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Molecular detection and genetic diversity of *Dientamoeba fragilis* and *Enterocytozoon bieneusi* in fecal samples submitted for routine parasitological examination

Thainá Valente Bertozzo[1,2], Érica Boarato David[3], Ana Paula Oliveira−Arbex[4], Semíramis Guimarães[1]✉

[1]Department of Parasitology, Institute of Bioscience, São Paulo State University (UNESP), Botucatu, SP, Brazil

[2]Tropical Diseases Postgraduate Program, Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil

[3]Sagrado Coração University (USC), Department of Health Sciences, Bauru, SP, Brazil

[4]Integrated Faculties of Bauru (FIB), Bauru, SP, Brazil

ABSTRACT

Objective: To detect *Dientamoeba* (*D.*) *fragilis* and *Enterocytozoon* (*E.*) *bieneusi* and to assess their genetic characteristics in stool samples submitted for routine examination in a clinical laboratory in Southeastern Brazil.

Methods: In this survey, 348 stool samples from female and male individuals with age ranging from 0 to ≥55 years were analyzed by PCR amplifying and sequencing based on the small subunit ribosomal RNA (SSU rRNA) gene of *D. fragilis* and the internal transcribed spacer of *E. bieneusi*.

Results: *D. fragilis* and *E. bieneusi* isolates were observed in 2.29% (8/348) and 4.59% (16/348) of the samples, respectively. These parasites were detected in stool samples from individuals of both genders, including young children under nine until adults over 55 years old. No statistically significant differences were found. All *D. fragilis* isolates were classified as genotype 1 and *E. bieneusi* isolates included genotypes D (*n*=15) and A (*n*=1).

Conclusions: The findings provide relevant findings on occurrence and genetic diversity of *D. fragilis* and *E. bieneusi*, pointing to the need for the diagnosis of these parasites in routine examinations in clinical laboratories. In addition to sensitive diagnostic methods, it is mandatory that these parasites be considered relevant for physicians and laboratory staff.

KEYWORDS: *Dientamoeba fragilis*; *Enterocytozoon bieneusi*; Clinical laboratory; PCR; Genotyping

1. Introduction

Intestinal parasitic infections are common causes of gastrointestinal disorders worldwide, however, the burden of disease is particularly high in developing countries where low-income populations are exposed to inadequate freshwater resources and poor sanitation and hygiene[1]. Along with diarrheal illness in humans, these infections represent serious implications for immunocompromised populations[1]. The importance of considering the diagnosis of intestinal parasites is a consensus, but unfortunately, the routine detection of some species is often ignored, mainly in developing settings. This situation includes the protozoan *Dientameba* (*D.*) *fragilis* and the microsporidian *Enterocytozoon* (*E.*) *bieneusi* since tests are currently not available in routine of diagnostic laboratories. *D. fragilis* has been commonly reported throughout the world, however the clinical significance of the infection is still debated[2]. Clinical presentations vary from asymptomatic carriage to gastrointestinal symptoms mainly of diarrhea[3]. Recently, investigations have implicated infection in progression and exacerbation of chronic gastrointestinal disorders, such as the irritable bowel syndrome[4].

✉To whom correspondence may be addressed. E-mail: guimaraes.viana@unesp.br

Microsporidia comprise a wide group of obligate intracellular eukaryotic parasites and *E. bieneusi* is the most common cause of human microsporidiosis[5]. *E. bieneusi* is the most common microsporidia specie in humans, responsible for opportunistic infections in AIDS patients and other immunodeficient individuals such as organ transplant recipients, patients with cancer, young children, the elderly, *etc*[6]. However, symptomatic and asymptomatic infections have been reported in immunocompetent individuals[5].

Traditional diagnosis of these parasites relies on methods involving permanent stained stool smears[1,3]. More recently, molecular assays are the most sensitive and specific tools available for diagnosis and when coupled with sequencing, it is allow to assess genetic diversity of isolates recovery from a wide range of hosts[3].

Based on differences in SSU rRNA sequences, there are two major *D. fragilis* genotypes, 1 and 2, with genotype 1 being the most common[7]. Regarding *E. bieneusi*, genotyping based on the internal transcribed spacer (ITS) has recognized ~500 genotypes phylogenetically arranged in 11 groups and a wide range of hosts related to humans[6].

Despite current advances, little effort is used toward routine laboratory diagnosis of *D. fragilis* and *E. bieneusi*, mainly in developing countries where these parasites are frequently dismissed by physicians. Thus, there is still the expectation for further insights into *D. fragilis* and *E. bieneusi* epidemiology where conditions ensure the parasite spreading. In this context, the present study describes a survey to evaluate the frequency and genetic diversity of both parasites in fecal samples submitted for routine parasitological examination in a clinical laboratory.

2. Materials and methods

2.1. Ethical statement

All procedures in the study were reviewed and approved by the Research Ethics Committees of the Botucatu Medical School, UNESP (protocol CAAE 90078218000005411). Leftover stool samples and available for disposal were used in this study. In this way, the clinical laboratory issued an authorization allowing the use of all samples left over after diagnostic procedures. Thus, all samples were encoded and used anonymously.

2.2. Study design and samples collection

Stool samples were obtained from the Clinical Laboratory of the Sacred Heart University in Bauru city, São Paulo, Brazil (22°18'54'' S, 49°03'39'' O), during a 3-month period between September and December 2018. Briefly, this laboratory performs microbiological/

parasitological, biochemical and haematological tests and it serves local and neighboring municipalities residents. In order to assemble representative data, the sample size and collection time frame were determined based on the number of parasitological routine examinations performing annually in the last three years.

Following routine processing, leftover stool samples without preservatives and available for disposal were used for this study. Prior to disposal of samples, technicians removed the identification label (patient's name and identification number, physician's name, *etc.*) of collection vials and a code number was assigned to each sample, thus, assuring the anonymity of patients. In addition, accessing to patients request form to obtain information on request details such as reason for referral and symptoms were not allowed. Only age and gender information could be provided. Soon after disposal, stool samples were transported in coolers to the research laboratory, where prior to molecular analysis, each stool sample was evaluated macroscopically in terms of consistency (formed, soft, or liquid) and presence of mucus (small amount of mucus, copious mucus or bloody mucus).

2.3. DNA isolation and molecular analysis

For DNA extraction, all samples were concentrated by centrifuge sedimentation technique with phosphate-buffered saline and the sediment was stored at -20 ℃ until use. To obtain high yield of DNA, fecal sediments were submitted to three cycles of freezing and thawing as follows: two cycles of five-minute incubations in liquid nitrogen and thawing in a water bath at 70 ℃, followed by one cycle of freezing in liquid nitrogen for five minutes and thawing at 95 ℃ for five minutes. Next, genomic DNA was extracted from 200 mg of fecal sample using the QIAamp® Fast DNA kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The resulting DNA was eluted in 200 μL of buffer, aliquoted and stored at -20 ℃ until PCR analysis. Each DNA specimen was analyzed for the presence of *D. fragilis* by using the primers Df-For (5'-TCAGGCTATAGGTCTTTCAGGA-3') and Df-Rev (5'-CATCTTCCTCCTGCTTAGACGC-3') for the amplification of a ~300 bp fragment of the *18S* rRNA gene[7,8]. Meanwhile for *E. bieneusi*, a set of nested primers was used to amplify the ITS region as well as a portion of the flanking large and small subunit ribosomal RNA genes (~390 bp) as previously described[9]. The outer primers were EBITS3 (5' GGTCATAGGGATGAAGAG 3') and EBITS4 (5' TTCGAGTTCTTTCGCGCTC 3'), while the inner primers were EBITS1 (5' GCTCTGAATATCTATGGCT 3') and EBITS2.4 (5' ATCGCCGACGGATCCAAGTG 3'). In all PCR reactions, positive (DNA recovery from *D. fragilis* and *E. bieneusi* positive samples and confirmed via amplicon sequencing) and negative controls (ultrapure water) were included.

2.4. Sequencing and phylogenetic analysis

All PCR products were purified using spin columns (QIAquick PCR purification kit, Qiagen, Hilden, Germany) and sequenced on both strands by a sequencing service (Biotechnology Institute, Sao Paulo State University), using the same set of primers as in PCR assays. Nucleotide sequences were aligned with each other and with reference sequences downloaded from GenBank, using Clustal X. *D. fragilis* and *E. bieneusi* genotypes were identified by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic reconstruction using Neighbour-Joining algorithm was performed using the software MEGA X version 10.1 (https://www.megasoftware.net). Bootstrap analysis was applied by using 1 000 replicates. The nucleotide sequences obtained in the present study were deposited in the GenBank database under accession numbers MN920432-MN920439 (*D. fragilis*) and MN922358-MN922369 (*E. bieneusi*).

2.5. Statistical analysis

Chi-square test or Fisher's exact test were applied to test associations between *D. fragilis* and *E. bieneusi* infections and some epidemiological variables. Statistical analyses were performed using SAS 9.3 and *P*<0.05 was considered as statistically significant.

3. Results

In total, 348 stool samples routinely processed in a clinical laboratory were included in the study. Based on consistency, all fecal samples were scored as formed stools, suggesting no gastrointestinal disorders. Among these samples, 57.18% (199/348) were from females and 42.82% (149/348) from males (Table 1). The median age of individuals who submitted fecal samples was 21.0 years (IQR 5.0-33.0), ranging from three months to 88 years. The subjects were grouped by age in the following categories: under nine years, 10 to 20 years, 21 to 55 years and up to over 55 years.

Table 1. Frequency of *Dientamoeba fragilis* and *Enterocytozoon bieneusi* according to gender and age of subjects referred to a clinical laboratory in Southeastern Brazil [*n* (%)].

Variables	*Dientamoeba fragilis*	*Enterocytozoon bieneusi*
Gender		
Female	4 (50.00)	9 (56.25)
Male	4 (50.00)	7 (43.75)
Age (years)		
<9	6 (75.00)	6 (37.50)
10-20	1 (12.50)	2 (12.50)
21-55	1 (12.50)	4 (25.00)
>55	-	4 (25.00)
Total	8	16
P[*]	0.368	0.060

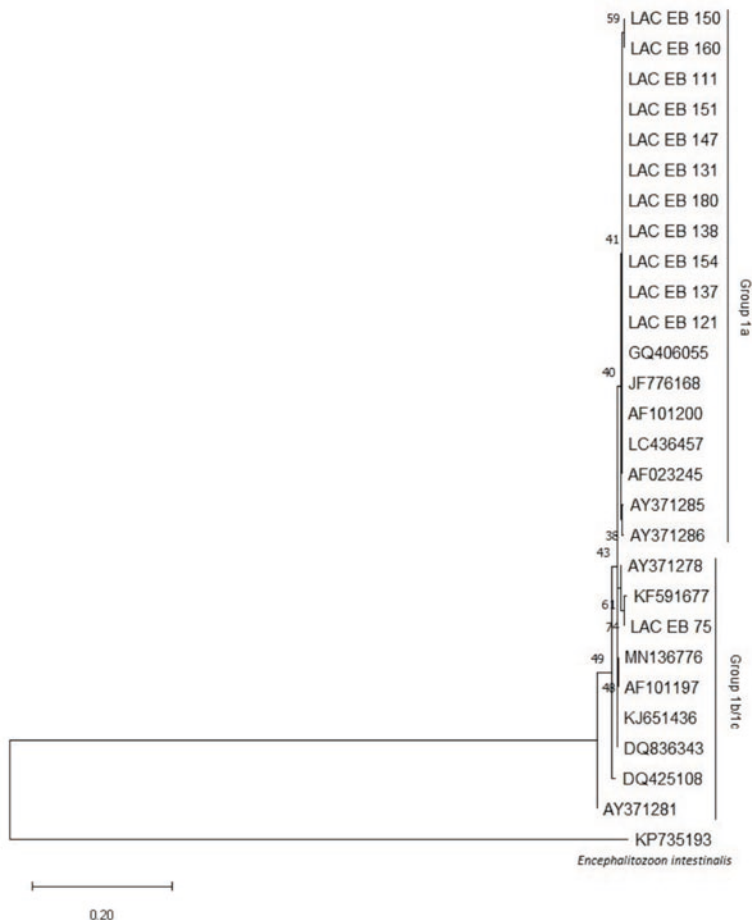[*]*Chi*-square (χ^2) test.

Figure 1. Neighbor-Joining reconstruction tree of *Enterocytozoon bieneusi* based on the nucleotide sequences of internal transcribed spacer region of the rRNA gene retrieved from this study compared with sequences of known assemblages from GenBank. Each reference sequence is identified by its accession number, source of origin, and genotype. The sequences identified in the present study were designated as LAC EB. The numbers along branches correspond to bootstrap values from 1 000 replicates.

Each fecal sample was screened for both *D. fragilis* and *E. bieneusi* by PCR reactions and a positive result was considered when the target locus was successfully amplified and sequenced. The PCR/ sequencing assays detected *D. fragilis* and *E. bieneusi* isolates in 2.29% (8/348) and 4.59% (16/348) of the samples, respectively. No coinfections of theses parasites were detected in any of the samples. By age, *E. bieneusi* positive cases included subjects distributed in all groups, including young children under nine years and older adults up 55 years (Table 1). The highest frequency was found in samples from children aged less than 10 years. Regarding to *D. fragilis*, at least one positive sample was detected among individuals in different age categories, except among those over 55 years. For both parasites, no statistically significant association was reported between the frequency and the variables analyzed.

Concerning *E. bieneusi* isolates, all 16 sequences were subtyped and two ITS distinct genotypes were identified: D and A. Genotype D was dominant and was detected in 15 isolates. Among these isolates, 60.00% (9/15) were identical to GenBank sequences and to each other. The remaining sequences showed similarity ranging between 99.73% and 99.46%. The only one isolate assigned as genotype A showed similarity of 99.19% to 98.65%. Novel genotypes were not identified in the present study. The phylogenetic relationship of 12 representative isolates from this study and some reference sequences from GenBank database were showed in Figure 1. The largest similarities were observed with representative sequences of genotypes clustered into group 1, subgroups 1a and 1b/1c.

Of the eight successfully sequenced *D. fragilis* isolates, all were identified as genotype 1 and showed 100% identity with previously published sequence (AY730405). In addition, these same isolates were identical to each other.

4. Discussion

D. fragilis and *E. bieneusi* are common pathogens responsible for diarrheal disease in humans, however, both have been frequently found in asymptomatic carriages. To the best of our knowledge, the present study is the first report of molecular detection and genetic characterization of *D. fragilis* and *E. bieneusi* isolates detected in fecal samples submitted to parasitological examination in a clinical laboratory in Latin America.

Here, the overall frequency rates of *D. fragilis* and *E. bieneusi* were 2.29% (8/348) and 4.59% (16/348), respectively. Both were consistent with those in previous reports, however, differences depending on the country, study cohort and the performance of diagnostic methods employed[2,4,10].

Based on different surveys, the prevalence of *D. fragilis* ranges from 0.2% to 82.0%, and in contrast to other intestinal protozoa, is generally higher in developed countries[10]. However, prevalence data have also been reported in developing regions where adequate sanitation is not available[2]. In South America, information is still limited, but some studies have reported rates >20%[10].

Regarding *E. bieneusi*, this parasite has been increasingly reported in HIV-seropositive persons in both developed and developing countries, however, with access to antiretroviral therapy, infection rates in HIV/AIDS patients have reduced significantly with prevalence ranging from 0% to 42%[1]. In recent years, studies involving other immunocompromised individuals such as cancer patients reported rates between 1.7% and 40.0%. In HIV-seronegative persons with and without diarrhea, the parasite was detected in 5.35% to 58.10% of the fecal samples examined[5].

Analysis of frequency of positive samples for *D. fragilis* and *E. bieneusi* according to age and gender showed no statistically significant differences. However, it is noteworthy that these parasites were detected in stool samples from individuals of both genders, including young children under nine until adults over 55 years old. With respect to age distribution and *Dientamoeba* infection, studies have reported different trends[2], however, findings should be interpreted with care, since the results may be confounded by study design[11]. Some investigations suggest it is more common in adults and teen than young children[2]. In Denmark, Stensvold and colleagues[11] found the highest incidence in patients aged 16 to 20 years. More recently in Turkey, in a report by Aykur *et al*.[12], *Dientamoeba* was found to be higher in the young between 10 and 19 years. In relation to *E. bieneusi*, immunodeficiency when associated to younger age is considered as a risk factor for intestinal microsporidiosis[5].

Regarding *D. fragilis* genotyping, our results showed that all the eight isolates recovered from fecal samples were successfully sequenced and assigned to only genotype 1. All nucleotide sequences showed high identity (100%) with each other and also when compared to GenBank sequences. Of the only two known *Dientamoeba* SSU rRNA genotypes (1 and 2), genotype 1 is the most common in different geographical areas and little variability has been found among its isolates[13]. Interestingly, in the first study to characterize human samples from South America, only genotype 1 was identified among dwellers of fisher villages in Brazil[8].

The analysis of the ITS sequences of *E. bieneusi* found genotype D as the most common genotype, detected in 15 samples. Genotype D (syn. CEbc, PigEBITS9, WL8, Peru9, and PtEb VI) is one of the most common genotypes in humans[6]. Among the 11 ITS phylogenetic groups, genotype D is within Group 1, which exhibits phenotypic difference in host specificity and zoonotic potential[6]. In recent years, this genotype has been reported in transplant recipients, cancer patients and in environmental samples such as various water types[5,6]. The genotype A detected in only one fecal sample belongs

to the phylogenetic Group 1 and it is a human adapted genotype, but not as frequent as genotype D.

In the present study, the absence of information on reasons for referral to the clinical laboratory, including the clinical and immunological status of subjects, implied a limitation, hampering attempts to verify a likely association between the infection by both parasites and these variables could not be considered. However, it is undeniable that the findings presented here provide relevant findings on occurrence and genetic diversity of *D. fragilis* and *E. bieneusi*. In addition, they reinforce that these parasites cannot be ruled out routine examination in clinical laboratories. Thus, in addition to sensitive techniques and well-trained laboratory staff, it is mandatory that these parasites be considered relevant for physicians. Currently, the possibility of infection must be raised for transplant recipients, cancer patients, chronic gastrointestinal disorders, such as irritable bowel syndrome, *etc*.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Authors' contributions

SG and EBD conceived and designed the study protocol. TVB collected the study samples. TVB performed all the laboratory tests, and APOA helped in the genotyping. TVB, EBD, APOA and SG analyzed data and interpreted results. SG supervised the laboratory work and data analysis. All authors participated in drafting the article. SG and TVB wrote the final form.

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