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The high co-existence rate of *Blastocystis* and *Dientamoeba fragilis* in human faecal samples and the analysis of demographic and clinical findings

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ABSTRACT

Aim: *Blastocystis* and *Dientamoeba fragilis (D. fragilis)* are among the most common protozoon species in human faecal samples. The cross-sectional studies have reported the frequencies in a variety of populations. However, we have very limited information about the co-existence rate of those protozoans. The study aimed to compare *D. fragilis* frequency in *Blastocystis* positive and negative faecal samples in order to determine the co-existence rate. The secondary objective was to analyse demographic characteristics and gastrointestinal (GI) symptoms in relation to both infections.

Material and Method: In the present study, we defined a study group that included 100 *Blastocystis* positive faecal samples and a control group that included 100 *Blastocystis* negative samples. The frequency of *D. fragilis* in samples was determined with a PCR assay specific to the small-subunit ribosomal RNA (SS rRNA) gene. A positive control of *D. fragilis* was used and the samples with amplification of the expected size (863 bp) were considered as positive. In addition to the statistical comparison of frequencies, the descriptive and clinical findings of cases were analysed retrospectively with Pearson chi-square or ANOVA tests.

Results: The frequency of *D. fragilis* was 21% in *Blastocystis* positive group and it was 10% in *Blastocystis* negative group. There was statistically significant difference in terms of *D. fragilis* positivity between the groups ($p < 0.05$). Age, gender and GI symptoms did not reveal a significant difference between the following groups: only *Blastocystis* infected (n=77), only *D. fragilis* infected (n=11), infected with both protozoans (n=34) and non-infected individuals (n=89) ($p > 0.05$).

Conclusion: Our study highlighted the high co-existence of *D. fragilis* and *Blastocystis* in human faecal samples. A possible explanation for this finding may be the faecal-oral transmission of these protozoans. In addition, analysis of clinical findings was supported common asymptomatic colonisation of *Blastocystis* and *D. fragilis*.

Keywords: *Blastocystis*, *Dientamoeba fragilis*, co-existence, clinical findings

INTRODUCTION

Dientamoeba fragilis (D. fragilis) is a common, globally distributed, enteric protozoon in humans. It was initially classified in amoebas, but the exact taxonomic position was found after phylogenetic and ultra-structural studies. It was finally defined as a member of trichomonads (1). Presence of non-motile pre-cyst and cyst forms in human faecal samples was recently confirmed (2). It colonizes large intestines of humans and some other non-human hosts such as livestock and pet animals. Frequency of *D. fragilis* in many countries has been studied using a variety of diagnostic methods. In common, higher frequencies were reported in developed countries as compared to undeveloped countries unlike other intestinal protozoans (3). Prevalence of *D. fragilis* greatly varied (between 0.4% and 82.9%) in those studies and influenced by the diagnostic methods, study groups, sample size, and geographical location (2). Despite continuous reports emerging over last 100 years, it is still often ignored as a pathogen "neglected parasite", and routine testing often not conducted by diagnostic laboratories. A study from the Netherlands reported that after implementation of faecal PCR, the number of reported *D. fragilis* cases increased 20 folds. In addition, the symptoms in *D. fragilis* infected group lasted longer when compared to *Giardia intestinalis* infected group and complete resolution of symptoms was noted after eradication of the parasite in faecal samples (4). The previous studies mostly performed in industrialized countries such as the Netherlands and Denmark using molecular techniques and they reported frequencies reaching up to 71% in particular age groups (5). In Brazil, *D. fragilis* was detected in 10.3% of children; the other protozoans were *Blastocystis* (14.1%), *Endolimax nana*

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(13.5%), *Entamoeba coli* (12.2%), and *G. intestinalis* with a frequency of 10.9% (6). A study found that frequency of *D. fragilis* (5.2%) was lower than *Blastocystis* (9.6%) but higher than of *G. intestinalis* (2%) among symptomatic and asymptomatic population in Sydney (7). The most common transmission way of intestinal, parasites is faecal-oral and they are mostly defined as food-borne pathogens. World Health Organisation (WHO) reported that the foodborne parasitic diseases including *Entamoeba histolytica*, *G. intestinalis* and *Cryptosporidium spp*. are focal and cause significant morbidity and mortality in vulnerable populations, however, *D. fragilis* was not listed (8).

Previous studies on *D. fragilis* in Turkey reported that the frequencies varied from 0% to 26.9% (9, 10). However, a systematic and comprehensive understanding of *D. fragilis* prevalence is still lacking in our country. The pathogenic or opportunistic role of *D. fragilis* in human diseases has been a controversial issue for a long period of time. Most of the infected individuals do not represent clinical symptoms and higher frequencies have been reported in healthy group as compared to symptomatic group. Although initially described as a non-pathogen, *D. fragilis* has been associated with wide-ranging symptoms. Symptomatic cases represent primarily non-specific gastrointestinal (GI) symptoms mostly abdominal pain or intermittent diarrhoea (7). In addition, many other symptoms including malaise, nausea, anorexia, fatigue, poor weight gain, and unexplained eosinophilia (almost half of the positive cases) are also attributed to *D. fragilis* infection. The symptoms may persist or re-occur in some patients until application of effective treatment (2).

Blastocystis is an intestinal anaerobic protozoan of humans and many other non-human species. Following the long-term taxonomic studies, *Blastocystis* was included in the group of Stramenopiles. The colonization in humans and the absence of a flagellated differentiates *Blastocystis* from others in this group (11). *Blastocystis* has a global distribution and has been reported as the most common protozoon in human faecal samples in many studies (12). *Blastocystis* prevalence is higher in undeveloped countries, and frequencies reaching up to 100% have been reported in Senegal. The evaluation of prevalence studies revealed estimation that 1-2 billion people around the world had *Blastocystis* infection (13). A study from Sweden evaluated retrospectively the intestinal parasite frequency for 10-year period, and found that 4.2% of intestinal parasite prevalence, all of them were positive for *Blastocystis*. However, it was noted that most had an immigration history (14). *Giardia* and *Blastocystis* were the most common protozoan species in a study from Australia (15). A study identified GI pathogens in children with diarrhoea reported 2.9% *Blastocystis* carriage and none in asymptomatic group in United Arab Emirates (16). A systematic review in China estimated overall 3.3% *Blastocystis* prevalence in the country and noted great variations between cities in terms of *Blastocystis* frequency, from 0.8% to 100% (17). *Blastocystis* prevalence in Turkey was reported at rates ranging from 1,4% to 23,5% (18, 19). There is lot of controversy regarding the pathogenicity, genetic diversity, life cycle, diagnosis and treatment of *Blastocystis* (12). Similar to *D. fragilis* the role of *Blastocystis* in the aetiology of particular GI diseases such as irritable bowel syndrome (IBS) and ulcerative colitis (UC) is an important area of interest. Recently, controversial findings suggest that both infections implicate the development of IBS. A systematic review with meta-analysis examined the possible link and reported a correlation with *Blastocystis* (21). However, there is great need for future studies to reveal the actual mechanisms. Some defined *Blastocystis* as a pathogen, an opportunistic pathogen, or a non-pathogenic microorganism. Currently, it is thought that *Blastocystis* pathogenicity is multifactorial and complicated phenomenon that depends on *Blastocystis* strains, host characteristics, therefore it is hard to explain the pathogenicity over a single feature (12).

Both *Blastocystis* and *D. fragilis* are common microorganisms in human faecal samples, worldwide. However, there is limited data about their co-existence and few studies directly investigated this relationship. The aim of the present study was to determine the co-existence of *Blastocystis* and *D. fragilis* in human faecal samples with molecular methods. In addition, we aimed to analyse some demographic characteristics and GI symptoms related to *Blastocystis* and/or *D. fragilis* infections.

MATERIAL AND METHOD

The study was reviewed and applied by No-Interventional Clinical Research Ethics Committee of Aydın Adnan Menderes University Faculty of Medicine (Date: 17.02.2021, Decision No: 2021/37). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

The study included two groups: *Blastocystis* positive 100 and *Blastocystis* negative 100 DNA from faecal samples. All of the 200 individuals were scanned retrospectively for the presence of any other intestinal protozoans or helminths and negatives with direct microscopy were included in the study. Genomic DNAs were previously isolated from faecal samples of the individuals during routine coprological examination in parasitology laboratory in Aydın Adnan Menderes University, Training and Research Hospital. A commercial kit (QIAamp DNA Stool mini kit, Germany) was used to isolate genomic DNA directly from fresh faecal samples. *Blastocystis* positivity was

detected by amplification of 18S rRNA gene of *Blastocystis* with the primers RD5 and BhRDr as previously reported (22). *Blastocystis* isolates were confirmed by submission of partial 18S rRNA sequences to MLST database (http://pubmlst.org) and with neighbour-joining method including reference sequences.

Determination of *D. fragilis* Positivity

Positivity of *D. fragilis* was studied with amplification of the small-subunit rRNA (SSU rRNA) (23). Reaction was set in 30-ul volume: 1 µl of template DNA, Taq DNA polymerase (0.3 U), dNTPs (0.2 mM), the primers (0.4 pmol), $MgCl_2$ and 1× Taq buffer with $(NH_4)2SO_4$. The primers DF400 (TAT CGG AGG TGG TAA TGA CC) and DF1250 (CAT CTT CCT CCT GCT TAG ACG) were used in the assay. PCR cycle was as follows: initial denaturation at 94°C for 3 min, 30 cycles (1 min at 94°C, 1.5 min at 57°C, 2 min at 72°C) and final extension at 72°C for 7 min. The PCR amplicons analysed by electrophoresis on 1% agarose gel and visualized with the UV imaging system (Vilber Lourmat, France). A previous *D. fragilis* isolate (ADUDf101), confirmed with partial sequence of SSU rDNA, was used as positive control in our experiment.

Statistical Analysis

The descriptive data was presented using Statistical Package for the Social Sciences, SPSS (IBM, USA) vs. 21.0. A chi-square test of independence was performed to show the relation between *Blastocystis* and *D. fragilis* co-existence rate. The sociodemographic characteristics (gender and age) were evaluated also analysed using Pearson chi-square. The common GI findings were compared between four groups (*Blastocystis* infected, *D. fragilis* infected, non-infected and both infected) with ANOVA.

RESULTS

The positive rate of *D. fragilis* was 23% in *Blastocystis* positive samples and it was 11% in *Blastocystis* negative samples (**Figure**, **Table 1**). *D. fragilis* positivity was significantly higher in *Blastocystis* infected individuals as compared to non-infected cases χ^2 (1, N=200)=0.1028, p=.0238. The age of study population (n=200) varied from 1 to 84 with the average of 37.2±23.5. Males accounted for the %53 (n=106) of individuals and females for %47 (n=94).

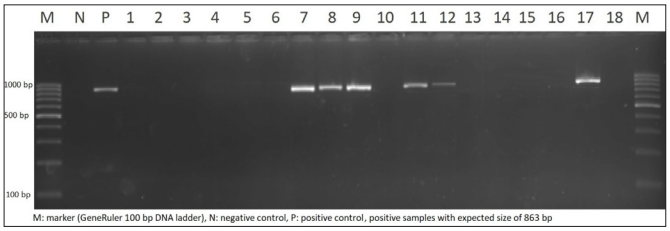

Figure. Agarose gel electrophoresis of amplicons from *D. fragilis* 18 S rRNA PCR

Table 1. The comparison of *D. fragilis* frequency in *Blastocystis* positive and negative samples

	***D. fragilis* PCR**		
	Positive n (%)	**Negative n (%)**	**Total**
Blastocystis			
Positive	23 (23)	77 (77)	100
Negative	11 (11)	89 (89)	100
Total	34 (17)	156 (78)	200

The faecal samples were sent from many different clinical departments: gastroenterology and hepatology (n=53, 26.5%), child health and diseases (n=40, 20%), dermatology (n=31, 15.5%), general internal medicine (n=20, 10%), allergy and immunology (n=17, 8.5%), oncology (n=10, 5%), infectious diseases (n=8, 4%), chest diseases (n=5, 2.5%), and the other departments (n=16, 8%) including haematology, urology, family medicine, rheumatology, otolaryngology, and nephrology.

The clinical features and diagnosis greatly varied in the study population: abdominal pain (n=39, 19.5%), diarrhoea (n=25, 12.5%), allergy (n=25, 12.5%), flatulence (n=24, 12%), constipation (n=18, 9%), pruritus (n=17, 8.5%), nausea-vomiting (n=16, 8%), general medical examination (n=14, 7%), malnutrition-developmental delay (n=13, 6.5%), urticeria (n=12, 6%), gastroesophageal reflux (n=11, 5.5%), vitamin-D deficiency (n=9, 4.5%), anaemia (n=7, 3.5%), colitis (n=4, 2%), dermatitis (n=4, 2%), dyspepsia (n=4, 2%), and the others; skin rash, dysuria, cramping, GI haemorrhage, lassitude, myalgia, and cellulitis in single patients. In addition, 12 (6%) of the studied population were cancer patients, 10 (5%) were ulcerative colitis patients, six (3%) had irritable bowel syndrome, four had Crohn's disease, four (2%) had urinary system infections, two (1%) had pneumonia, two (1%) had obesity treatment, two (1%) had renal failure, one had diabetes, and one had rheumatoid arthritis.

In the present study, we found 77 cases with single *Blastocystis* infection, 11 cases with single *D. fragilis* infection, 34 cases with both of *Blastocystis* and *D. fragilis* and 89 non-infected cases. These groups were compared for demographic characteristic in statistical analysis and no significant relation was found regarding gender and age (**Table 2**). We also analysed common GI findings including abdominal pain, diarrhoea, constipation, flatulence, and nausea-vomiting between the groups, none of these symptoms were significantly different between the groups (**Table 3**).

Table 3. The analysis of common gastrointestinal symptoms between the groups

	Abdominal pain*	Diarrhoea*	Constipation*	Flatulence*	Nausea- vomiting*
Blastocystis infected only (n=77)	15 (19.5)	10 (12.9)	5 (6.4)	8 (10.3)	5 (6.5)
D. fragilis infected only (n=11)	3 (27.3)	2 (18.1)	1 (9.1)	2 (18.1)	1 (9.1)
Blastocystis and *D. fragilis* infected (n=34)	6 (17.6)	4 (11.7)	3 (8.8)	6 (17.6)	2 (5.9)
Non-infected with both of them (n=89)	15 (16.9)	9 (10.1)	9 (10.1)	12 (13.4)	8 (9)
Chi square (p value)	0.782 (0.852)**	0.775 (0.855)**	4.873 (0.181)**	1.354 (0.716)**	0.557 (0.906)**

* number of positives and (%) in the groups ** not significant at p<0.05 level

Table 2. The comparison of some demographics of studied population between the groups

	N	Age* (mean±sd)	Gender**	
			Female (n, %)	Male (n, %)
Blastocystis infected only	77	32.2±24	37 (48.1)	40 (51.9)
D. fragilis infected only	11	42.9±28.1	5 (45.5)	6 (54.5)
Blastocystis and *D. fragilis* infected	34	39.6±22	14 (41.2)	20 (58.8)
Non-infected with both of them	89	36.4±21.4	38 (42.7)	51 (57.3)

N: number, Sd: standard deviation, *not significant, ANOVA: F (3, 207)=1.31, p=.271; ** not significant, χ^2= 0.666, p= .881

DISCUSSION

In the present study, we have tested the frequency of *D. fragilis* in *Blastocystis* positive and negative individuals with molecular methods. The findings indicate significantly high infection rate of *D. fragilis* in *Blastocystis* positive cases. In the literature, previous studies reported some data related to our findings. However, few studies directly aimed to study the co-existence rate with molecular methods. A study investigated the frequency of intestinal parasites in 580 children with diarrhoea and they reported a correlation between *Blastocystis* and *D. fragilis*. The frequency of *D. fragilis* was 2.7% among children without *Blastocystis* infection. However, the rate was 29.6% among children with *Blastocystis* infection (24). A study from Iran investigated prevalence of intestinal parasites with conventional parasitological methods and reported that 21 (17%) of 125 *Blastocystis* infected individuals were also positive for *D. fragilis* (25). In the Netherlands, *D. fragilis* was detected as the most frequent protozoon in faecal samples of 163 paediatric patients and the combination of *D. fragilis* and *Blastocystis* accounted for almost 50% of them (26). Another study found that *Blastocystis* frequency was 42% in *D. fragilis* positive cases (9 out of 21). *D. fragilis* was detected as the most common parasite species in faecal samples of studied population (27). Similarly, frequency of *D. fragilis* was studied in patients with GI symptoms and 23.7% of *D. fragilis* positives had *Blastocystis* infection (28). The most common protozoon was *D. fragilis* in *Blastocystis* infected individuals, 24% (53 of 221 *Blastocystis* infected cases). In addition, a significant correlation was found between *Blastocystis* and *D. fragilis* (29).

The proposed mode of transmission is faecal-oral for both *Blastocystis* and *D. fragilis* (2,11). Therefore, they share a common source of infection for enteric protozoans. Parallel to our findings, a study among IBS patients found that *Blastocystis* carriage was a risk factor and increased the odds for *D. fragilis* infection (30). In addition, another hypothesis about transmission of *D. fragilis* is the carriage with pinworm eggs (2), our study did not include Enterobius vermicularis positive faecal samples as well as other intestinal parasites. Therefore, we could eliminate possible effects related to this type of transmission in our study.

In our study, the overall positive rate of *D. fragilis* was 17% in Aydin. In general, the finding was in accordance with the reported frequencies from other cities of Turkey. The frequency of *D. fragilis* was studied in faecal samples collected from 121 individuals, of them 101 had GI complaints and remaining 20 cases were in control group. The overall positive rate of *D. fragilis* was 13% with iron haematoxylin staining (31). Another study from Istanbul determined 16.7% positivity of *D. fragilis* and they found a statistically significant difference between healthy individuals and patients in terms of *D. fragilis* positivity (9). In Manisa, *D. fragilis* positivity was studied with different culture methods and *D. fragilis* trophozoites were determined in 11 of 104 (10.6%) samples with Robinson's medium (32). A study from Izmir investigated *D. fragilis* positivity in 490 faecal samples with real-time PCR; they found that 59 (12%) patients were infected with *D. fragilis* (28).

A limitation of our study was the possible cross-reaction of PCR testing with other trichomonads in human faecal samples. There is currently no PCR testing protocol for laboratory detection of *D. fragilis* that is approved by U.S. Food and Drug Administration (FDA) and the validation of the tests are still in progress. It was reported that PCR method in our study was tested against various other protozoan parasites including *Blastocystis*, *Entamoeba spp*, *E. hartmanni*, *Giardia intestinalis*, *Endolimax nana*,

Iodamoeba butschlii, *Cryptosporidium spp*., *Cyclospora spp*., *Chilomastix mesnili*, *Enteromonas hominis* and no amplification was detected with having 100% specificity (23). However, recently, it was reported that conventional PCR for *D. fragilis* may result in cross reactions with other trichomonads (2). In addition, the sensitivity of PCR testing with DF400 and DF1250 primers was 93.5% in fresh faecal specimens (23).

Another limitation of the current study was the lack of permanent staining because of the retrospective nature of the study. Faecal samples were initially tested for the presence of *Blastocystis* and subjected to genomic DNA isolation. Therefore, we could present only wet-mount examination (native-Lugol's iodine) of faecal samples from hospital record. At the beginning of the study, we excluded the samples that were positive for other intestinal protozoa. It was reported that the nuclear structure of *D. fragilis* is visible when permanent staining methods are used (2). In general, molecular methods are more sensitive than examination of wet-mount preparations. A study reported frequency of *D. fragilis* with direct smear, formalin-ether concentration, culture, permanent staining and amplification of SSU rRNA and 5.8S rRNA genes. The positive rates with the methods were as follows: 0%, 0%, 1%, 5%, 6% and 13.5%, respectively (33).

In the present study, when we compared age, gender and common GI symptoms between the four groups, no statistically significant difference was noted. A number of studies reported no relation between gender and *Blastocystis* infection, as well as *D. fragilis* infection, supporting our findings (33, 34). A case control study reported that GI symptoms were more common in cases without *D. fragilis* or *Blastocystis*. In addition, they reported that both *D. fragilis* and *Blastocystis* frequency was higher in healthy controls than in cases with symptoms (35). However, some reported a correlation with age of cases and *Blastocystis* infection in particular age groups (19). Despite the relatively small size of study population, the findings on GI can be attributed to the general characteristics of both *Blastocystis* and *D. fragilis* infection. Because, there is growing body of literature that reported that these two infections are mostly asymptomatic and a small ratio of infected individuals represent GI symptoms (2,11). Diarrhoea, abdominal pain, constipation and urticarial findings were reported in symptomatic cases of *Blastocystis* infection (11-13). Similar to *Blastocystis*, non-specific GI symptoms including abdominal pain, cramps and diarrhoea were reported in symptomatic cases of *D. fragilis* (36). Nausea, vomiting, fever and eosinophilia have also been observed in cases with *D. fragilis* infection (2,37). Parallel to these findings, recent developments in the study of microbiota research revealed that the existence of *Blastocystis* and *D. fragilis* may be related to a healthy intestinal flora (38,39). It was reported that, the colonization of both *D. fragilis* and *Blastocystis*, unlike bacterial composition, diverged between healthy controls and irritable bowel syndrome (IBS) patients. Their colonization was associated with rich and diverse bacterial microbiota; but the association changed in patients with IBS (40).

CONCLUSION

The present study reported a significantly high frequency of *D. fragilis* in *Blastocystis* positive faecal samples. The current finding highlighted the importance of faecal-oral transmission of these two protozoa. The analysis of clinical findings emphasises common asymptomatic colonisation of these protozoans. However, the correlation found in our study may not directly indicate a causality and represent a direct relationship of these two pathogens. This finding provides new insights for future research that includes randomized-controlled studies with larger sample size.

ETHICAL DECLARATIONS

Ethical Committee Approval: The study was reviewed and applied by Non-Interventional Clinical Research Ethics Committee of Aydın Adnan Menderes University Faculty of Medicine (Date: 17.02.2021, Decision No: 2021/37).

Informed Consent: Because of the experimental, retrospective and non-invasive nature of the study design, no written informed consent form was obtained from patients.

Conflict of Interest Statement: The authors declare that they have no conflicts of interest.

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Author Contributions: All the authors participated in the design, in the experimental parts, and in the data analysis of the study. All authors approved the final version of the manuscript.

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