

Detection of *Dientamoeba fragilis* among diarrheal patients referred to Tabriz health care centers by nested PCR

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Received 18 October 2012; received in revised form 3 January 2013; accepted 14 January 2013

Abstract. *Dientamoeba fragilis* is a protozoan parasite of the human large intestine which is implicated as a cause of gastrointestinal diseases. The diagnosis of this parasite in direct smear preparations is difficult due to the lack of a cyst stage. The permanent staining method is generally used for diagnosis of *D. fragilis*, but the technique is laborious and time consuming. The purpose of this study was to evaluate the performance of PCR for detection of *D. fragilis* in clinical specimen of health care center in Tabriz, northwest of Iran. Stool samples of 1000 patients were collected from different laboratories and were immediately examined via wet mount and permanent staining methods. All positive samples and 55 randomly selected negative samples were studied by PCR technique. Using direct smear examination, no positive sample was found among 1000 stool samples, whereas 21 (2.1%) positive and 26 suspicious cases were reported in stained smears. PCR screening indicated that from 21 positive cases, 17 were positive by primary PCR, whereas nested PCR detected all 21 positive cases as well as 3 new positive samples from the suspicious cases (overall 24 (2.4%) positive samples), yet all negative cases remained negative through both stages of PCR amplifications. In comparison with nested PCR (if considered as gold standard), primary PCR showed 81% sensitivity and 100% specificity and those of microscopy was determined to be 87.5% and 100%, respectively. Considering the favorable sensitivity and specificity of nested PCR and its other advantages such as relative simplicity and speed this technique is proposed for rapid diagnosis of *D. fragilis* in clinical samples.

INTRODUCTION

Dientamoeba fragilis is a worldwide occurring protozoan parasite found in human's mucosal crypts of large intestine. *Dientamoeba fragilis* is a flagellate protozoan with no identified cyst stages (Schwartz & Nelson, 2003; Windsor *et al.*, 2006). It is capable of infecting the entire large intestine from cecum to rectum. The transmission mode of this parasite has so far remained unknown. However, fecal–oral transmission or the involvement of a vector,

possibly *Enterobius vermicularis* (pin worm), have been proposed (Burrows & Swerdlow, 1956; Yang & Scholten, 1977). *Dientamoeba fragilis* is often considered a harmless commensal organism but recent studies demonstrated that the infection caused by this parasite, may elicit various symptoms, which in most cases disappear with the elimination of the parasite by treatment with appropriate anti-microbial agents (Stark *et al.*, 2010). Diarrhoea, abdominal pain, abnormal stools, fatigue, loss of appetite, and weight loss are among the

symptoms experienced (Norberg *et al.*, 2003). Because of the lack of any cyst stage, diagnosis of the parasite can be carried out on freshly passed stool which needs the use of fixatives and permanent stains (Johnson & Clark, 2000; Windsor *et al.*, 2005). This aspect along with the extremely variable genetic diversity among different strains of *D. fragilis* has led to extremely diverse estimations of its prevalence rates, which is reported to vary between 0.4% and above 91% (Peek *et al.*, 2004; Stark *et al.*, 2005b). Definitive diagnosis of *D. fragilis* requires permanent staining of fixed fecal smears to reveal the characteristic structures of cell nuclei. The techniques are time consuming and require experienced laboratory personnel to discriminate between *D. fragilis* and other non-pathogenic protozoa such as *Endolimax nana*, as false-positive results may occur (Johnson *et al.*, 2004; Stark *et al.*, 2006b). Several studies have indicated that culture is more sensitive than permanent staining for diagnosis of *D. fragilis*. However, cultivation is technically difficult, time consuming, and not offered by routine diagnostic laboratories (Clark & Diamond, 2002). Molecular techniques offer the potential of a highly sensitive and specific alternative to traditional diagnostic approaches such as microscopy. It has been shown that regions of ribosomal DNA (rDNA) are ideal targets for PCR development, as they exhibit interspecies variability; While yet, these regions are among the most ubiquitous and conserved DNA sequences in nature (Stark *et al.*, 2005b, 2006a). In Iran, the reported prevalence rate determined by microscopy have been variable ranging from 0.5% to 2% in various parts of Iran (Rezaian & Hooshyar, 2006; Solaymani-Mohammadi *et al.*, 2006; Kia *et al.*, 2008; Ghazanchari *et al.*, 2012). Particularly in Tabriz, there has been only one limited study that estimated 13% prevalence rate of *D. fragilis* by permanent staining (Jamali, 2004). Our study is the first in Tabriz and second (after the study by Ghazanchari *et al.*) in Iran that makes use of both microscopy and nested PCR to determine the prevalence of *D. fragilis*.

MATERIALS AND METHODS

Stool samples of 1000 patients, collected from different laboratories of Tabriz, were investigated for intestinal parasites. A questionnaire was completed for each case, concerning clinical symptoms. Fresh stool specimens (within 30 min of passage) were examined microscopically and immediately fixed in PVA (Poly Vinyl Alcohol) fixative. Fixed specimens were transported to protozoology laboratory of Tabriz faculty of Medicine and stained with trichrome, a permanent stain. Definitive diagnosis of *D. fragilis* was based on the morphology of parasites observed in the permanently stained smears. The organisms that fulfilled this criterion were considered to be *D. fragilis* and their relevant stool specimens were described as 'positive samples'. All microscopically examined samples that were reported positive or suspicious, along with randomly selected negative specimens underwent DNA extraction. Stool specimen sediments (200 µl) were used for DNA extraction (Peymani *et al.*, 2012). This was achieved by means of stool DNA extraction kit (Bioneer, Korea) according to the manufacturer's instruction. PCR amplification with *Taq* DNA polymerase was done under standard conditions using the following primers (Stark *et al.*, 2005 b): DF400: 5'- TATCGGAGGTGGTAATGACC-3' and DF1250: 5- CATCTTCCTCCT GCTTA GACG-3'. These primers amplify trichomonad small-subunit 18S-rRNA genes. Amplification was achieved through 40 cycles including one cycle of 94°C for 4 min and, thirty-eight cycles of 94°C for 1 min, 58°C for 1 min, and one 72°C for 1 min followed by final extension cycle at 72°C for 5 min. The desired 886 bp fragments, amplified via PCR, were visualized through 1% agarose gel electrophoresis and ethidium bromide staining. The nested PCR was performed on 1µl of the primary PCR product using primers (designed in this study): DFF2: 5'-CGGGATAGATCTATTTTCATGGC-3' and DFR2: 5'-CCAACGGCCATGCACCACC-3'. Amplification was achieved through 30

cycles including one cycle of 94°C for 2 min and , thirty cycles of 94°C for 30 sec, 58°C for 30 sec, and one 72°C for 30 sec followed by final extension cycle at 72°C for 5 min. The desired 403 bp fragment, amplified via PCR, was detected through 1.5% agarose gel electrophoresis, using appropriate size marker.

RESULTS

Patients and clinical symptoms

One thousand samples from people with an age range between 6 months to 70 years (mean= 28.5 years) of age were included and examined for intestinal parasites. The results of this study demonstrated that infection caused by *D. fragilis* evokes gastro-intestinal symptoms among them diarrhoea and abdominal pain were the most common symptoms reported.

Direct smear examination and permanent staining

Screening by wet mount method failed to detect any *D. fragilis*. Thus, definitive diagnosis of *D. fragilis* was based on the morphology of parasites observed in the permanently stained smears. In stained smears, 21 positive samples (2.1%) and 26 suspicious cases were recorded (Table 1).

Primary PCR and nested PCR

Screening by primary PCR detected 17 cases of the 21 positive samples. However, none of the suspicious or negative samples was recorded as positive by this technique. In nested PCR, all of 21 trichrome positive samples and 3 cases of suspicious samples were recorded as positive. None of 55 randomly selected negative samples were positive by this method. Overall, 24 cases out of 1000 samples (2.4%) were detected as positive by nested PCR (Table 2; Figures 1 & 2).

Other parasites found

There were also other parasites present in our samples, some of them in co-infection with *D. fragilis*. *Entamoeba coli*, *Giardia*

intestinalis, *Iodamoeba butschlii*, and *E. nana* were found in this study. *Entamoeba coli* also showed the highest co-infection rate with *D. fragilis* (Table 3).

Table 1. Clinical signs of patients infected by *D. fragilis*

Clinical signs	Positive cases	Suspected cases
Diarrhoea	21	17
Abdominal pain	19	8
Loss of appetite	11	5
Constipation	2	0

Table 2. Comparison of trichrome, PCR and nested PCR methods for detection of *D. fragilis*

	Trichrome	
	Positive cases	Suspected cases
Primary PCR	17	0
Nested PCR	21	3

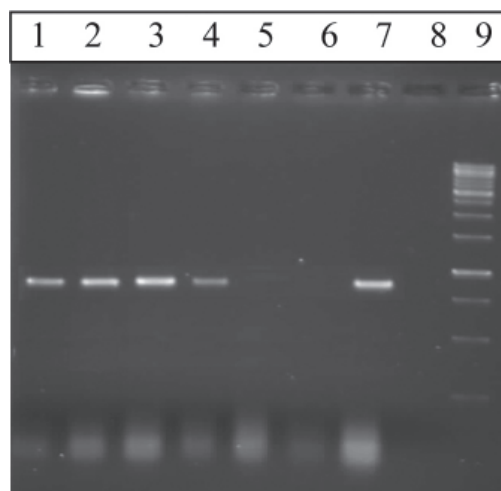


Figure 1. Results of primary PCR for detection of *D. fragilis*

Lane 1-4: 4 positive samples, lane 5 & 6: negative samples, lane 7: positive control, lane 8: no DNA, lane 9: 1kb DNA ladder

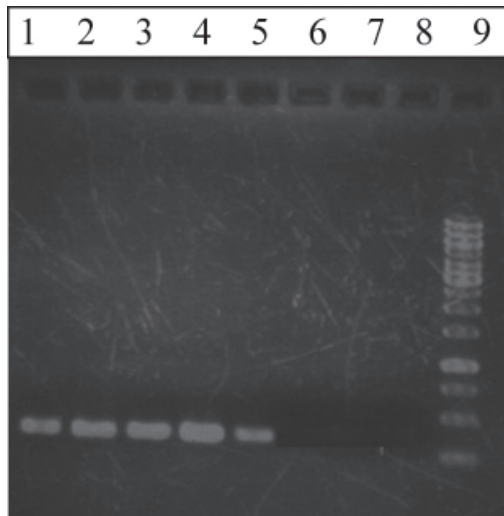


Figure 2. Results of nested PCR for detection of *D. fragilis*. Lane 1-5: positive samples; lane 6 and 7, negative samples; lane 8, negative control; Lane 9, 1kb DNA ladder

Table 3. The frequency of different parasites and their co- infection with *D. fragilis*

Parasites	Pos. (%)	Co- inf. (%)
<i>Entamoeba coli</i>	39 (3.9)	5 (0.5)
<i>Giardia intestinalis</i>	38 (3.8)	0
<i>Iodamoeba butschlii</i>	29 (2.9)	4 (0.4)
<i>Endolimax nana</i>	27 (2.7)	3 (0.3)
<i>Entamoeba histolytica</i>	2 (0.2)	0
<i>Blastocystis hominis</i>	17 (1.7)	2 (0.2)
Oxiurus Egg	3 (0.3)	0
Ascaris Egg	2 (0.2)	0
Trichocephal Egg	1 (0.1)	0

DISCUSSION

Dientamoeba fragilis is a pathogenic protozoan parasite of the human large intestine. Due to lack of any cyst stages, diagnosis of *D. fragilis* via direct smear examination has been unreliable. Microscopy, using fixative and permanent staining, is time consuming and a high level of expertise is required for screening and differentiation of the parasites. Molecular techniques, therefore, could be highly sensitive and specific alternatives for conventional diagnostic approaches such as

microscopy (Peek *et al.*, 2004). Only few studies to date have investigated the potential of fecal DNA detection as a diagnostic method for *D. fragilis* infection through PCR. The aim of this study was to evaluate the performance of PCR method for detection of *D. fragilis* among diarrheal patients referred to Tabriz health care centers, in Iran. By trichrome staining, the prevalence of *D. fragilis* was found to be 2.1% (21 patients out of 1000). In comparison, primary PCR was positive in 17 cases, whereas nested PCR (secondary PCR) not only confirmed all the 21 trichrome staining positive samples (100% sensitivity), it also found 3 other positive cases from suspicious samples. Thus, the prevalence rate was 2.4% after nested PCR amplification. Considering nested PCR as gold standard, microscopy showed 87.5% sensitivity and 100% specificity whereas the sensitivity and specificity of primary PCR were 81% and 100%. These findings are consistent with those of other studies which have reported PCR sensitivities of 85% to 93.5% and specificities of 93% to 100% (Stark *et al.*, 2005b, 2006a; Verweij *et al.*, 2007). Stark *et al.* reported that microscopy had 92.4% sensitivity and 98.7% specificity (Stark *et al.*, 2006a), which is almost in agreement with our results. There may be explanations for relatively high sensitivity of microscopy and unexpected lower sensitivity of PCR. In the context of microscopy, the amount of time, professional effort, and care routinely devoted by microscopists to fulfill the accurate diagnosis of the desired parasite, might be the cause (Stark *et al.*, 2006a). Since *D. fragilis* generates no cyst, it dies quickly after being excreted. Consequently, any delay in DNA extraction and PCR amplification can lead to loss of the DNA content, as DNA molecules degraded with time lapse. This may account for the lowered sensitivity of PCR technique and highlights the importance of using fresh stool specimens and swift performance. Among the parasites found other than *D. fragilis*, *E. coli* was the most frequent, with thirty nine cases (3.9%) detected by wet mount method; *Giardia* (3.8%), *I. butschlii* (2.9%), and *E. nana* (2.7%) were respectively the 2nd, 3rd and 4th common parasites in our samples, respectively.

The transmission mode of *D. fragilis* has so far been unclear. The high incidence of *D. fragilis* and *E. vermicularis* occurring together suggested that *E. vermicularis* may be involved as a vector in *D. fragilis* transmission (Burrows & Swerdlow, 1956; Yang & Scholten, 1977). However, results of some recent studies have contradicted this presumptive correlation (Stark *et al.*, 2005a, 2010). In our study, no *E. vermicularis* was found in *D. fragilis* positive samples and the data indicating co-infection of *D. fragilis* with some other parasites does not show any significant relation as well.

Several studies suggested that infection by *D. fragilis* may evoke various clinical symptoms (Cerva *et al.*, 1991; Cuffari *et al.*, 1998; Windsor & Macfarlane, 2005; Lagace-Wiens *et al.*, 2006; Rayan *et al.*, 2007). Our results, also, indicated that this parasite was associated with different clinical signs most frequently with diarrhoea, abdominal pain, fatigue, loss of appetite, and flatus. Twenty-one and 19 patients out of the 24 patients who were positive for *D. fragilis*, had diarrhoea and abdominal pain, respectively. Hence, we strongly recommend that patients who complain of diarrhoea and/or abdominal pain be checked for infection by *D. fragilis*.

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