Infection, Genetics and Evolution 37 (2016) 49–56

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Research paper

Molecular genotyping and sub-genotyping of *Cryptosporidium* spp. isolates from symptomatic individuals attending two major public hospitals in Madrid, Spain

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ARTICLE INFO

Article history:
Received 18 September 2015
Received in revised form 9 October 2015
Accepted 26 October 2015
Available online 28 October 2015

Keywords:
Cryptosporidium
Cryptosporidiosis
Human
Molecular characterization
Spain
Symptomatic patients

ABSTRACT

Infections by members of the protozoan genus *Cryptosporidium* are among the most common causes of human gastrointestinal illness worldwide. In Spain cryptosporidiosis is not a compulsory notifiable disease, so the actual burden of the infection in both clinical and general populations remains largely unknown. We present here data on the diversity and frequency of the *Cryptosporidium* species and sub-genotypes identified in symptomatic individuals seeking medical care in two major hospitals in Madrid, Spain, between December 2013 and January 2015. Initial detection of the parasite was conducted on a total of 122 stool samples collected from 120 patients by microscopy with modified Ziehl-Neelsen and/or immunochromatographic tests. We used immunofluorescence, PCR-based methods and sequence analyses of the 60-kDa (*GP60*) glycoprotein and the small subunit ribosomal RNA (*SSU* rRNA) genes for confirmatory purposes and to characterize *Cryptosporidium* isolates. A total of 110 patients were confirmed with cryptosporidiosis. Overall, 101 isolates were successfully sub-genotyped at the *GP60* locus, and an additional seven at the *SSU* rRNA locus. The analyses of all amplicons defined 10 distinct sequence types representing the *GP60* family sub-genotypes IbA10G2 (78.7%), IeA11G3T3 (3.7%) of *C. hominis*, and the *GP60* family sub-types IIaA15G2R1 (5.6%), IIaA18G6R1 (0.9%), IIcA5G3a (0.9%), IIdA18G1 (0.9%), IIdA19G1 (0.9%), IIdA21G1 (0.9%), and IIdA22G1 (0.9%) of *C. parvum*. A single isolate was assigned to *C. felis* (0.9%), two *C. parvum* isolates (1.9%) could not be characterized at the sub-genotype level and an additional four isolates (3.7%) were not typable. These results strongly suggest that transmission of cryptosporidiosis is mostly anthroponotic in origin in the clinical sample under study. We expect that our molecular epidemiological data will make a significant contribution to unravel the actual epidemiological situation of cryptosporidiosis in Spain, providing health care and policy makers with solid baseline information to unavoidably improve the national surveillance system and allocate additional resources to research, diagnosis, and treatment of cryptosporidiosis.

1. Introduction

Members of the genus *Cryptosporidium* (phylum Apicomplexa, class Coccidea, order Eimeriidae) are ubiquitous protozoa that are being increasingly recognized as a major cause of human diarrhoeal disease globally (Fletcher et al., 2012; Shirley et al., 2012). Reported prevalences of cryptosporidiosis are in the range of 1% in developed countries to 5–10% in less favoured settings (Checkley et al., 2015), although these figures very likely represent an underestimation of the actual burden of the disease (McDonald et al., 2001; Scallan et al., 2011). Cryptosporidiosis takes the largest toll on young children living in low-income settings. Because of its link with poverty and large socioeconomic impact in endemic, deprived regions, cryptosporidiosis was included in the 'Neglected Diseases Initiative' launched by the World Health Organization in 2004 (Savioli et al., 2006). In immunocompetent subjects, cryptosporidiosis typically results in a transient (2–3 weeks), self-limiting illness characterized by watery diarrhoea, abdominal pain, and less frequently, nausea, vomiting, fever, and weight loss. However, chronic or protracted diarrhoea are frequently reported in immunocompromised patients (Bouzid et al., 2013).

Transmission of *Cryptosporidium* occurs via the faecal-oral route, either indirectly by accidental ingestion of contaminated water or raw

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http://dx.doi.org/10.1016/j.meegid.2015.10.026
1567-1348/

produce, or by direct contact with infected individuals, animals or fomites (Ryan et al., 2014). Not surprisingly, *Cryptosporidium* has been reported as one of the major contributors to food- and waterborne outbreaks of diarrhoea in developed countries (Baldursson and Karanis, 2011; Lee and Greig, 2010).

Based on morphological, biological, molecular, and phylogenetic differences, at least 27 *Cryptosporidium* species are currently regarded as valid by most authoritative scholars (Ryan et al., 2014; Xiao, 2010). Among them, *C. hominis* and *C. parvum* are responsible for more than 90% of human infections worldwide (Xiao, 2010). Less common species include *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum*, and *C. viatorum*, whereas human cryptosporidiosis cases by *C. muris*, *C. suis*, *C. fayeri*, *C. andersoni*, *C. bovis*, *C. scrofarum*, *C. tyzzeri*, *C. erinacei*, and *Cryptosporidium* horse, skunk, and chipmunk I genotypes are only sporadically found (Ryan et al., 2014; Xiao, 2010).

In Spain *Cryptosporidium* has been identified in production, companion, and captive animal species, wildlife, humans and environmental (mainly water) samples (Navarro-i-Martinez et al., 2011). Human cryptosporidiosis cases have been well-documented in young children attending day care centres and primary schools (Cardona et al., 2011; Mateo et al., 2014; García-Rodríguez et al., 1990; Rodríguez-Hernández et al., 1996), paediatric clinical populations (Clavel et al., 1996; García-Rodríguez et al., 1989), HIV-positive patients (Clavel et al., 1995; López-Vélez et al., 1995), and inmate populations (Alonso-Sanz et al., 1995). Recorded infection rates typically varied from 1% to 18%, depending on the age group, immunological and clinical status of the population considered (Navarro-i-Martinez et al., 2011). Additionally, a limited number of *Cryptosporidium* outbreaks allegedly linked to waterborne (Galmes et al., 2003; Rodríguez-Salinas et al., 2000), person-to-person (Artieda et al., 2012; Goñi et al., in press) or unknown (Fuentes et al., 2015) transmission have also been characterized to a varied extent in the last 15 years. In contrast with this relative wealth of epidemiological data, molecular information is far scarcer. In an attempt to overcome this limitation, we present here data on the diversity and frequency of the *Cryptosporidium* species and subgenotypes identified in symptomatic individuals seeking medical care in two major hospitals in the autonomous region of Madrid, Spain, over a 14-month period of time.

2. Materials and methods

2.1. Ethical statement

Written informed consent was not required for this study because the stool samples used were exclusively intended for routine clinical diagnostic procedures at hospital settings. Gathered or generated socio-demographic or clinical data were conveniently anonymized prior to any analysis to preserve the identity of the patients involved. This study has been approved by the Research Ethics Committee of the Carlos III Health Institute under reference number CEI PI 34_2014.

2.2. Population and study design

In this longitudinal epidemiological study we assessed the presence of *Cryptosporidium* spp. in patients attended at the University Hospitals Puerta de Hierro-Majadahonda (UHPHM) and Severo Ochoa (UHSO) during December 2013 to January 2015. Both UHPHM and UHSO are major public hospitals serving an estimated catchment population of 857,000 people in the Madrid area. Outpatient admissions and inpatients presenting symptoms compatible with cryptosporidiosis (acute or persistent diarrhoea, abdominal pain, weight loss, cramps, fever, nausea, and vomiting) were requested to provide a stool sample for parasitological analyses. Simultaneously, clinical (symptoms, general immune status, concomitant infections) and basic socio-demographic (gender, age, place of birth, history of travelling abroad) data were retrieved from the hospital medical records of each patient with presumptive cryptosporidiosis, as diagnosed in clinical laboratories by routine methods (see below).

2.3. Stool samples collection and initial diagnosis of Cryptosporidium spp.

At least a single fresh stool sample was collected per patient. When possible, sequential stool samples from individual patients were pooled. Obtained samples were labelled with anonymized study codes and stored at 4 °C until further analyses. The detection of *Cryptosporidium* was based on conventional microscopy (CM) and/or an immunochromatographic test (ICT). Stool samples (~1 g) were processed at each hospital setting using the concentration systems Parasitrap® (Biosepar GmbH, Germany) or Para-Pak® PLUS (Meridian Bioscience, Luckenwalde, Germany). Faecal smears were then produced, stained with the modified Ziehl–Neelsen method, and microscopically examined at 400× magnification, switching to 1000× magnification when structures morphologically compatible with *Cryptosporidium* oocysts were suspected. A commercially available solid-phase qualitative ICT for the rapid simultaneous detection of *G. duodenalis* and *Cryptosporidium* spp. (Cer Test Biotec S.L., Zaragoza, Spain) was also used according to the manufacturer's instructions. From those stool samples that tested positive or probable for *Cryptosporidium* by conventional microscopy and/or ICT a new, fresh aliquot was sent to the National Centre for Microbiology, Majadahonda (Madrid) for further diagnostic confirmation and genotyping analyses.

2.4. Direct fluorescent antibody test (DFAT)

A direct fluorescent antibody test was used to confirm presumptive microscopy/ICT positive results. Briefly, stool samples (~1 g) were processed using the concentration system PARASEP Midi® (Grifols Movaco, Barcelona, España) according to the manufacturer's instructions. Five microliters of concentrated faecal material were placed on welled slides. Smears were air-dried, methanol fixed, and stained with fluorescein-labelled mouse monoclonal antibodies directed against *Cryptosporidium* oocysts and *Giardia* cysts (Crypto/Giardia Cel, Cellabs, Sydney, Australia). Samples were examined on a Zeiss fluorescence microscopy equipped with a MC63 camera system at 400× magnification. Known positive and negative controls were routinely included in each sample batch.

2.5. DNA extraction and purification

Total DNA was extracted from ~200 mg of a new aliquot of faecal material using the QIAamp® DNA stool mini test kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified DNA samples (200 μL) were stored at −20 °C until further use in downstream molecular analysis.

2.6. Molecular detection and sub-typing of C. hominis and C. parvum isolates at the GP60 locus

Because *C. hominis* and *C. parvum* are the *Cryptosporidium* species expected to cause most (>90%) human infections (Xiao, 2010), we adopted as first-line diagnostic strategy a PCR method primarily targeting the 5′ end of the highly polymorphic 60 kDa glycoprotein (*GP60*) of the parasite. This region contains a variable number of tandem repeats of the serine-coding trinucleotide (TCA/TCG/TCT) which allow categorization of *C. hominis* and *C. parvum* isolates within distinct *GP60* sub-genotype families (Strong et al., 2000). To date this is the preferred and most accurate sub-typing tool used in *Cryptosporidium* investigations, although available PCR primers are currently limited to a number of *Cryptosporidium* species including *C. hominis*, *C. parvum*, *C. meleagridis* (Ryan et al., 2014; Sulaiman et al., 2005), *C. ubiquitum* (Li et al., 2014), and *C. viatorum* (Stensvold et al., 2015). This strategy allowed us not only to detect the presence of the above mentioned