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Genetic characterizations of *Cryptosporidium* spp. and *Giardia duodenalis* in humans in Henan, China

Rongjun Wang [a], Xiaosan Zhang [a], Huili Zhu [a], Longxian Zhang [a,b,c,*], Yaoyu Feng [d], Fuchun Jian [a], Changshen Ning [a], Meng Qi [a], Yang Zhou [a], Kanda Fu [e], Yaqiang Wang [e], Yanru Sun [a], Qiang Wang [a], Lihua Xiao [c,**]

[a] College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou 450002, China
[b] Atlanta Research and Education Foundation, 1670 Clairmont Road, Decatur, GA 30033, USA
[c] Division of Foodborne, Waterborne, and Environmental Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA
[d] School of Resource and Environmental Engineering, East China University of Science and Technology, Shanghai 200237, China
[e] Inspection Division of Huaihe Hospital, Henan University, Kaifeng 475000, China

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ABSTRACT

Cryptosporidium and *Giardia* infections are common causes of diarrhea worldwide. To better understand the transmission of human cryptosporidiosis and giardiasis in Henan, China, 10 *Cryptosporidium*-positive specimens and 18 *Giardia*-positive specimens were characterized at the species/genotype and subtype levels. *Cryptosporidium* specimens were analyzed by DNA sequencing of the small subunit rRNA and 60 kDa glycoprotein genes. Among those genotyped, nine belonged to *C. hominis* and one *C. felis*, with the former belonging to three subtype families: Ia, Ib, and Id. The three Ib subtypes identified, IbA16G2, IbA19G2, and IbA20G2, were very different from the two common Ib subtypes (IbA9G3 and IbA10G2) found in other areas of the world. The distribution of *Giardia duodenalis* genotypes and subtypes was assessed by sequence analysis of the triosephosphate isomerase (tpi) gene. The assemblages A (eight belonging to A-I and four A-II) and B (belonging to six new subtypes) were found in 12 and six specimens, respectively. More systematic studies are needed to understand the transmission of *Cryptosporidium* and *G. duodenalis* in humans in China.

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1. Introduction

Cryptosporidium and *Giardia* infections are significant causes of diarrhea in humans worldwide. The diarrhea caused by cryptosporidiosis may become profuse, chronic, or even life-threatening, particularly in immunocompromised persons. Both parasites have a broad range of hosts and can be transmitted by the fecal-oral route, via either direct contact or ingestion of contaminated food or water (Xiao et al., 2004; Smith et al., 2006).

Using genotyping tools, it has been shown that eight *Cryptosporidium* species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. canis*, *C. felis*, *C. suis*, *C. muris*, and *C. andersoni*) and at least six genotypes (cervine, skunk, chipmunk I, horse, rabbit genotype, and pig genotype II) can infect humans, with *C. hominis* and *C. parvum* responsible for most clinical cases. The distribution of *C. hominis* and *C. parvum* in humans differs in geographic regions (Xiao, 2010). In addition, DNA sequence analysis of the 60 kDa glycoprotein (gp60) gene has shown the complexity of *Cryptosporidium* transmission in endemic areas. Among the five common *C. hominis* subtype families, Ia, Ib, Id, Ie, and If, three to four subtype families were each seen in humans in India, Peru, New Orleans, Malawi, South Africa, Kuwait, and Portugal. In contrast, only one or two *C. parvum* subtype families (IIa and IIc) were seen in humans in the same areas (Alves et al., 2003, 2006; Peng et al., 2003; Sulaiman et al., 2005; Gatei et al., 2007).

In giardiasis, only *Giardia duodenalis* (synonym *G. lamblia* or *G. intestinalis*) is recovered from humans and most other mammals (Adam, 2001). Isolates of *G. duodenalis* are classified into seven common 'assemblages' or genotypes: A–G, based on the characterization of the triosephosphate isomerase (tpi), small subunit (SSU) rRNA, β-giardin, glutamate dehydrogenase (gdh), and other genes (Thompson et al., 2000; Sulaiman et al., 2003; Read et al., 2004). Assemblages A and B infect humans and a broad range of other hosts, including livestock, cats, dogs, and wild mammals. The assemblage A is further divided into two major subassemblages, I

* Corresponding author at: College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou 450002, China. Fax: +86 371 63558180.
** Corresponding author. Fax: +1 (770) 488 4454.
E-mail addresses: zhanglx8999@yahoo.com.cn (L. Zhang), lxiao@cdc.gov (L. Xiao).

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and II, and the less common III. Many subtypes are present in the assemblage B (Feng et al., 2008; Geurden et al., 2009; Mahdy et al., 2009). Assemblages C, D, E, F, and G appear to be mostly restricted to companion animals, livestock, and rodents (Monis et al., 2003; Sulaiman et al., 2003).

In China, the number of molecular epidemiological studies of cryptosporidiosis and giardiasis in humans is small. Peng et al. characterized genetically five specimens (all belonged to *C. hominis*) from children in Tianjing, three specimens were characterized by gp60 sequence analysis and three subtypes (IbA23G2, IdA14, and IeA13G3T3) were identified (Peng et al., 2001). Four Cryptosporidium specimens from HIV-infected patients in Taiwan were identified as *C. hominis* (two cases), *C. felis* (one case), and *C. meleagridis* (one case) (Hung et al., 2007). A report by Yong et al. based on sequence analysis of the SSU rRNA gene of eight *G. duodenalis* specimens from Anhui Province showed the presence of assemblages A and B in four patients each (Yong et al., 2000). Another study by Lu et al. based on sequence analysis of the tpi gene of three specimens showed the presence of two assemblage B and one A-I mixed with assemblage B (Lu et al., 2002). Chen et al. reported that three *G. duodenalis* specimens from humans in Hebei Province belonged to subtype A-II (Chen et al., 2001). Therefore, the molecular epidemiology of human cryptosporidiosis and giardiasis in China is still unclear. In this study, to better understand the transmission of cryptosporidiosis and giardiasis, 10 *Cryptosporidium* specimens and 18 *Giardia* specimens from humans in Henan were genetically characterized.

2. Materials and methods

2.1. Stool specimens

The 10 *Cryptosporidium*-positive specimens and 18 *Giardia*-positive specimens from humans were obtained from a survey conducted in hospitals of Zhengzhou, Kaifeng, and Linzhou in Henan Province between June 2007 and December 2008. All specimens were stored at 4 °C in 2.5% (w/v) potassium dichromate solution or frozen at −80 °C without preservatives prior to being used in molecular characterizations.

2.2. DNA extraction

Genomic DNA was isolated from ~200 μL of stool specimens using the QIAamp® DNA stool kit (QIAGEN Inc., Valencia, CA) and the previously described procedures, after washing of specimens three times with distilled water by centrifugation at 1000*g* for 10 min, oocyst lysis by alkaline digestion, and crude DNA extraction by the phenol–chloroform method (Xiao et al., 2002). The extracted DNA was eluted in 200 μL of the AE elution buffer and stored at −20 °C.

2.3. Cryptosporidium genotyping and subtyping

Cryptosporidium spp. were genotyped by PCR amplification of a ~830-bp fragment of the small subunit (SSU) rRNA gene and restriction fragment length polymorphism (RFLP) analysis of the PCR products using restriction enzymes SspI and VspI (Xiao et al., 2001). To confirm the genotype identification, all PCR products were sequenced.

Subtyping of *C. hominis* was done by DNA sequencing of the gp60 gene. A fragment of ~855-bp of the gp60 gene was amplified by nested PCR (Alves et al., 2003). The previously established nomenclature system was used to determine subtype families and subtypes within each family (Sulaiman et al., 2005; Xiao, 2010).

2.4. Giardia genotyping and subtyping

G. duodenalis genotypes and subtypes were determined by DNA sequence analysis of the tpi gene. A nested PCR was used to amplify the partial tpi gene (~530-bp) of *Giardia* specimens (Sulaiman et al., 2003). Sequencing of tpi gene was done as described below. Genotype and subtype identities of *G. duodenalis* were established by direct comparison of the acquired sequences with reference sequences.

2.5. DNA sequence analysis

All PCR products were sequenced on an ABI PRISMTM 3730 XL DNA Analyzer (Applied Biosystems, USA), using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequence accuracy was confirmed by two-directional sequencing and by sequencing a new PCR product if necessary. The SSU rRNA and gp60 sequences of *Cryptosporidium* and tpi sequences of *Giardia* obtained in this study were aligned with reference sequences downloaded from GenBank using the program ClustalX 1.83. Nucleotide sequences of representative subtypes have been deposited in the GenBank under Accession Nos. FJ707310–FJ707316 (*Cryptosporidium*) and GU564274–GU564284 (*Giardia*).

3. Results

3.1. Cryptosporidium genotypes and subtypes

The 10 *Cryptosporidium*-positive specimens were successfully amplified at the SSU rRNA locus. RFLP analysis of the 10 PCR products suggested the presence of *C. hominis* in nine specimens and *C. felis* in one. This was confirmed by DNA sequence analysis of the PCR products.

Cryptosporidium hominis was further subtyped by gp60 gene sequence analysis. Three subtype families were identified in the nine *C. hominis*-positive specimens: Ia (11.1%), Ib (66.7%), and Id (22.2%). Among subtype family Ib, three subtypes were found: IbA16G2 (in one case), IbA19G2 (in two cases), and IbA20G2 (in three cases). In contrast, only one subtype each was present in subtype families Ia (IaA9R3 in one case) and Id (IdA21 in two cases) (Table 1).

3.2. Giardia genotypes and subtypes

All 18 *Giardia*-positive specimens produced the expected tpi PCR products. Alignment of the tpi sequences obtained with reference sequences indicated the presence of two genotypes of *G. duodenalis*; 12 specimens were identified as *G. duodenalis* assemblage A and six as assemblage B (Table 2). Among the three cities examined in this study, Kaifeng appeared to have more assemblage B infections than Zhengzhou and Linzhou ($P < 0.01$ between Kaifeng and Linzhou areas by Chi-square analysis, Table 2).

Of the 12 assemblage A specimens, eight produced sequences that were identical to the A-I (GenBank Accession No. AF069556), and the remaining four produced sequences identical to the A-II (GenBank Accession No. AF069557). In contrast, the six assemblage B specimens produced sequences that were not identical to any known assemblage B subtypes, thus representing six new subtypes, named B1–B6 for convenience (Table 2).

4. Discussion

In this study, 90% of *Cryptosporidium* specimens were identified as *C. hominis*. Previously, five human *Cryptosporidium* isolates from Tianjing also belonged to *C. hominis* (Peng et al., 2001) and two of four *Cryptosporidium*-positive specimens from Taiwan had