

## Prevalence and genetic characterization of *Dientamoeba fragilis* in asymptomatic children attending daycare centers

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### ABSTRACT

In order to provide additional data on the prevalence and genetic diversity of *Dientamoeba fragilis* in human populations, we conducted a study in children from low-income communities in Sao Paulo State, Brazil. Fecal samples from daycare center attendees up to 6 years old (n=156) and staff members (n=18) were submitted to PCR and sequencing of *D. fragilis* as well as to microscopic examination for the presence of other intestinal parasites. All children assessed were asymptomatic and 10.3% (16/156) were positive for *D. fragilis*. No worker was found to be positive. An association between *Dientamoeba* and coinfection with other intestinal parasites was observed. Concerning the genetic diversity, 14 and only two isolates were genotype 1 and genotype 2, respectively. Our findings outline interesting aspects: (1) asymptomatic children as carriers of *Dientamoeba* in communities in which environmental conditions ensure parasite transmission and, (2) association between *Dientamoeba* infection in young children and coinfection with other enteric parasites, reinforcing its transmission via the fecal–oral route.

**KEYWORDS:** *Dientamoeba fragilis*. Prevalence. Children. PCR. Genotypes.

### INTRODUCTION

*Dientamoeba fragilis*, a trichomonad protozoan, is a common parasite of the human gastrointestinal tract, yet our knowledge on its biology, pathogenicity and epidemiology is largely unclear<sup>1-4</sup>. Transmission likely occurs via the fecal-oral route, although the recently described cystic form of the protozoan is rarely identified in human stool samples<sup>3</sup>. Another suggested route of transmission is via eggs of the pinworm *Enterobius vermicularis*, which may act as a carrier<sup>5</sup>.

*D. fragilis* infection ranges from the asymptomatic carrier status to gastrointestinal symptoms including abdominal pain, intermittent or persistent diarrhea, poor weight gain and a possible eosinophilic inflammatory response triggered off in the intestinal mucosa<sup>1,4</sup>. Recently, the infection has been implicated in the progression and exacerbation of chronic gastrointestinal disorders, such as the irritable bowel syndrome<sup>6,7</sup>. On the other hand, this parasite is frequently detected in healthy subjects, making it difficult to establish a correlation between infection and clinical symptoms<sup>8</sup>.

Diagnosis of *D. fragilis* traditionally relies upon the microscopic detection of trophozoites in permanently stained smears. Although it requires the proper processing of the samples and trained personnel to recognize a parasite that lacks specific morphologic features, microscopy is still useful for the parasitological

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diagnosis, particularly in developing countries<sup>1,9</sup>. Molecular assays have been developed as a diagnostic alternative to microscopy<sup>1-4</sup>. In addition to provide higher sensitivity and specificity, molecular methods are useful to assess the genetic diversity of *D. fragilis*. Based on differences in a few genetic markers, in particular within the small subunit ribosomal (SSU rDNA), two genotypes were described, genotypes 1 and 2, with a striking predominance of the former<sup>3</sup>.

Globally, the prevalence of *D. fragilis* infection varies from as low as 0.2% to as high as 80%, depending upon the population studied, the region and the diagnostic procedures employed<sup>2,3</sup>. Most studies on *Dientamoeba* prevalence come from high-income countries, however prevalence data have also been reported in developing regions where adequate sanitation is not available<sup>2,4</sup>. Recently, surveys based on microscopy and/or molecular assays have been carried out in low-resource communities in Asia<sup>10</sup>, Middle East<sup>11</sup> and Africa<sup>12</sup>. In Latin America, information is still limited<sup>13-16</sup>, including in Brazil, where there have been only four studies on this subject<sup>17-20</sup>. Among these studies, two focused on HIV patients<sup>17,18</sup>, one on dwellers of fisher villages<sup>19</sup> and the other on subjects referred to a clinical laboratory<sup>20</sup>.

Probably, the prevalence rates of dientamoebiasis are higher than those reported worldwide, mainly where clinicians and laboratories have neglected its occurrence. Given the need to gather more information on *D. fragilis*, particularly in low-resource communities of developing areas, the present study has focused on the prevalence and genetic diversity of *D. fragilis* in children attending daycare centers.

## MATERIALS AND METHODS

This survey was conducted with children attending two daycare centers that serve low-resource communities in Botucatu, Sao Paulo State, Southeastern Brazil (22°53'09''S 48°26'42''O). Three stool samples per child were collected on alternate days in a week period. Staff members were also asked to provide samples. Fecal specimens of each individual were pooled and concentrated (800 x g for 3 min with PBS). An aliquot of fecal sediment was examined for the presence of helminthes and protozoa (except *D. fragilis*), using centrifugation-sedimentation and zinc sulfate flotation techniques. The remaining sediment was stored at -20 °C for DNA extraction. This study was approved by the Research Ethics Committee of Botucatu's Medical School, UNESP (CAAE 56883616.8.0000.5411). During meetings to explain the study, a written informed assent was obtained from all children prior to sample collection as well as a consent form from their parents/guardians who

were interviewed with a structured questionnaire covering some epidemiological and clinical information.

For molecular analyses, DNA was extracted from fecal sediments using the QIAamp® Fast DNA kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. DNA specimens were assessed for the presence of *D. fragilis* by using a standard protocol for the amplification of an approximately 300 base pair (bp) fragment from the 18S rRNA gene, as previously described<sup>21</sup>. Positive (DNA from *D. fragilis* positive samples previously sequenced) and negative controls (ultrapure water) were included in all reactions. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced on both strands by a sequencing service (Macrogen, Inc., Seoul, Korea). Nucleotide sequences were aligned with each other and with reference sequences downloaded from GenBank using the Clustal X<sup>22</sup> and *D. fragilis* genotypes were identified by the BLAST software (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, Maryland, USA). Phylogenetic analyses were conducted using the MEGA X software<sup>23</sup>. Representative sequences were deposited in GenBank under the accession N° MN183755-MN183767.

The chi-square test was applied for assessing associations between *Dientamoeba* and the variables. All analyses were performed using the SPSS statistical software, version 21.0 (SPSS Inc., Chicago, IL, USA), and the level of significance was set at  $p < 0.05$ .

## RESULTS

A total of 156 children, 72 (46.2%) boys and 84 (54.8%) girls were enrolled in this survey with ages ranged from one to 72 months (0 to six years). The mean ( $\pm$ SD) age of children was 33( $\pm$ 18) months and with one-to 24-month old children accounting for 53% of the total. One hundred and twenty children (76.9%) lived in urban areas and the remaining 36 participants (23.1%) in rural communities. All children were considered asymptomatic, since no episodes of diarrhea or other gastrointestinal symptoms were reported from two months prior to stool collection until the end of the survey. As most children spent all day at the daycare centers, staff members could confirm the absence of diarrhea among these children.

By PCR, *Dientamoeba fragilis* was detected in 10.3% (16/156) of children. Other intestinal parasites could be found by microscopy, and their frequencies were: *Blastocystis* spp. 14.1% (22/156), *Endolimax nana* 13.5% (21/156), *Entamoeba coli* 12.2% (19/156), *Giardia duodenalis* 10.9% (17/156), and *Trichuris trichiura* and

*Enterobius vermicularis* with 0.64% (1/156) each. The overall rate of intestinal parasites, as detected by PCR and microscopic examination, was 42.9% (67/156). Single and mixed infections were detected in 44 (65.7%) and 23 (34.3%) of children, respectively (Figure 1). Out of 18 staff members that provided fecal specimens, *Blastocystis* spp. (2/18), *E. coli* (2/18) and *E. nana* (1/18) were found, while *D. fragilis* was not detected in this group.

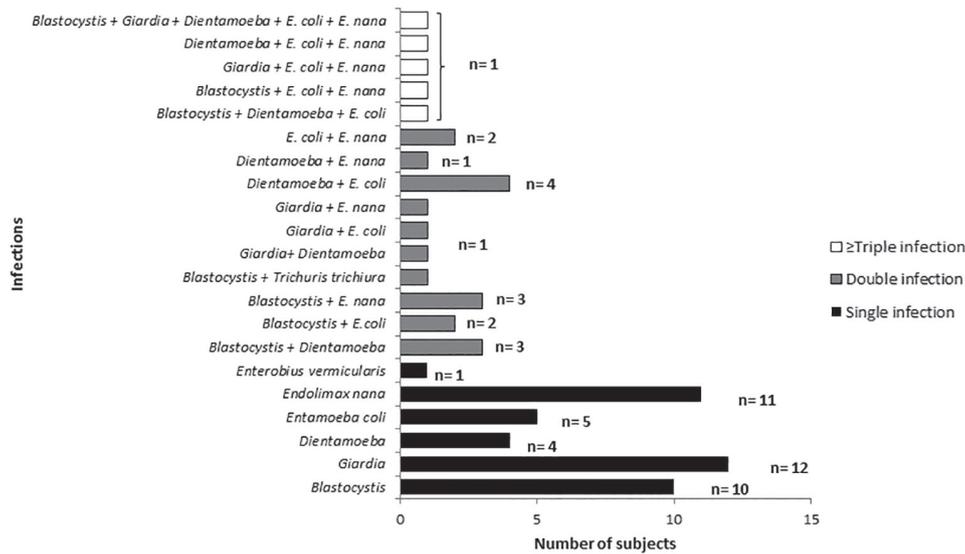
*D. fragilis* was observed among children in all age groups, and the prevalence was higher in children aged 48 to 72 months (Table 1). No significant association was found according to gender, age and household location. On the other hand, the association between *D. fragilis* infection and coinfection with other intestinal parasites was observed

in 52.2% (95% CI, 0.12-0.35, p<0.0001) (Table 1). Overall, 75% (12/16) of children positive for *D. fragilis* also tested positive for other intestinal parasites (Table 1, Figure 1).

The sequencing analysis of 16 isolates revealed genotype 1 in 87.5% of the samples (14/16) and genotype 2 in 12.5% (2/16) (Figure 2). The phylogenetic analysis showed a clear distribution of these isolates into two distinct clusters, and most of them were 100% identical to publicly available DNA sequences.

**DISCUSSION**

In low-income regions, surveillance of *D. fragilis* is probably a low priority, so that sensitive techniques

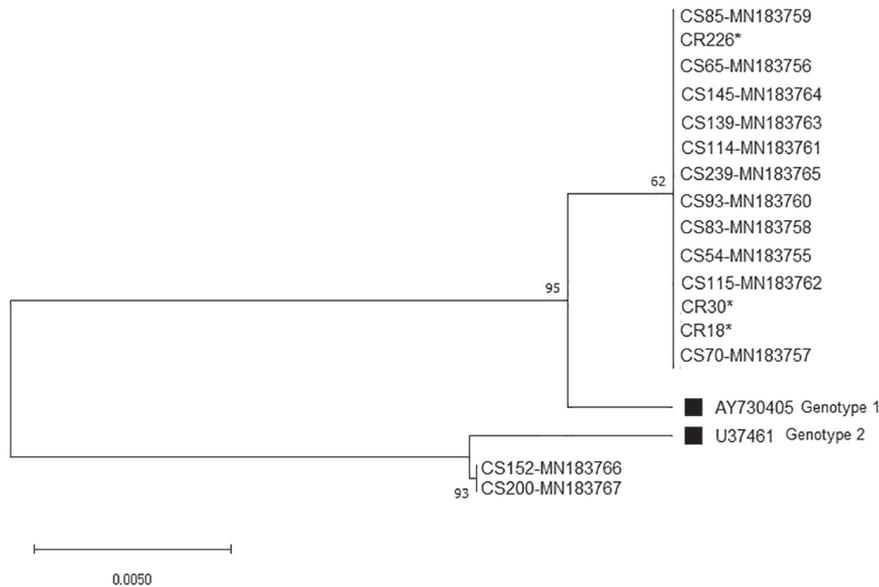


**Figure 1** - Distribution of *Dientamoeba fragilis* and other parasitic infections in children attending daycare centers.

**Table 1** - Prevalence of *Dientamoeba fragilis* infection among children attending daycare centers, by gender, age, the household location and the presence of coinfection.

Variable	Children attending daycare			95% CI	p value*
	N°. infected	N°. examined	Prevalence (%)		
<b>Gender</b>					
Female	9	84	10.7	(0.05-0.15)	0.839
Male	7	72	9.7		
<b>Age (months)</b>					
0-23	3	53	5.66	(34.6 – 35.1)	0.245
24-35	3	35	8.57		
36-47	2	23	8.69		
48-72	8	45	17.77		
<b>Household</b>					
Urban	12	120	10.0	(0.17-3.54)	0.847
Rural	4	36	11.0		
<b>Coinfection</b>					
Yes	12	23	52.2	(0.12 – 0.35)	<0.0001
No	4	44	9.1		

\*X<sup>2</sup> test



**Figure 2** - Neighbor-Joining (NJ) reconstruction tree of *Dientamoeba fragilis* based on the nucleotide sequences of the 18S rRNA gene retrieved from this study, in comparison with reference sequences of known genotypes from GenBank (black squares). The numbers on the branches are percent bootstrapping values from 10,000 replicates. Each sequence retrieved in the study is identified by its accession number and/or the code number of the sample. \*Sequences CR226, CR30 and CR18 were not submitted to the GenBank.

in routine diagnostic laboratories are scarcely used, contributing to a largely incomplete epidemiological picture. Few studies on *D. fragilis* prevalence have been published in Latin America, and to date, investigations were performed in Argentina<sup>13</sup> Mexico<sup>14</sup>, Venezuela<sup>15</sup>, Cuba<sup>16</sup> and Brazil<sup>17-20</sup>. Infection rates of <2% to 40% were found, which may be explained by differences in the surveyed populations, geographic locations and diagnostic methods<sup>2-4</sup>. In the current study, *D. fragilis* was detected in 10.3% of daycare children living in low socioeconomic communities. Notably, in another molecular based-survey, a closer prevalence rate (15%) was observed in dwellers of fisher villages<sup>19</sup> located in distinct areas within the same municipality of this survey. More recently, *D. fragilis* was detected by PCR in 2.29% of stool samples submitted to a reference laboratory in a neighboring municipality<sup>20</sup>.

Our results revealed the occurrence of *Dientamoeba* in daycare attendees up to six years old with higher frequencies among children aged four to six years (48 to 72 months), even though a significant difference was not found. These findings are consistent with a recent survey in Denmark, which illustrated that *Dientamoeba* infection is commonly acquired at an early age, emphasizing that among children aged zero to six years, older age children are at higher risk for testing positive<sup>24</sup>. Here, among staff members, none was positive for *D. fragilis*. Until today, no consensus exists regarding the age group distribution of *Dientamoeba* infection, and conflicting reports have led to different trends<sup>4</sup>. Some reports suggest that children

are frequent carriers of *Dientamoeba*, while other investigations imply that this parasite is more common in adults<sup>4</sup>. Interestingly, other reports have suggested a link of transmission between children and their adults caregivers<sup>4</sup>. Nonetheless, it is opportune to stress that as *Dientamoeba* is probably transmitted via the fecal-oral route, factors such as the sanitation level in the communities and poor hygiene practices of younger children can make them more predisposed to higher rates of infection<sup>4</sup>.

In the present survey, children who tested positive for *D. fragilis* were asymptomatic. Currently, the evidence that this parasite can cause diarrhea and/or other gastrointestinal symptoms is still inconclusive, mainly in young children<sup>8</sup>. Although some studies reported the association between *Dientamoeba* and diarrhea, in others, infected individuals remained asymptomatic<sup>8</sup>. Previous molecular-based studies found higher prevalence rates in asymptomatic children attending daycare centers and primary/secondary schools and there was no correlation suggesting that gastrointestinal symptoms are a common outcome of this infection<sup>24-26</sup>. These investigations were conducted in developed countries, and reports from poor-resource countries are still scarce. However, it is likely that *Dientamoeba* is not rare in regions in which inadequate sanitation and poor hygiene practices predispose populations to infections caused by enteric parasites.

Here, coinfections were detected in 34.3% (23/67) of children who tested positive for any intestinal parasite. Particularly in children positive for *D. fragilis*, 75% (12/16)

of them were positive for other intestinal parasites and a significant association between *Dientamoeba* infection and coinfections was observed. *D. fragilis*-positive individuals have been often coinfecting by other enteric protozoa, especially *Blastocystis*<sup>12,27,28</sup>. The role of coinfections is unclear but their occurrence reinforces the idea that *Dientamoeba* is transmitted via the fecal-oral route<sup>4</sup>.

The isolates recovered from children were assigned to genotypes 1 and 2, with predominance of the former. Yet, studies have only reported genotypes 1 and 2 associated with human infections from symptomatic and asymptomatic people of different age groups and in different geographical areas, with a wide predominance of genotype 1<sup>12,19,20,29,30</sup>. Recently, Cacciò *et al.*<sup>30</sup>, employing a panel of markers, have genotyped 111 isolates of human origin from Italy, Denmark, Brazil and Australia, from symptomatic and asymptomatic people of different age groups and samples collected at different time points. These authors showed that excepting for one isolate, the remaining belonged to genotype 1. The reasons why genotype 1 has a striking predominance are unclear, but the genetic diversity of *D. fragilis* has been suggested as a factor influencing differences in the clinical outcomes of infections<sup>1,2,4</sup>. Infections with genotype 1 range from asymptomatic to symptomatic ones while there are very little data on genotype 2<sup>4</sup>. Thus, no correlation has been made between genotypes and the presence or absence of disease.

To date, many issues concerning *D. fragilis* are still unclear, including their relevance to public health. In the present study, although the surveyed population and the number of isolates genotyped were relatively small, data assembled herein provide pertinent insights on occurrence and genetic diversity of *Dientamoeba* infection in asymptomatic young children attending daycares and living in communities where the low pattern of hygiene practices increases the risk of infections with enteric parasites. In addition, our findings reinforce the fact that the presence of *Dientamoeba* is not always associated with symptoms and the infection is not as rare as it has been reported in healthy subjects.

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