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Genetic Characterization of *Cryptosporidium* Isolates from Humans in Northern Portugal

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CRYPTOSPORIDIUM spp. are apicomplexan parasites that infect a wide range of vertebrates and humans worldwide. The parasite causes diarrhea in humans and is of public health concern (Fayer 2004; Hunter and Thompson 2005); it also causes significant morbidity and mortality in animals. Older people, young children, and immunocompromised people are very susceptible to infection. Persons with immune deficiencies have illness of long duration and high severity depending on the extent of cellular immunity impairment (Fayer 2004). The severity of illness increases as CD4 T-cell number decreases. Immunocompromised individuals can experience chronic infection often lasting several months (Fayer 2004). The number of oocysts excreted by an infected individual can vary greatly. U.S. Center for Disease Control reported in 1986 that 3.6% of almost 20,000 immunocompromised individuals had cryptosporidiosis with fatality of 61% (Fayer 2004; Hunter and Thompson 2005). In Europe, detection of oocysts in feces and anti-*Cryptosporidium* antibodies in human sera showed prevalence between 0.1% and 14.1%, much lower than that observed in less developed nations. In Portugal, some studies estimated 8% prevalence of cryptosporidiosis in AIDS patients (Matos et al. 2004), although the samples were only from Lisbon. In the present study data were obtained from other Portuguese regions, mainly those in the northern part of the country.

Multiple transmission routes occur in *Cryptosporidium* spp.: animal-to-human, human-to-human, foodborne, and waterborne. Thus, the epidemiology of cryptosporidiosis is difficult to study and is now best done by molecular techniques (Fayer 2004; Fayer, Santin, and Trout 2005). These molecular approaches recognized *Cryptosporidium hominis* and *Cryptosporidium parvum* as the major causes of cryptosporidiosis in both immunocompromised and immunocompetent humans (McLauchlin et al. 1999). Although *C. hominis* is involved and maintained in the anthroponotic cycle of infection, other species infect both animals and humans, clearly showing zoonotic potential. *Cryptosporidium parvum* is the most frequently reported zoonotic *Cryptosporidium* species and due to its high prevalence in cattle, these animals are important sources for environmental contamination (Fayer 2004; Fayer et al. 2005). The aim of this study was to identify the genotypes of *Cryptosporidium* spp. present in the feces of adult immunocompromised humans in northern Portugal. We also tested genotype analysis by using two different genetic loci: the *18SrRNA* and *hsp70* gene of *Cryptosporidium*.

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MATERIAL AND METHODS

The study was carried out in northern Portugal. A total of 526 fecal samples from immunocompromised and immunocompetent people were randomly collected from the Joaquim Urbano hospital in Porto and delivered to our laboratory. The samples were processed by acid-fast staining followed by PCR analysis. The intensity of infection was scored in acid-fast-positive samples: using +—low (0–5), ++—medium (5–30), and +++—high (> 30) oocysts in the microscopic field observed at 200 × magnification.

Oocysts from the fecal samples were isolated using a cesium chloride-based technique (unpublished) (Fig. 1), and DNA was extracted using a commercial kit (QIAmp DNA Mini Kit, Qiagen, Valencia, CA). Polymerase chain reaction was performed to amplify a fragment of the *hsp70* gene (LeChevallier et al. 2003) and a fragment of the *18SrRNA* gene of *Cryptosporidium* spp. (Xiao et al. 1999). Amplification products from both genes were separated by horizontal gel electrophoresis on a 1.4% agarose gel (Sigma Chemical Co.) containing 0.5 µg of ethidium bromide/ml (Sigma Chemical Co.) and were visualized under UV light. Gel images were captured by using a gel documentation system (GelDoc, BioRad). A separate 1.4% low-melting agarose gel electrophoresis was performed to purify the products using a commercial kit GFXTM PCR DNA and Gel Band Purification Kit (Amersham Biosciences). Product bands were eluted in 50 µl of distilled water. DNA fragments were sequenced in both directions by a commercial laboratory (MWG Biotech, Ebersberg, Germany) using forward and reverse internal primers of *18SrRNA* gene and primers of *hsp70* gene. Sequencing of both the *hsp70* and *18SrRNA* genes were subjected to BLAST analysis against a database (GenBank at www.ncbi.nlm.nih.gov/BLAST), and corrected using the ChromasPro[®] software. Alignment was performed with the ProSeq[®] 2.0 software.

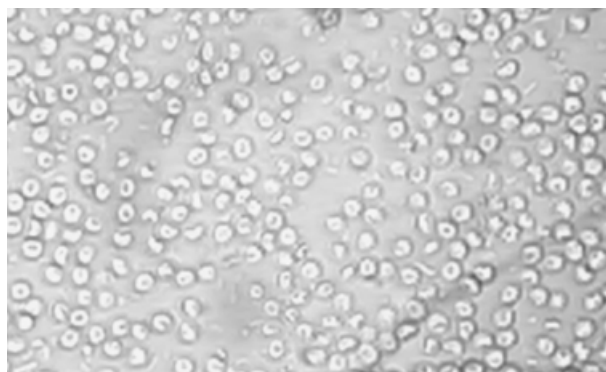


Fig. 1. Oocysts recovered from feces using a cesium chloride-based technique.

RESULTS AND DISCUSSION

Cryptosporidium spp. oocysts were detected in fecal samples obtained from 14 out of 526 individuals (2.7%) in this study of immunocompromised (65%) and immunocompetent (35%) individuals. All fecal samples (100%) containing *Cryptosporidium* oocysts came from immunocompromised adults. One of these cases was diagnosed as concurrently infected with *Giardia duodenalis*. Symptoms of diarrhoea were observed in four out of the five individuals infected with *C. parvum*, in one individual infected with *Cryptosporidium meleagridi*, and in both individuals infected with *C. hominis*. The degree of oocyst shedding varied greatly. Samples with *C. parvum* infection had medium and high numbers of oocysts (++ and +++). Samples with *C. hominis* had low and medium numbers of oocysts (+ and ++), and those with *C. meleagridi* had low numbers of oocysts. *Cryptosporidium* spp. oocysts was not detected in fecal samples from immunocompetent individuals, which is possibly due to limited sensitivity using acid-fast staining and the relatively small number of samples analyzed.

Using PCR-based approaches, *C. parvum* was identified in five isolated organism preparations, *C. meleagridis* was detected in two other preparations, and *C. hominis* was detected in another two. Sequence analysis of the isolates revealed 100% homology with *C. parvum* (AF221528 and AF112571), *C. hominis* (AF093492 and XM661662) and *C. meleagridis* (AY166839 and DQ201831). Sequencing of both the *hsp70* and *18S rRNA* genes showed the same *Cryptosporidium* species assignments.

This is the first study on *Cryptosporidium* spp. infections in immunocompromised individuals in northern Portugal, which estimates a 4% prevalence of cryptosporidiosis in HIV patients living in this region of the country. It was estimated that the prevalence of cryptosporidiosis among HIV patients was 8% in

other regions of Portugal (Matos et al. 2004). In the present survey, HIV patients infected with *C. parvum* appear to be higher than those with *C. hominis* and *C. meleagridis*. However, additional specimens must be analyzed to obtain a better picture of relative species prevalence.

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