Journal of Clinical Microbiology, Mar. 2000, p. 1180–1183 Vol. 38, No. 3
0095-1137/00/$04.00+0
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Molecular Characterization of *Cryptosporidium* Isolates Obtained from Human Immunodeficiency Virus-Infected Individuals Living in Switzerland, Kenya, and the United States

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Received 27 September 1999/Returned for modification 1 December 1999/Accepted 21 December 1999

A total of 22 *Cryptosporidium* isolates from human immunodeficiency virus-infected patients from Kenya, Switzerland, and the United States were examined at three genetic loci: the 18S ribosomal DNA, HSP-70, and acetyl coenzyme A synthetase genes. Four distinct *Cryptosporidium* genotypes were identified: (i) the *Cryptosporidium parvum* "human" genotype, (ii) the *C. parvum* "cattle" genotype, (iii) *Cryptosporidium felis*, and (iv) *Cryptosporidium meleagridis*. This is the first report of *C. meleagridis* in a human host. These results and those of others indicate that immunocompromised individuals are susceptible to a wide range of *Cryptosporidium* species and genotypes. Future studies are required to understand the full public health significance of *Cryptosporidium* genotypes and species in immunocompromised hosts.

Cryptosporidium is an important enteric pathogen that causes diarrheal illness in humans and animals (4). In immunocompetent individuals, infection is usually self-limiting, but in immunocompromised individuals receiving immunosuppressive drugs and human immunodeficiency virus (HIV)-infected individuals, persistent infections which can be life threatening may develop (3). *Cryptosporidium* may account for 10 to 20% of the cases of diarrhea in HIV-infected patients living in developed countries and as much as 50% in underprivileged countries (12, 16). Among HIV-infected individuals who contracted cryptosporidiosis as a result of the infamous outbreak in Milwaukee, Wis., in 1993, the outcome of disease was severe, with greater than 50% mortality within 6 months to a year after the outbreak (I. Gilson, B. P. Buggy, C. F. Brunnitt, M. Busalacchi, and K. Ivantic, Int. Conf. AIDS, 1994).

The genus *Cryptosporidium* is a member of the phylum Apicomplexa along with the related genera *Toxoplasma*, *Eimeria*, and *Plasmodium*. To date, eight named species of *Cryptosporidium* have been proposed as valid. These include *C. parvum* from many mammals, *C. muris* from rodents and ruminants, *C. felis* from cats, *C. wrairi* from guinea pigs, *C. meleagridis* and *C. baileyi* from birds, *C. serpentis* from reptiles, and *C. nasorum* from fish (4, 9).

The causative agent of cryptosporidiosis in humans and a range of mammalian species is the species *C. parvum*. Recently, it has become increasingly clear, however, that *C. parvum* is not a single species but is composed of a number of distinct genotypes: a "human" genotype which has so far been found only in humans; a "cattle" genotype which is found in domestic livestock such as cattle, sheep, and goats and which can also infect humans; a "mouse" genotype which has been found in mice from around the world and more recently in bats; a "pig" genotype which is found in pigs; a "marsupial" genotype that has been found in koalas and kangaroos; and "dog" and "ferret" genotypes which have to date been found only in the respective hosts (7, 9, 18). Only the human and cattle genotypes have so far been identified in immunocompetent humans, and the public health significance of the remaining genotypes is unknown.

The susceptibilities of immunocompromised individuals to genotypes of *C. parvum* and to other species of *Cryptosporidium* have not been extensively studied. The aim of the project described here was to genetically characterize isolates of *Cryptosporidium* from HIV-infected individuals in order to determine the public health significance of genotypes of *C. parvum* and other species of *Cryptosporidium*.

MATERIALS AND METHODS

Sources of parasite isolates, diagnosis, and clinical information. The sources of the parasite isolates are listed in Table 1 (see the Results section). Swiss isolates were collected between February 1992 and July 1995; Kenyan isolates were collected between January 1998 and January 1999. Isolates of *Cryptosporidium* from the United States were collected from 1997 to 1998. Stool specimens were examined by Kinyoun carbol-fuchsin modified acid-fast staining, and an aliquot of each positive stool was stored either in 2.5% dichromate at 4°C or without preservatives at −80°C until molecular analysis. In Switzerland, sociodemographic, clinical, and immunological data for the patients were prospectively collected as part of the Swiss HIV Cohort Study (5), and the patients filled in a questionnaire on risks associated with diarrhea at the time of stool examination (16).

DNA purification and PCR amplification of 18S rDNA and acetyl-CoA synthetase genes. DNA was purified as described previously (8). The amplification conditions used to amplify a 298-bp portion of the 18S ribosomal DNA (rDNA) gene were as described previously (6). The primers and their sequences used to amplify a 390-bp product from the acetyl coenzyme A (acetyl-CoA) synthetase gene were as described previously (7). TAQ Extender (Stratagene, La Jolla, Calif.) was included in all reaction mixtures to minimize PCR error.

PCR amplification of the HSP-70 gene. A two-step nested PCR protocol was used to amplify the heat shock gene (HSP-70 gene) from genomic DNA for nucleotide sequencing. For the primary PCR, a PCR product of ~2,015 bp was amplified with a forward primer (5′-ATG TCT GAA GGT CCA GCT ATT GGT ATT GA-3′) and a reverse primer (5′-TTA GTC GAC CTC TTC AAC AGT TGG-3′). The PCR mixture consisted of 50 ng of genomic DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, 1× PCR buffer (Perkin-Elmer), 3.0 mM $MgCl_2$, 5.0 U of *Taq* polymerase (GIBCO BRL), and 40

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TABLE 1. Isolates and genotypes of *Cryptosporidium* used in this study

Isolate code	Geographical location	Source[a]	Genotype by analysis of:		
			18S rDNA gene	Acetyl-CoA synthetase gene	HSP-70 gene
CH-H66	Switzerland	USZ	Cattle	Cattle	
CH-H67	Switzerland	USZ	*C. felis*		
CH-H68	Switzerland	USZ	Cattle	Cattle	Cattle
CH-H69	Switzerland	USZ	Cattle	Cattle	
CH-H71	Switzerland	USZ	*C. felis*		
CH-H73	Switzerland	USZ	Cattle	Cattle	
CH-H74	Switzerland	USZ	Cattle	Cattle	Cattle
CH-H75	Switzerland	USZ	Cattle	Cattle	
CH-H77	Switzerland	USZ	*C. felis*		
CH-H78	Switzerland	USZ	*C. meleagridis*		
CH-H79	Switzerland	USZ	Human	Human	Human
CH-H80	Switzerland	USZ	Cattle		
CH-H81	Switzerland	USZ	Human	Human	
Ke-H271	Kenya	KEMRI	Cattle	Cattle	Cattle
Ke-H289	Kenya	KEMRI	Human	Human	
Ke-H392	Kenya	KEMRI	*C. meleagridis*	*C. meleagridis*	*C. meleagridis*
Ke-H375	Kenya	KEMRI	Human	Human	Human
Ke-H155	Kenya	KEMRI	Human	Human	Human
Ke-H189	Kenya	KEMRI	Human	Human	Human
US-HNO2	United States	CDC	*C. felis*		*C. felis*
US-HNO21	United States	CDC	*C. felis*		*C. felis*
US-HNO36/38	United States	CDC	*C. felis*		*C. felis*

[a] USZ, University Hospital, Zurich, Switzerland; KEMRI, Kenya Medical Research Institute; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.

ng of forward and reverse primers in a total reaction volume of 100 μl. Thirty-five PCR cycles (94°C for 45 s, 55°C for 45 s, 72°C for 60 s) were carried out in a Perkin-Elmer Gene Amp PCR 9700 thermocycler with an initial hot start (94°C for 5 min) and a final extension (72°C for 10 min). For the secondary PCR, a fragment of ~1,950 bp was amplified by using 2.5 μl of primary PCR product and nested forward (5′-TA/CT TCA TG/CT GTT GGT GTA TGG AGA AA-3′) and nested reverse (5′-CAA CAG TTG GAC CAT TAG ATC C-3′) primers. The PCR conditions for the secondary PCR were identical to those for the primary PCR, except that the annealing temperature was 45°C.

Sequencing of PCR products. PCR products were purified by using Qiagen spin columns (Qiagen, Hilden, Germany) and were sequenced by using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturers' instructions. PCR products were sequenced in both directions. Sequences were analyzed by using SeqEd, version 1.0.3 (Applied Biosystems).

RESULTS

Sequence analysis of the 18S rDNA gene. The 18S rDNA gene locus of all 22 isolates was analyzed. DNA sequence analysis at this locus identified four distinct genotypes, with the cattle genotype being the most common genotype identified (8 of 22) (Table 1). Six isolates were of the human genotype. Interestingly, a total of six isolates exhibited the *C. felis* genotype. Three of these isolates were from Switzerland and three were from the United States. Two isolates (isolates CZ-H78 and Ke-H392), one from Switzerland and one from Kenya, were identified as *C. meleagridis*.

Sequence analysis of the acetyl-CoA synthetase gene. The acetyl-CoA synthetase gene locus of 14 isolates was examined. A number of isolates, particularly those isolates which exhibited the *C. felis* genotype, did not produce a product with the acetyl-CoA synthetase gene-specific primers, presumably due to genetic differences between *C. felis* and *C. parvum* (H/C) at the primer binding sites. Sequence analysis of this locus identified three distinct genotypes: the cattle genotype, the human genotype, and a distinct genotype exhibited by one isolate (isolate Ke-H392) which typed as *C. meleagridis* at the 18S rDNA gene locus. The genotyping results for the acetyl-CoA synthetase gene locus were in agreement with those for the 18S rDNA gene locus (Table 1).

Sequence analysis of the HSP-70 gene. The HSP-70 gene locus of a smaller subset of isolates ($n = 11$) was analyzed. Four distinct genotypes were identified at this locus: human, cattle, *C. felis*, and *C. meleagridis* (Table 1). Genotyping results for this locus were in agreement with those for the 18S rDNA and acetyl-CoA synthetase gene loci.

Clinical information. The majority of the clinical information available in this study was obtained for HIV-infected patients from Zurich, Switzerland. These patients exhibited a variety of other concurrent AIDS-defining infections including microsporidiosis, toxoplasmosis, and Kaposi's sarcoma (Table 2). For all patients, $CD4^+$ lymphocyte counts were low and ranged from 0 to 110 per μl. The main mode of HIV acquisition was via sexual contact. All patients were experiencing chronic diarrhea that had lasted at least 4 weeks at the time of evaluation. No concurrent infection was found in the three *C. felis*-infected patients whose isolates were provided by the Centers for Disease Control and Prevention. In particular, patient US-HNO36/38 was positive twice for *C. felis* over a 5.5-month span and had diarrhea for the entire time.

DISCUSSION

Numerous studies of *Cryptosporidium* infections in HIV-infected individuals have been conducted; however, few have genotyped the *Cryptosporidium* isolates from these patients in order to obtain more predictive epidemiological data as to the sources of the infections. In this study, *Cryptosporidium* isolates from HIV-infected individuals from Switzerland, Kenya, and the United States were analyzed at three genetic loci: the 18S rDNA, HSP-70, and acetyl-CoA synthetase gene loci. The results revealed that the majority of the patients (64%) were infected with the human and cattle *C. parvum* genotypes. However, a number of patients were infected with *C. felis* (27%) or *C. meleagridis* (9%). This is the first report of *C. meleagridis* infection in a human host.

Few genotyping studies have been conducted with isolates of *Cryptosporidium* from HIV-infected patients (2, 13, 15, 17). In

TABLE 2. Clinical and epidemiological data for HIV-infected patients with *Cryptosporidium* infections from Zurich and the United States

Isolate code	Mode of HIV acquisition	Other concurrent AIDS-defining infections	CD4 lymphocyte count (no./μl)	Genotype, species	Pets
CZ-H66	Homosexual activity	*Candida* esophagitis	20	Cattle, *C. parvum*	Cat, dog
CZ-H67	Homosexual activity	None	60	*C. felis*	Cat
CZ-H68	Homosexual activity	Toxoplasmosis, Kaposi's sarcoma	20	Cattle, *C. parvum*	None
CZ-H69	Intravenous drug use	*Candida* esophagitis	20	Cattle, *C. parvum*	Cat
CZ-H71	Homosexual activity	Esophagitis, microsporidiosis	30	*C. felis*	None
CZ-H73	Homosexual activity	Cytomegalovirus retinitis	10	Cattle, *C. parvum*	None
CZ-H74	Homosexual activity	Microsporidiosis	110	Cattle, *C. parvum*	Dog
CZ-H75	Homosexual activity	Kaposi's sarcoma, cytomegalovirus retinitis	20	Cattle, *C. parvum*	None
CZ-H77	Intravenous drug use	Disseminated nontuberculous mybobacteriosis	0	*C. felis*	Cat
CZ-H78	Homosexual activity	Disseminated nontuberculous mycobacteriosis, Kaposi's sarcoma	0	*C. meleagridis*	Cat
CZ-H79	Homosexual activity	None	10	Human, *C. parvum*	Cat
CZ-H80	Homosexual activity	Isosporiasis, *Candida* esophagitis	40	Cattle, *C. parvum*	Snake
CZ-H81	Homosexual activity	Tuberculosis, Kaposi's sarcoma, cytomegalovirus retinitis	54	Human, *C. parvum*	None
US-HNO2	Intravenous drug use	None (history of syphilis)	48	*C. felis*	None
US-HNO21	Homosexual activity	None (history of Kaposi's sarcoma, *Candida* esophagitis)	38	*C. felis*	Cat, bird, dog
US-HNO36/38	Congenital transmission		3	*C. felis*	

1998, Widmer et al. (17) conducted multilocus analysis with *C. parvum* isolates from HIV-infected patients. Isolates from five of the patients tested exhibited the human genotype and isolates from two patients exhibited the cattle genotype (17). No genotypic information was obtained for isolates from two subjects; the oocysts from one failed to be amplified by any of the PCR primers used, and the second isolate was amplified with only the 18S rDNA gene-specific primers (17). A possible reason for this is that the oocysts detected in these two patients were not of the human or cattle genotype. Experience in our laboratories has shown that primers that are specific for *Cryptosporidium* but that have been designed on the basis of the human and cattle genotypes frequently do not amplify more genetically diverse genotypes such as the pig or dog genotypes of *C. parvum* or other species of *Cryptosporidium*.

A more recent study genotyped the 18S rDNA gene locus of 10 *Cryptosporidium* isolates from HIV-infected individuals (13). In that study, one isolate exhibited the cattle genotype, five isolates exhibited the human genotype, three isolates exhibited the *C. felis* genotype, and one isolate exhibited the newly identified dog genotype (13). For some patients, multiple specimens collected over 12 months were available, and for these patients the same *Cryptosporidium* genotype persisted throughout the course of the patient's infection (13).

Epidemiological investigations on the mode of transmission or the possible sources of human cryptosporidial infections are difficult because there are two possible pathogenetic mechanisms of cryptosporidial disease: first, HIV-infected patients with clinically manifest cryptosporidiosis may have very recently acquired cryptosporidial infection (a new exogenous infection); and second, infection may have occurred earlier, possibly prior to the state of immunodeficiency, and the infection was activated due to the progression of the immunodeficiency (an endogenous activation of latent infection).

In the present study, three of the six patients infected with *C. felis* had reported having a cat as a pet. It is therefore likely that they acquired their infections from their pets. For the remaining three patients infected with *C. felis*, no pets were recorded. However, it is possible that these patients had been exposed to cats at some time prior to the onset of clinically apparent cryptosporidiosis. One of the patients infected with *C. meleagridis* reported having only a cat as a pet, but clinical information was not obtained for the second patient who was infected with *C. meleagridis*. *C. meleagridis* normally infects turkeys (4). However, this species has recently been confirmed in an Indian ring-necked parrot, which is a common aviary bird with a worldwide distribution (10). *C. meleagridis* may therefore have a much wider host range than was previously thought, and it is possible that these patients acquired their cryptosporidial infections via contact with aviary birds.

Recent studies have reported that among HIV-infected individuals, those with $CD4^+$ lymphocyte counts of $<100 \times 10^6$/liter are at increased risk of contracting clinical cryptosporidiosis (12, 14, 16). All the patients in the present study were suffering from chronic diarrhea, and the majority of the patients in this study had other concurrent AIDS-defining infections, such as toxoplasmosis or microsporidiosis (Table 2). The majority of patients had $CD4^+$ lymphocyte counts which were $<100 \times 10^6$/liter, and in most patients the counts were less than 50×10^6/liter. In two patients (CZ-H77 and CZ-H78), the $CD4^+$ lymphocyte counts were 0 (Table 2). However, the small number of samples available does not allow any hypotheses regarding the different clinical courses to be made.

The majority of *Cryptosporidium* isolates from HIV-infected individuals from Switzerland exhibited the cattle genotype (54%). In previous studies with immunocompetent human-derived *Cryptosporidium* isolates from Perth, Western Australia, the human genotype was the most common genotype identified (83%; $n = 36$) (8). Whether there are differences in the predominance and distribution of the human and cattle genotypes between different geographic areas and whether the cattle genotype is more predominant among HIV-infected persons are issues which warrant future investigation.

Six of the isolates examined in this study were from Kenya; four of these were of the human genotype, and the remaining two (isolates Ke-H271 and Ke-H392) were of the cattle genotype and *C. meleagridis*, respectively. Little is known about the prevalence of *Cryptosporidium* in African countries, and this is the first time that *Cryptosporidium* isolates from Kenyan HIV-infected patients have been analyzed genetically. Recent research, however, indicates that the prevalence of *Cryptosporidium* in African HIV-infected patients may be high. A prospective cross-sectional study of 75 consecutive HIV-seropositive adult patients admitted to a government hospital in Nairobi, Kenya, revealed that *Cryptosporidium* was the most common pathogen (17%). Thirty-one (41%) patients died, and detection of *Cryptosporidium* oocysts was the single most significant predictor of death ($\chi^2 = 5.2$; $P < 0.05$) (11). These results are in agreement with those of an earlier study performed in Nairobi, Kenya (1). The study reported that *Shigella*

flexneri, *Salmonella enterica* serovar Typhimurium, and *C. parvum* were the most common fecal pathogens among HIV-infected and non-HIV-infected individuals studied (1).

In conclusion, the results of the present study and those of other studies indicate that immunocompromised individuals are susceptible to a wide range of *Cryptosporidium* species and genotypes and that host factors must play a role in controlling susceptibility to these divergent parasites. Future studies with larger numbers of AIDS patients for whom more extensive clinical information is available are required in order to understand the full public health significance of *Cryptosporidium* species and genotypes in immunocompromised hosts.

ACKNOWLEDGMENTS

We thank Anne Morse, Jeff Inungu, and S. K. Kang'ethe for assistance.

This research was supported by the Public Health Research and Development Committee of the National Health and Medical Research Council of Australia and the Centers for Disease Control and Prevention Opportunistic Infections Fund.

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