acid reagents, solutions of SYBR Gold stain should be poured through activated charcoal before disposal. The charcoal must then be incinerated to destroy the dye (Molecular Probes 1999).

To effectively trial this or other improved stains a PCR needs to be set up with a DNA titration. This new limit of detection would be determined by visualisation of amplicons run out on 8 % PAGE gels. A back calculation then needs to be carried out to determine the number of oocyst(s) which this DNA equates. A true titration of oocysts needed for the assay would be to use a micro-manipulator and physically add the contents of 1 –10 oocysts into a PCR tube prior to the amplification. The use of the micro-manipulator would also eliminate any PCR inhibitors which can be present when DNA is extracted from environmental isolates.

Another application if completed should lead to a better understanding of where the primers are binding in different isolates and amplify each chromosome independently. In order to carry out this study whole intact chromosomes, from several *Cryptosporidium* isolates, needs to be extracted and purified. The amplifying of each chromosome is useful in the determination if the fingerprints produced are a composite of all 8 chromosomes or if the fingerprint produced is from the amplification of a few chromosomes. It would also be possible to map the M1 primer to individual chromosomes and its distance relationship with the T primer. The T primer would be present on all *C. parvum* chromosomes, but the number of T binding domains may vary from chromosome to chromosome for each *Cryptosporidium* isolate. Furthermore the T primer binding domains may differ between isolates. A fuller understanding of the binding domains of both primers would lead to a better understanding of how the PCR is working and generating of DNA fingerprints. An additional benefit would be a better understanding of how the teleomerease enzyme behaves in different isolates.

With a detection limit in the order of 1-10 oocysts, trials and method modifications can be completed so M1 + T PCR may work for environmental isolates. To complete a true tracing and tracking of *Cryptosporidium*, the PCR method will need to efficiently amply a low number of oocysts from a variety of environmental sources *i.e.* concentrates from water samples. Once the protocol is able to routinely amply *Cryptosporidium* DNA from water samples *etc.* matching of DNA fingerprints found in clinical samples can occur. This enables the source of an outbreak to be traced. Should another water contamination incident occurred similar to that of Sydney mid-late 1998,

the M1 + T PCR method could match the fingerprints from any suspect clinical isolates to environmental samples and possibly detect the outbreak source. When used with a range of source samples it may show where the contamination incident originated *e.g.*. breakdown in treatment at a water treatment plant or sewage treatment plant, or from a natural or endemic human source(s).

## 5.4 Conclusions

The PCR typing protocol which has been developed is effective in sub-species typing of at least *C. parvum, C. hominis*. The T primer, is a 2 times teleomere repeat based on the most frequent repeat unit found in the telomeric region of *C. parvum* (Liu *et al.* 1998). When this primer is used alone in a PCR, it does not yield products. The *Cryptosporidium* specificity is reliant upon the M1 being in the 3' area of a chromosome and in close proximity to the T primers. When combined they are able to be used to differentiate *Cryptosporidium* spp. from different sampling locations rather than just the host origin. The PCR method developed is a single PCR-PAGE procedure as follows:-

- DNA 2µl (DNA concentration was 100ng$\mu L^{-1}$)
- dNTP 1µl (initial concentration was 10mM)
- Microsatellite 1 primer 3 µL (10 pM $GAA_5$)
- Telomere 2 primer 3 µL (10 pM G $(CCTAAA)_2$)
- 10x Buffer 4µl
- Mg 8µl (initial concentration was 25mM)
- Red Hot *Taq* 5U

The final volume of the PCR was 40µl.

The PCR cycling conditions:
94 °C (30 seconds), followed by 30 cycles of 92 °C (20 seconds), 30 – 45 °C (20 seconds), 72 °C (30 seconds). A final anneal of primers was preformed for 45 seconds at 45 °C followed by 5min at 72 °C .

The method has successfully be used to produce DNA fingerprints for *Cryptosporidium* from both animal (Chapter 3) and human (Chapter 4) origin genotypes. Effective fingerprints were produced from samples of three types including widely spaced geographic sources. These included multiple hosts at the same dairy herd location,

both in samples at the same date and sample dates extending over a four year period, as well as samples of a defined and passage-propagated isolate. It distinguishes between unrelated sources within a narrow geographical area (*e.g.* 20 km radius). Reproducible fingerprints were achieved for each isolate (up to 10) taken from the same location on the same sampling date. The fingerprints observed in samples from Syd-SW appeared to change over the full four-year period of observation. Comparison to samples of the defined "Iowa" strain over a comparable period (2 years) revealed no change. Examination of individual isolates over a longer period may shed light on the long-term stability of the target region of the *Cryptosporidium* genome. The PCR method is applicable to DNA extracted from faeces without oocyst isolation. That is stable for a single homogenous source over time. With the human isolates from outbreaks the M1 + T PCR PAGE distinguishes between related and unrelated cases among an epidemiologic contact group.

Based on results reported here, the differentiation provided by the primer set in combination with apparent gene region stability establishes a useful procedure for epidemiologic investigation of outbreaks and related infections. Current applications of the protocol is useful for the water industry in the determination of clinical human isolates which form part of outbreaks *i.e.* swimming pool, veterinary industry tracking at dairy farms and outbreaks within domestic animals as well as handlers. When the developments described in section 5.4 of this chapter are completed, additional applications may then be added; such as being able to detect *Cryptosporidium* fingerprints from environmental samples. Being able to apply this PCR method to the type of environmental samples would give a tracking and tracing tool. That could be applied to the identification of an outbreak source. Furthermore, the application could also be used for the tracing of *Cryptosporidium* isolate stability in environmental sources, *e.g.* longitudinal studies at farms. An environmental water application could be to use the technique as a method for identification of sources of *Cryptosporidium* within a catchment or during water associated outbreaks of *Cryptosporidium*. The later application would be used in conjunction with widely used genotyping methods to fully characterise isolates which are responsible for the contamination event. A further use for the M1 + T PCR method would be to identify the strain or strains within an outbreak group or to monitor the stability of an isolate at an environmental location.

The application of the M1 + T PCR is useful in the determination of sub-species identification of *Cryptosporidium* isolates as it is able to identify isolates from different locations as well as identifying isolates from an outbreak as having the same fingerprint.

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# Appendix 1


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|---|---|---|
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Table A1.1: Sydney South West isolates

| Collection date | ID number | Birth date | Age (in Days) | Isolation date |
|---|---|---|---|---|
| Nov-97 | Crypto 1 | unknown | unknown | Nov-97 |
| | Crypto 2 | unknown | unknown | Nov-97 |
| | Crypto 3 | unknown | unknown | Nov-97 |
| | Crypto 4 | unknown | unknown | Nov-97 |
| 20-Oct-98 | 6172 | unknown | unknown | 26-Oct-98 |
| | 6184 | unknown | unknown | 26-Oct-98 |
| | 6206 | unknown | unknown | 26-Oct-98 |
| | 655 | 04-Oct-98 | 16 | 23-Oct-98 |
| | 658 | 06-Oct-98 | 14 | 23-Oct-98 |
| | 672 | 10-Oct-98 | 10 | 23-Oct-98 |
| | 676 | 10-Oct-98 | 10 | 23-Oct-98 |
| | 2156 | 14-Oct-98 | 6 | 23-Oct-98 |
| | 2569 | 14-Oct-98 | 6 | 23-Oct-98 |
| | 6200 | unknown | unknown | 26-Oct-98 |
| | 6217 | unknown | unknown | 26-Oct-98 |
| | 6497 | unknown | unknown | 26-Oct-98 |
| | 6518 | unknown | unknown | 26-Oct-98 |
| | 6534 | unknown | unknown | 26-Oct-98 |
| | 6568 | unknown | unknown | 26-Oct-98 |
| | 6604 | unknown | unknown | 26-Oct-98 |
| | 6619 | unknown | unknown | 26-Oct-98 |
| | 6667 | unknown | unknown | 26-Oct-98 |
| | 6689 | unknown | unknown | 26-Oct-98 |

Table A1.1: Sydney South West isolates (continued)

| Collection date | ID number | Birth date | Age (in Days) | Isolation date |
|---|---|---|---|---|
| | 6768 | unknown | unknown | 23-Oct-98 |
| | 6814 | unknown | unknown | 23-Oct-98 |
| | 6826 | unknown | unknown | 23-Oct-98 |
| | 6831 | unknown | unknown | 23-Oct-98 |
| | 6846 | unknown | unknown | 23-Oct-98 |
| | 6876 | unknown | unknown | 23-Oct-98 |
| | 6899 | unknown | unknown | 23-Oct-98 |
| | 6901 | unknown | unknown | 23-Oct-98 |
| | 6943 | unknown | unknown | 23-Oct-98 |
| | 6948 | unknown | unknown | 23-Oct-98 |
| 30-June-99 | 1907 | 12-May-99 | 49 | 15-Jul-99 |
| | 1915 | 15-May-99 | 46 | 15-Jul-99 |
| | 1952 | 27-May-99 | 34 | 15-Jul-99 |
| | 1992 | 9-Jun-99 | 21 | 15-Jul-99 |
| | 1996 | 10-Jun-99 | 20 | 15-Jul-99 |
| | 1999 | 11-Jun-99 | 19 | 15-Jul-99 |
| | 2000 | 11-Jun-99 | 19 | 15-Jul-99 |
| | 2156 | 13-Jun-99 | 17 | 15-Jul-99 |
| | 2163 | 15-Jun-99 | 15 | 15-Jul-99 |
| | 2186 | 15-Jun-99 | 15 | 15-Jul-99 |
| | 2189 | 19-Jun-99 | 11 | 15-Jul-99 |
| | 5630 | 6-Jun-99 | 24 | 15-Jul-99 |

Table A1.1: Sydney South West isolates (continued)

| Collection date | ID number | Birth date | Age (in Days) | Isolation date |
|---|---|---|---|---|
| 22-Sep-99 | 2407 | 03-Aug-99 | 50 | 24-Sep-99 |
| | 2437 | 3-Aug-99 | 50 | 24-Sep-99 |
| | 2486 | 16-Aug-99 | 37 | 24-Sep-99 |
| | 2486 | 24-Aug-99 | 29 | 24-Sep-99 |
| | 2522 | 27-Aug-99 | 26 | 24-Sep-99 |
| | 2525 | 30-Aug-99 | 23 | 24-Sep-99 |
| | 2526 | 31-Aug-99 | 22 | 24-Sep-99 |
| | 2535 | 31-Aug-99 | 22 | 24-Sep-99 |
| 03-May-00 | 4105 | 05-Apr-00 | 28 | 17-May-00 |
| | 4158 | 11-Apr-00 | 22 | 8-May-00 |
| | 6943 | 22-Apr-00 | 11 | 5-May-00 |
| | 6948 | 22-Apr-00 | 11 | 9-May-00 |
| | 4250 | 25-Apr-00 | 8 | 17-May-00 |
| | 4159 | 11-Apr-00 | 22 | 17-May-00 |
| | 4160 | 11-Apr-00 | 22 | 17-May-00 |
| | 4165 | 11-Apr-00 | 22 | 17-May-00 |
| | 4170 | 11-Apr-00 | 22 | 17-May-00 |
| | 6821 | 11-Apr-00 | 22 | 17-May-00 |
| 22-Aug-00 | 5345 | 19-Jul-00 | 34 | 24-Aug-00 |
| | 7402 | 29-Jul-00 | 24 | 24-Aug-00 |
| | 7431 | 03-Aug-00 | 19 | 24-Aug-00 |
| | 7430 | 06-Aug-00 | 16 | 24-Aug-00 |
| | 5751 | unknown | unknown | 24-Aug-00 |
| | 7440 | 09-Aug-00 | 13 | 24-Aug-00 |

Table A1.1: Sydney South West isolates (continued)

| Collection date | ID number | Birth date | Age (in Days) | Isolation date |
|---|---|---|---|---|
| | 5803 | 14-Aug-00 | 8 | 24-Aug-00 |
| | 7478 | 15-Aug-00 | 7 | 24-Aug-00 |
| | 7454 | 12-Aug-00 | 10 | 24-Aug-00 |
| | 5729 | 7-Aug-00 | 15 | 24-Aug-00 |
| 22-May-01 | 8203 | 2-May-01 | 20 | 25-May-01 |
| | 8204 | 3-May-01 | 19 | 25-May-01 |
| | 8300 | 5-May-01 | 17 | 25-May-01 |
| | 8307 | 6-May-01 | 16 | 25-May-01 |
| | 8309 | 6-May-01 | 16 | 25-May-01 |
| | 8315 | 6-May-01 | 16 | 25-May-01 |
| | 8132 | 7-May-01 | 15 | 25-May-01 |
| | 8136 | 7-May-01 | 15 | 25-May-01 |
| | 8319 | 7-May-01 | 15 | 25-May-01 |
| | 8137 | 8-May-01 | 14 | 25-May-01 |
| 6-Aug-01 | 9367 | 18-7-01 | 22 | 9-Aug-01 |
| | 9354 | 23-7-01 | 17 | 9-Aug-01 |
| | 9351 | 23-7-01 | 17 | 9-Aug-01 |
| | 9348 | 24-7-01 | 16 | 9-Aug-01 |
| | 9340 | 25-7-01 | 15 | 9-Aug-01 |
| | 9338 | 26-7-01 | 14 | 9-Aug-01 |
| | 9337 | 26-7-01 | 14 | 9-Aug-01 |
| | 9328 | 27-7-01 | 13 | 9-Aug-01 |
| | 9321 | 28-7-01 | 12 | 9-Aug-01 |
| | 9320 | 28-7-01 | 12 | 9-Aug-01 |

Table A1.2: Sydney North West isolates

| Collection date | ID number | Birth date | Age (in Days) | Isolation date |
|---|---|---|---|---|
| 18-Jan-99 | 4313 | 02-Dec-99 | 47 | 20-Jan-99 |
| | 4316 | 17-Dec-99 | 32 | 20-Jan-99 |
| | 4325 | 09-Jan-99 | 9 | 20-Jan-99 |
| | 4326 | 6-Jan-99 | 12 | 20-Jan-99 |
| | 4314 | 10-Dec-99 | 39 | 20-Jan-99 |
| | 4322 | 2-Jan-99 | 16 | 20-Jan-99 |
| 24-Jan-00 | 4721 | 27-Dec-99 | 28 | 19-Jan-00 |
| | 4708 | 29-Dec-99 | 26 | 19-Jan-00 |
| | 4680 | 4-Jan-00 | 20 | 19-Jan-00 |
| | 4671 | 8-Jan-00 | 16 | 19-Jan-00 |
| | 4670 | 8-Jan-00 | 16 | 19-Jan-00 |
| | 4663 | 9-Jan-00 | 15 | 19-Jan-00 |
| 25-Aug-00 | 4923 | 4-Aug-00 | 21 | 31-Aug-00 |
| | 4926 | 5-Aug-00 | 20 | 31-Aug-00 |
| | 4928 | 7-Aug-00 | 18 | 31-Aug-00 |
| | 4930 | 7-Aug-00 | 18 | 31-Aug-00 |
| | 4927 | 8-Aug-00 | 17 | 31-Aug-00 |
| | 493 | 8-Aug-00 | 17 | 31-Aug-00 |
| | 4935a | 11-Aug-00 | 14 | 31-Aug-00 |
| | 4922 | 3-Aug-00 | 22 | 31-Aug-00 |
| | 4936 | 12-Aug-00 | 13 | 31-Aug-00 |
| | 4940 | 13-Aug-00 | 12 | 31-Aug-00 |

Table A1.2: Sydney North West isolates (continue)

| Collection date | ID number | Birth date | Age (in Days) | Isolation date |
|---|---|---|---|---|
| 12-Feb-01 | 5102 | 26-Jan-01 | 18 | 14-Feb-01 |
| | 5105 | 26-Jan-01 | 18 | 14-Feb-01 |
| | 5112 | 29-Jan-01 | 15 | 14-Feb-01 |
| | 5114 | 30-Jan-01 | 14 | 14-Feb-01 |
| | 5118 | 1-Feb-01 | 12 | 14-Feb-01 |
| | 5120 | 1-Feb-01 | 12 | 14-Feb-01 |
| 25-Aug-01 | 5409 | 19-Jul-00 | 37 | 31-Aug-00 |
| | 5419 | 23-Jul-00 | 33 | 31-Aug-00 |
| | 5423 | 04-Aug-00 | 21 | 31-Aug-00 |
| | 5426 | 05-Aug-00 | 20 | 31-Aug-00 |
| | 5438 | 6-Aug-00 | 19 | 31-Aug-00 |
| | 5430 | 07-Aug-00 | 18 | 31-Aug-00 |
| | 5434 | 08-Aug-00 | 17 | 31-Aug-00 |
| | 5438 | 9-Aug-00 | 16 | 31-Aug-00 |

Table A1.3: New South Wales Central West isolates

| Collection date | ID number | Birth date | Age (in Days) | Isolation date |
|---|---|---|---|---|
| NSW C 1 01-Feb-99 | 7993 | 13-Jan-99 | 18 | 15-Feb-99 |
| | 7999 | 17-Jan-99 | 14 | 15-Feb-99 |
| | 8000 | 14-Jan-99 | 17 | 15-Feb-99 |
| | 8005 | 16-Jan-99 | 15 | 17-Feb-99 |
| NSW C 2 02-Feb-99 | W6 | unknown | Unknown | 17-Feb-99 |
| | W7 | Unknown | Unknown | 17-Feb-99 |
| | W9 | Unknown | Unknown | 17-Feb-99 |
| | W10 | unknown | Unknown | 17-Feb-99 |

Table A1.4: Western New South Wales isolates

| Collection date | Isolate Source | ID number | Isolation date |
|---|---|---|---|
| 22-Sep-00 | sheep 1 | SW 1 | 03-Oct-00 |
| | sheep 2 | SW 2 | 03-Oct-00 |
| | sheep 3 | SW 3 | 03-Oct-00 |
| | sheep 4 | SW 4 | 03-Oct-00 |
| | sheep 5 | SW 5 | 03-Oct-00 |
| | sheep 6 | SW 6 | 03-Oct-00 |
| 22-Sep-00 | Red Kangaroo 1 | RRW 1 | 04-Oct-00 |
| | Red Kangaroo 2 | RRW 2 | 04-Oct-00 |
| | Red Kangaroo 3 | RRW 3 | 04-Oct-00 |
| | Red Kangaroo 4 | RRW 4 | 04-Oct-00 |
| | Red Kangaroo 5 | RRW 5 | 04-Oct-00 |
| | Red Kangaroo 6 | RRW 6 | 04-Oct-00 |
| | Red Kangaroo 7 | RRW 7 | 04-Oct-00 |
| | Red Kangaroo 8 | RRW 8 | 04-Oct-00 |
| | Red Kangaroo 9 | RRW 9 | 04-Oct-00 |
| | Red Kangaroo 10 | RRW 10 | 04-Oct-00 |
| | Red Kangaroo 11 | RRW 11 | 04-Oct-00 |
| | Red Kangaroo 12 | RRW 12 | 04-Oct-00 |
| 22-Sep-00 | Grey Kangaroo 1 | GRW 1 | 04-Oct-00 |
| | Grey Kangaroo 2 | GRW 2 | 04-Oct-00 |
| | Grey Kangaroo 3 | GRW 3 | 04-Oct-00 |
| | Grey Kangaroo 4 | GRW 4 | 04-Oct-00 |

Note: Ages were not provided all animals isolates used were from adult animals

Table A1.5: Victorian Sheep isolates

| Collection date | ID number | Oocyst Collection date | Age (in Days) | Isolation date |
|---|---|---|---|---|
| Vic A<br>5-10-00 | 2369 | 21-Aug-00 | 44 | 06-Oct-00 |
| | 2372 | 28-Aug-00 | 37 | 06-Oct-00 |
| | 2373 | 30-Aug-00 | 35 | 06-Oct-00 |
| | 2374 | 26-Aug-00 | 39 | 06-Oct-00 |
| | 2375 | 31-Aug-00 | 34 | 06-Oct-00 |
| | 2376 | 26-Aug-00 | 39 | 06-Oct-00 |
| | 2378 | 01-Sep-00 | 33 | 06-Oct-00 |
| | 2381 | 03-Sep-00 | 31 | 06-Oct-00 |
| | 2382 | 29-08-00 | 36 | 06-Oct-00 |
| | 2383 | 30-Aug-00 | 35 | 06-Oct-00 |
| | 2386 | 04-Sep-00 | 30 | 06-Oct-00 |
| | 2390 | 13-Sep-00 | 21 | 06-Oct-00 |
| Vic B<br>06-10-00 | 1 | 06-10-00 | 2yrs | 23-Oct-00 |
| | 2 | 06-10-00 | 2yrs | 23-Oct-00 |
| | 3 | 06-10-00 | 2yrs | 23-Oct-00 |
| | 4 | 06-10-00 | 2yrs | 23-Oct-00 |
| | 5 | 06-10-00 | 5-10weeks | 17-Oct-00 |
| | 6 | 06-10-00 | 5-10weeks | 17-Oct-00 |
| | 7 | 06-10-00 | 5-10weeks | 17-Oct-00 |
| | 8 | 06-10-00 | 5-10weeks | 17-Oct-00 |
| | 9 | 06-10-00 | 5-10weeks | 17-Oct-00 |
| | 10 | 06-10-00 | 5-10weeks | 17-Oct-00 |
| | 11 | 06-10-00 | 5-10weeks | 17-Oct-00 |
| | 12 | 06-10-00 | 5-10weeks | 17-Oct-00 |

Table A1.6: Porcine *Cryptosporidium* isolates from South Eastern Australia

| Collection date | ID number | Age (in Weeks) | Isolation date |
|---|---|---|---|
| 21-Apr-99 | 6c | 6 weeks | 27-Apr-99 |
| 21-Apr-99 | 6e | 6 weeks | 27-Apr-99 |
| 21-Apr-99 | 7a | 7 weeks | 27-Apr-99 |
| 21-Apr-99 | 7d | 7weeks | 27-Apr-99 |
| 21-Apr-99 | 7e | 7weeks | 27-Apr-99 |

Table A1.7: *C. parvum* isolates from outside Australia

| Collection date | ID number | Oocyst Collection date | Age (in Days) | Isolation date |
|---|---|---|---|---|
| Europe | 661/99 | Nov 1999 | unknown | unknown |
| | 679/00 | Feb 2000 | unknown | unknown |
| Iowa | UAI 1 | September 1998 | unknown | unknown |
| | UAI 2 | September 1998 | unknown | unknown |
| | UAI 3 | September 1998 | unknown | unknown |
| | UAI 4 | September 1998 | unknown | unknown |
| | UAI 5 | September 1998 | unknown | unknown |
| | UAI 6 | September 1998 | unknown | unknown |
| | UAI 7 | Feb 1999 – Nov 1999 | unknown | unknown |
| | UAI 8 | Feb 1999 – Nov 1999 | unknown | unknown |
| | UAI 9 | Feb 1999 – Nov 1999 | unknown | unknown |
| | UAI 10 | Feb 1999 – Nov 1999 | unknown | unknown |
| | UAI 11 | Feb 1999 – Nov 1999 | unknown | unknown |
| | UAI 12 | Feb 1999 – Nov 1999 | unknown | unknown |
| | UAI 13 | Feb 1999 – Nov 1999 | unknown | unknown |
| | UAI 14 | Jan 2000 – Oct 2000 | unknown | unknown |
| | UAI 15 | Jan 2000 – Oct 2000 | unknown | unknown |
| | UAI 16 | Jan 2000 – Oct 2000 | unknown | unknown |
| | UAI 17 | Jan 2000 – Oct 2000 | unknown | unknown |
| | UAI 18 | Jan 2000 – Oct 2000 | unknown | unknown |
| | UAI 19 | Jan 2000 – Oct 2000 | unknown | unknown |
| | UAI 20 | Jan 2000 – Oct 2000 | unknown | unknown |

Table A1.7: *C. parvum* Isolates from outside Australia

| Collection date | ID number | Oocyst Collection date | Age (in Days) | Isolation date |
|---|---|---|---|---|
| Iowa | UAI 21 | Feb 2001 – Aug 2001 | unknown | unknown |
| | UAI 22 | Feb 2001 – Aug 2001 | unknown | unknown |
| | UAI 23 | Feb 2001 – Aug 2001 | unknown | unknown |
| | UAI 24 | Feb 2001 – Aug 2001 | unknown | unknown |
| | UAI 25 | Feb 2001 – Aug 2001 | unknown | unknown |
| | UAI 26 | Feb 2001 – Aug 2001 | unknown | unknown |

# Appendix 2


| | | |
|---|---|---|
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Table A2.1: Western Australian Daycare centre isolates

| **Sample Location**[1] | **Reference Number** |
|---|---|
| Nursery | WA 1 |
| | WA 2 |
| | WA 3 |
| | WA 4 |
| | WA 5 |
| | WA 6 |
| | WA 7 |
| | WA 8 |
| | WA 9 |
| | WA 10 |

1. All isolates were purified DNA samples and provided by Dr Una Ryan from Murdoch University Fremantle, Western Australia, Australia

Table A2.2: South Australian human isolates – sporadic cases

| **Reference Number** | **Sex** | **Age** | **Date Ref** |
|---|---|---|---|
| SA 1 | F | 20-Apr-45 | 9-Feb-00 |
| SA 2 | F | 20-Apr-45 | 9-Feb-00 |
| SA 3 | F | 12-Feb-70 | 14-Feb-00 |
| SA 4 | M | 27-Jun-97 | 11-Feb-00 |
| SA 5 | F | unknown | 14-Feb-00 |
| SA 6 | M | 23-Apr-98 | 1-Mar-00 |
| SA 7 | F | 15-Nov-94 | 9-Mar-00 |
| SA 8 | M | 5-Mar-98 | 14-Mar-00 |
| SA 9 | M | 24-Nov-76 | 29-Mar-00 |
| SA 10 | F | 22-Jul-59 | 30-Mar-00 |
| SA 11 | F | 2-Jul-71 | 7-Apr-00 |
| SA 12 | F | 26-Dec-94 | 12-Apr-00 |

1 Genotype determined by TRAP-C1, as described in Chapter 3.3.3

Table A2.3: England/Wales Daycare centre, human *C. hominis* isolates

| **Reference Number** | **Date Ref** |
|---|---|
| DC 1 | 10-00 |
| DC 2 | 10-00 |
| DC 3 | 10-00 |
| DC 4 | 10-00 |
| DC 5 | 10-00 |
| DC 6 | 10-00 |
| DC 7 | 11-00 |
| DC 8 | 11-00 |
| DC 9 | 11-00 |
| DC 10 | 11-00 |
| DC 11 | 11-00 |
| DC 12 | 11-00 |
| DC 13 | 11-00 |
| DC 14 | 11-00 |

1 Genotype determined by PHLS *Cryptosporidium* Reference Unit

Table A2.4: England/Wales human *Cryptosporidium* sporadic isolates

| **Reference Number** | **Date Ref** | **Genotype**[1] |
|---|---|---|
| UK Sp 1 | 18-09-00 | *C. hominis* |
| UK Sp 2 | 18-09-00 | *C. parvum* |
| UK Sp 3 | 18-09-00 | *C. hominis* |
| UK Sp 4 | 18-09-00 | *C. hominis* |
| UK Sp 5 | 18-09-00 | *C. hominis* |
| UK Sp 6 | 21-09-00 | *C. hominis* |
| UK Sp 7 | 21-09-00 | *C. parvum* |
| UK Sp 8 | 27-10-00 | *C. hominis* |
| UK Sp 9 | 27-10-00 | *C. hominis* |
| UK Sp 10 | 23-11-00 | *C. hominis* |

1 Genotype determined by PHLS *Cryptosporidium* Reference Unit

Table A2.5: England/Wales human *C. parvum* isolates group 1

| **Reference Number** | **Date Ref** |
|---|---|
| Sth UK T2 1 | 03/2000 |
| Sth UK T2 2 | 03/2000 |
| Sth UK T2 3 | 03/2000 |
| Sth UK T2 4 | 03/2000 |
| Sth UK T2 5 | 03/2000 |
| Sth UK T2 6 | 03/2000 |
| Sth UK T2 7 | 03/2000 |
| Sth UK T2 8 | 03/2000 |
| Sth UK T2 9 | 03/2000 |
| Sth UK T2 10 | 03/2000 |
| Sth UK T2 11 | 03/2000 |
| Sth UK T2 12 | 03/2000 |
| Sth UK T2 13 | 03/2000 |
| Sth UK T2 14 | 03/2000 |
| Sth UK T2 15 | 03/2000 |
| Sth UK T2 16 | 03/2000 |
| Sth UK T2 17 | 03/2000 |
| Sth UK T2 18 | 03/2000 |

1 Genotype determined by PHLS *Cryptosporidium* Reference Unit

Table A2.6: England/Wales human *C. parvum* isolates group 2

| **Reference Number** | **Date Ref** |
|---|---|
| Sth UK T2 B 1 | 03/2000 |
| Sth UK T2 B 2 | 03/2000 |
| Sth UK T2 B 3 | 03/2000 |

1 Genotype determined by PHLS *Cryptosporidium* Reference Unit

Table A2.7: North UK human *Cryptosporidium* isolates

| **Reference Number** | **Date Ref** |
|---|---|
| UK Nth T1 1 | 09/2000 |
| UK Nth T1 2 | 09/2000 |
| UK Nth T1 3 | 09/2000 |
| UK Nth T1 4 | 09/2000 |
| UK Nth T1 5 | 09/2000 |
| UK Nth T1 6 | 09/2000 |
| UK Nth T1 7 | 09/2000 |
| UK Nth T1 8 | 09/2000 |
| UK Nth T1 9 | 09/2000 |

1 Genotype determined by Scottish Parasite Diagnostic Laboratory

Table A2.8: North UK human *C. parvum* isolates

| **Reference Number** | **Date Ref** |
|---|---|
| UK Nth T2 1 | 09/2000 |
| UK Nth T2 2 | 09/2000 |
| UK Nth T2 3 | 09/2000 |
| UK Nth T2 4 | 09/2000 |
| UK Nth T2 5 | 09/2000 |
| UK Nth T2 6 | 09/2000 |
| UK Nth T2 7 | 09/2000 |
| UK Nth T2 8 | 09/2000 |

1 Genotype determined by Scottish Parasite Diagnostic Laboratory