Direct Amplification and Genotyping of Dientamoeba fragilis from Human Stool Specimens

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Dientamoeba fragilis is a globally occurring parasite that has been recognized as a causative agent of gastrointestinal symptoms. A single-round PCR was developed to detect D. fragilis DNA directly from human stool samples. The genetic diversity of D. fragilis from 93 patients and 6 asymptomatic carriers was examined by PCR followed by restriction fragment length polymorphism and sequencing of part of the small-subunit rRNA gene. The data show that D. fragilis sequences can be studied directly from fecal specimens despite the absence of a cyst stage and without the need for prior culturing. In addition, the results suggest strongly that D. fragilis shows remarkably little variation in its small-subunit RNA gene.

Dientamoeba fragilis is a protozoan parasite found in the mucosal crypts of the large intestines of humans. Originally D. fragilis was considered an ameba, but based on ultrastructural characteristics (2), antibody data (8), and phylogenetic data originating from 16S-like rRNA gene sequences, it has been established that it is a trichomonad (20), with no identified cyst stage. Most recent literature accepts that D. fragilis is an important enteric pathogen (7, 10, 18), with an estimated incidence of symptomatic infection of between 4 and 91% (11, 21, 25, 26). Symptoms include abdominal pain, bloating, and diarrhea.

Because of the lack of a cyst stage, diagnosis can be performed only on freshly passed stool or by the use of fixatives and permanent stains. In addition, day-to-day shedding is highly variable, which imposes the need for multiple sampling (24). These features have likely led to an underestimation of the prevalence of D. fragilis, which is reported to vary between 0.2% and more than 19% depending upon the population studied (3, 9, 15, 16, 26).

Despite the relatively high prevalence of D. fragilis and its apparent role in patients presenting with gastrointestinal complaints, surprisingly little is known about its pathogenicity, route of transmission, epidemiology, and genetics. Because only some infected persons experience symptoms, it is possible that D. fragilis is a heterogeneous species with nonpathogenic and pathogenic variants with similar morphology but different pathogenicities. This has been suggested for other lumendwelling protozoa such as Giardia duodenalis (13) and demonstrated for Entamoeba histolytica/Entamoeba dispar (4, 6). In a first report on variability in D. fragilis, the 16S-like ribosomal subunit DNA sequence of cultured D. fragilis from a small number of patients was analyzed by restriction fragment length polymorphism (RFLP) (14). Two of the 11 isolates gave a different restriction fragment pattern, indicating that there was genetic diversity in these D. fragilis cultures.

The cumbersome and time-consuming techniques to maintain D. fragilis in culture in combination with the rapid disappearance of trophozoites from fresh feces have probably precluded more extensive and detailed studies of the genetic variability in D. fragilis. In addition, culture is not suitable for molecular analysis of D. fragilis from large numbers of stool samples directly in a routine setting. In this report we describe a DNA isolation and PCR procedure that efficiently and specifically detects D. fragilis in nonpreserved stool specimens and can be used for RFLP analysis to detect the two known genetic variants. Based on these PCR/RFLP analyses and sequencing of part of the small-subunit (ssu) rRNA genes, D. fragilis is a clonal species with little variation.

MATERIALS AND METHODS

Stool specimens. Microscopically, D. fragilis-positive stool specimens were collected from 93 patients, including 28 children (<18 years), 50 adults, and 15 travelers (all adults) presenting with gastrointestinal symptoms. In addition, stool specimens microscopically positive for D. fragilis from six nonrelated asymptomatic carriers with no gastrointestinal complaints during the past 2 months were included. Specimens were collected and processed with the Triple Feces Test (TFT) (24). The TFT includes three tubes, two containing sodium acetate-acetic acid-formalin (SAF) fixative, TFT1 and TFT3, and one that is empty, TFT2. Patients collected stools on 3 consecutive days in TFT1 (SAF), TFT2 (unpreserved), and TFT3 (SAF), after which the whole set was returned to the laboratory for analysis. Because of the use of SAF in the first (TFT1) and third (TFT3) samples, reliable microscopic diagnosis of D. fragilis could be performed (24). After detection of D. fragilis in TFT1 and/or TFT3 by chlorazol black dye permanent stain, the TFT2 sample was frozen at −20°C before subsequent DNA extraction. All stool samples were processed for analysis within 1 week.

DNA extraction. Aliquots of thawed stool samples (approximately 200 mg) from TFT2 were dissolved in 1 ml of 5.6 M guanidine thiocyanate, 18 mM EDTA, 1% Triton X-100, 25 mM Tris-HCl (pH 6.4) and vortexed for 1 min; 200 µl of the suspension was mixed with 200 µl of binding buffer (High Pure PCR Template Preparation kit; Roche) and 40 µl of proteinase K (20 mg/ml) and incubated at 70°C for 10 min. After centrifugation for 1 min at 14,000 × g, the supernatant was used for DNA extraction. The DNA was eluted in 200 µl of 10 mM Tris-HCl (pH 8.3). For each series of isolations, a mock DNA isolation containing no feces was performed. Trichomonas vaginalis was obtained from a vaginal swab followed by culturing in TYM. DNA of the T. vaginalis culture was isolated with the same protocol as for the TFT2 samples.
PCR method. Primer DF1 (5’CTC ATA ATC TAC TTG GAA CCA ATT3’; positions 100 to 123) and DF4 (5’TCA TAG TTT CTC TTA TTA GCC CC3’; positions 739 to 761) design was based on the sequence of the ssu rRNA gene of *D. fragilis* (EMBL nucleotide sequence database accession number U37461). Both primers are complementary to the *D. fragilis* sequence but have several mismatches at their 3’ ends compared to the *D. fragilis* rRNA genes from other trichomonads. For each series of patient-control samples, a negative control containing no input DNA was added. The PCR mixture (50 μl) contained 20 μl of DNA solution, 100 ng of each primer, 500 μM deoxynucleoside triphosphate mix, 5 μl of 10× PCR buffer (Promega), 6 μl of 25 mM MgCl₂, 5 μl of bovine serum albumin (5 mg/ml), 5 μl of α-casein (20 mg/ml) to relieve PCR inhibition by fecal substances, 0.2 μl (5 U/μl) of Taq polymerase (Promega), and two drops of mineral oil. Forty cycles with denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min were performed. Amplification of the ssu rRNA gene with primers TRD5/TRD3 was performed as described (14). Amplification products were size fractionated on 1% agarose gels and visualized by ethidium bromide staining.

Restriction fragment length polymorphism. We digested 8 μl of the PCR mixture with 10 U of *Ddel* or *RsaI* (both from Roche) in a final volume of 15 μl for 1 h at 37°C. Samples were loaded on a 9% polyacrylamide gel run in 1x TBE (9 mM Tris, 10 mM boric acid, 1 mM EDTA [pH 8.3]) at 100 V for 1.5 h. After electrophoresis, gels were stained with ethidium bromide and photographed.

Cloning and sequencing. One microliter of a PCR mix with amplified DF1-DF4 fragments of *D. fragilis* genotype 1 was cloned in the PCR cloning TA vector, as described by the manufacturer (Invitrogen). After transformation to *Escherichia coli* DH5α and analysis of individual clones, a plasmid containing a single insert was identified. DNA of this plasmid was quantified and used for the sensitivity test of the DF1-DF4 PCR analysis. For direct sequencing of PCR products, an Applied Biosystems Prism 310 dye terminator sensitivity test of the DF1-DF4 PCR analysis. For direct sequencing of PCR products, an Applied Biosystems Prism 310 dye terminator fluorescence-based genetic analyzer was used. PCR products were sequenced from both sides with primers DF1 and DF4. Homology searches were done with the Blast program with default settings (at http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignments were performed with the CLUSTAL W program with default settings (at http://www.ebi.ac.uk/clustalw/).

**RESULTS**

DNA isolated from stool samples of an initial group of 11 patients containing microscopy-detected *Dientamoeba fragilis* was amplified by PCR with the primers and conditions described by Johnson and Clark (14). This PCR amplifies the complete coding region of the ssu rRNA gene and produces an amplicon of approximately 1.7 kbp. Only 3 of the 11 samples tested gave a product of the expected size, while many additional nonspecific bands were observed for all patients’ samples. Amplification of DNA extracted from cultured *D. fragilis* (kindly provided by C. G. Clark) or cultured *Trichomonas vaginalis* showed only the 1.7-kbp product. This indicated that the only PCR method published for the detection of *D. fragilis* (14) was not species specific and inefficient for direct amplification from stool specimens, while subsequent RFLP analysis of the amplicon would be severely hampered by the presence of the nonspecific bands. No inhibitory effect of fecal material could be detected, as DNA extracted from two *D. fragilis*-negative stool samples spiked with *D. fragilis* chromosomal DNA gave bands of the same intensity as without DNA from *D. fragilis*-negative feces.

To obtain a more sensitive and species-specific PCR for the detection of *D. fragilis*, primers DF1 and DF4 were designed, amplifying the region from positions 100 to 761 of the ssu rRNA gene. Both primers contain several consecutive nucleotides at their 3’ ends that match only the *D. fragilis* sequence but not the other known ssu rRNA genes of closely related *Trichomonas* species. PCR analysis of DNA extracted from cultured *D. fragilis* or *T. vaginalis* with primers DF1 and DF4 showed amplification of *D. fragilis* DNA only. This 662-bp amplicon contained two *RsaI* restriction sites; the *RsaI* site at position 313 is species specific and not present in other trichomonads, while one of the four *Ddel* restriction sites present in the amplicon (position 644) could be used to discriminate between the two known genetic variants in *D. fragilis* (14).

To determine the sensitivity of the PCR, the 662-bp amplicon was cloned and a known number of copies were then amplified with the same conditions as for the patients’ samples. This indicated that the detection limit was 10 plasmid copies or an equivalent of approximately 0.1 *D. fragilis* trophozoite. *D. fragilis* does not have a cyst stage, and trophozoites are known to degenerate within a few hours of stool passage. The PCR assay developed was therefore further evaluated with four microscopically *D. fragilis*-positive stool samples stored at room temperature for various time intervals. In all four samples *D. fragilis* DNA could be detected up to 1 week, after which the signal could no longer be observed or became very weak.

PCR amplification with primer set DF1/DF4 of the DNA samples from the 11 patients mentioned above yielded a single product of the expected size in 9 samples. Restriction enzyme digestion of these PCR products with *RsaI* produced identical patterns (Fig. 1, top panel) that were as predicted from the gene sequence. Digestion with *Ddel* revealed a pattern that was distinct for the two known RFLP haplotypes of *D. fragilis* (Fig. 1, bottom panel; G2 and G1, lanes 10 and 11, respectively) and could be used to analyze the variation in the patients’ stool samples. *D. fragilis* amplified directly from the first nine patient stool samples were all of genotype 1.

To further study the genetic diversity of *D. fragilis* in the
human population, stool samples from additional patient
groups and controls, microscopically positive for
*D. fragilis*, were analyzed. These included children (≤18 years) and adults
with gastrointestinal complaints, travelers returning from trop-
cal countries, and asymptomatic carriers. The sample taken on
the second day of the TFT test (TFT2) does not contain a
fixative and can be used for DNA isolation and subsequent
PCR analyses. TFT2 samples subjected to PCR originated
from TFTs were either TFT1 or TFT3 positive for
*D. fragilis*, or TFT1 and TFT3 positive for
*D. fragilis* by microscopic exami-
nation.

Interestingly, intermittent shedding of *D. fragilis* observed in
the TFT was also observed by PCR analyses. In cases where
both TFT1 and TFT3 were positive for
*D. fragilis*, the PCR
detected *D. fragilis* in TFT2 in a high percentage (74%), while
in the cases where only TFT1 or TFT3 were positive, the
percentage of positive TFT2 was much lower (18%). In total,
59 samples were found positive by PCR for
*D. fragilis* (20 of 28
children [71%], 24 of 50 adults [48%], 9 of 15 travelers [60%],
all adults), and 6 of 6 asymptomatic carriers [100%, all adults]
and subjected to RFLP analysis by digestion with *DdeI*. Again
all samples were of genotype 1.

Of the 59 PCR-positive samples, 16 patients (seven children and
nine adults) and four asymptomatic carriers were selected
for sequence analysis of the PCR product. In parallel, the
sequence of the coding region of the ssu rRNA gene of
*D. fragilis* genotypes 1 and 2 was determined. The alignment of
these sequences indicated that there was no variation in the
sequences obtained from the patients or asymptomatic carriers
and that all were identical to genotype 1 (Fig. 2). In addition
to several nucleotide changes, the sequence of *D. fragilis* genotype
2 contained a *DdeI* site at position 644, as expected from the
RFLP haplotyping. The sequence divergence between the ssu
RNA genes of genotypes 1 and 2 was approximately 2%, based
on the 10 nucleotide differences in the 558 bp of the ssu rRNA
genes.

**DISCUSSION**

In the present study we analyzed the DNA of *Dientamoeba
fragilis* directly from feces of various groups of patients and
asymptomatic carriers without the need for prior culturing of
the organism. Culturing of *D. fragilis* has been reported to be
notoriously difficult, and the species has never been cultivated
axenically (5). The absence of a cyst stage and the rapid de-
generation of *D. fragilis* after it leaves its host are probably also
important factors that have impaired progress in research of
this globally occurring parasite. Our knowledge of *D. fragilis*
is deficient in many aspects; nothing is known about the patho-
genicity and route of transmission, while the genetic data are
confined to a single partially sequenced ssu rRNA gene. Our
data indicate that it is now possible to study *D. fragilis* sequences
directly from stool samples and that intact DNA could

**FIG. 2.** Sequence alignment of part of the ssu rRNA gene sequence (between positions 140 and 698) from *D. fragilis* isolated from 16 patient
samples and four asymptomatic controls (isolates) from cultured *D. fragilis* with genotype 1 and from a cloned *D. fragilis* ssu rRNA gene with
 genotype 2. The nucleotide differences between genotypes 1 and 2 are indicated in bold, and the polymorphic *DdeI* restriction site (CTTAG)
present in genotype 2 is underlined.
still be recovered from feces that have been at room temperature for 1 week.

Because *D. fragilis* is reported to give symptoms in only some infected persons (4 to 91%), it is possible that the species contains genetic variants with different pathogenic potential. Genetic variation of intestinal protozoa in humans is not without precedent; * Blastocystis hominis* displays a marked heterogeneity in ssu rRNA gene sequences, but these have not been linked to clinical symptoms (1, 2). *Giardia lamblia* also displays genetic variability at various loci (23), which are also possibly associated with different gastrointestinal complaints (13). The most-studied and best-known example of genetic variation of a intestinal protozoan “species” which was linked to clinical presentation is that of *Entamoeba histolytica*/*E. dispar*. Infection with *E. histolytica* may lead to severe disease, whereas carriers of *E. dispar* remain asymptomatic. Although microscopic techniques are unable to discriminate between these two morphologically identical variants, DNA-based detection techniques have now firmly established that amoebiasis is caused by *Entamoeba histolytica*, while *Entamoeba dispar* is not pathogenic (4, 6).

As a first step towards the identification of genetic variants in *D. fragilis*, Johnson and Clark (14) analyzed nine short-term cultures obtained from stool samples of patients positive for *D. fragilis* (no asymptomatic carriers of *D. fragilis* were analyzed). After amplification of the ssu rRNA gene and RFLP analysis, it appeared that two genetically different variants could be identified in this small number of isolates of patients with gastrointestinal symptoms. These two variants differed by three restriction enzyme sites in their ssu rRNA gene. Although no sequence data were presented, this suggested a sequence divergence of approximately 2% between the two *D. fragilis* variants (14). In the current study, part of the ssu rRNA gene of *D. fragilis* was directly analyzed from stool samples of several different patient groups, including children and adults with gastrointestinal symptoms, travelers, and asymptomatic carriers.

Although the PCR fragment used in the current study is smaller (661 bp) than the PCR product of 1,674 bp described by Johnson and Clark (14), it appeared to be far more efficient and specific for direct analysis of *D. fragilis* from stool specimens, and it could be used to differentiate between the two described genotypes, as it contained one of the polymorphic *Dde*I sites. The genotype could be established from all PCR-positive samples (n = 59) through RFLP analysis. Remarkably, we were unable to find any variation in RFLP pattern in these groups, and all corresponded to genotype 1. RFLP of PCR products is an easy-to-use technique that is commonly employed to identify genetic variation between closely related organisms. The obvious disadvantage is that it will detect sequence variation only within the recognition sites of the restriction enzymes that mostly cover only 4 or 6 bp.

* D. fragilis* contains three stretches of almost exclusively adenine/uracil in its ssu RNA sequence at positions 564 to 616, 685 to 733, and 1385 to 1411 that are absent from ssu RNA sequences from closely related trichomonads and are responsible for the reduced G/C content and increased length of the ssu RNA of *D. fragilis* (20). These adenine/uracil expansion fragments are known to be hypervariable in eukaryotic rRNA but are difficult to access for variability by RFLP. Two of these adenine/uracil expansion segments are present within the DF1/DF4 PCR fragment (positions 564 to 618 and 685 to 733), and to further study the possible sequence variation in *D. fragilis*, the DF1/DF4 PCR fragment was sequenced for 16 patients and four asymptomatic carriers. The adenine/uracil segment at positions 564 to 618 could be sequenced from both strands and did not show any variation among patient- and carrier-derived sequences. Importantly, this sequence was identical for genotype 1 and genotype 2 except for a single transversion from A to T at position 659. This again indicated that there was no variation in the sequences obtained by direct amplification of *D. fragilis* from fecal samples. The estimated sequence divergence between the two ssu rRNA genotypes observed by Johnson and Clark (14) was 2%, which correlates well with our sequencing data, in which 10 of the 558 bp analyzed were different between genotypes 1 and 2.

Our findings that *D. fragilis* displays only a single genotype in fecal samples of various patient groups, including travelers, suggest that genotype 2 is either very rare or that culturing of *D. fragilis* leads to a strong bias in favor of the more rare genotype 2.

Variations in rRNA gene sequences are used only as markers to analyze speciation and search for phylogenetic associations in closely related species. The variation itself is unlikely to have any influence on the pathogenic potential of parasites. It could therefore be worth studying variation in genes in *D. fragilis* which are potentially involved in pathogenicity. Similar studies have been performed on *Entamoeba histolytica* and resulted in the identification of polymorphic gene products that are correlated with virulence in amebiasis (17, 19) and gene products that could be used to determine the geographic origin of isolates and routes of transmission (12, 27, 28). Similar studies on *D. fragilis* should begin with identifying such protein coding genes, as to date only the coding region of the ssu RNA gene has been published (20). The findings presented in the current paper indicate that variation in such newly identified genes could be directly analyzed from large numbers of human fecal samples without the need for prior culturing of *D. fragilis*.

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REFERENCES


