Transmission of *Dientamoeba fragilis*: pinworm or cysts?

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Keywords

Dientamoeba, trichomonad, life cycle, transmission, cyst, *Enterobius*

Recently, conflicting evidence has been published on the mode of transmission of the trichomonad *Dientamoeba fragilis*. Detection of *D. fragilis* DNA inside *Enterobius vermicularis* eggs agrees with the prediction of Dobell in 1940 that the eggs of a nematode act as a vector for transmission. However, the identification of a cyst stage of *D. fragilis* in the stool of rodents infected with a human isolate has also been reported, and this implies a life cycle similar to those of most other intestinal protistan parasites. Herein, we discuss the recent data, identify gaps in the experimental evidence, and propose a method for determining which view of the life cycle of this organism is correct.

Dientamoeba: basic information is elusive despite its ubiquity

Dientamoeba fragilis (see Glossary) is an intestinal trichomonad parasite that has lost its microtubular cytoskeleton and flagella, leading to an amoeboid lifestyle [1]. Its life cycle has

remained a mystery since its description 95 years ago because only a fragile trophozoite stage and no cyst stage has been described, unlike most other intestinal protists where a cyst is essential for transmission of the infection. Three recent publications address the major gap in the *D. fragilis* life cycle, namely its mode of transmission, but come to two completely different conclusions; one identifies a previously unknown typical cyst form [2], whereas the other two find *D. fragilis* DNA inside nematode eggs [3, 4], implying that these act as a vector for transmission instead. We summarise and evaluate the data presented by the various authors and discuss what experimental work is still needed to resolve the conflict between the two conclusions.

History and *Histomonas*

Because it is an intestinal parasite, one might assume that, like most other intestinal protozoa, *D. fragilis* requires a cyst stage to survive in the external environment. However, until very recently, although there have been a few inconclusive reports of pseudocysts, precysts, or cysts of *D. fragilis* (see references in [1]), it has been generally accepted that no cyst form exists for this parasite. Indeed, Clifford Dobell said, "although a prolonged and very careful search has been made for the cysts of this organism, none have ever been found," [5] and, later, "many careful workers in many different countries have now studied scores of natural infections and thousands of cultures, but no one of us has ever found anything that could plausibly be interpreted as a cyst of *Dientamoeba*" [6]. Anyone who has read the original work of Dobell will know how rigorous his microscopic work was.

The absence of a cyst stage would usually cast doubt on direct faecal-oral transmission. Dobell ingested cultured trophozoites of *D. fragilis* on multiple occasions, but was never able to find the organism in his stool [6]. Attempts to infect non-human primates also failed. Dobell was the first to draw parallels between *Dientamoeba* and *Histomonas*, a pathogen of turkeys; he noted that because *Histomonas* does not have a cyst stage and is transmitted via the eggs of the avian nematode *Heterakis gallinae* (syn. *gallinarum*), perhaps *Dientamoeba* is transmitted via the eggs of a human

nematode. The close relationship between *Dientamoeba* and *Histomonas* was eventually confirmed by phylogenetic analyses of small subunit ribosomal RNA gene sequences [7] and, more recently, by actin and elongation factor 1-alpha sequences [8] (Figure 1).

The link to *Enterobius*

Dobell believed that the vector for *Dientamoeba* could be *Trichuris* or *Ascaris* eggs but, for many years now, *Enterobius vermicularis* (pinworm) eggs have been the leading candidate as the vector for *D. fragilis* transmission. This is consistent with the continued presence of *E. vermicularis*, especially in children, in many countries where *D. fragilis* infection remains common whereas other nematodes are increasingly rare or absent. Moreover, pinworm and *D. fragilis* infections can be epidemiologically linked in several ways (Box 1). Burrows and Swerdlow [9] were the first to find a higher incidence than expected of coinfection with *D. fragilis* and *E. vermicularis*. They also observed small structures in the eggs that resembled *D. fragilis,* although they were unable to establish trophozoite cultures from pinworm eggs. Testing the *Enterobius* theory, Ockert [10] successfully infected himself with *Dientamoeba* by ingesting 150 pinworm eggs from a coinfected carrier; the infection persisted for several weeks.

Since then many additional studies have reported a higher rate of coinfection than expected between these two parasites [11-16]. Some studies report no association between *D. fragilis* and *E. vermicularis* (see [17] for references); however, most often these studies are either small-scale or employ diagnostic tools inappropriate for the detection of *E. vermicularis* (stool microscopy instead of adhesive tape test). It should also be noted that, in principle, ingestion of an infected *E. vermicularis* egg could lead to establishment of *D. fragilis* infection without producing a pinworm infection, or the latter could spontaneously resolve, leaving a *D. fragilis* infection behind.

Proof of the presence of *Dientamoeba* within *Enterobius* eggs would be a major point in favour of the nematode egg vector theory of *D. fragilis* transmission, and this has been the focus of two recent publications [3, 4]. The first molecular investigation of this possibility dates back to 2005 [18] but, working with a small number of samples, the authors were not able to detect *D. fragilis* DNA inside the eggs. However, studies of large numbers of samples detected *Dientamoeba* DNA inside *Enterobius* eggs with varying frequencies [3-4]. Eggs were carefully prepared by sterilisation to avoid the possibility of surface contamination with extra-ova *D. fragilis* DNA.

Does this prove the case for *Enterobius* egg transmission of *D. fragilis*? The sceptic will point out that the presence of DNA does not mean the presence of live organisms. Burrows and Swerdlow [9] were unable to establish cultures of *D. fragilis* from *E. vermicularis* eggs and the most recent authors did not attempt this confirmation step [3, 4].

How solid is the evidence for egg transmission of *Histomonas*?

The whole construct of nematode egg transmission of *D. fragilis* rests on the parallels with *Histomonas*; thus, it is therefore essential to know how solid the evidence is for the requirement of *H. gallinae* in *Histomonas* transmission. For many years, experimental infection of birds with *Histomonas* has employed, among other methods, oral administration of eggs or other stages of *H. gallinae* containing *Histomonas* [19] . The interaction between the two organisms has been investigated at the morphological level [20]. The method by which *Histomonas* ends up in the egg involves ingestion of trophozoites by adult female *Heterakis* in the intestine, followed by penetration of first the ovary and then the immature egg by *Histomonas* trophozoites. Infected eggs would be shed, then ingested by a new host and an intestinal infection established, following either hatching of *Heterakis* larvae or active egress through the egg surface by *Histomonas* trophozoites. The assumption is that infection of *Enterobius* eggs by *Dientamoeba* would follow a similar process.

104 It should be noted, however, that *Histomonas* can spread between turkeys and from turkeys to
105 chickens in the absence of the nematode [19,21,22], and it is therefore clear that nematode eggs are
106 not an essential requirement for successful transmission. Of relevance here is that, in recent years,
107 there have been several studies reporting the development of cyst-like structures in cultures of
108 *Histomonas* [23-25], and it has been proposed that they may also develop *in vivo*, the implication
109 being that these forms could be responsible for direct transmission of *Histomonas* between hosts in
110 the absence of nematodes.
111
112 **Cysts of *Dientamoeba*?**
113 If *Histomonas* produces cysts, why should this not also be true of *Dientamoeba*? Is there any
114 evidence for cysts in this parasite? As mentioned above, there have been sporadic reports over the
115 years of cyst-like structures but nothing definitive. However, apparently *bona fide D. fragilis* cysts
116 with thick walls have been reported recently [2], and the authors propose these to be the missing
117 link in transmission of *D. fragilis* between hosts. This discovery comes as a great surprise to many in
118 the field of parasitology who for years have been teaching students about the absence of cysts and
119 possible nematode-dependent transmission of *D. fragilis*, and would no doubt be a source of great
120 consternation to Dobell were he alive today.
121
122 So which life cycle is right? Is it possible that both are correct, or neither of them? Before attempting
123 to answer these questions, we need to look in more detail at the experiments that led to these very
124 different conclusions.
125
126 **The evidence**
127 In the egg studies, *E. vermicularis* eggs of human origin from adhesive tape samples, swabs, or
128 female adult worms were surface-sterilised using hypochlorite [3, 4] or extensively washed [4]
129 before DNA extraction and PCR. Notably, DNA was extracted from the last buffer solution used to

wash the eggs, and this was shown by PCR to be negative for *D. fragilis* in every [3] or almost every [4] case. DNA was extracted from individual [3] or pooled [4] eggs, and *D. fragilis* was detected by PCR and sequencing in many but not all of the samples tested.

In the cyst study, mice to be infected orally with cultured trophozoites "were confirmed as specific pathogen free by microscopy and PCR" before infection, although it is not explicitly stated for which organisms the mice were screened [2]. Animals were examined for a week before the experiment using iron-haematoxylin staining of stool fixed in sodium acetate formalin (SAF), and stool was tested by PCR for the presence of *D. fragilis* DNA. Mice infected with trophozoites began shedding cysts within a day after challenge and shed them intermittently for up to 6 months. Cysts transferred to rats and other mice using stool suspensions led to shedding of cysts by these hosts, but confirmation by PCR of the continued presence of *D. fragilis* was not mentioned. Rats did not shed cysts after being infected orally with *D. fragilis* trophozoites.

A point worth noting in this study is the link between the cyst and *D. fragilis*. Cysts were not purified and sterilised before DNA extraction; instead, DNA was purified from whole stool for analysis [2]. This means that the link between the *D. fragilis*-positive PCR result and the cyst is unproven. The possibility remains, for example, that *D. fragilis* did colonise the gut, and was responsible for the PCR result, but that the cyst was from another organism. The authors state that cyst shedding was intermittent, although no detail of frequency is given, and therefore perhaps shedding did not occur during pre-screening of the animals before infection; in some cases, for example, detection of *Giardia* infection by microscopy has required examination of seven or more stool samples. Another issue is morphological; the cysts illustrated are morphologically very different from *Histomonas* cysts, and the appearance of the nucleus in the cyst is unlike that in images of *D. fragilis* trophozoites published previously [26,27]. However, the absence of any evidence for such cysts in humans is probably the main difficulty. Unless humans are a dead-end host for *D. fragilis,* in which no cysts are

produced and all human infections occur *de novo,* presumably originating from rodents, it seems inconceivable that *D. fragilis* cysts in humans would have been missed by all parasitologists to date. In addition, natural *D. fragilis* infection has not been reported in rodents despite survey work [28]; there is therefore no evidence of a zoonotic transmission source either.

Is it possible that neither life cycle is correct? Certainly, there are related intestinal trichomonads for which no cyst stage has been described and where there has been no hint of nematode involvement, such as *Tritrichomonas*. In such species, pseudocysts without thick walls are known to develop in response to stress [29] and are thought to be involved in transmission. These do not resemble the thick-walled cyst proposed for *D. fragilis*. Could both life cycles be correct? The precedent of *Histomonas* described above suggests that the answer is yes, but at present we would suggest that no life cycle is proven for *D. fragilis* (Box 2).

Concluding remarks: closing the loop

To make or break the link between the cyst and *D. fragilis* there is a variety of options; for instance, it should be possible to stain the cysts specifically by fluorescent in situ hybridisation using *Dientamoeba*-specific oligonucleotide probes that hybridise to the ribosomal RNA. With suitable controls, this approach could give unambiguous results. The fact that there is a thick cyst wall should not be an insurmountable barrier because this approach has been successful for *Giardia, Cryptosporidium*, and microsporidia [30-33].

Two experimental approaches could prove or disprove the proposed life cycles of *Dientamoeba.* To be involved in transmission, the cysts and/or eggs must contain viable *D. fragilis* organisms. Viability can be demonstrated either by infecting naïve hosts or by establishing the organisms in culture.

Culture is likely to be the cheaper and simpler alternative. It is important that no extra-cyst or extra-ovum organisms could be responsible for any culture obtained, which means that pure cysts/eggs need to be treated to destroy any external organisms. The medium into which the material is inoculated must be capable of supporting trophozoite growth. To mirror a natural infection, inclusion of acid treatment and enzymatic exposure may be necessary to mimic transit through the stomach and duodenum, and stimulate the trophozoite to emerge from the egg/cyst when placed in culture medium, although experience with other intestinal protist parasites suggests that such treatment is not always necessary. The identity of any resulting eukaryotes growing in culture would require verification by PCR and sequencing to confirm that they are indeed *D. fragilis*.

Should culture prove unsuccessful, then perhaps experimental infections may be the only option. Fortunately, humans may not be needed as hosts because naturally occurring *D. fragilis* infections in pigs have been described [34, 35], and gnotobiotic pigs are available. Again, the inoculation material would need to be freed of extra-cyst or extra-ovum organisms before use and the hosts checked extensively for pre-existing infections.

A negative result cannot rule out one or both proposed transmission methods definitively because establishing *D. fragilis* in culture has a variable success rate and the requirements for establishing *D. fragilis in vivo* are unknown. Neither can a positive result for one rule out the other proposed method of transmission. However, if one or both sources of material give rise to cultures or infection with *D. fragilis* we feel that this will confirm a missing link in the evidence for the life cycle of *Dientamoeba fragilis*.

Acknowledgements

We would like to thank Drs. Bobbi S. Pritt and Marianne Lebbad for helpful discussions and comments on this manuscript.

Glossary

Adhesive tape test: Also known as transparent adhesive test, cellophane tape test, or Scotch tape test. The gold standard diagnostic test for detecting pinworm (*Enterobius vermicularis*) infection. The tape is pressed against the anus and perianal area of the patient causing pinworm eggs to stick to the tape surface; this allows detection (and collection) by simple light microscopy.

Amoeboid: Cells of no fixed shape where movement involves protrusion of cytoplasm of the cell to form pseudopodia are referred to as amoeboid.

Bimodal age distribution: A frequency distribution, in this case of infection, that shows peaks at two different ages.

Cyst: The cyst stage typically enables a parasite to survive outside the host and is hence also the infective stage. It is usually characterised by a thick and resistant cell wall. Excystation or hatching of cysts releases trophozoites.

***Dientamoeba fragilis*:** A unicellular intestinal trichomonad parasite common in humans, also found in some non-human primates and pigs. Two genotypes are known, one of which appears to be rare.

***Enterobius vermicularis*:** A human intestinal nematode common in children and, to a lesser degree, in their caregivers. Commonly known as pinworm, the adult female deposits its eggs in the perianal area. Infection is a common cause of anal itching, which facilitates transmission of the worm by eggs become trapped under fingernails, in clothes, etc.

Gnotobiotic: Gnotobiotic animals include 'germ-free' animals and in this context animals for which the intestinal flora is known.

***Heterakis gallinae* (syn. *gallinarum*):** A parasitic nematode of the caecum of galliform birds (chickens, turkeys, etc.).

***Histomonas meleagridis*:** A unicellular amoeboflagellate intestinal trichomonad parasite of birds; the cause of histomoniasis (or blackhead disease) in poultry.

Iron-haematoxylin stain: One of several stains used to make a permanent stained slide for detecting and quantitating parasites, in particular protozoa in human faecal samples.

Parabasalid: A member of a group of primarily flagellated protists, most of which form commensal or parasitic relationships with animals. Includes the trichomonads.

Precyst and pseudocyst: In this context, precyst refers to an immature cyst stage whereas pseudocyst refers to a cell for that may resemble a precyst but may or may not have a role in the life cycle of the organism. Both, in general, lack the thick wall of the cyst stage.

Trichomonad: A member of the Trichomonadida subgroup of parabasalid protists.

***Tritrichomonas*:** A genus of trichomonad flagellates that are commensals or parasites of mammals and amphibia. Examples include *Tritrichomonas foetus*, *T. augusta* and *T. muris*.

Trophozoite: Also known as the 'vegetative stage', this term is used to denote the feeding and dividing form many protozoan parasites. Trophozoites are usually non-infectious.

Box 1. Epidemiological considerations

Apart from a higher level of coinfection than expected, the epidemiologies of *D. fragilis* and *E. vermicularis* have other similarities. *D. fragilis* carriage shows a bimodal age distribution, peaking children aged 7 years and women aged 40 (mothers) [36], suggesting the occurrence of child to child and child to parent transmission. Similar figures have been reported for *E. vermicularis* [37-39], and data from Statens Serum Institut (Röser *et al*., unpublished) show congruent age distributions for *D. fragilis* (Figure I) and *E. vermicularis* (Figure II). Although the prevalence of *E. vermicularis* may seem low in adults, this does not preclude pinworm eggs being the vector of *D. fragilis*, because many pinworm infections go unnoticed or may fail to establish in adults. In addition, the intake of mebendazole, an anthelminthic drug, which in Denmark is used almost exclusively to treat pinworm infection, is significantly associated with higher risk of *D. fragilis* carriage (Röser et al., unpublished).

The findings are consistent with *D. fragilis* transmission by *E. vermicularis*, but the mechanism of transmission cannot be proven by epidemiological association alone, and the age distribution is also reminiscent of *Giardia*, for example [40], which is transmitted through cysts.

Box 1 Figure legends

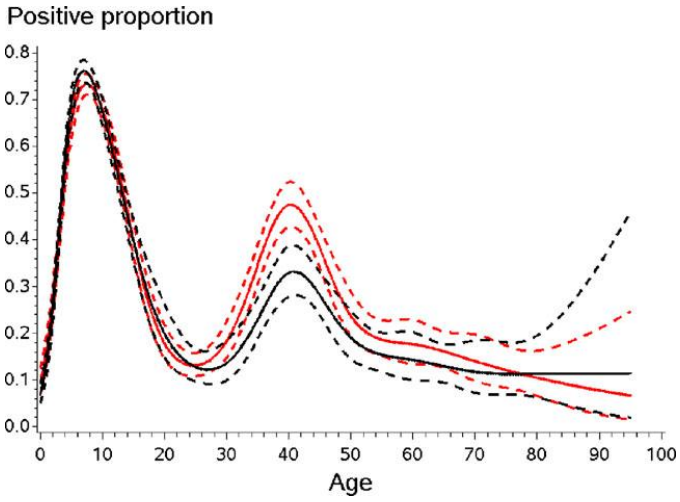

Figure I. Proportion of patients positive for *D. fragilis* in various age groups. The solid lines denote positive proportions; the dotted lines denote confidence intervals. Females are shown in red and males in black. . The *x*-axis shows age in years; the *y*-axis shows the positive proportion. Two distinct peaks in the positive proportion can be observed at 7 and 40 years of age, with a significant gender-dependent difference at ~ 40 years of age, with females having the highest positive proportion. Reproduced with permission from [36].

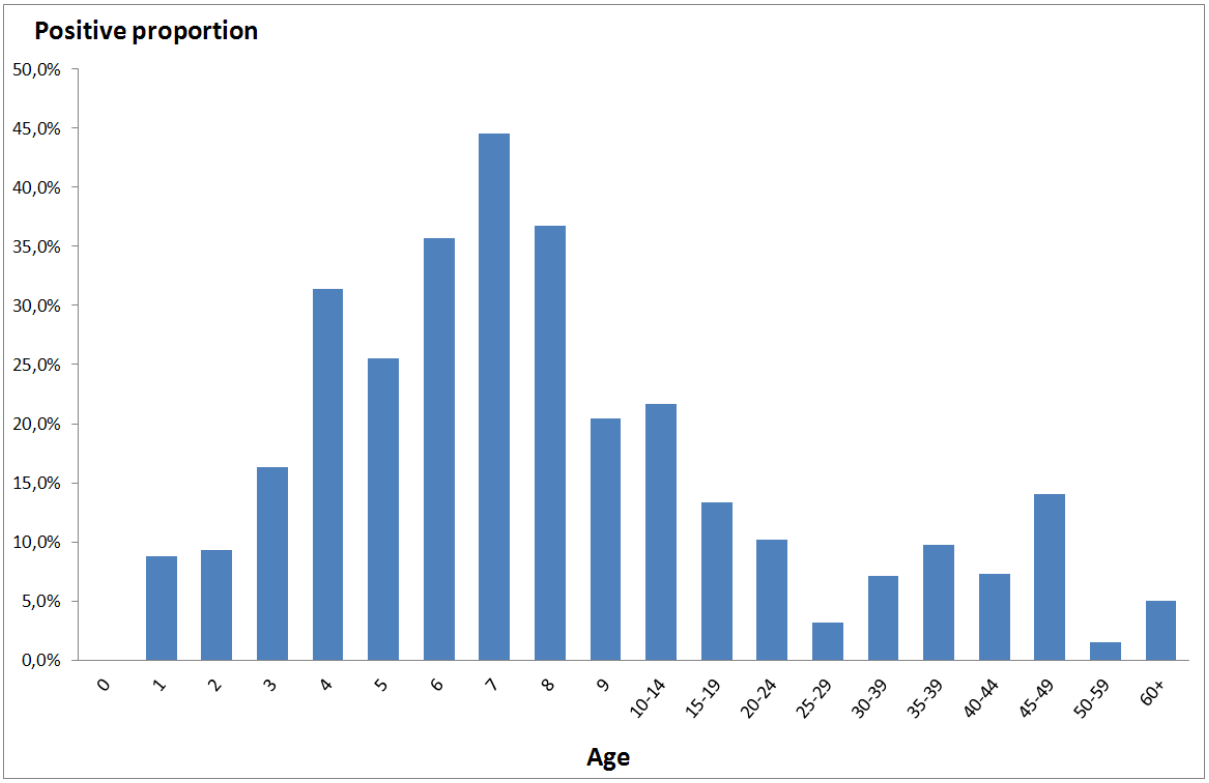

Figure II. Proportion of patients positive for *E. vermicularis* in various age groups. Data are from Statens Serum Institut from 2000-2012; the material includes >4500 routine adhesive tape test samples collected from patients. The *x*-axis shows age in years (0—9) or in 5 year intervals (10—60+); the *y*-axis shows the positive proportion in percent. Peak proportion is seen at year 7, with a secondary increase around years 35-49.

Box 2. Outstanding questions

- Is *D. fragilis* transmitted by cysts, by nematode eggs, and/or by other means?
- Do multiple modes of transmission exist, and if so what circumstances determine which mode is used?
- If *D. fragilis* produces cysts, why have these never been reported in humans?
- Can *D. fragilis* cultures be obtained from *D. fragilis* DNA-containing *Enterobius* eggs or cysts from rodents?
- Can experimental *D. fragilis* infections be produced from surface-sterilized eggs or cysts?

Figure legends

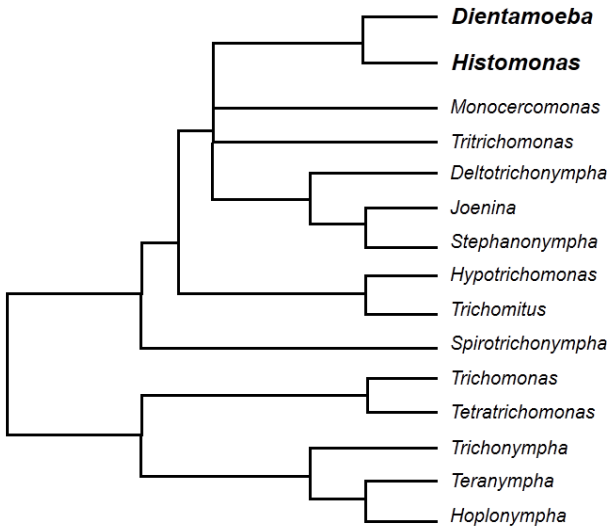

Figure 1. Phylogenetic relationships of *Dientamoeba* and *Histomonas*. The phylogenetic tree of actin and elongation factor 1-alpha sequences [8] has been redrawn and simplified to illustrate the relationships of *Dientamoeba* and *Histomonas* to each other and to other parabasalids. Tree nodes with low support have been collapsed for simplicity.

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