Multilocus Genotyping of Cryptosporidium sp. Isolates from Human Immunodeficiency Virus-Infected Individuals in South India

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Received 21 September 2005/Accepted 28 September 2005

This study characterized cryptosporidial infections in 48 human immunodeficiency virus-infected individuals in India by multilocus genotyping. Cryptosporidium hominis, C. parvum, C. felis, C. muris, and C. meleagridis were identified. Cpgp40/15 PCR-restriction fragment length polymorphism identified six subgenotypes. Cryptosporidial diarrhea was associated with decreased CD4 counts, below 200 (P = 0.009), but not high viral loads.

Human cryptosporidiosis, in most parts of the world, is mainly caused by Cryptosporidium hominis (previously known as C. parvum genotype I or human genotype) and Cryptosporidium parvum (genotype II or bovine genotype) (9). C. hominis is found almost exclusively in humans, whereas C. parvum is found in domestic livestock, wild animals, and humans (1, 2, 9). Studies using PCR-restriction fragment length polymorphism (RFLP) and sequence analysis of several genes have identified genetic differences among isolates, but genotyping at this level alone is insufficiently discriminatory because of significant genetic polymorphisms between isolates of the same genotype. Several recent studies have revealed tremendous heterogeneity among genotype 1 isolates at the Cpgp40/15 locus (10, 13, 19) which is far greater than that of any other Cryptosporidium genetic locus examined to date (10, 16, 17).

In this study, we used these recently developed tools to identify the species and subgenotypes of Cryptosporidium isolates from human immunodeficiency virus (HIV)-infected adults with and without diarrhea in southern India. The study was approved by the Christian Medical College and Tufts-New England Medical Center Institutional Review Boards.

(These data were presented, in part, at the American Society for Tropical Medicine and Hygiene 53rd Annual Conference in Miami, Fla., November 2004.)

Study population and screening. HIV-infected adults attending a clinic in Vellore were screened between September 2002 and August 2004. Stool samples from asymptomatic patients (n = 423) were screened for parasites, and samples from patients with diarrhea (n = 111) were screened for parasitic and bacterial enteric pathogens. Cryptosporidium oocysts were identified using modified acid-fast staining. CD4 counts were carried out using a Capcellia CD4/CD8 kit (Bio-Rad) or flow cytometry (Molecular Diagnostics, Mumbai, India). Viral load estimation was carried out using the Amplicor HIV-1 viral RNA kit (Roche Diagnostics). Table 1 lists the parasites identified from the 534 HIV-positive individuals screened. The difference in proportions of Cryptosporidium infection among diarrheal patients (25.2%; 95% confidence interval, 17.8 to 33.9) versus nondiarrheal patients (4.7%; 95% confidence interval, 3.0 to 7.1) was statistically significant (P < 0.001).

CD4 counts were measured for 33 patients positive for Cryptosporidium spp. The median CD4 count among symptomatic patients was 145 cells/μl (range, 44 to 745), while the median for asymptomatic patients was 312 cells/μl (range, 113 to 686). Among symptomatic Cryptosporidium sp. infections, 74% had CD4 counts of <200 cells/μl, while among asymptomatic infections only 22% had CD4 counts of <200 cells/μl. The difference was statistically significant (P = 0.01). Viral load estimations for 35 patients showed that 28 out of 35 had high viral loads of >100,000 copies of RNA/ml, but the correlation between the presence of diarrhea and high viral load was not statistically significant.

DNA isolation, species determination, and multilocus genotyping. DNA extraction by the QIAamp Stool DNA minikit (QIAGEN Inc., Valencia, CA) was followed by nested PCR-RFLP analysis at the loci for the small-subunit (SSU) rRNA, the thrombospondin-related adhesive protein of Cryptosporidium (TRAP C1), the Cryptosporidium oocyst wall protein (COWP), and Cpgp40/15, performed using previously described primers and conditions (5, 7, 10, 14, 15). The results of the PCR-RFLP analysis at these four loci are shown in Table 2. A subset of strains was sequenced, and the sequences were deposited in GenBank under accession numbers DQ067564 to DQ067570.

Subgenotyping by PCR-RFLP analysis of the Cpgp40/15 locus. Among 31 C. hominis isolates, the distribution of subgenotypes was Ia (6 out of 31; 19.3%), Ib (8 out of 31; 25.8%), Ic (3 out of 31; 9.7%), Id (4 out of 31; 12.9%), and If (8 out of 31; 25.8%), with one mixed infection with subgenotypes Ic and Id. Among C. parvum isolates, five typed as genotype II, of which four were IIa or IIb, which have similar-sized fragments, and one was Iic. Of the remaining four C. parvum isolates, three typed as Ic and one typed as Ig.

The C. parvum mouse/ferret...
isolate typed as Ib. The *C. meleagridis* isolate, which was confirmed by sequencing, had an RFLP pattern that differed from those of the genotype I and II subgenotypes (Fig. 1).

In the present study, 48 *Cryptosporidium* isolates from human samples were analyzed at the SSU rRNA, COWP, TRAP C1, and Cpgp40/15 gene loci. The sensitivities of the PCR amplification of the SSU rRNA, COWP, and TRAP-C genes of *Cryptosporidium* were 100%, 39.5%, and 44.1%, respectively, which is lower for the COWP and TRAP genes than reported in the United Kingdom (11, 12), which has reported sensitivities of 97, 91, and 66%, respectively. The sensitivity of Cpgp40/15 amplification is 97.5% in our study for *Cryptosporidium* parasites, similar to other reports (10, 19).

In this study, despite the relatively small number of isolates characterized, we identified at least five species, a greater diversity than has been reported from anywhere else in the world, excepting a recent report from Thailand (8), where anthropotrophic and zoonotic species were almost equally common in HIV-infected individuals. A large study from Peru identified six species, but the majority of isolates were *C. hominis* (4).

The difference in mean CD4 count between asymptomatic and symptomatic patients was less marked in this study than in studies in the United States, where the mean counts were 312 and 57 cells/μL, respectively (6), and another in Uganda, where patients with cryptosporidiosis had a median CD4 count of 16 cells/μL (3). The median CD4 count of 145 cells/μL can be compared with the only other Indian study that evaluated parasitic diarrhea in HIV-infected individuals and also measured CD4 counts, where the mean CD4 count in patients with parasitic diarrhea was 226 cells/μL (18).

Subgenotyping of the isolates showed that types Ib and If were the most common alleles at the Cpgp40/15 locus. The finding that three isolates that typed as Ic at the Cpgp40/15 locus typed as *C. parvum* (type II) at the SSU rRNA, TRAP C1, and COWP loci has been reported previously (2, 10, 13), and it has been suggested that this allele should be renamed IIc, even though so far this allele has been found exclusively in humans. In addition, two other *C. parvum* isolates displayed the alleles Ib and Ig at the Cpgp40/15 locus. These results need to be confirmed by sequencing but raise the possibility that the discordant alleles were derived by sexual recombination between genotype I and II parasites, as has been proposed earlier (10, 16).

Little is known about circulation and transmission patterns of cryptosporidiosis in developing countries. The application of genetic characterization of *Cryptosporidium* and field epidemiology is likely to lead to more rational approaches to disease control.

**Nucleotide sequence accession numbers.** The sequences determined in the course of this work were deposited in GenBank under accession numbers DQ067564 to DQ067570.

This work was supported by NIH Fogarty International Center grant D43TW00237.

None of the authors have any financial interest in any commercial company represented in this study nor any other potential conflicts of interest.

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