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MOLECULAR CHARACTERIZATION OF *Cryptosporidium* spp. FROM HIV INFECTED PATIENTS FROM AN URBAN AREA OF BRAZIL

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SUMMARY

Cryptosporidium spp. are important cause of enteric disease in humans, but may also infect animals. This study describes the relative frequency of several *Cryptosporidium* species found in human specimens from HIV infected patients in the São Paulo municipality obtained from January to July 2007. Sequence analysis of the products of nested-PCR based on small subunit rRNA and *Cryptosporidium* oocyst wall protein coding genes revealed 17 (63.0%) isolates of *C. hominis*, four (14.8%) *C. parvum*, five (18.5%) *C. felis* and one (3.7%) *C. canis*. These findings suggest that, in urban environments of Brazil, the cat adapted *C. felis* may play a potential role in the zoonotic transmission of cryptosporidiosis whereas the anthroponotic transmission of cryptosporidiosis caused by *C. hominis* seems to predominate.

KEYWORDS: *Cryptosporidium* spp.; Molecular characterization; Human; HIV; Zoonosis; Brazil.

INTRODUCTION

Cryptosporidium spp. are protozoan parasites of a wide range of vertebrates, including mammals, birds, reptiles, amphibians and fish[14]. Currently, there are at least 20 recognized species and 40 genotypes of *Cryptosporidium*[13]. Among them, five species, *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium meleagridis*, *Cryptosporidium felis* and *Cryptosporidium canis* may infect immunocompetent and immunocompromised humans[11,14]. The use of small subunit rRNA-based genotyping tools revealed the presence of *C. canis*, *C. felis* and *C. meleagridis* in AIDS patients of several parts of the world in addition to the frequently found *C. hominis* and *C. parvum*[15]. To compare the differences in genetic diversity among *Cryptosporidium* species, DNA sequences of other markers such as, the *Cryptosporidium* oocyst wall protein (COWP) gene[7,8,9], and the 70 kDa heat shock protein (HSP70) gene[10] have also been used. In Brazil, few studies on the relative frequency of *Cryptosporidium* genotypes found in humans have been published, with *C. parvum* and *C. hominis* being incriminated as the most prevalent genotypes[1,2,3,4,5]. In this paper, we aimed to reveal and discuss the relative frequencies of *Cryptosporidium* in human patients from an infectious diseases hospital in the municipality of São Paulo, São Paulo State, Brazil.

MATERIAL AND METHODS

Oocysts of *Cryptosporidium* spp. were obtained from diarrheic feces of naturally infected human patients with HIV, irrespective of their age, sex or any other risk factor which could be associated with the infection. We analyzed all the *Cryptosporidium* isolates recovered from January to July 2007 from human patients from a hospital specialized in infectious diseases in the municipality of São Paulo, São Paulo State, Brazil. Twenty seven stool samples were examined from January to July 2007 for oocysts of *Cryptosporidium* spp. by a conventional sucrose flotation method. Floated material was transferred to a slide and examined by light microscopy. When 4-5 µm sized oocysts were observed, the slide was washed and the DNA was extracted from material as previously described[6].

To amplify fragments of the small subunit rRNA and *Cryptosporidium* oocyst wall protein (COWP) coding genes, two nested-PCR protocols were used[8,12]. The nested-PCR products were sequenced using the secondary PCR primers and the Big Dye chemistry (Applied Biosystems, Foster City, California). Sequencing products were analyzed on a ABI377 automated sequencer. Both strands of each nested-PCR products were sequenced at least twice to increase the confidence of sequencing. The sequences were assembled and the contig formed with the phred-base calling and the phrap-assembly tool available in the suite Codoncode aligner v.1.5.2. (Codoncode Corp. Dedham, MA, USA).

Nucleotide sequences were deposited in Genbank under the accession numbers FJ232996 to FJ233040.

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RESULTS AND DISCUSSION

From the 27 samples, 18 were positive by the nested-PCR based on 18S rRNA primers and by the nested PCR based on COWP primers. Nine samples were positive by only one PCR, being six positive by nested-PCR based on 18S rRNA and three positive by the nested PCR based on COWP (Table 1).

Table 1
Molecular identification of isolates of *Cryptosporidium* spp. from humans of the São Paulo municipality, São Paulo State

Isolate	rRNA % identity[1]	COWP % identity[2]	Identification
01	100	**neg**	*C. felis*
02	100	**ND**	*C. parvum*
04	100	100	*C. hominis*
13	100	100	*C. hominis*
14	100	100	*C. hominis*
15	100	100	*C. hominis*
16	100	100	*C. hominis*
17	**ND**	100	*C. hominis*
18	100	100	*C. hominis*
24	**ND**	100	*C. hominis*
25	**ND**	100	*C. parvum*
27	100	**neg**	*C. felis*
28	100	**neg**	*C. felis*
29	100	100	*C. hominis*
30	100	**neg**	*C. felis*
32	100	100	*C. hominis*
33	100	100	*C. hominis*
35	99.9	99.8	*C. canis*
39	100	**neg**	*C. felis*
51	100	100	*C. parvum*
83	100	100	*C. parvum*
03B	99.7	100	*C. hominis*
09B	99.7	100	*C. hominis*
01B	100	100	*C. hominis*
04B	100	100	*C. hominis*
07B	100	100	*C. hominis*
03	100	100	*C. hominis*

[1] % identity of fragments of 18S rRNA coding sequences of each isolate to homologous sequences of reference isolates. [2] % identity of fragments of COWP coding sequences of each isolate to homologous sequences of reference isolates. Reference sequences of 18S rRNA: AF093489, AF093490, AY120909, DQ836340 for *C. hominis*, *C. parvum, C. canis* and *C. felis*, respectively. Reference sequences of COWP coding sequences: AF266265, AF266273 and AF266274 for *C. hominis*, *C. parvum* and *C. canis*, respectively. **ND:** Not done. **Neg:** negative by PCR

After analysis of the chromatograms, ~700 and 450 bp sequences were obtained from products of the 18S rRNA and COWP genes of each sample. The resulted sequences were aligned and compared with references sequences obtained from GenBank. From the results, 17 (63.0%) isolates were identified as *C. hominis*, four (14.8%) as *C. parvum*, five (18.5%) as *C. felis*, and one (3.7%) as *C. canis*. None of the sequences were discrepant from the well known species available in the GenBank and novel genotypes were not found (Table 1).

The isolates 1, 27, 28, 30 and 39 identified as *C. felis* were negative when tested by the COWP-based nested-PCR. DNA of the isolates 2, 17, 24, 25 was amplified by only one PCR assay because a small amount of material was available. Thus, the genotyping of these samples was accomplished by using only one molecular marker. The failure in amplifying fragments of COWP coding genes from *C. felis* might be caused by nucleotide diversity in the primer regions at this locus which prevented the anchorage of the primers for PCR amplification. In fact, *C. felis* is the most divergent species of *Cryptosporidium* when compared to *C. canis*, *C. parvum* and *C. hominis*.

Although the present study was not based on a statistically defined sampling, it showed that the relative frequencies of the genotypes of *Cryptosporidium* spp. in human patients of São Paulo city are somewhat similar to those found in other populations in developing countries. Developing and industrialized countries differ markedly in the proportion of infections caused by zoonotic species with the latter having more cases related to zoonotic transmissions[15]. In the present survey, a lower proportion of the isolates (37.0%) consisted of species potentially involved in zoonotic transmission.

However, it is noteworthy stressing that the source of infection is not possible to be inferred solely based on the description of the genotypic identification of the parasite. In fact, anthroponotic transmission of seemingly zoonotic *Cryptosporidium* species was already described in many parts of the world.

Two surveys for *Cryptosporidium* spp. infection in human population of South America reveal that *C. hominis* infections predominate over those caused by *C. parvum*, the two most common species found in humans[15]. In Peru, among 85 samples, 67 were identified as *C. hominis*, eight as *C. parvum*, one as *C. felis* and two as *C. canis*. In Venezuela, among 10 isolates, eight were revealed *C. hominis*, one *C. parvum* and one *C. canis*.

In Brazil, recent studies revealed that *C. hominis* predominate over *C. parvum*. BUSHEN *et al.* reported that among 42 isolates from children, 24 were *C. hominis* (57.1%) and 18 were *C. parvum* (42.9%)[2]. Multilocus genotyping of *Cryptosporidium* spp. associated with diarrhea outbreak in a day care unit by DNA sequencing analysis of fragments coding for 18S, COWP and a microssatelite locus (ML1) revealed the presence of *C. hominis* in all the 29 samples[4]. *Cryptosporidium* isolates identified in fourteen stool samples from five HIV patients and nine immunocompetent children revealed two *C. parvum*, eight *C. hominis* and two *C. meleagridis*[1]. In a larger survey[5] involving 83 patients attended at a reference hospital in the São Paulo municipality, the majority of samples (88.5%) were *C. hominis* positive whereas zoonotic genotypes other than *C. parvum* were not identified. In this study, it is noteworthy mentioning that more than 60% of the isolates detected by microscopy remained unidentified.

The major difference of our results in comparison to those of other surveys in Brazil is the higher frequency of non-*parvum* and non-*hominis* (22.2%) *Cryptosporidium* spp. It is noteworthy mentioning that the occurrence of uncommon genotypes in humans may be due to patients who were HIV positive, hence more susceptible to infection than immunocompetent patients. In addition, the sampling methods used in each study may explain such a high difference in the relative frequencies of genotypes.

Among the species of *Cryptosporidium* spp. less frequently found in humans, three can be highlighted, *C. meleagridis*, *C. felis* and *C. canis*, the last one being the rarest[15]. In the present study, *C. meleagridis* was not found, *C. canis* was detected only once whereas *C. felis* was detected in a frequency very similar to *C. parvum*. These findings suggest that, in urban environments of Brazil, the cat adapted *C. felis* may play a potential role in the zoonotic transmission of cryptosporidiosis.

RESUMO

Caracterização molecular de *Cryptosporidium* spp. de pacientes de área urbana do Brasil infectados por HIV

Cryptosporidium spp. são importantes causas de doenças entéricas em humanos, mas podem também ser encontrados em animais. O presente estudo descreve a frequência relativa de diversas espécies de *Cryptosporidium* em amostras de humanos da cidade de São Paulo, Brasil, obtidas de janeiro a julho de 2007. Análises de sequências de produtos de *nested* PCR direcionadas ao genes codificadores da menor unidade ribosomal e da proteina de parede de oocistos revelaram 17 (63,0%) isolados de *C. hominis*, quatro (14,8%) *C. parvum*, cinco (18,5%) *C. felis*, e um (3,7%) *C. canis*. Estes resultados sugerem que, em ambientes urbanos no Brasil, o genótipo adaptado ao gato pode desempenhar potencial papel na transmissão zoonótica de criptosporidiose, enquanto a transmissão antroponótica da criptosporidiose causada pelo *C. hominis* parece predominar.

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ETHICAL APPROVAL

This survey was approved by the ethical committee for scientific research of the Instituto Emílio Ribas, São Paulo, SP, Brazil.

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