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Longitudinal multi-locus molecular characterisation of sporadic Australian human clinical cases of cryptosporidiosis from 2005 to 2008

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ABSTRACT

Cryptosporidium is a gastrointestinal parasite that is recognised as a significant cause of non-viral diarrhea in both developing and industrialised countries. In the present study, a longitudinal analysis of 248 faecal specimens from Australian humans with gastrointestinal symptoms from 2005 to 2008 was conducted. Sequence analysis of the 18S rRNA gene locus and the 60 kDa glycoprotein (*gp60*) gene locus revealed that 195 (78.6%) of the cases were due to infection with *Cryptosporidium hominis*, 49 (19.8%) with *Cryptosporidium parvum* and four (1.6%) with *Cryptosporidium meleagridis*. A total of eight *gp60* subtype families were identified; five *C. hominis* subtype families (Ib, Id, Ie, If and Ig), and two *C. parvum* subtype families (IIa and IId). The Id subtype family was the most common *C. hominis* subtype family identified in 45.7% of isolates, followed by the Ig subtype family (30.3%) and the Ib subtype family (20%). The most common *C. parvum* subtype was IIaA18G3R1, identified in 65.3% of isolates. The more rare zoonotic IId A15G1 subtype was identified in one isolate. Statistical analysis showed that the Id subtype was associated with abdominal pain ($p < 0.05$) and that in sporadic cryptosporidiosis, children aged 5 and below were 1.91 times and 1.88 times more likely to be infected with subtype Id (RR 1.91; 95% CI, 1.7–2.89; $p < 0.05$) and Ig (RR 1.88; 95% CI, 1.10–3.24; $p < 0.05$) compared to children aged 5 and above. A subset of isolates were also analysed at the variable CP47 and MSC6-7 gene loci. Findings from this study suggest that anthroponotic transmission of *Cryptosporidium* plays a major role in the epidemiology of cryptosporidiosis in Western Australian humans.

1. Introduction

Cryptosporidiosis is a gastrointestinal disease in humans and animals caused by the protozoan parasite *Cryptosporidium*. The disease, characterised by self-limiting diarrhoea in immunocompetent individuals, may be chronic and life-threatening to those that are immunocompromised (Hunter and Nichols, 2002; Hunter et al., 2007). Of the 21 valid *Cryptosporidium* species, *Cryptosporidium hominis* and *Cryptosporidium parvum* are responsible for the majority of infections in humans, although *Cryptosporidium meleagridis Cryptosporidium felis* and *Cryptosporidium canis* and to a lesser extent, *Cryptosporidium muris*, *Cryptosporidium suis* and several genotypes have also been reported in humans (Xiao and Fayer, 2008; Xiao, 2010).

Oocysts of most *Cryptosporidium* spp. are morphologically similar and therefore they can only be distinguished by molecular means, usually at conserved loci such as the 18S rRNA or the actin gene. Several fingerprinting tools have been developed to examine the population structure and transmission dynamics of *C. parvum* and *C. hominis* including sequencing of the 60-kDa glycoprotein (*gp60*) gene, which has shown to be useful in tracking source of infection of *C. parvum* and *C. hominis* (Strong et al., 2000; Mallon et al., 2003a; Peng et al., 2003a; Widmer et al., 2004; Xiao and Ryan, 2004; Sulaiman et al., 2005; Alves et al., 2006; Feltus et al., 2006; Gatei et al., 2006; Trotz-Williams et al., 2006; Leoni et al., 2007; Thompson et al., 2007; Ng et al., 2008; O'Brien et al., 2008).

Cryptosporidiosis was listed as a nationally notifiable disease in Australia in 2001 (Communicable Disease Surveillance: Highlights for 2nd quarter 2001, 2001) and since then, incidence of the disease in Australia has been increasing steadily with 2009 recording the highest number of cases nationally compared to that of the previous 5 years (National Notifiable Diseases Surveillance System (NNDSS), http://www.health.gov.au). In Western Australia (WA), 625 cases of cryptosporidiosis were notified in 2007, which was almost three times that of notification rates for the previous 4 years.

Molecular studies of *Cryptosporidium* species infecting humans in WA, New South Wales (NSW), Victoria (VIC) and South Australia (SA) have identified three *Cryptosporidium* species; *C. hominis*, *C. parvum* and *C. meleagridis* with *C. hominis* being the most

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frequently identified species of the three (Robertson et al., 2002; Chalmers et al., 2005; Jex et al., 2007, 2008; Ng et al., 2008; O'Brien et al., 2008; Alagappan et al., 2009; Waldron et al., 2009a,b). The published studies to date however were conducted on limited numbers of sporadic cases of cryptosporidiosis and no longitudinal molecular studies of cryptosporidiosis have been conducted in Australia. The aim of the present study therefore, was to examine samples from sporadic cases of cryptosporidiosis in WA humans over a 3-year period (May 2005–March 2008) to identify the species and subtypes of *Cryptosporidium* causing sporadic human cryptosporidiosis to better understand the transmission dynamics and distribution of the parasite in WA.

2. Materials and methods

2.1. Specimens and DNA extraction

A total of 254 microscopy positive human faecal specimens from sporadic cases of cryptosporidiosis were collected from various pathology centres in WA from May 2005 to March 2008. All samples were stored at 4 °C prior to molecular analysis. Patient epidemiological information for most of the specimens were collected (age, $n = 234$; location, $n = 230$; symptoms or clinical signs, $n = 209$; collection date, $n = 234$). Total DNA was extracted using a QIAmp DNA Stool Kit (Qiagen, Germany).

2.2. PCR amplification and DNA sequencing

Initial genotyping of the samples were carried out by PCR-RFLP of an ~830 bp fragment of the *Cryptosporidium* 18S gene locus as described by Xiao et al. (2001), using restriction analysis of the PCR product by the VspI (Promega, USA) to discriminate between *C. hominis* and *C. parvum*. For samples that failed to amplify or produced ambiguous banding patterns, a two-step nested PCR and sequencing of a ~540 bp product the 18S gene locus was carried out (Ryan et al., 2003).

Cryptosporidium hominis and *C. parvum* positive samples were sub-typed at the *gp60* gene locus using a two-step nested PCR that amplifies a ~830 bp fragment (Strong et al., 2000; Sulaiman et al., 2005). Secondary PCR products were purified and sequenced as described by Ng et al. (2006).

Multilocus sequence typing was carried out on a smaller number ($n = 13$) of *C. hominis* and *C. parvum* positive clinical human isolates at the MSC6-7 gene locus (serine repeat antigen) and the CP47 gene locus (47-kDa protein) and subtypes were assigned according to Gatei et al. (2007). Briefly, at the MSC6-7 gene locus, subtypes were assigned based on the minisatellite TGATGATGAT(G)GAACC(T) in the repeat region and single nucleotide polymorphisms (SNPs) in the non-repeat region. For the CP47 gene locus, *C. hominis* was assigned as type I and classified based on the number of TAA repeats, (coded as A) and TGA/TAG repeats, (coded as G), with the following digit showing the number of trinucleotide repeats (Gatei et al., 2007). Isolates were selected for analysis at the MSC6-7 and CP47 gene loci based on their *gp60* subtype family; Ib (1), Id (3), Ie (2), Ig (3) and four IIa subtypes, A17G2R1 (1), A18G3R1 (2) and A19G4R1 (1). Amplification of both gene loci was carried out as described in Gatei et al. (2006).

Nucleotide sequences were analyzed using ChromasPro version 2.3 (http://www.technelysium.com.au) and aligned using ClustalW (http://clustalw.genome.jp). Distance estimation was conducted using TREECON (Van de Peer and De Wachter, 1994), based on evolutionary distances calculated with the Kimura-2 model and grouped using neighbour joining. Parsimony analysis was conducted using MEGA version 3.1 (MEGA3.1: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, AZ). The confidence of groupings was assessed by bootstrapping, using 1000 replicates.

2.3. Statistical analysis

Statistical analysis was performed using SPSS 17.0 (Statistical Package for the Social Sciences) for Macintosh OS X (SPSS Inc. Chicago, USA) to determine if there was any association between subtypes and within subtype families versus age, gender and clinical symptoms.

3. Results

3.1. Cryptosporidium species

From the 254 samples received between May 2005 and March 2008, 248 samples were amplified at the 18S rRNA locus. Analysis identified 195/248 (78.6%) as *C. hominis*, 49/248 (19.8%) as *C. parvum* and 4/248 (1.6%) as *C. meleagridis* (Table 1).

Of the 77 positive specimens received in 2005/2006, 74 were amplified at the 18S rRNA gene locus. *C. hominis* was identified in 62.2% of the isolates, *C. parvum* in 33.8% and *C. meleagridis* in 4.1% as (Table 1). Infection with *C. hominis* was the highest amongst patients aged 0–5 years in 87.2% of cases, compared to infection *C. parvum* and *C. meleagridis*, in 10.3% and 2.6% of cases, respectively (Table 2). Cases of *Cryptosporidium* were more prevalent in regional areas of Western Australia at 81.2% compared to urban areas at 18.8%, of which *C. hominis* was identified in 90.9% of these cases (Table 3).

In 2006/2007, a total of 107/109 isolates received were amplified at the 18S rRNA gene locus, with 90.7% of the isolates identified as *C. hominis*, 8.4% as *C. parvum* and 0.9% as *C. meleagridis* (Table 1). *C. hominis* was most prevalent across all age groups, of which 60.2% of cases were from the age group 0–5, 21.2% from 16 to 39 years and 6.8% and 3.9%, respectively, for 6–15 and >40 years. The single *C. meleagridis* case identified, was from the 16–39 age group (Table 2). There were a higher number of *Cryptosporidium* cases from regional areas of Western Australia (80.6%), compared to urban areas (19.4%), with *C. hominis* infection identified in 85% of urban and 94% of regional cases, respectively (Table 3).

From the 68 isolates received in 2007/2008, 67 isolates successfully amplified at the 18S rRNA gene locus, of which *C. hominis* was identified in 77.6% of the samples and *C. parvum* in 22.4% of the samples. No *C. meleagridis* were identified from specimens received in this year. Most cases (60.7%) were from patients aged 5 and below and of these, 94.1% were *C. hominis*. The majority of specimens received were from regional areas, with *C. hominis* being more prevalent than *C. parvum* in regional areas (90% of cases) (Table 3).

3.2. Gp60 sub-genotyping of C. hominis and C. parvum

A total of 243 isolates, previously typed at the 18S locus, were successfully sub-typed at the gp*60* locus. Representative sequences from each subtype were deposited in GenBank under the Accession Nos. GU933438–GU933458. The four *C. meleagridis* and one *C. hominis* isolates failed to amplify at the *gp60* gene locus. A total of five *C. hominis* subtype families, Ib (39/243), Id (88/243), Ie (4/243), If (3/243) and Ig (60/243), and two *C. parvum* subtype families, IIa (48/243) and IId (1/243) were identified. Within the subtype families, two Id subtypes (IdA15G1 and IdA16), two Ig subtypes (IgA17 and IgA19) and five different IIa subtypes (IIaA17G2R1, IIaA18G3R1, IIaA19G4R1, IIaA20G3R1 and IIaA21G3R1) were identified (Table 4 and Fig. 1a). The most common *C. hominis* subtype identified was the IdA15G1 subtype in 45.1% of the *C. hominis*