Molecular and Spatial Epidemiology of Cryptosporidiosis in Children in a Semiurban Community in South India[▽]

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Cryptosporidium spp. are a leading cause of diarrhea in Indian children, but there are no data for prevalent species or subgenotypes. Genetic characterization of *Cryptosporidium* spp. by PCR-restriction fragment length polymorphism and spatial analysis of cases using Geographical Information Systems technology was carried out for 53 children with cryptosporidial diarrhea in an urban slum. The two most common species were *C. hominis* (81%) and *C. parvum* (12%). Other species identified were *C. felis* and *C. parvum* (mouse genotype). Five subgenotypes were identified at the *Cpgp40/15* locus. Subgenotype Ia predominated among *C. hominis* isolates, and all *C. parvum* isolates were subgenotype Ic. *C. hominis* infection was associated with a greater severity of diarrhea. Sequencing of the *Cpgp40/15* alleles of *C. felis* and *C. parvum* (mouse genotype) revealed similarities to subgenotype IIa and *C. meleagridis*, respectively. Space-time analysis revealed two clusters of infection due to *C. hominis* Ia, with a peak in February 2005. This is the first study to demonstrate space-time clustering of a single subgenotype of *C. hominis* in a setting where cryptosporidiosis is endemic. Molecular characterization and spatial analysis have the potential to further the understanding of disease and transmission in the community.

Cryptosporidiosis is a major cause of diarrhea in children with and without human immunodeficiency virus (HIV) infection in developing countries (8, 16, 21, 27, 30). In these countries, cryptosporidial infection in early childhood has been reported to be associated with subsequent impairment in growth, physical fitness, and cognitive function (5, 20). In India, *Cryptosporidium* spp. are a leading cause of infectious diarrhea in children, with reported positivity rates ranging from 1.1% to 18.9% (11, 22, 24, 28).

The epidemiology of cryptosporidiosis in humans is not completely understood due to the existence of multiple transmission routes such as person-to-person, animal-to-person, water-borne, food-borne, and possible airborne transmission (1). More recently, genetic characterization of *Cryptosporidium* spp. at polymorphic loci has facilitated the development of molecular approaches with which to study the epidemiology of cryptosporidiosis (35, 36). Extensive polymorphisms in the *Cpgp40/15* gene have been widely used to define at least eight allelic subgroups or subgenotypes of the parasite (14, 32). The majority of human infections, including those in developing countries, are caused by *C. hominis* (14, 17, 19, 29, 31, 33). There have been several studies of the prevalence rates of cryptosporidial infections in children in India, but there is only one report of the species or genotypes of *Cryptosporidium* spp. from children in eastern India (3), and none from southern India. Furthermore, there have been no studies of the genetic characterization of *Cryptosporidium* sp. infections in children in a well-defined community setting.

Geographical Information Systems (GIS) methods have previously been used to map the locations of residences of sporadic cases as well as in ecosystem studies of cryptosporidiosis (7, 10). The use of GIS to study the spatial distribution of cases has been found to be useful in identifying geographical variation but not necessarily for identifying the reasons for this variation. In this study, we determined the species, genotypes, and subgenotypes of *Cryptosporidium* sp. infections in a well-defined cohort of children in a semiurban community in Vellore, South India. A spatial analysis of children with cryptosporidial diarrhea was also carried out in order to study the transmission dynamics of cryptosporidiosis in this community.

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MATERIALS AND METHODS

Study population, sample collection, and screening. The study subjects are part of an ongoing birth cohort of 452 children recruited for a study of rotaviral infections from a semiurban slum area with a population of approximately 33,390 in Vellore, in South India. The study was approved by the Institutional Review Board of Christian Medical College, and informed consent was obtained from the parents. The children were enrolled at birth and followed for 3 years on a twice-weekly basis. Demographic and birth details were recorded at baseline. In this study, an episode was defined as at least 1 day of diarrhea (with the occurrence of three or more watery stools in a 24-h period) preceded and followed by 2 or more days without diarrhea. The episode was considered to have ended on the day bowel movements returned to normal (18). Children with diarrhea were assessed clinically, and details of the number of stools passed per day, any associated fever or vomiting, and treatment given were recorded daily until the cessation of diarrhea. These data were collected to calculate the Vesikari score, a scale used in rotaviral infection diarrhea to assess severity (23). Fecal samples collected from 1,949 of a total of 1,989 episodes of diarrhea over 14,584 child-months (the sum of months of follow-up of all children) of follow-up from April 2002 to January 2006 were screened for *Cryptosporidium* spp. by microscopic examination of modified acid-fast-stained smears, and these samples were also

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FIG. 1. (A) Restriction fragment length polymorphism patterns are shown at the following locus for *Cryptosporidium* spp. after nested PCR amplification: SSU rRNA locus and digestion with SspI (lanes 1 to 4) and VspI (lanes 6 to 9). Lanes 1 and 6, *C. hominis*; lanes 2 and 7, *C. parvum*; lanes 3 and 8, *C. parvum* (mouse); lanes 4 and 9, *C. felis*; lane 10, 1-kb marker. (B) *Cpgp40/15* locus and digestion with AluI: lane 1, *C. hominis* Ia; lane 2, *C. parvum* Ic; lane 3, *C. hominis* Id; lane 4, *C. hominis* Ib; lane 5, *C. hominis* Ie; lane 6, *C. felis*; lane 7, *C. parvum* (mouse); lane 8, 1-kb marker.

tested for other bacterial and parasitic diarrheal pathogens by culture and microscopy and for rotavirus by enzyme-linked immunosorbent assay (Dako Cytomation rotavirus IDEIA, Ely, United Kingdom).

DNA isolation, identification of species, genotyping, and subgenotyping. Briefly, DNA extracted from fecal samples positive for *Cryptosporidium* spp. by microscopy with a QIAamp stool DNA minikit (QIAGEN Inc, Valencia, CA) was subjected to PCR-restriction fragment length polymorphism (RFLP) at the small-subunit (SSU) rRNA locus using enzymes SspI and VspI for species and genotype determination (34) and PCR-RFLP at the *Cpgp40/15* locus using enzymes AluI and RsaI for subgenotyping (14) as previously described. Samples negative for SSU rRNA by PCR were analyzed by PCR-RFLP at the *COWP* and *TRAP-C* loci (25, 26). In samples where ambiguous results were obtained by PCR-RFLP at the *Cpgp40/15* locus, the PCR products were purified using a QIAquick kit (QIAGEN Inc, Valencia, CA), sequenced by the dye terminator method, and compared with sequences of a known subgenotype using a ClustalW alignment algorithm of the AlignX program of Vector NTI, Suite 8 (Informax, North Bethesda, MD). The secondary PCR products of two *C. felis* samples, one obtained from an HIV-infected adult from a previous study (19) and one from a child in the present study, were cloned into a PCR 2.1-TOPO vector (Invitrogen Corp., Carlsbad, CA), and the inserts were sequenced. The *Cpgp40/15* PCR product of the *C. parvum* (mouse) was also sequenced and analyzed. Phylogenetic analysis was performed using the maximum-likelihood method with BioEdit Sequence Alignment Editor version 5.0.0 (6).

Spatial analysis. The Poisson probability model (12) was used to identify any spatial or space-time clusters for high rates with the maximum spatial cluster size of 50% of the total population. Spatial analysis was carried out for cryptosporidial infections occurring from birth until January 2006 to detect any clustering of episodes by using SaTScan version 6.0 (13). The streets and the study houses of the urban slum area had been previously mapped and geographically referenced using ArcView GIS 3.3 (Environmental Systems Research, Inc., CA). The way points and track points were collected using a GPS Garmin V and downloaded as layers using GPS Utility 4.10.4. (GPS Utility Ltd., Southampton, England).

Statistical analyses. Data generated during this study were double entered using Epi Info 6.4. software (CDC, Atlanta, GA) and analyzed using STATA version 9.0 (Stata Corp., College Station, TX). Statistical comparisons were made using Fisher's exact and chi-square tests.

Nucleotide sequence accession numbers. Sequences from this study were deposited in GenBank with accession numbers DQ 848995 to -97.

RESULTS

Cases. Of the 1,949 diarrheal episodes screened, one or more diarrheal agents was detected in 591 episodes. *Cryptosporidium* spp. alone accounted for 7.61% of episodes and was the third most common etiologic agent after rotavirus and *Giardia* spp. Fifty-eight diarrheal episodes associated with *Cryptosporidium* spp. were identified in 53 children, with 5 children having two episodes each. Mixed infections from rotavirus, *Shigella flexneri*, *Vibrio cholerae*, or *Giardia* were identified

in four children. In the children with repeated episodes of cryptosporidial diarrhea, the duration between the episodes ranged from 10 days to more than 1 year. Fifty-four of these episodes were treated with oral rehydration. None of the children with cryptosporidial diarrhea was hospitalized or required intravenous rehydration. In 7 of the 58 episodes, the child was found to be dehydrated clinically, based on assessments carried out in the field. A total of 5 of 452 children in the cohort died during the period of follow-up, with one death each due to congenital anomalies and a seizure disorder and three deaths due to diarrhea, but these diarrheal episodes were not associated with cryptosporidiosis.

Species and subgenotypes. The species most commonly identified was *C. hominis* (47/58, 81%), followed by *C. parvum* in (7/58, 12.1%) and *C. felis* (3/58, 5.2%). One sample, which was negative by SSU (18S) rRNA PCR, was identified by *COWP* PCR. On comparison of the three PCR-based techniques with microscopy-positive samples, others have found a sensitivity of 97, 91, and 66% for the for the 18S rRNA, *COWP*, and *TRAP-C1* gene fragments, respectively (15). Given this sensitivity, it is possible that 1 of 58 samples, tested twice, could be negative by SSU PCR but positive by *COWP* PCR. One sample with *C. parvum* (mouse genotype) was also identified (Fig. 1A). Subgenotyping of the 47 *C. hominis*-positive samples at the *Cpgp40/15* locus showed that subgenotype Ia was the most common (35/47, 74.5%), followed by Id (8/47, 17%), Ie (3/47, 6.4%), and Ib (1/58, 1.7%). Subgenotyping of the *C. parvum* samples revealed that all seven were subgenotype Ic (Fig. 1B). In the five children with repeat infections, both infections were caused by *C. hominis* subgenotype Ia in four children and the remaining child had *C. felis* and *C. hominis* Ia infections.

Cryptosporidium felis and *C. parvum* (mouse genotype) samples could not be classified into any previously described subgenotypes by PCR-RFLP at the *Cpgp40/15* locus (Fig. 1B). Phylogenetic analysis of the *Cpgp40/15* sequences revealed that the *C. felis* *Cpgp40/15* allele was most closely related to the *C. parvum* IIa subgenotype (identities of 0.759/100% similarity over 795 bp) (Fig. 2), while the *C. parvum* (mouse genotype) allele was most closely related to the *C. meleagridis* allele (0.611/96% identity over 326 bp).

FIG. 2. Phylogenetic analyses of *C. felis* (sample 9 and 26) and *C. parvum* (mouse) *Cpgp40/15* alleles using the maximum-likelihood method performed with BioEdit Sequence Alignment Editor version 5.0.0. (Sequences from this study have been compared with the following sequences obtained from GenBank: AF440621-22, AF440624-26, AF440628-30, AF114166, AF440633-34, and AF 401498.)

Association of clinical characteristics with *Cryptosporidium* spp. and subgenotypes. There were no significant differences in demographic (age, gender, birth order, educational status of the mother, or occupation of the father) or clinical (nutritional status assessed by weight and height for age, vomiting, fever, and hospital visits) characteristics between *C. hominis*-infected children and those infected with other species (Table 1) or between subgenotype Ia-infected children and those infected with other subtypes. However, *C. hominis*-infected children had a significantly greater severity of diarrhea ($P < 0.05$, Fisher's exact test). There was also a trend toward a longer average duration of diarrhea in *C. hominis*-infected children ($P = 0.09$) than in those infected with other species (Table 1). However, there was no increase in the severity of diarrheal symptoms in children who were coinfecting with rotavirus, *Shigella flexneri*, *Vibrio cholerae*, and *Giardia*. Diarrhea associated with subgenotype Ia, the most commonly identified subgenotype, was not more severe than that associated with the other subgenotypes ($P = 0.77$). In the five children with repeated episodes of cryptosporidial diarrhea, the severity of diarrhea was increased in one case, decreased in two cases, and remained the same in the remaining two children during the second episode.

GIS analysis. There were two significant space-time clusters of cryptosporidial diarrhea, one during February to March 2005 and the other during June to August 2003. The most likely space-time cluster involved six cases within a 0.07-km radius during the time frame of 1 February 2005 to 31 March 2005 (relative risk [RR], 133.8; $P = 0.002$). The second likely

cluster involved eight cases during the time frame of 1 June 2003 to 31 August 2003 (RR, 22.9; $P = 0.002$). The calendar time distribution of cryptosporidial diarrhea during the follow-up period relative to the total diarrheal episodes experienced by the cohort also showed a peak in February 2005, with 16% of all diarrheal episodes in that month associated with *Cryptosporidium* spp. When children infected with *C. hominis* subgenotype Ia alone were analyzed, a similar pattern was found, in which the most likely space-time cluster involved eight cases that occurred within an area of 0.26 km radius during the same time period (RR, 48.9; $P = 0.001$), and the second most likely cluster involved six cases that occurred during 1 June 2003 to 31 August 2003 (RR, 62.4; $P = 0.001$) (Fig. 3). There was no clustering when the other subtypes were analyzed. Based on data collected during twice-weekly visits, we found that the cases belonged to different communities and that the children attended different schools, so we have no reason to suspect person-to-person transmission. No clustering was seen with the seven children with *C. parvum* infection. In order to identify potential zoonotic transmission of infection, the households and those of neighbors of the children with *C. parvum* and *C. felis* infections were questioned for the presence

TABLE 1. Association of demographic and clinical features with *Cryptosporidium* spp.

Patient data	Incidence (%) of infection by		<i>P</i> value
	<i>C. hominis</i> (47 strains)	Other species (11 strains)	
Demographic features			
Age in mo at diarrhea onset (range) ^a	14.4 (9.6–23.1)	10.3 (7.3–24.4)	0.17
Gender			
Male	22 (47)	7 (64)	0.50
Female	25 (53)	4 (36)	
Birth order			
First	11 (23)	2 (18)	1.0
Others	36 (77)	9 (82)	
Education of the mother			
None	12 (26)	6 (55)	0.11
Primary	17 (31)	4 (36)	
More than primary	18 (38)	1 (9)	
Occupation of the father			
Unskilled	17 (36)	5 (45)	0.73
Skilled	30 (64)	6 (55)	
Clinical features (no. [%] of patients)			
Associated fever	4 (9)	2 (18)	0.33
Associated vomiting			
No	41 (87)	9 (82)	0.64
Yes	6 (13)	2 (18)	
Malnutrition			
Weight ^b	14 (30)	4 (36)	0.72
Height ^c	22 (47)	3 (27)	0.32
% of patients with a diarrhea severity score of ^d			
1–3	5	5	0.005
4–5	12	4	
>6	29	2	
No. of days of duration (range) ^e	3 (2–5)	2 (2–3)	0.09

^a Median (25th to 75th percentile) no. of months, derived from Mann-Whitney test.

^b Weight for age <2 standard deviations.

^c Height for age <2 standard deviations.

^d Maximum no. of stools/day are given as percentages, derived by Fisher's exact test.

^e Median (25th to 75th percentile) no. of days, derived by the Mann-Whitney test.

FIG. 3. Spatial analysis with SaTScan version 6.0 of cases with *C. hominis* Ia infection, revealing two space-time clusters in the community.

of cattle or domestic pets, but no history of exposure to these animals was found.

The association between cryptosporidial diarrhea and the water source was also studied. In the community, five different overhead tanks supplied water to different areas. The tank-specific infection rates revealed that 7 of the 27 households which were supplied by a particular tank (tank 1) had cases of *C. hominis* Ia infection, which was a significantly higher rate than in areas supplied by the other tanks (chi-square test, $P = 0.001$). There was also a tank (tank 2) that was associated with only one episode of cryptosporidial diarrhea. Water samples from the area were tested for coliforms, and the most probable number/ml (MPN) was assessed. Of the three samples collected from the Kaspia region during January to March 2005, two had an MPN of >360 , indicating a very high level of contamination that suggested a possible water-related transmission for this cluster. The technology and reagents for testing water samples for cryptosporidial oocysts are not readily available in India, and this test was not carried out.

DISCUSSION

This is the first study to genetically characterize *Cryptosporidium* spp. identified in children in a community setting in India and to compare the association of species and subgenotype with clinical features, and it is also the first study to carry

out spatial and temporal analyses of cryptosporidial diarrhea in India.

Compared to our previous, hospital-based study of *Cryptosporidium* spp. from HIV-infected adults in the same area (19), there were significant differences between the proportions of potentially zoonotic species in the two populations (chi-square test, $P < 0.001$), with fewer zoonotic species found in children in the community than in HIV-infected adults in the hospital. The *Cpgp40/15* subgenotypes were also more diverse among the HIV-infected adults than among the children. The predominant subgenotypes among the *C. hominis* samples were Ib and If in HIV-infected adults, whereas Ia predominated in children. All *C. parvum* samples from children were Ic at the *Cpgp40/15* locus, whereas several different subgenotypes, including IIa, IIb, and Ic, were seen in *C. parvum* samples from HIV-infected adults. These data suggest differences in transmission patterns or susceptibility to disease in these two populations, which merits further study of potential sources of infection in a developing country. In a previous report, a probability proportional to size cluster survey of approximately 3,000 samples from individuals in the 15- to 40-year-old age group was carried out to estimate the community prevalence of antibodies to human immunodeficiency virus in rural and urban Vellore. This study showed that the prevalence of antibodies to HIV in the population was 0.66% (9). The program to prevent HIV transmission from parents to children has been in place since 2000, with all HIV-infected pregnant women throughout the state receiving antiretroviral therapy (http://www.unicef.org/india/hiv_aids_278.htm). Even in the absence of such a program, expected rates in children, who could possibly have had vertical transmission of infection, would be less than one-third of that in the adult population, or 0.22% in children or 1 child in the cohort of 452 recruited. Therefore, we do not believe that immunosuppression due to HIV could have been a significant risk factor for cryptosporidiosis in the 53 infected children or for recurrent diarrhea in the cohort.

Data for subgenotype distribution are scarce and are derived mainly from hospital-based studies and from outbreaks (2–4, 19); this is the first community-based study to subgenotype cryptosporidial samples in a developing country. This is also the first nonoutbreak study to show the predominance of one subgenotype, Ia, in the community. A previous study from Malawi involving community-acquired diarrhea in children showed the presence of six different subtypes among *C. hominis* samples (21). In the present study, diarrhea due to *C. hominis* infection was also found to be more severe than diarrhea due to other species in this community. A study from Peru reported longer periods of oocyst shedding in *C. hominis*-infected patients (33), but there has been no previous report of association with the severity of diarrhea.

The *C. parvum* samples were all subgenotype Ic, a subgenotype associated with anthroponotic transmission (35). In children infected with this species, no potential sources of zoonotic transmission were found. The finding that all *C. parvum* samples subgenotyped as Ic at the *Cpgp40/15* locus, suggesting discordant alleles at the SSU rRNA locus, has been reported previously (14, 19, 21); and it has been suggested that this allele should be renamed IIc. These data collectively suggest a predominantly anthroponotic transmission cycle of *Cryptosporo-*

ridium spp. in this community, although the sources of infection were not conclusively determined.

The application of spatial and temporal analyses revealed two significant clusters of infections due to subgenotype Ia in children, which had not been identified with routine surveillance. Previous studies involving GIS and *Cryptosporidium* spp. have focused on the epidemiology of sporadic cases of cryptosporidiosis in the northwest of England and Wales (7) and have demonstrated contamination sites in watershed environments in Kenya and Ecuador (10). The present study is the first to apply GIS technology to document the epidemiology of cryptosporidiosis in a well-defined community where the disease is endemic. Additionally, an area of the community supplied with water from a particular overhead tank (tank 1) was found to have a significantly higher incidence of cryptosporidiosis. In the area where one cluster of cases was identified by GIS technology, very high levels of fecal contamination of water samples were detected at the same time point and suggest the possibility of waterborne transmission. However, a conclusive demonstration of this requires the identification of oocysts of the same subgenotype in the source water. Previous studies of waterborne outbreaks have found an association with *C. hominis* subgenotype Ib (2, 4, 37). In contrast, the subgenotype seen in the children who potentially acquired cryptosporidiosis from tank 1 was Ia. Only 1 of the 47 *C. hominis* samples subgenotyped in this study was Ib, suggesting that waterborne transmission of subgenotype Ib may not be a significant mode of acquisition of disease in this area and that there may be a geographic variation in genotypes associated with waterborne transmission. Alternatively, it is possible that subgenotype Ib is less prevalent in India than in other transmission sites due to other as-yet-undefined reasons.

These data provide new evidence to support the need for the use of molecular and geospatial tools to investigate potential sources of infection and to study transmission patterns in the community in order to apply relevant interventional measures for the prevention of disease.

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