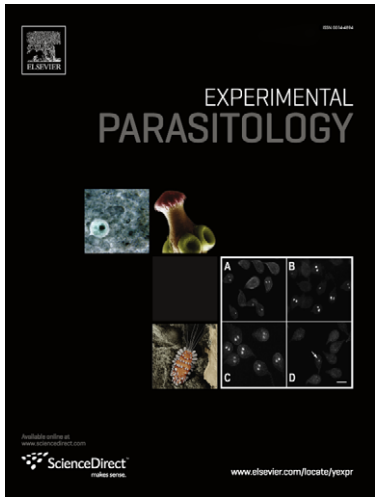

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PII: S0014-4894(10)00067-6
DOI: 10.1016/j.exppara.2010.02.012
Reference: YEXPR 5940

To appear in: *Experimental Parasitology*

Received Date: 21 September 2009
Revised Date: 18 February 2010
Accepted Date: 23 February 2010

Please cite this article as: Ng, J.S.Y., Pingault, N., Gibbs, R., Koehler, A., Ryan, U., Molecular Characterisation of *Cryptosporidium* outbreaks in Western and South Australia, *Experimental Parasitology* (2010), doi: 10.1016/j.exppara.2010.02.012

Molecular Characterisation of *Cryptosporidium* outbreaks in Western and South Australia.

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Abstract

Molecular typing at the 18S rRNA and *Gp60* loci was conducted on *Cryptosporidium*-positive stool samples from cases collected during 2007 Western Australian and South Australian outbreaks of cryptosporidiosis. Analysis of 48 Western Australian samples identified that all isolates were *C. hominis* and were from five different *Gp60 C. hominis* subtype families. The IbA10G2 subtype was most common across all age groups (37/48). In South Australia, analysis of 24 outbreak samples, identified 21 *C. hominis* isolates, two *C. parvum* isolates and one sample with both *C. hominis* and *C. parvum.* All *C. hominis* isolates were identified as the IbA10G2 subtype.

Keywords: *Cryptosporidium*; Outbreak; *Gp60*; 18S rRNA; Glycoprotein; Western Australia; South Australia.

1. Introduction

Cryptosporidium species are parasites that infect a wide range of hosts including humans, domestic and wild animals. In most cases, including those in humans, *Cryptosporidium* infection results in gastrointestinal problems such as severe diarrhoea. Currently 21 species of *Cryptosporidium* are recognised as valid and at least eight of them have been reported in humans; *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. muris*, and *C. suis*, and the *Cryptosporidium* cervine genotype. Of these, *C. hominis* and *C. parvum* are responsible for the majority of infections in humans (Xiao and Fayer, 2008; Xiao, 2009).

To understand the transmission dynamics of *Cryptosporidium*, various fingerprinting/subtyping tools have been developed, one of the most popular of which is sequence analysis of the 60 kDa glycoprotein (*Gp60*, also called *gp15/40*). It is the most polymorphic marker identified so far in the *Cryptosporidium* genome (Gatei et al., 2006; Leoni et al., 2007; Wielinga et al., 2008). It is also biologically relevant as it encodes surface glycoproteins (gp45 and gp15), both of which are implicated in zoite attachment to and invasion of enterocytes (Strong et al., 2000).

In Australia, *Cryptosporidium* is a notifiable disease and evidence to date suggests that *C. hominis* is the predominant species infecting humans but that there is a higher prevalence of *C. parvum* in the human population in New South Wales (NSW) and South Australia (SA) than in Western Australia (WA) (Robertson et al., 2002; Chalmers et al., 2005; Jex et al., 2007; Ng et al., 2008; O'Brien et al., 2008; Morgan et al., 2008; Jex, et al., 2008; Alagappan et al., 2008; Waldron et al., 2009a; 2009b).

From January to May 2007, there was a large increase in the number of notified cryptosporidiosis cases in WA, with 476 cases reported. This was three times the mean notification rate from the previous four years (Gibbs et al., 2008). Cases ranged in age from 4

months to 89 years (median 8 years), with 43% of cases in the <5 year age group. No gender disparity was observed, with 49% of cases male and 51% female. The majority of cases (67%) resided in the Perth metropolitan area. Non-Aboriginal people represented 69% of notified cases, with 13% of cases from Aboriginal people, predominantly from the north of WA. Indigenous status was unknown for 18% of cases. The total number of cases reported for 2007 was 625, which was a rate of 30 cases per 100,000 population (Gibbs et al., 2008). The demography of cases from this outbreak year was similar to cases from non-outbreak years (2004, 2005 and 2006) with the exception of geography and indigenous status. In the outbreak year the proportion of cases from the metropolitan area was higher, and the proportion of indigenous cases was lower.

Similarly in SA in 2007, there was a large increase in the number of notified cases of cryptosporidiosis between January and the end of April with 393 cases reported (Anon, 2007). Cases ranged in age from <1 month to 87 years (median 10 years), with 32% of cases within the 0-4 year age group. No gender disparity was observed, with 45% of cases male and 55% female. The majority of cases (78%) resided in the Adelaide metropolitan area. Non-Aboriginal people represented 82% of notified cases, with 1% of cases from Aboriginal people. Indigenous status was unknown for 17% of cases. The total number of cases reported for 2007 was 449 cases, which was over twice the notification for the preceding year and seven times the number of cases for the subsequent year (Anon, 2007).

The aim of the present study was to identify the *Cryptosporidium* species present in the WA and SA outbreaks and to subtype the isolates at the *Gp60* locus to determine the subtypes present.

2. Materials and methods

2.1 Specimens and DNA extraction

A total of 72 faecal samples associated with the *Cryptosporidium* outbreaks were received from various pathology centers, of which 48 were from Western Australia and 25 were from South Australia. Total DNA was extracted immediately upon receipt, using a QIAmp DNA Stool Kit (Qiagen, Hilden, Germany) and stored at -20°C.

2.2 PCR Amplification and DNA sequencing

Initial genotyping of the samples were carried out by a two-step nested PCR of the 18S rRNA locus (Ryan et al., 2003). The secondary PCR products were purified using a Power Soil DNA Kit (MolBio, Carlsbad, California, USA) according to manufacturer's protocol and sequenced using an ABI Prism Dye Terminator Kit (Applied Biosystems, Foster City, California).

Isolates were sub-genotyped at the *Gp60* gene locus using a two-step nested PCR which amplifies a secondary PCR fragment of ~832bp as previously described (Strong et al, 2000). Purification and sequencing of the amplified DNA fragments were carried out as described above.

Nucleotide sequences were analyzed using Chromas lite version 2.0 (http://www.technelysium.com.au) and aligned using Clustal W (http://clustalw.genome.jp).

3. Results

3.1 Western Australia

All of 48 isolates from WA were identified as *C. hominis* at the 18S locus (Table I). At the *Gp60* locus, five different *C. hominis Gp60* subtype families were identified; Ib, Id, Ie, If and Ig (Table I). Within the *C. hominis* Ib family, IbA10G2 was the only subtype identified and was the most common subtype identified in the WA cases genotyped (37/48). The second most common *Gp60* subtype family was the *C. hominis* Id family (6/48). Three subtypes were identified within the *C. hominis* Id family; IdA16 (3/6), IdA15G1 (2/6) and IdA17 (1/6). The other subtypes identified included *C. hominis* IeA11G3T3 (1/48), *C. hominis* IfA12G1 (1/48) and *C. hominis* IgA17 (3/48).

Although not systematically sampled, the samples genotyped were representative of the overall outbreak in terms of age and gender distribution (Table II). A lower proportion of samples from rural cases were typed compared to the overall outbreak, which may have decreased the probability of identifying *C. parvum*. The majority of the samples typed (94%) were collected during the outbreak period again demonstrating that the typing results were, for the most part, representative of the overall outbreak.

As genotyping was conducted towards the end of the epidemiological investigation, only a quarter of those cases that had isolates genotyped were interviewed. Therefore it was not possible to examine subtype specific risk factors. However, 171 interviews were conducted out of the 476 notified cases. All interviewed cases, resided in the metropolitan area. The case series identified that in the two weeks prior to onset of symptoms, 64% of cases swam in a pool, 53% had contact with pets, 22% were children that attended day care centres, 16% reported contact with a person with a diarrhoeal illness, 14% had contact with

124 farm animals and 10% drank water from a private (untreated) water supply (Gibbs et al.
125 2008). Eleven of the interviewed cases were hospitalized as a result of their illness.

126 The age range for these 48 WA isolates genotyped was <1 to 76 (Table I). The
127 highest prevalence was amongst children <5 years of age (39.6%) but was also common
128 amongst older age groups with the second highest peak, amongst the 16-39 age group (Table
129 I). The IbA10G2 subtype was identified across all age groups.

130

131 *3.2 South Australia*

132

133 Of the 24 isolates from SA, 21 were *C. hominis*, 2 were *C. parvum* and 1 isolate was a
134 mixed *C. hominis/C. parvum* infection (Table I). At the *Gp60* locus, all 21 of the *C. hominis*
135 isolates belonged to the *C. hominis* IbA10G2 subtype. The two *C. parvum* isolates belonged
136 to the *C. parvum* IIaA18G3R1 subtype. Isolate SA17, which was a mixed *C. parvum/C.*
137 *hominis* infection exhibited the *C. hominis* IbA10G2 and the *C. parvum* IIaA19G3R1
138 subtypes respectively.

139 The age range for the 24 SA isolates was <1 to 74 (Table II). The highest prevalence
140 occurred in the 16-29 age-group (37.5%), followed by the <5 age group (29.2%). As with the
141 WA outbreak, the IbA10G2 subtype was distributed across all age-groups (Table I). Of the 24
142 isolates examined, 30% were from regional locations in SA and the remainder (n = 15) were
143 from metropolitan Adelaide. Of the 8 isolates from rural locations, only one was identified as
144 *C. parvum*.

145 Limited epidemiological investigations were conducted in SA. Initial phone interviews
146 with 30 individuals identified that 20 people had used public swimming pools, 3 had used
147 private swimming pools, 1 drank rainwater, 1 swam in the Murray River, 1 swam in a farm
148 dam, 1 had fallen into a septic tank and 3 had family members with gastrointestinal illness
149 consistent with cryptosporidiosis, but did not submit faecal specimens for testing.

3.3 Gp60 nucleotide sequence accession numbers

Representative *Gp60* subtypes identified during the present study have been deposited in the GenBank database under accession numbers GU810905-GU810914.

4. Discussion

In the present study, the anthroponotic *C. hominis* was the most prevalent *Cryptosporidium* species identified in both cryptosporidiosis outbreaks in WA and SA. In WA, all isolates genotyped (48) were *C. hominis*, whereas in SA, *C. hominis* was identified in 87.5% (21/24) of isolates, *C. parvum* was identified in 8.3% of samples (2/24) and a mixed *C. hominis/C. parvum* infection was identified in one patient.

There was a broad spread of age groups infected during both the WA and SA outbreaks. In WA, 32% of cases were in children less than 5 years of age, with a second peak (27%) in the 16-39 age group. In SA, the highest infection rate was amongst the 16-39 age group (37.5%), followed by the <5 age group (29.2%). Cryptosporidial infections are predominately reported in children <5 years of age (Fayer, 2007), but the present results reveal a higher peak of infection in the 16-39 age group in SA and highlight the fact that all age-groups are susceptible to infection with *Cryptosporidium.* The peak in the 16-39 age group, may be due to adults and teenagers caring for infected children becoming infected themselves.

A case series conducted in WA identified that 64% of interviewed cases swam in a pool prior to onset of illness. Twenty-three SA cases reported swimming in a pool prior to onset of illness. While these data indicate that swimming pools could be a source of infection, it is important to note that neither state conducted a case-control study. Therefore, the

proportion of non-infected people that swam in that time frame was unknown, and it could not be determined whether swimming was a significant risk factor *Cryptosporidium* infection.

Waterborne transmission of *Cryptosporidium* through swimming in contaminated pools is now recognized as an important transmission route for *Cryptosporidium* and approximately 50.3% (83) of the reported outbreaks of cryptosporidiosis worldwide have been due to contamination of swimming pools (Karanis et al., 2007). Contamination in swimming pools is often associated with accidental faecal contamination and poorly constructed and/or maintained plumbing (Joce et al., 1991). It is possible that outbreaks begin in swimming pools but then become transmitted from human to human through contact with children, fomites etc. To date, no studies have been conducted in Australia on *Cryptosporidium* contamination levels and genotypes in public swimming pools. This work is urgently needed to better understand the transmission dynamics in the human population in Australia.

In the present study, all the WA isolates genotyped were *C. hominis*, whereas in the SA isolates, *C. parvum* was detected in 3 isolates. This supports previous research reporting a higher prevalence of *C. parvum* in the human population in NSW, Victoria and SA than in WA (Robertson, et al., 2002; Chalmers et al, 2005; Jex et al., 2007; Ng et al., 2008; O'Brien et al., 2008; Jex, et al., 2008; Alagappan et al., 2008; Waldron et al., 2009a; 2009b). For example, in NSW, a recent study of 69 sporadic cases of cryptosporidiosis identified *C. hominis* in ~53% (37/69) and *C. parvum* in ~46% (32/69) (Waldron et al., 2009a). Why this appears to be the case is difficult to determine. In the present study, an analysis of the SA outbreak samples indicated that only one of the *C. parvum* isolates identified from SA was from a rural location.

Determining the prevalence of *C. hominis* and *C. parvum* in human populations is important as recent reports strongly suggest that there are different clinical manifestations between *C. parvum* and *C. hominis*, particularly in children. In a study of sporadic

cryptosporidiosis in the UK, illness was more severe in persons infected with *C. hominis* than in those infected with *C. parvum* (Hunter et al., 2004a; 2004b). In addition, *C. hominis* but not *C. parvum* was associated with an increased risk of non-intestinal symptoms such as joint pain eye pain, headaches, dizzy spells and fatigue (Hunter et al., 2004b). In a study of children infected with *Cryptosporidium* in Brazil, *C. hominis* infections were associated with higher oocyst shedding in stools as well as with greater shortfalls in growth in the post-infection period even in the absence of symptoms (Bushen et al., 2007). A 4-year longitudinal birth cohort of 533 children in Peru, reported that C. *hominis* was associated with diarrhea, nausea, vomiting, general malaise, and increased oocyst shedding intensity and duration. In contrast, *C. parvum*, *C. meleagridis*, *C. canis*, and *C. felis* were associated with diarrhea only (Cama et al., 2008).

Gp60 subtyping indicated that the *C. hominis* IbA10G2 subtype was the most prevalent subtype identified in both the WA and SA outbreaks. The *C. hominis* Ib subtype family is the most frequently detected *Cryptosporidium* spp. in waterborne outbreaks of cryptosporidiosis in industrialized nations (Xiao, 2009). The *C. hominis* IbA10G2 subtype has been common in sporadic human cryptosporidiosis cases in the UK, US, Peru, Slovenia and South Africa (Peng et al., 2001; Leav et al., 2002; Zhou et al., 2003; Chalmers et al., 2005; Alves et al., 2006; Soba and Logar, 2008). The IbA10G2 subtype is also common in NSW (Ng et al., 2008; Waldron et al., 2009b), and is the subtype responsible for the 2009 NSW outbreak (Waldron et al., 2009c). Previous studies in WA and SA have reported a prevalence of ~20-50% of the IbA10G2 subtype amongst sporadic C. hominis infections (Jex et al., 2008; O'Brien et al., 2008). Few studies have examined the association between specific *Gp60* subtype families and clinical symptoms. A recent study has reported that while all *C. hominis* subtype families were associated with diarrhoea, only *C. hominis* subtype family Ib was also associated with nausea, vomiting, and general malaise (Cama et al., 2008).

The *C. hominis* subtypes Id, Ig, Ie and If were also detected in the WA outbreak

isolates. Id subtypes are common in industralised countries and the Ig subtype has previously been reported in a drinking-water associated outbreak in Northern Ireland (Glaberman et al., 2002). Subtype If is less common. All the Id, Ig, and If subtypes identified in the present study, have been previously reported in WA human isolates (O'Brien et al., 2008). Subtype IeA11G3T3 found in a single isolate from WA, is a cause of sporadic cryptosporidiosis around the world (Xiao, 2009). Amongst the SA *C. parvum* isolates, subtype IIaA18G3R1was identified in 2/3 *C. parvum* positive human isolates. This subtype has been identified as the most common subtype in cattle in Perth, WA (O'Brien et al., 2008), Tamworth, NSW (Ng et al., 2008), Canada (Trotz-Williams et al., 2006) and Northern Ireland (Thompson et al., 2007).

In conclusion, the *C. hominis* IbA10G2 was the pre-dominant subtype identified in both the WA and SA outbreak samples. No conclusive link between the outbreak and a source of contamination was determined.

Acknowledgements

The authors gratefully acknowledge the participation of the Departments of Microbiology, PathWest Laboratory Medicine WA, Princess Margaret Hospital for Children and Fremantle Hospital, Western Diagnostic Pathology, Clinipath Pathology, Saint John of God Pathology; Ms Bethany Lord and the population health nurses, Department of Health WA; and staff at the Institute of Medical and Veterinary Science (IMVS) SA in this study.

The OzFoodNet enhanced foodborne disease surveillance program is funded by the Australian Government Department of Health and Ageing.

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Table I. Distribution of *Cryptosporidium* species and subtypes in different age groups in WA and SA outbreaks, for samples that were genotyped.

State	Age range (yrs)	No of cases	%	Species	*Gp60* subtype	No. of subtypes
WA	<5	23	47.9	*C. hominis*	Ib A10G2	18
					Id A16	1
					Id A17	1
					1g A17	3
	5-15	6	12.5		1b A10G2	5
					1d A16	1
	16-39	13	27.1		1b A10G2	10
					1d A16	1
					1d A15G1	2
	40-90	6	12.5		1b A10G2	4
					1e A11G3T3	1
					IfA21G1	1
Total		**48**				**48**
SA	<5	7	29.2	*C. hominis*	Ib A10G2	7
	5-15	3	12.5	*C. hominis*	Ib A10G2	3
	16-39	9	37.5	*C. hominis*	Ib A10G2	8
				+ C. parvum	IIa A18G3R1	1
	40-90	4	16.7	*C. hominis*	1b A10G2	3
				+ C. parvum	IIa A19G4R1*	1
	UK	1	4.1	*C. parvum*	IIa A18G3R1	1
Total		**24**				**24**

UK = age unknown

*mixed *C. parvum* IIa A19G4R1 and *C. hominis* Ib A10G2 infection.

Table II. Comparison of demographic information for samples that were genotyped and overall outbreak data for WA and SA.

	Demographic Information	**Overall outbreak**	**Samples typed**
WA	Age range	0-89 years	0-76 years
	Median age	8 years	6.5 years
	< 5 years old (%)	43	46
	Male (%)	49	42
	Female (%)	51	58
	Urban (%)	67	79
	Rural (%)	33	19
	Date of specimen collection	1/1/2007 31/5/2007	13/3/2007 15/8/2007
SA	Age range	0-87 years	0-74 years
	Median age	10 years	25 years
	<5 years old (%)	32	29
	Male (%)	45	35
	Female (%)	55	65
	Urban (%)	78	70
	Rural (%)	22	30
	Date of specimen collection	N/A	N/A

NA = not available.