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ORIGINAL RESEARCH

MULTILOCUS GENOTYPING OF *CRYPTOSPORIDIUM HOMINIS* ASSOCIATED WITH DIARRHEA OUTBREAK IN A DAY CARE UNIT IN SÃO PAULO

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A number of species of *Cryptosporidium* are associated with diarrhea worldwide. Little data exists regarding the genotypes and species of *Cryptosporidium* associated with cases of infections in Brazil.

PURPOSE: In the present study, we ascertained by molecular methods the species and the genotype of *Cryptosporidium* sp from a diarrhea outbreak diagnosed in a day care at the Hospital Clínicas, São Paulo University Medical School.

MATERIALS AND METHODS: Specific identification and typing of the isolates associated with the outbreak was done by DNA sequencing analysis of fragments amplified by polymerase chain reaction (PCR) from 3 different *Cryptosporidium* loci: the SSUrRNA coding region, the *Cryptosporidium* oocyst wall protein (COWP) gene, and the microsatellite locus 1 (ML1), a tandem GAG-trinucleotide repeat containing substitutions that differentiate the genotypes of *Cryptosporidium parvum* and *Cryptosporidium hominis*.

RESULTS: A total of 29 positive samples from the outbreak were studied by the molecular methods described. Our study revealed the presence of a single genotype of *Cryptosporidium hominis* in all samples.

CONCLUSION: The molecular analysis reinforced the hypothesis that the transmission of *Cryptosporidium hominis* during the period the samples were collected occurred in an outbreak pattern, possibly by person-to-person contact through the fecal-oral route. As far as we know, this is the first time that molecular tools have been used to identify the species and the genotype of isolates showing the presence of the ML1 genotype in samples from Brazilian patients.

KEYWORDS: Diarrhea. *Cryptosporidium hominis*. Parasite. Intestine. Brazil.

INTRODUCTION

Cryptosporidium sp is a coccidian genus belonging to the phylum apicomplexa.[1] This genus includes at least 6 species knowingly associated with intestinal disease in humans that include *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium felis*, *Cryptosporidium meleagridis*, *Cryptosporidium canis*, and *Cryptosporidium muris*.[2–6] *Cryptosporidium hominis* and *C. parvum* are the two most prevalent species causing human disease, associated with both outbreaks and sporadic cases.[6,7] The other species have been associated only with sporadic cases in immunocompromized persons, but also in individuals not showing signs of immunodeficiency.[7,8]

Cryptosporidiosis may be transmitted directly through person-to-person or person-to-animal contact, or through water or food contaminated by symptomatic or asymptomatic human or animal carriers passing *Cryptosporidium* oocysts.[6,9-15] Massive outbreaks may occur when oocysts end up in water reservoirs serving highly populated communities, such as in the cryptosporidiosis outbreak in the city of Milwaukee, WI, USA, in 1993.[16–18] Serological surveys conducted worldwide, including in Brazil, have shown the presence of anti-*Cryptosporidium* antibodies in a large number

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of children living in slums and in infants less than 1 year old living in rural areas.[19–21] This suggests that exposure to *Cryptosporidium* oocysts is more frequent in individuals living under inadequate sanitation conditions. Studies conducted in different regions of Brazil have shown that *Cryptosporidium* sp is associated with symptomatic intestinal disease in HIV infected individuals and in children suffering of acute diarrhea, including those attending day care.[22-28] Environmental contamination with *Cryptosporidium* oocysts has been observed in rivers and public water reservoirs serving different regions in Sao Paulo.[29–32] Nevertheless, no massive cryptosporidiosis outbreaks associated with consumption of water in these regions have been reported. In the state of São Paulo, data gathered on waterborne and foodborne epidemics reported to the Center of Epidemiological Vigilance (CVE) indicated that 10 outbreaks of cryptosporidiosis took place within the period of 1995 to 2003.[33] In the present study, we used molecular methods to identify the species and the genotype of *Cryptosporidium* associated with an outbreak that occurred between March and May, 2001, which involved children attending day care at the Hospital das Clínicas, the teaching hospital of the São Paulo University Medical College.

MATERIALS AND METHODS

Study Site

Hospital das Clínicas is a 3,271- bed hospital employing 9,888 health care providers, serving patients referred from the Public Health System, and a number of patients referred from private health care providers. The hospital manages a day care center to serve its workers, enrolling children from 4 months to 4 years of age. This center enrolls children based on age or child development, separating them into 7 groups: Nursery I and II (N I and N II) with 46 and 59 children; Mini Group I and II (MG I and MG II) with 41 and 49 children and Toddlers I, II, and III (T I, T II, and T III) with 53, 45, and 35 children respectively.

Stool samples from 224 of the 328 children (68.3%) attending this day care were collected during the outbreak period in March and May, 2001. The stool samples were processed by concentration methods for ova and parasite (O&P) examination[34–36]; smears were stained with Kinyoun's modified acid-fast stain for identification of *Cryptosporidium* sp.[37] One part of the selected positive samples was mixed with 3 parts of 2.5% of potassium dichromate and kept at 4ºC until DNA extraction was performed for molecular analysis.

DNA was extracted from aliquots of approximately 300 µl of each stool specimen using a modification of the FastDNA® method (Q-Biogene, Carlsbad, CA.) as previously described.[38] The extracted DNA samples were stored at 4°C and used for polymerase chain reaction (PCR) amplification.

PCR Amplification

A PCR was performed to amplify fragments from 3 different loci of the *Cryptosporidium* sp genome, ie, 18S rRNA, COWP, and microsatellite locus 1 (ML1), following the procedures described below. The PCR products were analyzed by electrophoresis on 2% SeaKem GTG agarose (Cat. No. 50074, FMC Bioproducts, Rockland, Maine), stained with ethidium bromide and visualized on an ultraviolet transilluminator.

Amplification of a fragment from the SSUrRNA coding region

The primer pair named CPBDIAGF/CPBDIAGR that produces fragments of 435, 438, and 455 base pairs (bp) from the *C. parvum*, *C. hominis* (formerly know as *C. parvum genotype 1*), and *C. felis*, respectively, was used for amplification of a variable region of the SSU-rRNA.[39] Sequencing of this region can distinguish between all the *Cryptosporidium* species and genotypes described to date. Polymerase chain reactions with these primers were performed as previously described.[40]

Amplification of fragments from the COWP gene

Two primer pairs, CRY12/CRY14 and CRY15/CRY9, were used to amplify 2 distinct fragments of the COWP gene. The primer pair CRY12/CRY14 was used to amplify a fragment from the C-terminal domain of this gene. Amplification with this primer pair produces fragments that vary from 568 and 571 bp if used on templates from *C. parvum* and *C. hominis*, respectively. The primer pair CRY15/CRY9 was used to amplify a fragment of the N-terminal domain of the same gene. Amplification with these primers produces a DNA fragment of 553 bp from both *C. parvum* and *C. hominis* templates.[41] Amplifications with primer pairs CRY12/CRY14 and CRY9/CRY15 were performed as described previously,[41] the only differences being primer concentration (50 pmoles) and reaction cycles (45 times instead of 30).

Amplification of locus ML1

ML1 was amplified using primers GAGF (5' CTAAAAATGGTGGAGAATATTC 3') and GAGR (5' CAACAAAATCTATATCCTC 3'); the procedure has been

described elsewhere.[42] These primers amplify a fragment of approximately 240 bp arranged in tandem GAG repeats. Previous analysis of this locus has revealed polymorphisms that discriminate 2 and 4 genotypes of *C. hominis* and *C. parvum*, respectively.

DNA Sequencing and Analysis

Amplification products were purified by using the Stratagene PCR purification kit (Cat. No. 400773, Stratagene, La Jolla, Calif.). Sequencing reactions were performed using the Perkin Elmer Big Dye kit (Cat. No. 4303149, PE Biosystems, Foster City, California), and sequence data were obtained by using the Perkin Elmer ABI 3100 automatic DNA sequencer. Sequences were assembled using the program SeqMan II (DNASTAR Inc., Madison, Wisc.). Genotype identification of the ML1 was done by aligning unknowns with master sequences retrieved from GenBank using the program, Clustal X v.18.[43] For the other loci analyzed, genotype identification was done by using the GeneStudio suite (GeneStudio Inc., Suwanee, Georgia).

Epidemiologic investigation

The investigation included interviews with children's mothers and staff as well as analysis of sanitary conditions of the day care center. Children with diarrhea were defined as those with liquid or semi-liquid stools. The risk factors were determined using all patients who were positive for *Cryptosporidium* as confirmed by PCR and compared with 100 negative-control patients. We considered the same risk factors as those observed by the hospital surveillance group.[44]

We developed an analytical study (retrospective cohort) for *Cryptosporidium* processing the distribution of frequencies among 2 groups for the qualitative variables, including sex, diarrhea, class, exposure to filtered tap water, use of an earth filter, use of a nursing bottle, use of diapers, ingestion of raw vegetables, and contact among children in the cafeteria and recreation area, using Pearson's chi square test.[45] Fisher's exact test was used when the expected values were less than 5. We considered the value $P < 0.05$ as statistically significant. Then we calculated the relative risks and their confidence intervals. For ages, we used the Mann-Whitney's nonparametric test,[46] and $P < 0.05$ was considered statistically significant.

RESULTS

Study Site

The day care at Hospital das Clinicas had some diarrhea control measures, including dismissal of children with diarrhea, assigning different employees to different functions, such as changing diapers and handling food. Employees conducted such activities in different areas, performing appropriate disposal of diapers and hand washing between procedures. There were distinct restrooms for children and staff, and the water and sewage services used by the day care were the same used by the entire Hospital.

Laboratory Investigation

Of the 224 children with stool samples analyzed for parasites, 47 (21%) were negative and 26 (11.6%) were positive for *Giardia lamblia*, while 23 (10.3%) were positive for other parasites, ie, *Isospora belli*, *Trichuris trichiuris*, *Entamoeba coli*, *Entamoeba hartmani*, *Enterobius vermicularis*, and *Ascaris lumbricoides*. For *Cryptosporidium* sp, 29 (12.9%) stool samples were positive by microscopic analysis as well as by PCR and were used for analytical epidemiology.

PCR amplification and DNA sequencing analysis for molecular typing

Cryptosporidium DNA was amplified by PCR from the 29 stool samples using 4 different primer pairs that generate a) 2 independent fragments from the C and N terminal domains of the COWP gene (primer pairs CRY12/CRY14 and CRY15/CRY9, respectively); b) 1 fragment from the SSUrRNA coding region (primers CPBDIAGF/CPBDIAGR); and c) the ML1 (primers GAGF and GAGR).

Genotyping of *Cryptosporidium* was based on sequencing of all fragments amplified for speciation and for identification of species genotype. The expected fragment size was amplified in all samples using the primers described above. Table 1 summarizes the results obtained from this analysis. Not all samples were amplified with all primers, which might indicate a difference of amplification efficiency among these tools. The only species found in this set was *C. hominis*. Data obtained from the ML1, which can distinguish between the genotypes of *C. hominis* and *C. parvum*, revealed the presence of only 1 genotype, ie, ML1 genotype1, in 45% of the 29 samples positive for *C. hominis*, which was subjected to ML1 analysis.

Descriptive and Analytical Epidemiology

A total of 180 cases met the diarrhea illness definition (general attack rate = 54.9%): 19 (10.5%) cases occurred in March, 152 (84%) in April, and 9 (5.5%) in May. Of these, 30 cases were from class N I (attack rate [AR] =

Table 1 - Analysis of 29 stool samples positive for *Cryptosporidium* collected from children attending in the day care at the Clinical Hospital, Sao Paulo Brazil

Sample	COWP-C-terminal	COWP-N-terminal	SSUrRNA	ML1
1	*C. hominis*	*C. hominis*	*C. hominis*	G1
2	*C. hominis*	*C. hominis*	*C. hominis*	G1
3	*C. hominis*	*C. hominis*	*C. hominis*	G1
4	*C. hominis*	*C. hominis*	*C. hominis*	G1
5	*C. hominis*	*C. hominis*	*C. hominis*	G1
6	*C. hominis*	Negative	*C. hominis*	G1
7	*C. hominis*	*C. hominis*	Negative	Negative
8	*C. hominis*	*C. hominis*	*C. hominis*	Pos-ND
9	*C. hominis*	*C. hominis*	Negative	Negative
10	*C. hominis*	*C. hominis*	*C. hominis*	G1
11	*C. hominis*	*C. hominis*	*C. hominis*	Pos-ND
12	*C. hominis*	*C. hominis*	*C. hominis*	Pos-ND
13	*C. hominis*	*C. hominis*	*C. hominis*	Pos-ND
14	*C. hominis*	*C. hominis*	*C. hominis*	Pos-ND
15	*C. hominis*	*C. hominis*	*C. hominis*	Pos-ND
16	*C. hominis*	*C. hominis*	*C. hominis*	Pos-ND
17	*C. hominis*	*C. hominis*	*C. hominis*	Pos-ND
18	*C. hominis*	*C. hominis*	*C. hominis*	Pos-ND
19	*C. hominis*	*C. hominis*	*C. hominis*	G1
20	*C. hominis*	*C. hominis*	*C. hominis*	Pos-ND
21	*C. hominis*	*C. hominis*	*C. hominis*	G1
22	*C. hominis*	*C. hominis*	*C. hominis*	G1
23	*C. hominis*	*C. hominis*	*C. hominis*	G1
24	*C. hominis*	*C. hominis*	*C. hominis*	Negative
25	*C. hominis*	*C. hominis*	*C. hominis*	G1
26	*C. hominis*	*C. hominis*	*C. hominis*	Negative
27	*C. hominis*	*C. hominis*	*C. hominis*	G1
28	*C. hominis*	*C. hominis*	Pos-ND	Negative
29	*C. hominis*	*C. hominis*	Pos-ND	Negative

Legend: ML1 = microsatellite locus 1; G1-Genotype 1 for the ML1; Pos-ND- positive amplification, with no genotyping data due to PCR amplicons of substandard quality for sequencing analysis; ND-not done due to lack of enough sample for molecular analysis.

65.2%), 33 from N II (AR = 55.9%), 20 from MG I (AR = 48.8%), 34 MG II (AR = 69.4%), 25 T I (AR = 47.2%), 28 T II (AR = 62.2%), and 10 T III (AR = 28.6%). We analyzed 164 cases (91.1), and the presence of *Cryptosporidium* was statistically significant in all classes ($P = 0.001$), with a higher concentration in MG I (Table 2).We observed no significant association of *C. hominis* with sex, diarrhea, use of a nursing bottle, and contact with other children during recreation (results summarized in Table 3). Children using diapers and drinking water filtered through an earth filter were at higher risk for acquiring *C. hominis* infection compared to the control group (28.4% versus 12.5%; $P = 0.037$ and 39.1% versus 13.3%; $P = 0.001$, respectively). However, risk for acquiring *C. hominis* infection was lower for children who used filtered tap water and ingested raw vegetables ($P < 0.05$). For children with *C. hominis* who drank filtered tap water, the frequency was 13.3% versus 39.1% compared to children who did not drink filtered tap water. Children who ingested raw vegetables had a *C. hominis* infection frequency of 12.5% versus 28.4% for children who did not eat raw vegetables ($P = 0.037$). There was no significant difference in ages between infected and uninfected children ($P = 0.316$).

DISCUSSION

Cryptosporidium is an ubiquitous parasite found worldwide, Brazil included.[19,20,22–30,32,33] Despite the number of reports indicating the association of this parasite with diarrhea in different settings and the presence of this parasite in the environment in different regions of this country, only 1 molecular assessment has been previously performed, in clinical samples collected in Fortaleza, Brazil.[47] We used previously described molecular tools [39,41,42] to analyze samples collected from children attending a day care at the Hospital das Clínicas, from March to May 2001, during which an outbreak of cryptosporidiosis took place. These tools allowed us to identify that the species associated with all 29 PCR-confirmed cases was *C. hominis*. In addition, a genetic marker that can distinguish among genotypes of *C. hominis*, the ML1[42] was also used. The usefulness of the ML1 as a marker to identify *C. hominis* strains with a dis-

Table 2 - Distribution of laboratory positive results for *Cryptosporidium* confirmed by PCR and collected from children with symptoms of intestinal disease and asymptomatic children by classes - diarrhea outbreak in daycare at the Clinical Hospital, Sao Paulo Brazil, March-May 2001

	Children with diarrhea		Children asymptomatic		Total		
	Children tested	Children positive	Children tested	Children positive	Children tested	Children positive	Attack rate (%)
N I	26	2	6	0	32	2	6.3
N II	28	2	9	1	37	3	8.1
MG I	19	9	6	5	25	14	56.0
MG II	29	4	5	0	34	4	11.8
T I	24	1	16	0	40	1	2.5
T II	28	3	9	1	37	4	10.8
T III	10	0	9	1	19	1	5.3
Total	164	21	60	8	224	29	12.9

Table 3 - Association between positive results for *Cryptosporidium hominis* and some risk factors

			Positive PCR No	Positive PCR Yes	Pearson's chi square test (*P*)	Risk Relative	Confidence Interval (CI) 95%
Diarrhea	**No**	**n**	28	08	0.965	ND	ND
		%	77.8	22.2			
	Yes	**n**	72	21			
		%	77.4	22.6			
Sex	**Female**	**n**	50	10	0.140	ND	ND
		%	83.3	16.7			
	Male	**n**	50	19			
		%	72.5	27.5			
Exposure tofiltered tap water	**No**	**n**	28	18	0.001	0.24	0.10 – 0.57
		%	60.9	39.1			
	Yes	**n**	72	11			
		%	86.7	13.3			
Exposure toearth filter	**No**	**n**	72	11	0.001	4.21	1.78 – 10.02
		%	86.7	13.3			
	Yes	**n**	28	18			
		%	60.9	39.1			
Nursingbottle	**No**	**n**	70	24	0.174	ND	ND
		%	74.5	25.5			
	Yes	**n**	30	5			
		%	85.7	14.3			
Diaper	**No**	**n**	42	6	0.037	2.78	1.04 – 7.41
		%	87.5	12.5			
	Yes	**n**	58	23			
		%	71.6	28.4			
Ingestionof raw vegetables	**No**	**n**	58	23	0.037	0.36	0.14 – 0.96
		%	71.6	28.4			
	Yes	**n**	42	6			
		%	87.5	12.5			
Recreationwith otherchildren	**No**	**n**	30	5	0.174	ND	ND
		%	85.7	14.3			
	Yes	**N**	70	24			
		%	74.5	25.5			

tinct biological or clinical profile has not been ascertained. Other authors who have studied the ML1 in *C. hominis* have not found a large number of genotypes in geographically distinct samples, indicating that this marker may not vary a lot in *C. hominis*.[14,42,48] Nevertheless, several consistent genotypes of ML1 have been identified in *C. parvum*, which is not as host-specific as *C. hominis*. Our study confirms these findings, since we have only identified one ML1 *C. hominis* genotype, the ML1 H1, in 45% of these samples.

The molecular analysis reinforced the hypothesis that the transmission of *C. hominis* during the period the samples were collected occurred in an outbreak pattern, possibly by person-to-person contact through the fecal-oral route. The hypothesis of a diarrhea outbreak in the day care center was confirmed by epidemiological analysis, which showed that *Cryptosporidium* infection concentrated mainly in class MG I, as well as in class MG II, attended by 14- to 27-month-old children who had started to walk, promoting contact with other children. We observed that the exposure to filtered tap water and the ingestion of raw vegetables were not risk factors for acquiring cryptosporidiosis in this group, a fact also observed in a previous study.[48] As far as we know, this is the first time that molecular tools have been used to identify the species and the genotype of isolates associated with cases of cryptosporidiosis in Brazil. It is also the first report showing the presence of the ML1 genotype in samples from Brazilian patients. The ML1 serves to strengthen the species identification obtained by other molecular markers in *C. hominis*. However, there are not enough data in the literature ascertaining its usefulness as a molecular epidemiologic tool when used separately. Its function as a marker to

identify *C. hominis* strains that have distinct biological or clinical profile has not been ascertained either. Our findings support the use of the ML1 as a confirmatory marker for molecular identification of *C. hominis* and support other findings that suggest that ML1 has low variability within this species.[14,42,48] Because this is a preliminary assessment, it is probably correct to state that this is not the only *C. hominis* genotype associated with cases of cryptosporidiosis in Brazil. This uncertainty indicates the need for more studies of this kind to be conducted in this country.

RESUMO

Gonçalves EM do N, da Silva AJ, Eduardo MB de P, Uemura IH, Moura INS, Castilho VLP et al. Genotipagem de multilocus de *Cryptosporidium hominis* associado a surto diarréico em creche de São Paulo. Clinics. 2006;61(2):119-26.

Mundialmente, diferentes espécies de *Cryptosporidium* estão relacionadas com doenças diarréicas. No Brasil há poucos dados sobre os genótipos das espécies de *Cryptosporidium* associadas a infecções.

OBJETIVO: No presente estudo, caracterizamos, por métodos moleculares, a espécie e o genótipo de *Cryptosporidium* sp diagnosticado em surto diarréico ocorrido na creche do Hospital das Clínicas, São Paulo, Brasil.

MATERIAL E MÉTODOS: Identificação específica e tipagem dos isolados associados ao surto foram feitos a partir do seqüenciamento de fragmentos de DNA amplificados por PCR dos seguintes loci: a região que codifica o SSUrRNA, o gene que codifica uma proteína do envoltório dos oocistos de *Cryptosporidium* (COWP), e o locus de microsatélite ML1, representado por seqüências repetitiva de três nucleotídeos GAG contendo substituições que diferem entre os genótipos de *Cryptosporidium parvum* e *Cryptosporidium hominis*.

RESULTADOS: Um total de 29 amostras positivas para *Cryptosporidium* associadas ao surto diarréico foi analisado com base nos métodos moleculares acima descritos. O estudo revelou a presença do genótipo ML1 de *Cryptosporidium hominis*.

DISCUSSÃO: A análise molecular reforçou a hipótese de que a transmissão de *Cryptosporidium hominis* durante o surto diarréico ocorreu de pessoa a pessoa através da rota fecal oral. Esta é a primeira vez que ferramentas moleculares são utilizadas para identificação de espécies e genótipos de isolados acusando a presença do genótipo ML1 em pacientes brasileiros.

UNITERMOS: Diarréia. *Cryptosporidium hominis*. Parasita. Intestinal. Brasil.

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