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Characterization of *Giardia duodenalis* by polymerase-chain-reaction fingerprinting

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Abstract The development of a polymerase chain reaction (PCR) based fingerprinting method for the characterization of *Giardia duodenalis* isolates is described. The method uses three different PCRs; one is specific for the A (''Polish'') major group of *G. duodenalis* isolates, another is specific for the B (''Belgian'') group of isolates; and one amplifies a fragment of the glutamate dehydrogenase gene present in all *G. duodenalis* isolates. The PCRs perform highly sensitively on DNA from cultured trophozoites. Isolates were further characterized by restriction-fragment-length polymorphism (RFLP) analysis of the PCR products. In this way, representative isolates from the A and B groups could be grouped together into a number of subgroups. The stability of the genotypes with time and the reproducibility of the two methods were tested on cloned and subcloned lines from a number of isolates and proved to be highly satisfactory. The PCR/RFLP method was evaluated on cysts derived from a number of human patients. It is concluded that the PCR fingerprinting method described in this paper provides a reliable characterization method for *Giardia* isolates and has the potential to be used as a direct method of typing *G. duodenalis* cysts from feces.

Abbreviations *PCR* Polymerase chain reaction · *RFLP* restriction-fragment-length polymorphism · *RAPD* random amplification of polymorphic DNA · *PAGE* polyacrylamide gelelectrophoresis · *AMPPD* 3-(2′-spiroadamantane)-4-methoxy-4(3″-phosphoryloxy)-phenyl-1,2-dioxetane · *Dig* digoxiginin · *GDH* glutamate dehydrogenase

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Introduction

Giardia is a flagellated intestinal parasite found in a wide range of hosts. It is ubiquitous and is the causative agent of giardiasis, one of the most prevalent intestinal diseases in humans (Meloni et al. 1995). On the basis of morphological differences a limited number of species have been recognized, e.g., *G. agilis*, *G. ardeae*, *G. duodenalis*, *G. muris*, and *G. psittaci* (Filice 1952; Erlandsen and Bemrick 1987; Erlandsen et al. 1990). The parasite found in mammals is known as *G. duodenalis* (synonyms: *G. lamblia* and *G. intestinalis*). Genetic characterization has revealed considerable heterogeneity within the species *G. duodenalis* (Nash et al. 1985; Andrews et al. 1989; Meloni et al. 1989, 1995; Homan et al. 1992; Monis et al. 1996).

A number of recent studies are in general agreement with the observation that *G. duodenalis* isolated from mammals, including humans, consists of at least two groups that differ to a great extent in the DNA sequence of a number of genes (Homan et al. 1992; Nash 1992; Weiss et al. 1992; Upcroft et al. 1994; van Keulen et al. 1995; Mayrhofer et al. 1995). Isolates from the two groups, formerly termed ''Polish'' and ''Belgian'' (Homan et al. 1992), have been relabeled A and B (Monis et al. 1996). Within the two groups, isolates have been reported to be heterogeneous as well (Homan et al. 1992; Ey et al. 1993; Mayrhofer et al. 1995; Monis et al. 1996). Recently, Hopkins et al. (1997) described two genetic groups, different from the A and B groups, isolated exclusively from dogs.

A correlation between the genetic classification of *G. duodenalis* into the A and B groups or subgroups and the host range or virulence has never been detected (Meloni et al. 1995; Monis et al. 1996). A possible reason for this is that genetic markers for these characteristics have been missed by the approaches used. Another possibility is that most of the *Giardia* isolates that have been tested to date have been heavily selected by their cultivation and, hence, do not represent the diversity

present in the various hosts. Axenization of *Giardia* is generally known to be a highly selective process, producing only a fraction of the *Giardia* population present in patients' material. In the most successful excystation experiments, only up to 50% of the starting batches result in viable trophozoites (Kasprzak and Majewska 1985; Hautus et al. 1988; Thompson et al. 1990). An example is the notorious problem of obtaining *G. duodenalis* cultures from dogs. Moreover, there are several reports on selection during the cultivation of trophozoites; different genotypes were produced from a single batch of cysts, depending on the growth conditions used (Andrews et al. 1992; Mayrhofer et al. 1992), or even when different samples obtained from a single patient were cultured under identical conditions (Butcher et al. 1994; Upcroft and Upcroft 1994). From these studies it can be concluded that a single host can harbor mixed cultures and that in these cases a single genotype is selected during cultivation. Both the low success rate of excystation and the selection during cultivation of trophozoites clearly weaken the classification systems based on cultured *Giardia*. Direct characterization of *Giardia* cysts will be necessary before we can investigate the extent of selection that occurs during cultivation.

In this paper we describe the development of a sensitive PCR-based fingerprinting assay that can be used to characterize low quantities of *Giardia* DNA. Three PCRs were produced that amplify polymorphic parts of the *Giardia* genome, and the PCR fragments were analyzed by RFLP analysis for the presence of genetic markers that differentiate *G. duodenalis* isolates. The method was tested on a number of cysts isolated from human feces.

Materials and methods

Origin of *Giardia duodenalis* isolates

The majority of *Giardia* isolates that were used in this study have been described elsewhere and have been typed by DNA analysis as members of the "Belgian" or "Polish" group (Table 1; Homan et al. 1992; van Belkum et al. 1993). Isolates from human subjects were collected in Dutch hospitals [codes AMC and Nij (Homan et al. 1992; van Belkum et al. 1993) and the CAM-1 isolate from a member of the Dutch military returning from Cambodia], from Belgium (LD-18; Gordts et al. 1984), Poland (HP-98; Kasprzak and Majewska 1985), Israel [KC-8; isolated by Korman et al. (1986) and described by De Jonckheere and Gordts (1987)], the United States (Portl-1; Meyer 1976), and Australia (BAH-8; Meloni et al. 1988). Isolate BAC-1 was isolated from a cat by Meloni et al. (1988).

Cysts obtained from stool samples collected from patients aged from 0 to 65 years who had consulted their general practitioner with diarrheal complaints of more than 1 week's duration or intermittent diarrhea were kindly provided by Dr. T. Mank (Stichting Artsenlaboratorium Haarlem) and Dr. T. Kortbeek (National Institute of Public Health and the Environment, Bilthoven).

Cloning and subcloning of *G. duodenalis* isolates

Cloning and subcloning of parasites was essentially carried out as described elsewhere (Binz et al. 1991). In brief, 1 µl of diluted trophozoite suspension (0.3 parasite/µl) dropped onto one-third of a coverslip was examined microscopically. Drops containing single parasites were immersed in 4 ml of Keisters modified Diamond's TY1-S-33 medium supplemented with 0.8 g of bovine bile/l and 10% bovine serum (Diamond et al. 1978), penicillin (100 IU/ml), streptomycin (75 IU/ml), and gentamycin (50 µg/ml). After 10–12 days of incubation at 37 °C, larger volumes were inoculated if dividing trophozoites were observed. Inoculation experiments were aimed at obtaining 10 clones, 1 of which was selected for further inoculation of 20 subclones. The number of subclones ultimately produced ranged from 4 to 10.

DNA isolation, gel electrophoresis, and Southern-blot analysis

The isolation of *Giardia* trophozoite DNA, electrophoresis on 1.5% agarose gels, Southern blotting, and hybridization were performed as previously described (Sambrook et al. 1989). A PCR fragment derived from isolate LD-18 was used as the probe in the hybridization experiment. It was purified from agarose gels using the Prep-a-Gene DNA purification kit (BioRad, Veenendaal, The Netherlands) and digoxigenin (dig)-labeled with the dig-deoxyuridine triphosphate (dig-dUTP), labeling and detection kit (Boehringer, Mannheim) according to the conditions recommended by the manufacturers. Hybridization was performed at 68°C and dig-labeled hybrids were visualized with 3-(2′-spiroadamantane)-4-methoxy-4(3″-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD) using the conditions recommended in the dig-dUTP labeling and detection kit from Boehringer (Mannheim). Molecular-weight markers (unlabeled and dig-labeled) were obtained from Boehringher (Mannheim).

DNA isolation from cysts

Cysts derived from human patients with diarrheal complaints were isolated and concentrated from feces according to the method of Hautus et al. (1988). For DNA isolation the cysts were suspended in lysis buffer provided in the XTRAXTM DNA extraction kit

Table 1 Classification of *Giardia duodenalis* isolates from A/B and GDH PCRs by RFLP (Dde1) of PCR products. Groups were compared with the classifications of Monis et al. (1996) and Homan et al. (1992) (–Not done)

Isolate	A/B DdeI	GDH Dde1	Monis et al. (1996)	Homan et al. (RFLP) (1992)
KC-8	A1	a1	A-II	4
CAM-1	A1	a1	–	–
AMC-1	A1	a1	A-II	6
AMC-6	A1	a1	A-I	2
AMC-7	A1	al	A-I	2
AMC-12	A1	a1	A-II	5
Nij-1	A1	a1	A-II	9
HP-98	A2	a1	A-I	1
Nij-2	A2	a1	A-I	–
Portl-1	A2	a1	A-I	1
BAC-1	A3	a1	–	8
AMC-13	A4	a1	A-II	3
AMC-3	B1	b2	not A	12
AMC-4	B1	b1	not A	15
AMC-5	B1	b1	not A	15
AMC-10	B1	b5	–	16
Nij-3	B1	b1	–	–
Nij-4	B1	b1	B	–
Nij-5	B1	b1	B	–
AMC-9	B1	b2	not A	14
BAH-8	B2	b2	B	17
LD-18	B3	b2	not A	10
AMC-2	B4	b3	not A	13
AMC-8	B5	b4	–	11

(Gull Laboratories, Salt Lake City, Utah) and were treated for 2 min in a minibeadbeater (Biospec. Bartlesville, Okla.) using 0.5-mm zirconium beads. After centrifugation the supernatant was further treated according to the procedure provided in the XTRAXTM DNA extraction kit.

Molecular cloning

Procedures for ligation, transformation, and clone selection were performed as described by Sambrook et al. (1989). The isolation of the highly polymorphic "Polish" (A)- and "Belgian" (B)- specific DNA clones (pGH835 and pGL524, respectively) has been described elsewhere (Homan et al. 1992). Both DNA clones were subcloned and partially sequenced to produce sequence information for the selection of specific primers. Subcloning of the Pst-1-digested insert from clones pGH835 and pGL524 in low-melting-point agarose was performed as described by Sambrook et al. (1989). Subclones were selected for positive hybridization with DNA from A or B group isolates in a Southern blot. A 3.2-kDa insert from clone pGH835 was used to produce nested deletions by the Erase-a-base system (Promega, Leiden, The Netherlands) after Kpn 1 and Xba 1 digestion. Nested deletions were partially sequenced with the Prism Ready Reaction Dye Primer Cycle Sequencing kit (Applied Biosystems, Maarssen, The Netherlands) using fluorescent M13 primers. Sequencing was performed on an Applied Biosystems 373A DNA sequencer. From the sequence, two A-specific primer pairs were selected: the forward primer P1F – 5′CTGCAGGGGCAAGGCGTAGAT3′ – and the reverse primer P3R – 5′CCACCGTGCCAGTCTTCTGGG3′. The region of the forward primer and 20 bp downstream of the primer showed 95% identity with the major *Giardia* trophozoite surface antigen TSA 417 (bases 2328–2368), whereas the reverse primer did not produce any similarity after a BLAST search.

A 0.8-kb fragment from clone pGL524 was sequenced and the sequence was used for the selection of two B-specific primers: the forward primer B1F: – 5′CTGCAGTAACACTGGCAAG3′ – and the reverse primer B3R – 5′CTGCAGAGTTCTCCGCAGCG3′. No similarity was found after a similarity search using BLAST.

Polymerase chain reactions

After optimalization of PCRs with primer sets P1F/P3R and B1F/B3R for $MgCl_2$ concentration, pH, and annealing temperature the following conditions were found to be optimal for both PCRs: 0.5U Taq enzyme (Perkin Elmer), 60 m*M* TRIS, 15 m*M* $(NH_4)_2SO_4$, (pH 9.0), 100 µmol of each deoxynucleoside triphosphate (dNTP), 0.5 µ*M* forward and reverse primer, and 2 m*M* $MgCl_2$ in a 25-µl reaction mixture. Amplifications were performed on a Perkin Elmer thermal cycler under the following conditions for the "A" PCR: 94°C for 7 min, then 30 cycles comprising 1 min at 94 °C and 2 min at 72°C, followed by a final extension of 7 min at 72°C. For the "B" PCR the following conditions were used: 94°C for 7 min; then 35 cycles comprising 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final extension of 7 min at 72°C. From the sequence of the glutamate dehydrogenase gene (Yee and Dennis 1992), two primers were selected for use in a general *Giardia*-specific PCR. Forward primer GDH1 (ATCTTCGAGAGGATGCTTGAG) and reverse primer GDH4 (AGTACGCGACGCTGGGATACT) were used in the same PCR mixture described for the "A" and "B" PCRs and amplifications were performed under the following conditions in a Perkin Elmer thermal cycler: 94°C for 7 min; then 35 cyles comprising 1 min at 94°C, 1 min at 55 °C, and 1 min at 72°C; followed by a final extension of 7 min at 72°C.

Restriction enzyme analyses of PCR fragments

Aliquots 10 µl of PCR reaction mixture were digested at 37°C for 2.5 h using 4U restriction endonucleases (Boehringer Mannheim) in a 25-µl final volume of the appropriate digestion buffer. Restriction products were analyzed on ethidium bromide-stained 2% agarose gels. "B" PCR products were isolated from agarose gels prior to restriction analysis using the QIA quick gel extraction kit (Qiagen, Valencia, Calif.) according to the protocol provided by the manufacturer.

Results

Development of *Giardia*-specific PCRs

The "A"-specific PCR produced a single 800-bp product, whereas the "B"-specific PCR produced a major band of 1500 bp together with several minor lower-molecular-weight bands (see Figs. 1 and 2, respectively). Both the "A"- and "B"-specific PCRs were capable of detecting 1 pg of DNA in ethidium bromide-stained agarose gels (data not shown). For determination of the specificity of the two PCRs for their respective groups, DNA from several *Giardia* isolates belonging to the A and B groups were tested in both PCRs. Strains were selected that represented different genotypes (Homan et al. 1992).

The "A"-specific PCR produced the 800-bp band with DNA from all A-group isolates, whereas DNA from B-group isolates did not produce a band (Fig. 1). In the "B"-specific PCR, all B-group isolates produced

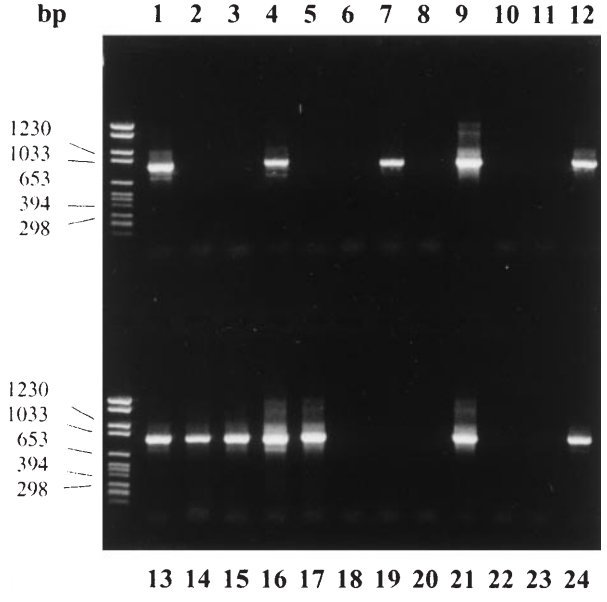


Fig. 1 Ethidium bromide-stained agarose gel of "A" PCR products from DNA of several *Giardia duodenalis* isolates. DNA (5 ng) from the following isolates was subjected to the "A" PCR (A and B indicate the distinction measured previously; Homan et al. 1992): *1* KC-8 (A), *2* Nij-5 clone 1 (cl 1) (B), *3* AMC-2 (B), *4* CAM-1 (A), *5* AMC-3 (B), *6* AMC-4 (B), *7* AMC-6 (A), *8* AMC-5 (B), *9* HP-98 (A), *10* LD-18 (B), *11* AMC-8 (B), *12* BAC-1 (A), *13* AMC-12 (A), *14* AMC-13 (A), *15* Nij-1 (A), *16* AMC-1 (A), *17* Portland-1 (A), *18* Nij-3 (B), *19* BAH-8 (B), *20* Nij-4 (B), *21* Nij-2 (A), *22* AMC-10 (B), *23* AMC-9 (B), *24* AMC-7 (A). Molecular-weight markers *from top to bottom* represent 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234–220, and 154 bp

the major 1500-bp band and some minor low-molecular-weight bands (ranging from 300 to 700 bp). The appearance of these low-molecular-weight bands was not influenced by a change in the pH, the $MgCl_2$ concentration, or the annealing temperature of the PCR reaction. In some of the B-group isolates a small variation in length was observed among the minor bands (AMC-2 ; Fig. 2, lane 3). Although minor low-molecular-weight bands were also visible in the PCR product from A-group DNA samples, the major 1500-bp band was not seen in any of the A-group isolates (Fig. 2). The specificity of the "B" PCR for B-group DNAs was confirmed by Southern-blot analysis using a dig-labeled B-specific PCR fragment as the probe. The probe strongly hybridized onto the 1500-bp product of B-group DNA, whereas PCR products derived from A-group DNA did not react (data not shown). As all DNA samples reacted in one of the two PCRs it was concluded that the observed specificity was not due to inhibition of the polymerase reaction and that both the "A" and "B" PCRs are highly specific for isolates in their respective groups.

A third PCR amplifying a 768-bp fragment of the GDH gene was used as a confirmation of the presence of *Giardia* in fecal samples. All isolates that were tested produced a band of the right size (data not shown).

Characterization of PCR fragments

For further characterization of the *Giardia* isolates within both the A group and the B group, PCR products from both groups were subjected to RFLP analysis. For minimization of complexity in the case of the "B" PCR the 1500-bp B PCR product was extracted from the agarose gel prior to digestion. PCR products from the A and B isolates previously used for the specificity tests (see Figs. 1, 2) were digested with Dde1 and the reaction products were analyzed on agarose gels. Figure. 3 shows that four distinctive patterns could be distinguished in isolates of the A group. The majority of the isolates (lanes 1–3, 6, 8, 9, 12) revealed a characteristic four-band pattern plus some minor high-molecular-weight bands (pattern A1). Isolates in lanes 3 and 12 were included into the A1 group, as the additional 160-bp band produced by these isolates did not appear reproducibly in other experiments. Three isolates (lanes 4, 10, 11) lacked the upper 400-bp band and exhibited a high-molecular-weight band of 650 bp (pattern A2). One isolate (lane 5) lacked the 400-bp band (pattern A3), and the isolate in lane 7 exhibited a 510-bp band instead of the 400-bp band (pattern A4). In the B-group isolates, five different patterns showing subtle differences were observed (Fig. 4). A large group (lanes 1, 3–5, 8, 10–12) repro-

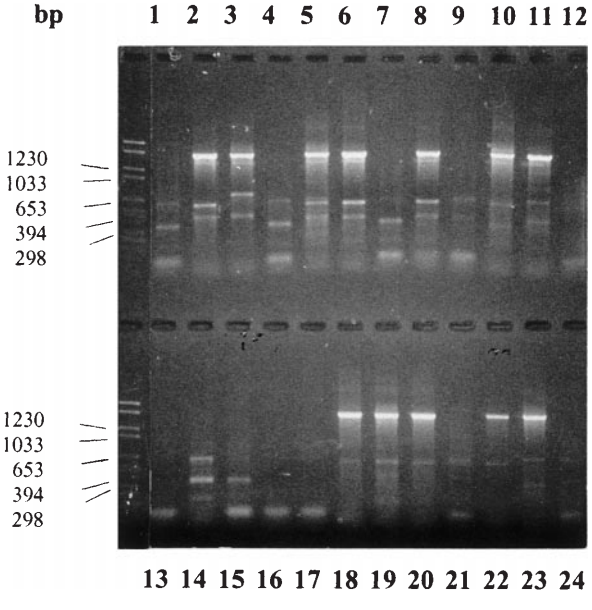


Fig. 2 Ethidium bromide-stained agarose gel of "B" PCR products from DNA of several *G. duodenalis* isolates. DNA (5 ng) was subjected to the "B" PCR. *Lane numbers* are identical to those given in Fig.1. Molecular-weight markers *from top to bottom* represent 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234–220, and 154 bp

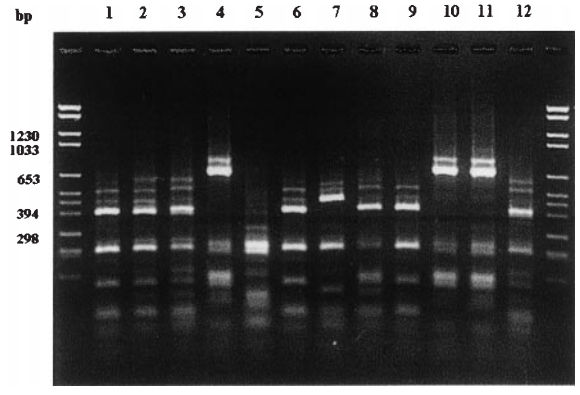


Fig. 3 Ethidium bromide-stained agarose gel of Dde1-digested PCR products from A isolates. PCR products from the following A isolates were analyzed: *1* KC-8, *2* CAM-1, *3* AMC-6, *4* HP-98, *5* BAC-1, *6* AMC-12, *7* AMC-13, *8* Nij-1, *9* AMC-1, *10* Portland-1, *11* Nij-2, *12* AMC-7. Molecular-weight markers *from top to bottom* represent 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234–220, and 154 bp

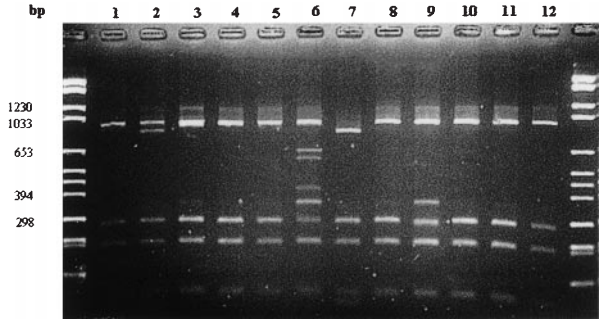


Fig. 4 Ethidium bromide-stained agarose gel of Dde1-digested PCR products from B isolates. PCR products from the following B isolates were analyzed: *1* Nij 5-cl1, *2* AMC-2, *3* AMC-3, *4* AMC-4, *5* AMC-5, *6* LD-18, *7* AMC-8, *8* Nij- 3, *9* BAH-8, *10* Nij-4, *11* AMC-10, *12* AMC 9. Molecular-weight markers *from top to bottom* represent 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234–220, and 154 bp

ducibly showed a characteristic major three-band pattern (pattern B1). The isolate in lane 9 produced an extra band of around 350 bp (pattern B2), whereas the isolate in lane 6 produced several other extra bands (pattern B3), the isolate in lane 2 produced an extra band just beneath the 950-bp band (pattern B4), and in lane 7 the 950-bp band was replaced by a lower-molecular-weight band of approximately 800 bp (pattern B5; Fig. 4). The classification of isolates from both the A group and the B group into a number of subgroups on the basis of these patterns is shown in Table 1.

Dde1 restriction of the GDH PCR products also produced several patterns (Fig. 5). The product from all A isolates had identical band patterns (lanes 1, 4, 7, 9, 12–17, 21, 24; pattern a1), whereas five different patterns were observed within the B isolates (lanes 2, 6, 8, 18, 20: pattern b1; lanes 5, 10, 19, 23: pattern b2; lane 3: pattern b3; lane 11: pattern b4; lane 22: pattern b5).

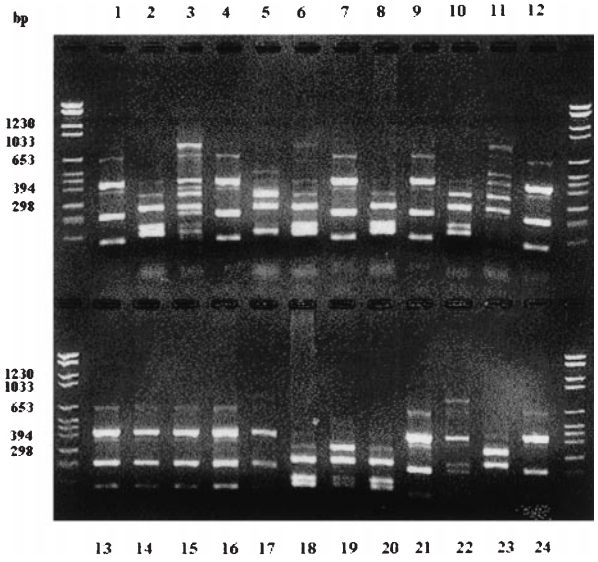


Fig. 5 Ethidium bromide-stained agarose gel of Dde1-digested GDH PCR products from the following A and B isolates. 1 KC-8 (A), 2 Nij-5 clone 1 (cl1) (B), 3 AMC-2 (B), 4 CAM-1 (A), 5 AMC-3 (B), 6 AMC-4 (B), 7 AMC-6 (A), 8 AMC-5 (B), 9 HP-98 (A), 10 LD-18 (B), 11 AMC-8 (B), 12 BAC-1(A), 13 AMC-12 (A), 14 AMC-13 (A), 15 Nij-1 (A), 16 AMC-1 (A), 17 Portland-1 (A), 18 Nij-3 (B), 19 BAH-8 (B), 20 Nij-4 (B), 21 Nij-2 (A), 22 AMC-10 (B), 23 AMC-9 (B), 24 AMC-7 (A). Molecular-weight markers *from top to bottom* represent 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234–220,and 154 bp

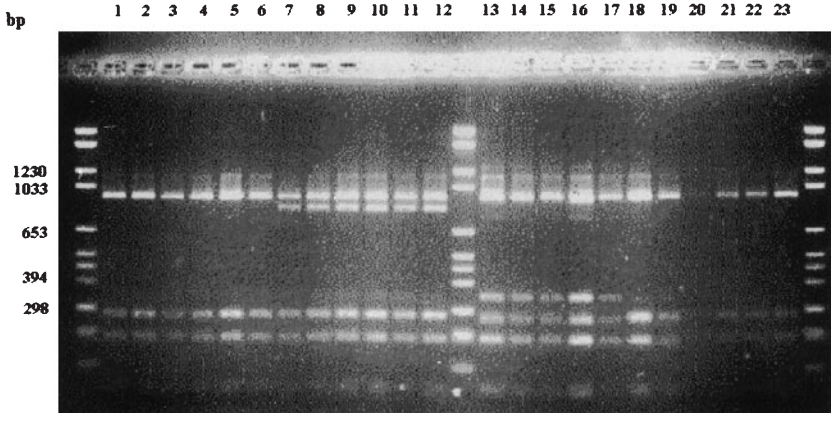
Analyses of clones and subclones of *G. duodenalis* isolates

The reproducibility of the characterization methods described in this report and the stability with time of the banding patterns produced was examined by comparison of a number of original isolates with cloned and subcloned lines obtained from them. One clone and five subclones from the A-type isolates Portland-1 and Nij-2 produced restriction fragment patterns that were identical to each other and to that of the original isolate (pattern A2; data not shown). The results obtained for clones and subclones of the B-type isolates AMC-4 (lanes 1–6), AMC-2 (lanes 7–12), BAH-8 (lanes 13–17), and Nij-5 cl2 (lanes 18–23) are shown in Fig. 6. The patterns obtained from the original isolates, clones, and subclones were consistently identical. The differences observed between the isolates were maintained in the subclones; Portland-1 and Nij-2 produced identical restriction patterns, as did AMC-4 and Nij-5, whereas AMC-2 and BAH-8 formed separate groups (cf. Fig. 6 with Fig. 4).

Fig. 6 Ethidium bromide-stained agarose gel of Dde1-digested "B" PCR products from subcloned B isolates. Clones and subclones from AMC 4, AMC 2, BAH 8, and Nij5 were subjected to the B-specific PCR followed by Dde1 restriction analysis. Isolates tested were: *1* AMC-4, *2* AMC-4cl5, *3* AMC-4cl5#4, *4* AMC-4cl5#6 *5* AMC-4cl5#7, *6*AMC-4cl5#9, *7* AMC-2, *8* AMC-2cl2, *9* AMC-2cl2#1, *10* AMC-2cl2#4, *11* AMC-2cl2#5, *12* AMC-2cl2#12, *13* BAH-8cl3#1, *14* BAH-8cl3#2, *15* BAH-8cl3#7, *16* BAH-8cl3#11, *17* BAH-8cl3#15, *18* Nij-5cl2, *19* Nij-5cl2#1, *20* Nij-5cl2#2, *21* Nij-5cl2#4, *22* Nij-5cl2#7, *23* Nij-5cl2#9. Molecular-weight markers *from top to bottom* represent 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234–220, and 154 bp

Characterization of *Giardia* cysts from human feces

DNA was extracted from cysts isolated from 15 different human fecal samples. The GDH PCR classified cysts as



either the A or the B type (Fig. 7). No mixture of patterns was evident, indicating that no mixture of the A and B types was present. All A-type cysts exhibited pattern a1 (lanes 4, 7–11, 13–15), whereas B types exhibited pattern b1 (lanes 1–3, 5, 6) except for the cyst isolation in lane 12, which exhibited pattern b2 (Fig. 7). Cysts classified as A and B by the GDH PCR were further analyzed using the "A" and "B" PCRs (Fig. 8, lanes 1–9 and 10–15, respectively). The A-type cyst isolate in lane 2 exhibited the A2 pattern, whereas the other cyst isolates produced pattern A1. All B-type cyst isolates exhibited pattern B1 except for the cyst isolate in lane 11, which produced an additional high-molecular-weight band, possibly due to partial digestion of the 1500-bp product (Fig. 8). No indication of mixtures of subgroups was detectable (Figs. 7, 8).

Discussion

The development of a PCR-based fingerprinting method that can differentiate strains of *Giardia duodenalis* is described. Due to its sensitivity and specificity it is the method of choice for direct characterization of cysts obtained from the environment and from feces. The method uses two different PCRs specific for the two major groups recognized in axenized *G. duodenalis* isolates and a general PCR amplifying the household gene glutamate dehydrogenase. Isolates could be differentiated by RFLP analysis of the products derived from both PCRs. The differentiation obtained was complementary. Isolates from the A group did not show any polymorphism in the Dde1-restricted GDH fragment, whereas the "A" PCR classified A isolates into five groups. Classification was more complex in the case of the B group of isolates: the "B" PCR classified isolates into five groups, most isolates having the B1 genotype. The GDH PCR also classified isolates into five groups, genotype b1 being the most dominant. Except for isolates AMC-3, AMC-9, and AMC-10, genotypes B1 and b1 are linked.

The differentiation obtained by the method described in this paper, though producing less resolution, is consistent with the classification based on the RFLP analysis of the *Giardia* genome (Homan et al.1992). It is also consistent with the division into A and B/non-A genotypes and the subgrouping of A isolates into groups I and II as defined by Andrews et al. (1989; also see Table 1). The indistinguishable isolates AMC-6 and AMC-7, however, form a notable exception. Whereas they were distinct from isolates HP-98, Nij-2, and Portl-1 as determined by the methods described in this report, they were grouped together with isolates HP-98, Nij-2, and Portland-1 in the A-I cluster as determined by polymorphic markers on the tsa417/tsp11 surface protein genes (Monis et al. 1996). The genetic background for the observed lack of correlation between the sets of independent markers observed for isolates AMC-6 and AMC-7 and for isolates AMC-3, AMC-9, and AMC-10 remains unclear.

The RFLP patterns produced by the "A" and "B" PCR products provide us with a reproducible and stable classification method for *G. duodenalis* isolates – reproducible because repeated assays resulted in identical results for a single isolate and, more importantly, because clones and subclones produced patterns identical to each other and to the original isolates. The stability of the markers produced by RFLP analysis was deduced from the results obtained with cloned and subcloned lines. Subclones and the original isolate are separated by numerous division cycles and time, as they were raised from single cloned individuals that, again, were derived from a single organism in the original isolate. Because all subclones and clones produced identical patterns for the most prominent bands the pattern is stable for at least the period studied (1–2 months). The conclusion that the patterns produced by RFLP analysis provide stable markers is reinforced by the finding that several isolates obtained from different patients and from a different origin produced identical genotypes.

The stability of genotypic markers for epidemiology studies is a matter of debate with regard to *Giardia*

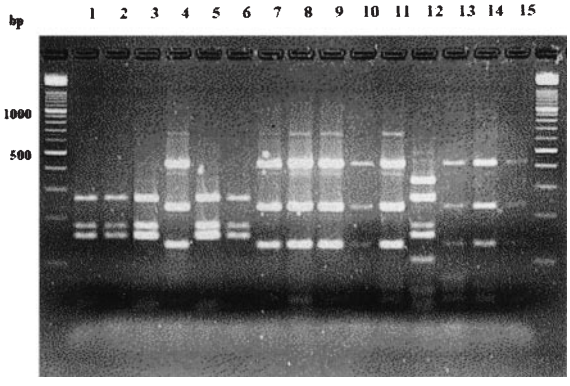


Fig. 7 Ethidium bromide-stained agarose gel of Dde1-digested GDH PCR products from DNA extracted from human-derived *Giardia* cysts. Lanes 1–15 Samples 12, 13, 15, 17, 18, 20, 23, 24, 25, S, vS, dR, M, 30, and 01. *A 100-bp ladder* (Boehringer Mannheim) serves as the molecular-weight marker

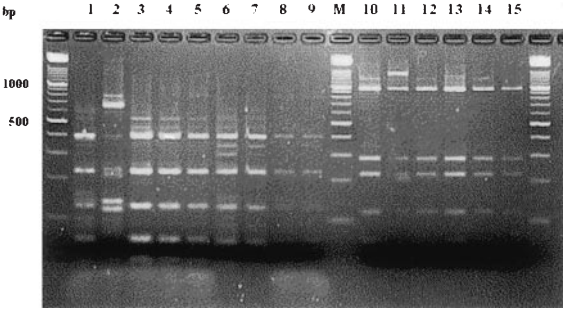


Fig. 8 Ethidium bromide-stained agarose gel of Dde1-digested "A" and "B" PCR products from DNA extracted from human-derived *Giardia* cysts. Lanes 1–9 "A" PCR on samples 17, 23, 24, 25, S, vS, M, 30, and 01. Lanes 10–15 "B" PCR on samples 12, 13, 15, 18, 20, and dR. *A 100-bp ladder* (Boehringer Mannheim) serves as the molecular-weight marker

(Carneby et al. 1994). The *Giardia* genome exhibits extensive genomic plasticity due to the frequent genomic rearrangements seen in *Giardia* chromosomes. A mutation rate of ~1% per cell per division has been reported for the rDNA-containing chromosomes (Le Blancq et al. 1991; Hou et al. 1995). Especially markers localized in repeated elements might be subject to short-term heterogeneity and are therefore not suitable for population or epidemiology studies. Carnaby et al. (1994) have presented results indicating that genetic heterogeneity in *Giardia* as visualised by M13 DNA fingerprinting is due to the genomic plasticity of the *Giardia* genome and arises in a relatively short period of cultivation (months).

RAPD and interrepeat PCR analyses of clones and subclones of *G. duodenalis* have also revealed the lability of at least some of the markers (van Belkum et al. 1993). By isoenzyme and RFLP analysis, several genotypes have been recognized in axenized *G. duodenalis* isolates (Nash et al. 1985; Andrews et al. 1989; De Jonckheere et al. 1990; Homan et al. 1992; Weiss et al. 1992; Ey et al. 1993; Meloni et al. 1995). However, care should be taken in the classification of *Giardia* isolates obtained from axenized cultures. Selection for certain genotypes of *G. duodenalis* due to the different growth conditions used for the establishment of *Giardia* cultures is well reported (Andrews et al. 1992; Mayrhofer et al. 1992). Moreover, besides the selection of genotypes in culture, a large number (~50%) of *Giardia* isolates are reported to be refractory to in vitro culture (Kasprzak and Majewska 1985; Hautus et al. 1988; Thompson et al. 1990). The conclusion that can presently be drawn is that cultivation of *Giardia* isolates, though an important step toward the understanding of a number of basic questions concerning *Giardia*, may have resulted in a biased insight into the extent of genetic variability of *Giardia* in different hosts and into the correlations between genotypes and biologically important parameters.

Direct characterization of cysts can solve important questions involving issues such as the presence of mixtures, the influence of genetic variability on the normal course of infection, the correlations between genotypes and host or pathogenicity, and the selection for irrelevant genotypes during cultivation. Future work will focus on the characterization of cysts directly from the host using a sensitive and specific assay as described in this paper. The preliminary results obtained in a limited number of human samples show that the method works well. No mixture of genotype could be detected, though the sensitivity of the method does yet not allow typing of a single cyst, the implication being that low numbers of cysts of other genotypes could be missed.

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