

## Intragenomic Variation in the Internal Transcribed Spacer 1 Region of *Dientamoeba fragilis* as a Molecular Epidemiological Marker<sup>∇</sup>

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*Dientamoeba fragilis* is a parasite that has been recognized to be a causative agent of gastrointestinal symptoms. Because in most studies only some infected persons experience symptoms, it is possible that *D. fragilis* is a heterogeneous species with variants that display similar morphologies but different pathogenicities. The search for genetic variation in *D. fragilis* was based on the small-subunit rRNA gene, which was not found to be useful for molecular epidemiology. In this report, we describe the isolation and characterization of additional rRNA gene cluster sequences, the internal transcribed spacer 1 (ITS-1)–5.8S rRNA gene–ITS-2 region. For comparative purposes, we also isolated the ITS-1–5.8S rRNA gene–ITS-2 region of *Histomonas meleagridis*, a protozoan parasite of birds and a close relative of *D. fragilis*. This region was found to be highly variable, and 11 different alleles of the ITS-1 sequence could be identified. Variation in the ITS-1 region was found to be intragenomic, with up to four different alleles in a single isolate. So-called C profiles were produced from the ITS-1 repertoire of single isolates. Analysis of the C profiles of isolates from nonrelated patients identified several clearly distinguishable strains of *D. fragilis*. Within families, it was shown that members can be infected with the same or different strains of *D. fragilis*. In conclusion, the ITS-1 region can serve as a molecular epidemiological tool for the subtyping of *D. fragilis* directly from feces. This may serve as a means of studying the transmission, geographical distribution, and relationships between strains and the pathogenicity of this parasite.

The protozoan *Dientamoeba fragilis* is found in the mucosal crypts of the large intestines of humans. For a long time, this parasite was ignored by most clinicians; but recently, evidence has been accumulating that *D. fragilis* is an important enteric pathogen associated with abdominal pain, bloating, diarrhea, and irritable bowel syndrome (4). Despite its global distribution and its apparent causative role in gastrointestinal complaints, very little is known about its pathogenicity, route of transmission, epidemiology, or genetics. This neglect of *D. fragilis* is partly due to the difficulties associated with the diagnosis of *D. fragilis* infection. The organism degenerates rapidly in passed stool and lacks a cyst stage, making microscopic detection possible only with freshly passed stool or by the use of fixatives and permanent stains. Recently, PCR-based methods that detect *D. fragilis* in a clinical sample without the need for prior culturing of the organism have been developed (9, 12, 16). These methods make use of the multicopy, small-subunit (ssu) rRNA gene, the sequence of *D. fragilis* that has been the best studied to date. The ssu rRNA gene is useful for the sensitive and specific detection of *D. fragilis* by diagnostic PCR analysis, but due to a lack of variability in both symptomatic and asymptomatic individuals, this target is not suitable for epidemiological studies. Although heterogeneity in the *D. fragilis* ssu rRNA gene sequence, which results in the differenti-

ation of genotypes 1 and 2, was reported (9), the latter genotype was found in only two cases and not in subsequent studies of larger groups of patients in The Netherlands (9), England (17), and Australia (13). This indicated that the ssu rRNA gene of *D. fragilis* displays insufficient variability to be used as a molecular epidemiological marker.

In an effort to detect genetic variations in *D. fragilis* that could be used as molecular epidemiological markers, we isolated and characterized part of the ribosomal gene cluster region containing internal transcribed spacer 1 (ITS-1), the 5.8S rRNA gene (5.8S gene), and ITS-2 of *D. fragilis* and the closely related avian parasite *Histomonas meleagridis*. The ITS-1–5.8S gene–ITS-2 region has been extensively used in phylogenetic studies and as a molecular epidemiological marker for other parasites. Concurrently, intragenomic variation in the ITS region of *D. fragilis* was observed by Windsor et al. (18), who dismissed this target as a marker for strain subtyping, as sequence traces derived from direct amplification would be too complex. However, in the closely related avian parasite *Histomonas meleagridis*, the complexity of ITS sequencing electropherograms was successfully reduced by a method called “C profiling” (14).

The present paper describes the identification of 11 different variants of the ITS-1 region of *D. fragilis*, confirming and extending the results obtained by Windsor et al. (18). As observed with *Histomonas*, analysis of the C nucleotide residues in these complex chromatograms produced chromatograms that were reproducible and easy to interpret. This method could be applied to generate a molecular epidemiological marker of *D. fragilis* directly from stool specimens that can be

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used to study the transmission and geographical distribution of strains, the relationships between strains, and the pathogenicity of this parasite.

#### MATERIALS AND METHODS

**Stool specimens and DNA extraction.** Intestinal parasites, including *D. fragilis*, were detected by the triple feces test (15). Stool specimens that were *D. fragilis* positive by microscopy were collected from patients presenting with gastrointestinal symptoms in routine clinical practice of the Academic Medical Center of Amsterdam. In addition, stool specimens microscopically positive for *D. fragilis* from asymptomatic carriers with no gastrointestinal complaints during the previous 2 months were included. Details of the processing of the fecal samples and subsequent DNA isolation were described earlier (9). A limiting dilution of *D. fragilis* trophozoites was obtained by 10-fold serial dilution of freshly passed stool samples from two patients in phosphate-buffered saline, followed by DNA extraction. *D. fragilis* genotype 2 DNA was isolated from strain Bi/pa (ATCC 30948). *Trichomonas vaginalis* was obtained from a vaginal swab specimen, followed by culturing in Trypticase-yeast extract-maltose. *Histomonas meleagridis* was cultured from the cecum of an infected turkey (14). DNA was isolated from the *T. vaginalis* and *H. meleagridis* cultures as described above for the *D. fragilis* samples.

**PCR method.** The design of forward primer ssu2 (5'-GGAATCCCTTGTAATGCGT-3') was based on the sequences of the ssu rRNA genes of *D. fragilis* (GenBank accession number U37461), *H. meleagridis* (GenBank accession number AF293056), and *T. vaginalis* (GenBank accession number AY677680). Reverse primer lsu1 (5'-AGTTCAGCGGGTCTTCCTG-3') was complementary to a conserved region in the 5' end of the large-subunit (lsu) rRNA genes of *Trichomonad* species. The ssu2 and lsu1 primer combination was used for PCR amplification of the ITS-1–5.8S gene–ITS-2 region. Reverse primer 5.8s1 (5'-TGTGAGGAGCCAAGACATCC-3') was complementary to the 5' end of the 5.8S rRNA gene of *D. fragilis*. The ssu2 and 5.8s1 primer combination was used for PCR amplification of the ITS-1 region. For each series of samples, a negative control containing no input DNA was added. The PCR mixture (50  $\mu$ l) contained 20  $\mu$ l of DNA solution, 100 ng of each primer, 0.5 mM of a deoxynucleoside triphosphate mixture, 5  $\mu$ l of 10 $\times$  PCR buffer (Promega), 6  $\mu$ l of 25 mM MgCl<sub>2</sub>, 5  $\mu$ l of bovine serum albumin (5 mg/ml), 5  $\mu$ l of  $\alpha$ -casein (20 mg/ml) to relieve PCR inhibition by fecal substances, and 0.2 U *Taq* polymerase (Promega). After 3 min of initial denaturation at 94°C, 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min were performed, followed by a final extension at 72°C for 5 min. The amplification products were size fractionated on 1% agarose gels and visualized by ethidium bromide staining.

The sensitivity of the assay was assessed in several PCR experiments with a range of dilutions (in Tris-EDTA buffer with bacteriophage lambda DNA) of a control pGEMT vector containing a single copy of ITS-1. It could be established how many copies were minimally needed under our assay conditions to still obtain a PCR signal. A minimum of 10 copies (representing one individual parasite containing 11 copies) was needed.

**Cloning and sequencing.** One microliter of the PCR product was ligated in the PCR cloning vector pGEMTeasy, as described by the manufacturer (Promega). After transformation into *Escherichia coli* DH5 $\alpha$ , individual clones were tested for the presence of an insert by a colony PCR for 25 cycles with either the primer combination ssu2 and lsu1 (specific for the ITS-1–5.8S gene–ITS-2 region) or the primer combination ssu2 and 5.8s1 (specific for the ITS-1 region). Individual clones with an insert were subjected to sequencing with primer ssu2. For direct sequencing of the ITS-1 PCR products, an Applied Biosystems Prism 310 dye terminator fluorescence-based genetic analyzer was used. Homology searches were done by using the BLAST program with the default settings (available at <http://www.ncbi.nlm.nih.gov/blast/>). Multiple-sequence alignments, C profiles, and insertion/deletion (indel) analysis were performed by using CodonCode software (CodonCode Corporation, Dedham, MA).

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this study for the *D. fragilis* ITS-1–5.8S gene–ITS-2 region appear in the European Molecular Biology Laboratory (EMBL) GenBank database under accession number DQ167586.

#### RESULTS

**Isolation of ITS-1–5.8S gene–ITS-2 region of *D. fragilis* and related species.** To obtain sequence information on the ITS-1–5.8S gene–ITS-2 ribosomal gene region of *D. fragilis*, several

PCR primer pairs were designed to amplify the sequences between the 3' end of the ssu rRNA gene and the 5' end of the lsu rRNA gene. The forward primers were complementary to the published ssu rRNA sequences of *D. fragilis*, *H. meleagridis*, and *T. vaginalis* (GenBank accession numbers U37461, AF293056, and AY677680 respectively) and contained several nucleotides at their 3' ends that did not match the ssu rRNA gene sequences of other parasites prevalent in the human gut (data not shown). We therefore designed reverse primers in a conserved region at the utmost 5' end of the lsu rRNA gene, based on published lsu rRNA gene sequences of trichomonads (data not shown). Only the primer combination ssu2 and lsu1 amplified a product of approximately 500 bp from DNA extracted from human feces containing *D. fragilis*, cultured *T. vaginalis* parasites, and cultured *H. meleagridis* parasites. These PCR products were excised from the gels, cloned, and sequenced. Sequence analysis showed that we indeed successfully cloned the ITS-1–5.8S gene–ITS-2 region of all three species. Both the ITS-1 region and the ITS-2 region of *D. fragilis* and *H. meleagridis* were found to be extremely AT rich (~94%), while the 5.8S rRNA genes had an AT content of approximately 60%. The putative 5' and 3' ends of the ITS-1, 5.8S rRNA gene, and ITS-2 were based on homology with 5.8S rRNA genes of other trichomonads (data not shown) and abrupt changes in the AT content of the sequence. The 5.8S rRNA genes of *D. fragilis* and *H. meleagridis* appeared to be truncated at the 3' end and were approximately 45 nucleotides shorter than those of the other trichomonads (data not shown).

**The ITS-1 sequence of *D. fragilis* is variable.** The PCR for amplification of the complete ITS-1–5.8S gene–ITS-2 region appeared to be too inefficient for the study of the genetic variability of *D. fragilis* parasites directly from human feces. We therefore focused on the ITS-1 region and developed a PCR using ssu rRNA-specific primer ssu2 and 5.8S rRNA gene-specific primer 5.8s1. This PCR was able to efficiently amplify the 3' end of the ssu rRNA and the complete ITS-1 region of *D. fragilis* directly from fecal DNA and from cultured *T. vaginalis* and *H. meleagridis* parasites (Fig. 1A). Analysis of the sequences of the PCR products from *D. fragilis* showed a full match with the published ssu rRNA gene sequence and an aberrant ITS-1 sequence, with many double base calls detected in the sequencing chromatograms (Fig. 1B). This suggested that the ITS-1 sequence of *D. fragilis* is heterogeneous and that several ITS-1 variants were present in a single fecal specimen. An aberrant ITS-1 sequence with many double base calls was also observed in *H. meleagridis* but not in two clinical isolates of *T. vaginalis* (data not shown). The PCR products of *D. fragilis* from fecal samples from eight nonrelated individuals were cloned to further delineate the sequence heterogeneity within the ITS-1 region. Full-length sequencing of individual clones showed that among these eight fecal samples, six samples each contained three ITS-1 alleles, while two and four alleles were detected in one fecal sample each. A total of 11 different ITS-1 alleles could be identified. On the basis of the divergence in the 3' part of the sequence, it was possible to separate these 11 ITS-1 alleles into two groups: alleles 1 to 5 and alleles 6 to 11 (Fig. 1c). Within these two groups of ITS-1 alleles, sequence variation was limited to single A/T indels. In addition to the sequences of *D. fragilis* genotype 1, we also analyzed the ITS-1 region of *D. fragilis* genotype 2 using the

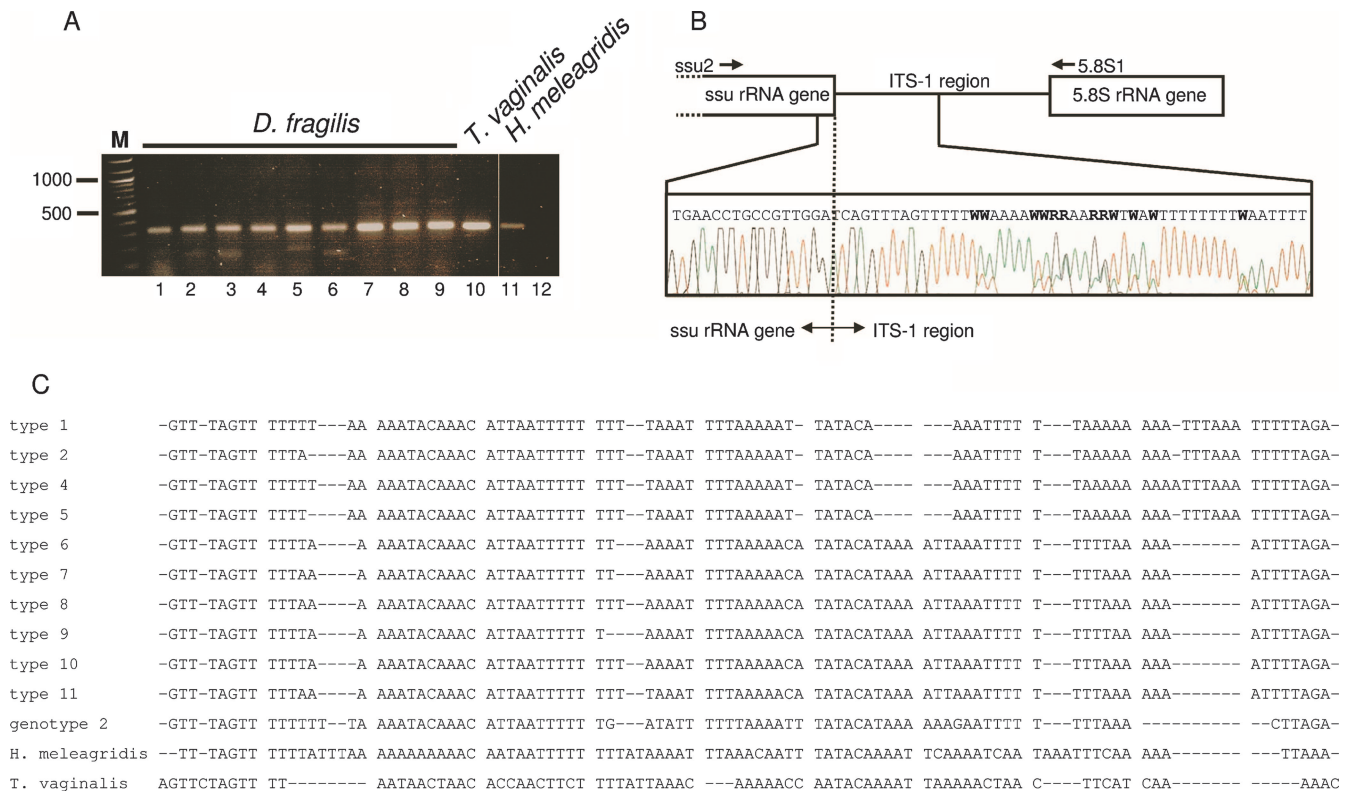


FIG. 1. (A) PCR amplification of the ITS-1 region of *D. fragilis* directly from human feces (lanes 1 to 9) and from cultured *T. vaginalis* (lane 10) and *H. meleagridis* (lane 11) parasites. Lane 12, results of PCR without template. The size marker (lane M) is a 100-bp ladder. (B) Schematic representation of the region amplified by primers ssu2 and 5.8S1. The partial sequencing chromatogram of the 3' end of the ssu RNA gene and the 5' end of the ITS-1 region of *D. fragilis* illustrates the ambiguities and double base calls in the ITS-1 sequence. (C) Sequence alignment of the 11 different cloned ITS-1 regions of *D. fragilis* from fecal samples from eight patients and cloned ITS-1 regions of *D. fragilis* genotype 2, *T. vaginalis*, and *H. meleagridis*. The *D. fragilis* ITS-1 regions can be divided into two groups (types 1 to 5 and types 6 to 11, respectively) on the basis of the presence of the C residue at position 52 and sequence divergence in the 3' part of the ITS-1 region.

same ssu2 and 5.8S1 primer combination. Similar to genotype 1, the ITS-1 region of genotype 2 displayed sequence ambiguity, indicating heterogeneous ITS-1 sequences. The cloned ITS-1 region of *D. fragilis* genotype 2 also showed a high AT content, but its sequence was different from the ITS-1 sequences of *D. fragilis* genotype 1 isolates (Fig. 1C, genotype 2).

**The variability of the ITS-1 sequence can be used as a molecular epidemiological marker for *D. fragilis*.** The presence of multiple ITS-1 alleles of *D. fragilis* in a single human fecal sample resulted in a complex and aberrant chromatogram during sequence analysis (Fig. 1B). A reduction of this complex chromatogram by deleting the peaks for the A, T, and G nucleotides gave a pattern of C nucleotides only that was relatively easy to interpret (Fig. 2). In this so-called C profile, each C residue is represented by multiple adjoining peaks. The multiple peaks for each C residue are caused by A/T nucleotide indels between the individual ITS-1 alleles. The relative heights of the individual peaks of the C profile depend on the relative abundance of the different ITS-1 alleles within a fecal sample. For example, the first two adjacent peaks in the C profiles shown in Fig. 2A correspond to the C residue at position 52 of ITS-1 types 6 to 11 (Fig. 1C). In the C profile of patient samples 1 and 2, these first two peaks were missing. This indicates that in these samples, only ITS-1 sequences missing the C residue at position 52 are present (Fig. 1C,

alleles [types] 1 to 5). The reproducibility of the C profile was evaluated by repeating the DNA extraction and sequence analysis of three fecal samples containing *D. fragilis*. This demonstrated that the C profile was indeed reproducible for a particular sample (data not shown).

Analysis of fecal samples containing *D. fragilis* from samples from 10 nonrelated patients showed four different C profiles, which were present in one, two, two, and five samples, respectively (Fig. 2A). In addition, we tested fecal samples containing *D. fragilis* from the members of two families. For the family whose results are shown in the top panel of Fig. 2B, both the father and the mother were infected with *D. fragilis* but showed no clinical signs. In contrast, their infected son (age, 6 years) did have gastrointestinal complaints. The samples from both parents displayed very similar C profiles, while the sample from their son had a different C profile. Interestingly, the C profile of the sample from the son (C profile 9) was very similar to the C profile of the sample from another patient (Fig. 2A, C profile 2). In the family whose results are shown in the bottom panel of Fig. 2B, the twin sisters (ages, 4 years) and their brother (age, 7 years) were asymptotically infected with *D. fragilis*. The twin sisters were infected with different *D. fragilis* variants (compare C profiles 7 and 8, respectively), while their brother was infected with the same variant as one of his sisters (compare C profiles 8 and 9).

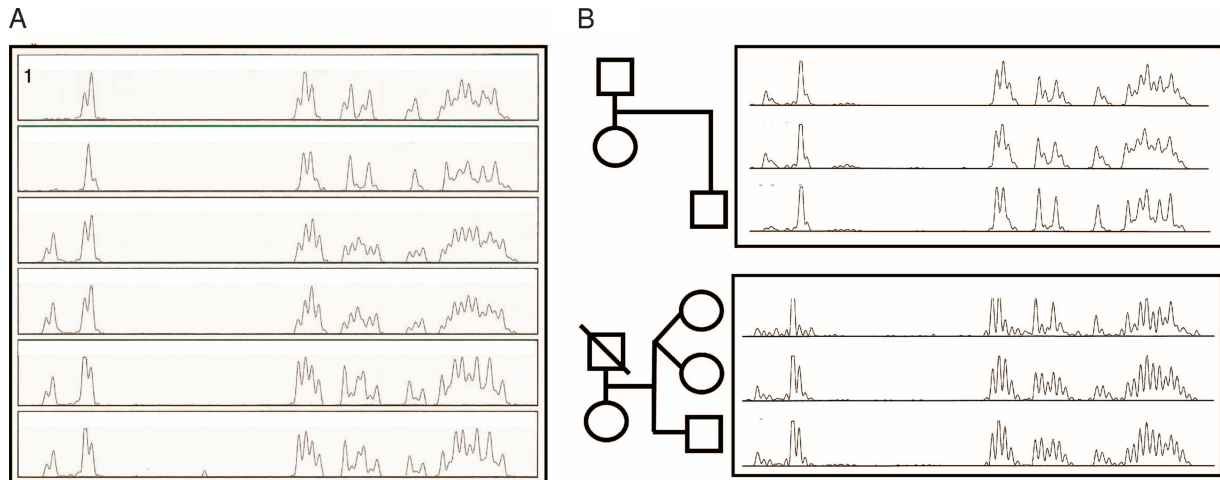


FIG. 2. (A) C profiling of the *D. fragilis* ITS-1 regions directly from fecal specimens of nonrelated patients. Rows 1, 2, 3, and 5, the four different C profiles identified; row 4, C profile from a patient similar to the C profile in row 3; row 6, C profile from a patient similar to the C profile in row 5. (B) C profiling of the *D. fragilis* ITS-1 region in families. C profiles from parents asymptotically infected with *D. fragilis* and their symptomatically infected son are shown in the top panel. The C profiles of twin sisters and their brother are shown in the bottom panel. All three children were asymptotically infected with *D. fragilis*.

**The ITS-1 variation in *D. fragilis* is intragenomic.** The results presented above show that a fecal sample from an infected individual contains different *D. fragilis* ITS-1 alleles. This can be explained either by the presence of multiple *D. fragilis* strains within a single fecal sample or by intragenomic variation of the ITS-1 alleles within a single *D. fragilis* trophozoite, or by a combination of both possibilities. To discriminate between these possibilities, a limiting dilution of freshly passed stool samples from two patients containing *D. fragilis* trophozoites was made. If different strains with different genotypes were present with different relative abundances, then the profile would change as the parasite populations were diluted out. DNA was extracted from each dilution step, and a C profile was produced from the nondiluted sample and from the  $10^{-1}$ ,  $10^{-5}$ , and  $10^{-6}$ -fold dilutions (Fig. 3). In the  $10^{-6}$ -fold dilution, which corresponded to the detection limit, no significant change in the C profile compared to that for the nondiluted sample could be observed. Similar results were obtained with

the sample from the second patient (data not shown). It is highly unlikely that more than one subtype of *D. fragilis* was present in both stool samples with equal abundances and that at the  $10^6$ -fold dilution the complete array of ITS-1 types would still be present. The weight of the evidence therefore indicates that ITS-1 sequence variation is of intragenomic origin only.

Similarly, variation in the ITS of strain Bi/pa (genotype 2) was observed. Since this culturable strain represents a clonal population, variation in this isolate must be intragenomic. Direct sequencing of a PCR product obtained from this strain yielded a complex chromatogram (Fig. 4), for which the CodonCode program predicted heterozygous indels. The sequencing of individual clones derived from the PCR product itself confirmed that the observed chromatogram was the result of sequence heterogeneity, including indel events in mononucleotide stretches in the sequence (Fig. 4).

## DISCUSSION

The search for genetic variation in *D. fragilis* that can be used for molecular epidemiology has been the topic of several recent papers by research groups from the United Kingdom, The Netherlands, and Australia (5, 9, 12, 13, 17). These studies focused on the detection of sequence heterogeneity in the *ssu* rRNA gene of *D. fragilis*, which, to date, is the only sequence information available for this organism. Surprisingly, all but 1 of the 154 *D. fragilis* *ssu* rRNA sequences obtained from clinical samples showed no heterogeneity, and all sequences were assigned to genotype 1. The genotype 2 *ssu* rRNA gene was found in only one clinical sample, and its sequence was identical to that of *D. fragilis* strain Bi/pa (ATCC 30948). From these observations, it was concluded that *D. fragilis* appears to be a clonal species and that the genotype 1/2 variation could not be used for epidemiological studies. In the current study we isolated the ITS-1–5.8S gene–ITS-2 region of *D. fragilis* and examined it for genetic variation. For reasons of comparison,

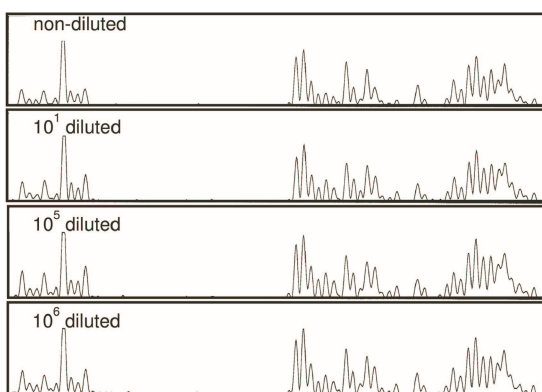


FIG. 3. Limiting dilution of a fecal sample containing *D. fragilis* trophozoites. The C profiles for a nondiluted sample and samples diluted  $10^1$ ,  $10^5$ , and  $10^6$  times are shown. No significant differences between nondiluted and diluted samples were observed.

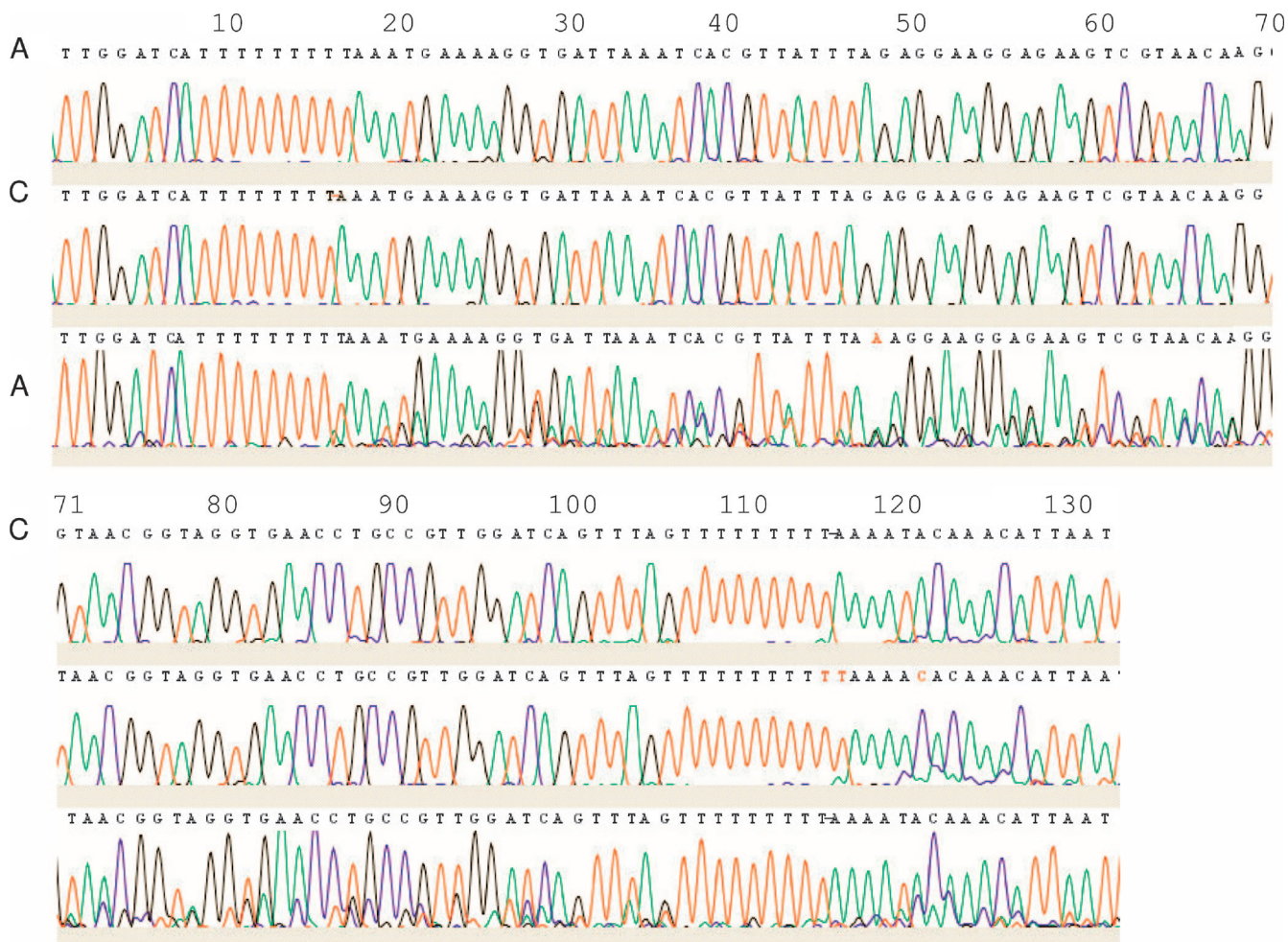


FIG. 4. Sequence chromatograms of two individual clones (clones a and b; top two rows) derived from the PCR product of strain Bi/pa (genotype 2) and the PCR product itself (c; bottom row). CodonCode predicts an indel at position 17, which corresponds to a difference in the stretch of T residues at about position 10 between the two clones (clone a, eight T residues; clone b, nine T residues). From this point onward, each peak is preceded by a smaller identical peak. Variation in the stretch of T residues at about position 110 (clone a, 9 T residues; clone b, 11 T residues) and the T-C transition at position 120 result in even more complexity after position 110.

the ITS-1-5.8S gene-ITS-2 region of the closely related bird parasite *H. meleagridis* was also isolated. The ITS regions of the ribosomal gene cluster have no function in the mature ribosome and are thus likely to sustain much more sequence variation than coding regions. ITS regions are among the markers that are the most frequently used to study intra- and interspecific evolution in all kinds of organisms, including human parasites (2, 6, 8). Analysis of the ITS-1-5.8S gene-ITS-2 region of *D. fragilis* showed that the 5.8S rRNA genes of *D. fragilis* and *H. meleagridis* are surprisingly short (111 bp) compared to the lengths of the 5.8S rRNA genes of other trichomonads (~150 bp). The high degree of homology between the 5.8S rRNA genes of *D. fragilis* and *H. meleagridis* further substantiates the observation that these organisms are closely related (4, 11). The ITS-1 and ITS-2 sequences were extremely AT rich and showed extensive variation in *D. fragilis*, both within and between clinical isolates. Cloning and sequencing of individual ITS-1 regions from *D. fragilis* revealed 11 different ITS-1 alleles which could be separated into two

different groups, confirming and extending the observations of Windsor et al. (18).

Up to four different *D. fragilis* ITS-1 sequences could be observed in a single fecal specimen. Although this could indicate polyparasitism, in which the host is infected with multiple parasite variants (8), limiting-dilution experiments with intact trophozoites demonstrated that the variation in the ITS-1 region is intragenomic. *H. meleagridis* also shows this intragenomic heterogeneity of the ITS-1 region (14), while the two clinical isolates of *T. vaginalis* were devoid of ITS-1 sequence variation. The intragenomic heterogeneity of the ITS regions has already been observed in many different organisms. The extent varies greatly between taxa and is thought to arise by mechanisms such as slipped-strand mispairing events (19).

With the C-profiling technique, we found four different types of *D. fragilis* parasites among 10 randomly chosen, non-related patients. This suggests that the variability that exists in *D. fragilis* is sufficient for the use of this simple technique for the generation of a molecular epidemiological marker. This

was substantiated by the analysis of two families infected with *D. fragilis*. In the first family, both parents were asymptotically infected with the same type, while their 6-year-old son had gastrointestinal complaints and was infected with a different, possibly more virulent strain also detected in another symptomatic subject. Twin sisters of the second family were infected with different types, even though these girls spent most of their time together and visited the same day care center. Interestingly, their brother was infected with the same type as one of his sisters. Clearly, more data from large groups of asymptomatic individuals and patients with different symptomatology are needed to establish a possible link between infection with a particular *D. fragilis* type and the clinical outcome of the infection. Moreover, more types may exist in different geographical locations.

The specificity of the ITS-1 PCR was not investigated in detail in this paper. However, the 204 bp of the ssu rRNA gene sequence that is part of the ~300-bp ITS-1 PCR amplicon was always found to be identical to the sequence of the 3' end of the *D. fragilis* genotype 1 ssu rRNA. DNA was not amplified from other (gut) protozoa, including *Entamoeba dispar*, *Entamoeba coli*, *Entamoeba hartmanni*, *Entamoeba histolytica*, *Blas-tocystis hominis*, *Saccharomyces cerevisiae*, and *Encephalitozoon hellem*, or from smear-negative controls (data not shown).

Our study clearly shows that the intragenomic variation of the ITS-1 region of *D. fragilis* can be used as a molecular epidemiological marker. Variation in this region could be directly analyzed from human feces by PCR amplification and sequencing without the need for prior culturing of the parasite. Techniques such as restriction fragment polymorphism analysis and single-strand conformation polymorphism analysis have also been successfully used for the detection of ITS sequence variations in human parasites (1, 3, 6, 7, 10). However, due to the extreme AT content and intragenomic variation of the *D. fragilis* ITS sequences, these techniques did not give satisfying results in our hands (data not shown). By analyzing only the peaks for C nucleotides from a sequencing chromatogram of the ITS-1 region of *D. fragilis* amplified directly from human feces, a profile that is relatively easy to interpret is obtained. Most research laboratories now have access to fluorescence-based sequence analyzers and should be able to create such C profiles. The ITS-1 C profile can be considered a fingerprint of *D. fragilis* which is found to be highly reproducible. This technique can now be used to discriminate between persistent infection and reinfection after drug treatment and for molecular epidemiological studies that are aimed at investigating the geographical distribution of *D. fragilis* strains, their transmis-

sion, and, most importantly, the possible correlation between various parasite strains and different degrees of virulence.

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