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Application of Indirect Immunofluorescence to Detection of *Dientamoeba fragilis* Trophozoites in Fecal Specimens

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An indirect fluorescent-antibody (IFA) assay was carried out to examine for the presence of *Dientamoeba fragilis* trophozoites in preserved fecal specimens. Antiserum to *D. fragilis* trophozoites was raised in a rabbit with a dixenic culture of *D. fragilis* (ATCC 30948) from the American Type Culture Collection. After absorption with *Klebsiella pneumoniae* and *Bacteroides vulgatus*, the immune rabbit serum was used for examination by the IFA assay. A total of 155 clinical samples were tested: 42 with no parasites, 9 with *D. fragilis*, and 104 with other parasites. The IFA assay identified seven *D. fragilis* organisms. Two specimens with doubtful IFA assay readings showed very scanty amounts of *D. fragilis* trophozoites on stained smears. There were no false-positive IFA assay readings. The IFA assay appeared to be a promising method because of its speed in screening. The specificity of the IFA assay indicates that other diagnostic tests such as an enzyme-linked immunosorbent assay could be developed to identify *D. fragilis* antigens in fecal specimens.

Dientamoeba fragilis is a protozoan parasite of the human large intestine. The organism was originally seen by Wenyon in 1909, but it was not recognized as a new species at that time. It was then described and named by Jepps and Dobell in 1918 (9). Since then it has been found in most parts of the world where careful surveys have been taken, with an estimated incidence of 1.4 to 53% (1, 2, 10, 12, 19, 23, 27).

Although *D. fragilis* is often considered a harmless commensal organism, a number of reports have suggested that infection by the parasite may evoke various symptoms, which, in most cases, disappear with the elimination of the parasite. Abnormal stools, diarrhea, abdominal pain, fatigue, loss of appetite, and weight loss were among the symptoms experienced (27). Fibrosis of the appendix (22), phagocytosis of erythrocytes (2), low-grade eosinophilia (19), the presence of *D. fragilis* in biliary tracts (23), and colitis (17) have also been reported. Wenrich et al. (25) found a higher incidence of gastrointestinal disorders among college students harboring *D. fragilis* than among those infected with *Entamoeba histolytica*. Sapero (15) reported that 27.3% of patients with *D. fragilis* infection presented with symptoms, and Steinitz et al. (20) reported symptoms in 15.1% of infected persons. An incidence of 25% symptomatic cases was reported by Yang and Scholten (27) in Canada.

Spencer et al. (19) conducted a retrospective study on 35 children in whom *D. fragilis* was the only parasite found in their gastrointestinal tracts. Gastrointestinal symptoms were present in 32 (91%) of these children. Diarrhea was the most common finding in patients with acute illness, whereas abdominal pain was more common in children with chronic symptoms. Therapy with diiodohydroxyquin or metronidazole was effective. Symptoms were eliminated or diminished on follow-up evaluation after treatment. From this association between therapy and symptomatic relief, the investigators stressed that *D. fragilis* should be considered pathogenic in those children with gastrointestinal symptoms. Similar findings were also reported in the adult population by the same group of investigators (18).

D. fragilis is classified as a flagellate (11), but it has no demonstrable flagella. It does not have a cyst stage. The only available method of laboratory diagnosis of *D. fragilis* is microscopic examination of stained smears of preserved fecal specimens for the characteristic trophozoites (8, 16). The objective of the study described here was to determine the usefulness of indirect fluorescent-antibody (IFA) assay for the identification of *D. fragilis*.

MATERIALS AND METHODS

***D. fragilis* strain and maintenance of culture.** A dixenic culture of *D. fragilis* (ATCC 30948) was acquired from the American Type Culture Collection (ATCC). It was maintained in TYGM-9 medium (ATCC medium 1171). The medium was dispensed aseptically to screw-cap tubes (16 by 125 mm) in 8-ml amounts. Immediately before use, 0.15 ml of rice starch solution was added aseptically to each tube. The rice starch solution was prepared by heat sterilizing 0.5 g of rice starch (BDH Inc., Toronto, Ontario, Canada) at 150°C for 2 h and adding 9.5 ml of sterile phosphate-buffered saline (FA buffer [pH 7.2]; Difco Laboratories, Detroit, Mich.) prior to use. After 3 to 4 days of incubation at 35°C and thorough mixing, *D. fragilis* was subcultured with the bacteria in the culture to two new tubes in 0.5- and 1.0-ml volumes. The maximum yield of *D. fragilis* trophozoites was estimated to be between 10^5 and 10^6 cells per ml, with a hemacytometer used for counting.

Bacteria in *D. fragilis* culture. The two bacteria listed by ATCC in the *D. fragilis* dixenic culture are *Klebsiella pneumoniae* and *Clostridium perfringens*. The presence of *K. pneumoniae* was evident, but we were unable to isolate *C. perfringens* from this dixenic culture. Instead, a *Bacteroides* sp. was isolated; the organism was identified as *Bacteroides vulgatus* by the Laboratory Centre for Disease Control, Ottawa, Ontario, Canada. The discrepancy in bacterial identification was discussed with the staff at ATCC, who indicated that they would further investigate this

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dixenic culture. The bacterial species that were cocultured helped to maintain *D. fragilis* on continuous passage.

Preparation of *D. fragilis* antigen. Twelve tubes of 3- to 4-day-old *D. fragilis* cultures were centrifuged at 600 × *g* for 8 min to deposit the *D. fragilis* trophozoites. To remove the bacteria in the supernatant, the tubes were washed three times with warm (prewarmed to 35°C) FA buffer containing piperacillin (200 μg/ml) and imipenem (100 μg/ml). These two antibiotics were active against the two bacterial species and were included in the preparation to protect the rabbit for immunization. After the final washing, the number of *D. fragilis* trophozoites was counted. The vaccine prepared by this procedure contained 3.4×10^6 trophozoites per ml. It was frozen and thawed once (frozen at −20°C and then thawed at 4°C) to disintegrate the trophozoites. After microscopic examination to confirm the disintegration of the trophozoites, the antigen preparation was kept at −20°C until use.

Production of antiserum. A New Zealand White rabbit of 2 kg in weight was used to produce the antiserum. Equal volumes of antigen preparation and complete Freund's adjuvant (Difco) were mixed. A 0.5-ml amount of the mixture was injected intramuscularly into the biceps femoris muscle. A booster (0.5-ml mixture of equal parts of antigen preparation and incomplete Freund's adjuvant [Difco] was given 1 month after the initial vaccination. The rabbit was bled 2 weeks after the booster.

Absorption of antiserum. The two bacterial isolates present in the culture (*K. pneumoniae* and *B. vulgatus*) were grown in TYGM-9 medium overnight at 35°C. They were washed three times in FA buffer by centrifugation at 1,000 × *g* for 10 min. Equal volumes of rabbit antiserum and bacterial suspension (to which 0.15 ml of rice starch suspension had been added after the final washing) were mixed and allowed to react for 1 h at 35°C, with gentle mixing every 10 min. At the end of 1 h, the mixture was placed in 1.5-ml Eppendorf tubes, and the tubes were spun in an Eppendorf microcentrifuge at 10,000 × *g* for 3 min to pellet the bacteria and the starch particles. The supernatant was removed, filter sterilized with a 0.22-μm-pore-size Millipore filter unit, and stored at −70°C. Because residual antibody toward the bacteria was detected by the IFA assay at low titers, the antiserum was absorbed a second time to ensure its specificity for *D. fragilis.*

Specimens. All fecal specimens for parasitological examination were collected in sodium acetate-acetic acid-formalin (SAF) fixative (28). Specimens collected for the present study included those received at the Children's Hospital of Eastern Ontario, the Ottawa Civic Hospital, Queensway-Carleton Hospital, and the Ottawa Public Health Laboratory. Because many samples stored at different laboratories were known positives, the findings reported here would not reflect the true prevalence of the parasites in our region.

Concentration and staining of specimens. Specimens collected in SAF fixative were strained through gauze into 15-ml centrifuge tubes and centrifuged at 600 × *g* for 2 min, and the deposit was washed twice in saline. For permanent staining, a drop of Mayer's albumin was placed on a glass slide and mixed with a drop of the sediment. The mixture was then spread with an applicator stick by using a "dabbing" motion to produce a smear of varying thickness. After drying at room temperature, the smear was stained with a modified iron hematoxylin-Kinyoun stain (13). To continue with the concentration procedure, 8 ml of 10% formalin was added to the remaining sediment. This was followed by the addition of 4 ml of ethyl acetate (29) and shaking for 1 min. After centrifugation, the plug of debris in the ethyl acetate layer was removed and the supernatant was decanted. The sediment was then examined microscopically for ova and parasites. The wet preparation of the concentrates was examined with a ×10 objective covering overlapping fields under a 22-by-22-mm coverslip. Confirmation of the findings was made with a ×25 or a ×40 objective. The average time of this examination procedure was about 5 min per preparation. The hematoxylin-Kinyoun-stained slide was mounted with a 22-by-40-mm coverslip and was examined with a ×50 oil immersion objective for protozoan parasites, which covered at least two-thirds of the mounted area. This procedure took approximately 15 min per slide.

IFA assay. The preimmunization rabbit serum (diluted 1 in 20 in FA buffer) and immune absorbed rabbit serum were tested by the IFA assay. Smears of samples known to be abundant with *D. fragilis* trophozoites and smears made from *D. fragilis* dixenic culture (preserved in SAF fixative) were used to assess the immunoassay. Teflon-coated welled slides of 6 mm in diameter (Cel-Line Associates Inc., Newfield, N.J.) were used to conserve the volume of reagents used. Twofold serial doubling dilutions of rabbit serum were made in FA buffer and were applied to the smears for 1 h at 35°C in a moist chamber. After three washings in FA buffer (5 min each), fluorescein-labelled sheep anti-rabbit immunoglobulin (Wellcome Diagnostics, Dartford, United Kingdom) diluted 1 in 40 in FA buffer was applied for 1 h. After the washings, the slides were mounted with buffered glycerol (Gull Laboratories, Inc., Salt Lake City, Utah) and were examined with a ×25 objective on a Leitz epifluorescence microscope with a 490-nm exciter filter, a 510-nm dichromatic beam splitter, and a 520-nm barrier filter. The preimmunization rabbit serum yielded a negative IFA assay reading (Fig. 1A). The immune absorbed rabbit serum gave a strong fluorescence (4+) with *D. fragilis* (Fig. 1B) up to a dilution of 1 in 256. A dilution of 1 in 128 of the immune absorbed rabbit serum was chosen for the detection of *D. fragilis* in clinical samples. The entire well was examined with a ×25 objective. Each examination was accomplished in less than 1 min.

RESULTS

Routine examination of ova and parasites. A total of 155 clinical samples were included in the present study. Concentration procedures and a modified iron hematoxylin-Kinyoun stain were used to examine the clinical samples for helminths and protozoal parasites. Parasites were not found in 42 specimens. *D. fragilis* was present in 9 samples, while other parasites were found in the remaining 104 specimens. In total, four species of helminths (*Enterobius vermicularis*, *Hymenolepis nana*, *Strongyloides stercoralis*, and *Trichuris trichiura*) and 11 species of protozoa (*Blastocystis hominis*, *Chilomastix mesnili*, *Cryptosporidium* spp., *D. fragilis*, *Entamoeba coli*, *Entamoeba hartmanni*, *Entamoeba histolytica*, *Endolimax nana*, *Giardia lamblia*, *Iodamoeba buetschlii*, and *Retortamonas intestinalis*) were found (Table 1). Infection by a single parasite was identified in 67 specimens, while mixed infections were observed in 46 samples. Of the nine specimens positive for *D. fragilis*, eight were "pure" infections. *E. nana* and *E. hartmanni* were also identified with *D. fragilis* in the remaining specimen.

IFA assay. The IFA assay was performed on all 155 clinical samples. Immunofluorescence was read by one of us (M.X.G.) who had no prior knowledge of the results of the routine parasitological examination. *D. fragilis* was identi-

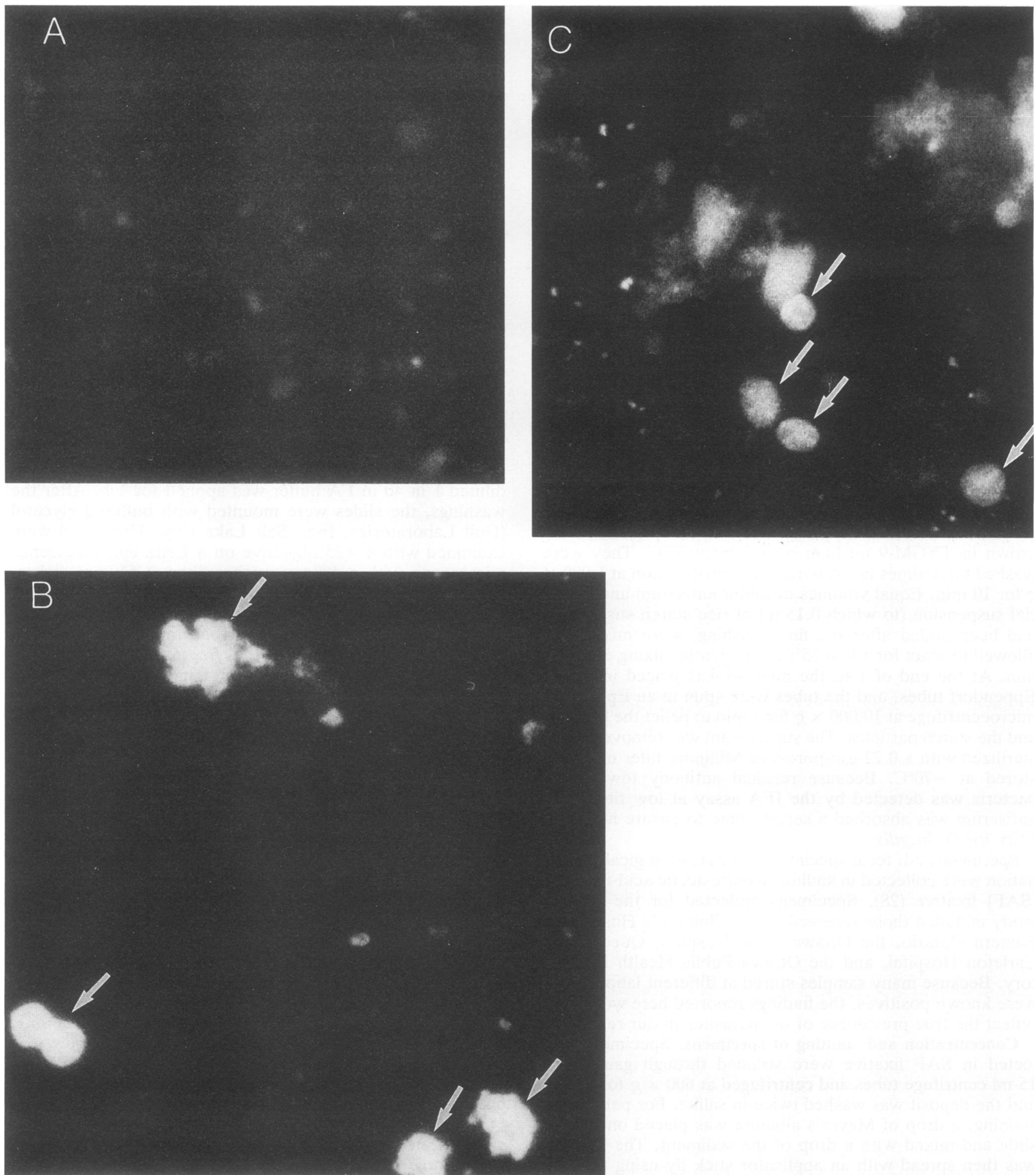

FIG. 1. *D. fragilis* trophozoites grown in culture and visualized by IFA staining with rabbit preimmunization serum (no fluorescence) (A) and immune rabbit serum (strong fluorescence; arrows) (B). (C) *D. fragilis* trophozoites in a clinical stool specimen showing fluorescence with immune rabbit serum. Final magnification, ×2,000.

TABLE 1. Helminths and protozoa recovered in 113 preserved fecal specimens

Organism	No. of occurrences[a]
Helminths	
Enterobius vermicularis	2
Hymenolepis nana	5
Strongyloides stercoralis	1
Trichuris trichiura	8
Protozoa	
Blastocystis hominis	25
Chilomastix mesnili	3
Cryptosporidium spp.	6
Dientamoeba fragilis	9
Entamoeba coli	32
Entamoeba hartmanni	12
Entamoeba histolytica	3
Endolimax nana	37
Giardia lamblia	24
Iodamoeba buetschlii	4
Retortamonas intestinalis	1

[a] A single parasite was identified in 67 specimens, while mixed infections were observed in 46 samples.

fied by the IFA assay in seven specimens in less than 1 min of examination time per specimen. The reaction was strong, as demonstrated by the bright (3+ to 4+) fluorescence (Fig. 1C). There were two specimens with doubtful readings by the IFA assay. On cross-reference with routine parasitology results, it was found that there were only very scanty amounts (less than five trophozoites) of *D. fragilis* on the stained smears. None of the helminths or protozoa (Table 1) cross-reacted with the immune absorbed rabbit serum. There was also no false-positive IFA reading with the 42 specimens in which no parasites could be found.

DISCUSSION

Since its first description in 1918, *D. fragilis* has been a frequent finding in most laboratories where careful parasitological examinations are performed. The organism does not have a cyst stage, and the trophozoites disintegrate rapidly in unpreserved fecal specimens. To date, the only reliable method for the laboratory diagnosis of *D. fragilis* is microscopic examination of stained smears of preserved stool specimens. Laboratories which do not use this procedure would almost certainly miss the diagnosis (8, 16).

While examination of stained smears enables the identification of *D. fragilis* and most other parasites, the procedure is lengthy and the microscopic examination is time-consuming because it takes 15 min or longer. Fluorescence microscopic methods have been developed for other parasites such as *G. lamblia* (26) and *Cryptosporidium* sp. (7). These methods are very useful as screening procedures because of their sensitivity and speed. Dwyer (5, 6) studied the antigenic relationship of *D. fragilis* and other protozoa by immunofluorescence (5) and gel diffusion (6). He found that *D. fragilis* was least related to *Entamoeba* spp., was more related to *Trichomonas*, and was most related to *Histomonas meleagridis*. However, the data suggested that cross-reactivity was minimal among species. The indication was that the antigenicity of *D. fragilis* was specific and that cross-reactivity with other parasites should not occur with clinical samples.

Axenic cultures of *Entamoeba* spp., *G. lamblia*, and *Trichomonas* spp. are readily available from ATCC. Unfortunately, this is not the case for *D. fragilis*. We have attempted to axenize *D. fragilis* by different means, including increasing the concentration of bovine serum to 15% (4); using of *Crithidium* sp. strain ATCC 50083 (3), preconditioned medium with bacterial growth, and heat-killed bacteria; and incubating cultures under anaerobic conditions (30). So far none of these approaches has been successful. It is unfortunate that a *D. fragilis* axenic culture is unavailable because its availability would simplify the procedure for obtaining a specific antiserum and absorption procedures would be obviated. The unavailability of an axenic culture would probably also explain why so little progress has been made in the study of *D. fragilis*. However, in the absence of an axenic culture, we have managed to produce an absorbed antiserum suitable for carrying out our immunofluorescence study to identify *D. fragilis* trophozoites in preserved fecal specimens.

In the IFA study described here, *D. fragilis* was identified in seven of nine specimens which were positive by routine microscopic examination. The two positive specimens missed by the IFA assay contained only very scanty amounts (less than five trophozoites) of *D. fragilis*. The miss by the IFA assay is not surprising when we consider that the surface area of the 6-mm-diameter circle on the slide for IFA examination is 28 mm^2, whereas the coverslipped area for routine microscopic examination is 22 by 40 mm (880 mm^2). If the entire surface area of the permanently stained smears is read, then the area examined is 31 times that for IFA examination. Theoretically, there should be at least 31 *D. fragilis* trophozoites on the smear for routine microscopic examination before the smear for IFA examination would be read as positive. Even if only one-third of the coverslipped area is examined, the surface area covered by routine microscopic examination would still be 10 times more than that examined by the IFA assay. We believe that if a larger amount of sample was spread onto an increased surface area for examination, the sensitivity of the IFA assay could be improved.

The IFA assay was highly specific for *D. fragilis* in the present study. None of the 4 species of helminths or the other 10 species of protozoa (Table 1) cross-reacted with *D. fragilis* trophozoites. There was also no false-positive IFA reading for any of the specimens in which no parasites could be found.

It has been more than 70 years since *D. fragilis* was first described. Over these years there has been little if any advancement in the laboratory diagnosis of this parasite. Published reports (1, 2, 10, 12, 19, 23, 27) show that the prevalence of *D. fragilis* equals or surpasses that of *G. lamblia*. In our laboratory, *D. fragilis* is one of the most frequently found parasites on a perennial basis. Clinical data also suggest that the pathogenicity of *D. fragilis* resembles that of *G. lamblia* (24). Yet, despite many recent advances in the study of *G. lamblia*, little knowledge has been gained about *D. fragilis*, including knowledge in the field of laboratory diagnosis. One of the main reasons for the lack of progress is probably the absence of an axenic culture. With a dixenic culture the presence of the two bacteria undoubtedly would cause undesirable complications in any type of experimental design. In the present study, we demonstrated that it is possible to raise an antiserum to *D. fragilis* in a mixed bacterial culture and its specificity can be obtained by absorption. This specific antiserum can then be used to identify *D. fragilis* trophozoites in preserved fecal specimens. To the best of our knowledge, this is the first study that has used immunological techniques to identify *D. fragi-*

lis in clinical samples, and the results of this preliminary study are very encouraging. The IFA assay might prove to be a useful screening method. The time of the IFA procedure could be reduced if a direct immunofluorescence technique were developed. There have been recent reports (14, 21) describing the successful use of enzyme-linked immunosorbent assay (ELISA) techniques to identify *G. lamblia* antigens in stool specimens. It is one of our objectives to develop an ELISA or a simpler method to identify *D. fragilis* antigens, preferably in both fresh and preserved fecal samples. As indicated in the study on the diagnosis of giardiasis in stool specimens (14), an enzyme immunoassay could detect *Giardia* infection in at least 30% more cases than microscopic examination. It is possible that this would also hold true for *D. fragilis*, and a similar antigen detection test might help to improve the underdiagnosis of this infection.

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