Experimental Parasitology 122 (2009) 124–127

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

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

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ARTICLE INFO

Article history:
Received 19 September 2008
Received in revised form 1 February 2009
Accepted 13 February 2009
Available online 20 February 2009

Keywords:
Cryptosporidium
Cryptosporidiosis
Diarrhoea
Human disease
GP60

ABSTRACT

Management and control of cryptosporidiosis in human 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.

1. 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 et al., 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 et al., 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., 2006; 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.

2. Materials and methods

2.1. Sample sources and DNA extraction

Sixty-nine human faecal samples, positive for *Cryptosporidium,* were obtained from Pathology laboratories in Sydney,

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doi:10.1016/j.exppara.2009.02.006

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., 2009). Five additional isolates were identified to species level using T-RFLP (Waldron et al., 2009) for inclusion in this study.

2.2. 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 4 mM $MgCl_2$, 200 nM 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 s, 58 °C 45 s and 72 °C for 1 min 30 s, with a final extension at 72 °C for 5 min. PCR products were visualised by 1.5% agarose gel electrophoresis and ethidium bromide staining (1 μg/ml).

2.3. Cloning and sequence analysis

PCR products containing the correct band size (~1000 bp) 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. *C. hominis* nucleotide sequences were submitted to GenBank under the accession numbers FJ861209–FJ861238 and *C. parvum* FJ861278–FJ861305.

3. 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 (data not shown). 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 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 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.

4. 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

Table 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