Detection of *Dientamoeba fragilis* in Portuguese children with acute gastroenteritis between 2011 and 2013

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SUMMARY

Dientamoeba fragilis is an inhabitant of human gastrointestinal tract with a worldwide distribution. The first description considered this protozoan a rare and harmless commensal, since then it has struggled to gain recognition as a pathogen. Commercial multiplex real-time PCR was used to detect *D. fragilis* in fecal samples from hospitalized children (⩽18 years) with acute gastrointestinal disease, admitted to two hospitals of Lisbon area, with different demographic characteristics. A total of 176 children were studied, 103 (58·5%) male, 144 (81·8%) children between 0 and 5 years and 32 (18·2%) above 6 years old. The overall protozoa frequency considering the four tested microorganisms were 8·5% (15/176), and the most frequently found protozoan was *D. fragilis*, 6·3% (11/176). *Dientamoeba fragilis* frequency was higher among older children (21·9%), than younger children (2·8%), and greater in boys (6·8%) than in girls (5·5%). All positive children presented with diarrhoea associated with vomiting, fever and abdominal pain. Infection was associated with the age of children ($P < 0{\cdot}001$), school attendance ($P = 0{\cdot}002$) and consumption of certain foods ($P = 0{\cdot}014$), e.g. cakes with crème and ham. The frequency of diantamoebiasis found in a cohort of hospitalized Portuguese children, with acute gastrointestinal disease, could be considered a very high value when compared with the protozoan frequency normally associated with this pathology.

Key words: *Dientamoeba fragilis*, frequency, acute gastroenteritis, hospitalized children, Portugal.

INTRODUCTION

Acute gastroenteritis (AGE) is a leading cause of morbidity and mortality, especially in paediatric age in developing countries (Ramani and Kang, 2009). In developed countries, it is estimated that AGE affect one-third of the population, children also being the most affected group (Verdu and Riddle, 2012). In Portugal, AGE is considered the second most common cause of paediatric hospital admissions (Lima and Dias, 2010), although information concerning its aetiology is scarce. *Entamoeba histolytica*, *Giardia duodenalis*, *Cryptosporidium parvum* and *Dientamoeba fragilis* are the four most commonly occurring diarrhoea causing parasitic protozoa (Stark *et al*. 2011); out of these, *G. duodenalis* is considered the most frequent (Schuurman *et al*. 2007). Initially, *D. fragilis* was considered a harmless commensal due to the absence of clinical symptoms in infected individuals. However, an increasing number of reports show that *D. fragilis* is a potential pathogenic human intestinal protozoan parasite (Stark *et al*. 2010, 2014), and if a patient with gastrointestinal disturbances harbour *D. fragilis* and no other pathogen, the parasite should be considered the aetiological agent and the patient treated for this infection (Barratt *et al*. 2011). Clinical symptoms associated with *D. fragilis* include: diarrhoea, abdominal pain, vomiting, fever, anorexia, nausea and flatulence, which usually disappear with the eradication of the parasite (Girginkardeşler *et al*. 2003).

The frequency of *Dientamoeba* varies by clinical groups, as well as the diagnostic techniques used. The frequency in children using light microscopy and culture varies between 0·2 and 82·9%, whilst frequency measured using PCR diagnosis in all types of fecal specimens, from both asymptomatic and symptomatic individual's ranges between 4 and 32% (Barratt *et al*. 2011). The aim of the present study was to estimate the frequency of *D. fragilis* in a cohort of hospitalized Portuguese children presenting with AGE, by multiplex real-time PCR.

MATERIAL AND METHODS

Patients

The study population, comprised of children under 18 years old, were admitted as inpatients to the

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paediatric hospital of Lisbon urban centre (hospital A) or to the paediatric service of a district general hospital located in the suburban Lisbon area (hospital B), between May 2011 and May 2013, with a diagnosis of AGE. Children who were taking antibiotics at the time of admission were excluded from the study. For each child, a stool specimen was collected in a sterile container and in a transport swab, with gel Cary-Blair (Oxoid, Hants, UK). The specimens were sent to the Infectious Diseases Department of the National Institute of Health, within 24 h of collection. A questionnaire with demographic, clinical and epidemiologic data followed the sample. Ethical approval for the study was obtained from the two ethical committees of the involved hospitals.

Parasite DNA amplification

DNA was extracted with the Specific B protocol, on the NucliSens easyMAG system using the NucliSens magnetic kit (bioMérieux, Marcy l' Étoile, France). Before DNA extraction, feces samples were pre-treated, by mixing the sample (500 μL) with Lysis buffer 1 (1·5 mL), followed by vortexing and centrifugation at 13 000 rpm, in order to recover at least 200 μL of supernatant fraction, to be used for DNA extraction.

A commercial multiplex real-time PCR assay targeting the 18S-ITS was performed for the qualitative detection and differentiation for *G. duodenalis*, *C. parvum* and *E. histolytica*, with fluorogenic target-specific hydrolysis probes and *D. fragilis* detection with an intercalating dye followed by melting curve analysis (RIDA®GENE Parasitic Stool Panel, R-Biopharm AG, Germany), according to the manufacturer's instructions. In each PCR reaction, the four DNA parasites were used as positive control and water as a negative control. Within each sample, an internal amplification DNA control was co-amplified for the detection of possible PCR-inhibitions after standardized adjustments of the analysis parameters (RIDA®GENE Color Compensation Kit, R-Biopharm AG) in the LightCycler®480II thermocycler (Roche Diagnostics, GmbH, Germany).

The PCR reactions were performed in a 20 μL reaction mixture consisting of 19·9 μL of the reaction mix, 0·1 μL Taq-polymerase (RIDA®GENE Parasitic Stool Panel, R-Biopharm AG) and 5 μL of template DNA. PCR Conditions consisted of an initial denaturation during 1 min at 95 °C, followed by 45 cycles of denaturation (95 °C for 15 s), annealing and extension (60 °C for 30 s). Fluorescence data were collected at the end of each cycle as a single acquisition. The melting curve program was performed at the end of each reaction and consisted of 60 °C for 5 s, and heating to 95 °C with continuous acquisition (0·14 acquisitions per degree Celsius).

Bacteria and virus detection

The transport swab was used for the detection of other potential aetiological agents, surveyed as follows: (a) multiplex real-time PCR for detection of the enteric virus Norovirus GI and GII, Astrovirus, Rotavirus, Adenovirus and Sapovirus, including an internal control (FTD Viral Gastroenteritis, Fast TrackDiagnostics, Luxembourg), according to the manufacturer's instructions; (b) culture on specific media for detection of *Campylobacter* spp., *Salmonella* spp., *Shigella, Yersinia* and *Escherichia coli*, and detection of *E. coli* pathogenicity factors by conventional multiplex PCR (Fujioka *et al.* 2013).

Statistical analyses

All statistics analyses were performed with SPSS version 22 (IBM®SPSS Statists, Chicago, USA). Results were analysed by the Fisher Exact test, and differences between two proportions were compared. A probability under 0·05 was considered significant. The variables were calculated with their adjusted odds ratios (OR), 95% confidence intervals (CI) and significance levels.

RESULTS

A total of 176 children with AGE were included in the study, 65·3% (115/176) were from the urban hospital, 103/176 children (58·5%) were male and the patients' mean age was 3·14 years (ranging from 10 months to 17 years). Most of the specimens (144/176; 81·8%) were obtained from children aged between 0 and 5 years and only 32 (18·2%) specimens from children aged above 6 years old.

The overall protozoa frequency, detected by PCR, of the four tested microorganisms was 8·5% (15/176), and the most frequently found was *D. fragilis* (Fig. 1), with a frequency of 6·3% (11/176). Two specimens were positive for *G. duodenalis* and another two for *C. parvum* (2/176, 1·1%, for each), while no positive specimens were found for *E. histolytica*. The four children with positive results for *G. duodenalis* and *C. parvum* were aged <4 years old and all presented diarrhoea among other symptoms.

Dientamoeba fragilis was significantly more frequent among older children (⩾6 years) than among younger children (21·9 *vs* 2·8%, $P < 0·001$; OR = 0·102; CI = 0·03–0·37). Boys had a higher percentage of infection (6·8%, 7/103) when compared with girls (5·5%, 4/73), with a 1/1·75 *ratio*, although not statistically significant (Table 1).

Most of the children (169/174; 97%) enrolled in this study presented with diarrhoea; vomiting (72·4%; 126/174) and fever (61·5%; 107/174) were also reported. Among children positive for *D. fragilis* those three symptoms were also the most frequent,

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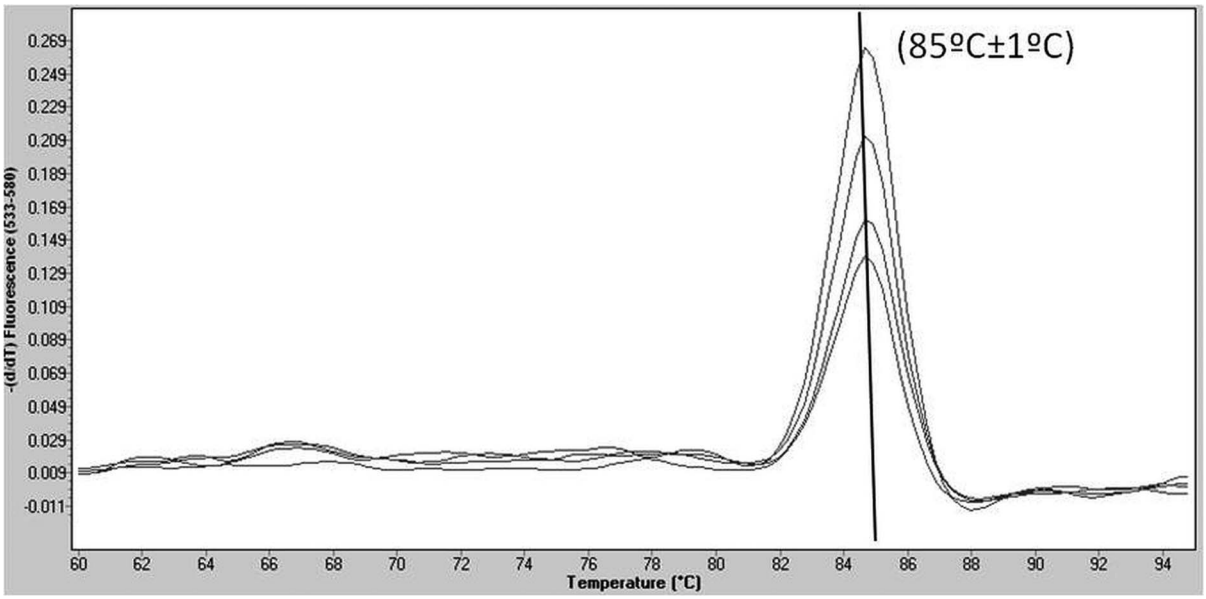

Fig. 1. Melting curve analyses of the PCR products obtained from the amplification of the *Dientamoeba fragilis* DNA with Lightcycler 480. The temperature (°C) is indicated on the *x*-axis, and the derivative of the fluorescence is indicated on the *y*-axis. The peaks indicate the melting points of the respective amplicons.

Table 1. Frequency of *Dientamoeba fragilis* infection according to demographic data and family characteristics

Variables (total *N*)	*N*	Positive results *n* (%)	*P*
Gender (176)			
Male	103	7 (6·8)	0·491
Female	73	4 (5·5)	
Age (176)			
0–5 years	144	4 (2·8)	<0·001
⩾6 years	32	7 (21·9)	
Hospital (176)			
Hospital A	115	8 (7·0)	0·750
Hospital B	61	3 (4·9)	
Date of collection (176)			
2011	66	8 (12·1)	0·039
2012	91	3 (3·3)	0·028
2013	19	0 (0·0)	0·014
Parents nationality (173)			
European	116	9 (7·8)	0·058
Other	57	1 (1·8)	
Attendance of day care/school (175)			
Yes	82	10 (12·2)	0·002
No	93	1 (1·1)	
Symptoms:			
Diarrhoea (174)			
Yes	169	11 (6·5)	0·719
No	5	0 (0·0)	
Fever (174)			
Yes	107	7 (6·5)	0·575
No	67	4 (6·0)	
Vomit (174)			
Yes	126	9 (7·1)	0·730
No	48	2 (4·2)	
Abdominal pain (174)			
Yes	49	6 (12·2)	0·053
No	125	5 (4·0)	
Consumption of contaminated food (169)			
Yes	9	3 (33·3)	0·014
No	160	8 (5·0)	

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Table 2. Results for bacterial and viral enteric agents among the positive children for *Dientamoeba fragilis*

Hospital	Gender	Age	Parents nationality	Bacteriology results	Virology results
A	F	15	Portuguese	Negative	Negative
A	M	9	Guyanese	Negative	Negative
A	M	4	Unknown	Negative	Negative
A	M	11	Portuguese	*Campylobacter jejuni*	Negative
A	M	10	Portuguese	*Campylobacter jejuni*	Negative
A	F	10	Portuguese	Negative	Rotavirus
B	F	12	Portuguese	Negative	Sapovirus
B	M	2	Portuguese	Negative	Adenovirus 41
A	F	1	Portuguese	*Escherichia coli* ETEC[a]	Norovirus II
A	M	2	Portuguese	Negative	Adenovirus and Sapovirus
B	M	13	Portuguese	Negative	Norovirus II and Parechovirus

[a] Enterotoxinogenic.

as well as abdominal pain (28%; 49/174), although none of the symptoms was significantly associated with this infection (Table 1).

Among the other variables analysed, *Dientamoeba* infection showed to be associated with school attendance ($P = 0{\cdot}002$; OR = 12·78; CI = 1·59–102·14) as well as with consumption of certain contaminated foods ($P = 0{\cdot}014$; OR = 9·5; CI = 2·00–45·10) such as cakes with crème and ham (Table 1).

A possible association of *D. fragilis* infection with ethnicity or recent travel abroad was also evaluated, but no correlation was found. Out of the 11 children who tested positive for *D. fragilis*, 10 were European descent and only one child who tested positive reported a recent travel history to Guinea and Senegal.

During 2011 and 2012, a seasonal trend for *D. fragilis* frequency was identified, with a higher frequency in the autumn (7/11; 64%) compared with the average for the rest of the year (4/11; 36%).

The fecal samples were also tested for bacterial and viral enteric agents (Table 2). Among the 11 positive samples for *D. fragilis*, three (27·3%) were negative for the other enteric agents. The remaining eight cases were positive for other enteric agents, with the following distribution: two specimens were positive for *Campylobacter jejuni*, one was positive for rotavirus, one for sapovirus, one for adenovirus 41, one specimen had a co-infection with *E. coli* enterotoxinogenic and norovirus GII, another had adenovirus 41 and sapovirus and the remaining specimen was positive for both norovirus GII and parechovirus.

DISCUSSION

To the best of our knowledge, the present study was the first study of diantamoebiasis in Portuguese children hospitalized with AGE. The multiplex real-time PCR protocol is considered an additional diagnostic tool for the rapid, sensitive and specific, detecting the most common protozoa pathogens (Stark *et al*. 2011).

In our study, the frequency of protozoa found was high, largely attributable to the frequency finding of *D. fragilis*. This frequency was higher in comparison with the frequency of *C. parvum* and of *E. histolytica* and of *G. duodenalis*, usually considered the most frequent protozoa.

Nevertheless, the frequency of *D. fragilis* observed in our study was lower than that observed at several other studies from across the world, including some developed and industrialized countries, with few cases parasite-related outbreaks and, with excellent hygiene standards (Stensvold *et al*. 2007). Frequency was recorded as 46% in Denmark (Röser *et al*. 2013), 32·2% in Sweden (Norberg *et al*. 2003), 66·7% in Italy and 61·9% in the Netherlands (Maas *et al*. 2013). Comparison with the findings of these studies is limited due to differences in the study design and diagnostic technique used. Similar values of frequency to those found in our study have been reported from Belgium 6·3% (Vandenberg *et al*. 2006), Italy 6·9% (Lacasella *et al*. 2013) and Tunisia 5·5% (Ayadi and Bahri, 1999) using light microscopy. In Australia, the reported frequency was about 5% using a PCR protocol (Stark *et al*. 2010). At a global level, the large variation on the frequency of *D. fragilis* reported makes difficult to interpret the results of our study. Nevertheless, our findings suggest that this protozoan should be included in the laboratory diagnosis of diarrhoeal.

Frequency was higher in older children (>6 years; $n = 7$), most of the cases occurring in children aged between 9 and 12 years ($n = 5$), following other studies which also reported higher frequency in Dutch children aged between 5 and 14 years (de Wit *et al*. 2001) and in Turkish children between 8 and 15 years (Girginkardeşler *et al*. 2003). In common with a large Danish study, frequency in Portuguese children with AGE showed a strong association with age, although Danish children presented a very well-defined peak at 7 years old (Röser *et al*. 2013), while in our study the age ranged between 9 and 12 years old.

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Females are more likely to harbour *D. fragilis* than males (Barratt *et al*. 2011) although in children one study report that this protozoan is more frequently encountered in boys aged 16–20 years when compared with females of the same age, a trend which was not observed in our study as well as in other studies (Lagacé-Wiens *et al*. 2006). The different distribution of *Dientamoeba* between genders could be linked to the different roles of males and females in different cultural groups or societies (Barratt *et al*. 2011). Our study found that *Dientamoeba* infection was almost twice as common in males compared with females although the association with gender was not statistically significant.

Co-detection of protozoa occurs relatively frequently especially with *Blastocystis hominis* (Lagacé-Wiens *et al*. 2006; Yakoob *et al*. 2010; Maas *et al*. 2013) and to a lesser extent with *E. histolytica* (Steinitz *et al*. 1970; Sargeaunt *et al*. 1980), *G. duodenalis* and *Cryptosporidium* sp. (Maas *et al*. 2013), suggesting a similar mode of transmission (Barratt *et al*. 2011). In our study, no co-detection with *G. duodenalis* and *Cryptosporidium* sp. was found within the patients and we did not test specimens for *B. hominis*.

Pathogenic enteric parasites include, among others, *Cryptosporidium* sp., *D. fragilis, E. histolytica* and *G. duodenalis* (Stark *et al*. 2014). The most frequent symptoms presented by the infected patients are: abdominal pain, diarrhoea, vomiting, nausea, anorexia, weight loss and fever (Stark *et al*. 2010). There are numerous reports from all over the world reporting an association between *D. fragilis* infection and various clinical symptoms, most commonly diarrhoea and abdominal pain (Norberg *et al*. 2003; Johnson *et al*. 2004; Vandenberg *et al*. 2007). In our study, no statistical association was observed with diarrhoea, vomiting, fever and abdominal pain, although all these symptoms were common among infected children.

The diagnosis of *D. fragilis* was not associated with a history of foreign travel for the majority of children studied; however, one had a history of travel outside Portugal to Guinea Bissau and Senegal. In addition, with the fact that most of the children are descended from European parents (82%), seems to indicate that most of the *D. fragilis* infections were probably acquired in Portugal.

The mode of transmission of this parasite is still unknown. Our results indicated a 12 times greater risk of acquiring this infection for children who attend school, suggesting probable human-to-human transmission. Despite further statistical analysis needed, our results are according to Bøås *et al*. (2012) also showing that 27% of infected children have a family member with same symptoms. Otherwise, a statistically significant association between the consumption of particular food items and *D. fragilis* frequency was also found leading us to consider food contamination as a potential source of the parasite.

Seasonality was associated with the risk of *D. fragilis* carriage in Denmark patients, showing a small increase in autumn (Röser *et al*. 2013). Our study also showed a slight increase in the number of *D. fragilis*-positive cases in autumn, especially during 2011 and 2012, with no statistical association. The incidence of cryptosporidiosis follows a seasonal pattern in Europe, with a peak during late summer and autumn (ECDC, 2013), it is possible that *D. fragilis* also follows a seasonal pattern, although further research is needed to confirm this findings.

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AUTHOR CONTRIBUTION

C.J. planned, performed laboratory work and wrote the manuscript; C.F. provided epidemiological support and critical revision of the manuscript; R.R. performed laboratory work; M.J.B. and C.E. performed the children inclusion on the study and collected the samples; M.O. was involved with conception of the study and critical revision of the manuscript.

CONFLICT OF INTEREST

None.

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