Molecular epidemiology of human cryptosporidiosis in New South Wales, Australia

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Declaration

The work described in this thesis was carried out in the Marsupial Laboratory at Macquarie University. All work described in this thesis is original and has not been submitted, in any other form, for a higher degree at any other university or institution.

Liette Waldron

April 2011

Infectious disease is one of the few genuine adventures left in the world. The dragons are all dead and the lance grows rusty in the chimney corner. ... About the only sporting proposition that remains unimpaired by the relentless domestication of a once free-living human species is the war against those ferocious little fellow creatures, which lurk in dark corners and stalk us in the bodies of rats, mice and all kinds of domestic animals; which fly and crawl with the insects, and waylay us in our food and drink and even in our love.

Hans Zinsser

Rats, Lice and History (1935)

Abstract

Cryptosporidium parasites have emerged as a significant threat to human and animal health. Initially considered as an opportunistic pathogen, *Cryptosporidium* is firmly established as one of the most common causes of global waterborne disease, capable of causing illness in both immune-compromised and immune-competent individuals. Essential to managing cryptosporidiosis and reducing risks of increasing prevalence is knowledge on the species contributing to disease and the potential zoonotic sources. The aim of this study was to investigate the molecular epidemiology of human cryptosporidiosis in New South Wales, Australia. Specifically, four aspects of cryptosporidiosis were investigated; the genetic diversity of *Cryptosporidium* causing disease in humans, the zoonotic role livestock and wildlife, the identification of demographic groups most at risk of disease and development of a molecular method that analyses parasite populations within the host.

Molecular analyses of 447 human faecal samples, collected between January 2008 and December 2010, showed sporadic human cryptosporidiosis is caused by four species; *C. hominis*, *C. parvum*, *C. andersoni* and *C. fayeri*. Sequence analysis of the *gp60* gene identified 5 subtype families and 31 subtypes. *Cryptosporidium hominis* IbA10G2 and *C. parvum* IIaA18G3R1 were the most frequent cause of sporadic disease, attributing to 59% and 16% of infections respectively. Between the months of January and April, 2009, NSW experienced the largest waterborne cryptosporidiosis outbreak reported in Australia to date. Molecular characterization identified the *C. hominis* IbA10G2 subtype as the causative parasite. In both sporadic and outbreak cases, an equal proportion of infections were found in males and females with cases most prevalent in the 0 – 4 year olds.

The zoonotic role of livestock and wildlife was determined by screening faecal samples from cattle (n = 205) and eastern grey kangaroos (n = 76). Screening of cattle faecal samples identified four *Cryptosporidium* species; *C. hominis*, *C. parvum*, *C. andersoni* and *C. ryanae*. Subtype IIaA18G3R1 was the most common cause of cryptosporidiosis in cattle, attributing to 47% of infections. Subtype IbA10G2 was also identified. Spatial analysis showed that cattle are an important zoonotic disease source. *Cryptosporidium* was not detected in kangaroo samples, indicating absence or low level infections.

Molecular characterisation of the *gp60* has facilitated contamination source tracking and increased understanding of the epidemiology of cryptosporidiosis. However the extent of genetic diversity observed, along with the exposure to the hosts' immune system, would support the hypotheses that significant selection pressure is placed on the *gp60*. A dual fluorescent terminal-restriction fragment length polymorphism analysis was developed to investigate the genetic diversity of *Cryptosporidium* subtype populations in a single host infection. This study showed subtype variation within infections and was the first to show that differences in the ratio of subtype populations occur between consecutive infections.

Results from this thesis show human cryptosporidiosis is a complex relationship involving humans, animals and the environment, both inside and outside the host. Successful future management of cryptosporidiosis, particularly in Australia, will require a strong knowledgebase on the species present in livestock and native fauna and an understanding of the factors that influence contact between these sources and humans.

Chapter Description

Chapter 1: General Introduction, research objectives and positioning of the chapters

I performed the review of literature and writing of the chapter. My supervisor Michelle Power provided constructive feedback.

Chapter 2: EpiReview: Epidemiology of cryptosporidiosis in Australia

I was responsible for undertaking the review of literature and writing the chapter. My supervisor Michelle Power gave direction and constructive feedback.

Chapter 3: Molecular epidemiology and spatial distribution of a waterborne cryptosporidiosis outbreak, Australia

Both myself and Cristel Cheung-Kwok-Sang, from the Marsupial laboratory, Macquarie University were responsible for collecting human faecal samples. I carried out all aspects of lab work including purification of oocysts, DNA extraction, PCR assays, restriction digests and sequence analysis. However, Cristel Cheung-Kwok-Sang assisted in 18S rRNA PCR-RFLP analyses. Patient information such as, age, gender and postal address was provided by NSW Health. I was responsible for the design of spatial and temporal analyses and was assisted by Borce Dimeski, from the University of Western Sydney, in generating the images. My co-supervisor Paul Beggs, from Macquarie University, provided input on spatial analyses. I carried out data analysis and wrote the manuscript with feedback provided by Michelle Power, Belinda Ferrari and Paul Beggs.

Chapter 4: Sporadic human cryptosporidiosis, Australia: Molecular epidemiology, spatio – temporal analysis and ecology

Human faecal sample collection was performed by both myself and Cristel Cheung-Kwok-Sang. I was responsible for organizing the supply of cattle faecal samples from Wagga Wagga with the help of Craig McConnell from Charles Sturt University, Wagga Wagga. I was responsible for all cattle field work in Sydney. I performed all lab work (as previously desribed). I designed the spatial and temporal analyses with assistance from Borce Dimeski. I carried out data analysis and interpretation, and wrote the manuscript with feedback provided by Michelle Power and Belinda Ferrari.

Chapter 5: Wildlife-associated *Cryptosporidium fayeri* in human, Australia

I performed human sampling, lab work (as previously described) and data analysis which included a phylogenetic analysis. I was responsible for the design of the kangaroo component and carried out all aspects of field work and lab analyses. I wrote the manuscript with constructive feedback from Michelle Power and Professor Michael Gillings.

Chapter 6: *Cryptosporidium gp60* dual fluorescence analysis: A novel platform to investigate infection dynamics

I was responsible for the design of the molecular methods described in this chapter, including the fluorescent primers, PCR assay optimization, and RFLP analysis. Mouse passage isolates were sourced from Una Ryan from Murdoch University, Perth. I was

responsible for the experimental design, data analysis and interpretation of results. I wrote the manuscript with critical feedback provided by Michelle Power.

Chapter 7: Conclusions

I summarized the major findings from the thesis and outlined implications for future research. Critical input and constructive feedback was provided by my supervisor Michelle Power.

Media

Newspaper Articles

The Sydney Morning Herald, February 27th, 2009

City faces wave of parasitic infections as pools harbour bug

Kate Benson
Medical Reporter

CONTAMINATED public pools have been blamed for Sydney facing an epidemic of cryptosporidiosis, a parasitic infection which causes severe cramping and diarrhoea.

NSW Health has been unable to pinpoint the pools harbouring the parasite but issued a public warning yesterday, urging people not to swim if they were suffering diarrhoea or had recently been ill.

The parasite, which can also cause fever, nausea and vomiting, cannot be killed by chlorine and is easily spread by swallowing contaminated water or coming into contact with someone who is infected.

A cryptosporidiosis epidemic swept across Sydney in 1998 when more than 1000 cases were confirmed, but NSW Health fears the city is on the verge of another crisis.

"We are seeing a huge upswing in the number of cases being reported so we're very concerned," NSW Health's director of communicable diseases, Jeremy McAnulty, said yesterday.

He said 157 cases had been reported in this month compared to 105 last month and 45 in December, the highest number recorded over summer in the past four years. Most of those infected had been younger than five.

A five-year-old boy from Lethbridge Park in Sydney's west was taken to hospital with cryptosporidiosis a year ago after drinking from his garden tap, but NSW Health believes the recent outbreak has been caused by an infected swimmer.

"Someone who has been suffering diarrhoea has gone swimming and probably had an accident, which has contaminated the water. There is no specific treatment for the condition," he said.

He urged parents to be cautious, particularly as school swimming carnivals are under way.

"When it comes to smaller children, parents should ensure they take regular toilet breaks while swimming and those not toilet-trained must wear waterproof tight-fitting pants over swimmers," he said. Nappies should not be changed by the pool.

Cryptosporidiosis can also be contracted by drinking contaminated water from creeks or lakes, or handling infected animals or their manure. Symptoms can set in between one and 12 days after infection.

The Sydney Morning Herald, Weekend Edition, February 28th, 2009

… but just when you thought it was safe to swim again

Kate Benson
Medical Reporter

NINETEEN public swimming pools are being investigated by the Health Department after the number of people suffering a severe abdominal infection soared to more than 200 yesterday, the highest number recorded in February for a decade.

NSW Health fears Sydney is on the brink of an epidemic of cryptosporidiosis, a parasitic infection that causes cramping, diarrhoea, vomiting and fever, after another 44 cases were reported yesterday. Half have been children.

The pools targeted included the Sydney Olympic Park Aquatic Centre at Homebush Bay, the Annette Kellerman Aquatic Centre in Enmore and the Manly Andrew Boy Charlton Swim Centre. Three pools in the Blue Mountains, 11 in the western suburbs and two in southern NSW have also been named.

The director of communicable diseases at NSW Health, Jeremy McAnulty, said yesterday none of the 19 pools had tested positive for cryptosporidium but sufferers had reported swimming at those venues before becoming ill.

He said all the pools had been ordered urgently to "super chlorinate" using 10 milligrams of chlorine per litre in a bid to stem the rising rates of infection.

"The message here is that if you've got diarrhoea or had diarrhoea in the past two weeks, stay out of pools because you could contaminate that pool and cause many other cases of crypto, maybe even thousands," Dr McAnulty said.

The public swimming pool and spa pool guidelines, devised by NSW Health, advise that if a swimmer has a bout of diarrhoea while in the water the faecal material should be removed using a fine mesh, nearby water should be disinfected immediately and the pool should be vacuumed and "super chlorinated" overnight.

Swimmers should be encouraged to shower before using the pool and those with open wounds, sores and rashes, infected eyes, or wearing bandages should be asked not to swim.

Public swimming pools are often havens for bugs, including mycobacterium marinum, which causes chronic skin ulceration, coagulase positive staphylococci, which infects skin, ears and noses, and shigella, salmonella and campylobacter, all causing vomiting and diarrhoea.

Giardia, which is found in human faeces, and naegleria fowleri, shown to cause the fatal disease primary amoebic meningo-encephalitis, can also be found in pool water, while viruses causing plantar warts, herpes, conjunctivitis, tinea and genital yeast infections have been detected on pool decks and surfaces in change rooms.

The biggest outbreak of cryptosporidiosis was in NSW in 1998 when more than 1000 cases were confirmed.

The Sydney Morning Herald, March 31st, 2009

Crypto clue: one person to blame for outbreak

Richard Macey

GENETIC testing has revealed that Sydney's cryptosporidium outbreak may have stemmed from a single infected person.

The finding means it is unlikely that an infected animal or environmental conditions, such as the weather, are to blame.

Since the beginning of last month, when the latest outbreak appeared, 628 people have fallen ill, compared with 482 in all of last year.

Macquarie University researchers say their testing is experimental, and unlikely to identify the origin of the current outbreak, but they hope their work will lead to the development of a world-leading, inexpensive automated test to quickly identify parasite strains involved in future outbreaks, allowing sources to be rapidly traced and infections contained.

Michelle Power, of Macquarie's biological sciences department, said cryptosporidium outbreaks were difficult to trace using existing technology.

Two key parasite "species" infected people – *Cryptosporidium hominis*, only contracted by direct human to human contact, and *Cryptosporidium parvum*, which can also be transmitted by animals. For each type there were about 40 different strains.

However, virtually nothing was known about which strains were responsible for most outbreaks. A decade after the contamination of Warragamba Dam, which forced Sydneysiders to boil their water, "we still just know that it was cryptosporidium", Dr Power said.

"To put it simply, we're looking for the easiest way to fingerprint the organisms."

With the new test it should be possible to compare results from individual patients to seek common patterns.

"We could look to see if they had all eaten at the same restaurant, or been to the same day-care centre or swimming pool."

The NSW Health Department has provided hundreds of fecal samples from the latest outbreak for testing by a Macquarie University parasite researcher, Liette Waldron.

"We have analysed about 250 samples from the current outbreak, and they are all the same strain" of *Cryptosporidium hominis*, Dr Power said.

If it had originated from different sources she would have expected to see multiple strains. When sporadic cases, reported over the past 12 months, were analysed they identified 24 strains, involving both the human and animal species.

Dr Power said people who were infected, but not necessarily appearing ill, usually had 5000 to 10,000 parasites per gram of faeces. However, some samples from the latest outbreak contained more than 1 million. "The more parasites you see, the more nasty it is."

Over the next two years the project, funded by the Australian Research Council and the Health Department, will "fingerprint" parasites from up to 4000 cryptosporidium patients.

Live Radio

1233 ABC Newcastle: Interview regarding the 2009 Sydney cryptosporidiosis outbreak

Publications

Waldron, L.S., Ferrari, B.C., Gillings, M.R. and Power, M.L. (2009). Terminal restriction length polymorphism for identification of *Cryptosporidium* species in human feces *Applied and Environmental Microbiology* 75:108-112.

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Waldron, L.S., Cheung-Kwok-Sang, C., Power, M.L. (2010). Wildlife-associated *Cryptosporidium fayeri* in human, Australia. *Emerging Infectious Diseases* 16, 2006 - 2007

Waldron, L.S., Ferrari, B.C., Cheung-Kwok-Sang, C., Beggs, P.J., Stephens, N., Power, M.L. (In Review). Molecular epidemiology and spatial distribution of a waterborne cryptosporidiosis outbreak, Australia. *Applied and Environmental Microbiology,* Submitted March 17th 2011.

Waldron, L.S., Dimeski, B., Beggs, P.J., Ferrari, B.C., Power, M.L. (In Review). Sporadic human cryptosporidiosis, Australia: Molecular epidemiology, spatio - temporal analysis and ecology. *Applied and Environmental Microbiology,* Submitted March 17th 2011.

Waldron, L.S. and Power, M.L. (In Review) Dual fluorescence analysis detects *gp60* subtype diversity in *Cryptosporidium* infections. *Infection, Genetics and Evolution*, Submitted April 1st 2011

Asher, A.J., **Waldron, L.S.** and Power, M.L. (In Review).Evaluation of a PCR protocol for sensitive detection of *Giardia intestinalis* in human faeces *Parasitology Research*, Submitted April 14th 2011

Conference oral presentations and poster

Waldron, L.S., Ferrari, B.C., Gillings, M.R. and Power, M.L. (2008). T-RFLP for identification of *Cryptosporidium* species in human faeces. *Australian Society for Microbiology*. Melbourne Convention and Exhibition Centre, Melbourne, Victoria. (poster)

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Acknowledgements

When first starting my PhD adventure, I recall asking myself 'What on earth am I thinking?' and 'will I be able to finish this?'. As I look back on my experience, I begin to see similarities between completing a PhD and a racing car team competing for the championship. It takes dedication, practice, patience, commitment, drive and a lot of support to reach the end of the season, stand on the podium and firmly grasp the champions' trophy. As I stand on my podium, holding my trophy, I see my support team. I would like to try put into words my gratitude to the people who have guided me and enabled me to be where I am today.

To my supervisor, Dr. Michelle Power, I am indebted to you for your guidance throughout my candidature. Thank you for helping me formulate my ideas and bring them to fruition in this thesis. Your encouragement enriched my entire PhD experience and you never once hesitated in throwing me in amongst the action. You gave me the confidence to be a successful student, demonstrator and lecturer. Your infectious passion for science and your relentless support truly made my PhD enjoyable. It is through your persistence, patience and encouragement that I finally see the bigger picture.

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To my fiancé, Dane, who without even knowing can melt away a day of failed experiments. Thank you for being my emotional pillar, reminding me that there is life after 5 pm and for keeping me sane over the last 6 months. Thank you for bringing me chocolate, vodka, leaving me alone, entertaining me, putting up with my moodiness and loving me.

I now look back on those first questions I asked myself, 'What on earth am I thinking?' and 'will I be able to finish this?'. The former question I may never know the answer to, however the later question I have do doubt of the answer. Yes.

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1. General Introduction and thesis rationale

1.1 *Cryptosporidium* and cryptosporidiosis

1.1.1 *Cryptosporidium*

Parasites of the genus *Cryptosporidium* are the most common cause of waterborne disease around the world. Although first described in 1907, by Ernest Edward Tyzzer, the human infective potential of *Cryptosporidium* was not recognised until 1976, when the first human cryptosporidiosis case was reported (Meisel et al., 1976). Increased cryptosporidiosis infections in the mid 1980's were synonymous with the human immunodeficiency virus (HIV) era. Initially, cryptosporidiosis was considered an opportunistic disease limited to the immune–compromised and due to the strong link with HIV, it was used as an initial diagnosis of the virus (Tzipori and Widmer, 2008). However, over the last 30 years, cryptosporidiosis has emerged as a significant human pathogen with a global distribution, capable of causing illness in both immune-compromised and immune-competent individuals, the young and the elderly. Transmission of *Cryptosporidium* is completed via the oocyst, the endogenous environmentally robust form of the parasite. The oocyst, approximately 5 μm in size, is excreted in a fully infective form and is resistant to the disinfectants used in water treatment (Clark, 1999; Ramirez et al., 2004). Chemical resistance, small size and immediate infectivity, coupled with a low infective dose has made *Cryptosporidium* one of the most difficult pathogens to control. *Cryptosporidium* parasites are a primary concern for water and health authorities in addition to the livestock industry which suffers significant economic losses from diseased animals

1.1.2 Cryptosporidiosis

Cryptosporidiosis is a disease of humans, domestic animals, livestock, wildlife, birds, reptiles and fish (Fayer, 2010). Disease initiates after the ingestion of infective oocysts which are transmitted via the faecal-oral route. Epidemiological investigations have elucidated the existence of numerous transmission pathways, with human-to-animal contact, human-to-human contact and waterborne contamination as the main routes for disease transmission (Chalmers et al., 2005). After ingestion, hosts factors such as temperature, pH and bile salts cause the excystation of four motile sporozoites from each oocyst (Feng et al., 2006). Sporozoites attach to and invade host epithelial cells, thereby initiating the cryptosporidiosis infection.

The severity of a cryptosporidial infection depends on the host immune system (Fayer and Ungar, 1986). For healthy individuals, cryptosporidiosis is a self limiting disease that lasts 5 -13 days followed by a full recovery (Chalmers and Davies, 2010). The most common symptom is persistent diarrhoea (98% of patients) which may be accompanied by abdominal pain (60-96% patients), vomiting (49-65% patients), fever (36-59% patients), and nausea (35% patients) (Ramirez et al., 2004; Hunter et al., 2007; Chalmers and Davies, 2010). Immunocompromised individuals, such as HIV sufferers, transplant patients and children, are susceptible to a debilitating and potentially fatal form of cryptosporidiosis manifested by overwhelming, and potentially prolonged cholera like diarrhoea (Clark, 1999). Severe weight loss, biliary tract disease, pancreatitis and respiratory disease have also been associated with cryptosporidiosis infections in HIV patients (Hunter and Nichols, 2002; Chalmers and Davies, 2010). *Cryptosporidium* infections account for 10-20% of diarrhoeal cases in HIV positive patients in developed countries and up to 50% in underdeveloped countries (Navin, 1999; Weber, 1999). A study on the prevalence of cryptosporidiosis among HIV infected and uninfected children with

persistent diarrhoea at Mulago Hospital in Uganda showed that 74% of HIV infected children had cryptosporidiosis compared to 6% of HIV negative children (Tumwine, 2005). The severity of cryptosporidiosis in HIV sufferers was highlighted after the 1993 Milwaukee outbreak, the largest reported to date, which had a 52% mortality rate of those with the disease (Rose, 1997). *Cryptosporidium* is the leading cause of enteric disease in children and has a reported association with impairment in growth, physical fitness and cognitive function (Gatei, 2006; Ajjampur et al., 2007). Prevention, risk reduction and rehydration therapy are the most important intervention and control strategies. Currently, there are no effective treatments for cryptosporidiosis.

1.2 *Cryptosporidium* species and the zoonotic public health threat

1.2.1 *Cryptosporidium* species

The *Cryptosporidium* genus is diverse, both genetically and in its host range. Due to the morphological similarities exhibited by this genus, molecular tools have been essential in *Cryptosporidium* species identification, taxonomy and epidemiology. To date, molecular analyses have identified 22 *Cryptosporidium* species and greater than 40 genotypes (Fayer, 2010). Molecular analyses have shown that the epidemiology of human cryptosporidiosis is a multifaceted relationship involving humans, domestic animals, livestock and wildlife. The close living proximity of these disease sources, combined with numerous *Cryptosporidium* species circulating through the environment, means human disease can initiate through multiple transmission pathways. For this reason, species identification has been pivotal in understanding the epidemiology of human cryptosporidiosis and in identifying disease transmission pathways.

Of the 22 *Cryptosporidium* species described, 90% of reported human infections are attributed to two species; the anthroponotic *C. hominis* and the zoonotic *C. parvum* (Morgan-

Ryan et al., 2002). An additional 8 species have also been flagged as public health threats, and include; *C. melegridis*, *C. canis*, *C. felis*, *C. suis*, *C. muris*, *C. ubiquitis*, *C. cuniculus* and *C. fayeri*. Each of these were once thought to be specific for turkeys, dogs, cats, pigs, mice, deer , rabbits and marsupials respectively (Ditrich et al., 1991; Pedraza-Diaz et al., 2000; Pedraza-Diaz et al., 2001; Xiao et al., 2001; Caccio et al., 2002; Chalmers et al., 2009; Waldron et al., 2010). The contribution of these species to human disease varies globally, and is often associated with seasonality, demographics, immune status and contact with reservoir hosts.

1.2.2 Anthroponotic transmission

Infections with the human specific *C. hominis*, which was first recognised as a separate species from *C. parvum* in 2002 (Morgan-Ryan et al., 2002), predominate throughout most of the world including, the United States, Australia, Kenya, Thailand, South Africa, Uganda, Peru, Malawi and India (Peng et al., 2001; Leav et al., 2002; Cama et al., 2003; Gatei et al., 2003; Peng et al., 2003; Akiyoshi et al., 2006; Gatei, 2006; Gatei et al., 2007; Chalmers et al., 2005; O'Brien et al., 2008; Jex et al., 2008). *Cryptosporidium hominis* transmission has been documented in day care centres, households, hospitals, drinking water supplies and public swimming pools (Current and Garcia, 1991; Casemore et al., 1994; Current, 1994; Ramirez et al., 2004). The Milwaukee 1993 outbreak was attributed to *C. hominis*.

1.2.3 Zoonotic transmission

Animal-to-human transmission of *C. parvum* is well documented and was originally considered the main transmission route for human cryptosporidiosis (Meinhardt et al., 1996). The ubiquitous nature of *C. parvum* in humans, wildlife, farm stock, and pets combined with its ability to cross host-species barriers has indicated numerous sources of infection (Casemore et al., 1985). Human infections with *C. parvum* are more prevalent in rural regions where contact with reservoir hosts is more frequent. This has been shown in rural localities in Europe, the Middle East, and the United Kingdom (Morgan, 2000; Glaberman et al., 2002; Gatei et al., 2003; Sulaiman et al., 2005; Cohen et al., 2006). Juvenile hosts, from cattle, sheep and pigs are major contributors of environmental contamination. In these cases, oocysts are shed in high numbers, up to 10^{10} per gram of faeces per day for up to 14 days (Blewett, 1989). Zoonotic transmission has been reported in children after exposure to infected calves and lambs, and in veterinarians and farm workers after occupational exposure (Blagburn and Current, 1983; Blacklow, 1985; Levine, 1988). However, due to the different infection capabilities of *C. parvum*, which can also be passed through human-to-human transmission, it is often difficult to determine whether infections are from anthroponotic or zoonotic disease sources.

Zoonotic infections with other *Cryptosporidium* species are increasing throughout the world, both in immune-compromised and immune-competent individuals. *Cryptosporidium meleagridis*, previously believed host specific to turkeys, is the third most common species causing illness in humans (Xiao, 2010). In Peru, the incidence of *C. meleagridis* is as high as *C. parvum* (Cama et al., 2008). Of recent concern is *C. cuniculus*, previously host specific to rabbits, which was responsible for a waterborne outbreak in England (Chalmers et al., 2009). The United Nations has

projected the human population to peak to 9.1 billion people by the year 2050, compared to the current 6.8 billion. Paralleling population growth is human expansion and urbanisation. The increased urbanisation that will be seen around the world, particularly in developed nations, will expose humans, domestic animals, livestock and wildlife to different and potentially pathogenic *Cryptosporidium* species. Therefore, the identification of the *Cryptosporidium* species persisting in these disease sources will be essential for future cryptosporidiosis management.

1.3 Diagnosis, species identification and intra-species diversity

1.3.1 Clinical diagnosis and species identification

Clinical diagnosis of cryptosporidiosis is performed by either visualising oocysts through microscopic examination of faecal smears with acid fast stains and immunofluorescence assays or by detecting oocyst wall antigens using enzyme immunoassays (Current and Garcia, 1991; Chalmers and Davies, 2010). The pivotal flaw in the current diagnostic assessment of cryptosporidiosis is that the species responsible for an infection is never identified.

Molecular methods, such as PCR, have benefited both clinical and environmental detection of *Cryptosporidium* (Morgan et al., 1998; Xiao et al., 1999a). Sequence variation within the 18S rRNA identified the extent of diversity of *Cryptosporidium* (Xiao et al., 1999b), resulting in the naming of the 22 current *Cryptosporidium* species (Morgan et al., 1998; Morgan et al., 1999; Xiao et al., 1999b; Xiao et al., 2002). The 18S rRNA is the gene preferentially used in molecular analyses for the identification of *Cryptosporidium* species and has been used in 86% of publications (Xiao, 2010). More specifically, a PCR- restriction fragment length polymorphism (PCR-RFLP) analysis targeting an ~830 bp fragment of the gene and using restriction enzymes *SspI* and *VspI* has been used in 60% of publications for the rapid identification of

Cryptosporidium species (Xiao et al., 1999a; Xiao, 2010). The application of PCR-RFLP to *Cryptosporidium* investigations has enabled detection and differentiation of *Cryptosporidium* parasites in environmental and faecal samples, and for the source of outbreaks to be identified (Xiao et al., 2000; Morgan-Ryan et al., 2002; Cohen et al., 2006).

1.3.2 Intra-species diversity

Extensive intra-species diversity has been observed in *Cryptosporidium* species through sequence analysis of the hypervariable *gp60* gene. The *gp60* gene is significant in the disease process, as it facilitates the movement of sporozoites and their subsequent attachment to and invasion of hosts epithelial cells (Winter et al., 2000). To date, it is the most polymorphic gene identified in *Cryptosporidium*, which epidemiological studies use to further classify *Cryptosporidium* species to the subtype family and subtype levels (Cevallos et al., 2000; Winter et al., 2000; Sulaiman et al., 2005). Nucleotide polymorphisms observed throughout the *gp60* gene has identified 6 *C. hominis* subtype families (denoted Ia – Ig, there is no Ic) and eleven *C. parvum* subtype families (IIa – IIl, there is no IIj) (Xiao, 2010). Six subtype families have also been identified in *C. meleagridis* (IIIa – IIIf) and *C. fayeri* (IVa – IVf) (Plutzer and Karanis, 2009; Power et al., 2009). The *gp60* also contains a poly-serine microsatellite region, polymorphisms in the number and form of serine-codons further characterises *Cryptosporidium* to the subtype level (Sulaiman et al., 2005).

Molecular analysis of the *gp60* gene has highlighted the importance of certain genetic variants to human health and facilitated the identification of transmission pathways and zoonotic disease contamination sources. *Cryptosporidium hominis* Ib and *C. parvum* IIa are the most frequently recorded subtypes in humans around the world, attributing to 47.4% and 25.5%

reported infections typed at the *gp60*, respectfully (Jex and Gasser, 2010). Typing to the subtype family level has also shown the unique transmission of *C. parvum* IIc, although recognized as a *C. parvum* species it follows a strict anthroponotic transmission. This finding highlighted the anthroponotic contamination source in urban areas in the United Sates, where IIc infections are more common than IIa (Xiao, 2010). Analysis of the *gp60* gene also showed cattle as an important zoonotic source for human disease. This was demonstrated in Portugal where *C. parvum* IIaA15G2R1 is the dominant *C. parvum* subtype infecting cattle and humans (Alves et al., 2006). Subtype classification has also highlighted the public health risk posed by particular *Cryptosporidium* subtypes. The *C. hominis* IbA10G2 subtype is a globally distributed subtype and is the most common cause of waterborne outbreaks and sporadic human cryptosporidiosis (Jex and Gasser, 2010; Xiao, 2010), attributing to 44.5% of reported human cases typed at the *gp60*.

The application of *gp60* gene typing to identify sources of infection and transmission routes is highly dependant on whether gp60 subtypes are stable in space and time (Mallon et al., 2003b). In a clonal organism subtype stability will be the case, whereas in an organism where genetic exchange plays a significant role, such subtypes will not remain stable. The obligatory sexual phase in the *Cryptosporidium* lifecycle, exposure of the *gp60* gene to the host's immune system, the high level of diversity observed within this gene, along with the constant discovery of new subtypes potentially indicates the instability of the *gp60* gene. Recombination between *C. parvum* genetic variants has been observed in experimentally infected mice (Feng et al., 2002). Investigation into *Cryptosporidium* population structure, and inherent population diversity, within human and animal infections will be essential for a complete understanding of the epidemiology of cryptosporidiosis.

1.4 Research objectives

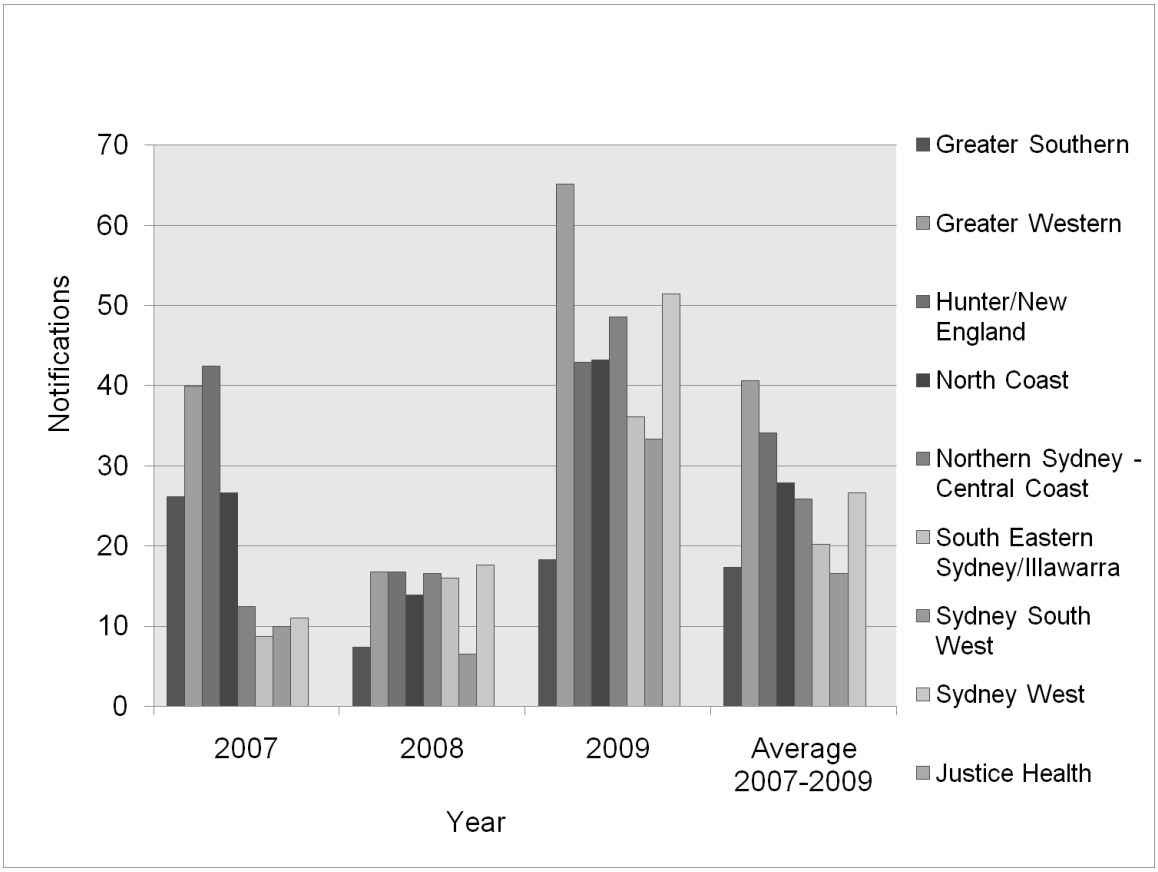
Cryptosporidiosis became a notifiable disease in NSW in 1996 under the *NSW Public Health Act 1991*. Notifications data has shown a significant rise in the incidence of human cryptosporidiosis in this Australian State. In 2003, the overall incidence of human cryptosporidiosis in NSW was 2.7/100,000 population, in 2006 it had risen to 10.5/100,000 population and in 2009 the incidence further increased to 19.8/100,000 population (www.nswhealth.gov.au). Numerous *Cryptosporidium* infections have been reported across NSW, particularly in regional areas. Crude notifications data per 100, 000 population between 2007 and 2009 show that in NSW the Greater Western, Hunter New England, Sydney West and North Coast regions were the most affected (Fig. 1.1). These regions in NSW are notable for beef and dairy farming, and recreational water activities in natural land areas such as national parks. Although cryptosporidiosis is common in rural areas, due to our limited understanding of the *Cryptosporidium* species occurring in humans and livestock in NSW, contamination sources have never been identified

Figure 1.1 New South Wales public health divisions

*** Data source: New South Wales Health communicable disease database**

(www.nswhealth.gov.au)

Figure 1.2 Cryptosporidiosis crude notifications per 100, 000 population by New South Wales health service area between 2007 and 2009*.



*** Data source: New South Wales Health communicable disease database (www.nswhealth.gov.au)**

Significant gaps exist in our knowledge of human cryptosporidiosis in NSW, particularly; the species of *Cryptosporidium* causing human disease, domesticated animal sources, and the demographic groups most at risk. Additionally, free roaming native wildlife, such as Kangaroos, live in close contact with livestock and have evolved to co-exist in the presence of humans. With 75% of zoonoses originating from wildlife (Jones et al., 2008), knowledge of the *Cryptosporidium* species they carry will become essential for determining potential roles of these wildlife hosts in *Cryptosporidium* transmission. Longitudinal studies, which investigate not only the *Cryptosporidium* species causing disease in humans and animals, but the seasonality, demographics and spatio-distribution will be required to fill the gap in our current understanding of the epidemiology of human cryptosporidiosis from an Australian perspective.

Specifically, this study aims to increase the understanding of cryptosporidiosis in Australia by:

1. Determining the genetic diversity of *Cryptosporidium* species and subtypes contributing to human cryptosporidiosis in NSW, Australia.
2. Examining the zoonotic role of livestock and native Australian fauna in human cryptosporidiosis
3. Identifying the NSW demographic groups most affected by cryptosporidiosis, and the geographical distribution and seasonal variation of *Cryptosporidium* species causing human disease.
4. Developing a rapid and sensitive epidemiological tool for the detection and genetic characterization of *Cryptosporidium* and to apply this methodology to investigate intra-species diversity within single host infections.

1.5 Positioning of thesis chapters

Chapter 2: EpiReview: Epidemiology of human cryptosporidiosis in Australia

L.S. Waldron and M.L. Power

Prepared for submission: *Communicable Diseases Intelligence*. This is a quarterly publication of the Surveillance Branch, Office of Health Protection, Australian Government Department of Health and Ageing.

This chapter presents an introductory review of the current knowledge of the *Cryptosporidium* species and subtypes causing human cryptosporidiosis in Australia and the potential zoonotic role of cattle. Cryptosporidiosis became a nationally notifiable disease in Australia in 2001. Notifications data shows that cryptosporidiosis is the third most common cause of gastrointestinal illness in Australia and that it is most prevalent in the 0-4 year olds. Waterborne transmission has been shown as a significant transmission source. Due to the lack of molecular work performed on samples from humans and animals in Australia, contamination sources have never been conclusively linked to disease cases. This paper highlights the gaps in the understanding of human cryptosporidiosis in Australia and proposes future research required to increase our knowledge on the public health risk posed by this parasite.

Chapter 3: Molecular epidemiology and spatial distribution of a waterborne cryptosporidiosis outbreak, Australia

L.S. Waldron, B. Dimeski, P.J. Beggs, B.C. Ferrari and M.L. Power

Submitted: *Applied and Environmental Microbiology*, March 17th 2011

New South Wales has experienced six waterborne outbreaks of cryptosporidiosis. However, between the months of January and April, 2009, NSW experienced the largest cryptosporidiosis

outbreak in Australia reported to date. The contamination event, which caused disease in 1131 individuals, was believed to be linked to public swimming pools in the Sydney area. This chapter describes the *Cryptosporidium* species and subtypes present in humans throughout the outbreak period and highlights the regions and demographic groups most affected.

Addresses objectives 1 and 3

Chapter 4: Sporadic human cryptosporidiosis, Australia: Molecular epidemiology, spatio – temporal analysis and ecology

L.S. Waldron, B.C. Ferrari, C. Cheung-Kwok-Sang, P.J. Beggs, N. Stephens and M.L. Power

Submitted: *Applied and Environmental Microbiology*, March 17th 2011

Zoonoses present a significant and growing threat to global human health (Jones et al., 2008). As populations grow and expand closer towards rural and wildlife boundaries, pathogen flow between the wildlife-urban-agricultural interface will increase. Knowledge of the *Cryptosporidium* species causing disease in humans, wildlife and livestock will therefore become essential for future prevention and management of the disease. This chapter presents the results from a longitudinal investigation into the *Cryptosporidium* species and subtypes causing disease in humans and cattle in NSW. The large number of human samples investigated in this study facilitated the identification of novel *Cryptosporidium* subtypes and showed the zoonotic potential of *Cryptosporidium* species not previously identified as a public health threat in Australia.

Addresses objectives 1, 2 and 3

Chapter 5: *Cryptosporidium fayeri* an emerging wildlife zoonoses

L.S. Waldron, C. Cheung-Kwok-Sang and M.L. Power

Emerging Infectious Diseases (2010) 16:2006 - 2007

Zoonoses from wildlife have attributed to 71.8% of emerging infectious diseases over the last 60 years (Jones et al., 2008). Continued urbanisation and intensifying food-animal production are creating a disease bridge that increases the contact between humans, livestock and wildlife. An increasing number of *Cryptosporidium* species are jumping the host-species barrier and becoming public health threats. *Cryptosporidium fayeri* was previously believed to be host specific to marsupials and was a species which did not pose a public health threat. This chapter shows the zoonotic potential of *C. fayeri* and documents the first human infection.

Addresses objective 2

Chapter 6: Dual fluorescence analysis detects *gp60* subtype diversity in *Cryptosporidium* infections.

L.S. Waldron and M.L. Power

Infection, Genetics and Evolution (2011) 11:1388 - 1395

The *gp60* is one of the most widely used genes in *Cryptosporidium* research and is used to classify species to the subtype family and subtype levels. Sequence analysis of this gene has highlighted extensive intra-species diversity, facilitated contamination source tracking and increased understanding of the epidemiology of cryptosporidiosis. However, based on the high degree of polymorphism exhibited by this gene, its involvement in the disease process and its exposure to the hosts' immune system, this chapter investigated the hypothesis of subtype

population diversity within infections. A dual fluorescent terminal-restriction fragment length polymorphism (T-RFLP) method was developed for the investigation.

Addresses objective 4

Chapter 7: Conclusions

This concluding chapter discusses the major findings of the thesis and highlights waterborne transmission as a significant contributor to emerging infectious diseases. Future collaborations between government health, and water authorities and research institutes will be required to prevent emerging infectious diseases in both humans and animals. A novel surveillance strategy is proposed.

Chapter 8: Appendix

Appendix 8.1

Glycoprotein 60 diversity in *C. hominis* and *C. parvum* causing human cryptosporidiosis in NSW Australia

L.S. Waldron, B.C. Ferrari and M.L. Power

Experimental Parasitology (2009) 122: 124 – 27

Appendix 8.2

Terminal restriction fragment polymorphism for identification of *Cryptosporidium* species in human faeces

L.S. Waldron, B.C. Ferrari, M.R. Gillings and M.L. Power

Applied and Environmental Microbiology (2009) 75: 108 – 12

Two publications describing research from my Honours year that were written in the first year of my PhD are presented. The *gp60* PCR used to identify *Cryptosporidium* subtype families and subtypes throughout my PhD was optimized in my Honours work. I was the first to apply T-RFLP methodology to protozoa; this was also performed in my Honours.

Appendix 8.3

Evaluation of a PCR protocol for sensitive detection of *Giardia intestinalis* in human faeces

A.J. Asher, L.S. Waldron and M.L. Power

Parasitology Research, April 14th 2011

A third manuscript has been included in the appendix. This paper examines *Giardia* isolates from humans in NSW and has drawn heavily on methods developed for *Cryptosporidium* described in my publications. This paper was written by an Honours student that I co-supervised with my supervisor, Dr. Michelle Power.

2. EpiReview: Human cryptosporidiosis, an Australian perspective

2.1 Emerging infectious diseases, protozoa and Australia

2.1.1 Emerging infectious diseases

Emerging infectious diseases (EID) are defined as diseases that have newly appeared in a population or that have been known for some time but are rapidly increasing or threatening to increase in incidence or geographic range (Lashley, 2006). Infectious diseases have plagued mankind for centuries. The sixth century Justinian Plague, which wiped out half of the Roman population, was the first major pandemic reported (Perry and Fetherston, 1997; Achtman et al., 2004). Major pandemics followed between the fourteenth and eighteenth centuries, with the persistence of the Bubonic Plague, *Yersinia pestis*, and intermittent pandemics of Asiatic Cholera, *Vibrio cholerae* (Achtman et al., 2004; Smirnova et al., 2004). Medical advancements, vaccines and treatments were expected to have rid the world of infectious diseases by the twentieth century. In part, this was successful with the eradication of Smallpox, the ongoing eradication of Poliomyelitis and in the development of preventable vaccines for Cholera, Diptheria and Smallpox. However throughout the twentieth century both emerging diseases, such as AIDS, Ebola, SARS and Influenza, and re-emerging diseases, such as antibiotic resistant *Yersinia pestis*, threatened public health (Hinnebusch et al., 2002; Lashley, 2006).

Infectious diseases still remain the most complex and significant threat to global human health in the 21st century. Disease emergence is a multifaceted relationship, dictated by human actions, involving domestic pets, wildlife, livestock and the environment. Analysis of data generated over the last 60 years showed that EID events were dominated by zoonoses and that 71% of these were attributed to wildlife (Jones et al., 2008). Population growth, urbanisation, intensified agriculture and food production, and encroachment on wildlife habitats increase the

contact with wildlife zoonotic sources (Jones et al., 2008). In turn, this creates a disease bridge that facilitates the expansion and adaption of pathogens in a new host species. Such spill over events from wildlife arising from human population growth and urbanization were seen in the 1950's in Africa where expansion into jungle areas increased contact between humans and primates. Subsequently, this saw the emergence of HIV-1, from chimpanzees, and HIV-2, from sooty mangabes, in humans (Hahn et al., 2000). Population growth has forced an increase in the demand of food-animals; supply meets this demand by intensifying animal production, often in confined and crowded animal enclosures. Food-animal production farms are commonly located on the outskirts of cities and are therefore exposed to zoonotic wildlife sources. The emergence of Nipah virus in pigs from bats was attributed to intensive farming practices in a crowded and confined enclosure (Eaton et al., 2006; Gurley et al., 2007). Due to the crowded living conditions, the Nipah virus persisted in the pig population before expanding to humans. Adaption of the virus to humans enabled human to human transmission which subsequently led to a large scale outbreak in Malaysia (Daszak et al., 2006; Eaton et al., 2006).

2.1.2 *Cryptosporidium*

The majority of EID's have been attributed to bacteria, followed by viruses and protozoa (Jones et al., 2008). A protozoan parasite that has gained global public exposure as a notorious cause of waterborne disease in humans is *Cryptosporidium*. First emerging in the 1980's HIV era, *Cryptosporidium* was considered an opportunistic pathogen and a health threat only to the immune compromised (Tzipori and Widmer, 2008). However, over the last 30 years *Cryptosporidium* has emerged as the most common cause of waterborne disease and persists in both immune – compromised and immune – competent individuals. Clinical symptoms of

cryptosporidiosis, the manifestation of a *Cryptosporidium* infection, are diarrhoea, nausea, vomiting, cramps, fever and weight loss (Chalmers and Davies, 2010). Immune – compromised individuals suffer a more severe and prolonged cholera – like diarrhoea, and infections may be followed by biliary tract disease, pancreatitis and respiratory disease (Clark, 1999; Hunter and Nichols, 2002; Chalmers and Davies, 2010). Long term health effects such as, arthritis, joint and eye pain, dizziness and recurring headaches have been associated with cryptosporidial infections (Hunter et al., 2004). In children, malnutrition, growth faltering and cognitive impairments have been reported (Clark, 1999; Chalmers and Davies, 2010). The importance of *Cryptosporidium* as a significant human pathogen was highlighted in its classification as a "neglected parasite" by the World Health Organizations Neglected Diseases Initiative, 2004. Diseases under this category "exhibit a considerable and increasing global burden, and impair the ability of those infected to achieve their full potential, both developmentally and socio-economically' (Savioli et al., 2006; Chalmers and Davies, 2010).

Ninety percent of human infections are caused by *C. hominis* and *C. parvum* (Morgan-Ryan et al., 2002). Infections with zoonotic *Cryptosporidium* species, such as *C. meleagridis*, *C. felis, C. canis, C. muris, C. ubiquitum* and *C. suis* were believed to only occur in immune – compromised individuals, however over the last decade infections with these species are increasingly being reported in immune – competent individuals (Ditrich et al., 1991; Pedraza-Diaz et al., 2000; Pedraza-Diaz et al., 2001; Xiao et al., 2001; Caccio et al., 2002; Chalmers et al., 2009). For example, in some areas of South America, infections attributed to *C. meleagridis* equal those caused by *C. parvum* (Cama et al., 2008). Recently, *C. cuniculus*, previously thought to be host specific to rabbits, was recognized as a public health threat after it caused a waterborne outbreak in England (Chalmers et al., 2009). With populations, especially in the developed world,

expanding closer towards wildlife boundaries, the zoonotic threat that humans and wildlife pose to each other needs to be investigated.

2.1.3 Australia

EIDs are a global problem, however certain regions have been more prone to repeated outbreaks and disease emergence. Regions recently identified as EID hotspots were the northeastern United States, western Europe, Japan and southeastern Australia (Jones et al., 2008). Human population density and growth were significant disease drivers in these areas. Furthermore, zoonotic EIDs from wildlife were significantly correlated with increased wildlife biodiversity (Jones et al., 2008).

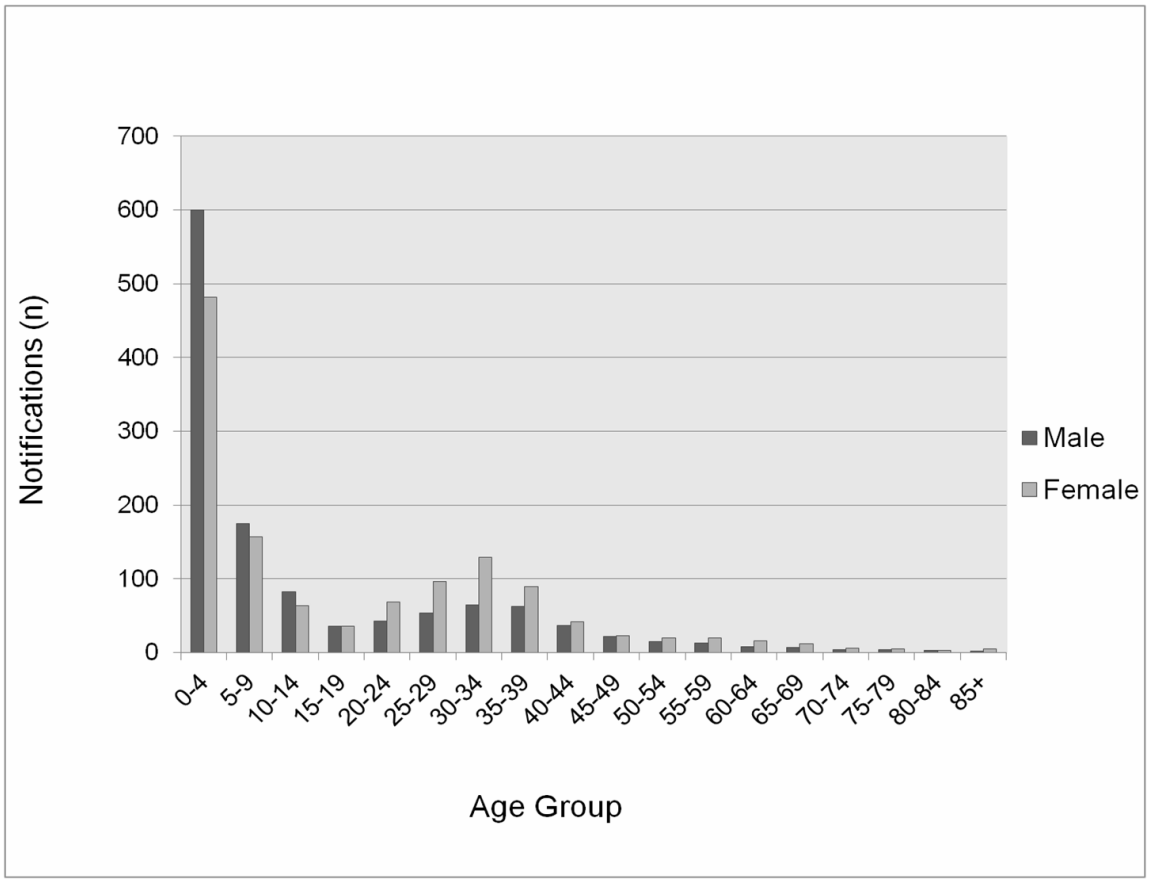
Isolated for over 35 million years, Australia is a unique country. It has evolved a unique ecosystem that is both diverse and abundant with wildlife. Eighty percent of the mammals, birds and reptiles found in Australia are endemic to this region (Williams et al., 2001). Australia has a human population of 23 million, 89% of which live in urban clusters bordering the coastlines (Australian Bureau of Statistics, www.abs.gov.au). Projections for Australia have estimated that the population will grow between 30.9 and 42.5 million people by 2056, and between 33.7 and 62.2 million people by 2101 (Australian Bureau of Statistics). To comfortably inhabit the additional 40 million people expected by 2101, coastal urbanized cities will need to expand inland, and urbanize rural-wildlife localities. In general, urbanized inhabitants have less contact with wildlife and livestock zoonotic disease sources; however population expansion past urban boundaries will expose both animals and humans to new, potentially harmful disease causing agents. It will become essential for research to analyze and understand pathogen transfer at the wildlife-urban-agricultural interface.

2.2 Epidemiology of human cryptosporidiosis in Australia

2.2.1 Cryptosporidiosis notifications

Cryptosporidiosis screening is not a routine test performed in Australian laboratories, being carried out only under a doctor's request. However, it is compulsory to report all positive cases to local government health authorities. Cryptosporidiosis has been a nationally notifiable disease in Australia since 2001. Notifications data from the National Notifiable Diseases Surveillance System (NNDSS) (http://www9.health.gov.au/cda/ Source/CDA-index.cfm) collected over the last decade shows that cryptosporidiosis is the third most common cause of gastrointestinal illness in Australia, exceeded only by campylobacteriosis and salmonellosis. Analysis of human cryptosporidiosis infections between 2001 and 2010 show the disease is most prevalent in the 0-4 and 5-9 age groups (Fig. 2.1) (NNDSS). The incidence in males is also higher than females at these ages. Notifications begin to peak again in the 20-44 age groups and a shift towards a higher incidence in females is observed.

Figure 2.1 Age and gender distribution of human cryptosporidiosis infections in Australia between 2001 and 2010*.

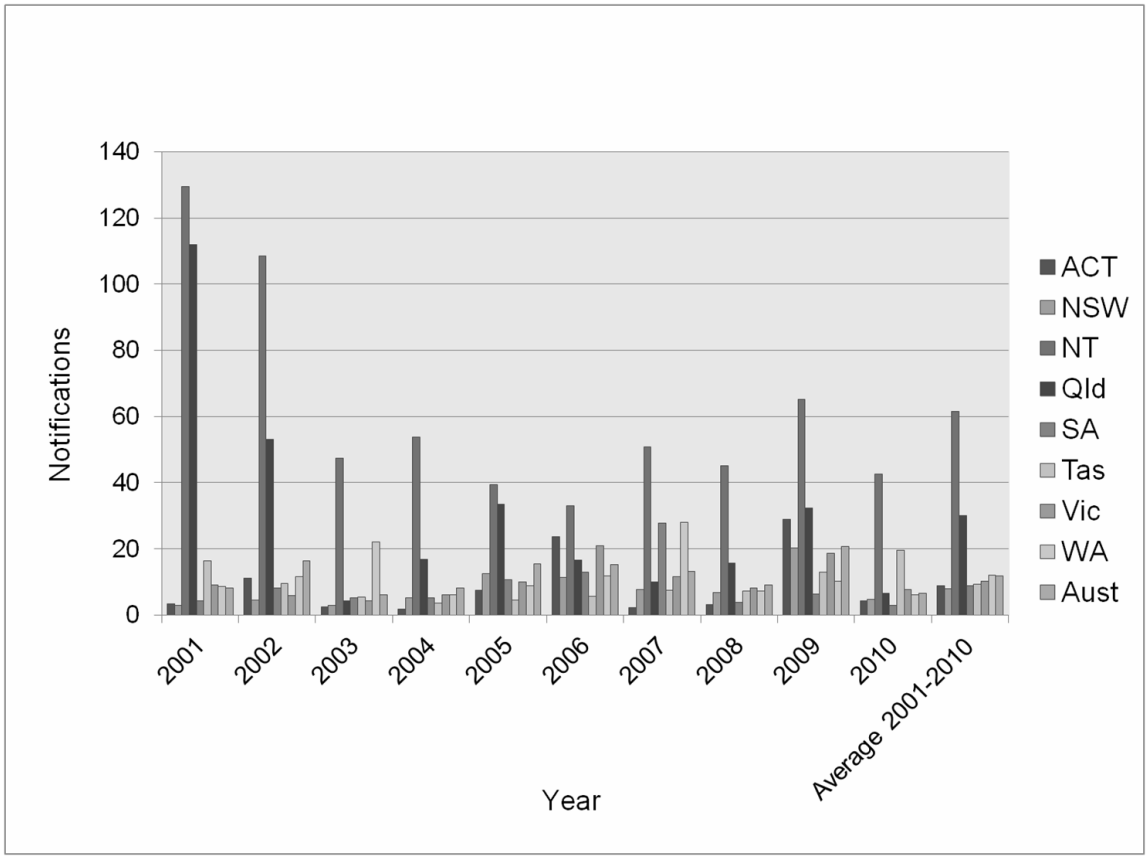


***Data source: National Notifiable Disease Surveillance System http://www9.health.gov.au /cda/ Source/CDA-index.cfm)**

In developed regions of the world, the prevalence of cryptosporidiosis ranges from 1-2% throughout Europe and 0.6-4.3% in North America (Fayer and Ungar, 1986; Tumwine, 2005). In contrast, prevalence rates in underdeveloped nations range between 5-32%. *Cryptosporidium* infections account for 6% of diarrhoeal cases in Uganda, 7.4% in Brazil, 13% in Tanzania, 17% in Egypt and as high as 32% in Guatemala (Newman, 1999; Laubach, 2004; Abdel-Messih et al., 2005; Tumwine, 2005). Notifications data shows that Australia has an average cryptosporidiosis rate of 11.92 per 100, 000 population (0.01% prevalence) between 2001 and 2010 (Fig. 2.2). The highest incidence was reported in 2009 where a rate of 20.7 per 100,000 population was reported (0.02% prevalence) (NNDSS).

Australia is divided into 6 states and 2 territories (Fig. 2.2). Notifications data for each of the states and territories shows that the Northern Territory and Queensland have the highest incidence of cryptosporidiosis in Australia, reporting rates of 61.56 (0.06% prevalence) and 30.1 (0.03% prevalence) per 100, 000 population respectively. Interestingly, New South Wales and Victoria, where the majority of Australia's populations reside, both recorded notifications below the national Australian average. New South Wales reported an average rate of 7.91 (0.007% prevalence) per 100, 000 population and Victoria, a rate of 10.24 (0.01% prevalence) per 100, 000 population.

Figure 2.2 Incidence of cryptosporidiosis throughout Australian States and Territories between 2001 and 2010*.



***Data source: National Notifiable Disease Surveillance System (http://www9.health.gov.au/cda/ Source/CDA-index.cfm)**

2.2.2 *Cryptosporidium* outbreaks

Australian interest in *Cryptosporidium* was initiated in 1998 when oocysts were detected in Sydney's main water supply (Stein, 2000). Three separate contamination events were reported between July and September, each leading to comprehensive Sydney wide boil water alerts (Stein, 2000). Although high levels of *Cryptosporidium* oocysts were detected on separate occasions, at different locations within the water distribution system, no significant increase in cryptosporidiosis notifications was observed (www.health.nsw.gov.au).

Numerous small outbreaks of cryptosporidiosis have been reported throughout Australia. Contamination sources have been linked to day care centers, animal petting farms, rural water supplies and most commonly public swimming pools. There have been at least seven large outbreaks of cryptosporidiosis reported by the state health authorities (Table 2.1). The first major human cryptosporidiosis outbreak Australia experienced was in Sydney, NSW in 1998, which occurred before the heavily publicized "great Sydney water crisis". The outbreak began in January and at least 1060 individuals were affected by the disease. The contamination source was linked to public swimming pools and it was believed to be Sydney wide. More recently Sydney experienced the largest waterborne outbreak reported in Australia to date. The outbreak occurred between January and April, 2009 and affected over 1131 individuals. Again, this was linked to public swimming pools. Victoria experienced a large waterborne outbreak of cryptosporidiosis in 2006 where 829 individuals fell ill with the disease. Outbreaks have also been recorded in South Australia and Western Australia, however these have been on a smaller scale (Table 2.1). All of the large cryptosporidiosis outbreaks have been linked to public swimming pool use. Due to Australian testing standards, *Cryptosporidium* oocysts identified from public swimming pools, or

from the Sydney water crisis, have never been identified to the species level. Consequently, contamination sources have never been identified.

Table 2.1 Cryptosporidiosis outbreaks in Australia by State and Territory, 1998-2010*

Australian State/Territory	Year	Individuals affected	Contamination source
New South Wales	1998	1060	Public swimming pool
	2005	254	Learn-to-swim pool
	2009	1131	Public swimming pool
South Australia	2007	228	Public swimming pool
Victoria	2001	192	Public swimming pool
	2006	829	Public swimming pool
Western Australia	2007	165	Public swimming pool

***Only outbreaks causing disease in greater the 100 individuals have been shown**

Data sources: New South Wales Health (www.health.nsw.gov.au); South Australia Health (www.health.sa.gov.au); Victoria Health (www.health.vic.gov.au); Western Australia Health (www.health.wa.gov.au).

2.3 Molecular epidemiology

Due to the morphological similarity of *Cryptosporidium* species, molecular technologies are essential for their differentiation, a reason why clinical samples only report a presence-absence result. Advancements in molecular technologies have facilitated the identification of *Cryptosporidium* species, and to a finer scale subtype families and subtypes.

Molecular studies in Australia have been conducted in four states; South Australia, Victoria Western Australia and to a lesser extent New South Wales (Chalmers et al., 2005; Jex et al., 2007; Jex et al., 2008; Ng et al., 2008; O'Brien et al., 2008; Waldron et al., 2009a; Waldron et al., 2009b; Ng et al., 2010a; Ng et al., 2010b). Species identification performed on clinical isolates show that Australia has a higher prevalence of *C. hominis*, which has attributed to 74% of infections (Table 2.2). This is likely due to the high level of urbanization in Australia. Infections with *C. parvum* were identified in 25.1% of isolates and *C. meleagridis* in 0.9% of infections. *Cryptosporidium meleagridis* was first identified in Victoria, where it has subsequently been identified in 3 patients (Jex et al., 2007; Pangasa et al., 2009). Four *C. meleagridis* infections have also been identified in Western Australia (Ng et al., 2010a). *Cryptosporidium hominis* is the most common cause of disease in all states, except in NSW where *C. hominis* and *C. parvum* contribute equally to disease (Waldron et al., 2009a). However, due to the small sample size, and the short study period, this needs to be further investigated.

Analysis of the hypervariable *gp60* gene has provided insight on the genetic diversity of *Cryptosporidium* contributing to human disease in Australia, with nine subtype families identified (Table 2.2). The most common subtype families identified in Australian infections are *C. hominis* Ib and *C. parvum* IIa, which attributed to 35.2% and 18% of infections investigated at the *gp60* gene, respectively (Table 2.2). *Cryptosporidium hominis* Ib remained the most common

subtype family in all states, except in Western Australia where the majority of infections were attributed to Id. A previous study analyzing the global prevalence of *Cryptosporidium* subtype families showed that *C. hominis* Ib and *C. parvum* IIa are the most common causing human disease, contributing to 47.4% and 25.5% of infections typed at the *gp60*, respectively (Jex and Gasser, 2010). An unusual find was the prevalence of *C. hominis* Ig which has a global prevalence of 0.1% (Jex and Gasser, 2010), however has a 10% prevalence in Australia and attributed to 17.8% of infections in WA.

Table 2.2 Prevalence and diversity of *Cryptosporidium* species and subtype families causing human disease in Australia

Species	Subtype family	Reports (n)	NSW (%)	SA (%)	Vic (%)	WA (%)	Australia (%)
C. hominis		**563**	**52.6**	**70.3**	**71**	**82.2**	**74**
	Ia	6	2.6	3	0	0.3	0.7
	Ib	268	39.5	64.4	30.3	29.2	35.2
	Id	114	7.9	0	0.4	31.2	15
	Ie	8	1.3	2	0	1.5	1.1
	If	9	1.3	1	0	2	1.2
	Ig	61	0	0	0	17.8	8
	unknown	97	0	0	40.2	0	12.7
C. parvum		**191**	**47.3**	**29.7**	**27.8**	**17**	**25.1**
	IIa	137	44.7	28.7	7.5	16.3	18
	IIc	8	1.3	1	2.1	0.3	1.1
	IId	2	1.3	0	0	0.3	0.3
	unknown	44	0	0	18.3	0	5.8
C. meleagridis		**7**	**0**	**0**	**1.2**	**1.2**	**0.9**

Data Sources: Chalmers et al., 2005; Jex et al., 2007; Jex et al., 2008; Ng et al., 2008; Waldron et al., 2009b; Ng et al., 2010a; Ng et al., 2010b

Forty-three subtypes have been identified from Australian human cryptosporidiosis infections (Table 2.3 and 2.4). *Cryptosporidium hominis* subtype IbA10G2 was the most prevalent subtype throughout Australia, attributing to 31.5% of infections typed at the *gp60* (Table 2.3). This is a globally distributed subtype and it has been the most common cause of global waterborne outbreaks and sporadic cryptosporidiosis, attributing to 44.5% of human cryptosporidiosis.

Table 2.3 A summary of the diversity of *Cryptosporidium hominis* subtypes causing human disease in Australia and their prevalence

Subtype family	Subtype	Report (n)	NSW (%)	SA (%)	Vic (%)	WA (%)	Australia (%)
Ia	A10R4	1	1.3	0	0	0	0.1
	A17R1	3	0	3	0	0	0.4
	A17R3	1	1.3	0	0	0	0.1
	A23	1	0	0	0	0.3	0.1
Ib	A5G2T3	1	0	0	0	0.3	0.1
	A6G3	1	1.3	0	0	0	0.1
	A9G2	3	2.6	0	0	0.3	0.4
	A9G2T1	1	0	0	0	0.3	0.1
	A9G3	21	0	20.8	0	0	2.8
	A10G2	240	35.6	43.6	29.9	28.3	31.5
	A18G1	1	0	0	0.4	0	0.1
Id	A15	2	2.6	0	0	0	0.3
	A15G1	99	1.3	0	0.4	28.3	13
	A16	6	0	0	0	1.7	0.8
	A17	1	0	0	0	0.3	0.1
	A25T1	1	1.3	0	0	0	0.1
	A25	3	0	0	0	0.9	0.4
	A26	2	2.6	0	0	0	0.3
Ie	A11G3T3	5	1.4	0	0	1.2	0.7
	A12G3T3	3	0	2	0	0.3	0.4
If	A11G1T1	1	0	0	0	0.3	0.1
	A12G1	7	1.3	1	0	1.5	0.9
	A21G1	1	0	0	0	0.3	0.1
Ig	A17	60	0	0	0	17.5	7.9
	A19	1	0	0	0	0.3	0.1

Data Sources: Chalmers et al., 2005; Jex et al., 2007; Jex et al., 2008; Ng et al., 2008; Waldron et al., 2009b; Ng et al., 2010a; Ng et al., 2010b

A high level of subtype diversity is seen in *C. parvum* IIa isolates, with 17 subtypes identified (Table 2.4). *Cryptosporidium parvum* is a generalist species, capable of causing disease in a wide variety of vertebrates. The numerous subtypes detected from humans in Australia indicate numerous potential disease hosts, transmission pathways and contamination sources. Commonly identified IIa subtypes, such as IIaA18G3R1 and IIaA20G3R1 which attributed to 9.5% and 2.6% of Australian infections respectively, are also frequently identified in Australian cattle.

Table 2.4 A summary of the diversity of *Cryptosporidium parvum* subtypes causing human disease in Australia and their prevalence

Subtype family	Subtype	Report (n)	NSW (%)	SA (%)	Vic (%)	WA (%)	Australia (%)
IIa	A15G2R1	3	1.3	0	0	0.6	0.4
	A16G3R1	2	2.6	0	0	0	0.3
	A16G4R1	1	1.3	0	0	0	0.1
	A17G2R1	14	2.6	0	0	3.5	1.8
	A17G3R1	4	5.3	0	0	0	0.5
	A17G4R1	2	2.6	0	0	0	0.3
	A18G3R1	72	14.8	16.8	3.7	10.2	9.5
	A19G2R1	1	0	0	0	0.3	0.1
	A19G3R1	4	2.6	1	0	0.3	0.5
	A19G4R1	4	0	1	0	0.9	0.5
	A20G2R1	1	1.3	0	0	0	0.1
	A20G3R1	20	5.3	7.9	2.9	0.3	2.6
	A20G5R1	3	4	0	0	0	0.4
	A21G2R1	1	0	0	0	0.3	0.1
	A22G3R1	2	1.3	0	0.4	0	0.3
	A22G4R1	2	0	2	0	0	0.3
	A23G3R1	1	0	0	0.4	0	0.1
IIc	A5G3a	8	1.3	1	2.1	0.3	1.1
IId	A15G1	1	0	0	0	0.3	0.1
	A24G1	1	1.3	0	0	0	0.1

Data Sources: (Chalmers et al., 2005; Jex et al., 2007; Jex et al., 2008; Ng et al., 2008; Waldron et al., 2009b; Ng et al., 2010a; Ng et al., 2010b)

2.4 Zoonotic sources

Due to the ability of *C. parvum* to pass through both zoonotic and anthroponotic transmission cycles, it is difficult to determine the original disease contamination source. Further complicating *C. parvum* contamination source tracking are the numerous potential hosts, such as dogs, deer, goats, horses, humans, pigs, rodents, sheep and cattle. Cattle are thought to maintain asymptomatic cryptosporidiosis infections and thereby serve as a major reservoir for *Cryptosporidium*. Australia has a potentially massive *Cryptosporidium* reservoir, with over 23.3 million beef cattle and 1.6 million dairy cows (Australian Bureau of Statistics). Farms adjacent to recreational waterways, throughout catchments zones and in urban regions all pose a threat to human health.

Genetic characterization of *Cryptosporidium* from cattle in Australia has been conducted in Western Australia, Victoria and to a smaller extent in New South Wales (Becher et al., 2004; Ng et al., 2008; O'Brien et al., 2008; Nolan et al., 2009; Ralston et al., 2010). Cattle in W.A. show the presence of both *C. parvum* and *C. andersoni* whilst in Victoria and NSW, studies to date indicate that cattle are only shedding *C. parvum*. The most common subtype, identified in 43.1% of cattle, is IIaA19G3R1 (Table 2.5). Subtype IIaA19G3R1 has only been identified in cattle from Victoria, however the incidence of this subtype in humans is highest in NSW, where it attributed to 2.6% of infections. The absence of this subtype in cattle in NSW indicates the anthroponotic transmission of this subtype. However this subtype may not have been detected in cattle farmed in NSW due to the small sample sizes that have been examined. Subtypes IIaA16G3R1 and IIaA18G3R1 were the next most frequently detected subtypes in cattle in Australia, each being identified in 5.3% of samples. Subtype IIaA16G3R1 has only ever been reported in cattle in Australia (Nolan et al., 2009). The presence of this unique subtype in cattle

and humans in the Australian environment, particularly NSW, highlights the need for comprehensive Australian studies to be conducted in order to identify risk factors specific to the Australian environment.

Table 2.5 A summary of the diversity of *Cryptosporidium* species and subtypes in cattle in Australia and their prevalence

Species	Subtype	Report (n)	NSW (%)	Vic (%)	WA (%)	Australia (%)
C. parvum		**168**	**100**	**100**	**62.3**	**89.4**
	IIa A15G2R1	2	0	0	3.8	1.1
	IIa A16G3R1	10	23.1	5.7	0	5.3
	IIa A17G2R1	2	7.7	0.8	0	1.1
	IIaA18G2R1	3	0	2.5	0	1.6
	IIa A18G3R1	10	38.5	0	9.4	5.3
	IIaA18G4R1	1	0	0.8	0	0.5
	IIa A19G3R1	81	0	66.4	0	43.1
	IIa A19G4R1	2	15.4	0	0	1.1
	IIa A20G2R1	9	0	7.4	0	4.8
	IIa A20G3R1	2	7.7	0.8	0	1.1
	IIa A20G4R1	9	0	7.4	0	4.8
	IIa A21G3R1	2	7.7	0.8	0	1.1
	IIa A23G3R1	9	0	7.4	0	4.8
C. andersoni		**20**	**0**	**0**	**37.7**	**10.6**

Data Sources: Becher et al., 2004; Ng et al., 2008; O'Brien et al., 2008; Nolan et al., 2009; Ralston et al., 2010

2.4 Conclusion

In the coming century, zoonoses originating from wildlife will pose the most significant threat to human health. As populations expand, the contact between humans, domestic animals, livestock and wildlife will increase and facilitate the establishment of different *Cryptosporidium* species in new hosts. It will become increasingly important for species identification to be performed on clinically diagnosed cryptosporidiosis as this will enable implementation of successful cryptosporidiosis management strategies. Targeted research, in known hotspot zones, that analyze the pathogen transfer between the wildlife-urban-agricultural interfaces will become essential for the implementation of successful cryptosporidiosis management and surveillance strategies. Additionally, the molecular interactions between *Cryptosporidium* and the host, and potentially between *Cryptosporidium* genetic variants within the host, need to be thoroughly investigated. Understanding the molecular drivers of a cryptosporidial infection will finally enable the development of a successful treatment.

3. Molecular epidemiology and spatial distribution of a waterborne cryptosporidiosis outbreak, Australia

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3.1 Abstract

Cryptosporidiosis is one of the most common waterborne diseases reported worldwide. Outbreaks of this gastrointestinal disease, which is caused by the *Cryptosporidium* parasite, are often attributed to public swimming pools and municipal water supplies. Between the months of January and April, 2009 New South Wales (NSW), Australia, experienced the largest waterborne cryptosporidiosis outbreak reported in Australia to date. Throughout the contamination event 1141 individuals became infected with *Cryptosporidium*. Health Authorities in NSW indicated public swimming pool use was a contributing factor to the outbreak. To identify the *Cryptosporidium* species responsible for the outbreak, faecal samples from infected patients were collected from hospitals and pathology companies throughout NSW for genetic analyses. Genetic characterization of *Cryptosporidium* oocysts from the faecal samples identified the anthroponotic *Cryptosporidium hominis* IbA10G2 subtype as the causative parasite. Equal proportions of infections were found in males and females, and an increased susceptibility in the 0 – 4 age group was observed. Spatio – temporal analysis indicated the outbreak was primarily confined to the densely populated coastal cities of Sydney and Newcastle.

3.2 Introduction

Emerging infectious diseases (EIDs) cause significant impacts on human and animal health. Protozoan parasites are the third most common cause of EIDs, attributing to 10.7% of EID events over the last 64 years (Jones et al., 2008). Protozoan parasites of the genus *Cryptosporidium*, responsible for the gastrointestinal illness cryptosporidiosis, are one of the most important disease causing agents in humans. Although 22 *Cryptosporidium* species have been described, over 90% of all reported human cryptosporidiosis infections are attributed to only two species; the anthroponotic *C. hominis* and the zoonotic *C. parvum* (Morgan-Ryan et al., 2002). *Cryptosporidium meleagridis*, *C. canis*, *C. felis*, *C. suis*, *C. muris*, *C. fayeri*, *C. ubiquitum* and *C. cuniculus* are also considered human pathogens (Chalmers and Davies, 2010; Fayer, 2010; Robinson et al., 2010; Waldron et al., 2010). *Cryptosporidium* is transmitted between hosts via the oocyst, an environmentally robust endogenous life-cycle stage that is resistant to the disinfectants used in water treatment. The oocyst is excreted in a fully infective form, and transmission is completed through the faecal-oral route. Chemical resistance and immediate infectivity, coupled with a low infective dose makes *Cryptosporidium* a significant threat to human health.

Waterborne disease transmission is the most common pathway for spread of cryptosporidiosis (Chalmers et al., 2005). The majority of waterborne outbreaks have been attributed to both *C. hominis* and *C. parvum* but a recent outbreak in Northhamptonshire, England, was caused by the *C. cuniculus* (Chalmers et al., 2009; Xiao, 2010). Species identification requires molecular tools due to morphological similarities exhibited within the *Cryptosporidium* genus. Numerous genetic markers are used for species specific *Cryptosporidium* identification. However, the *gp60* locus, which encodes the glycoprotein 60 a

highly variable surface antigen, is preferentially utilised in epidemiological investigations. The extensive sequence variation in the *gp60* enables grouping of *Cryptosporidium* into subtype families. For *C. hominis* 6 subtype families have been identified and for *C. parvum* there are 11 subtype families (Xiao, 2010). Analysis of a microsatellite region that encodes serine repeats is used to further characterise *C. hominis* and *C. parvum* to what is termed the subtype level.

Differences in virulence and clinical manifestations have been observed between *Cryptosporidium* species and the subtype families. *Cryptosporidium hominis* infections, which are commonly associated with non-intestinal sequelae, are more virulent than those from *C. parvum* (Hunter et al., 2004). All *C. hominis* infections cause diarrhoea, however subtype family Ib is the most virulent, and is associated with nausea, vomiting and general malaise (Cama et al., 2008). The number of waterborne outbreaks caused by *C. hominis* Ib, and particularly the IbA10G2 subtype, support the high virulence of this subtype family (Xiao, 2010).

Despite good hygiene, sanitary living conditions, safe food and water supplies, and access to medicine (immunisations and antibiotics), Australia's east coast has been identified as an EID hotspot (Jones et al., 2008). A myriad of bacterial, viral and protozoal infectious agents have contributed to this hot spot status. New South Wales (NSW), Australia has experienced numerous six waterborne outbreaks of cryptosporidiosis, all of which have been attributed to public swimming pool use (Lemmon et al., 1996; Stafford et al., 2000; Peauch, 2001; Peuch et al., 2001; Menzies, 2002; Black and McAnulty, 2006). A notifiable disease in NSW since 1996 (*New South Wales Public Health Act, 1991*), data shows that cryptosporidiosis infections are increasing, particularly during the warmer months from of November to March (http://www.health.nsw.gov.au/data/diseases/ cryptosporidiosis.asp). Between January and April 2009, 1141 cryptosporidiosis cases were reported to the NSW Health Communicable Disease

Branch, representing a 313% increase in incidence compared to the same period the previous year. Contaminated public swimming pools were identified as the source of the outbreak and up to 19 pools were hyperchlorinated as a result (NSW Health per comm.). The aim of this research was to undertake a genetic analysis to identify the *Cryptosporidium* species and subtypes responsible for the 2009 outbreak and to relate this to patient demographics. A spatial based approach was included in the study to investigate the geographical extent of the outbreak.

3.3 Materials and methods

3.3.1Samples sources, parasite enumeration and DNA extraction

Five hundred and eighty nine faecal samples, positive for *Cryptosporidium* were obtained from hospitals and pathology companies in NSW, Australia. Patient name was removed from samples to maintain privacy. All specimens were identified as *Cryptosporidium* positive by the pathology companies using the Remel ProSpecT *Giardia*/*Cryptosporidium* microplate assay. Oocysts were purified from faeces using a sucrose flotation gradient (Truong and Ferrari, 2006) and DNA extracted using PrepGem (Zygem Corporation Ltd, Hamilton, New Zealand) (Ferrari et al., 2007). Oocysts were stained with the *Cryptosporidium* specific antibody CRY104 labeled with FITC (Biotech Frontiers, Sydney, Australia) and enumerated by flowcytometry using a FACSCalibur-sort flow cytometer (BD Biosciences, Sydney, Australia)(Bennett et al., 1999).

3.3.2 Identification of Cryptosporidium species by PCR-RFLP analysis

Cryptosporidium species were identified using RFLP of a diagnostic fragment of the 18S rRNA. The fragment was amplified using a previously described nested PCR (Xiao et al., 1999b). Primary and secondary reactions (50 µL) contained 6 mM $MgCl_2$, 200 µM dNTPs, 200 nM of each primer, 1 U of RedTaq® polymerase (Thermo Scientific, Australia) and 2 µl of DNA template for the primary reaction, and 1 µl of the primary PCR product in the secondary reaction. A total of 35 cycles, each consisting of 94°C for 45 s, 56°C for 45 s and 72°C for 1 min, with an initial denaturation of 94°C for 3 min and a final extension step of 72°C for 7 min were performed for both primary and secondary reactions. PCR controls included a negative sample containing PCR water only and a positive sample containing *C. parvum* DNA. Reactions were run on Eppendorf Mastercycler Personal instruments (Eppendorf, North Ryde, Australia).

Amplicons were resolved by electrophoresis on 2% w/v agarose gel containing Sybr Safe (Invitrogen, Mulgrave, Australia) and visualised under UV light. Secondary products of the correct size (832-835 bp depending on species) were purified using the QIAquick PCR Purification Kit (Qiagen, Melbourne, Australia) following manufacturer's instructions.

Restriction fragment length polymorphism (RFLP) analysis of the 18S rRNA amplicon was performed using a previously described protocol with the restriction enzyme *VspI* (10 units/µl, New England BioLabs) (Xiao et al., 1999b). Digested fragments were resolved on 3.5% w/v agarose gels at 100V for 50 min. The RFLP patterns were visualised under UV light after pre-staining with Sybr Safe according to manufacturers instructions.

3.3.3 *gp60* amplification

Cryptosporidium subtype family and subtype identification were determined using a nested PCR targeting the hypervariable *gp60* gene (Waldron et al., 2009b). Primary and secondary reactions contained 4 mM $MgCl_2$, 200 nM dNTPs, 200 nM of each forward and reverse primer and 1 U of RedTaq® polymerase and 2 µl of DNA template for the primary reaction, and 1 µl of the primary PCR product in the secondary reaction. Reaction conditions comprised an initial denaturation at 94ºC for 3 min followed by 35 cycles of 94ºC for 45 s, 58ºC for 45 s and 72ºC for 1 min 30 s, with a final extension at 72ºC for 7 min. Amplicons were separated by 2% w/v agarose gel containing Sybr safe and were visualised under UV light. Reactions containing the correct size fragment (~1000 bp) were purified as described above and sequenced using an ABI 3130xl genetic analyser (Applied Biosystems, Foster City, California) with the BigDye™ terminator kit (Applied Biosystems).

Nucleotide sequences were analysed using Geneious v4.8.2 (Biomatters Ltd, Auckland, New Zealand). Isolates were assigned a subtype according to the nomenclature system described by Sulaiman et al. (2005) (Sulaiman et al., 2005). The *gp60* sequences generated from this study were submitted to GenBank ID: JF727750 – JF727762.

3.3.4 Patient information and spatial analysis

Patient information, such as age, gender and residential postcode, was obtained for each sample from NSW Health. To examine the spatial distribution of the outbreak, the numbers of patients infected with the various *Cryptosporidium* subtypes were mapped. Analysis of the distribution of the outbreak over the four month period was achieved by using the pathology sample date. Samples were divided into their respective months and mapped with their corresponding postcode. Geographical mapping was completed using ESRI ArcGIS Version 10.0 (http://esriaustralia.com.au/esri/default.html) in conjunction with New South Wales digital postal boundary postcodes 2006 (ABS, http://www.abs.gov.au/AUSSTATS/abs@.nsf/Details Page/2923.0.30.0012006).

A chi – squared statistical analysis was performed on the relative number of males and females, in addition to the various age groups, affected throughout the outbreak.

3.4 Results

3.4.1 *Cryptosporidium* Species, subtypes and parasite enumeration

Positive results for PCR of the 18S rRNA were obtained for 521/589 (88%) isolates. RFLP analysis showed that 502/521 (96%) patients were infected with *C. hominis* and 19/521 (4%) were infected with *C. parvum*.

Amplification of the *gp60* was successful for 473/521 (91%) of isolates typed at the 18S rRNA (Table 3.1). Sequence analysis of the *C. hominis* isolates identified four subtype families: Ia (1/473), Ib (453/473), Id (2/473) and Ie (1/473). The remaining isolates were identified to *C. parvum* subtype families IIa (15/473) and IId (1/473). Analysis of the microsatellite serine region identified 13 subtypes, 5 in *C. hominis* and 8 in *C. parvum*. The most common subtype, *C. hominis* IbA10G2, was identified in 449/473 (95%) isolates. The next most frequently detected subtype was *C. parvum* IIaA18G3R1which was identified in 8/473 (2%) isolates.

The number of oocysts/g in faeces was determined using flow cytometry. Patients infected with *C. hominis* were shedding higher parasitic numbers compared to those infected with *C. parvum*. Oocysts counts ranged from 10^2 to 10^7 /g in cases identified as shedding *C. hominis*, and from 10^2 to10^6 oocysts/g in *C. parvum* cases (Table 3.1). The majority of cases identified as *C. hominis* were shedding parasite numbers between 10^5 and 10^6, with an additional 73 patients shedding oocysts of 10^7 or greater (Table 3.1). Seventy-two of these cases were infected with the *C. hominis* IbA10G2 subtype. In contrast, the majority of *C. parvum* infected patients were shedding parasite numbers, between 10^3 and 10^4 oocysts/g of faeces.

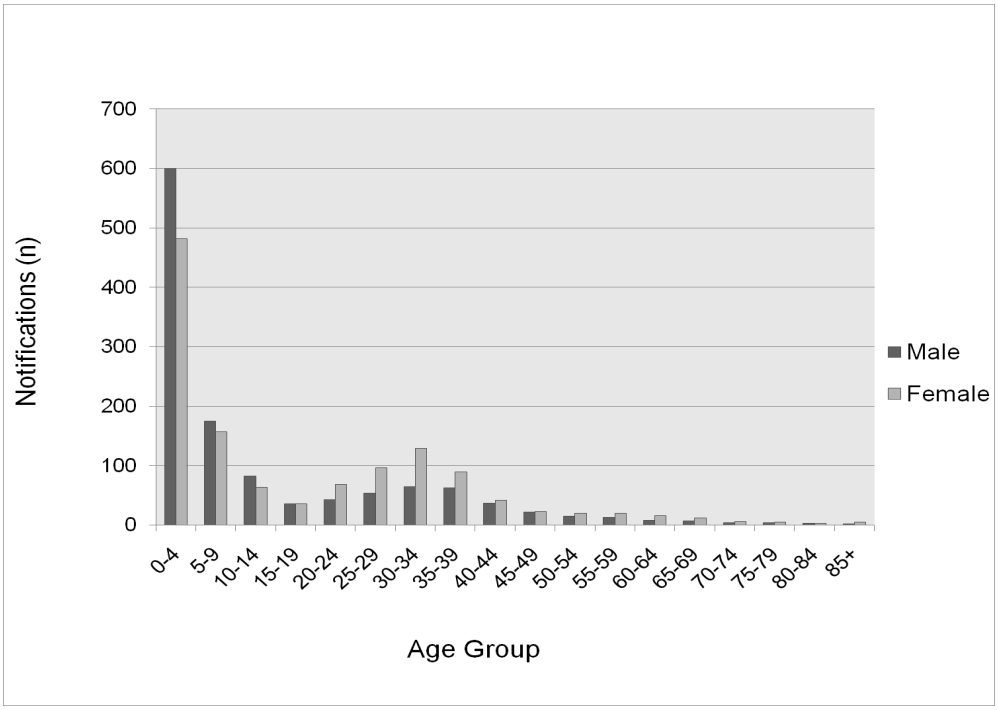
Table 3.1 Summary of oocyst numbers in faeces and the *Cryptosporidium* species and subtypes causing human illness in the January-April 2009 outbreak, NSW, Australia

			oocysts/g faeces			
***Cryptosporidium* species**	**gp60 subtype**	**Frequency**	**$\leq 10^2$**	**10^3 -10^4**	**10^5 - 10^6**	**$\geq 10^7$**
C. hominis	IaA26	1	1	0	0	0
	IbA9G3	4	0	0	3	1
	IbA10G2	449	36	118	223	72
	IdA17G1	2	0	0	2	0
	IeA12G3T2	1	0	0	1	0
Subtotal	**5**	**457**	**37**	**118**	**229**	**73**
C. parvum	IIaA15G2R1	1	0	0	1	0
	IIaA17G2R1	2	0	1	1	0
	IIaA17G4R1	1	0	0	1	0
	IIaA18G3R1	8	1	5	2	0
	IIaA19G6R1	1	1	0	0	0
	IIaA20G3R1	1	0	1	0	0
	IIaA22G3R1	1	0	0	1	0
	IIdA23G1	1	0	1	0	0
Subtotal	**8**	**16**	**2**	**8**	**6**	**0**
Total	**13**	**473**	**39**	**126**	**235**	**73**

3.4.2 Age and gender of patients infected with *C. hominis* IbA10G2

Information on patient age and gender were obtained for 387/521 (74%) samples (Fig 3.1). No gender bias was observed in outbreak cases (Chi-square = 0.26, df = 1, p-value = 0.61). The number of female cases was 199/387 (51%) and 188/387 (49%) for males. A bias in susceptibility of age was identified, with the 0 – 4 year age category most commonly affected group for both males (Chi-square = 579.66, df = 1, p-value = <<0.01) and females (Chi-square = 468.79, df = 1, p-value = <<0.01). Of all cases 88/199 (44%) female and 98/188 (52%) male were represented in the 0-4 year age group. Combined, the 0-4 age category attributed to 186/387 (48%) of all *C. hominis* IbA10G2 infections. Infections for both genders were also high in the 5 – 9, 30 – 34 and 35 – 39 age groups. In the 0 – 4 and 5 – 9 year age groups males were more affected than females. Females had a higher incidence than males in the 30 – 34 and 35 – 39 age groups.

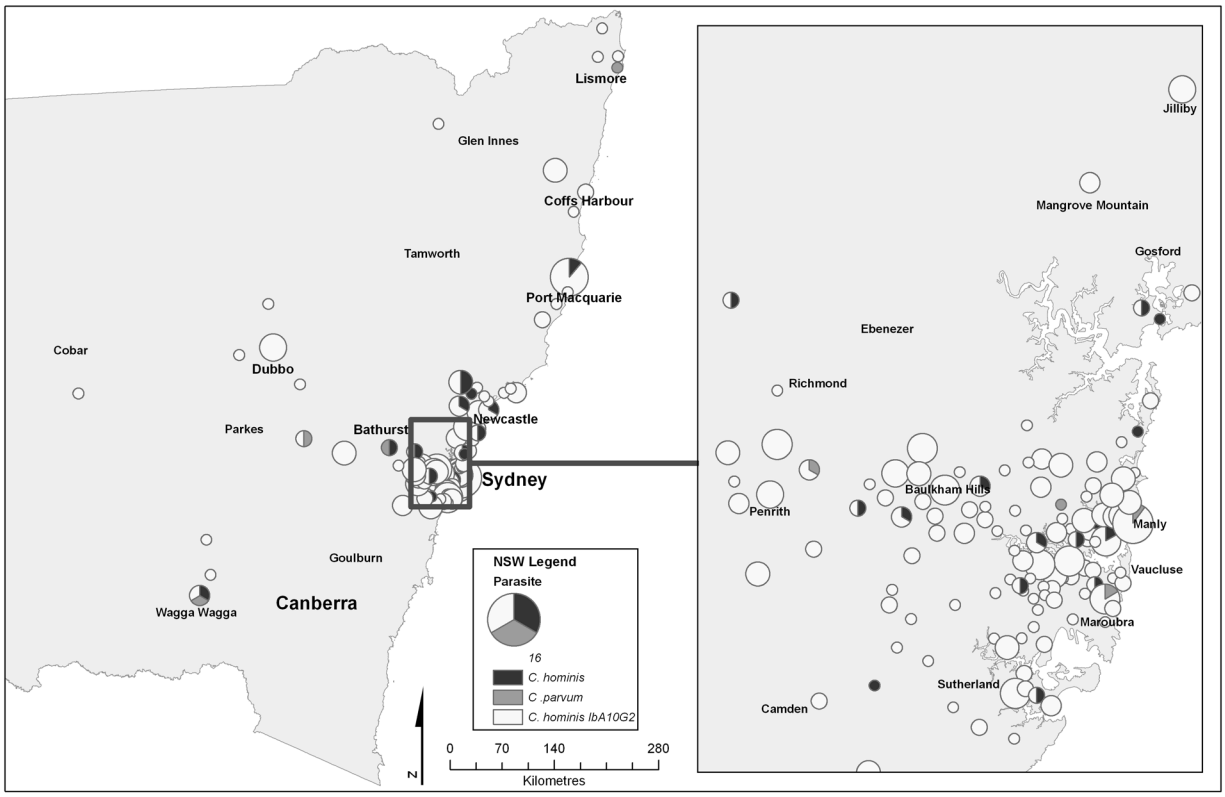
Figure 3.1 Age and gender distribution of patients infected with *C. hominis* IbA10G2 in the January-April 2009 *Cryptosporidium* outbreak, NSW, Australia.



3.4.3 Spatial distribution

The postal codes for the locations from which isolates originated were obtained for 367/473 (78%) of the isolates successfully typed at the *gp60* gene. Analysis of the NSW state-wide map for the entire outbreak period (January – April 2009) showed a disease cluster centered in Sydney, with extensions 200 km north towards Newcastle (Fig. 3.2). Isolated clusters in two costal communities north of Sydney, Port Macquarie and Coffs Harbour, were also observed along with a cluster centered within the Orana rural community, 400 km north-west of Sydney. An analysis of the greater Sydney region shows that the disease was widespread and not localised to any particular suburban area. However, clusters were seen through the inner west and northern suburbs of Sydney (Fig. 3.2).

Figure 3.2 Spatial distribution by postcode areas of the patients infected with the *C. hominis* IbA10G2 subtype, other *C. hominis* subtypes and *C. parvum* in NSW and Sydney, between January and April 2009. The size of the circle represents the number of cases.



Spatio-temporal analysis depicted a monthly view of the spread of *Cryptosporidium* infections throughout the state during the January to April period (Fig. 3.3). Following the *C. hominis* IbA10G2 subtype (shown in white), January infections showed that there were three disease clusters centered around Sydney-Newcastle, Port Macquarie and Coffs Harbour. At this time, infections had not spread inland towards the rural areas. Peaking in February, the Sydney-Newcastle cluster increased, and the appearance of clusters in the rural areas north-west of Sydney occurred. The Port Macquarie incidence remained constant, while the third cluster previously present in Coffs Harbour had disappeared. By March, the outbreak was contracting back towards Sydney and Newcastle with rural areas becoming less impacted. Decreased infections occurred in April and the clusters dissipated, becoming centralised around both the Sydney and Newcastle cities. The remaining *C. hominis* subtypes (shown in black) were located throughout the heavily populated urbanised regions of Sydney and Newcastle. *Cryptosporidium parvum* infections (shown in white) were recorded sporadically in both urban and rural areas.

Figure 3.3 Temporal analysis by month of the patients infected with the *C. hominis* IbA10G2 subtype, other *C. hominis* subtypes and *C. parvum* in NSW, between January and April 2009. The size of the circle represents the number of cases.

3.5 Discussion

A survey of global EID events indicated the east coast of Australia to be an emerging disease hotspot (Jones et al., 2008). Targeted surveillance and screening for potential disease outbreaks in hotspot zones is essential for identification and protection of population groups most at risk of disease. New South Wales, Australia has experienced six waterborne cryptosporidiosis outbreaks that have been linked to public swimming pools (Lemmon et al., 1996; Stafford et al., 2000; Peauch, 2001; Peuch et al., 2001; Menzies, 2002; Black and McAnulty, 2006). This study was based upon the recent 2009 outbreak, which is the largest reported outbreak to date in Australia. A molecular and spatial based approach was applied to investigate the *Cryptosporidium* species and subtype responsible for this outbreak, and to identify the population groups most affected.

One third of the 22 million Australian residents live within NSW, making this State the most populated region in Australia. Within NSW, 63% of the population lives in the states capital, Sydney. A growing city, Sydney, encompasses both urban and rural localities and has a population of 4.6 million (2009 census data, Australian Bureau of Statistics (ABS), www.abs.com.au). Newcastle is the second largest city within NSW, similarly to Sydney it is a metropolis comprised of urban and rural areas and has a current population of 354,054 (ABS). Spatial analysis performed on isolates obtained during the outbreak period showed the highest incidence of cryptosporidiosis was in the cities of Sydney and Newcastle. The higher incidence in these areas was likely associated with the higher population densities; this must be taken into consideration when interpreting the data. Although infections in Sydney were widespread, the densely populated inner west and north-west area were the most affected throughout the outbreak. Temporal analysis showed the outbreak originated in the urban cities and expanded inland to the rural areas of NSW. These results are consistent with previous studies in that *C.*

hominis infections are more common in urbanised city regions due to high population densities that provide stable anthroponotic transmission pathways (Xiao and Ryan, 2004; Xiao, 2010). Isolated clusters of *C. hominis* IbA10G2 infections in northern NSW were visualized in the early stage of the outbreak. With *C. hominis* IbA10G2 as the dominant parasite causing human cryptosporidiosis in NSW, it remains unclear whether these cases were attributed to sporadic infections or if they were part of a separate outbreak.

The outbreak was caused by the anthroponotic *C. hominis* IbA10G2 which was identified in 95% of disease cases. This subtype has a global distribution and has attributed to 44.5% of global human *Cryptosporidium* infections that have been studied to the subtype level (Jex et al., 2008; O'Brien et al., 2008; Waldron et al., 2009b; Jex and Gasser, 2010) . The *C. hominis* IbA10G2 sub-type is also the most common cause of sporadic human infections in Australia (Jex et al., 2008; O'Brien et al., 2008; Waldron et al., 2009b; Jex and Gasser, 2010) and has been the agent responsible for large waterborne cryptosporidiosis outbreaks in the United States, Northern Ireland and France (Glaberman et al., 2002; Cohen et al., 2006; Xiao and Ryan, 2008b). Most notably, *C. hominis* IbA10G2 caused the 1993 Milwaukee waterborne cryptosporidiosis outbreak which affected over 400 000 individuals and recorded a total outbreak cost of $96.2 million (Corso et al., 2003). Close to a decade later, this subtype was still the most common *Cryptosporidium* parasite detected in raw Milwaukee wastewater (Zhou et al., 2003).

Cryptosporidium hominis is a ‘specialist’ pathogen, specifically adapted to the human host. Conversely *C. parvum* is a ‘generalist’, capable of causing infections in humans, pigs, horses and a variety of ruminant species. ‘Specialist’ pathogens invoke more virulent infections than those of their ‘generalist’ counterparts (Hunter et al., 2004). Patients infected with *C. hominis*, particularly the IbA10G2 subtype, were shedding higher numbers of oocysts than those infected

with *C. parvum*. For the majority of *C. hominis* IbA10G2 infections oocyst shedding was between 10^5 – 10^7 oocysts/g. The high oocyst shedding intensity of *C. hominis* IbA10G2 may have facilitated the rapid spread of the parasite and contributed to the extent of the outbreak. Conversely, the majority of *C. parvum* infections in the same period were shedding between 10^3 – 10^4 oocysts/g of faeces. *Cryptosporidium parvum* cases were considered to be sporadic and not associated with the outbreak. Human cryptosporidiosis infections typically last between seven to 14 days, throughout this period oocyst shedding varies significantly (Chalmers and Davies, 2010). The fecal samples analysed in this study would have been obtained at varying points in an infection and hence a range of oocysts numbers being shed would be expected.

The increasing incidence of cryptosporidiosis in Australia indicates that this is an emerging disease. To reduce the risks of human illness, implementation of *Cryptosporidium* specific pool water treatments and monitoring systems are required. At this stage these technologies are not available, and increased public education is the current resource important to preventing future cryptosporidiosis outbreaks. Based on the dominance, persistence, distribution and virulence of the IbA10G2 subtype in human populations, and its association with waterborne outbreaks, this subtype should be considered a significant threat to global human health.

3.6 Acknowledgements

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4. Sporadic human cryptosporidiosis, Australia: Molecular epidemiology, spatio – temporal analysis and ecology

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4.1 Abstract

Parasites from the *Cryptosporidium* genus are the most common cause of waterborne disease around the world. Successful management and prevention of this emerging disease requires knowledge on the diversity of species causing human disease and their zoonotic sources. This study employed a spatio – temporal approach to investigate sporadic human cryptosporidiosis in New South Wales (NSW), Australia, between January 2008 and December 2010. Analysis of 261 human fecal samples showed sporadic human cryptosporidiosis is caused by four species; *C. hominis*, *C. parvum*, *C. andersoni* and *C. fayeri*. Sequence analysis of the *gp60* gene identified 5 subtype families and 31 subtypes. *Cryptosporidium hominis* IbA10G2 and *C. parvum* IIaA18G3R1 were the most frequent cause of human cryptosporidiosis in NSW, attributing to 59% and 16% of infections respectively. To determine the role of cattle in sporadic human infections in NSW, 205 cattle faecal samples were analysed. Four *Cryptosporidium* species were identified; *C. hominis*, *C. parvum*, *C. bovis* and *C. ryanae*. Subtype IIaA18G3R1 was the most common cause of cryptosporidiosis in cattle, attributing to 47% of infections. Subtype IbA10G2 was also identified in cattle isolates. Results showed infections were most prevalent in the 0 – 4 year olds. No gender bias or regional segregation between the distribution of *C. hominis* and *C. parvum* infections was observed.

4.2 Introduction

Cryptosporidium species are capable of initiating gastrointestinal disease in over 200 vertebrate species from various taxonomic groups, such as fish, birds, mammals and reptiles (Fayer, 2010). The emergence of human cryptosporidiosis in the mid 1980s coincided with the human immunodeficiency virus (HIV) era. Initially, it was considered a disease limited to the immune–compromised and due to the strong link with HIV, it was used as an initial diagnosis of the virus (Tzipori and Widmer, 2008). However, over the last 20 years, cryptosporidiosis has emerged as a significant human pathogen with a global distribution, capable of casing illness in both immune-compromised and immune-competent individuals. Cryptosporidiosis is a primary concern for water and health authorities in addition to the livestock industry which suffers significant economic losses from diseased animals.

Genetic heterogeneity exhibited within the *Cryptosporidium* genus has been highlighted by molecular analyses, which are essential for the differentiation of *Cryptosporidium* species. To date, 22 species and greater than 40 genotypes of *Cryptosporidium* have been described (Fayer, 2010; Robinson et al., 2010). DNA analysis of *Cryptosporidium* from humans has shown the anthroponotic *C. hominis* and the zoonotic *C. parvum* as the most common cause of human cryptosporidial infections, attributing to 90% of reported cases (Morgan-Ryan et al., 2002). However, due to the sensitivity of molecular analyses, in conjunction with the growing number of human samples analysed, 8 additional species have been identified as a public health threat and include *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris*, *C. fayeri*, *C. ubiquitum* and *C. cuniculus* (Chalmers and Davies, 2010; Robinson et al., 2010; Waldron et al., 2010). The contribution of these species to human disease varies globally, and is often associated with seasonality, demographics, immune status and contact with reservoir hosts.

Further intra–species variation has been observed in *Cryptosporidium* isolates through sequence analysis of the hypervariable *gp60* gene, which further classifies species to the subtype family and subtype levels. Sequence variation observed in the *gp60* gene has identified 6 *C. hominis* subtype families (designated with the Roman numeral I) and eleven *C. parvum* subtype families (Roman numeral II) (Xiao, 2010). Six subtype families have also been identified in *C. meleagridis* (Roman numeral III) and *C. fayeri* (Roman numeral IV) (Plutzer and Karanis, 2009; Power et al., 2009). Within a microsatellite region of the *gp60* gene, variation in the number and form of serine-codons further characterises *Cryptosporidium* to the subtype level (Sulaiman et al., 2005). Molecular analysis of the *gp60* gene has facilitated the identification of transmission pathways, zoonotic disease contamination sources and highlighted the importance of certain genetic variants to human health. For example, analysis of the *gp60* gene has shown cattle are an important zoonotic source for human disease. In Australia, *C. parvum* IIaA18G3R1 is the dominant subtype infecting cattle. The identification of IIaA18G3R1 in humans was first reported in Australia and it has now become the dominant *C. parvum* subtype causing Australian sporadic cryptosporidiosis (Jex et al., 2008; O'Brien et al., 2008; Waldron et al., 2009b). Cattle zoonotic sources have also been shown in Portugal where *C. parvum* IIaA15G2R1 is the most common subtype in cattle and humans (Alves et al., 2006). Subtype classification has also highlighted the public health risk posed by particular *Cryptosporidium* subtypes. The *C. hominis* IbA10G2 subtype is a globally distributed subtype and is the most common cause of waterborne outbreaks and sporadic human cryptosporidiosis (Jex and Gasser, 2010; Xiao, 2010).

Cryptosporidiosis has been a notifiable disease in New South Wales (NSW) since 1996 (NSW Public Health Act, 1991). Notifications data has shown a significant rise in the incidence of human cryptosporidiosis in NSW. In 2003, the overall incidence of human cryptosporidiosis

was 2.7/100,000, in 2006 it had risen to 10.5/100,000 and in 2009 the incidence further increased to 19.8/100,000 (www.nswhealth.gov.au). Previous studies conducted on sporadic human cryptosporidiosis in NSW show that *C. hominis* and *C. parvum* contribute equally to disease, with *C. hominis* IbA10G2 and *C. parvum* IIaA18G3R1 identified as the most common subtypes (Waldron et al., 2009b). Here we perform a longitudinal investigation into sporadic human cryptosporidiosis in NSW and examine transmission pathways and demographic groups most at risk of disease. This knowledge is essential for the management of cryptosporidiosis in Australia and for a global understanding of disease impacts of this parasite.

4.3 Materials and methods

4.3.1 Sample sources, parasite enumeration and DNA extraction

Two hundred and five faecal samples were collected from beef and dairy cattle throughout NSW. In total, calves from seven dairy farms were sampled, 1 farm in Camden (n = 75) and 6 farms in Wagga Wagga (farm 1, n = 14; farm 2, n = 10; farm 3, n = 19; farm 4, n = 10; farm 5, n = 7; farm 6, n=10; total n = 70). In addition, one beef farm in Richmond (n = 60) was also investigated. Typical for beef cattle, adults and juveiles were housed in a mixed pen, from this the age of cattle was not determined. Sampling occurred in the spring, 2010, with the exception of the Camden farm which was also sampled in 2008. DNA extraction from cattle faecal samples was performed using the Bioline isolate faecal DNA Kit (Bioline, Sydney, Australia) following the manufacturers instructions.

Four hundred and forty seven human faecal samples, positive for *Cryptosporidium,* were obtained from hospitals and pathology companies in NSW, Australia, between January 2008 and December 2010. Samples collected between January and April 2009 were attributed to a waterborne outbreak in NSW and were excluded from this sporadic cryptosporidiosis study. Oocysts were purified from faeces using a sucrose flotation gradient (Truong and Ferrari, 2006) and DNA extracted from purified oocysts using *PrepGem*® (Zygem Corporation Ltd, Hamilton, New Zealand) (Ferrari et al., 2007). Oocysts were fluorescently stained with the *Cryptosporidium*-specific antibody CRY104 labeled with FITC (Biotech Frontiers, Sydney, Australia) and enumerated by flow cytometry using a FACSCalibur-sort flow cytometer (BD Biosciences, Sydney, Australia) (Bennett et al., 1999).

4.3.2 Identification of *Cryptosporidium* species by PCR-RFLP analysis

Cryptosporidium species were identified using a previously described PCR- RFLP protocol targeting an 18S rRNA gene fragment (Xiao et al., 1999b). Primary and secondary reactions contained 6 mM $MgCl_2$, 200 µM dNTPs, 200 nM of each primer and 1 U of RedTaq® polymerase (Thermo Scientific, Australia). DNA template was 2 µl in the primary reaction and 1 µl of the primary PCR product was used as template in the secondary reaction. Reaction conditions comprised an initial denaturation of 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for 1 min, and a final extension step of 72°C for 7 min. All PCRs performed included a negative control containing PCR water and a positive control containing *C. parvum* DNA. Reactions were run on Eppendorf Mastercycler Personal instruments (Eppendorf, North Ryde, Australia). PCR products were resolved by electrophoresis using a 2% w/v agarose gel containing Sybr Safe (Invitrogen, Mulgrave, Australia) and visualised under UV light.

Secondary products of the correct size (~830 bp) were subjected to an RFLP analysis. Restriction analysis of *Cryptosporidium* 18S rRNA gene amplicons from cattle was performed using a previously described protocol with the restriction enzymes *SspI* and *MboII* (New England BioLabs) (Feng et al., 2007). Restriction analysis of 18S rRNA gene amplicons from human samples was performed using a previously described protocol with the restriction enzyme *VspI* (Xiao et al., 1999b). Restriction fragments were separated on 3.5% w/v agarose gels containing Sybr Safe at 100V and patterns were visualised under UV light.

4.3.3 *Gp60* amplification

Cryptosporidium subtype family and subtype identification were determined using a previously described nested PCR targeting the *gp60* gene (Waldron et al., 2009b). Primary and secondary reactions contained 4 mM $MgCl_2$, 200 nM dNTPs, 200 nM of each forward and reverse primer

and 1 U of RedTaq® polymerase. Primary PCR reactions contained 2 µl of DNA template and secondary reactions used 1 µl of the primary PCR product as the DNA template. Reaction conditions comprised an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 1 min 30 s, with a final extension at 72°C for 7 min. PCR products were separated by 2% w/v agarose gel containing Sybr safe and were visualised under UV light. Products containing the correct size fragment (~1000 bp) were purified using the QIAquick PCR Purification Kit (Qiagen, Melbourne, Australia) following the manufacturer's instructions and sequenced using an ABI 3130xl genetic analyser (Applied Biosystems, Foster City, California) with the BigDye™ terminator kit (Applied Biosystems).

Nucleotide sequences were analysed using Geneious v4.8.2 (Biomatters Ltd, Auckland, New Zealand). Isolates were assigned a subtype according to the nomenclature system as described previously (Sulaiman et al., 2005).

4.3.4 Parasite enumeration and cloning of *C. hominis* IbA10G2 from cattle

Oocysts from three cattle faecal samples, which contained *C. hominis* IbA10G2 were purified using a sucrose flotation gradient method (Truong and Ferrari, 2006) and enumerated by flow cytometry using a FACSCalibur-sort flow cytometer (BD Biosciences) after fluorescent staining with CRY104-FITC (Biotech Frontiers) (Bennett et al., 1999). Purified *gp60* PCR products were cloned using the TOPO-TA vector cloning system (Invitrogen, Australia) and plasmid DNA was recovered using the Qiagen plasmid kit (Qiagen, Melbourne, Australia). Sequencing was performed using an ABI 3130xl genetic analyser (Applied Biosystems, Foster City, California) with the BigDye™ terminator kit (Applied Biosystems).

4.3.5 Patient information and spatial analysis

Patient data (age, gender and residential postcode) was obtained for each sample from NSW Health. Spatial analyses were achieved using ESRI ArcGIS Version 10.0 (http://esriaustralia.com.au/esri/ default.html) in conjunction with New South Wales digital postal boundary postcodes 2006 (ABS, http://www.abs.gov.au/AUSSTATS/abs@.nsf/Details Page/2923.0.30.0012006). To examine the spatial distribution of sporadic cases, the numbers of patients infected with the various *Cryptosporidium* subtypes were mapped. Cattle farms sampled in this study were also incorporated in the spatial analysis. The temporal analyses were performed using the pathology date provided for each sample; a map was then generated for each year of the study in addition to each of the four seasons. To determine if a gender or age bias occurred in sporadic cryptosporidiosis, a chi-squared analysis was performed.

4.3.6 Nucleotide sequences

Cryptosporidium gp60 nucleotide sequences generated from cattle were submitted under GenBank ID: JF727776 – JF727779. *Gp60* nucleotide sequences from human *Cryptosporidium* infections were submitted under GenBank ID: JF727783 – JF727809. Cloned *C. hominis gp60* sequences from cattle were submitted under GenBank ID: JF727780 – JF727782.

4.4 Results

4.4.1 *Cryptosporidium* species and subtypes in cattle

Cattle fecal samples exhibiting positive PCR products for the target 18S rRNA gene fragment were obtained for 62/205 (30%) samples. RFLP analysis showed that 45/62 (73%) cattle were infected with *C. parvum*, 11/62 (18%) with *C. bovis*, 3/62 (5%) with *C. hominis* and 1/62 (2%) with *C. ryanae*. Mixed infections with *C. parvum*/*C. bovis* and *C. ryanae*/*C. bovis* were both reported in a single cattle sample (Table 4.1). *Cryptosporidium parvum* was the most common species detected on all farms, except Wagga Wagga farms 5 and 6 where *C. bovis* incidence was higher. *Cryptosporidium bovis* and *C. ryanae* were not detected on the Camden farm in 2008, but were present in 2010. *Cryptosporidium hominis* was identified on two farms, Richmond (n = 2) and Wagga Wagga farm 6 (n = 1). To determine if cattle positive for *C. hominis* were shedding oocysts, oocysts loads were determined by EFM and showed that the Richmond cattle contained parasite loads of 300 and 148 oocysts/g of faeces and the Wagga Wagga farm 6 cow was shedding 50 oocysts/g of faeces.

Amplification of the *gp60* gene was successful for 39/49 (80%) *C. parvum* and *C. hominis* samples typed at the 18S rRNA gene (Table 4.1). Two subtype families were detected in the cattle samples; *C. parvum* IIa (36/39) and *C. hominis* Ib (3/39). These two families comprised five subtypes with the most common subtype IIaA18G3R1 identified in 29/39 (74%) isolates. *Cryptosporidium hominis gp60* products were cloned to confirm this finding. Sequence analysis of *C. hominis* identified subtype IbA10G2 in all 3 isolates.

Table 4.1 Summary of the Cryptosporidium species and subtypes identified in cattle, NSW, Australia

4.4.2 *Cryptosporidium* species, subtypes and parasite enumeration in humans

Positive PCR products for the 18S rRNA gene were obtained for 261/447 (58%) faecal samples. RFLP analysis showed the presence of four species: *C. hominis* (172/261, 66%); *C. parvum* (87/261, 33%); *C. andersoni* (1/261, 0.5%); and *C. fayeri* (1/261, 0.5%) (Waldron et al., 2010).

Amplification of the *gp60* gene was successful for 245/261 (94%) isolates successfully typed at the 18S rRNA gene (Table 4.2). Three *C. hominis* subtype families were identified: Ia (4/245), Ib (156/245) and If (4/245). Analysis of the microsatellite serine region identified eleven *C. hominis* subtypes. The most commonly identified subtype, identified in 147/245 (60%) isolates, was *C. hominis* IbA10G2. Within *C. parvum* isolates, two subtype families were identified: IIa (79/245) and IId (1/245). Twenty *C. parvum* subtypes were detected, the most common being IIaA18G3R1 which was present in 37/245 (15%) isolates. Typing of the *C. fayeri* isolate at the *gp60* locus identified subtype IVaA10G3T1R1 (Waldron et al., 2010).

Parasite counts (oocysts/g), determined by flow cytometry, ranged between $\leq 10^2$ to $\geq 10^7$ oocysts/g in both *C. parvum* and *C. hominis* infected patients (Table 4.2). The majority of *C. hominis* IbA10G2 and *C. parvum* IIaA18G3R1 cases were shedding oocyst numbers between 10^5 and 10^6 oocysts/g.

Table 4.2 Summary of oocyst numbers in faeces and the *Cryptosporidium* species and subtypes causing sporadic human illness January 2008 – December 2010, NSW, Australia

			oocysts/g faeces			
***Cryptosporidium* species**	**Gp60 subtype**	**Frequency**	**≤ 10^2**	**10^3 -10^4**	**10^5 - 10^6**	**≥ 10^7**
C. hominis	IaA11R1	1	0	1	0	0
	IaA13R1	1	0	1	0	0
	IaA14R1	1	0	0	1	0
	IaA32R1	1	0	1	0	0
	IbA6G3	4	1	0	3	0
	IbA7G3	1	0	0	1	0
	IbA9G3	4	0	2	2	0
	IbA10G2	147	19	53	71	4
	IfA14G1	1	0	0	1	0
	IfA19G1	1	0	1	0	0
	IfA20G1	2	0	1	1	0
Subtotal	**11**	**164**	**20**	**60**	**80**	**4**
C. parvum	IIaA10G3	2	0	0	2	0
	IIaA14G3	1	0	0	1	0
	IIaA14G3R1	1	0	0	1	0
	IIaA15G1R1	1	0	1	0	0
	IIaA15G2R1	1	0	1	0	0
	IIaA15G4R1	1	1	0	0	0
	IIaA16G3R1	7	0	3	4	0
	IIaA17G2R1	3	0	2	1	0
	IIaA17G3R1	1	0	0	1	0
	IIaA17G4R1	3	0	1	2	0
	IIaA18G3R1	37	0	15	20	2
	IIaA19G2R1	3	0	2	1	0
	IIaA19G3R1	5	0	2	3	0
	IIaA19G4R1	3	1	1	1	0
	IIaA20G3R1	4	2	1	0	1
	IIaA20G5R1	2	0	1	1	0
	IIaA21G3R1	1	0	1	0	0
	IIaA22G3R1	2	0	1	1	0
	IIaA23G3R1	1	0	0	0	1
	IIdA24G1	1	0	0	1	0
Subtotal	**20**	**80**	**4**	**32**	**40**	**4**
C. fayeri	IVaA10G3T1R1	1	0	0	1	0
Subtotal	**1**	**1**	**0**	**0**	**1**	**0**
Total	**32**	**245**	**24**	**92**	**121**	**8**

4.4.3 Age and gender of patients infected with *Cryptosporidium*

Information on patient age and gender was obtained for 216/261 (83%) samples. Results showed that both genders were affected approximately equally (Chi-square = 1.5, df = 1, p-value = 0.247),, with 117/216 (54%) patients being female and 99/216 (46%) male (Table 4.3). Cryptosporidiosis was bimodal, with the 0-9 and 25-39 age categories showing the highest incidence. Overall, the 0-4 year age group was the most commonly affected group for both genders, attributing to 42/117 (36%) of female (Chi-square = 168.27, df = 1, p-value = <<0.01) and 51/99 (52%) of male (Chi-square = 297.07, df = 1, p-value = <<0.01) total infections. Combined, the 0-4 age group attributed to 93/216 (43%) of all *Cryptosporidium* infections and both females 78/117 (67%) and males 67/99 (68%) were most likely to be infected with *C. hominis*; this observation was seen in all ages groups except for the 20-24, 45-49, and 50-54 groups where the numbers were low.

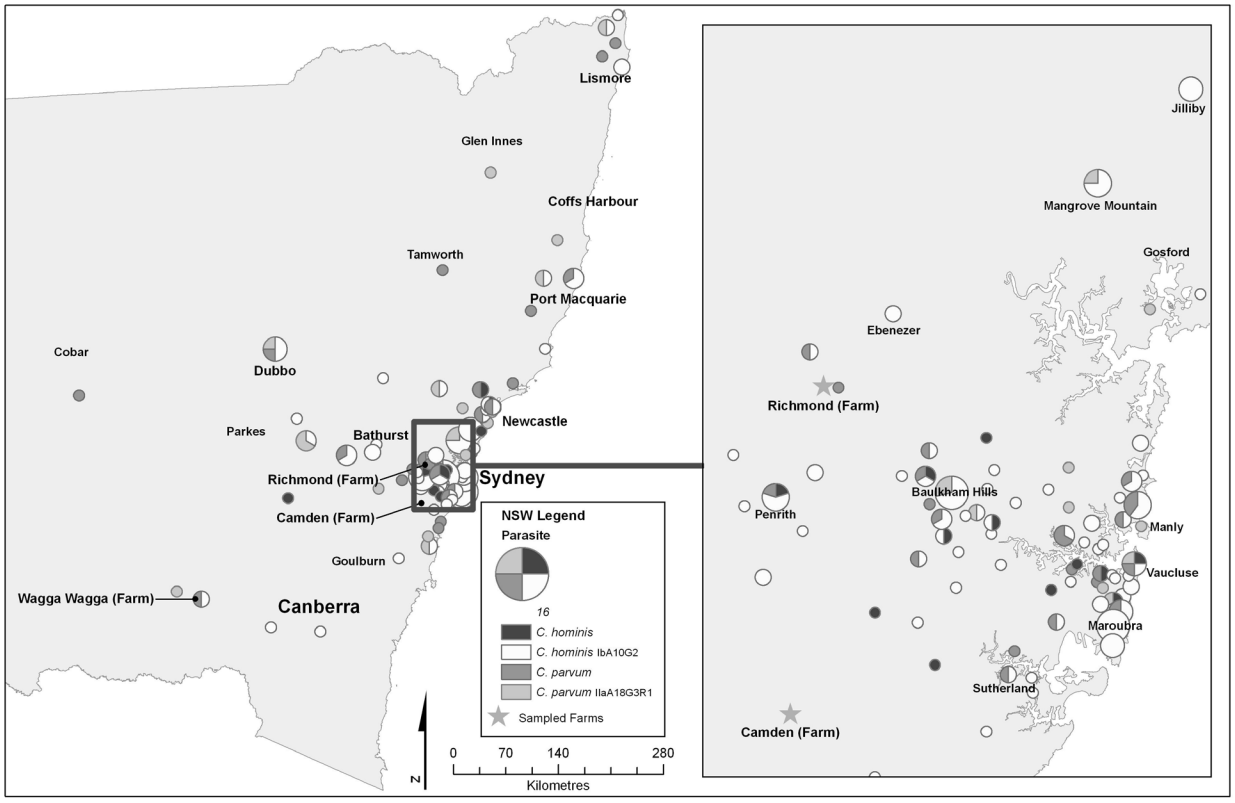
Table 4.3 Age and gender distribution of sporadic cryptosporidiosis infections between January 2008 – December 2010, NSW, Australia

4.4.4 Spatial distribution of infections, species and subtypes between January 2008 – December 2010.

Postal codes for the locations from which isolates originated were obtained for 209/245 (85%) isolates successfully typed at the *gp60* gene. Analysis of the NSW state-wide map showed sporadic cryptosporidiosis was confined to the east, north-east, south-east and central regions of NSW (Fig. 4.1). The western areas of NSW reported no cryptosporidiosis infections and were excluded from the maps. Disease clusters were observed in Sydney – Newcastle, Dubbo, Bathurst, Port Macquarie and in the north-eastern region of the state. Within Sydney, infections predominated in the eastern and the north–western suburbs, which were localized in the Baulkham Hills region. Clustering in the Penrith, Mangrove Mountain and Jilliby regions was also observed.

Geographical segregation between the distribution of *C. hominis* and *C. parvum* was not observed, that is, both species were found in urban and rural areas throughout the state; however, *C. parvum* infections became more prominent further inland (Fig. 4.1). Within Sydney, *C. hominis* and *C. parvum* infections were widespread, however *C. hominis* IbA10G2 was the dominant parasite causing Sydney infections.

Figure 4.1 Spatial distribution by postcode areas of the patients infected with the *C. hominis* IbA10G2 subtype, other *C. hominis* subtypes, *C. parvum* IIaA18G3R1 and other *C. parvum* subtypes in NSW and Sydney, between January 2008 and December 2010. The size of the circle represents the number of cases.



4.4.5 Spatio-temporal distribution of infections, species and subtypes

Postal codes and sample dates were obtained for 209/245 (85%) of samples successfully typed at the *gp60* gene. To determine the yearly distribution of sporadic cryptosporidiosis, samples were divided into their respective years: 2008, 104 samples; 2009, 67 samples; 2010, 38 samples. The distribution of sporadic infections was widespread throughout 2008, 2009 and 2010 (Fig. 4.2). Clusters in urban coastal cities, such as Sydney, Newcastle, Port Macquarie and in the north-eastern areas of the state remained constant. Cluster differences in regional areas, such as Wagga Wagga, Dubbo and Bathurst, were observed over the three year period. Clusters which were identified in Dubbo and Wagga Wagga in 2008 and 2009 were absent in both areas in 2010. While Bathurst clusters were observed in 2008 and 2010, but not in 2009. Infections within Sydney were also widespread. Urban areas surrounding Penrith and the eastern and north–western suburbs maintained the highest number of infections. Large clusters were reported in the semi–rural areas of Mangrove Mountain and Jilliby in 2008 and 2009, but were absent in 2010.

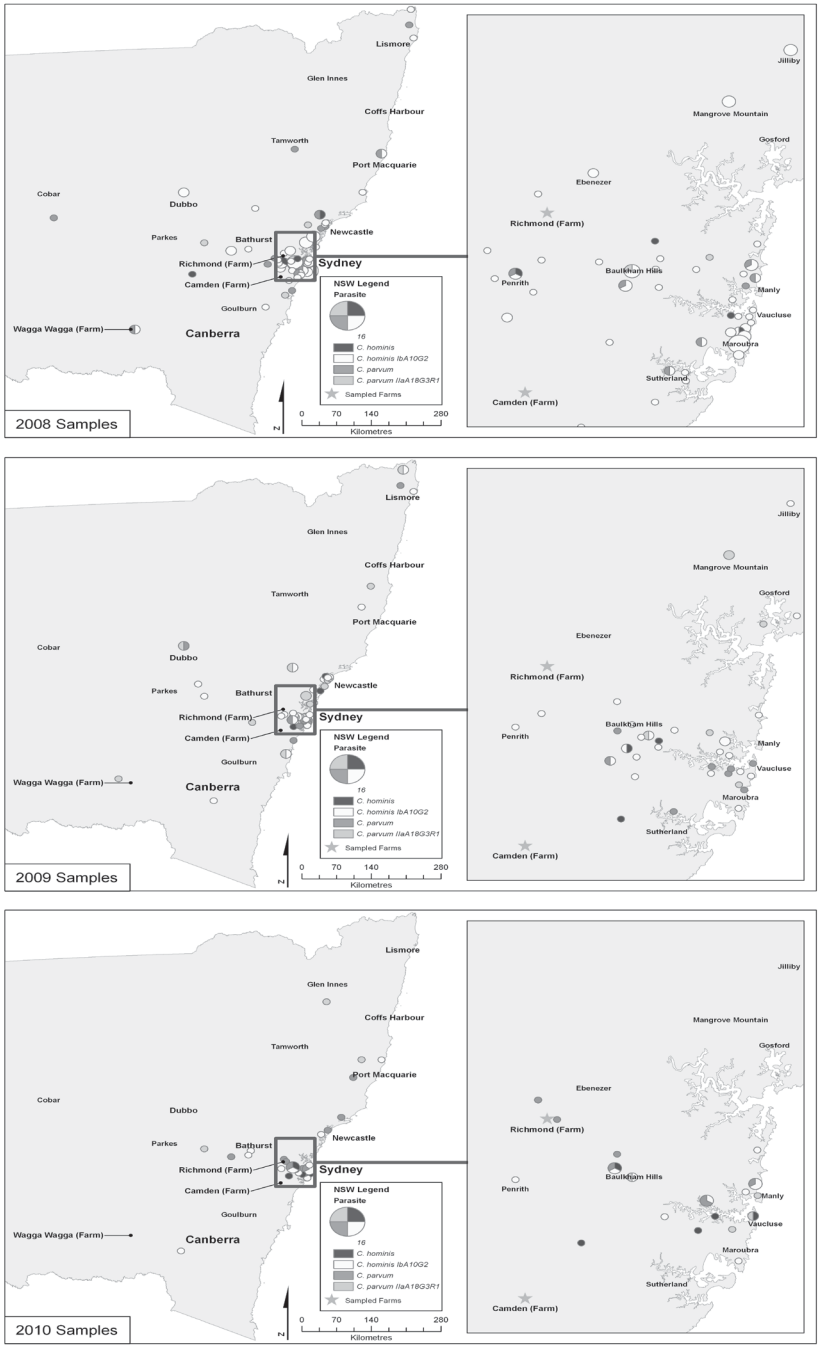
Spatio–temporal analysis of the state–wide species distribution showed *C. hominis* infections were more common than *C. parvum* infections in 2008. Large *C. hominis* IbA10G2 clusters were seen in Sydney, Newcastle, Bathurst, Dubbo and in the north-eastern regions of the state (Fig. 4.2). Infections with *C. parvum* IIaA18G3R1 and other *C. parvum* subtypes increased in the Newcastle region and on the outskirts of Sydney. Port Macquarie and Wagga Wagga both showed clusters made up of *C. hominis* IbA10G2 and other *C. parvum* subtypes. Within Sydney, *C. hominis* IbA10G2 infections dominated the eastern and north–western suburbs, Mangrove Mountain, Jilliby and Penrith.

In 2009, *C. parvum* infections were more common than *C. hominis*. State–wide analyses showed an increased incidence of *C. parvum* IIaA18G3R1 and other *C. parvum* subtypes in

Sydney, Newcastle, Dubbo, Wagga Wagga and in the north-eastern regions of NSW. The increased *C. parvum* infection trend continued within Sydney. *Cryptosporidium parvum* replaced the *C. hominis* IbA10G2 clusters previously seen in Mangrove Mountain and the eastern and north-western suburbs. The incidence of *C. hominis* IbA10G2 remained high in Jilliby.

Both *C. parvum* and *C. hominis* were contributing equally to sporadic disease in 2010. Bathurst remained dominated by *C. hominis* IbA10G2 whilst regions surrounding Port Macquarie exhibited both *C. hominis* and *C. parvum* infections. Isolated *C. hominis* and *C. parvum* clusters were seen throughout Sydney. However, two *C. parvum* subtype clusters appeared in the Richmond area.

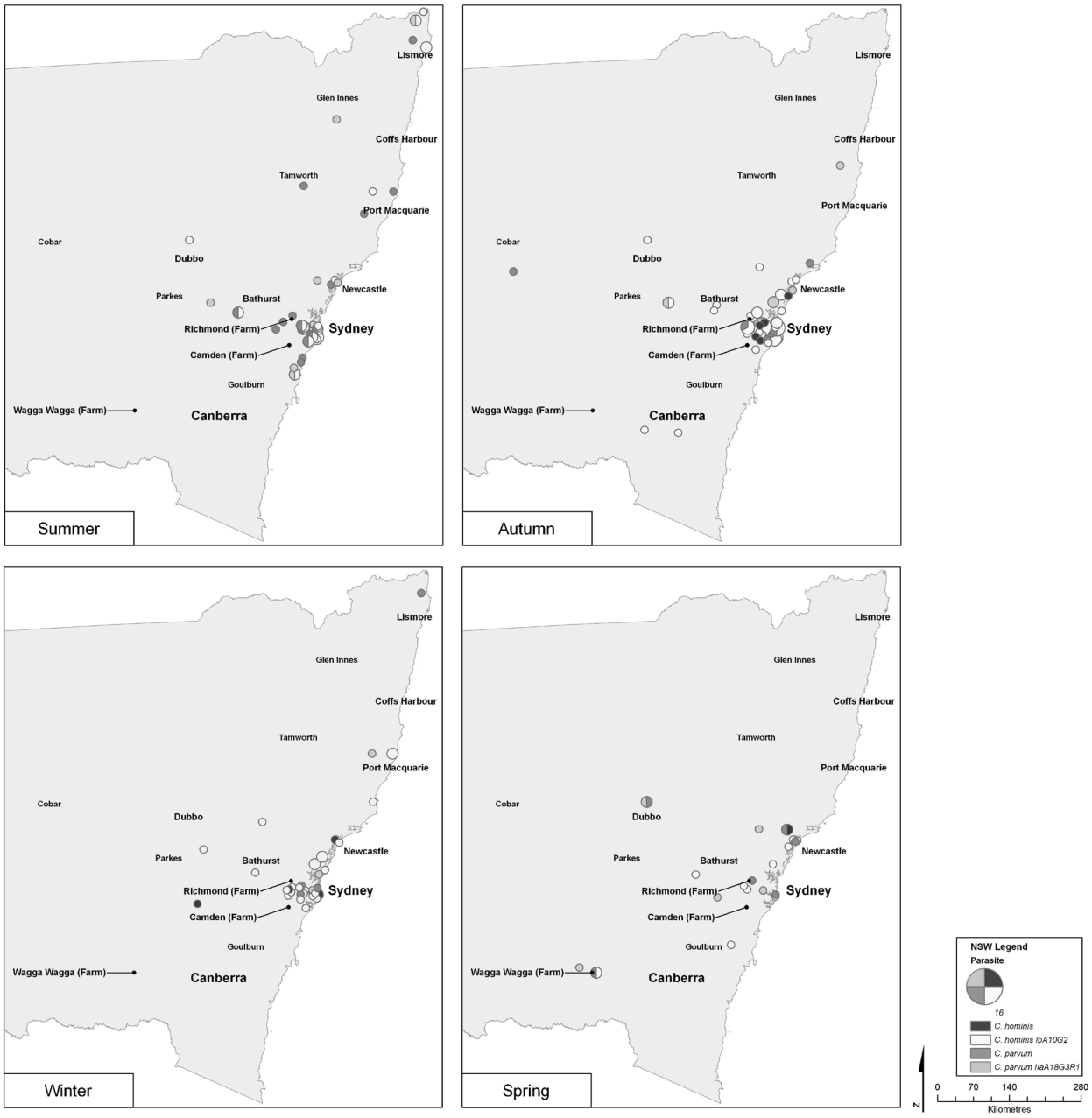
Figure 4.2 Temporal analysis by year of the patients infected with the *C. hominis* IbA10G2 subtype, other *C. hominis* subtypes, *C. parvum* IIaA18G3R1 and other *C. parvum* subtypes in NSW and Sydney, between January 2008 and December 2010. The size of the circle represents the number of cases.



4.4.6 Seasonal distribution of infections, species and subtypes

To determine the seasonal distribution of sporadic cryptosporidiosis, samples were divided into their respective seasons: summer, 59 samples; autumn, 85 samples; winter, 43 samples; spring, 22 samples. Summer sporadic cases were concentrated along the coastal areas of Sydney – Newcastle and in the north-eastern regions of the state (Fig 4.3). Both *C. hominis* and *C. parvum* were represented equally and all *C. hominis* infections were attributed to subtype IbA10G2. A high incidence of *C. parvum* infections was seen in the Richmond area. Autumn infections increased in western Sydney regions and along the coast between Sydney and Newcastle. *Cryptosporidium hominis* IbA10G2 shows a higher incidence than *C. parvum* infections. Infections with other *C. hominis* subtypes also appeared in western Sydney regions. Dubbo remained dominated by *C. hominis* IbA10G2 throughout both summer and autumn. Winter infections showed small clusters that were confined to the Sydney region. *Cryptosporidium hominis* IbA10G2 was the most common parasite. Spring infections were more dispersed throughout Sydney and the surrounding areas and an increased incidence was seen in Wagga Wagga and Dubbo. Spring showed a higher incidence of *C. parvum* throughout the state. Dubbo, previously dominated by *C. hominis* IbA10G2, was affected by *C. parvum* IIaA18G3R1 and other *C. parvum* subtypes. This trend was repeated in Wagga Wagga and Richmond.

Figure 4.3 Temporal analysis by season of the patients infected with the *C. hominis* IbA10G2 subtype, other *C. hominis* subtypes, *C. parvum* IIaA18G3R1 and other *C. parvum* subtypes in NSW and Sydney, between January 2008 and December 2010. The size of the circle represents the number of cases.



4.5 Discussion

Essential to managing cryptosporidiosis and reducing risks of continued disease prevalence is knowledge on the species contributing to disease and the potential zoonotic sources. This study used a spatial-based approach to investigate sporadic human cryptosporidiosis between January 2008 and December 2010.

Sixty–six percent of human infections in this study were caused by *C. hominis.* This observation is consistent with previous Australian studies investigating sporadic human cryptosporidiosis (O'Brien et al., 2008). No gender bias or regional segregation between the distribution of *C. hominis* and *C. parvum* infections was detected. Results indicated that both genders were most likely to be infected with *C. hominis* and that the disease was most prevalent in the 0 – 4 year age group.

The diversity within the *gp60* of *C. hominis* and *C. parvum* isolates was extensive with 5 subtype families and 31 subtypes within those families identified. The most common subtype was *C. hominis* IbA10G2 which was identified in 60% of samples. This subtype is the most common cause of sporadic cryptosporidiosis around the world, attributing to 44.5% of total reported infections (Jex and Gasser, 2010). Subtype IbA10G2 was also previously identified as the most common cause of sporadic disease in Australia and was the cause of the 2009 NSW waterborne outbreak (Jex et al., 2008; O'Brien et al., 2008; Waldron et al., 2009b)(L.S. Waldron, B.C. Ferrari, C. Cheung-Kwok-Sang, P.J. Beggs, N. Stephens and M.L. Power, Submitted for publication). The second most frequently detected *Cryptosporidium* subtype from humans was *C. parvum* IIaA18G3R1 which was identified in 15% of isolates. The occurrence of this subtype in humans was first reported in Australia and has previously been identified as the most common *C.*

parvum subtype causing sporadic cryptosporidiosis in NSW (Chalmers et al., 2005; Jex et al., 2007; Jex et al., 2008; O'Brien et al., 2008; Waldron et al., 2009b).

The epidemiology of human cryptosporidiosis is a complex interplay between humans, domestic animals, livestock, wildlife and the environment. Knowledge of the *Cryptosporidium* species infecting these sources and understanding the factors influencing contact between these sources is essential to cryptosporidiosis management. Analysis of *Cryptosporidium* from cattle samples was included in this investigation to determine their zoonotic potential for contribution to human cryptosporidiosis. Results showed that all subtypes identified in cattle were a frequent cause of sporadic human cryptosporidiosis in NSW. Of particular concern are the *C. parvum* IIaA18G3R1 and *C. hominis* IbA10G2 subtypes which were the most frequently detected subtypes in humans in NSW, with *C. parvum* IIaA18G3R1 attributing to 47% of cattle infections. This observation has been previously identified in Australian cattle in both Perth and Tamworth (Ng et al., 2008; O'Brien et al., 2008). Interestingly, *C. hominis* IbA10G2 was detected on two farms, in the regional area of Wagga Wagga and in the semi–rural locality of Richmond, Sydney. *Cryptosporidium hominis* is thought to be host-specific for humans, so the identification of this species in cattle was unexpected. However, the detection of a DNA sequence in a sample is not indicative that an infection is occurring. To ascertain that *C. hominis* infections were present in cattle, oocyst presence was confirmed by fluorescent microscopy which revealed that all cattle infected with *C. hominis* were shedding between $10^1 – 10^2$ oocysts/g, indicating low level infections. Unfortunately the *C. andersoni* infection, which was identified in one human sample by molecular analysis, was unable to be confirmed by flow cytometry or microscopy due to limited sample material Increasingly, diverse species of *Cryptosporidium* are being recognised as having zoonotic capabilities. Knowledge of the zoonotic threats that different *Cryptosporidium*

species pose to vertebrate hosts and how they circulate through the environment is essential to future disease management.

Expanding human populations, urbanisation and intensifying agricultural practices will influence the transmission of *Cryptosporidium* through the environment and will bring disease sources into closer contact. Visualisation of the geographic distribution of *Cryptosporidium* species and infections in zoonotic sources facilitates the identification of hotspot zones and the different disease risks posed to these areas. Sporadic human cryptosporidiosis was highest in the urban coastal cities of Sydney, Newcastle and Port Macquarie in addition to regional inland areas of Wagga Wagga, Bathurst and Dubbo. Both *C. hominis* and *C. parvum* were prevalent in these areas. Due to the different infection capabilities of *C. parvum*, it was undetermined whether infections were from anthroponotic or zoonotic disease sources. Seasonal differences were shown between the prevalence of *C. hominis* and *C. parvum* infections. *Cryptosporidium hominis* infections, particularly with subtype IbA10G2, dominated throughout the state and within the Sydney region in winter. Conversely, the majority of spring infections were attributed to *C. parvum*. This was most pronounced in the regional areas of Dubbo, Wagga Wagga and Richmond. The increased incidence of *C. parvum* in these rural areas was likely attributed to the calving season. Cattle samples analysed in this study were sampled in spring and showed the presence of *C. hominis* and *C. parvum* subtypes frequently identified in humans. Spring spatial analysis of sporadic human infections showed disease clusters in Wagga Wagga and Richmond where infections were caused by *C. parvum* subtypes identified in cattle in those regions. As human populations expand towards and into more rural areas, farm management practices will become pivotal for controlling zoonotic disease transmission, especially when community-shared recreational water is in close proximity.

Spatial analyses showed 7 disease hotspot zones throughout NSW: 4 in the urban coastal cities of Sydney, Newcastle, Port Macquarie and Lismore, and 3 in the regional areas of Wagga Wagga, Dubbo and Bathurst. All areas were equally impacted by *C. hominis* and *C. parvum*, except for Bathurst which maintained *C. hominis* infections over the 3 year study period. From the recent finding of *C. hominis* infections in cattle from NSW, cattle in Bathurst need to be investigated to determine if they are contributing to human disease in this regional locality. Within Sydney, Mangrove Mountain was identified as a hotspot zone. This is a recreational area with numerous camping grounds, recreational water activities, agriculture and wildlife. Incidentally, this was also the location of the human *C. fayeri* infection. Screening of wildlife and livestock in this area needs to be conducted to determine the zoonotic disease risks posed to both humans and animals.

Cryptosporidium is widespread in NSW and has complex transmission pathways involving humans, cattle and native Australian wildlife. Urbanisation, increased agriculture and population expansion will all contribute to the continued emergence of this disease in NSW. From this, understanding the contact and transmission pathways that occur between different hosts, in addition to knowing the parasites present in these sources, will become an essential component for disease management. This study has provided initial data on hotspot zones and population groups most at risk of disease in NSW. Targeted surveillance in these regions, and understanding human–animal contact, will enable the development and implementation of successful disease prevention measures.

4.6 Acknowledgements

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5. *Cryptosporidium fayeri* an emerging zoonoses

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Wildlife-associated *Cryptosporidium fayeri* in human, Australia

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5.1 Abstract

As urban populations expand towards wildlife boundaries, contact with potential zoonotic sources will increase. The frequency of zoonotic infections by the genus *Cryptosporidium* is essentially unknown. Increasing numbers of human infections with *Cryptosporidium* species previously considered to be specific to other vertebrate hosts are being reported. Here, the first human infection with *Cryptosporidium fayeri*, a species considered to be specific to marsupials, is documented. To determine the zoonotic role of marsupials in human cryptosporidiosis, wildlife inhabiting the area where the human infection occurred were screened for the presence of *Cryptosporidium*. The variable hosts specificities of *Cryptosporidium* species and their transmission between humans, domestic animals and wildlife makes it a parasite of concern to many authorities and industries. This study takes the first step in the surveillance and control of emerging zoonoses.

5.2 Introduction

Molecular tools have become essential for *Cryptosporidium* species identification, taxonomy and epidemiology due to morphological similarities exhibited by species within this genus. Molecular analyses have now identified 22 *Cryptosporidium* species and more than 40 genotypes across all vertebrate classes (Fayer, 2010). The myriad of potential *Cryptosporidium* hosts, in conjunction with the robustness of the infectious stage (oocyst) means diverse *Cryptosporidium* species constantly circulate through the environment. This increases the potential for disease to initiate from a diversity of contamination sources. Molecular analyses have shown that the epidemiology of human cryptosporidiosis is a complex relationship between humans, domestic animals, livestock, wildlife and the environment.

Human cryptosporidiosis is a global problem causing illness in the young, elderly, immunocompromised and immunocompetent individuals in both developed and developing nations. The two most common etiologic agents, responsible for 90% of reported human infections are *Cryptosporidium hominis* and *Cryptosporidium parvum* (Morgan-Ryan et al., 2002; Xiao and Ryan, 2004). Additional species identified as human pathogens are *C. melegridis*, *C. canis*, *C. felis*, *C. suis*, *C. muris*, *C. ubiquitis* and *C. cuniculus*. Each of these were once thought to be specific for turkeys, dogs, cats, pigs, mice, deer and rabbits respectively (Ditrich et al., 1991; Pedraza-Diaz et al., 2000; Pedraza-Diaz et al., 2001; Xiao et al., 2001; Caccio et al., 2002; Chalmers et al., 2009). Incidental findings of *C. andersoni*, *C. hominis* monkey genotype, *C. parvum* mouse genotype and *Cryptosporidium* cervine (W4), chipmunk I (W17), skunk and horse

genotypes have also been reported in humans (Chalmers et al., 2009). The pathogenicity of these zoonotic species and genotypes to humans remains unclear.

Of all emerging infectious diseases (EID), zoonoses from wildlife represent the most significant, growing threat to global health. Over the last 60 years wildlife zoonoses have attributed to 71% of global EID events (Jones et al., 2008). High levels of wildlife biodiversity, land use practices such as agriculture, and expanding human populations are all correlated with the increasing incidence of wildlife zoonoses (Jones et al., 2008). Population expansion towards wildlife boundaries places a demand on research to identify *Cryptosporidium* species present in wildlife sources and to analyse the risks posed to human and animal health. However, there are limited investigations that have analysed wildlife inhabiting the urban fringes of expanding cities. Within Australia, *Cryptosporidium* species have been identified in 14 Australian marsupial hosts and includes *C. fayeri*, *C. macropodum*, *C. muris* and the brushtail possum I and II genotypes (Hill et al., 2008; Power, 2010). Native Australian marsupials naturally inhabit water catchments and have evolved to co-exist in the presence of humans, for example possums residing in urban areas and kangaroos in agricultural and recreational zones.

This study documents the first human *C. fayeri* infection, a finding which highlights the importance of understanding the factors that increase contact between humans and wildlife. Additionally, eastern grey kangaroos (*Macropus giganteus*), an abundant *C. fayeri* host, in the region where the patient was infected were screened for *Cryptosporidium*. This takes the first step in the surveillance and control of emerging zoonoses and in the development of predictive approaches to disease emergence.

5.3 Materials and methods

5.3.1 Study site

The patient was residing in a National Park on the east coast of New South Wales, Australia. Kangaroo faecal samples were collected from two recreational areas and one rural area that bordered the National park. Recreational areas, Webbs Creek and Milli Creek are camping grounds with a variety of recreational water activities. Both of these areas are inhabited by an abundance of free ranging Australian Eastern Grey Kangaroos. Mangrove Creek, the third site, was a rural residential area bordered by the National park.

5.3.2 Sample sources, DNA extraction and parasite enumeration

The human specimen, MQ1022, was collected from a 29 year old female in July 2009. The faecal sample tested *Cryptosporidium* positive by a pathology company in Sydney, Australia using the Remel ProSpecT *Giardia*/*Cryptosporidium* microplate assay. Oocysts were purified from faeces using a sucrose flotation gradient (Truong and Ferrari, 2006) and DNA extracted from purified oocysts using *PrepGem*® (Zygem Corporation Ltd, Hamilton, New Zealand) (Ferrari et al., 2007). Oocysts were fluorescently stained with the *Cryptosporidium*-specific antibody CRY104 labeled with FITC (Biotech Frontiers, Sydney, Australia) and enumerated using epi-fluorescence microscopy at 400X magnification.

Kangaroo faecal samples, collected in March 2011, were collected opportunistically off the ground. Samples were determined to be fresh by the morphology and appearance of moisture. In total, 76 faecal samples were collected; Webbs Creek, n = 64; Milli Creek, n = 8 and Mangrove

Creek, n = 4. DNA extraction from Kangaroo samples was performed using the Bioline isolate faecal DNA Kit (Bioline, Sydney, Australia) following the manufacturers instructions.

5.3.3 Identification of *Cryptosporidium* species

Cryptosporidium species were identified using a previously described PCR- RFLP protocol targeting the 18S rRNA gene fragment (Xiao et al., 1999b). Primary and secondary reactions contained 6 mM $MgCl_2$, 200 µM dNTPs, 200 nM of each primer and1 U of RedTaq® polymerase (Thermo Scientific, Australia). For the primary reaction 2 µl of DNA was used and 1 µl of the primary PCR product served as the DNA template in the secondary reaction. Reaction conditions comprised an initial denaturation of 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for 1 min, and a final extension step of 72°C for 7 min. All PCR's performed included a negative control containing PCR water and a positive control containing *C. parvum* DNA. Reactions were run on Eppendorf Mastercycler Personal instruments (Eppendorf, North Ryde, Australia). PCR products were resolved by electrophoresis on 2% w/v agarose gel containing Sybr Safe (Invitrogen, Mulgrave, Australia) and visualised under UV light. Secondary products of the correct size (~830 bp) were purified using the QIAquick PCR Purification Kit (Qiagen, Melbourne, Australia) following the manufacturers instruction.

To identify species, and the possibility of a mixed infection, purified 18S rRNA PCR products were cloned using the TOPO-TA vector cloning system (Invitrogen, Australia). Plasmids from 50 clones were recovered using the Qiagen plasmid kit (Qiagen, Melbourne, Australia) and digested with the enzyme *SspI* (New England Biolabs)(Xiao et al., 1999b). Restriction fragments were separated on 3.5% w/v agarose gels containing Sybr Safe at 100V and patterns were visualised under UV light. Samples displaying unique RFLP patterns were sequenced using an ABI 3130xl genetic analyser (Applied Biosystems, Foster City, California)

with the BigDye™ terminator kit (Applied Biosystems). Nucleotide sequences were analysed using Geneious v4.8.2 (Biomatters Ltd, Auckland, New Zealand).

5.3.4 PCR competency of kangaroo faecal samples

PCR competencey of kangaroo DNA samples was performed to ensure the removal of inhibitors through the faecal purification process. Analysis was performed by making a spiked-mixture containing 10 µl of the purified DNA and 5 µl of control *C. parvum* DNA. This mixture served as the DNA template and was analysed by the nested 18S rRNA method described previously. PCR products were resolved by electrophoresis on 2% w/v agarose gel containing Sybr Safe.

5.3.5 Actin amplification

A previously described nested actin amplification was performed to confirm the identification of *Cryptosporidium* species. Primary and secondary reactions contained 3 mM $MgCl_2$, 200 µM dNTPs, 200 nM of each forward and reverse primer and 1 U of RedTaq® polymerase. Primary reactions used 2 µl of DNA template, 1 µl of the primary PCR product served as the DNA template in the secondary reaction. Reaction conditions comprised an initial denaturation at 94ºC for 5 min followed by 35 cycles of 94ºC for 45 s, 50ºC for 45 s and 72ºC for 1 min, with a final extension at 72ºC for 10 min. PCR products were separated by 2% w/v agarose gel containing Sybr safe and were visualised under UV light. Products containing the correct size fragment (~1066 bp) were purified, sequenced and analysed as previously described.

5.3.6 *gp60* amplification

Cryptosporidium subtype family and subtype identification was determined using a previously described nested PCR targeting the hypervariable *gp60* gene (Waldron et al., 2009b). Primary and secondary reactions contained 4 mM $MgCl_2$, 200 µM dNTPs, 200 nM of each forward and reverse primer and 1 U of RedTaq® polymerase. Primary reactions used 2 µl of DNA template and secondary reactions used 1 µl of the primary PCR as the DNA template. Reaction conditions comprised an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 58°C for 45 s and 72°C for 1 min 30 s, with a final extension at 72°C for 7 min. PCR products were separated by 2% w/v agarose gel containing Sybr safe and were visualised under UV light. Products containing the correct size fragment (~1000 bp) were purified, sequenced and analysed as described above. Isolates were assigned a subtype according to the nomenclature system previously described (Sulaiman et al., 2005).

Cryptosporidium nucleotide sequences generated in this study were submitted under GenBank ID: HQ008932 – HQ008924.

5.4 Results

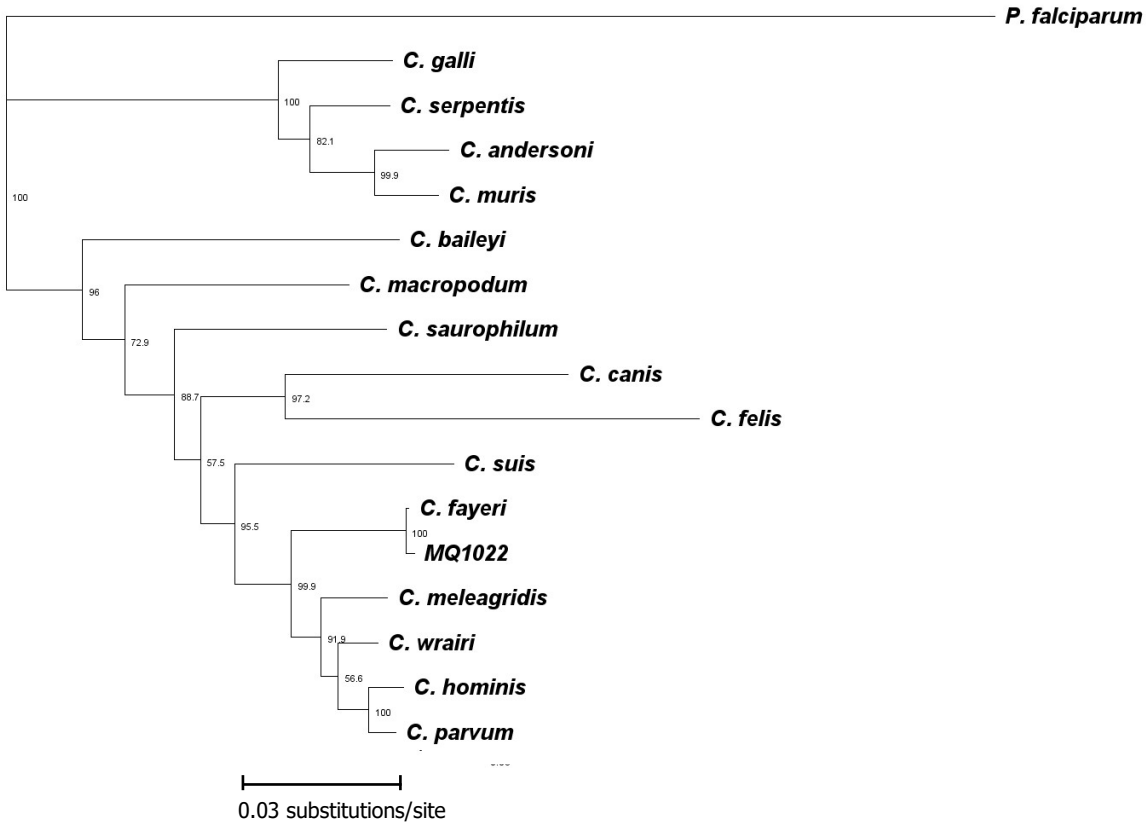
5.4.1 *Cryptosporidium* enumeration, and species and subtype identification in sample MQ1022

Prior to molecular analysis, *Cryptosporidium* oocysts were visualised using epi-fluorescence microscopy. A parasite count of 1.34 $x10^6$ oocysts /gram of faeces was determined for the human *Cryptosporidium* sample.

Sample MQ1022 generated a positive 18S rRNA PCR product which was subsequently purified and cloned. RFLP analysis of 50 purified plasmids identified two different *SspI* restriction profiles. Profile 1 contained fragment sizes of 33, 109, 247 and 441 bp and profile 2 had 33, 254 and 540 bp fragments. Sequencing of the purified plasmids identified the two profiles as *C. fayeri* type 1 and type 2. These sequences correspond to known heterogeneity within the 18S rRNA of *C. fayeri*.

Amplification of the actin gene was performed to confirm the identification of *C. fayeri*. Sample MQ1022 produced a positive actin PCR product and sequence analysis confirmed the 18S rRNA result, showing a 99.8% similarity to the *C. fayeri* actin gene (Figure 5.1).

Figure 5.1 Phylogenetic relationship between isolate MQ1022 and valid *Cryptosporidum* spp. inferred by neighbour joining. The tree was constructed by concatenating sequences of ssu rRNA and actin loci. *Plasmodium falciparum* was used as the outgroup for each locus. Bootstrap values (>50%) for 1,000 replicates are indicated.



Gp60 amplification produced a positive PCR product for MQ1022. The MQ1022 *gp60* sequence was 98% similar to *C. fayeri* subtype family IVa (Table 5.1). Analysis of the microsatellite region further characterized isolate MQ1022 to *C. fayeri* subtype IVaA9G4T1R1.

Table 5.1 The similarity of isolate MQ1022 to *C. fayeri gp60* subtype families

C. fayeri Subtype Family	Similarity to MQ1022 (%)
IVa	98.6
IVb	89.8
IVc	86.5
IVd	87.1
IVe	77.8
IVf	75.5

5.4.2 18S rRNA screening and PCR competency of kangaroo DNA samples

Amplification of the 18S rRNA was unsuccessful for 76/76 samples from kangaroos. To confirm that PCR inhibitors weren't present, *C. parvum* DNA was used to spike the samples. Amplification of the *C. parvum* spiked DNA was successful for 76/76 samples. Results showed that *Cryptosporidium* in the original kangaroo samples was either absent or present at a low level.

5.5 Discussion

Of all emerging infectious diseases, zoonoses from wildlife represent the most significant threat to global health. This study was the first to report a human *C. fayeri* infection, a species believed host specific to marsupials. Targeted screening of kangaroos in the locality of the human *C. fayeri* infection was conducted to determine the zoonotic role that these animals play in human cryptosporidiosis.

To date, *C. fayeri* has been identified in six Australian marsupial species. The *C. fayeri* infected patient was residing in a national park on Australia's east coast, and contact with native marsupials was frequent. The identification of *C. fayeri* in a human patient is a concern for water catchment authorities in the Sydney region. The main water supply for Sydney, Warragamba Dam, covers an area of 9, 050 square kilometers and is surrounded by national forest inhabited by diverse and abundant marsupials. A previous study investigating *Cryptosporidium* in a wild eastern grey Kangaroo population reported a prevalence of 6.72% and oocyst shedding ranged from 20/g of faeces to as high as 2.0 x10^6/g faeces (Power et al., 2005). Subtype IVaA9G4T1R1 identified from the patient in this study has been characterized from Eastern Grey Kangaroos in Warragamba Dam (Power et al., 2009). Throughout the year large groups of eastern grey kangaroos graze within riparian zones in the catchment. Such close proximity to the water presents a high possibility for water contamination with oocysts from these animals.

Population expansion towards wildlife boundaries will increase the contact between humans, domestic pets, livestock and wildlife. This places a demand on research to analyse pathogen transfer at wildlife boundaries. Kangaroos inhabiting the National Park where the

patient was residing at the time of infection were screened for *Cryptosporidium*. *Cryptosporidium* was not detected in any of the samples indicating either the absence of *Cryptosporidium* or low level infections. Due to the negative results, the source of the human infection was not determined. Seasonal patterns of oocyst shedding have been reported in both captive and wild marsupials. The peak occurs throughout the autumn months, coinciding with the weaning of juvenile offspring. In a similar way to cattle, which maintain asymptomatic cryptosporidiosis infections and serve as a reservoir for *Cryptosporidium*, adult eastern grey kangaroos also maintain low level asymptomatic infections. Additionally, due to naïve immune systems cryptosporidiosis is more pronounced in juvenile hosts. Communal grazing of eastern grey kangaroos, in combination with constant defecating, exposes susceptible juveniles to the *Cryptosporidium* parasite. Kangaroo sampling was conducted in early autumn and the majority of faecal specimens collected were large in size, indicating they were from adults. A second round of sampling in the later months of autumn may have increased the juvenile sample size, in turn increasing the chance of detecting *Cryptosporidium*. The kangaroo population size in this region is unknown and it is possible individuals were sampled multiple times, thereby biasing the molecular results. Multiple sampling rounds would not only have increased the chances of identifying *Cryptosporidium* but would also have limited the bias of single point sampling. The site analysed in this study is a rural environment comprising of livestock, wildlife, recreational water, camping facilities and human residents serviced by septic tanks. The potential for a spill over event, between communally grazing cattle and kangaroos or from humans to these animals, will facilitate the establishment of a *Cryptosporidium* species in a new host. *Cryptosporidium hominis*, previously considered host specific to humans, has been identified in cattle. From this, research needs to consider the potential threat that humans pose to native wildlife.

The *Cryptosporidium* genus is diverse, both in species and suitable hosts. The mechanisms of host specificity remain unknown, however there is an increased frequency of *Cryptosporidium* species crossing the host barrier and becoming zoonoses. This increase indicates that host specificity is not as clear cut as previously thought. The emergence of *C. fayeri* as a zoonotic species and the etiologic agent responsible for a recent human infection represents a significant concern for human health. Classification for *Cryptosporidium* relies heavily on molecular typing of isolates from an individual animal representative of a host group. Assumptions made on host specificity using such limited data biases knowledge of the biology of this parasite. Host specificity is critical for understanding the risks posed to human and animal health by *Cryptosporidium* species but current trends are creating confusion in an already taxonomically problematic genus.

5.6 Acknowledgments

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6. Fluorescence analysis detects *gp60* subtype diversity in *Cryptosporidium* infections

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Keywords: Key words: *Cryptosporidium;* T-RFLP; *gp60*; Fluorescence; infection

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6.1 Abstract

Ninety percent of human cryptosporidiosis infections are attributed to two species; the anthroponotic *Cryptosporidium hominis* and the zoonotic *C. parvum*. Sequence analysis of the hypervariable *gp60* gene, which is used to classify *Cryptosporidium* to the subtype level, has highlighted extensive intra-species diversity within both *C. hominis* and *C. parvum*. The *gp60* has also facilitated contamination source tracking and increased understanding of the epidemiology of cryptosporidiosis. Two surface glycoprotein's, the gp40 and gp15 are encoded in the *gp60* gene; both are exposed to the hosts' immune system and play a pivotal role in the disease initiation process. The extent of genetic diversity observed within the *gp60* would support the hypotheses of significant selection pressure placed on the gp40 and gp15. This study used a dual fluorescent terminal-restriction fragment length polymorphism (T-RFLP) analysis to investigate the genetic diversity of *Cryptosporidium* subtype populations in a single host infection. Terminal-RFLP showed subtype variation within one human *Cryptosporidium* sample and mouse samples from seven consecutive passages with *C. parvum*. Furthermore, this was the first study to show that differences in the ratio of subtype populations occur between infections. T-RFLP has provide a novel platform to study infection populations and to begin to investigate the impact of the hosts immune system on the *gp60* gene

6.2 Introduction

Molecular tools are essential to differentiate species within the morphologically indistinguishable *Cryptosporidium* genus. To date, DNA sequence analysis has facilitated the identification of 22 *Cryptosporidium* species and greater than 40 genotypes (Fayer, 2010; Robinson et al., 2010). Of the 22 described species, 10 have been reported in humans, however the majority of human infections are caused by two species; the anthroponotic *Cryptosporidium hominis* and the zoonotic *Cryptosporidium parvum* (Morgan-Ryan et al., 2002; Chalmers et al., 2009; Waldron et al., 2010)

Extensive intra-species diversity within *C. hominis* and *C. parvum* has been observed through sequence analysis of the hypervariable *gp60* gene. The *gp60* gene encodes a larger precursor glycoprotein, the gp60 (also called S60 or gp40/15) which is proteolitically cleaved into two mature glycoprotein's, the gp40 and gp15 (O'Connor et al., 2007). Localised to the outer membrane of the invasive form of the parasite, the sporozoite, these two cleavage products are significant in the disease process. The gp15, the C-terminal cleavage product, is anchored to the sporozoite membrane by a glycosylphosphatidyl inositol (GPI) moiety and is the most immunodominant antigen identified in *Cryptosporidium* to date (O'Connor et al., 2007; Ajjampur et al., 2011). Gp40, The N-terminal cleavage product is not directly linked to the sporozoite membrane and is predicted to be soluble (O'Connor et al., 2007). Association between the gp15 and gp40 cleavage products is not completely understood but it is believed they form a protein complex that facilitates the linkage between the sporozoite and the hosts' epithelial cells (Winter et al., 2000). The attachment and ensuing invasion of host cells is mediated by the gp40 cleavage

product (Strong et al., 2000; Winter et al., 2000). The *gp60* gene is the most polymorphic gene identified in *Cryptosporidium* to date. Epidemiological studies have taken advantage of this extensive variation and use the gene as an identification tool to further classify *Cryptosporidium* species to the subtype family and subtype levels (Cevallos et al., 2000; Winter et al., 2000; Sulaiman et al., 2005). To date 6 *C. hominis* subtype families and 11 *C. parvum* subtype families have been identified according to nucleotide polymorphisms located in conserved regions of the *gp60* gene (Xiao, 2010). Within the N-terminus region of the gp40 cleavage product there is a hypervariable microsatellite region that contains serine amino acid polymorphisms. The number and sequence of the serine codons vary significantly and are utilised to identify *Cryptosporidium* subtypes (Sulaiman et al., 2005).

The extent of genetic diversity observed in the gp40 and the continuing identification of new subtypes would support the hypotheses of significant selection pressure on this protein complex. Such evolutionary selection would create a high mutation rate at the population level, seen as subtype variability, within an infection. Diversity at the population level has not been reported but such studies require analysis of individuals. The common approach to examining the *gp60* is PCR amplicon sequencing, a method focussed to the population level, which results in identification of the predominant subtype within a population.

Here, we apply a dual fluorescence terminal restriction fragment length polymorphism (T-RFLP) to the *Cryptosporidium gp60* gene and apply this methodology to examine the genetic diversity of *Cryptosporidium* populations of a single host infection.

6.3 Materials and methods

6.3.1 T-RFLP development

The *gp60* sequences of *C. hominis* and *C. parvum* isolates representing eight different *gp60* subtype families (GenBank accession numbers FJ839873 – FJ839883) (Waldron et al., 2009b) were used to determine the expected lengths of Terminal-Restriction Fragments (T-RFs) generated after digestion with *RsaI*

DNA samples of these eight isolates were used to validate the T-RFLP protocol that comprised a nested protocol and restriction digestion with *RsaI* (Chalmers et al., 2007a). The primers S60.F728 and S60.OutR (Waldron et al, 2009b) were used in the primary amplification. The secondary PCR utilized the previously described S60.ATGF forward primer (Waldron et al., 2009b) in addition to the new reverse primer DV60r 5'-GCVACTTCRATTCYYYTACCACC. Both secondary primers were fluorescently labeled at the 5' end. The forward primer, S60.ATGF, which targets a region in the gp40, was labeled with 6-carboxyfluorescein (6-FAM) (Proligo, Lismore, Australia) and the reverse primer DV60r, which targets within the gp15 region, was labeled with PET (Applied Biosystems, Foster City, USA). Reaction conditions were optimized for primer annealing temperature and for dual fluorescence using gradient PCR.

Primary and secondary reactions contained 4 mM $MgCl_2$, 200 µM dNTPs, 200 nM of each forward and reverse primer, 2% dimethyl sulfoxide and 1 U of Accutaq (Sigma-Aldrich, Sydney, Australia). Primary reactions included 2 µl of DNA template, and secondary reactions included 1 µl of the primary PCR as the DNA template. The cycling conditions comprised an initial denaturation at 94ºC for 3 min, followed by 35 cycles of 94ºC for 45s, 58ºC for 45s, and 68ºC for

1 min 30s, and a final extension step of 68ºC for 7 min. PCR products were separated by 2% w/v agarose gel containing Sybr safe (Invitrogen, Mulgrave, Australia) and visualised under UV light.

Secondary products that generated the expected amplicon size (~800 bp) were purified and subjected to restriction digestion. Purified secondary products (5 µL) were digested with *RsaI* (10 U/µL; Roche Diagnostics, Mannheim, Germany) with the supplied Buffer L in a 37ºC water bath for 2 hrs. Digests were run on 3.5% w/v agarose gels containing Sybr safe before being analysed at the Macquarie University DNA sequencing facility as previously described (Waldron et al., 2009a). T-RF sizes were determined from the internal size standard Liz1200 using the GeneScan software, version 4.0 (Applied Biosystems).

6.3.2 Subtype family and subtype identification by T-RFLP

The optimized T-RFLP methodology was evaluated using 99 *Cryptosporidium* samples. Ninety-two human and cattle samples, previously identified to the species and *gp60* subtype levels (Waldron et al., 2009b; Waldron et al., In Review-a; Waldron et al., In Review-b) were used to develop T-RFLP. These samples represented a variety of subtypes; Ia (2/92), Ib (58/92), Id (2/92), Ie (2/92), If (3/90), IIa (22/92), IIc (1/92), IId (2/92).

An additional seven samples, *C. parvum* oocysts from consecutive passages through neonatal mice (*Mus musculus*), were obtained from Murdoch University, Perth, Australia. DNA was extracted from oocysts using the Bioline isolate fecal DNA kit (Bioline, Sydney, Australia) and the subtype identification was performed using a previously described nested *gp60* PCR protocol (Waldron et al., 2009b).

6.3.3 Population analysis

During validation of T-RFLP multiple 6-FAM peaks, indicating the presence of multiple subtypes, were visualized in one human sample and the seven mouse samples. These were selected for further analysis to determine what the additional peaks were. Samples (n = 8) were cloned using the TOPO-TA vector cloning system (Invitrogen, Australia). For the human sample, 50 clones were selected for T-RFLP. Twenty-five clones were analysed for each mouse sample. Plasmids were purified from clones displaying unique peak profiles using the Qiagen plasmid kit (Qiagen, Melbourne, Australia). Samples were then sequenced using an ABI 3130xl genetic analyser (Applied Biosystems, Foster City, California) with the BigDye™ terminator kit (Applied Biosystems). Sequence traces were checked for ambiguities and manually corrected using Geneious v4.8.2 (Biomatters Ltd, Auckland, New Zealand). The samples were assigned a subtype according to the nomenclature system as described previously (2005).

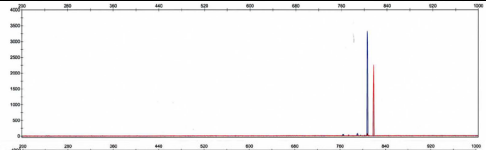
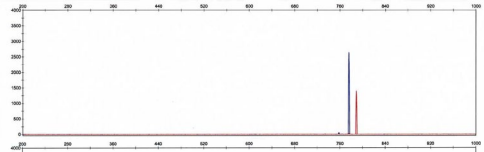
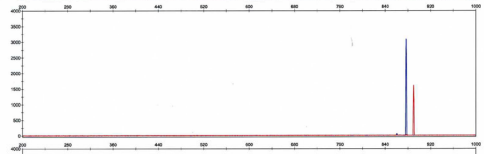
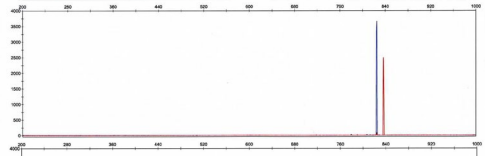
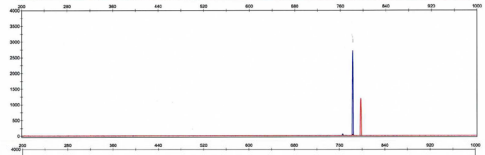
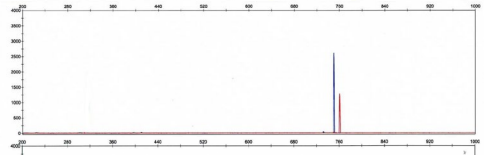
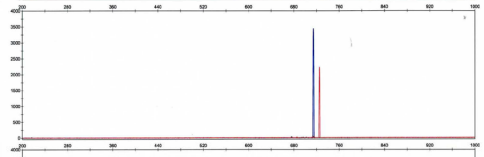
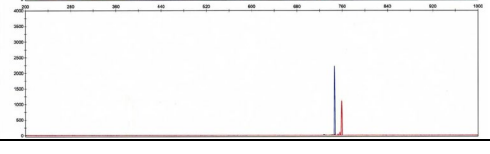
Cryptosporidium gp60 sequences generated in his study were submitted to GenBank. *Gp60* sequences obtained from the mouse samples (section 2.1) were submitted under GenBank ID: JF727763 – JF727769 and cloned *Cryptosporidium* samples (section 2.3) under GenBank ID: JF727770 – JF727775.

6.4 Results

6.4.1 Subtype family identification by T-RFLP analysis

The optimal protocol for PCR, determined using gradient PCR (data not shown) and the restriction digestion methods were evaluated to ensure that dual fluorescence was maintained through these processes. Red peaks and blue peaks corresponding to subtype families and subtypes respectively, were recorded for 8 samples representing different *Cryptosporidium* subtype families after capillary electrophoresis (Table 6.1).

Table 6.1 Detection of dual fluorescence in the fluorescent *gp60* PCR reaction using 6-FAM and PET.

Species	Subtype family	Electropherogram
C. hominis	Ia	
	Ib	
	Id	
	Ie	
	If	
C. parvum	IIa	
	IIc	
	IId	

Expected T-RFs for eight subtype families; *C. hominis* Ia, Ib, Id, Ie, and If and *C. parvum* subtypes IIa, IIc and IId were determined using *gp60* sequences (Table 6.2). Based on existing sequence data subtype families could be differentiated by the 3' T-RF. The same 3' T-RF was modeled for *C. hominis* Id and If and suggested that these two families may not be differentiated using T-RFLP. As per modeled data differentiation was achieved for 6/8 subtype families, the remaining two subtype families, *C. hominis* Id and If, both produced T-RF's of 312 bp (Table 6.2).

Table 6.2 *Cryptosporidium* subtype family identification by T-RFLP analysis of the *gp60* gene with *RsaI*.

6.4.2 Repeatability of T-RFLP subtype family identification and subtype differentiation

The ability to consistently differentiate *Cryptosporidium* subtype families by T-RFLP analysis was evaluated using 99 previously identified samples; 92 *Cryptosporidium* samples from humans and cattle and 7 mouse samples. Fluorescent PCR amplification of *Cryptosporidium* from humans and cattle was successful for 92/92 samples. Terminal-RF peaks identified 2 samples as Ia; 58 identified as Ib; 2 as Id; 2 as Ie; 3 as If; 22 as IIa; 1 as IIc; and 2 as IId. Results from the T-RFLP analysis were consistent with sequence data for each sample.

Before T-RFLP analysis was performed on the seven mouse samples, *gp60* subtype identification by DNA sequence analysis was conducted. Sequence analysis identified all mouse samples as *C. parvum* IIaA15G2R1. Subsequent T-RFLP analysis produced T-RF peaks of 309 bp which corresponded to the expected peak size for this subtype family.

Subtypes were differentiated by the peaks of the 5' T-RFs, fluorescently labeled with 6-FAM. Twenty-one subtypes were analysed by T-RFLP in this study (Table 6.3). Analysis of subtype peaks showed T-RFLP differentiated between subtypes based upon the number of serine codons and R repeats within the gp40 region. Subtypes containing the same number of codons displayed the same size 5' T-RF peak (Table 6.3). Single subtype peaks were visualized in 91/99 samples. Multiple subtype peaks were seen in 8/99 samples indicating the presence of different subtypes. This was seen in one human isolate, MQ1029 and all seven mouse isolates (Table 6.4 and Table 6.5).

Table 6.3 The number of serine codons present in each subtype and the corresponding 6-FAM fluorescent peak produced by T-RFLP analysis.

Species	Subtype	No. of serine codons	5' T-RF peak (bp)
C. hominis	IaAl1R3	11	409
	IaA17R2	17	385
	IbA9G3	12	387
	IbA10G2	12	387
	IdA17G1	25	466
	IdA24T1	18	489
	IeA11G3T3	17	435
	IeA12G3T2	17	436
	IfA12G1	13	390
	IfA19G1	20	397
	IfA20G1	21	400
C. parvum	IIaA14G3R1	17	217
	IIaA15G2R1	17	217
	IIaA17G3R1	20	224
	IIaA17G4R1	21	227
	IIaA18G3R1	21	227
	IIaA19G3R1	22	231
	IIaA20G3R1	23	231
	IIcA5G3	8	265
	IIdA23G1	24	282
	IIdA24G1	25	286

Table 6.4 T-RFLP screening of a sporadic human *C. parvum* infection shows three subtypes present within an infection, indicated by T-RF peaks between 214 – 227 bp.

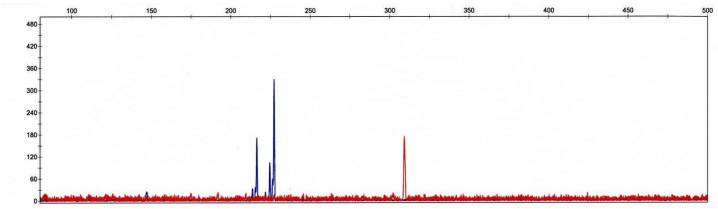
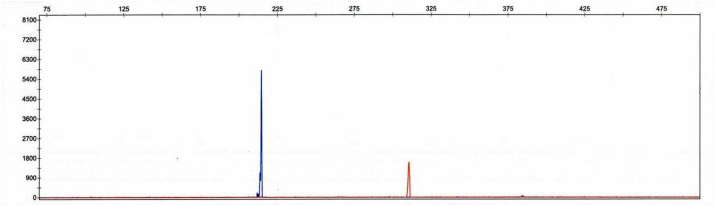
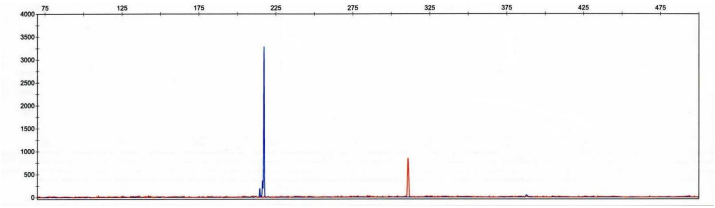
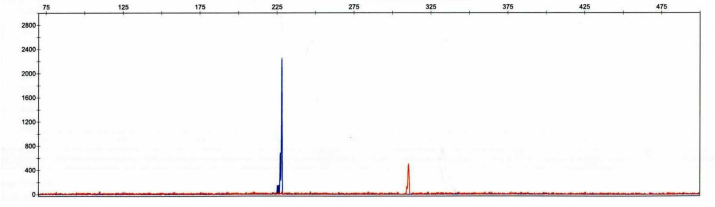
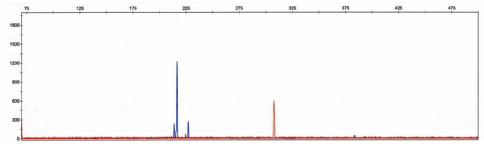
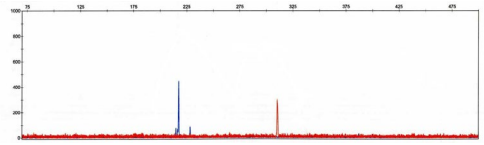
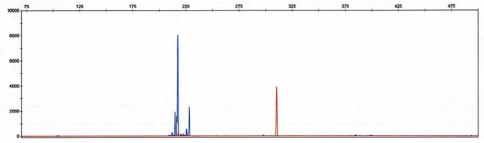
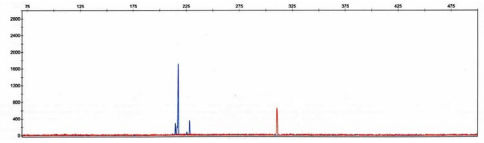
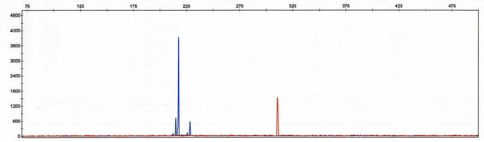
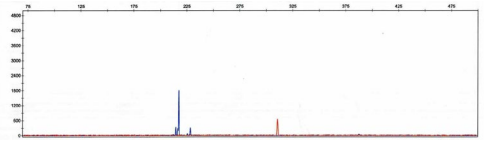
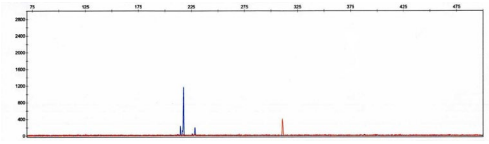
Isolate	gp60 sequence	electropherogram
MQ1029	IIaA18G3R1	
Peak profile 1	IIaA14G2R1	
Peak profile 2	IIaA15G2R1	
Peak profile 3	IIaA18G3R1	

Table 6.5 Molecular analysis of seven consecutive *C. parvum* mouse passages by *gp60* DNA sequencing and T-RFLP analysis with *RsaI*. Multiple subtypes are present within mouse samples, this is indicated by the multiple peaks occurring between 214 – 227 bp.

		Initial T-RFLP screen
Mouse isolate	gp60 subtype	electropherogram
1	IIaA15G2R1	
2	IIaA15G2R1	
3	IIaA15G2R1	
4	IIaA15G2R1	
5	IIaA15G2R1	
6	IIaA15G2R1	

7	IIaA15G2R1	

6.4.3 Cloning and T-RFLP analysis of samples with multiple subtype peaks

Isolate MQ1029 was identified as *C. parvum* IIaA18G3R1 from a previous study (Waldron et al., In Review-a). Cloning of MQ1029 was performed to isolate single *gp60* amplicons to facilitate identification of the subtypes corresponding to each 6 – FAM peak. PCR screening of 50 clones identified 36/50 (72%) were positive for the gp60 insert. T-RFLP analysis of the positive clones identified three different peak profiles; profile 1, 214 bp peak identified in 1/36 (3%) clones; profile 2, 217 bp peak identified in 6/36 (17%) clones; profile 3, 227 bp peak identified in 29/36 clones (80%) (Table 6.4). Sequencing was performed on three clones from each peak profile to identify the corresponding *Cryptosporidium* subtype. Profile 1 was identified as subtype IIaA14G2R1, profile 2 as IIaA15G2R1 and profile 3 as IIaA18G3R1 (Table 6.4).

Twenty-five clones were analysed for each of the seven mouse samples to identify the subtypes corresponding to each 6 – FAM peak. PCR screening of clones identified 166/175 (95%) were positive for the gp60 insert (Table 6.5). T-RFLP analysis of clones identified 3 different peak profiles; profile 1, 214 bp peak; profile 2, 217 – 218 bp peak; profile 3, 227 – 228 bp peak (Table 6.5). Subtypes were identified by sequencing three clones corresponding to each of the peak profiles. Profile 1 was identified as subtype IIaA14G2R1, profile 2 as IIaA15G2R1 and profile 3 as IIaA18G3R1. The frequency of each profile differed between mouse isolates, except between mouse isolates 5 and 6 which were identical (Table 6.6). Profile 2, corresponding to subtype IIaA15G2R1 was the most frequently detected profile in all mouse isolates, attributing to 133/166 (80%) of clones.

Table 6.6 Differences in subtype populations between seven mouse samples. Each sample represents the next consecutive passage through a mouse.

	Number of clones with a particular peak profile			
Mouse sample	**Profile 1 IIaA14G2R1**	**Profile 2 IIaA15G2R1**	**Profile 3 IIaA18G3R1**	**Total positive clones**
1	2	22	1	25
2	1	17	5	23
3	2	17	5	24
4	3	16	2	21
5	0	21	3	24
6	0	21	3	24
7	3	19	3	25
Total	**11**	**133**	**22**	**166**

6.5 Discussion

This study investigated subtype variation within *Cryptosporidium* infections to determine if selection at the subtype population level is occurring. Information regarding the stability of the *gp60* gene is essential as the gene is employed in *Cryptosporidium* identification and has a wide application in contamination source tracking and in understanding the epidemiology of cryptosporidiosis (Xiao, 2010).

Terminal-RFLP differentiated between *Cryptosporidium* subtype families in addition to highlighting subtype diversity within a sample. Simultaneous subtype family and subtype differentiation was accomplished using dual fluorescence and performing a restriction digest. This generated two fluorescently labelled T-RF's, which when analysed through an automated DNA sequencer, generated a profile containing two peaks, a non-variable subtype family – specific peak and a variable subtype – specific peak. Although there are 6 subtype families in *C. hominis* and 11 in *C. parvum*, this study optimized T-RFLP on the subtype families that most frequently cause human cryptosporidiosis; *C. hominis* Ia, Ib, Id, Ie, If and *C. parvum* IIa, IIc and IId (Jex and Gasser, 2010; Xiao, 2010). *Cryptosporidium hominis* Ib and *C. parvum* IIa are the two most frequently detected subtype families in human infections around the world, attributing to 47% and 26% of total reported infections (jex 2010). T-RFLP analysis unambiguously differentiated between these two subtypes, identifying the *C. hominis* Ib – specific peak at 194 bp and the *C. parvum* IIa – specific peak at 309 bp.

T-RFLP has been applied in the bacterial world to assess diversity within microbial populations. In a novel application of this technology, T-RFLP was used to assess the diversity of *Cryptosporidium* subtypes present within an infection. Subtypes were differentiated according to the number of serine codons and rare repeats located in the gp40 cleavage product. The higher the number of serine and rare repeats, the higher the bp size of the subtype peak. Due to the sensitivity of T-RFLP, subtypes differing by one serine codon were differentiated by T-RF peaks. The presence of multiple subtype peaks within a sample indicated that multiple subtypes were present within the original infection. One human isolate and all seven mouse isolates exhibited subtype diversity. For the human sample, *C. parvum* IIaA18G3R1 was originally identified as the *Cryptosporidium* subtype, however T-RFLP showed diversity within the sample and detected three subtypes;IIaA14G2R1, IIaA15G2R1 and IIaA18G3R1. Subtypes IIaA14G2R1 and IIaA15G2R1 were identified in 3% and 17% of clones respectfully. Subtype IIaA18G3R1 is the most common *C. parvum* subtype causing sporadic human illness in Australia, IIaA14G2R1 and IIaA15G2R1 are also identified in Australian sporadic human infections, however in lower numbers (Jex et al., 2008; O'Brien et al., 2008; Waldron et al., 2009b; Waldron et al., In Review-a). Although only one human sample displayed subtype diversity, T-RFLP differentiated on the number, not the form of serine codons. Subtypes such as *C. parvum* IIaA17G4R1 and IIaA18G3R1, although differing in the form of serine codons, have the same number and thereby produce the same subtype T-RF of 227 bp. Subtype diversity is potentially present in more samples, however remained undetected due to subtypes displaying the same size T-RF.

Here we show that differences in the ratio of subtype populations occur between infections. This was highlighted in the seven *Cryptosporidium* samples from mice, which represented consecutive *C. parvum* passages. Initial DNA sequencing of the *gp60* identified all seven isolates

as *C. parvum* IIaA15G2R1, however screening by T-RFLP showed the presence of three different subtypes; IIaA14G2R1, IIaA15G2R1 and IIaA18G3R1. The ratio of these subtypes was different between each of the mouse samples, except passages 5 and 6. Results showed that the inoculation population was different to the population excreted by the mouse. This indicates that selection at the subtype population level is occurring within *Cryptosporidium* infections. To gain a deeper insight into parasite adaptability in response to the host, parasite population analyses from challenge tests where a single host is exposed repeatedly to a parasite population need to be performed.

The application of T-RFLP in *Cryptosporidium* research has provided a platform to investigate evolutionary changes in parasite populations within an infection. Here we demonstrate diversity at the subtype level within a *Cryptosporidium* population from a single host. However, whether the observed diversity is a byproduct of selective pressure enforced by the host's immune system still needs to be determined.

6.6 Acknowledgements

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7. Conclusions

Infectious diseases are the most complex and significant threat to global human and animal health in the 21st century. As populations grow and expand into new areas, humans and animals will be exposed to new and potentially pathogenic bacteria, viruses and protozoa. Knowledge on the pathogen flow between humans, livestock and wildlife will therefore become essential in the development of preventative disease management and surveillance strategies.

Cryptosporidium is firmly established as a significant human pathogen, however the zoonotic threat and emergence of new human pathogenic species highlights the need for targeted screening of humans and animals in high risk areas. This study aimed to fill the knowledge gap in the understanding of human cryptosporidiosis in NSW, Australia, an area recently identified as an emerging infectious disease hotspot (Jones et al., 2008). The major outcome of this study is the provision of a comprehensive analysis of the *Cryptosporidium* species and subtypes causing disease in humans, their geographical distribution throughout NSW, and the identification of animals as significant zoonotic sources. Prior to this investigation, *Cryptosporidium* species causing human and animal disease in NSW had only been analysed in 76 human samples and 15 cattle samples (Ng et al., 2008; Waldron et al., 2009a; Waldron et al., 2009b). The zoonotic role of cattle could not be conclusively linked to human disease as human samples were collected from the Sydney and Orange regions, whilst cattle samples were collected 500 km north of

Orange and Sydney in Tamworth (Ng et al., 2008),. This study has provided *Cryptosporidium* species and subtype information on 718 and 717 human isolates respectively. Spatial analysis of samples from humans in NSW enabled targeted sampling of cattle (n = 200) and kangaroos (n =76) in areas which showed these animals as a zoonotic risk.

7.1 Anthroponotic transmission

Previous molecular analyses indicated that *C. hominis* and *C. parvum* contributed equally to human disease in NSW during 2008 (Waldron et al., 2009a). Analyses of samples from 2009 through to 2011, showed that human cryptosporidiosis infections in NSW were primarily attributed to *C. hominis*, which was identified in 86.4% (n = 660) of infections. This result is consistent with previous Australian studies investigating human cryptosporidiosis in South Australia, Victoria and Western Australia (Chalmers et al., 2005; Jex et al., 2007; Jex et al., 2008; O'Brien et al., 2008; Pangasa et al., 2009; Ng et al., 2010a; Ng et al., 2010b). The higher prevalence of *C. hominis* throughout Australia is likely attributed to the high degree of urbanisation, which accounts for 89% of the Australian population (Australian Bureau of Statistics). *Cryptosporidium hominis* infections predominate throughout most of the world including, the United States, Kenya, Thailand, South Africa, Uganda, Peru, Malawi and India (Peng et al., 2001; Leav et al., 2002; Cama et al., 2003; Gatei et al., 2003; Peng et al., 2003; Chalmers et al., 2005; Akiyoshi et al., 2006; Gatei, 2006; Gatei et al., 2007); Public swimming pools, day-care centers and clustered living conditions facilitate the human-to-human transmission of *C. hominis* (Current and Garcia, 1991; Casemore et al., 1994; Current, 1994; Ramirez et al., 2004).

Cryptosporidium has accounted for 50.8% (n = 165) of global waterborne outbreaks, furthermore 50.3% (n = 83) of these outbreaks have been attributed to recreational water (Karanis et al., 2007). The significant role of public swimming pools in the transmission of *C. hominis* in NSW was highlighted in chapter 3, which described the 2009 Sydney cryptosporidiosis outbreak. Thirteen pools were treated for the parasite, but this was not before 1131 individuals fell ill. Spatial analysis of the outbreak showed the majority of cases clustered within the Sydney region. With 65% of the NSW population residing in Sydney, it was not an unusual result. Patient data showed the disease was most prevalent in the 0-4 and 5-9 age groups. The hot summer, increased water activities and high shedding intensities between 10^5 and 10^6 oocysts/g in the majority of patients, facilitated the waterborne spread of the parasite. Data showed smaller peaks in the 30-34 and 35-39 age groups indicating secondary human-to-human transmission events. The significant public health threat from repeated outbreaks, in addition to the substantial cost incurred by the industry from chemical disinfectants and pool closures, highlights the need for *Cryptosporidium* specific pool water treatments and monitoring systems. However, these technologies are yet to be developed. Until these technologies are available, increased public awareness and education of adults, children, pool owners and swim instructors, is required. Mandatory showering, '*Cryptosporidium* Awareness' and hygiene signage and the sale of 'aquatic nappies' at public swimming pools, will help to reduce repeated outbreaks.

Cryptosporidium hominis Ib was the most prevalent subtype family identified in humans from this study, attributing to 84.8% (n = 609) of infections. Subtype family Ib has a global distribution and has contributed to 47.4% of human infections typed at the *gp60* gene (Jex and Gasser, 2010). The threat of subtype family Ib to public health was highlighted in the 1993 Milwaukee outbreak in the United States which affected over 400,000 individuals and potentially

caused up to 112 deaths (MacKenzie et al., 1994; Zhou et al., 2003; Karanis et al., 2007). To date, this remains the largest waterborne outbreak attributed to protozoa around the world (Karanis et al., 2007).

Of particular concern in NSW is subtype IbA10G2 which was identified in 83% (n = 596) of human samples from this study and was the parasite responsible for the 2009 outbreak. Studies performed in South Australia and Western Australia have also reported this parasite as the cause of waterborne outbreaks (Ng et al., 2010b). On a global scale, IbA10G2 has attributed to 44.5% of human infections typed at the *gp60* (Jex and Gasser, 2010) and has caused large scale waterborne cryptosporidiosis outbreaks in the United States, Northern Ireland and France (Glaberman et al., 2002; Cohen et al., 2006; Xiao and Ryan, 2008b). The global dominance, persistence, distribution and virulence of IbA10G2 indicate that it is a significant public health threat.

The advantage of conducting a large scale study is that it facilitates the identification of unusual species and subtypes capable of causing human disease. Within *C. hominis*, 7 novel subtypes were identified; IaA11R1, IaA13R1, IaA14R1, IaA26R1, IaA32R1, IbA7G3 and IdA17G1. Evidence supports that geographical segregation of subtypes occurs around the world (Xiao, 2010). However with increasing travel, migration and trade, subtypes previously believed to be geographically isolated will be distributed around the globe where they may or may not pose a significant health threat. *Cryptosporidium hominis* is a "specialist pathogen" which invokes a more virulent host response than their "generalist pathogen" counterpart, *C. parvum* (Hunter et al., 2004). The emergence of new *C. hominis* subtypes is a significant health threat and it will place a demand on future research to investigate the mechanisms which give rise to new *Cryptosporidium* subtypes.

7.3 Zoonotic transmission

Analysis of data from the past 60 years showed that EID events were dominated by zoonoses and that 71% of these were attributed to wildlife (Jones et al., 2008). As populations grow and expand closer to rural and wildlife boundaries, the pathogen flow between humans and animals will increase, enabling opportunity for pathogen establishment in new host species. Australia potentially has a large reservoir of zoonotic sources of *Cryptosporidium*. There are abundant and diverse wildlife species in addition to introduced pest animals and domestic species. Cattle, a primary host implicated in zoonotic transmission, are estimated to number 25 million animals; 23.3 million beef cattle and 1.6 million dairy cows (Australian Bureau of Statistics, www.abs.gov.au). The identification of *C. parvum*, *C. andersoni* and *C. fayeri* in human infections in this study confirms the zoonotic risk posed to humans in Australia.

Cryptosporidium parvum was the second most frequently detected species in humans, contributing to 13.4% of infections. *Cryptosporidium parvum* IIa was identified in 13.1% of samples, which made it the second most frequently, detected subtype family in this study. This is consistent with a global report which showed IIa as the second most prevalent subtype family, attributing to 25.5% of human infections typed at the *gp60* (Jex and Gasser, 2010). The most frequently detected *C. parvum* subtype in this study was IIaA18G3R1 which attributed to 6.3% of infections. The occurrence of this subtype in humans was first reported in Australia and has previously been identified as the most common *C. parvum* subtype causing sporadic cryptosporidiosis in NSW (Chalmers et al., 2005; Jex et al., 2007; Jex et al., 2008; O'Brien et al., 2008; Waldron et al., 2009b). The proportion of human *C. parvum* infections which originate from zoonotic sources remains unclear, largely due to the ability of *C. parvum* to pass through

both anthroponotic and zoonotic transmission pathways. Zoonotic infections with *C. parvum* have been shown to be higher in rural regions of the world where contact with animal sources is more frequent. This has been shown in rural localities the Middle East, United Kingdom and Europe (Morgan, 2000; Glaberman et al., 2002; Gatei et al., 2003; Sulaiman et al., 2005; Cohen et al., 2006) Zoonotic infections with *C. parvum* have also been attributed to recreational water and petting farms (Mathieu et al., 2004; Gormley et al., 2010).

Spatio-temporal analysis conducted on human infections in this study showed that *C. parvum* infections increased in Spring, a time which co-insides with calving. This was most pronounced in rural regions such as Wagga Wagga, Dubbo, and on the outskirts of Sydney. Molecular characterisation of *Cryptosporidium* from cattle in these regions showed the presence of subtypes IIaA16G3R1, IIaA18G3R1 and IIaA20G3R1. All of which were previously identified from humans in these localities. Further demonstrating the zoonotic role of cattle in human disease was the identification of a human *C. andersoni* infection. This species has previously been identified from immune-competent individuals in England (Leoni et al., 2006) and HIV positive patients in France (Guyot et al., 2001) but has not previously been identified in humans in Australia. Although *C. andersoni* was not detected in cattle in NSW, it is frequently found in cattle in Western Australia, attributing to 37.7% of infections (Ralston et al., 2010). Results from this study support the hypothesis that cattle are a significant contamination source for human cryptosporidiosis. The unique *C. parvum* subtypes identified in only humans and cattle from Australia, such as IIaA16G3R1, highlight the need for comprehensive investigations to be conducted in Australia so that Australian specific control measures can be implemented.

7.3 Future research

Pools and recreational water are the most frequent transmission sources for acquiring a cryptosporidiosis infection (Karanis et al., 2007). Contamination of public swimming pools is often associated with an accidental faecal release, particularly in the toddler and wading pools. Despite the numerous global outbreaks linked to public pools, there are limited studies, none in Australia, that have investigated the *Cryptosporidium* species, subtypes and oocyst concentrations in the water. Such studies are required to better understand the transmission dynamics and public health risks in individual areas. Pool water studies will not only help to prevent cryptosporidiosis, but also infections attributed to *Giardia*, *Entamoeba*, *Balantidium*, *Cyclospora* and *Naegleria*, which have attributed to pool related outbreaks around the world (Karanis et al., 2007). Contamination in recreational waterways is often associated with urban and rural run-off, storm water, and human and animal waste. Investigations carried out on raw waste water in Milwaukee showed the complexity *Cryptosporidium* species present in waterways with the detection of *C. hominis*, *C. parvum*, *C. andersoni C. canis, C. felis* and *C. muris* (Zhou et al., 2003). These results demonstrate the significant role of recreational water in the emergence of zoonotic *Cryptosporidium* infections.

Host specificity of *Cryptosporidium* species has become a topical subject in recent years as an increasing number are jumping the host-species barrier. The most recent examples of this are seen by the wildlife associated *C. fayeri* infection in an Australian human (Waldron et al., 2010), the waterborne outbreak caused by the presumed rabbit-host specific *C. cuniculus* (Chalmers et al., 2009). However, the vice-versa effect of humans as zoonotic disease sources needs to be considered, as the fourth most common subtype detected in cattle was *C. hominis* IbA10G2. Investigating the mechanisms that influence host specificity, enable a zoonotic potential or cause increased virulence needs to be the next step undertaken by *Cryptosporidium* researchers. New

molecular platforms, such as 454 sequencing, provide unique avenues to explore host-parasite interactions. Understanding how *Cryptosporidium* behaves inside the host will also be a pivotal step for the development of cryptosporidiosis treatments. Since its first description in 2000 (Winter et al., 2000), the *gp60* has been the gene preferentially used by researchers for *Cryptosporidium* subtype analysis. Use of the *gp60* gene has provided an unambiguous typing method, enabled easy comparisons between global epidemiological surveys and has highlighted the public health significance of certain genetic variants. The *gp60* gene is most frequently used in a single-locus approach, a method reliant on the stability of this gene in space and time. As shown in this study through the use of T-RFLP, numerous subtypes are present within a *C. parvum* infection. Furthermore, subtype population ratios were shown to change within the parasite population through subsequent experimental infections in successive passages. It remains unclear if subtype population changes inside the host are a rapid evolutionary response to the host's immune system, however T-RFLP provides the opportunity to investigate this. Due to the obligatory sexual phase in the lifecycle of *Cryptosporidium*, genetic exchange has been shown to play a significant role in generating genetic diversity (Feng et al., 2002; Mallon et al., 2003b; Widmer, 2009). From this, it was concluded that when used in a single-locus approach, the gp60 gene is not a reliable marker of *Cryptosporidium* population structure (Widmer, 2009). Future studies need to employ a multilocus genotyping approach to completely understand cryptosporidiosis epidemiology and population structure, to design the next drug treatment and to overcome the bias of a single-locus approach.

Australia lacks a unified notifications and surveillance system. Individual states, with their numerous health area divisions, notify communicable diseases to their local health department. However, publically available data is often not up to date, missing in years and in some instances

not presented at all. Government authorities and research institutes need to create a unified system. The National Notifiable Diseases Surveillance System provides an up to date state count for each communicable disease, however it does not divide each state into their respective health area networks. With State and Territory areas ranging between 2, 358 and 2, 529, 875 km^2, which in some cases is equivalent to the size of small countries such as Peru and Sudan, pin pointing disease or emerging disease hotspots is impossible. Research has shown that zoonotic diseases represent an increasing and very significant threat to global health (Jones et al., 2008). Population growth, urbanisation, intensified food-animal production and agriculture will increase the pathogen flow between the wildlife-urban-agricultural interfaces. A unified surveillance system incorporating human and animal disease notifications and the regions in which they occur will provide a unique collaboration between government agencies and research institutes. Such collaboration will facilitate the identification of regions where the next bacterial, viral or protozoal infectious disease threat is likely to emerge.

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8. Appendix

8.1. Glycoprotein 60 diversity in *C. hominis* and *C. parvum* causing human cryptosporidiosis in NSW Australia

8.2 Terminal restriction fragment polymorphism for identification of *Cryptosporidium* species in human faeces

8.3 Evaluation of a PCR protocol for sensitive detection of Giardia intestinalis in human faeces

8.1 Glycoprotein 60 diversity in *C. hominis* and *C. parvum* causing human cryptosporidiosis in NSW Australia

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8.1.1 Abstract

Management and control of cryptosporidiosis in humans requires knowledge of *Cryptosporidium* species contributing to human disease. Markers that are able to provide information below the species level have become important tools for source tracking. Using the hypervariable surface antigen, glycoprotein 60 (GP60), *C. hominis* (n = 37) and *C. parvum* (n = 32) isolates from cryptosporidiosis cases in New South Wales, Australia, were characterised. Extensive variation was observed within this locus and the isolates could be divided into 8 families and 24 different subtypes. The subtypes identified have global distributions and indicate that anthroponotic and zoonotic transmission routes contribute to sporadic human cryptosporidiosis in NSW

8.1.2 Introduction

Over the last 20 years, *Cryptosporidium* has emerged as one of the three most common enteropathogens causing diarrhoeal disease in humans (Petri, 2005). Although cryptosporidiosis cases in humans have been attributed to eight species or genotypes, (Morgan-Ryan et al., 2002; Xiao and Ryan, 2004; Misic and Abe, 2007), two species, *C. hominis* and *C. parvum,* cause significant impacts to human health (Morgan-Ryan et al., 2002; Xiao, 2004; Misic and Abe, 2007).

In Australia cryptosporidiosis has been a notifiable disease since 1996 (*New South Wales Public Health Act 1991).* Notifications data has shown a significant rise in the incidence of human cryptosporidiosis in New South Wales (NSW). In 2003 the incidence of human cryptosporidiosis was 203 and in 2006 it had risen to 777 (www.nswhealth.gov.au). Routine diagnoses performed in Australian diagnostic laboratories are presence/absence tests, and as a result, data on *Cryptosporidium* species causing human disease remains unknown. This is essential information for understanding the dynamics of human cryptosporidiosis and for effective management and prevention of this disease.

Identification of *Cryptosporidium* species requires molecular analyses. A multilocus sequencing approach has been applied to *Cryptosporidium* epidemiology and population structure studies using loci such as the 18S rDNA, HSP70, COWP and actin (Learmonth, 2004); (Misic and Abe, 2007); (Mallon et al., 2003). However, these conserved loci are not useful gene targets for assessing intra-species variation. A highly polymorphic surface-expressed antigen, glycoprotein 60 (GP60) (Cevallos et al., 2000); (Strong et al., 2000; Winter et al., 2000), is used for investigations of intra-species diversity of *Cryptosporidium*. The GP60 gene encodes a 60 kDa precursor protein that is cleaved into two sub-units, GP15 and GP40 (Strong et al., 2000). Characterisation of the GP60 for *C. hominis* and *C. parvum* has shown significant inter- and intra-species polymorphisms. This variation has facilitated assignment of family groups within each species. To date there are 7 described subtype families for *C. hominis* and 9 for *C. parvum* and (Strong et al., 2000); (Glaberman et al., 2001); (Peng et al., 2001); (Akiyoshi et al., 2006); (Abe et al., 2006); (Gatei et al., 2007) . Sub-typing *Cryptosporidium* using the GP60 gene has been useful for source tracking, transmission dynamics and population structure (Alves et al., 2006b); (Trotz-Williams et al., 2006); (Cama et al., 2007); (O'Brien et al., 2008)

In this study faecal samples from humans were analysed by PCR to identify variation in *Cryptosporidium* contributing to human cryptosporidiosis in NSW, Australia. Identification of *Cryptosporidium* species and subtypes will assist public health units in managing the increasing incidence of this disease in the human population.

8.1.3 Material and Methods

8.1.3.1 Sample sources and DNA extraction

Sixty-nine human fecal samples, positive for *Cryptosporidium,* were obtained from Pathology laboratories in Sydney, Australia. Specimens were identified as *Cryptosporidium* positive by pathology companies using the Remel ProSpecT *Giardia*/*Cryptosporidium* microplate assay. Oocysts were purified from faeces using a modified sucrose floatation method (Truong and Ferrari, 2006) and DNA extracted using PrepGem as previously described (Ferrari et al., 2007). Sixty-four *Cryptosporidium* isolates used in this study were previously identified as *C. parvum* (n = 30) and *C. hominis* (n = 34) using terminal restriction fragment length polymorphism (T-RFLP) as described (Waldron et al., 2009a). Five additional isolates were identified to species level using T-RFLP (Waldron et al., 2009a) for inclusion in this study.

8.1.3.2 GP60 amplification

Amplification of the GP60 locus was performed using a nested PCR. The primary reaction comprised the forward primer S60.F728 5'- ACCACATTTTACCCACACATC and reverse primer

S60.OutR 5'- TCCTC ACTCGATCTAGCTCA. Primers used for the secondary reaction were S60.ATGF 5'- ATGAGATTGTCGCTCATT ATCG and S60.StopR 5'- TTACAACACGAATAAGGCTGC. Primary and secondary reactions contained 4mM $MgCl_2$, 200nM dNTPs, 200 nM of each forward and reverse primer and 1 U of RedTaq® polymerase (Integrated Sciences, Sydney, Australia). Reaction conditions comprised an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C 45 sec, 58°C 45 sec and 72°C for 1 min 30 sec, with a final extension at 72°C for 5 min. PCR products were visualised by 1.5% agarose gel electrophoresis and ethidium bromide staining (1µg/ml).

8.1.3.3 Cloning and sequence analysis

PCR products containing the correct band size (~1000bp) were purified using the Qiagen spin column PCR Purification Kit (Qiagen, Melbourne, Australia) and cloned using the TOPO-TA vector cloning system (Invitrogen, Australia). Plasmid DNA was recovered using the Qiagen plasmid kit (Qiagen, Melbourne, Australia). Sequencing was performed using an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, California) with the BigDye™ terminator kit (Applied Biosystems). Nucleotide sequences representing GP60 families of *C. parvum* and *C. hominis* were retrieved from GenBank (www.ncbi.nlm.nih.gov) and aligned against isolates from this study using ClustalW (Thompson et al., 1994).

Isolates were assigned a subtype according to the nomenclature system described by Sulaiman et al. (2005). A conserved region is used to assign the family group, indicated by the roman numeral I for *C. hominis* and II for *C. parvum* followed by a letter. Naming of subtypes is based on differences in the number and form of serine codons in the microsatellite region at the 5' end of the gene (TCA/TCG/TCT) and an additional repeat region downstream of the serine repeat.

Representative isolates from each family group were cloned and sequenced bi-directionally to obtain the complete ORF, accession numbers FJ839873-FJ839883. *Cryptosporidium hominis* nucleotide sequences were submitted to GenBank under the accession numbers FJ861209-FJ861238 and *C. parvum* FJ861278-FJ861305

8.1.4 Results

In addition to the 64 isolates described in Waldron et al., 2009, species identification of 5 additional *Cryptosporidium* isolates using T-RFLP increased the isolates in this study to 69 (*C. hominis* n = 37 and C. *parvum* n = 32). Amplification of the GP60 locus resulted in a clean band for all 69 isolates tested. Nucleotide sequences were obtained for all 69 isolates, sequences for 46 isolates were from cloned amplicons and 23 isolates were obtained directly from the PCR amplicon.

Allocation of the 69 isolates to family groups identified 5 *C. hominis* and 3 *C. parvum* groups. For *C. hominis* isolates, sequence analysis placed them into family groups Ia (n = 2), Ib (n = 28), Id (n = 5), Ie (n = 1) and If (n = 1). The 37 *C. hominis* isolates were further typed into 10 subtypes according to the variation in serine codons. (table 8.1). Subtype IbA10G2 was the most frequent (n = 25) followed by IbA9G2 (n = 2), IdA26 (n = 2) and IdA15 (n = 2). The 6 remaining *C. hominis* subtypes were represented by a single isolate. Sequence analysis of the 32 *C. parvum* isolates identified 3 families, IIa (n = 30), IIc (n = 1) and IId (n = 1) and subtyping at the GP60 locus identified 14 subtypes (Table 8.1). Subtype IIaA18G3R1 (n = 10) was the most common,

followed by IIaA17G3R1 (n = 4), IIaA20G5R1 (n = 3), IIaA20G3R1 (n = 3), IIaA17G4R1 (n = 2) and IIaA16G3R1 (n = 2). The remaining 8 *C. parvum* subtypes were represented by a single isolate.

For representative isolates (n = 11) coverage of the GP60 ORF from start to stop codons, approximately 1000 bp, was obtained for *C. hominis* families Ia, Ib, Id, Ie and If and *C. parvum* families IIa, IIc and IId**.**

Table 8.1 Summary of the *Cryptosporidium* species and genotypes identified in humans in NSW

Cryptosporidium species	***gp60* subtype**	**Frequency**
C. hominis	IbA10G2	25
	IbA9G2	2
	IdA26	2
	IdA15	2
	IaA17R3	1
	IaA10R4	1
	IbA6G3	1
	IdA24T1	1
	IeA11G3T3	1
	IfA12G1	1
Total	**10**	**37**
C. parvum	IIaA18G3R1	10
	IIaA17G3R1	4
	IIaA20G5R1	3
	IIaA20G3R1	3
	IIaA17G4R1	2
	IIaA16G3R1	2
	IIaA22G3R1	1
	IIaA20G2R1	1
	IIa19G3R1	1
	IIaA17G2R1	1
	IIaA16G4R1	1

	IIaA15G2R1	1
	IIcA5G3a	1
	IIdA24G1	1
Total	**14**	**32**

8.1.5 Discussion

Epidemiological surveys assessing the contribution of different species to human cryptosporidiosis show that different species predominate in different geographical areas. *Cryptosporidium hominis* has been described as the dominant species in many urbanised regions of the world, including the United States, Africa, and India (Peng et al., 2001; Leav et al., 2002; Cama et al., 2003; Akiyoshi et al., 2006; Gatei et al., 2007). Recent studies in Australia (Western and South Australia) have also identified *C. hominis* as the predominant species in sporadic cases of human cryptosporidiosis (Chalmers et al., 2005); (O'Brien et al., 2008); (Jex et al., 2008). For *C. parvum*, human infections are more prevalent in rural regions where contact with reservoir hosts such as cattle and sheep are frequent. This has been shown in rural localities in Europe, the Middle East, and the United Kingdom (Morgan, 2000; Glaberman et al., 2002; Gatei et al., 2003; Sulaiman et al., 2005; Cohen et al., 2006). Previous typing of the isolates in this study indicated that *C. hominis* and *C. parvum* contribute equally to human cryptosporidiosis in NSW (Waldron

et al., 2009a). Here, sub-typing of the GP60 supports this finding and provides insight on the intra-species variation of *Cryptosporidium* causing human disease.

The diversity within the GP60 of the 69 human isolates was extensive with 8 different families and 24 subtypes within those families identified. The most common *C. hominis* subtype, IbA10G2, was recently identified as the most common subtype causing sporadic human disease in Perth, Australia (O'Brien et al., 2008) and in Tamworth, a country area in NSW (Ng et al., 2008). Subtype IbA10G2 has a global distribution and has been identified as the causative agent of outbreaks in the United Kingdom (Chalmers et al., 2007b) and the United States (Xiao and Ryan, 2008a) and is a source of sporadic cryptosporidiosis in Portugal, South Africa, and Kuwait (Leav et al., 2002; Alves et al., 2003; Zhou et al., 2003; Chalmers et al., 2005; Sulaiman et al., 2005; Alves et al., 2006b); (O'Brien et al., 2008). Subtypes in this study, IbA9G2 and IdA16 have also been recorded from humans in Perth (O'Brien et al., 2008). Subtype IeA11G3T3 found in a single isolate is a cause of sporadic cryptosporidiosis around the world and was the parasite responsible for an outbreak in Wales (Chalmers et al., 2007b).

Diversity within the GP60 was also evident for *C. parvum* isolates with 3 families and 14 subtypes identified in human isolates from NSW. The most common *C. parvum* subtype, IIaA18G3R1 in this study has also been identified as the most common subtype in cattle in Perth (O'Brien et al., 2008), Tamworth (Ng et al., 2008), Canada (Trotz-Williams et al., 2006) and Northern Ireland (Thompson et al., 2007). Interestingly the occurrence of this subtype in humans has only ever been reported in Australia (Chalmers et al., 2005; Jex et al., 2007; Jex et al., 2008; O'Brien et al., 2008). The remaining IIa subtypes found in this study have a wide distribution within Australia and around the world (Sulaiman et al., 2005; Alves et al., 2006b; Feltus et al., 2006; Trotz-Williams et al., 2006). The Sydney region encompasses both urban and rural

environments, and rural upland locations in catchment and recreational waters. In these areas farming may present a source of *C. parvum* to waterways.

The majority of *Cryptosporidium* GP60 sequences in GenBank represent partial sequence data with complete ORFs available for *C. parvum* IIa (Strong et al., 2000) and *C.fayeri* families (Power et al., 2009). The heterogeneity in the GP60 makes it a poor gene for phylogenetic inferences, but knowledge of both nucleotide and protein diversity will enable a better understanding of the epidemiology of this parasite. In this study full length nucleotide sequences were obtained for each GP60 family identified for *C. hominis* and *C. parvum*. The nested PCR protocol used in this study will enable full GP60 ORF sequences for other *C. hominis* and *C. parvum* families. At present DNA sequencing of the GP60 locus is commonly used for typing *C. parvum* and *C. hominis* isolates. For a large epidemiological study sequencing can be quite costly and therefore such studies will require cost effective, high throughput tools for GP60 analysis.

This study has provided preliminary data on GP60 diversity within *Cryptosporidium* isolates from humans in NSW. A large epidemiology study that combines parasite data with geographic and demographic information is necessary to determine factors contributing to the increasing incidence of human cryptosporidiosis in NSW, Australia.

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8.2 Terminal restriction length polymorphism for identification of *Cryptosporidium* species from human faeces

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8.2.1 ABSTRACT

Effective management of human cryptosporidiosis requires efficient methods for detection and species identification of *Cryptosporidium* isolates. Identification of isolates to species is not routine for diagnostic assessment of cryptosporidiosis, which leads to uncertainty about the epidemiology of the *Cryptosporidium* species that cause human disease. We developed a rapid and reliable method for species identification of *Cryptosporidium* oocysts from human fecal samples, using terminal restriction fragment polymorphism (T-RFLP) analysis of 18S rDNA. This method generated diagnostic fragments unique to each species of interest. A panel of previously identified species were blind tested to validate the method, which ascribed the correct species identity in every case. T-RFLP profiles obtained from samples spiked with known

amounts of *C. hominis* and *C. parvum* oocysts generated the two expected diagnostic peaks. The detection limit for an individual species was 1% of the total DNA. This is the first application of T-RFLP to protozoa, which proves to be a rapid, repeatable and cost effective method for species identification.

8.2.2 Introduction

Over the last 20 years, *Cryptosporidium* has been identified as one of the three most common enteropathogens causing waterborne disease in both immune compromised and immunocompetent individuals (Petri, 2005). Human cryptosporidiosis has been reported in all regions of the world, with prevalence rates ranging from 1-30% (Fayer and Ungar, 1986; Laubach, 2004). Of the five *Cryptosporidium* species that can cause human disease, 90% of reported cases are caused by two species; the anthroponotic *Cryptosporidium hominis* and the zoonotic *Cryptosporidium parvum* (Morgan-Ryan et al., 2002; Xiao and Ryan, 2004; Misic and Abe, 2007).

Understanding the dynamics of human cryptosporidiosis requires species identification, so that transmission routes and contamination sources can be identified. *Cryptosporidium parvum* and *C. hominis* are morphologically indistinguishable, and consequently species identification can only be performed using molecular techniques. DNA sequencing is the 'gold standard' for identification, but is too time consuming and expensive for routine diagnostics. More rapid and cost effective methods, such as restriction fragment length polymorphism (RFLP) are often used (Xiao et al., 2000; Morgan-Ryan et al., 2002; Cohen et al., 2006). The efficacy of RFLP is dependent on the availability of diagnostic restriction sites and a suitably high titre of parasites. The diagnosis of low level or mixed infections is more difficult, and may underestimate the contribution of each species to human disease and misidentify contamination sources. Hence there is a need to develop sensitive and discriminatory diagnostic methods for identification of *Cryptosporidium* species.

Terminal RFLP (T-RFLP) is a fluorescence based PCR technology that has been applied to measure demographic complexity and biodiversity in bacterial communities, including those

from marine environments, soil, groundwater, gut microbiota and feces (Bruce, 1997; Liu et al., 1997; van der Maarel et al., 1998; Marsh, 1999; Moeseneder et al., 1999; Dunbar et al., 2000; Wang et al., 2004). T-RFLP analysis relies on polymorphisms in the position of restriction sites relative to a fluorescently tagged primer. The use of a single fluorescent primer limits the analysis to the terminal restriction fragment in a target DNA molecule (T-RF) (Marsh, 1999). Samples can be analysed using capillary electrophoresis on an automated DNA sequencer, allowing fine scale size resolution and quantification of the T-RF abundance (Dunbar et al., 2001). Variation in the size of T-RFs of different species enables differentiation.

Because T-RFLP is fast and sensitive, and is amenable to high throughput analysis (Dunbar et al., 2001; Wang et al., 2004; Fogarty and Voytek, 2005), it warrants investigation as a diagnostic tool for *Cryptosporidium*. This study describes the application of T-RFLP to identify *Cryptosporidium* species present in human fecal samples. T-RFLP proved to be a reliable and reproducible alternative method for detection and identification of *Cryptosporidium* species.

8.2.3 Materials and methods

8.2.3.1 Parasite sources

Seventy-two human fecal samples, positive for *Cryptosporidium,* were obtained from Pathology laboratories in Sydney, Australia. Specimens were identified as *Cryptosporidium* positive by pathology companies using the Remel ProSpecT *Giardia*/*Cryptosporidium* microplate assay. Samples were stored as whole feces without preservatives at 4°C until processing, within a month post-collection.

8.2.3.2 Oocyst purification and DNA extraction

Faecal samples were irradiated by exposure to 1,500 curies from a ^{60}Cobalt source for 30 min. *Cryptosporidium* oocysts were purified using a sucrose flotation gradient as described by Truong and Ferrari (Truong and Ferrari, 2006). Purified oocysts, stained with the *Cryptopsoridium* specific antibody CRY104 labelled with FITC (Biotech Fronteirs, North Ryde, Australia), were examined using epi-fluorescence microscopy with appropriate filters for FITC excitation (Axioscope, Zeiss, Germany) and enumerated at 400X magnification (Power et al., 2003). Genomic DNA was extracted from purified oocysts using *PrepGEM* (ZyGEM Corporation Ltd., Hamilton, New Zealand) as previously described (Ferrari et al., 2007)

8.2.3.3 *Cryptosporidium* species identification by PCR-RFLP

Amplification of the 18S rDNA was performed using a previously described nested PCR (Xiao et al., 1999b). Primary and secondary reactions contained 6 mM $MgCl_2$, 200 µM dNTPs, 200 nM of each primer, 1 U of Red Hot Taq (ABgene, Surrey, UK) and 2 µl of DNA template. A total of 35 cycles, each consisting of 94°C for 45 s, 56°C for 45 s and 72°C for 1 min, with an initial

denaturation of 94°C for 3 min and a final extension step of 72°C for 7 min were performed. The secondary PCR comprised 1 µl of the primary PCR product in a total volume of 50 µl. Cycling conditions for the secondary reactions were identical to those used for the primary PCR. All PCRs were performed with a negative control containing PCR water only and a positive control containing *C. parvum* DNA. Reactions were run on Eppendorf Mastercycler Personal instruments (Eppendorf, North Ryde, Australia) and products were resolved by electrophoresis on 1.5% w/v agarose gels and run in TBE. Secondary products that were the correct size fragment (832-835 bp depending on species) were purified using the QIAquick PCR Purification Kit (Qiagen, Melbourne, Australia) following manufacturer's instructions for the Spin Protocol.

RFLP analysis was performed using a previously described protocol with the restriction enzyme *VspI* (10 units/µl, New England BioLabs) (Xiao et al., 1999b). DNA fragments were separated on 3.5% w/v agarose gels at 100V for 50 min. The RFLP patterns of *C. hominis* and *C. parvum* were visualised under UV light after staining with ethidium bromide (0.5 µg/ml) for 30 min.

8.2.3.4 T-RFLP optimisation

Optimisation of the T-RFLP protocol was determined using *C. hominis* n = 6 and *C. parvum* n = 6. The 12 isolates had previously been characterised at two loci, 18S rDNA and glycoprotein 60, to confirm species (Waldron et al., 2009b). The 18S rDNA sequences of these isolates were used to determine the lengths of the expected T-RFs.

Cryptosporidium hominis and *C. parvum* were assessed independently to ensure efficient amplification of both species. Reaction optimisation for each species was determined by comparing 4 different blunt end Taq polymerases, *Pfu Turbo* (Stratagene, La Jolla, Califonia,

USA) *Accuzyme* (Bioline, London, UK), *Accutaq* (Sigma-Aldrich, Australia) and *Sahara* (Bioline, UK), with two different final $MgCl_2$ concentrations (4 mM and 6 mM). For fluorescence analysis the forward primer of the secondary reaction was fluorescently labelled with 5' 6-carboxyfluorescein (Proligo, Lismore, Australia).

8.2.3.5 T-RFLP nested PCR

The constituents for primary and secondary reactions were identical to that described previously but with *Red Hot Taq* substituted with *Accutaq* (1 U) and the addition of DMSO to a final concentration of 2%. Cycling conditions comprised a total of 35 cycles, each consisting of 94°C for 45 s, 56°C for 45 s and 68°C for 1 min, with an initial denaturation of 94°C for 3 min and a final extension step of 68°C for 7 min. Secondary PCRs that generated the expected products were purified and subjected to restriction digestion with *VspI*. The amount of DNA in the restriction digest for T-RFLP is 10 fold less than the amount used for RFLP due to the sensitivity of capillary electrophoresis.

Samples were analysed at the Macquarie University DNA sequencing facility. Prior to capillary electrophoresis, 10 µl of HiDi / Standards Master Mix comprising 9.9 µl of formamide and 0.1 µl of internal DNA standard LIZ500 (Applied Biosystems), was added to 1 µl of template, followed by denaturation at 95° for 5 min. The fluorescence of the T-RFs was detected using an ABI Prism 3130x1 genetic analyser (Applied Biosystems) in Genescan mode (8.5 Kv; 40-s injection; 60°C for 100 min) with a G5 filter. T-RF sizes were determined using GeneScan software version 4.0 (Applied Biosystems).

8.2.3.6 Minimum detection limit of T-RFLP

To determine the minimum detection limit (MDL) of T-RFLP, purified *C. hominis* and *C. parvum* oocysts were used to prepare suspensions with known oocyst numbers of each species. Oocysts were immuno-labelled with CRY104 as described above and sorted onto polycarbonate membranes using a FACSCalibur-sort flow cytometer (BD Biosciences, Sydney, Australia). *Cryptosporidium hominis* and *C. parvum* oocyst suspensions were prepared in triplicate and contained 1, 10, 100 and 1000 oocysts. To confirm oocyst numbers, membranes containing 1 or 10 oocysts were examined using epi-fluorescence microscopy as described above. DNA extraction and T-RFLP analysis was then conducted as previously described.

8.2.3.7 Mixed infection detection

Mixed species suspensions were used to determine the sensitivity of T-RFLP for the detection of mixed infections. Purified oocysts of *C. hominis* and *C. parvum* were immuno-labelled and sorted using FACS as previously described. Triplicate samples containing 500 oocysts were prepared in percentages of *C. hominis*: *C. parvum* 5:95, 10:90, 25:75, 50:50, 75:25, 90:10, 95:5. DNA was extracted using *prepGEM*™ and amplifications for T-RFLP analysis were performed as described above. To serve as a comparative model, RFLP was performed on mixed species suspensions as described by Xiao et al. (1999).

8.2.4 Results

8.2.4.1 Identification of *Cryptosporidium* species

Amplification of the 18S rDNA was successful for 71 of the 72 isolates analysed. Digestion of secondary PCR products with *VspI* enabled differentiation of *C. hominis* isolates from *C. parvum* isolates. *Cryptosporidium hominis* isolates had 69 bp and 530 bp fragments and *C. parvum* isolates had a single fragment of 627 bp (Fig. 8.1A). Digestion profiles identified 38 of 71 isolates as *C. hominis* (53%) and 33 of 71 isolates as *C. parvum* (46%).

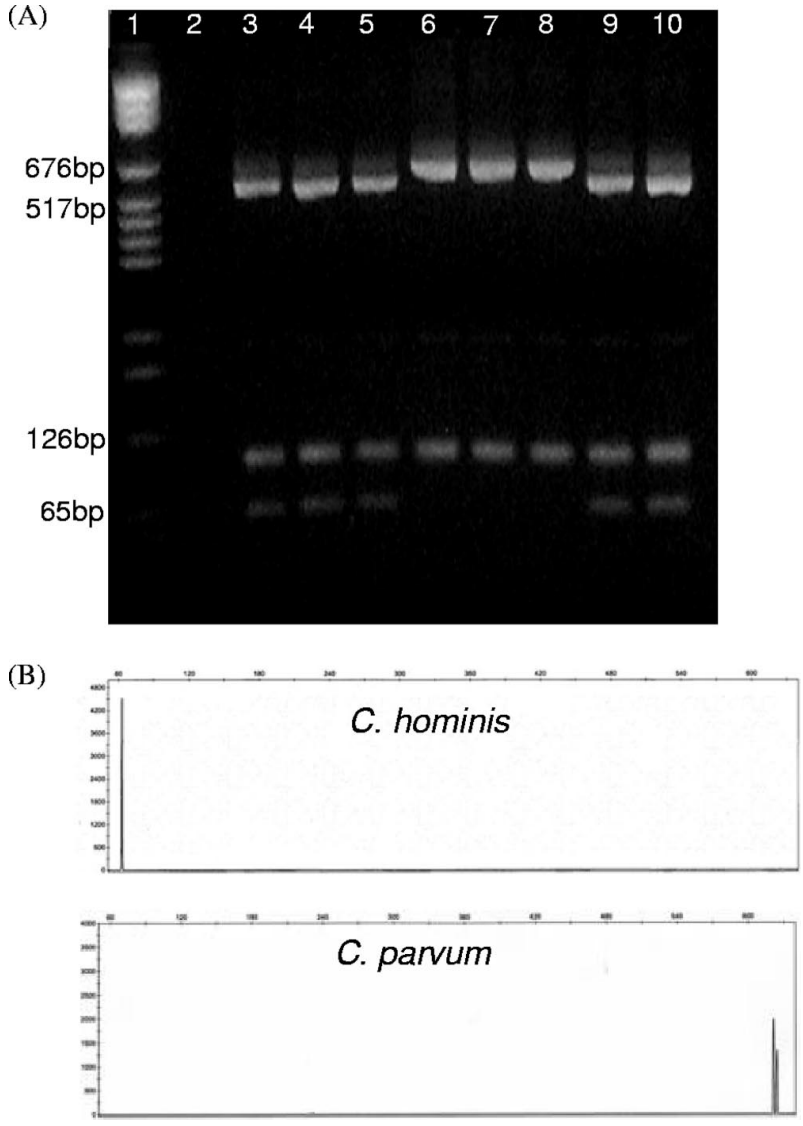
8.2.4.2 Species Identification by T-RFLP

Optimisation of T-RFLP protocol was determined using *C. hominis* n = and *C. parvum* n = 6. The 18S rDNA sequences of these isolates were used to determine the lengths of the expected T-RFs. For *C. hominis* the predicted length was 69 bp and for *C. parvum* 627 bp. Reactions were optimised for *taq* polymerase and $MgCl_2$ concentration and determined that the fluorescently tagged primer did not alter PCR conditions. The optimal taq that produced a smooth tight peak for both species was *Accutaq* with 6 mM magnesium, these conditions were adopted for subsequent T-RFLP reactions. Purification of the fluorescently labelled PCR products also facilitated clean GeneScan outputs and PCR clean was instigated in optimised protocol.

Terminal-RFLP differentiated *C. hominis* and *C. parvum* based on the fluorescently labelled 5' T-RF. *C. hominis* isolates generated a 64 bp T-RF and *C. parvum* isolates a 624 bp T-RF (Fig. 8.1B). A second T-RF with an estimated size of 621 bp was detected in the profiles of all *C. parvum* isolates. The intensity of the second peak was consistently less than the intensity of the 624 bp T-RF.

Comparison of T-RFLP analysis to RFLP produced consistent results and with each sample identified as having the same species by both methods. One sample, isolate 24, which failed to produce a visible RFLP product when resolved using gel electrophoresis, was identified as *C. parvum* by T-RFLP. Eight isolates failed to amplify by T-RFLP after 6 months of storage.

Figure 8.1Species identification of *Cryptosporidium* isolates by RFLP and T-RFLP analysis of the 18S rDNA with *VspI*. *Cryptosporidium hominis* isolates (lanes 3-5, 9 and 10) contain three fragments but are identified by the presence of the 69 bp and 530 bp fragments compared to *C. parvum* isolates (lanes 6-8) which are identified by a 627 bp fragment only. Terminal-RFLP identified *C. hominis* by a 64 bp peak and *C. parvum* by a 624 bp peak.



8.2.4.3 Minimum detection limits of T-RFLP

The MDL for T-RFLP was higher than the MDL for RFLP. For *C. parvum*, RFLP analysis was able to resolve DNA from a single oocyst but for *C. hominis* RFLP products were observed in only 1 of the 3 samples containing DNA from a single oocyst (Table 8.2). Despite using 10-fold less DNA template than RFLP, T-RFLP detected the expected T-RF for all three *C. hominis* samples which contained DNA from a single oocyst.

Table 8.2 Detection of *C. hominis* and *C. parvum* in samples with known oocyst numbers

	C. hominis detection / 3		*C. parvum* detection / 3	
Oocyst(s) in sample	RFLP	T-RFLP	RFLP	T-RFLP
1	3	3	1	3
10	3	3	3	3
100	3	3	3	3
1000	3	3	3	3

8.2.4.4 Mixed infection detection

For all ratios tested *C. parvum* DNA was detected by RFLP (Fig. 8.2) and T-RFLP (Table 8.3). *Cryptosporidium hominis* DNA was not detected by RFLP or T-RFLP when its DNA contribution was equal to or less than *C. parvum*. The sensitivity of T-RFLP enabled detection of *C. hominis* DNA in the suspension containing equal amounts of *C. hominis* and *C. parvum*.

Figure 8.2 (a) Analysis of mixed species suspensions identified *C. parvum* DNA in all *C. parvum*: *C. hominis* ratios. Lane 1, molecular weight marker; lane 2, negative control; lane 3, *C. hominis* control; lane 4, *C. parvum* control; lanes 5-7, 0.1:9.9; lanes 8-10, 0.5:9.5; lanes 11-13, 1:9; lanes 14-16, 2.5:7.5; lanes 17-19, 5:5. (b) *C. hominis* DNA could not be detected by RFLP in the majority of ratios except for sporadic amplification in the samples containing *C. hominis*: *C. parvum* ratio of 0.5:9.5 (lanes 8-10).

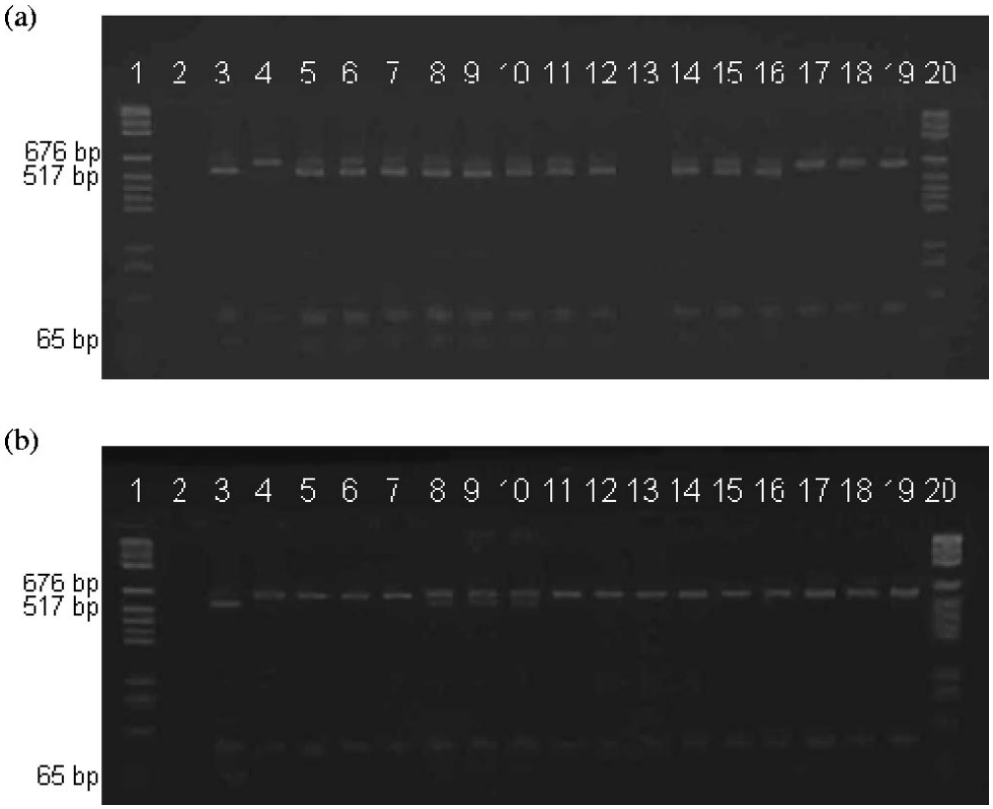


Table 8.3 Detection of *C. hominis* and *C. parvum* within mixed population ratios

	Positive detection / 3		
C. hominis :*C. parvum* ratio	*C. hominis* only	*C. parvum* only	Both
0.1:9.9	0	3	0
0.5:9.5	0	0	3
1:9	0	2	1
2.5:7.5	0	1	2
5:5	0	2	1
7.5:2.5	0	0	3
9:1	0	0	2
9.5:0.5	0	0	3
9.9:0.1	0	0	3

8.2.4.5 Repeatability of T-RFLP

The ability to consistently identify *Cryptosporidium* species by T-RFLP was determined by a blind screen analysis using 20 alphabetically coded samples. Amplification was successful for 19 of the 20 isolates analysed. Fourteen isolates contained a 64 bp T-RF and were identified as *C. hominis* and the remaining 5 as *C. parvum* due to detection of the 624 bp T-RF's. T-RFLP correctly typed all blind screen isolates to their respective species.

8.2.5 Discussion

This study developed and evaluated T-RFLP for *Cryptosporidium* species identification, and is the first to apply this methodology to protozoa. Routine laboratory diagnosis of *Cryptosporidium* oocysts does not discriminate between *Cryptosporidium* species (Kehl et al., 1995; Baveja, 1998). Species identification, a basic requirement for epidemiological research, is commonly performed using PCR-RFLP or DNA sequencing (Xiao et al., 1999b; Chalmers et al., 2007b). Although PCR-RFLP is capable of species detection and identification, it requires relatively high parasite loads for interpretation of restriction fragments resolved using agarose gel electrophoresis. Consequently, low-level infections elude diagnosis, and important species information required for effective management of the disease is lost. Terminal-RFLP was shown to be a rapid, sensitive and non subjective alternative method for *Cryptosporidium* species identification.

Although 5 *Cryptosporidium* species are known to cause disease in humans (Morgan-Ryan et al., 2002; Xiao and Ryan, 2004; Misic and Abe, 2007), this study developed T-RFLP on 18S rDNA to differentiate *C. hominis* and *C. parvum*. Terminal-RFLP unambiguously differentiated between *C. hominis* and *C. parvum* isolates, identifying the *C. hominis* specific peak at 64 bp and the *C. parvum* specific peak at 624 bp. Due to the heterogeneity within the 18S rRNA genes of *C. parvum*, electropherograms from these isolates displayed a second minor peak at 621 bp which was attributed to the second, Type B, copy of the 18S rDNA (Le Blancq et al., 1997). The mobility calculated by the GeneScan software was approximately the expected size for both species, 69 bp for *C. hominis* and 627 bp for *C. parvum*. To maintain accurate species identification and correct for run variation, a panel of reference controls should be included in each analysis run. The reliability and reproducibility of T-RFLP for *Cryptosporidium* species

identification is high with the T-RFs for all samples agreeing with previous sample identifications. The DNA extraction method used in this study is new (9) and no stability data has been reported. It is possible that failure of 8 isolates re-amplify was due to degradation over the 6 month storage. Evaluation of DNA stability after extraction with the PrepGem needs to be undertaken if this method is to be used for the preparation of *Cryptosporidium* libraries.

Mixed infections are not commonly detected in human cryptosporidiosis cases. It has been proposed that when *C. hominis* and *C. parvum* simultaneously infect the same host, *C. parvum* will displace *C. hominis* and establish itself as the predominant parasite (Akiyoshi et al., 2003). Mixed species analyses in this study indicate that preferential amplification of *C. parvum* DNA occurs regardless of its relative contribution to the DNA pool. Both RFLP and T-RFLP failed to consistently detect *C. hominis* DNA when its contribution to the DNA pool was equal to or less than *C. parvum*. Similar results have been recorded in a previous study which used different nested primer sets (Reed et al., 2002) and suggests that mixed infections may be occurring more frequently than recorded. Limitations of selective amplification of PCR mean that past human cryptosporidiosis studies have overlooked the contribution of *C. hominis*, and other pathogenic species, to human disease. This would have limited the identification of all possible disease sources.

Accurate identification of all species causing human cryptosporidiosis will increase our understanding of both disease dynamics and infection sources. Here, *C. hominis* and *C. parvum* were the only species used, but altering the restriction enzyme will enable T-RFLP to be used for identification of other *Cryptosporidium* species of concern to human health; such as *C. meleagridis*, *C. canis*, and *C. muris* which can be differentiated using *SspI* (Xiao et al., 1999a).

The implementation of T-RFLP to epidemiological studies will provide rapid, reproducible and accurate identification of *Cryptosporidium* infections. The MDL and high sensitivity of T-RFLP to a single oocyst will improve prevalence estimations. The methodology developed in this study will be used in an ongoing investigation of human cryptosporidiosis in NSW, Australia.

8.2.6 Acknowledgments

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8.3 Evaluation of a PCR protocol for sensitive detection of *Giardia intestinalis* in human faeces

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8.3.1Abstract

Giardia intestinalis is a protozoan parasite and a human pathogen. It is a leading cause of human diarrheal disease and a significant cause of morbidity worldwide. At the molecular level, *G. intestinalis* is a species complex, consisting of genetic assemblages (A to G) and sub-assemblage strains. The genotypes that cause human disease have been characterised to assemblages A and B, and include strains AI, AII, BIII and BIV. PCR amplification of diagnostic loci is used to genotype samples and is required to understand different transmission cycles within communities. A multi-locus approach is required for validation of *Giardia* genotyping and molecular diagnostic techniques that are efficient across numerous loci have not been established. This study evaluated several published protocols for the 18S small subunit ribosomal RNA and glutamate dehydrogenase genes and determined an optimal method for *G. intestinalis* identification. The protocol was highly sensitive for PCR amplification and an efficient method for testing clinical samples.

8.3.2 Introduction

Giardia intestinalis (syn. *G. duodenalis*, *G. lamblia*) is a flagellated, protozoan parasite and a leading cause of human diarrheal disease worldwide (Caccio et al. 2005; Read et al. 2004). Molecular data indicates that *G. intestinalis* is a species complex, consisting of seven genetic assemblages (A to G) that have been isolated from a range of mammalian hosts (Caccio and Ryan 2008; Monis et al. 1999). These assemblages also consist of numerous sub-assemblage strains (Caccio and Ryan 2008; Monis et al. 1999). Human disease appears to be only caused by assemblages A and B, including four strains; AI, AII, BIII and BIV (Monis et al. 1999). Transmission cycles for sub-assemblage strains have not been defined and both anthroponotic and zoonotic transmission is likely (Caccio and Ryan 2008). Clinical diagnosis of giardiasis is confirmed through immuno-detection assays and / or microscopic examination of faecal smears (Traub et al. 2005). These tests do not involve genetic characterisation and as such, strain information is not collected for human cases (Traub et al. 2005). Molecular analysis is the only means to identify assemblages and strains, and has become a central component of epidemiological research (Traub et al. 2005). Published protocols to extract and amplify *G. intestinalis* DNA are numerous, however no standard method exists (Caccio et al. 2005; Nantavisai et al. 2007). For clinical samples PCR remains problematic due to the presence of DNA polymerase inhibitors and degraded DNA in faecal material, non-specific binding of primers, and intermittent parasite shedding (Caccio and Ryan 2008; Nantavisai et al. 2007; Traub et al. 2005). To overcome PCR inhibition, many protocols incorporate cyst concentration prior to DNA extraction, however it is unclear what combination of molecular protocols is optimal for *Giardia* (Hopkins et al. 1997; Pelayo et al. 2008; Read et al. 2004). Robust, reproducible and

highly sensitive techniques are essential for comparisons of regional data and for estimating the contribution of different *G. intestinalis* strains to global human infection (Smith et al. 2006).

In this study, published molecular protocols for *G. intestinalis* identification were evaluated for both the small subunit ribosomal RNA (18S rRNA) and the glutamate dehydrogenase (*gdh*) genes. Molecular methods for five *Giardia* loci have been previously evaluated (Nantavisai et al. 2007) and DNA extraction using FTA filter paper (Whatman Bioscience, Cambridge, United Kingdom) in combination with 18S rRNA amplification was found to be the most sensitive method (Nantavisai et al. 2007). The 18S rRNA gene is highly conserved and can be used to assign *G. intestinalis* to the assemblage level (A-G). Analysis of clinical samples requires more variable diagnostic loci such as the *gdh* to characterise the sub-assemblage strains that infect humans (Read et al. 2004). To validate *G. intestinalis* genotyping, molecular techniques also need to be efficient across numerous loci. The purpose of the present study was to evaluate current molecular protocols for detection of *G. intestinalis* in human faeces at the 18S rRNA and *gdh* loci, and to determine an efficient and sensitive method, that improves processing of problematic clinical samples.

8.3.3 Materials and methods

8.3.3.1 Faecal samples and DNA extraction

Giardia positive faecal samples (n = 50) were obtained from pathology laboratories in Sydney, Australia and assigned a Macquarie University *Giardia* (MQG) number. Pathology companies had deemed samples positive using a Remel ProSpecT (Thermo Fisher Scientific© 2010) *Giardia*/*Cryptosporidium* microplate immunoassay, and confirmed this result by microscopic examination of faecal smears. Samples were irradiated with ^{60}Colbalt exposure, 1500 curies, for 30 minutes (Macquarie University) to enable processing in non-PC2 laboratories and stored at 4°C until further analysis.

The optimal method for extracting and amplifying *G. intestinalis* DNA at two loci was evaluated using two separate approaches and positive DNA controls that included; pure *G. intestinalis* DNA extracted from laboratory cultivated cysts (+ cysts) (Waterborne Ltd, New Orleans, LA, USA) and laboratory cultivated trophozoites (+ trophozoites). Spiked faecal material from four clinical samples (MQG17, MQG23, MQG24, MQG27) were also included to assess the clinical performance of each assay in the presence of PCR inhibitors in faecal material.

8.3.3.2 Evaluation of PCR protocols

The first approach evaluated several published PCR protocols following direct DNA extraction using an ISOLATE Fecal DNA Kit (Bioline, Sydney, Australia). Faecal material (50 mg) was directly added to a bead lysis tube and samples were spiked with 625 *G. intestinalis* cysts in PBS (5 µl) and DNA extraction was conducted according to the manufacturer's instructions.

To determine the most sensitive PCR method, all positive controls were amplified at the 18S rRNA locus (180 bp fragment) following the nested PCR protocol (Hopkins et al. 1997;

Read et al. 2002) and using primers RH11, RH4LM, GiAR18SiR and GiAR18SeR. Six DNA polymerases; *Tth* plus DNA polymerase (Fisher Biotech, Australia) (Hopkins et al. 1997; Read et al. 2002; Read et al. 2004), Red Hot *Taq* (ABgene, Surrey, United Kingdom) (Waldron et al. 2009), *Accutaq* (Sigma-Aldrich, Australia) (Waldron et al. 2009), *Sahara* DNA polymerase (Bioline, United Kingdom) (Waldron et al. 2009), Ampli*Taq* 360 DNA polymerase (Invitrogen) (Caccio et al. 2003; van der Giessen et al. 2006), GC-RICH PCR System, dNTPack (Roche Diagnostics, Indianapolis, IN) (Sulaiman et al. 2003) were tested. All reaction mixtures (25 µl) were prepared following the manufacturer's instructions. To optimise PCR chemistries, primer concentration was varied between 12.5 pmol and 20 pmol. For *Tth* plus, Red Hot *Taq*, *Accutaq* and *Sahara* DNA polymerases, $MgCl_2$ was varied between 1.5 and 6 mM, BSA between 0 and 1 µg/µl, and DMSO was trialled at 2% and 5%. For Ampli*Taq*, 360 GC-enhancer (manufacturer supplied) was tested at 0 µl, 5 µl and 10 µl. For the GC-RICH PCR System, dNTPack, GC-RICH Resolution Solution (manufacturer supplied) was tested at 0 M, 0.5 M, 1 M and 2 M. All reactions were performed in an Eppendorf Mastercycler (Eppendorf, North Ryde, Australia). PCR products were resolved by agarose gel electrophoresis (2% w/v, 110V for 30 min) in TBE (Tris, EDTA, boric acid, pH 8) with SYBR safe and product size was estimated against a HyperLadder™ II DNA marker (Bioline, Sydney, Australia). Secondary PCR products were compared under UV light and products were purified using a QIAquick PCR Purification Kit (Qiagen, Melbourne, Australia), following the manufacturer's instructions for the Microcentrifuge Bench Protocol. To evaluate the efficiency of each assay, the amplicon concentration (ng/µl) was measured using a NanoDrop® Spectrophotometer (NanoDrop Technologies, Inc., Wilmington DE, USA). To evaluate the differences in DNA concentrations for secondary PCR products, One-Way ANOVA and Tukey post hoc statistical tests were

performed. To evaluate *gdh* PCR, positive controls were amplified using the DNA polymerases that generated the highest 18S rRNA PCR yields, and an optimal PCR method for both loci was determined.

8.3.3.3 Evaluation of DNA extraction techniques

The second approach evaluated DNA extraction techniques using Immunomagnetic Separation (IMS) to concentrate cysts from faeces and remove DNA from PCR inhibitors (Power et al. 2003). The procedure for IMS, as outlined by Power et al. (2003) was followed using *Giardia*-specific mouse IgG1 monoclonal antibody, G203 (100 µg; AusFlow®) to prepare *Giardia*-specific paramagnetic beads. Following filtration of the faecal slurry (1 ml), each sample was spiked with 1250 *G. intestinalis* cysts in PBS (10 ul). Cyst capture involved a primary bead volume of 150 µl and antibody disassociation was not conducted (Power et al. 2003). Following cyst capture the supernatant was removed and cysts bound to beads were washed with water (160 µl; sterile) and resuspended by vortexing. Purified IMS suspensions (160 µl; sterile H_20) were then divided into two aliquots (80 µl), each containing 625 purified cysts (equivalent to spiked cyst loads in direct ISOLATE fecal DNA extractions). The samples were used for two DNA extraction methods, the ISOLATE Fecal DNA Kit (used previously for direct faecal DNA extractions) and PrepGEM (ZyGEM Corporation Ltd., Hamilton, New Zealand). For the ISOLATE Fecal DNA Kit, one aliquot (80 µl) of IMS purified cysts were directly added to a bead lysis tube and processed according to manufacturer's instructions. For PrepGEM DNA extraction, the second aliquot (80 µl) was suspended in 1X Buffer Gold and processed as described by Ferrari et al. (2007). DNA samples were amplified at the *gdh* locus alone using the optimal PCR chemistry (previously determined). The *gdh* gene was favoured as the higher

sequence diversity enables all human strains and all major assemblages to be delineated (Read et al. 2004). The *gdh* gene is also more difficult to amplify due to its single copy nature and higher nucleotide diversity, and as such DNA extraction was targeted at increasing PCR sensitivity for this gene (Caccio and Ryan 2008; Nantavisai et al. 2007). DNA concentration was measured for all isolates as previously described. A two tailed t-test for significance was performed on concentration measurements for secondary PCR products and an optimal molecular protocol for *G. intestinalis* identification was determined.

8.3.3.4 Assessment of clinical performance

Clinical performance of the optimal protocol was evaluated using fifty clinical samples and amplification of the 18S rRNA and *gdh* loci. The numbers of *Giardia* cysts per gram of faeces were calculated for each sample using IMS-flow cytometry (IMS-FC) to determine sensitivity of the protocol. IMS-FC was conducted according to the method described by Power et al. (2003) using G203-FITC pre-stain (8 µl; 50µg/ml), G203-FITC post stain (50 µl; 10 µg/ml) and a positive control containing *G. intestinalis* cysts were used to identify the FITC detection (FL2), compensation and threshold parametres for *Giardia* cysts. FC sorting was conducted on a FACSCALibur sort, flow cytometre (Becton Dickinson Biosciences, Sydney Australia) and analysed by Cellquest software (BD Biosciences, Sydney Australia). Cysts were collected on Isopore^{TM} membranes (13mm, 0.8µM; Millipore, Sydney, Australia) using a Sort Stage (MRL, Sydney, Australia) connected to the flow cytometre, as previously described (Power et al. 2003). Cysts were resolved under epifluorescent microscopy, 40X magnification (Zeiss Axioskop epifluorescent microscope, Carl Zeiss, Sydney Australia) and counted across 20 microscope fields per membrane.

8.3.4 Results

8.3.4.1 Evaluation of PCR protocols

The performance of six published PCR assays were evaluated by comparing the intensity of PCR products under UV light and the concentration of DNA generated by each assay. Amplification of the 18S rRNA gene was only successful for five of the six protocols tested. Ampli*taq* failed to generate any PCR products whilst Red Hot *Taq* and *Sahara* DNA polymerase generated comparatively lower PCR yields, as visualised under UV light and by comparing DNA concentration measurements (Table 8.4). 18S rRNA amplification using *Tth* plus DNA polymerase, following the PCR chemistry previously described (Hopkins et al. 1997; Read et al. 2002) and the GC-RICH PCR System, dNTPack (using 20 pmol of each primer and 0.5 M of GC-RICH Resolution Solution) generated the highest DNA concentrations (Table 8.4) and clearer and brighter amplicons (Figure 8.3). For the GC-RICH PCR System, dNTPack DNA concentration measurements were significantly higher than Red Hot *Taq* and *Sahara* (ANOVA main p-value < 0.00 and all Tukey p-values < 0.01), whilst *Tth* plus generated higher concentrations than Red Hot *Taq*, *Sahara* and *Accutaq* (Tukey p-values < 0.01). There was no statistical difference between concentration measurements for *Tth* plus and the GC-RICH PCR System, dNTPack. Whilst our results indicate both PCR methods were optimal, the GC-RICH PCR System, dNTPack was chosen to evaluate DNA extraction methods, *gdh* amplification and clinical performance. This method was favoured as the GC-RICH PCR System, dNTPack was more user-friendly, and reduced PCR preparation times.

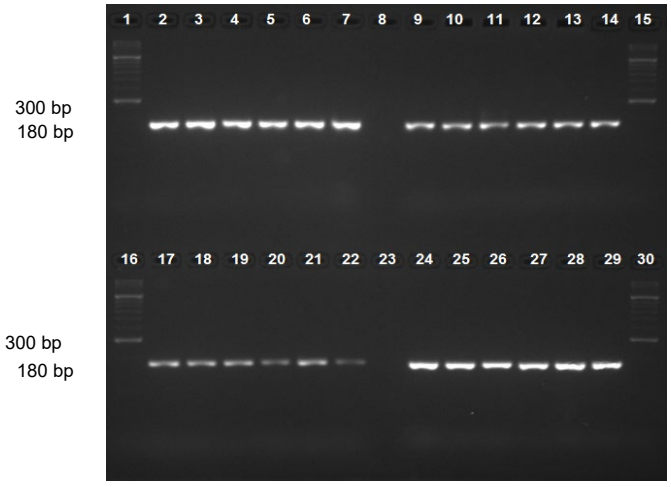
Table 8.4 Comparison of DNA concentrations generated by different molecular protocols for 18S rRNA and *gdh* amplification

Samples	*Tth* plus	18S rRNA PCR[a]				*gdh* PCR[b]	
		Red Hot *Taq*	*Sahara*	*Accutaq*	GC-RICH PCR System	IMS & PrepGEM	IMS & ISOLATE
+ cysts	15.5	8.4	9.8	11.5	12.8		
+ trophozoites	14.5	10.2	10.1	10.8	13.5		
MQG17	14.2	7.3	8.4	13	14.9	22.6	39.3
MQG23	12.7	6.2	7.4	11	12.2	11.8	30.1
MQG24	15.1	6.9	8.4	11.4	13.5	15.6	36.0
MQG27	16.7	6.8	6.5	11.6	13	20.3	40.7
Mean concentration	14.78	7.63	8.43	11.55	13.32	17.58	36.53
SD	1.35	1.45	1.38	0.77	0.92	4.83	4.71

[a] 180 bp fragment, 35 PCR cycles

[b] 432 bp fragment, 55 PCR cycles, amplified using GC-RICH PCR System

Figure 8.3Comparison of positive DNA controls and DNA polymerases that generated the highest 18S rRNA yields. Lanes 2–7, *Tth* plus DNA polymerase. Lane 2, cyst DNA; lane 3, trophozoite DNA, lanes 4–7, spiked clinical samples MQG17, MQG23, MQG24, MQG27; lane 8, no product added. Lanes 9–14, PCR using *Accutaq* DNA polymerase. Lane 9, cyst DNA; lane 10, trophozoite DNA; lanes 11- 14 spiked samples MQG17, MQG23, MQG24, MQG27; Lanes 17-22, PCR using *Sahara* DNA polymerase. Lane 17, cyst DNA; lane 18, trophozoite DNA; lanes 19-22, spiked samples MQG17, MQG23, MQG24, MQG27; lane 23, no product added. Lanes 24-29, PCR with GC-RICH PCR System. Lane 24, cyst DNA; lane 25, trophozoite DNA; lanes 26-29 spiked samples MQG17, MQG23, MQG24, MQG27



8.3.4.2 Evaluation of DNA extraction techniques

DNA concentration measurements were used to compare the efficiency of ISOLATE Fecal DNA extraction and PrepGEM DNA extraction, following IMS. The ISOLATE Fecal DNA Kit generated the highest DNA concentration measurements in *gdh* amplicons (Table 8.4) and the differences between the two techniques were statistically significant (t-test p-value < 0.00). *Gdh* concentrations measurements were also substantially higher than 18S rRNA measurements however product size and number of PCR cycles would account for this difference. The method was trialled on clinical samples however conducting IMS prior to DNA extraction resulted in PCR failures and IMS was removed from the protocol. The optimal molecular protocol was established from direct DNA extraction using the ISOLATE Fecal DNA Kit and GC-rich PCR chemistry using the GC-RICH PCR System, dNTPack.

8.3.4.3 Evaluation of clinical and diagnostic performance

Application of the optimal molecular protocol to fifty clinical samples resulted in successful amplification at both loci. For the 18S rRNA gene, 45 clinical samples were amplified and for the *gdh* gene, 47 samples were amplified. The numbers of cysts in clinical samples ranged from 10 to over 10^6 cysts per gram of faeces (Table 8.5). One sample that was PCR negative at both the 18S rRNA and *gdh* loci, was also negative by IMS-FC and microscopy, and for this sample, *G. intestinalis* cysts could not be identified (Table 8.5). Of the six that samples that were PCR negative at one locus, four samples contained *G. intestinalis* cysts, and in two of these cyst loading exceeded 10^4 cysts per gram of faeces (Table 2). An additional eight samples were negative by IMS-FC.

Table 8.5 Comparison of 18S rRNA and *gdh* PCR amplification with the number of cysts isolated from 49 clinical samples.

PCR amplification	> 10^3 cysts/gram faeces	200 – 10^3 cysts/gram faeces	< 200 cysts/gram faeces	IMS-FC negative	Total
18S rRNA & *gdh* positive	20	3	13	6	42
Positive 1 locus	2		2	2	6
Negative both loci				1	1

8.3.5 Discussion

The aim of this study was to evaluate molecular protocols for *G. intestinalis* identification and determine an optimal method for clinical samples. *G. intestinalis* is a significant cause of morbidity in humans worldwide, and identification of human infective strains through PCR detection and genotyping is the only means of documenting which strains are present within communities (Traub et al. 2005). This study analysed several published protocols for DNA extraction and PCR amplification. For PCR amplification protocols included; *Tth* plus DNA polymerase, Red Hot *Taq*, *Accutaq*, *Sahara* DNA polymerase, Ampli*Taq* and the GC-RICH PCR System, dNTPack. For DNA extraction IMS, the ISOLATE Fecal DNA Kit and PrepGEM were investigated. Once a sensitive PCR method was determined, our results showed that optimising DNA extraction to increase DNA template in clinical samples was more efficient than purifying DNA from faecal inhibitors. PCR using the GC-RICH PCR System, dNTPack or *Tth* plus was optimal for amplifying *Giardia* DNA and generated comparatively higher 18S rRNA PCR yields than other PCR methods. *Tth* plus is commonly used for *Giardia* (Hopkins et al. 1997; Read et al. 2004; Read et al. 2002) however we chose to use the GC-RICH PCR System, dNTPack to optimise a streamlined protocol and improve processing speed. *Tth* plus was difficult to use for both PCR reactions and gel electrophoresis. Reagents were sticky and solutions were difficult to aliquot, and time consuming to use. Although we preferred the GC-RICH PCR System, dNTPack, *Tth* plus is an inexpensive alternative (< 20% of GC-RICH PCR System, dNTPack price on a per unit basis). Based on our results, the sensitivity of the *Tth* protocol means it would be suitable for clinical analyses.

For DNA extraction the ISOLATE Fecal DNA Kit following IMS was the most sensitive method for spiked faecal samples and generated comparatively higher concentrations of DNA

than PrepGEM. When tested on clinical samples, conducting IMS resulted in PCR failures and may be the result of several factors including; non-specific binding of the monoclonal antibody; intermittent cyst shedding; and the exclusion of trophozoites through G203 specificity for cyst wall antigens (Ferrari et al. 1999; Power et al. 2003). The IMS recovery rate for *Giardia* was not determined in this study however our results suggest that trophozoites are an important source of *Giardia* DNA. Previous studies have reported reduced sensitivity following IMS (Pelayo et al. 2008; Wilke et al. 2009) and higher PCR success rates (>70%) using direct faecal DNA extraction (Wilke et al. 2009). Our analysis of faecal samples by IMS-FC showed that several samples contained low numbers of excreted cysts. Many clinical patients shed trophozoites with the rapid passage of intestinal contents (Nantavisai et al. 2007; Roxstrom-Lindquist et al. 2006) and maximising detection by extracting DNA from both cysts and trophozoites is beneficial.

The optimal molecular protocol for 18S rRNA and *gdh* amplification was established from direct DNA extraction using the ISOLATE Fecal DNA Kit followed by GC-rich PCR chemistry using the GC-RICH PCR System, dNTPack. This technique was a combination of previously described methods (Read et al. 2004; Sulaiman et al. 2003) and demonstrated high diagnostic performance when applied to fifty positive clinical samples; generating PCR success rates of 90% and 94% for the 18S rRNA and *gdh* loci respectively. The protocol also displayed high sensitivity and precision, amplifying DNA from as little as 10 cysts per gram of faeces. Cyst loads were highly variable in clinical samples, ranging from 10 cysts to 10^6 cysts per gram of faeces. Identification of high cyst loads ($>10^4$) in samples that were PCR negative at one locus demonstrated that even when cyst shedding is high, other factors such as DNA polymerase inhibition and the quality of intact DNA can prevent PCR amplification.

8.3.5.6 Conclusions

This study has evaluated published protocols *G. intestinalis* and determined an optimal method for clinical samples. Direct DNA extraction from faecal material is simple and rapid, and additional processing is not required. PCR amplification is successful in the presence of faecal inhibitors, eliminating the need for cyst concentration prior to DNA extraction. The high sensitivity of this protocol also reduces labour time by minimising the need to repeat assays and account for intermittent shedding, and only a small amount of faecal material (approximately 50 - 150 mg) is needed. A multi-locus approach is required for validation of *Giardia* genotyping and application of this protocol to two loci removes additional PCR optimisation steps that are normally conducted.

We view the assay as a tool for epidemiological investigations, which is of clinical and public health importance. Using PCR detection for routine diagnosis of giardiasis would be favourable and of significant value to understanding the biology of this important parasite. However, due to the expense and expertise required to perform such analyses, application of PCR in clinical diagnostic laboratories is not yet routine (Savioli et al. 2006). A standard method to extract and amplify *G. intestinalis* DNA is needed improve processing of problematic DNA samples, document infective strains and progress epidemiological knowledge. This study has highlighted that insensitive molecular techniques can produce false negative results, and may lead to inaccurate disease reporting.

The use of the protocol described here for molecular diagnostics and epidemiological investigations should provide a streamlined, efficient and sensitive method for examining clinical samples. It should offer a specific framework for *G. intestinalis* identification that is reproducible between laboratories. Application of this protocol should allow larger sample

screenings and identification of *G. intestinalis* strains, which is essential for understanding transmission cycles, infection sources and disease risks, and developing targeted disease management and surveillance relative to the different human infective *Giardia* strains.

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