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1 **Transmission of *Dientamoeba fragilis*: pinworm or cysts?**

2

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10

11 **Keywords**

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

13

14

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

23

24 ***Dientamoeba*: basic information is elusive despite its ubiquity**

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

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

35

36 **History and *Histomonas***

37 Because it is an intestinal parasite, one might assume that, like most other intestinal protozoa, *D.*
38 *fragilis* requires a cyst stage to survive in the external environment. However, until very recently,
39 although there have been a few inconclusive reports of pseudocysts, precysts, or cysts of *D. fragilis*
40 (see references in [1]), it has been generally accepted that no cyst form exists for this parasite.

41 Indeed, Clifford Dobell said, “although a prolonged and very careful search has been made for the
42 cysts of this organism, none have ever been found,” [5] and, later, “many careful workers in many
43 different countries have now studied scores of natural infections and thousands of cultures, but no
44 one of us has ever found anything that could plausibly be interpreted as a cyst of *Dientamoeba*” [6].
45 Anyone who has read the original work of Dobell will know how rigorous his microscopic work was.

46

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

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

56

57 **The link to *Enterobius***

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

69

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

77

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

85

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

90

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

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

103

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

130 wash the eggs, and this was shown by PCR to be negative for *D. fragilis* in every [3] or almost every
131 [4] case. DNA was extracted from individual [3] or pooled [4] eggs, and *D. fragilis* was detected by
132 PCR and sequencing in many but not all of the samples tested.

133

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

143

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

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

160

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

168

169 **Concluding remarks: closing the loop**

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

176

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

180

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

190

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

196

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

203

204 **Acknowledgements**

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206 comments on this manuscript.

207

208

209 **Glossary**

210

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

246

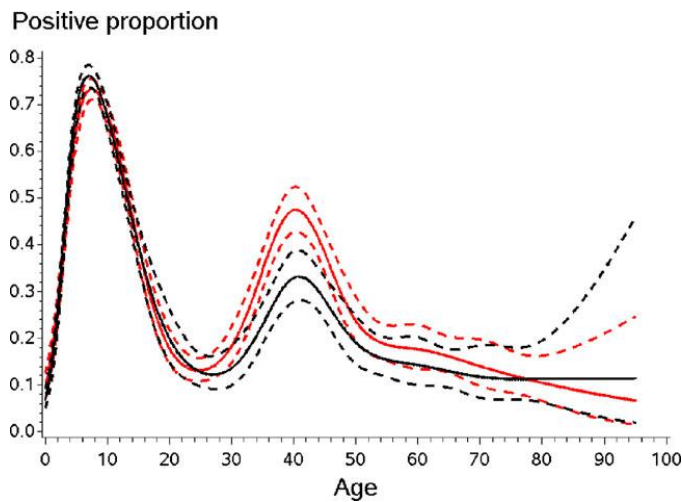
247 **Box 1. Epidemiological considerations**

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

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

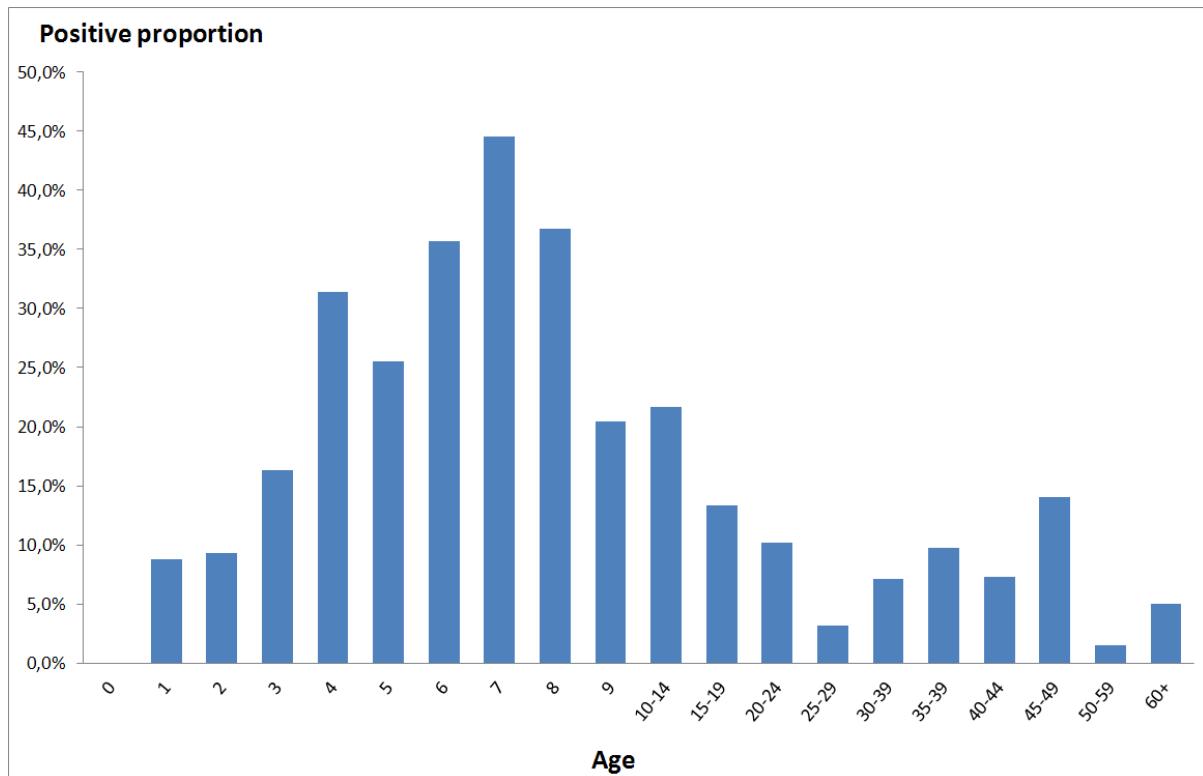
261

262 **Box 1 Figure legends**



263

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



270
 271 **Figure II.** Proportion of patients positive for *E. vermicularis* in various age groups. Data are from
 272 Statens Serum Institut from 2000-2012; the material includes >4500 routine adhesive tape test
 273 samples collected from patients. The x-axis shows age in years (0—9) or in 5 year intervals (10—
 274 60+); the y-axis shows the positive proportion in percent. Peak proportion is seen at year 7, with a
 275 secondary increase around years 35-49.

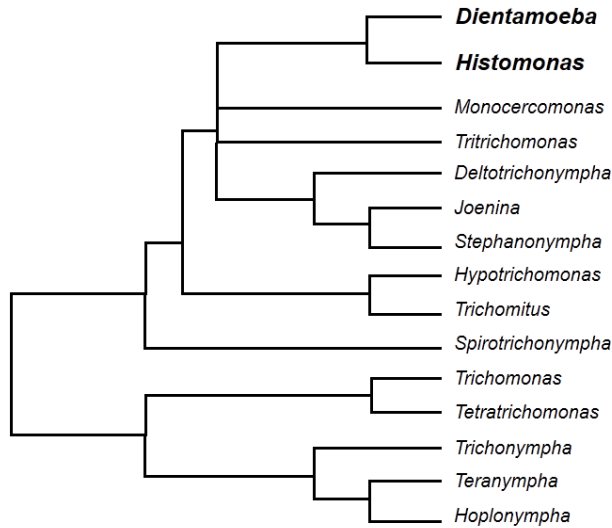
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277 **Box 2. Outstanding questions**

- 278
- Is *D. fragilis* transmitted by cysts, by nematode eggs, and/or by other means?
 - 279 • Do multiple modes of transmission exist, and if so what circumstances determine which
 280 mode is used?
 - 281 • If *D. fragilis* produces cysts, why have these never been reported in humans?
 - 282 • Can *D. fragilis* cultures be obtained from *D. fragilis* DNA-containing *Enterobius* eggs or cysts
 283 from rodents?
 - 284 • Can experimental *D. fragilis* infections be produced from surface-sterilized eggs or cysts?

285

286 **Figure legends**



287

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

292

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