Infection, Genetics and Evolution 31 (2015) 190–197

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Multilocus typing and population structure of *Cryptosporidium* from children in Zaragoza, Spain

Ana Ramo [a], Joaquín Quílez [a,*], Claudia Vergara-Castiblanco [a], Luis Monteagudo [b], Emilio Del Cacho [a], Antonio Clavel [c]

[a] Department of Animal Pathology, Faculty of Veterinary Sciences, University of Zaragoza, 50013 Zaragoza, Spain
[b] Department of Anatomy, Embryology and Genetics, Faculty of Veterinary Sciences, University of Zaragoza, 50013 Zaragoza, Spain
[c] Department of Microbiology, Preventive Medicine and Public Health, Faculty of Medicine, University of Zaragoza, 50009 Zaragoza, Spain

ARTICLE INFO

Article history:
Received 14 November 2014
Received in revised form 16 January 2015
Accepted 21 January 2015
Available online 7 February 2015

Keywords:
Cryptosporidium
Children
GP60 subtypes
Microsatellites
Minisatellites

ABSTRACT

A multilocus typing approach with eight variable-number tandem-repeat (VNTR) loci and the GP60 gene was used to analyze the inter- and intra-species variation of 44 *Cryptosporidium* isolates from pediatric patients in Zaragoza city (NE, Spain). Restriction and sequence analyses of the SSU rRNA gene revealed that *Cryptosporidium* transmission is mostly anthroponotic in this area, with the predominance of *Cryptosporidium hominis* (*n*: 41) over *Cryptosporidium parvum* (*n*: 3). GP60 subtyping showed limited genetic diversity and four subtypes were identified, including IbA10G2 (*n*: 35), IaA24R3 (*n*: 6), IIaA15G1R1 (*n*: 1) and IIaA15G2R1 (*n*: 2). Five out of eight VNTR loci showed a discriminatory power higher than the GP60 gene, although each locus had a predominant allele exhibited by more than 50% of isolates. All but four alleles were associated to either *C. hominis* or *C. parvum* and linked alleles at different loci were found. Multilocus typing substantially increased the discriminatory power (Hunter–Gaston index: 0.807, 95% CI, 0.683–0.926) and revealed that genetic diversity is much higher than that reported by GP60 sequencing, since 17 multilocus subtypes (MLTs) were identified. Nearly half of the specimens were allocated to a single major MLT. However, no more than three specimens were allocated to each of the remaining MLTs. Both phylogenetic and population analyses revealed a population clustering of *C. hominis* according to the GP60 subtype, which indicates the robustness of this marker to differentiate genetic subpopulations. Subpopulations had an overall clonal genetic structure, although traces of genetic flow between them were also observed.

1. Introduction

Protozoa of the genus *Cryptosporidium* are ubiquitous and significant enteropathogens of various classes of vertebrates and remain a major cause of diarrhoeal illness and waterborne outbreaks in humans worldwide. Human infection results predominantly in acute self-limiting gastroenteritis in immunocompetent individuals, but can prove fatal in those with T-cell deficiencies. In fact, the protozoan was one of the most common opportunistic intestinal parasites and a cause of chronic diarrhea and death in HIV-infected patients prior to introduction of Highly Active Antiretroviral Therapy (Bouzid et al., 2013).

Molecular techniques have revealed that *Cryptosporidium* is composed of multiple genetically distinct but morphologically indistinguishable species and genotypes. A total of 26 *Cryptosporidium* species have been named to date based on a combination of biological data and genetic characterization at multiple loci, including the small-subunit (SSU) rRNA and other functional genes (Chalmers and Katzer, 2013). Two species are of major significance to public health causing the majority of sporadic and outbreak related cases, including the zoonotic *Cryptosporidium parvum* and the human-adapted *Cryptosporidium hominis*, although other species have also been associated with human illness such as *Cryptosporidium meleagridis*, *Cryptosporidium felis*, *Cryptosporidium canis*, *Cryptosporidium ubiquitum*, *Cryptosporidium cuniculus* or *Cryptosporidium suis* (Xiao, 2010; Chalmers et al., 2011; Li et al., 2014). Extensive intra-species variation has been reported within some *Cryptosporidium* spp. using several subtyping tools. One of the most widely used is based on sequence analysis of the 60 kDa glycoprotein (GP60) gene, which enables the identification of

* Corresponding author at: Department of Animal Pathology, Faculty of Veterinary Sciences, University of Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain. Tel.: +34 976 762150; fax: +34 976 761612.
E-mail address: jquilez@unizar.es (J. Quílez).

http://dx.doi.org/10.1016/j.meegid.2015.01.023
1567-1348/

subtype families, as well as several subtypes within each family. Nevertheless, the GP60 gene is under selective pressure as it mediates parasite attachment to host cells and its use as a single locus subtyping method has been reported to underestimate genetic diversity where sexual reproduction occurs (Widmer and Lee, 2010).

The high variability of short variable-number tandem-repeat (VNTR) loci, also known as minisatellites and microsatellites, has made them the genetic markers of choice for addressing the population structure and transmission dynamics of *Cryptosporidium* in multilocus analyses (Xiao, 2010). Two types of approaches have been used to investigate the variability of these markers, including sequence analysis, which allows the detection of both length polymorphisms and nucleotide sequences, and fragment sizing of PCR-amplified products, which can be achieved by sequencing, capillary electrophoresis (CE) with labeled primers, and polyacrylamide or precast high resolution slab gels (Robinson and Chalmers, 2012). Comparative studies on the performance and consistency of these methods have demonstrated that automated fragment analysis by CE is a time- and cost-saving alternative to sequencing and has been validated for detecting mixed parasite subpopulations, although fragments of equal length but with nucleotide substitutions are not differentiated by the former technique (Díaz et al., 2012; Quílez et al., 2014).

In Spain, cryptosporidiosis has been a nationally notifiable disease since 2009 but routine testing is not carried out and many cases can remain unnoticed (Martín-Ampudia et al., 2012). Cryptosporidial infections have been reported as a cause of diarrhea in both sporadic cases and waterborne or person-to-person transmitted outbreaks in children and adults (Clavel et al., 1996; Galmes et al., 2003; Artieda et al., 2012). However, the identity of *Cryptosporidium* species and genotypes infecting humans in Spain is not well documented and few data on the intra-species diversity of this protozoan are available. Some molecular studies have revealed that *C. hominis* and *C. parvum* are responsible for most infections but minor species such as *C. meleagridis*, *C. felis* and *C. ubiquitum* have also been occasionally described (Llorente et al., 2007; Cieloszyk et al., 2012; Martín-Ampudia et al., 2012). The major *C. hominis* IbA10G2 has been found as the predominant circulating GP60 subtype, but rare subtypes such as IaA18R3 have also been reported (Fuentes et al., 2014). In the current study, a multilocus typing approach combining GP60 and eight VNTR loci was used to investigate the genetic polymorphisms exhibited among a group of *Cryptosporidium* isolates from an urban area in Spain, in order to explore the population structure of human *Cryptosporidium* isolates.

2. Materials and methods

2.1. Cryptosporidium isolates

A total of 44 human stool specimens microscopically positive for *Cryptosporidium* were used in this study. Fecal samples were collected in 2010 from pediatric patients aged 1–5 years with no known immunocomprising conditions originating from Zaragoza city (NE, Spain). Five samples were selected from a diarrheic outbreak occurring in a day-care center and 39 samples were from sporadic cases. Only 1 specimen per patient was included in the study.

2.2. DNA extraction and Cryptosporidium species differentiation

Oocyst suspensions from whole fecal specimens were prepared by saturated salt flotation, resuspended in distilled water and stored at 4 °C as previously described (Elwin et al., 2001). Total DNA was extracted from 200 µl oocyst suspensions by three cycles of freezing with liquid nitrogen (1 min) and thawing at 100 °C (5 min), followed by incubation at 56 °C for 30 min in lysis buffer containing proteinase K and purification over a spin column according to the manufacturer's instructions (QIAamp DNA mini-kit; Qiagen). DNA extracts were stored at −20 °C.

Cryptosporidium species were determined by nested PCR of a small-subunit (SSU) rRNA gene fragment and RFLP analysis with the endonucleases *Ssp*I and *Vsp*I using primers previously described (Xiao et al., 2001). PCR products and restriction fragments were subjected to electrophoresis in 1.5% or 2% agarose gels, respectively, and visualized by staining with GelRed nucleic acid gel stain (Biotium, Hayward, CA). A subset of twenty isolates was selected for confirmation of the RFLP results by DNA sequence analysis.

2.3. Multilocus sequence typing

Three gene loci were targeted for sequence analyses, including the GP60 locus and the VNTR loci CP47 (47 kDa protein) and MSC6-7 (serine repeat antigen). These markers were amplified by nested PCRs using protocols described previously (Alves et al., 2003; Gatei et al., 2007). The secondary PCR products, as well as selected SSU rRNA products, were purified and sequenced in both directions on a MegaBACE 500 sequencer (Amersham Biosciences) according to the manufacturer's instructions. The sense and antisense strand sequences were aligned and edited using Bioedit version 7.0.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and consensus sequences analyzed using BLASTN searches of the NCBI databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alleles at GP60 and CP47 loci were named according to the nomenclature proposed by Sulaiman et al. (2005) and Gatei et al. (2007), respectively. Alleles at the MSC6-7 locus were differentiated based on both the fragment length due to insertions or deletions of the minisatellite TGATGATGAT(G)GAACC(T) in the repeat region as well as single nucleotide polymorphisms in the non-repeat region. All alleles were translated into numbers for multilocus analyses. A Neighbor-Joining tree was generated for each locus using TreeCon (http://bioinformatics.psb.ugent.be/downloads/psb/Userman/treeconw.html) based on genetic distances calculated by the Kimura two-parameter model (Van de Peer and De Wachter, 1994). The reliability of branches was assessed by bootstrap analysis using 1000 replicates. In addition to an individual tree for each marker, sequences from GP60, CP47 and MSC6-7 were concatenated to produce a more accurate phylogeny (Gadagkar et al., 2005).

2.4. Multilocus fragment typing

An automated CE-based DNA fragment analysis tool combining three fluorochromes was used to type *Cryptosporidium* isolates at six additional VNTR markers, including two minisatellite (MSB, MS5) and four microsatellite (ML1, ML2, TP14, 5B12) loci. Primers and conditions were as previously described (Quílez et al., 2011). Briefly, the fragments were amplified by using simple (MSB, ML2 and 5B12), heminested (MS5 and ML1) and nested (TP14) PCRs. In order to allocate alleles with overlapping peaks to a specific locus, the reverse primers used in simple PCRs and the internal reverse primers used in heminested and nested PCRs were 5′ labeled with HEX (4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein), FAM (6-carboxyfluorescein), or TAMRA (6-carboxytetramethylrhodamine), according to the predicted fragment size.

PCR products were separated by electrophoresis in 1.5% agarose gels and visualized by staining with GelRed nucleic acid gel stain (Biotium, Hayward, CA) to confirm DNA amplification. According to the amplicon intensity, 0.5–2 µl samples of the mini- and