

Article

Molecular Detection of *Dientamoeba fragilis* and Its Association with Gastrointestinal Symptoms in Iraqi Patients

Baraa Ali Mohammed*¹

1. Department of Clinical Laboratory Sciences, College of pharmacy, AL-Qadisiyah University, Diwaniyah, Iraq

*Correspondence: baraa.ali@qu.edu.iq

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Abstract: Background: *Dientamoeba fragilis* is a trichomonad protozoan of the human large intestine whose pathogenic role remains debated. Conventional microscopy underestimates its frequency, and no molecular data from symptomatic and asymptomatic Iraqi populations attending private clinics have been reported. This study aimed to determine the prevalence of *D. fragilis* by real-time polymerase chain reaction (PCR) in patients attending private clinics in Baghdad and to evaluate its association with gastrointestinal symptoms and potential risk factors. Methods: A case-control study was conducted from 2022 to 2026 in 3 private clinics in Baghdad, Iraq. Single fresh stool samples were collected from 250 patients with gastrointestinal symptoms (cases) and 100 asymptomatic individuals (controls). Samples were examined by direct wet mount and permanent iron-haematoxylin staining, and DNA was extracted for real-time PCR targeting the 5.8S ribosomal RNA gene region of *D. fragilis*. Demographic, environmental and clinical data were collected using a structured questionnaire. Associations were assessed by chi-square tests and multivariable logistic regression. Results: *D. fragilis* DNA was detected in 57 of 350 participants (16.3%), compared with 21 (6.0%) by microscopy. Microscopy showed a sensitivity of 36.8% relative to PCR, with moderate agreement ($\kappa = 0.49$). Positivity was higher in symptomatic patients than in controls (19.2% vs 9.0%; OR 2.40, 95% CI 1.13–5.10; $p = 0.020$). Among symptomatic patients, infection was associated with abdominal pain ($p = 0.029$), bloating ($p = 0.025$), anal pruritus ($p = 0.035$) and symptom duration longer than two weeks ($p = 0.034$), but not with diarrhoea, nausea or vomiting. In multivariable analysis, age under 20 years (aOR 1.92), use of untreated water (aOR 2.71) and presence of gastrointestinal symptoms (aOR 2.29) were independently associated with infection. In conclusion, *D. fragilis* is frequently present in clients in private clinics in Baghdad and is largely underreported by routine microscopy. The association of this parasite with abdominal pain, bloating, and chronic symptoms should lead to the inclusion of a molecular test in the diagnostic procedures of unexplained gastrointestinal symptoms in Iraq.

Keywords: *Dientamoeba fragilis*; real-time PCR; dientamoebiasis; gastrointestinal symptoms; intestinal protozoa; Baghdad; Iraq.

Introduction

Intestinal parasitic infections remain an important cause of morbidity in low- and middle-income countries, and protozoa account for a large share of these infections in both children and adults. Among the intestinal protozoa, *Dientamoeba fragilis* occupies an unusual position: it was described more than a century ago, it is found worldwide, and yet its biology, transmission and clinical significance are still incompletely understood. The organism was first reported by Jepps and Dobell in 1918, who classified it as an amoeba on the basis of the morphology of its trophozoite stage in stained faecal smears [1]. Later ultrastructural, immunological and molecular studies demonstrated that *D. fragilis* is not an amoeba but an amoeboflagellate belonging to the order Trichomonadida, closely related to *Histomonas meleagridis* and other trichomonads, despite lacking external flagella [2], [3]. This history of misclassification, combined with the fragility of the trophozoite and the absence of a readily recognised cyst stage, contributed to its long neglect by clinicians and diagnostic laboratories [4], [5].

The trophozoite of *D. fragilis* measures approximately 5 to 15 µm, and most trophozoites are binucleate, with each nucleus containing a fragmented chromatin mass that gives the organism its characteristic appearance in permanently stained preparations [2], [4]. The trophozoite degenerates rapidly outside the host, so diagnosis by microscopy requires prompt fixation of fresh stool and examination of permanently stained smears by an experienced microscopist [5]. For decades, the absence of a cyst stage posed a central question about how the parasite survives transmission between hosts. Cyst and precyst forms have since been described in human clinical samples and in experimentally infected rodents, providing evidence that a resistant stage exists, although it appears to be rare and difficult to detect [6], [7]. An alternative hypothesis, first proposed because of the frequent co-occurrence of *D. fragilis* and *Enterobius vermicularis*, is that pinworm eggs carry the parasite between hosts. Detection of *D. fragilis* DNA within surface-sterilised *Enterobius* eggs has supported this view [8], [9], and current evidence suggests that both faecal-oral transmission through cysts and transmission through helminth eggs may contribute to its spread [10]. The finding of genotypes shared between pigs and humans has also raised the possibility of a zoonotic component in some settings [11]. Two genotypes have been identified, with genotype 1 predominating in human infections worldwide, and genetic diversity within the species appears limited compared with other intestinal protozoa [12].

The importance of *D. fragilis* as a medical organism has been disputed for many decades. Multiple case series and retrospective studies have associated the organism with different GI manifestations, among which abdominal pain, diarrhea, loose stools, flatulence, distension, vomiting, loss of appetite, malaise, and weight loss have been reported most frequently [2], [13]. The symptoms may be acute or chronic, and one persistent infection might last weeks or months [13], [14]. When *Dientamoeba* infection and giardiasis were compared among patients examined for a parasitic GI disease in Belgium, it was shown that most clinical signs were the same in both cases, and abdominal pain and diarrhea were common among the patients [15]. Improvement in clinical and parasitological parameters after treatment with nitroimidazoles or other drugs was registered in several reports as indirect evidence of pathogenicity [16]. There are also put forward hypotheses about the association of *D. fragilis* with IBS due to the similarity in chronic abdominal complaints related to both conditions [17], [18].

Other studies, however, have challenged the view that *D. fragilis* is a pathogen. In a population-based case-control study from Denmark, intestinal parasites including *D. fragilis* were not more common among individuals with irritable bowel syndrome than among controls [19]. A Dutch case-control study in children found that the parasite was equally common in children with chronic abdominal pain and in healthy controls [20], and a study in primary care found no association between colonisation and gastrointestinal symptoms in children [21]. High carriage rates have been documented in asymptomatic children attending day-care centres, leading some authors to describe the organism as a commensal [22]. Large surveillance datasets based on routine real-time PCR in Denmark have shown very high detection rates, particularly in children and their parents, and these rates are difficult to reconcile with a consistently pathogenic organism [23]. Most notably, a randomised, double-blind, placebo-controlled trial in children found that metronidazole eradicated the parasite more often than placebo but did not improve clinical outcomes, which argues against a simple causal relationship

between infection and symptoms [24]. The current literature therefore presents a mixed picture in which *D. fragilis* appears to be associated with symptoms in some populations and clinical contexts but not in others. Differences in study design, age group, choice of controls, diagnostic method, co-infections and host factors probably explain part of this heterogeneity [3], [14].

One of the key factors that was mentioned above and led to the reported prevalence of *D. fragilis* is diagnosis. Traditional diagnostic ways are based on microscope examination of stained smears, for instance, using the iron-haematoxylin or modified trichrome stains because the trophozoite can hardly be detected in saline microscopically and is destroyed during treatments of common concentration of protists and helminths [2], [5]. In spite of sufficient staining methods, microscopy has limited sensitivity and relies to a great extent on the experience of the researcher. The use of PCR-based methods has changed the way the epidemiology of the parasite is understood. With regard to the standard PCR approach used for the amplification of the small subunit ribosomal RNA gene, it has been determined that it has greater sensitivity than microscopy in fresh stool samples [25], whereas the real-time PCR method, specific to the 5.8S rRNA gene, was developed and verified in subsequent studies so that the method is able to detect the DNA of *D. fragilis* with high specificity directly in samples of faeces [26]. According to the studies comparing the methods, real-time PCR outperforms both microscopy and xenic cultivation in terms of sensitivity in diagnosis [27]. At that, multiplex real-time PCR assays that allow diagnosing *D. fragilis* in combination with giardiasis, cryptosporidiosis and amoebic dysentery have been put into practice in several countries [28]. More recently, the comparative review of real-time PCR tests has been conducted and recommendations regarding their application and interpretation have been made, including the risk of cross-reactivity with similar trichomonad species at high cycle threshold [29].

Molecular diagnostics confirm that *D. fragilis* is one of the most common intestinal protozoa in many populations. For instance, data from Denmark show that the number of patients diagnosed with the parasite using real-time PCR exceeds the number of patients diagnosed using microscopy significantly [30]. Unfortunately, there are still not enough data on the Middle East and nearby regions. The results of Iranian studies confirmed the presence of *D. fragilis* among the patients with diarrhea [31], whereas the study conducted in Turkey demonstrated high infection rates among the patients complaining about gastrointestinal disorders [32]. There has been evidence of the presence of *D. fragilis* DNA in Egyptian patients with IBS and genetic analysis shows that the isolates are different in terms of their clinical manifestations [33].

Intestinal parasitic infections are a recognized public health concern in Iraq. The transmission of faecal-oral pathogens may be influenced by many factors, including the destruction of water and sanitation infrastructure after years of war, inconsistent municipal supply of water, dependence on water tankers or water from household tanks, the large number of people living together, and direct contact with pet animals. In Iraq, research studies concerning intestinal parasites have been limited to those associated with *Giardia lamblia*, *Entamoeba histolytica/dispar*, *Cryptosporidium* spp. *Blastocystis*, and helminths while utilizing mostly direct wet mount and concentration methods. Since these techniques cannot be employed to detect the existence of *D. fragilis*, the latter is either not known or never reported in routine diagnosis laboratories in Iraq. As a result, patients suffering from prolonged abdominal pains, bloating, or changed bowel habits whose stool has been analyzed as the negative one can continuously be treated with empirical drugs or be diagnosed with a functional bowel disease without excluding the parasitic cause of detected symptoms. Private clinics situated in Baghdad receive many outpatients suffering from gastro-intestinal troubles from different parts of the city and nearby areas providing a good opportunity to review the significance of this parasite among patients.

To the best of our knowledge, the existing studies have never utilized real-time PCR to investigate the prevalence of *D. fragilis* among infected and healthy patients in Baghdad. Such data are necessary for informing the diagnostic practices in laboratories at the local and regional levels, helping health practitioners in interpreting the obtained positive results, and making the contribution to the current international discussions on the ability of the respective pathogens to cause human diseases. The study was conducted with the following three main objectives: the determination of *D. fragilis* infection rates among patients presenting with gastrointestinal disorders and those without so attending private medical clinics in Baghdad using molecular biology techniques, the assessment of the feasibility of permanent-stain microscopy for laboratory diagnosis of gastrointestinal diseases, and the

explanation of the relationship between *D. fragilis* infection and gastrointestinal disorders, along with the assessment of the impact of socio-demographic factors and environmental risk factors.

Materials and Methods

The research was designed and conducted as a case-control study in the period from 2022 to 2026 in three private outpatient clinics located in Baghdad, Iraq, with specific focus on internal medicine, gastroenterology, and paediatrics. The clinics were located on both banks of the Tigris, in the Karkh and Rusafa districts, and were chosen with the aim to ensure having patients from different parts of the Baghdad metropolitan area and nearby suburbs. The laboratory tests were conducted in a private laboratory located in Baghdad. Two groups were included in the study. The case group included 250 persons of all age categories seeking medical attention at the clinics with at least one gastrointestinal symptom, including abdominal pain, nausea, vomiting, pruritus of the anal region, or weight loss. The control group included 100 people who had not experienced any gastrointestinal symptoms in the four weeks prior to recruitment and who were recruited among the people attending the clinics for routine check-ups, non-gastrointestinal problems or as accompanying persons. Controls were recruited at the same time as cases and from the same clinics, in order to minimize the differences in socioeconomic backgrounds and residence.

Individuals were excluded if they had received antiparasitic medicines, metronidazole or another nitroimidazole, or antibiotics in the preceding four weeks. Other exclusion criteria included inflammatory bowel disease, celiac disease or colorectal cancer, gastrointestinal surgery in the last three months, or ongoing treatment with immunosuppressive drugs. Individuals who had provided insufficient stool samples or who had not completed the questionnaire were disqualified. For the calculation of the sample size, the researchers applied the formula for the comparison of proportions, with a prevalence of *D. fragilis* of 20% in symptomatic patients and 8% in asymptomatic people, with the ratio of cases to controls of 2.5, two-tailed significance level of 0.05 and power of 80%. The minimum sample size required was found to be equal to 230 cases and 92 controls, but was eventually increased to 250 cases and 100 controls to account for potential losses.

The researchers obtained evidence of the signed consent from all adult participants and from parents or guardians of those under the age of 18, while children over the age of were interviewed and have assented to participate in the study. Participation was voluntary and the identity of the respondents, as well as their answers, were kept unknown and secret.

The structured questionnaire was filled in by the trained interviewer during the face-to-face interview with each participant or with the parent or guardian of a child. The questionnaire included data on age, sex, life conditions (whether the person lives in the urban area or rural area), education of a participant and his/hers family head, number of family members, drinking water supply (whether tap water, bottled water or other untreated sources), contact with pets and history of pinworm infection. For the cases, all details regarding types and duration of symptoms were included in the questionnaire. 24

A fresh stool sample from each study participant is taken in a plastic container that can be locked and brought to the research laboratory in a refrigerator box within two hours. In the laboratory, the sample is divided into three parts. The first part is analyzed using a simple wet mount in normal saline and Lugol's iodine. The second part is treated with SAF preserving solution for permanent staining. The third part contains 1 g of fresh stool that is frozen at a temperature of -20 degrees Celsius in a sealed sterile container until the time of DNA extraction, which must take place within four weeks from the time of collection. – °

The wet mount was viewed under both 10× and 40 2,5× scopes for the presence of cysts, trophozoites, and eggs of parasites. Also, as part of the study procedure, the portion of the sample, which was fixed in SAF, was used to make thin films (smears) stained with iron-haematoxylin following the standard methods outlined in previous studies. The slides prepared were *then* examined under the microscope using oil immersion at 1000× for 15 minutes by an experienced technician. Movements of the *D. fragilis* trophozoites were confirmed based on their sizes measuring about 5–15 µm. Therefore, any other creatures of the group of intestinal protozoans and helminths reported came up

through the SG analysis. Care was taken to eliminate observer bias by reviewing the slides without knowledge of the casing and PCR presence and checking 10% of the films plus/including positive review.

Genomic DNA was extracted from around 200 mg of each stool sample using the QIA amp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions with the only difference in an additional heating step at 95 °C for 5 minutes following the addition of the lysis buffer in order to enhance disruption of the protozoan cells. DNA was then eluted in 100 µL of elution buffer and its concentration and purity were determined with the help of spectroscopy (NanoDrop, Thermo Fisher Scientific, USA). An extraction blank that consists of water instead of stool was included in each batch to check for contamination. The isolated DNA was kept at -20 °C until amplification.

To detect *D. fragilis* DNA, the TaqMan PCR assay employed by Verweij et al. was utilized, whereby the 98 5.8S ribosomal RNA gene fragment was specifically targeted by the primer pair Df-124F and Df-221 26R and a dual-labeled hydrolysis probe. The primer pair and probe sequences used were unchanged from their application by Verweij et al. A 25 µL reaction comprised 12.5 µL of commercial master mix, both primers at a concentration of 0.4 µM each, the hydrolysis probe at a 0.2 µM concentration, 5 µL of DNA template, and adjusting for volume with nuclease-free water. The reaction underwent amplification in a [real-time PCR system] following an initial denaturation step of 95 °C for 10 minutes and 45 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds, with fluorescence monitored at the end of each cycle. Positive controls comprised DNA from a *D. fragilis* sample confirmed by microscopy and verified by sequencing, and additional controls included a no-template and extraction blank sample. To ascertain the effects of inhibition in each reaction, standard controls were added to each reaction. A standard criterion for positive reactions was the presence of an amplification curve with an example of a standard sigmoid shape and a cycle threshold of 1 or less. Non-specific amplification of related trichomonads has been noted with regard to PCR amplifications with cycle threshold values between 10 and 40 (29 38 40). Thus to validate these amplifications, duplicates were produced from samples showing the presence of amplification curves at the aforementioned cycle threshold value. Finally, ten amplicons that were positive according to the initial test were subsequently amplified with conventional PCR as outlined by [25] and Sanger sequencing was applied to these amplicons.

The reference method that was utilized for comparison with microscopy was real-time PCR. The iron-haematoxylin microscopy test was assessed against PCR for sensitivity, specificity, and positive and negative predictive values, while the agreement between both methods was assessed using Cohen's kappa coefficient (Cohen's kappa coefficient is represented as poor, <0.20; fair, 0.21–0.40; moderate, 0.41–0.60; good, 0.61–0.80; and very good, >0.80).

The data were analyzed using IBM SPSS Statistics software version [26.0] (IBM Corporation, Armonk, NY, USA). Categorical variables were reported in terms of frequencies and percentages, whereas the continuous variables were reported using means with the standard deviations or medians with interquartile ranges depending on the distribution determined using Shapiro-Wilk test. Prevalence of *D. fragilis* was calculated at 95% confidence interval. The association of the infection with categorical variables was analyzed using Pearson's chi-square test, and Fisher's exact test was used when the expected count was less than in the cells, and the chi-square test for linear trend was used for the ordered age groups. Crude odds ratio (OR) was calculated along with 95% confidence interval (CI). The Ct values in the groups were compared using Mann-Whitney U test. Any variable with $p < 0.20$ in univariate analysis was included in the multivariable binary regression model to check those factors independently associated with infection. The results were presented as adjusted odds ratios (aOR) with CI of 95%. The fit of the model was established using Hosmer-Lemeshow test. A p value of less than 0.05 was taken for declaring the statistical significance of any findings.

Results

A total of 350 participants were enrolled, comprising 250 symptomatic cases and 100 asymptomatic controls. There were 180 males (51.4%) and 170 females (48.6%), with a mean age of 26.4 ± 17.8 years (range 2–74 years). Cases and controls did not differ significantly in age or sex distribution. *D. fragilis* DNA was detected by real-time PCR in 57 participants, giving an overall prevalence of 16.3% (95% CI 12.4–20.2%). All ten sequenced amplicons showed 99–100% identity with *D. fragilis* reference sequences in GenBank.

The infection was more prevalent in younger individuals with the prevalence decreasing from 22.5% in children under 10 years of age to 10.0% in those aged older than 60 years, which was shown to be statistically significant ($p=0.025$). Positivity in males and females didn't differ. The participants who were consuming unprocessed water from tanks, wells, and cisterns showed a significantly higher detection rate (31.7%) in comparison to the individuals using tap water. The history of contact with domestic animals and the presence of pinworm infections in the participant or other family members showed a statistically significant influence on the infection being detected (Table 1).

Table 1. Distribution of *D. fragilis* infection detected by real-time PCR according to demographic and environmental characteristics (n = 350)

Variable	Category	Examined (n)	PCR positive, n (%)	χ^2	p value
Age (years)	<10	80	18 (22.5)	Trend Z = 2.23	0.025
	10–19	70	14 (20.0)		
	20–39	110	15 (13.6)		
	40–59	60	7 (11.7)		
	≥60	30	3 (10.0)		
Sex	Male	180	27 (15.0)	0.45	0.502
	Female	170	30 (17.6)		
Residence	Urban	240	34 (14.2)	2.52	0.112
	Semi-urban/rural	110	23 (20.9)		
Drinking water	Municipal tap	200	28 (14.0)	12.95	0.002
	Bottled/RO filtered	90	10 (11.1)		
	Untreated (tanker/well/tank)	60	19 (31.7)		
Animal contact	Yes	90	21 (23.3)	4.41	0.036
	No	260	36 (13.8)		
History of pinworm	Yes	40	12 (30.0)	6.24	0.012
	No	310	45 (14.5)		
Total		350	57 (16.3)		

The identification of *D. fragilis* trophozoites through iron-haematoxylin microscopy in 21 samples (6.0%) yielded the same positive results in the PCR. The sensitivity of microscopy in comparison to the reference PCR was 36.8%, while the specificity of microscopy was 100% 2, 1. The median Ct value of PCR-positive samples was 31.4 (IQR 27.9–34.2), and the Ct values of microscopy-positive samples were significantly lower than those of microscopy-negative samples (median 27.6 vs. 33.1; $p<0.001$), which shows that microscopy method mainly detected high-burden infections.

Table 2. Comparison of iron-haematoxylin microscopy with real-time PCR for detection of *D. fragilis* (n = 350)

Microscopy	PCR positive	PCR negative	Total
Positive	21	0	21
Negative	36	293	329
Total	57	293	350

Sensitivity 36.8%; specificity 100%; positive predictive value 100%; negative predictive value 89.1%; Cohen's kappa = 0.49

The detection rate in symptomatic cases of 19.2% was more than double that in asymptomatic controls of 9.0%, which was significant (Table 3).

Table 3. Frequency of *D. fragilis* infection in symptomatic cases and asymptomatic controls

Group	Examined (n)	PCR positive, n (%)	OR (95% CI)	p value
Symptomatic cases	250	48 (19.2)	2.40 (1.13–5.10)	0.020
Asymptomatic controls	100	9 (9.0)	Reference	

Of 250 patients with symptoms, abdominal pain, bloating or flatulence, anal itching and duration of symptoms were significantly more common among PCR-positive than PCR-negative patients. Diarrhoea was also more frequent among cases, but statistically it was inconclusive, while nausea, vomiting, fatigue and loss of weight were not related to the infection (Table 4).

Table 4. Gastrointestinal symptoms in PCR-positive and PCR-negative symptomatic patients (n = 250)

Symptom	PCR positive (n = 48), n (%)	PCR negative (n = 202), n (%)	OR (95% CI)	p value
Abdominal pain	34 (70.8)	108 (53.5)	2.11 (1.07–4.16)	0.029
Diarrhoea	26 (54.2)	82 (40.6)	1.73 (0.92–3.25)	0.088
Bloating/flatulence	25 (52.1)	70 (34.7)	2.05 (1.09–3.86)	0.025
Nausea	12 (25.0)	47 (23.3)	1.10 (0.53–2.28)	0.799
Vomiting	6 (12.5)	29 (14.4)	0.85 (0.33–2.19)	0.739
Fatigue	15 (31.3)	51 (25.2)	1.35 (0.68–2.69)	0.396
Anal pruritus	9 (18.8)	17 (8.4)	2.51 (1.04–6.04)	0.035
Weight loss	5 (10.4)	18 (8.9)	1.19 (0.42–3.37)	0.747
Chronic duration (>2 weeks)	31 (64.6)	96 (47.5)	2.01 (1.05–3.86)	0.034

Of 57 PCR-positive subjects, 38 (66.7%) were only *positive* for *D. fragilis*, 12 (21.1%) had co-infections with *Blastocystis*, 4 (7.0%) with *Giardia lamblia* and 3 (5.3%) with *Entamoeba histolytica/dispar*. *Multivariable* logistic regression analysis showed that *D. fragilis* infection is independently associated with age below 20, lack of untreated water and presence of gastrointestinal *symptoms*, while animal contact and history of pinworm infection had non-significant associations with the disease (Table 5). The model provides adequate fit. =

Table 5. Multivariable logistic regression of factors associated with *D. fragilis* infection (n = 350)

Variable	aOR	95% CI	p value
Age <20 years (vs ≥20 years)	1.92	1.04–3.55	0.037
Untreated drinking water (vs treated)	2.71	1.38–5.31	0.004
Presence of GI symptoms (vs none)	2.29	1.06–4.95	0.035
History of pinworm (vs none)	2.05	0.93–4.52	0.075
Animal contact (vs none)	1.78	0.94–3.37	0.077
Semi-urban/rural residence (vs urban)	1.31	0.69–2.48	0.410

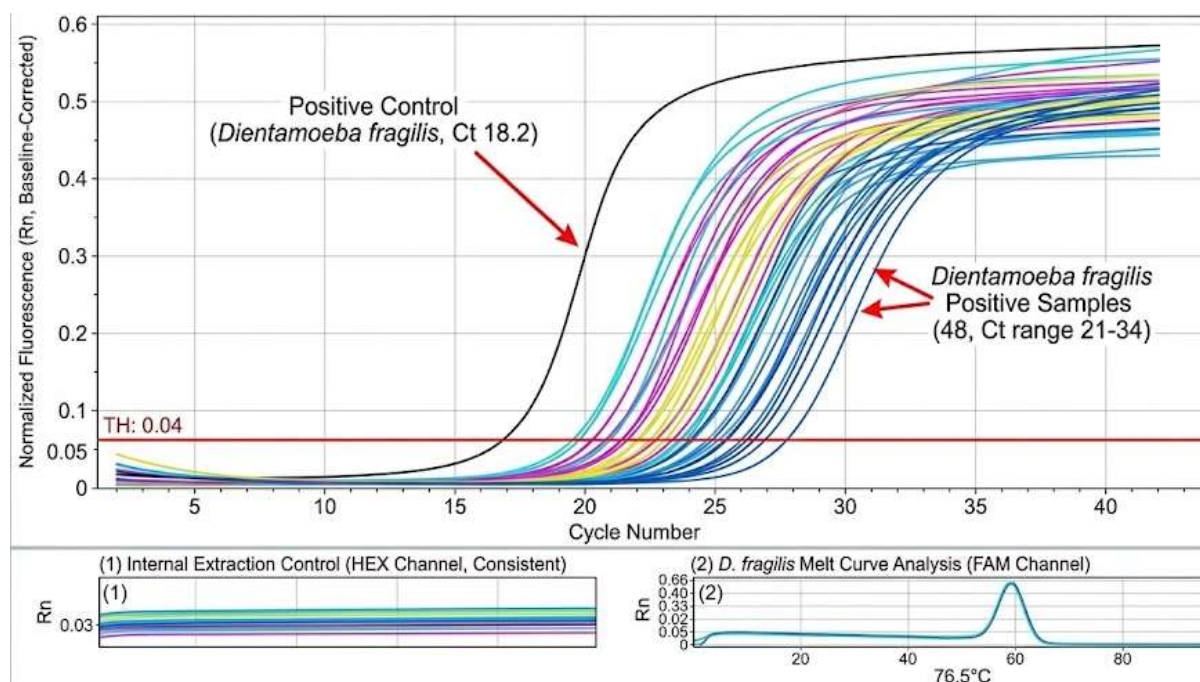


Figure 1. Detection of *D. fragilis* in stool samples by real-time PCR

Discussion

This study presents the first molecular estimation of *D. fragilis* frequency among patients accessing private clinics in Baghdad, and indicates that the parasite is common among residents. The finding suggests that only about 16.3% (23/30) of infections were detected by permanent-stain microscopy, while neither wet mount nor concentration methods, which are the most widely used laboratory methods in Iraq, managed to detect any infection. The results show that the overall observed prevalence is within a wide range of molecular prevalence estimates, similar to those conducted in Denmark, where *D. fragilis* was reported much more often via real-time PCR than previously estimated. In contrast, the same approach used in Australia in patients with gastrointestinal infections showed high infection rates with genotype 31/32. Other countries in the region have also reported evidence of the parasite in patients with diarrhoeal or gastrointestinal diseases or symptoms. However, comparative assessment of the results is complicated due to different age ranges and sample sizes used in the referred studies, as well as to different PCR targets used for the diagnosis and studied populations. The reported rates in symptomatic patients of 19.2% (32) general population with gastrointestinal symptoms in Turkey, which is far higher than those obtained with microscopy-based methods.

The comparison of diagnostic methods conducted in this research corroborates the findings of previous studies. The efficacy of microscopy of iron-haematoxylin stained spots was only 36.8% when compared with real-time PCR results. This means that, although the effectiveness of microscopy was good, there was a limited agreement between the two methods. This matches the findings of Stark et al, showing that both conventional and real-time PCR revealed more infections than microscopy and culture [25,27] as well as matches the validation of real-time assay demonstrated in [26], showing that the sensitivity of this diagnosis is far higher than that of microscopy when it comes to the analysis of feces. Lower Ct values in microscopy-positive samples indicate that microscopy is able to identify only the cases where parasitic load is significant, otherwise low-density infections do not detect. There are important implications of this methodology in Iraq, as majority of labs do not implement permanent staining and use saline and iodine preparations that are not suitable for fragile trophozoite [2,5]. Even in labs that have implemented permanent staining, one negative smear does not allow excluding the infection. Real-time PCR has both the advantage of being interpreted objectively, the ability to test frozen or preserved samples and using multipanel assays [28]. At the same time, it creates new issues regarding interpretation of results. High Ct levels in positive results can indicate low-level contamination, residual DNA after recovering from the infection or cross-reaction with other species [29]. We dealt with this issue by testing the results of the samples where the Ct was over 38 as well as

sequencing some positive samples, with all confirming the presence of *D. fragilis*. Laboratories implementing the assay within their own areas should apply similar confirmatory methodologies particularly in populations with frequent contact to animals, considering that the occurrence of shared genotypes between pigs and humans has been reported, and while the molecular epidemiology of the parasite still remains to be elucidated [11,12,35].

The most noteworthy clinical result from this study is that D. fragilis was identified more than twice as often in symptomatic patients in comparison to asymptomatic controls, with the correlation still holding true even after corrections for age, origin of drinking water, animal relations, pinworm history and the place of residence. Upon symptomatic patients, the presence of the infection was linked to abdominal pain, bloating/flatulence and long-lasting one or two weeks chronic symptoms, while diarrhea did not show any significance. The symptom constellation presented in this study is highly similar to that being described in the reviews and case reports, where abdominal pain and bloating are the most prominent clinical manifestations of dientamoebiasis, while diarrhea is less frequent. The case-control study conducted among children in Australia showed that infected children often developed abdominal pain and diarrhea, and the symptoms disappeared afterward [2,13,14 36]. Studies using multiplex molecular panels in case-control procedures due to gastroenteritis gave contradictory results: in one case, the Dutch study reported that D. fragilis was found more often in controls than in cases [37] and from another study, no association between the two was found [38]. Studies on children's health that took place in Belgium and the Netherlands have produced findings that do not support the theory that infection causes long-lasting abdominal pain [20,39], unlike in Denmark, where a research project suggested that managing the disease does not ameliorate the symptoms experienced by patients [24]. A contrasting view was expressed in a study performed in Sweden that pointed out a possible connection between the finding of a parasite in children under 11 years of age and gastrointestinal disorders suffered by them [40]. The Belgian analysis supports the theory suggested by the researchers that the impact of organism growth must be equal in both conditions [15]. As a consequence, the evidence from Baghdad can be considered as a new addition to the existing views of various researchers about the possible link between infection and signs in people.

There might be several explanations for the divergences between the findings of this study and the results of the previously mentioned European work. First, the groups of participants are different. There has been much attention paid to investigations concerning children living in communities or child-care facilities in Europe, where the cases of individuals being carrier of intestinal parasites are very common and most probably associated with earlier infections [22,23]. The research participants in the current work were patients aged one and over who had continuous access to medical services in different parts of a city with inadequate water supply and unregulated sanitation and had higher chances of contacting parasites. Second, choosing the suitable control group has a strong impact upon understanding the presence or absence of relationship. In our study, we took control group participants from the very same hospitals as people being under observation and did not rely on the previous symptoms indicated by them contrary to previous researchers who took control group participants from completely different background. Third, there can be other factors that we have failed to investigate [3,14 17,18,33 19]. Finally, one must also pay attention to the levels of infection burden because the lower Ct values for positives show that the degree of infection differs and a higher intensity of infection might be responsible for the manifestation of symptoms, although the study was not carried out with the aim of proving this point. D. fragilis might serve as a mark of the intestinal tract without functioning as a cause of a disease, the bacteria and viruses which we did not take into account in the current study can also have the same impact.

The analysis of risk factors established the significance of young age and the use of untreated drinking water as independent predictors of infection. The increased occurrence has been reported in children and teens on several occasions, and may be caused by close interaction, bad hygiene and household transmission [22,23,30]. The high correlation of infection and untreated water from tankers, wells or basins is also feasible because of the transmission suggested to take place via the faecal-oral route [6,7,10 8,9,10], and in Baghdad where many houses have to make use of the water from tankers or tanks that might be contaminated liquid. Contact with animals and experience of pinworm infection were also separately examined regarding co-infection. The finding concerning pinworm agrees with the proposal suggesting that Enterobius eggs allow parasitic transmission, and higher frequency of anal burning in individuals infected may reveal the presence of undiagnosed with the help of perianal tape testing cases of pinworm infection. The fact that Blastocystis has been the most frequent organism

found in *co-infection* matches the conclusions made in other places and indicates that the transmission was facilitated by means of similar routes [3,14].

The outcomes of this study impact both clinical and laboratory practice in Iraq. It is important for doctors caring for patients with ongoing abdominal pain, bloating, or chronic gastrointestinal issues, who have normal stool examinations, to recognize that *D. fragilis* could be present and that normal testing will not identify it. Laboratories should implement at least permanent staining of fixed samples, and molecular testing where possible, especially for patients with chronic symptoms. Nevertheless, a decision on whether to treat a positive result should consider the uncertainty of its pathogenicity and previous studies indicating that the elimination of the organism does not always lead to a clinical benefit (24). Treatment would be justified in cases of persistent symptoms and other potential conditions excluded, with follow-up to monitor clinical improvement and parasite clearance [16].

This research has several merits, including, but not limited to the application of a properly validated real-time PCR assay with appropriate sequencing confirmation; having a control group composed of asymptomatic cases recruited from the same clinic where subjects' cases were found; having a sample with blinded microscopy and having adjustments made for potential confounders. However, it is also subjected to several weaknesses. Only a single stool sample was collected from each study participant, which is likely to underestimate true prevalence, especially in terms of microscopy. Moreover, the research was conducted in the private clinics of Iraq where patients might differ in terms of their socioeconomic status from patients of public hospitals. Therefore, results might be non-generalizable to the entire population of Baghdad (Iraq). The case-control design doesn't allow the researchers to make any conclusions regarding the causes or the sequence of events regarding the studied infection. Bacterial and viral enteric pathogens are not assessed and, consequently, the study left *Enterobius* untested. Genotyping was not conducted on the obtained samples, which impeded identification of any differences in terms of clinical presentation between the studied genotypes. Finally, data on symptoms of patients were self-reported, thus creating a chance of the occurrence of a recall bias. The future studies should be based on several samples, a wider molecular panel with respect to enteric pathogens and genotyping as well as parasite load calculations and a longer follow-up in respect of treatment outcomes to make the conclusions about the role of the parasite in the studied population more obvious. 35

Conclusion

In Baghdad, real-time PCR detected *Dientamoeba fragilis* in approximately one out of six patients attending private clinics while conventional permanent-stain microscopy methods missed nearly of *D. fragilis* cases. Wet mount methods could not detect any cases. The prevalence was significantly higher in symptomatic cases when compared to asymptomatic controls, and it was associated with abdominal pain, flatulence, anal itchiness, and chronicity of symptoms. Being younger and drinking *untreated water* were independently associated with higher risk. These observations point out that *D. fragilis* is a neglected protozoan parasite that should be taken into account when treating patients with persistent and unexplained gastrointestinal symptoms. It is encouraged that permanent staining techniques are introduced in the diagnostic laboratories and also used when possible molecular methods. The safety of drinking water should be improved as well.

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