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DNA of *Dientamoeba fragilis* detected within surface-sterilized eggs of *Enterobius vermicularis*

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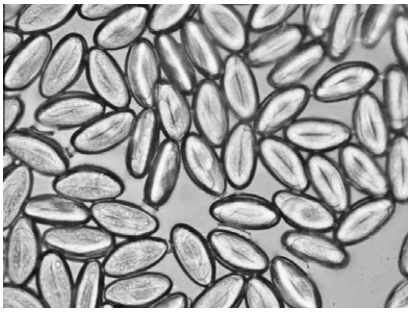
HIGHLIGHTS

- Eggs of *Enterobius vermicularis* were surface-sterilized using hypochlorite.
- DNA was extracted from individual eggs.
- *Dientamoeba fragilis* and *E. vermicularis* PCR was performed on DNA extracts.
- Sequencing of PCR amplicons showed evidence of *D. fragilis*-specific DNA.
- Results support hypothesis of *D. fragilis* transmission by *E. vermicularis*.

GRAPHICAL ABSTRACT



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ABSTRACT

With no evidence of a cyst stage, the mode of transmission of *Dientamoeba fragilis*, an intestinal protozoon of common occurrence and suggested pathogenicity, is incompletely known. Numerous studies have suggested that eggs of intestinal nematodes, primarily *Enterobius vermicularis* (pinworm), can serve as vectors for *D. fragilis*, although attempts to culture *D. fragilis* from pinworm eggs have been unsuccessful and data from epidemiological studies on *D. fragilis*/pinworm co-infection have been conflicting.

The aim of this study was to investigate whether we could detect *D. fragilis* DNA from pinworm eggs collected from routine diagnostic samples (cellophane tape) and surface-sterilised by hypochlorite. DNA was extracted from individual eggs and tested by PCR using *D. fragilis*- and *E. vermicularis*-specific primers; amplicons were sequenced for confirmation.

In cellophane tape samples from 64 patients with unknown *D. fragilis* status we detected *D. fragilis* DNA in 12/238 (5%) eggs, and in a patient known to harbour *D. fragilis* we detected *D. fragilis* DNA in 39/99 (39%) eggs.

The finding of *D. fragilis* DNA within eggs of *E. vermicularis* strongly supports the hypothesis of *D. fragilis*-transmission by pinworm and has implications for antimicrobial intervention as well as control and public health measures.

1. Introduction

Dientamoeba fragilis is a common yet little studied intestinal protozoon suspected of causing gastrointestinal illness, particularly in children (Windsor and Johnson, 1999; Johnson et al., 2004; Stark et al., 2010a; de Wit et al., 2001). Despite being discovered nearly a

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century ago (Jepps and Dobell, 1918), research on *D. fragilis* has been scarce, in part due to diagnostic difficulties (Calderaro et al., 2010), but also due to its uncertain pathogenicity and clinical significance (Gijsbers et al., 2011). While some recent publications advocate for the inclusion of this flagellate in the list of enteric pathogens to be tested for in patients with gastrointestinal illness (Stark et al., 2010a; Barratt et al., 2011a), there is still a lack of conclusive evidence of pathogenicity and the mechanisms hereof.

Since *D. fragilis* has no known cyst stage and trophozoites degrade rapidly in stool samples (<48 h) (Hakansson, 1936; Barratt et al., 2011b), traditional microscopy of faecal concentrates is unlikely to detect *D. fragilis* (Johnson et al., 2004), and molecular diagnostics are preferred because of higher sensitivity and specificity (Stensvold and Nielsen, 2012; Stark et al., 2010b). A recent review on reported prevalences of *D. fragilis* worldwide showed a prevalence of 0.3–52%, depending on cohort investigated and diagnostic method used (Barratt et al., 2011a). In Danish patients suspected of enteroparasitic disease, Stensvold et al. (2007a,b) found a *D. fragilis* prevalence of 11.7% using a permanent staining technique (Stensvold et al., 2007a), however, a recent Danish study using real-time PCR found a *D. fragilis* prevalence of 10–70%, depending on age group (unpublished data).

The mode of transmission for *D. fragilis* is still unknown, and there is neither evidence of a (pseudo-)cyst stage for *D. fragilis*, nor has faecal–oral transmission by trophozoites been shown to occur (Johnson et al., 2004; Barratt et al., 2011b).

For decades it has been suggested that eggs of nematodes, particularly *Enterobius vermicularis* (pinworm), could serve as vectors for *D. fragilis* (Burrows and Swerdlow, 1956; Ockert, 1972; Girginkardesler et al., 2008) (Fig. 1).

Transmission of protozoa via helminth eggs has previously been shown for the bird helminth *Heterakis gallinae* (s. *gallinarum*) and the protozoon *Histomonas meleagridis* (Smith and Graybill, 1920; Ruff et al., 1970), an organism phylogenetically closely related to *D. fragilis* (Gerbod et al., 2001). Like *D. fragilis*, pinworm has a global distribution and a high prevalence among children (Burkhart and Burkhart, 2005; Lacroix and Sorensen, 2000; Barratt et al., 2011a), and previous studies have used epidemiological data to test for association between *D. fragilis* and *E. vermicularis*. However such studies can neither confirm nor reject vector transmission of *D. fragilis* by pinworm, as they are cross-sectional and cannot take into account differences in infection duration, making determination of cause and effect difficult.

The lifecycle and transmission of *D. fragilis* was recently reviewed by Barratt et al. (2011a), who found the pinworm vector hypothesis controversial but plausible, and called for studies employing PCR, DNA sequencing and electron microscopy to substantiate findings (Barratt et al., 2011b).

In a previous attempt to test this hypothesis using molecular diagnostics, Menghi et al. (2005) successfully amplified pinworm and *D. fragilis* DNA from a solution of pinworm eggs, but were unable to amplify *D. fragilis* DNA once the eggs had been treated with DNase, indicating that the amplified *D. fragilis* DNA had been located on the surface of the eggs only, and not within (Menghi et al., 2005). In the present study we present a method that enables reliable detection of *D. fragilis* DNA within the eggs of helminths. Moreover, we report for the first time the successful detection of *D. fragilis* DNA inside eggs of *E. vermicularis*, endorsing the hypothesis that pinworm may indeed serve as a vector for *D. fragilis*.

2. Materials and methods

2.1. Method development

Since our aim was to be able to detect DNA of *D. fragilis* potentially lodged inside individual pinworm eggs, a method was developed for surface-sterilizing and collecting individual eggs, while preserving sufficient DNA within the egg to be detectable by PCR.

Hypochlorite (HClO) is a bactericidal agent known to induce damage to DNA, RNA, and polynucleotides (Hawkins and Davies, 2002), and has previously been used to remove the uterine, but not the chitin layer, of nematode eggs (Oksanen et al., 1990).

In order to determine the HClO concentrations sufficient to degrade DNA, we prepared eight solutions of 1 mL HClO (10^{-2}–10^{-9}) (Sodium Hypochlorite 1%, BDH PROLABO), to which we added 1 μL (undiluted) DNA extracted from an adult female pinworm using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) with a 100 μL eluate. From these initial eight HClO-DNA solutions we then prepared 10-, 100- and 1,000-fold dilutions, using DNase/RNase

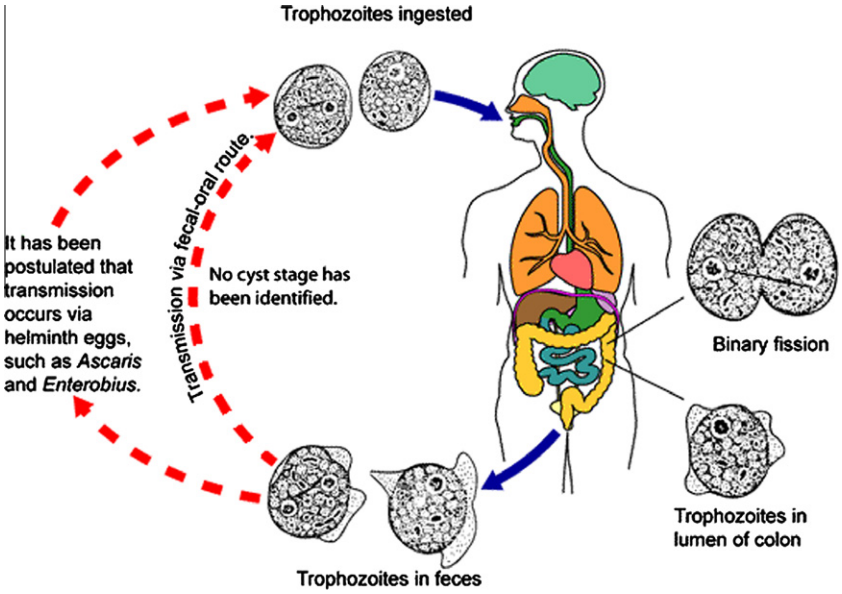

Fig. 1. The presumed life cycle of *D. fragilis*. Transmission of *D. fragilis* by faecal–oral route and via nematode eggs (e.g., *Ascaris*, *Enterobius* spp.). There is currently no evidence of an infective cyst or a pseudocyst stage for *D. fragilis*, nor has faecal–oral transmission by trophozoites, the only known stage of *D. fragilis*, been shown to occur. Life cycle image courtesy of CDC–DPDx; shown with modifications.

Table 1
Hypochlorite trials; determination of hypochlorite (HClO) concentrations sufficient to degrade DNA of *E. vermicularis*. Row A1 shows initial concentrations of HClO (1% = 10^{-2}), row A2 shows initial concentration of pinworm DNA (1 μL undiluted DNA added to 1 ml 1% HClO i.e. 0.1% = 10^{-3}) and row A3 shows obtained PCR products/presence of gel band. Rows B1–B3, C1–C3 and D1–D3 show 10-, 100- and 1000-fold dilutions (using DNase/RNase free water) of the HClO/pinworm DNA solutions from row A1–A2 and obtained PCR products. Rows E1–E2 show obtained PCR products from the pinworm DNA concentrations used, without added HClO.

Initial solutions									
A1	HOCl conc.	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
A2	Pinworm DNA conc.	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}
A3	PCR product	–	–	+	+	+	+	+	+
10-fold dilution									
B1	HOCl conc.	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
B2	Pinworm DNA conc.	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
B3	PCR product	–	–	+	+	+	+	+	+
100-fold dilution									
C1	HOCl conc.	10^{-4}	10^{-5}	10^{-6}	10^{-75}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
C2	Pinworm DNA conc.	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}
C3	PCR product	–	–	+	+	+	+	+	+
1000-fold dilution									
D1	HOCl conc.	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
D2	Pinworm DNA conc.	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}
D3	PCR product	–	–	+	+	+	+	+	+
Pinworm DNA conc.									
E1	Pinworm DNA conc.	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
E2	PCR product	+	+	+	+	+	+	+	

Table 2
Primers used for amplification of DNA from *D. fragilis* and *E. vermicularis*.

Organism	Primer name	Primer sequence	Gene	PCR product size (in base pairs (bp))
D. fragilis	DFpn_1f	5′-GCC AAG GAA GCA CAC TAT GG-3′	5′-Terminal of the SSU rRNA gene (GenBank accession No. AY730405)	364 bp
	DFpn_364r	5′-GTA AGT TTC GCG CCT GCT-3′		
E. vermicularis	EVpn_1f	5′-CAA CAC TTG CAC GTC TCT TCA-3′	5′-Terminal end of the 5S region of rRNA gene	195 bp
	EVpn_195r	5′-ATT GCT CGT TTG CCG ATT AT-3′		

free water, in order to show if subsequent lowering of the HClO concentration would result in PCR amplification (Table 1).

We then incubated all solutions for 10 min at room temperature, performing PCR with a 5 μL template and standard PCR conditions and using primers targeting *E. vermicularis* (Table 2).

Two methods were employed for washing eggs after HClO incubation and for DNA isolation/membrane rupture. The first method included washing the entire HClO-egg solution (multiple-egg washing) by centrifugation, aspirating the supernatant and washing the pellet with DNase/RNase-free water to lower the HClO concentration below 10^{-4} (=0.01%), the threshold concentration as determined in our hypochlorite trials. Single eggs were then subjected to proteinase K treatment and subsequently PCR was performed (see below). The second method employed washing single eggs (single-egg washing) by transferring a single egg (within 1 μL 10^{-2} HClO) into a solution of lysis buffer and proteinase K (both provided with the QIAamp DNA Mini kit, QIAGEN, Hilden, Germany), performing the extraction according to the instructions of the manufacturer, which also lowered the HClO conc. below 10^{-4} before PCR.

Both methods proved feasible and enabled successful amplification and sequencing of *E. vermicularis* DNA, though multiple-egg washing resulted in a greater loss of eggs (through aspiration), and single-egg washing proved more laborious. We also noted that proteinase K was unable to rupture eggs not treated with HClO, and that washing with DNase/RNase-free water was not sufficient to remove DNA contaminants from eggs (data not shown).

2.2. Samples

Between Dec. 1st 2010 and Dec. 1st 2011 we collected clinical samples from Danish patients (cellophane tape) at Statens Serum Institut. We analysed each sample for egg density using light microscopy, discarding samples with <10 eggs per visual field, as it proved difficult to harvest a sufficient number of eggs from such samples. We collected a total of 64 individual samples from 64 patients with unknown *D. fragilis*-status, and 1 sample with a high egg load (>1,000 eggs per visual field) from a patient known to harbour *D. fragilis* (confirmed by real-time PCR as previously described (Stensvold et al., 2007b; Stensvold and Nielsen, 2012)). The cellophane tape samples were processed averagely 36 days after sampling.

2.3. Egg recovery and surface sterilization

Eggs were scraped off cellophane tape samples using a scalpel and transferred to a small glass jar containing 1 mL 10^{-2} HClO and incubated for 10 min at room temperature.

2.4. Egg washing and DNA isolation

In samples from patients with unknown *D. fragilis*-status, we used single-egg washing, transferring individual eggs suspended in 1 μL 10^{-2} HClO into a 1.5 mL Eppendorf tube, using a micropipette and a light microscope (Nikon SMZ6454, Nikon Nordic, Copenhagen, Denmark), thus visually confirming the presence of one egg. Between 1 and 16 individual eggs from each sample were collected, depending on the number of eggs harvested into the glass jar. To each tube, 180 μL of ATL buffer and 20 μL of proteinase K (QIAamp DNA Mini kit) were added, and the remaining steps in the DNA extraction were carried out according to the recommendations of the manufacturer; however, only 20 μL AE buffer was used to elute the DNA in the final step.

In the sample from the patient known to harbour *D. fragilis*, we used multiple-egg washing (Section 2.1), transferring the entire

content of the glass jar to a 1.5 mL Eppendorf tube, and washing × 3 with 1 mL DNase/RNase free water (centrifugation at 1,500*g* for 5 min). After the final wash, we aspirated 10 μL of the washing water from the supernatant, and used this as control for DNA contamination of the water, potentially caused by hatched or crushed eggs. Individual eggs were transferred in 1 μL of fluid into 0.2 mL PCR tubes using a micropipette and a light microscope, visually confirming the presence of one egg, and preparing a total of 100 single eggs. To each PCR tube we added 1 μL Proteinase K (QIAGEN, Hilden, Germany) and 8 μL water, spun the mixture (containing one egg in 10 μL) briefly (5 s), and incubated it at 50 °C for 30 min, then 100 °C for 15 min.

2.5. PCR and sequencing

For conventional PCR, we used the following conditions: initial denaturation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s; and a final elongation step at 72 °C for 5 min (Primus HT, Biotech, Ebersberg, Germany). From DNA extracted by use of the Qiagen protocol, we used a template of 4 μL (of 20 μL eluate from one pinworm egg) and from the proteinase K extractions, we used 5 μL (of 10 μL solution from one pinworm egg). PCR components were as follows: 2.5 μL of 10× PCR Rxn Buffer (Invitrogen®, Tåstrup, Denmark), 1 μL of dNTPs (1.25 mM/base, Roche®, Hvidovre, Denmark), 1.75 μL of $MgCl_2$ (50 mM, SIGMA®, Brøndby, Denmark), 1 μL of each primer (10 pmol/μL, TAG Copenhagen A/S, Copenhagen, Denmark), 0.2 μL of Platinum® Taq DNA Polymerase (5 U/μL, Invitrogen®, Tåstrup, Denmark), in a total volume of 25 μL. Positive (DNA) and negative (H_2O and water from the washing step) controls were included in all runs. For electrophoresis we used 5 μL of the 25 μL PCR volume, and the remaining 20 μL were used for sequencing, which was done at least uni-directionally (Eurofins MWG Operon, Ebersberg, Germany).

3. Results

3.1. Hypochlorite trials

We found that a solution of 1% HClO was sufficient to prevent PCR amplification of *E. vermicularis* DNA, and that agarose gel bands indicating positive PCR reactions re-appeared at a threshold concentration of 10^{-4} HClO. Also, subsequent lowering of the HClO concentration did not result in PCR amplification, showing that that failing amplification was due to DNA degradation and not PCR inhibition by HClO (Table 2).

3.2. Samples from patients with unknown D. fragilis status

A total of 238 single eggs were collected from 64 patients, and 14/238 (6%) eggs tested positive for *D. fragilis* (Table 2). Of these, 11 specific amplicons were successfully sequenced and shown to be identical to GenBank acc. no. AY730405 (*D. fragilis* genotype 1) (Johnson and Clark, 2000); one was identical to GenBank U37461 (*D. fragilis* genotype 2) and in the remaining 2/14, the genotype could not be called because of insufficient sequence quality. In total, 8/64 (13%) patients were found to harbour *D. fragilis* DNA within pinworm eggs collected from their sample.

3.3. Sample from patient known to harbour D. fragilis

A total of 100 single eggs were collected and 39/100 (39%) tested positive for *D. fragilis* by PCR. Of these, 39/39 (100%) had specific amplicons successfully sequenced and shown to be identical to GenBank AY730405 (*D. fragilis* genotype 1). No *E. vermicularis* or *D. fragilis*-specific products were produced by PCR on DNA extracted from washing water, and 99/100 of the single eggs tested positive for *E. vermicularis* by PCR. We also used a *D. fragilis* real-time PCR on a selection of eggs, showing a mean Ct of 35.6 (30, 3–40, 9) for *D. fragilis* positive eggs (data not shown), indicating only few gene copies present within individual eggs.

4. Discussion

The present study is, to our knowledge, the first to demonstrate the presence of *D. fragilis* DNA inside eggs of *E. vermicularis*, allowing us to presume that *D. fragilis* can be transmitted by a vector, in this case via eggs of *E. vermicularis*.

The rationale behind this presumption is the repeated detection of *D. fragilis* DNA from pinworm eggs, which had been surface-sterilized prior to DNA extraction using a solution of hypochlorite in concentrations shown to render DNA non-amplifiable due to DNA damage. In addition, since water from the final washing steps was PCR-negative for both *E. vermicularis* and *D. fragilis*, we conclude that *D. fragilis* DNA amplified from DNA extracts from the surface-sterilised eggs must have been present inside the pinworm eggs and not on the surface. It is possible that intact *D. fragilis* trophozoites (or an undetected cyst stage) can stick to the surface of an egg, as suggested by Menghi et al. (2005), who noted amoeboid-like structures on the surface of the eggs. However, we consider this unlikely, given that *D. fragilis* normally degrades rapidly (<48 h) once passed from the intestine, and since the mean time from sampling to processing of the cellophane tape samples was 36 days. Also, any organism adherent to the surface of the egg would still be subjected to the damaging effect of the hypochlorite.

Menghi et al. (2005) collected eggs by emptying the uteri of five female pinworms, four of which had been collected from patients with *D. fragilis* co-infection, and one from a patient without *D. fragilis* co-infection. The authors divided the egg solutions, treating one part with DNase to degrade DNA present on the surface and left another solution untreated. The DNase treated eggs were ruptured using a combination of NaOH/2-mercaptoethanol incubation and high-velocity centrifugation (10,000 rpm), and PCR was performed on both solutions using *E. vermicularis*- and *D. fragilis*-specific primers, showing amplification of both pinworm and *D. fragilis* DNA from eggs untreated with DNAse, but only amplification of pinworm DNA, once the eggs had been treated with DNase, indicating that amplified *D. fragilis* DNA did not stem from within the eggs (Menghi et al., 2005).

The fact that the authors' detected *D. fragilis* DNA prior to the DNase treatment of the eggs does not necessarily imply that the worms investigated were responsible for transmission of *D. fragilis*, as the detected DNA could have originated from *D. fragilis*-positive faeces. The authors commented on the risk of contamination, but dismissed it, as all worms had successively been washed with water and stored in RPMI 1640 (a cell culture medium). However, in the present study we found that washing with DNase/RNase free water instead of HClO was not sufficient to remove DNA contaminants from the eggs, and have no reason to assume this would not be the case for an adult worm. Moreover, in the present study we detected *D. fragilis* DNA in only a fraction of the eggs (39%) from a co-infected patient, and it could be hypothesised that the fraction of eggs harbouring *D. fragilis* could vary, depending on the pinworm and/or *D. fragilis* genotype.

The findings of the present study also warrant a consideration of possible DNA contamination. Faecal content could contaminate the cellophane tape samples used; however, since we harvested eggs directly into HClO in concentrations shown to damage DNA, and since we subsequently transferred eggs individually in as little

as 1 μL of fluid, we consider this mode of contamination unlikely. Contamination by ruptured/hatched egg contents is also a possibility, as our multiple-egg washing protocol employed several successive centrifugation steps of the egg solution. It could be speculated that such a treatment could damage a portion of the eggs, allowing DNA to contaminate the egg-solution. To address this, we performed PCR on the water from the last washing step, which tested negative for both pinworm and *D. fragilis* DNA. However, as was the case for the high density sample, the wash water was removed first, and the time-consuming process of picking up the eggs individually was done afterwards, with the possibility of an untimely hatching of an egg causing contamination of the egg-solution. However, any such false-positive eggs would still represent DNA originally found within a pinworm egg, while the reported fraction of pinworm eggs harbouring *D. fragilis* in a co-infected patient (39%) might be inflated.

Epidemiological studies reporting either higher than expected co-infection of pinworm and *D. fragilis*, or no association at all, should be interpreted with caution. Since such studies rarely adjust for age, and given that both parasites occur more commonly in children than adults, the observed co-infection can represent a significant source of bias when not age-adjusted. Also, without a longitudinal setup, a difference in duration of infection becomes important, as a shorter time to spontaneous remission of one of the parasites could potentially mask an association. Not all *Enterobius* may harbour *D. fragilis* and even if they do, the present data suggest that eggs from such worms may not all harbour *D. fragilis*.

While our findings strongly support the hypothesis of *D. fragilis* transmission by a pinworm vector, many issues remain to be resolved regarding the lifecycle of *D. fragilis*. First, does the presence of *D. fragilis* DNA represent viable *D. fragilis* that can be cultured from the pinworm eggs?

If so, is *Enterobius* a 'dead end' or a paratenic (transport) host? How does *D. fragilis* exit the eggs? Does *D. fragilis* require transmission by a vector to complete its life cycle and does *D. fragilis* undergo development and multiplication in pinworms? Do other nematodes harbour *D. fragilis*, and if so, could they serve as vectors?

Using microscopy, Sukanahaketu et al. (1977) reported the presence *D. fragilis* trophozoites within eggs of *Ascaris lumbricoides* (Sukanahaketu, 1977), but confirmation using molecular diagnostics or electron microscopy remains to be demonstrated.

As stated by Barratt et al. (2011a), protozoa closely related to *D. fragilis* have been reported to display both pseudocysts and cyst-like structures (trichomonads) and direct transmission of trophozoites remain at least a theoretical possibility (Barratt et al., 2011b), though more research is needed.

Transmission of *D. fragilis* by pinworm would provide an enticing explanation for the high prevalence of *D. fragilis*, particularly among children, who also have the highest prevalence of pinworm. While the present findings might warrant consideration on the use of antimicrobial intervention and control measures related to *D. fragilis* and *E. vermicularis*, the overall uncertainty of the proposed pathogenicity in *D. fragilis* should give clinicians pause before new strategies are implemented.

Future studies should aim to generate information on the viability, longevity, potential development and multiplication of *D. fragilis* in pinworm eggs, with an essential first step being the successful culture of live *D. fragilis* trophozoites from a pinworm egg or successful transmission of *D. fragilis* in an animal model using pinworm eggs.

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