Are microscopy and culture enough to diagnose *Dientamoeba fragilis*: A preliminary study

Original Article

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ABSTRACT

Background: Detection of *D. fragilis* relies upon microscopic examination of permanently stained fixed stool smears, and/or culture. Although molecular diagnostic techniques, were developed for several pathogens, those for *D. fragilis* are not used routinely.
Objective: To evaluate usefulness of nested PCR in diagnosis of *D. fragilis*.
Patients and Methods: Fresh stool samples were collected from 100 children aged 6-12 y complaining of gastrointestinal disturbances. Samples were subjected to microscopic examination of iron hematoxylin stained smears, culture in Loeffler's medium and nPCR.
Results: The study detected *D. fragilis* molecularly in 4% of samples (4/100) and by microscopy and culture on Loeffler's medium in 2% (2/100). Molecular assay showed 100% sensitivity and specificity compared to microscopy (50%, and 95%, respectively), and Loeffler's culture medium (50%, and 100% respectively).
Conclusion: Nested PCR offers superior accuracy over microscopy and culture for diagnosis. In cases where PCR is not accessible in a diagnostic laboratory, at least two alternative diagnostic methods should be employed.

Keywords: diagnosis; dientamoebiasis; intestinal protozoa; loeffler's culture; molecular assay; trichomonad.

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INTRODUCTION

The enteric protozoan, *D. fragilis* remains vague and ignored[1]. After prolonged debate, and utilizing molecular studies, it was classified as a trichomonad flagellate, although it lacked external flagella[2]. Among the detected three forms of *D. fragilis*, trophozoites, the most common, are spherical or amoeboid and typically binucleate with variable diameter from 5 to 15 µm[3]. The most prominent hypothesis that was supported by several studies on parasite transmission is its transfer *via E. vermicularis* eggs[2,4].

Since the discovery of *D. fragilis*, arguments concerning its pathogenicity continued, mainly because the majority of dientamoebiasis cases were asymptomatic[3,5]. The lack of a suitable animal model obstructed research on its pathogenicity for decades. Numerous potential pathogenicity markers were described, involving amoeba pore-like proteins and immunomodulatory proteins[6,7]. However, members of the cathepsin L-like cysteine protease family were the most abundant virulence factor transcripts detected. Many of the cysteine protease transcripts recognized in *D. fragilis* closely resembled cytotoxic cysteine proteases previously identified as virulence factors in *T. vaginalis*[6].

The diagnosis of *D. fragilis* traditionally relied upon light microscopic examination of permanently stained stool smears. Sodium acetate acetic acid formalin (SAF) used in conjunction with modified iron hematoxylin was reportedly the best combination[8,9]. Meanwhile, biphasic Loeffler's medium in a microaerophilic atmosphere was recorded as the optimal environment for *D. fragilis* growth, especially when a new liquid overlay consisting of Earle's balanced salt solution was added[8]. However, molecular techniques, such as PCR are becoming the methods of choice, even though these tests are not used routinely by most diagnostic laboratories[10,11]. In addition to quantitative real-time PCR, nPCR assays were designed to be more successful than conventional PCR, especially for amplifying large-sized DNA products[12].

The present study aimed to detect *D. fragilis* among Egyptian children complaining of gastrointestinal illness by nPCR in comparison to direct microscopy and culture as an alternative.

PATIENTS AND METHODS

This cross-sectional study was conducted at the Medical Parasitology Department, Faculty of Medicine, Ain Shams University, and the Laboratory of Molecular Medical Parasitology (LMMP), Faculty of Medicine, Cairo University during the period from June 2017 to June 2018.

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Study design: Fresh stool samples were randomly collected from 100 children complaining of gastrointestinal disturbances. Stool samples were divided into three portions for microscopic examination, culture in a biphasic xenic medium, and nPCR.

Study population: Children, aged 6-12 y living in Naser Center, Beni-Suef Governorate, Egypt, and complaining of gastrointestinal disturbances. Exclusion criteria included acute watery diarrhoea, and fever to exclude the possibility of viral aetiology.

Collected samples: Stool samples were collected during community service campaigns over a period from June to August 2017. Samples were immediately transported in an ice box to our laboratory and divided into three portions for microscopic examination, culture in a biphasic xenic medium, and nPCR.

Microscopic examination: The first portion was processed in direct saline and/or iodine mounts, concentrated by the formol-ethyl acetate technique, and then stained by the modified Ziehl Nelsen stain (MZN). It was also stained with iron hematoxylin after SAF preservation[13].

Culture (Loeffler's medium): Approximately 10 mg of unpreserved stool sample was inoculated into Loeffler's medium and incubated at 37°C as previously described[14]. Confirmation of amoebic growth was performed with iron hematoxylin stained smears.

Molecular assay

1. **Extraction of DNA:** After thawing the fresh frozen stool samples, DNA extraction was performed using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with some modifications. The DNA concentrations were determined by using an ultraviolet (UV) spectrophotometer at a wavelength of 260 nm[15].

2. **Primers:** The primers used for the first step were DF400 (5´TATCGGAGGTGGTAATGACC3´) and DF1250 (5´CATCTTCCTCCTGCTTAGACG3´) to amplify SSU 18S-rRNA genes. The nested PCR primers were DFF2c (5'CGGGGATAGATCTATTTCATGGC3´) and DFR2c (5'CCAACGGCCATGCACCACC3´)[16]. Primers were titrated to determine the optimal concentrations using the stepwise increases in concentrations (50, 100, 200 and 400 nm).

3. **Amplification and detection:** We performed two-step amplification using the Arktik Thermal Cycler (Thermo Scientific, Germany). Using gel electrophoresis and UV trans-illumination, PCR products were visualized on a 1.5% agarose gel after ethidium bromide staining[17].

Statistical analysis: Data were analysed using SPSS package version 20 (SPSS Inc., Chicago, IL). Numerical data were expressed as the mean and standard deviation (SD). Qualitative data were expressed as frequencies and percentages. The Chi-square (X^2) test of 0.05 significance level was used to compare proportions between qualitative parameters. The degree of agreement among nPCR, microscopy and Loeffler's culture medium was determined using the kappa test.

Ethical considerations: Approval was obtained from the Research Ethical Committee of Ain Shams University with code number (MSO 02/2022) in accordance with the ethical standards put forth by the 1964 Declaration of Helsinki. Verbal consent was obtained from parents or guardians after clearly explaining the study objectives. All infected children received the appropriate treatment.

RESULTS

Out of 100 iron-hematoxylin stained stool smears, 7 samples (7%) were positive for *D. fragilis*. Out of 100 fecal samples that were cultured in Loeffler's culture medium, 2 (2%) were positive for *D. fragilis* trophozoites (Fig. 1). Out of the 100 DNA extracted stool samples, 4 samples (4%) were successfully amplified, while 96 samples gave negative results (Fig. 2).

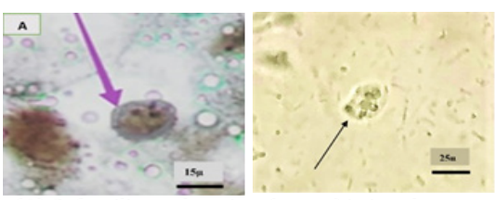

Fig. 1. (A) *D. fragilis* trophozoites (arrow) in iron hematoxylin stained stool smear with magnification (×1000), **(B)** *D. fragilis* trophozoite (arrow) in Loeffler's culture medium with highly refractive rice starch granules observed in the trophozoite cytosol.

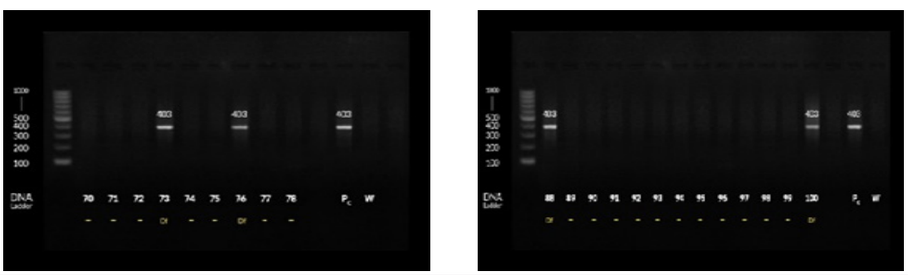

Fig. 2. Agarose gel electrophoresis showing PCR products of *D. fragilis* 18s rRNA gene (403 bp); **Pc:** Positive control; **W:** Negative control. Samples 73, 76, 88, and 100 are positive.

Out of 100 stool samples, 75 (75%) were parasite-free, and 25 (25%) had parasitic infections other than *D. fragilis* upon examination by the direct smear (saline and iodine wet mount). Of these, 8 had *G. lamblia*, 2 had *H. nana*, 12 had *Blastocystis* spp., and 3 had mixed infections (*G. lamblia* with *Blastocystis* spp.). After performing the formalin-ethyl acetate concentration technique, 63 (63%) were parasite-free and 37 (37%) had other parasitic infections, *i.e.*, 12 of the cases who had negative results by the direct smear method showed parasitic infections based on the concentration technique, staining with iron-hematoxylin and modified Ziehl Nelsen stains. They included 8 patients with *Microsporidium* spp., 3 patients with *Blastocystis* spp., and one patient with *G. lamblia* (Table 1).

Out of 100 stool samples, only one was positive by the three diagnostic methods. One case was positive by nPCR, and microscopy. One case was positive by nPCR, and culture. Discrepant results occurred in 6 samples; one was positive only by nPCR, and 5 cases were positive only by light microscopy (Table 2).

Table 1. Intestinal parasites other than *D. fragilis* in stool samples.

Intestinal parasites other than *D. fragilis*	Positive direct smear	Positive concentration technique
	No. (%)	No. (%)
G. lamblia	8 (8)	9 (9)
H. nana	2 (2)	2 (2)
***Blastocystis* spp.**	12 (12)	15 (15)
***G. lamblia and Blastocystis* spp.**	3 (3)	3 (3)
Microsporidium spp.	ND	8 (8)*
Total	**25 (25)**	**37 (37)**

ND: Not detected; ***:** Detected by MZN.

Table 2. Results of microscopy, culture and nPCR for *D. fragilis*.

No.	Microscopy	Culture	nPCR
1	+	+	+
1	+	-	+
1	-	+	+
1	-	-	+
5	+	-	-
91	-	-	-

Table (3) compares the results of microscopy and culture to those of nPCR (as a gold standard) for the detection of *D. fragilis* in stool samples. Out of 4 PCR positive cases, microscopy detected only 2 true positive cases and out of the 96 PCR true negative cases, microscopy showed 5 false positives, with a sensitivity and specificity of 50% and 95%, respectively. Additionally, culture detected 2 true positive cases with 50% sensitivity and 100% specificity. There was moderate agreement between nPCR and both culture and microscopy (K=0.41). There was a significant difference (P=0.003) between nPCR and both culture and microscopy for the detection of *D. fragilis* infection.

Table 3. Comparative evaluation of microscopy and culture with nPCR.

	nPCR		Sensitivity	Specificity	Statistical analysis	
	Positive (n=4)	Negative (n=96)			*P* value	*K* value
Microscopy						
Positive	2	5	50%	95%	0.003*	0.41@
Negative	2	91				
Culture						
Positive	2	0	50%	100%	0.003*	0.41@
Negative	2	96				

***:** Significant (P<0.05); **@:** Moderate agreement (0.41-0.60).

DISCUSSION

Parasitic infections of the gastrointestinal tract are widespread globally, particularly in Egypt, a developing country[18]. Treating dientamoebiasis mostly results in obvious clinical improvements. Therefore, detection of *D. fragilis* is clinically important for directing treatment, and preventing disease in the patients complaining of gastrointestinal symptoms without the presence of other detectable pathogens[19]. Traditional methods for detecting and differentiating the causative organisms, such as formalin-ether concentration and stained smears, as previously reported, lack accuracy compared to molecular assays[20]. This was demonstrated in an Egyptian study that employed molecular diagnosis of *D. fragilis* (using conventional PCR) in a localized area of Kafr El-Sheikh[11]. Furthermore, the use of nPCR increases the sensitivity to detect samples with low copy number DNA in comparison to any single round PCR[21]. Subsequently, nPCR was used in the present cross-sectional study to detect *D. fragilis* from children complaining of gastrointestinal disturbances, then

results were compared to those of light microscopy and culture.

The results of the microscopic examination showed that *D. fragilis* was not detected using wet mounts (with saline and iodine-stained) or formalin-ethyl acetate concentration techniques. However, using the iron hematoxylin stain yielded 7 positive samples, 5 from them were false-positive. This observation aligns with another study which indicated that prompt fixation and permanent staining are essential for the detection of *D. fragilis*[9]. However, morphological identification of *D. fragilis* trophozoites can be easily overlooked due to the pale staining of their nuclei, making them difficult to be distinguished from other protozoa such as *E. nana* and the amoeboid forms of *Blastocystis* spp.[19,22,23].

Regarding the culture media, several xenic culture systems were evaluated for *D. fragilis*. Loeffler's medium was selected because it is recommended as the optimal medium for cultivating *D. fragilis* under microaerophilic conditions at 42°C[14]. Results revealed 2 positive samples. This low sensitivity may be attributed to delays in the delivery of stool samples, overgrowth of other protozoa in the samples that inhibit *D. fragilis* replication[24], or limited growth in the initial culture tubes. Therefore, at least two passages should be performed in clinically suspected cases of dientamoebiasis. It has been reported that the detection rate of culture among patients with known *D. fragilis* infection was 40% and 80% after the first and second passages, respectively[24].

The comparative evaluation of microscopy to nPCR for the diagnosis of *D. fragilis*-infected patients revealed that microscopy had a sensitivity of 50% with moderate agreement and significant difference. These findings align with other studies that have reported low sensitivity of microscopy compared to nPCR[9,25]. After addition of the culture technique that also showed moderate agreement and significant difference in comparison to nPCR, the rate increases to 75%. It was claimed that the detection rate of *D. fragilis* has significantly improved with the use of culture methods. An Egyptian study conducted in 2015/2016 among 150 cases of irritable bowel syndrome reported an increase in the detection rate from 1.3% to 2% following the combination of microscopy and culture[26]. Additionally, in a study evaluating the role of culture in the absence of molecular methods, 104 samples from patients with negative smears were cultivated on three different culture media to detect *D. fragilis*. The detection rates increased from 0% to 10.6%[24]. The ability of nPCR to detect *D. fragilis* in one sample detected by microscopy and another one detected by culture was attributed to the fact that molecular techniques can detect the DNA from non-intact or non-viable organisms[27].

In conclusion, molecular techniques to detect *D. fragilis* unveiled that sole use of microscopy, or culture had underestimated detection rates. However, PCR is mostly an unaffordable technique for many laboratories as it requires expensive tools and large maintained supply of consumable products. If PCR is unavailable in a diagnostic laboratory, it is advisable to employ at least two different diagnostic methods for the detection of *D. fragilis* infection.

Authors contribution: Abou-Gamra MM proposed the study topic, designed the plan of work, supervised the practical part, and revised the manuscript. Tawfik RAS shared in designing the plan of work, helped with the practical part, analysed the data and revised the manuscript. Nazeer JT shared in choosing the topic and designing the plan of work, analysed the data, and wrote the manuscript. Sara FHA performed the practical part, analysed the data and shared in writing the manuscript. All authors accepted the final version of the manuscript before publication.

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