Distinct genetic groups of *Giardia intestinalis* distinguished by restriction fragment length polymorphisms

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The taxonomic status of the parasitic protozoal species *Giardia intestinalis* depends on the morphological similarity of all *Giardia* isolated from humans and the presumption that *Giardia* are host-specific. On the basis of electrophoretic data derived from examination of 26 enzyme loci in Australian isolates, it has been proposed that *G. intestinalis* is a species complex comprising three or four genetically distinct (but morphologically cryptic) species. These received the tentative designations of genetic groups I–IV (R. H. Andrews, M. Adams, P. F. L. Boreham, G. Mayrhofer & B. P. Meloni. *International Journal for Parasitology* 19, 183–190, 1989). In the present study, two unrelated DNA probes (one specific for a gene encoding a trophozoite surface protein, the other detecting a non-coding repetitive sequence within the *G. intestinalis* genome) were used in Southern hybridization analyses to examine 10 axenic isolates of *G. intestinalis*, established from diverse geographical regions in Australia, together with the Portland-1 isolate from the USA. Both probes identified every isolate unambiguously as belonging to one or other of two genetic clusters. Electrophoretic analysis of the same samples indicated that these clusters correspond to the previously defined genetic groups I and II. No heterogeneity was apparent within the seven group I isolates using either probe. However, when probed with the repetitive sequence, the four isolates belonging to group II exhibited small differences in banding patterns, suggesting that this group may be less homogeneous than group I. The consistency of the Southern blot data with the allozyme groupings confirms group I and group II as distinct genetic subgroups of *G. intestinalis* and establishes that selected restriction sites can be used as taxonomic markers for the purposes of a genetically based systematics for *G. intestinalis*.

Introduction

Present-day members of the genus *Giardia* are believed to have evolved from ancestral organisms that formed an early branch point in the lineage of eukaryotic cells (Sogin *et al.*, 1989; Kabnick & Peattie, 1991; Lanzen-dörfer *et al.*, 1992). They are all anaerobic, flagellate protozoa that parasitize the intestines of vertebrates. The systematics of the genus is based on a limited number of morphological characters that are visible by light microscopy (Filice, 1952), to which have been added recently several species-specific ultrastructural characters (Erlandsen & Bemrick, 1987; Feely, 1988;

Erlandsen *et al.*, 1990). The following species have been described (reviewed by Adam, 1991): *G. agilis* (amphibia), *G. muris* (rodents, birds, reptiles), *G. duodenalis* (mammals, birds, reptiles), *G. microti* (voles and muskrats), *G. ardeae* (great blue heron) and *G. psittaci* (birds). An earlier taxonomy based on presumed host-specificity has no sound epidemiological or experimental basis (Thompson *et al.*, 1990; Adam, 1991). Nevertheless, it is too early to discard the principle of host-specificity or host range as having little taxonomic value. The great diversity of vertebrates that are hosts to *Giardia* in many parts of the world, and the ancient lineage of the genus, could allow greater heterogeneity to exist at the genetic level than is obvious from morphological examination.

The species *G. intestinalis* (syn. *G. lamblia*) is a relic of the taxonomy based on presumed host-specificity and as such consists of all *Giardia* that parasitize man. *G. intestinalis* falls within the morphological description of the species *G. duodenalis* and the genetic relationship between human isolates and isolates from animals is a

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Abbreviation: RFLP, restriction fragment length polymorphism(s).

subject of continuing interest. Furthermore, *G. intestinalis* isolates exhibit heterogeneity for a number of phenotypic and genotypic characters, including surface antigens (Smith *et al.*, 1982; Nash & Keister, 1985), isoenzymes (Bertram *et al.*, 1983; Baveja *et al.*, 1986; Meloni *et al.*, 1988, 1989; Abaza *et al.*, 1991), DNA restriction patterns (Nash *et al.*, 1985; de Jonckheere *et al.*, 1989; Meloni *et al.*, 1989; Archibald *et al.*, 1991), chromosome patterns (Adam *et al.*, 1988; Upcroft *et al.*, 1989; Campbell *et al.*, 1990; de Jonckheere *et al.*, 1990) and sensitivity to therapeutic agents (Boreham *et al.*, 1987; Upcroft *et al.*, 1990). Recently, 26 allozymic characters were examined in axenic isolates of *G. intestinalis* from Australia and the isolates fell into four distinct genetic groups (Andrews *et al.*, 1989). The definition of 'species' is difficult in asexual protozoa but the level of fixed genetic differences between these groups was greater than the differences measured by this technique between species in a variety of sexual metazoan genera (Richardson *et al.*, 1986). It was therefore proposed that *G. intestinalis* was a species complex comprising at least three or four morphologically cryptic species.

Studies at the DNA level employing sequence analysis of ribosomal RNA genes (van Keulen *et al.*, 1991), hybridization of DNA probes representative of individual genes (Campbell *et al.*, 1990) and polymerase chain reaction (PCR) analysis of the gene for β -giardin (Mahbubani *et al.*, 1992) have exploited in different ways the potential of DNA analysis as a genetic tool in establishing the systematics of *Giardia*. This potential is based on the vast number of genetic characters provided by the base sequence of genomic DNA. The studies have uniformly supported the separate species status of *G. duodenalis*, *G. muris* and *G. ardeae*. However, the hybridization techniques employed by Campbell *et al.* (1990) found overall similarity between isolates of *G. duodenalis* from human and animal sources, indicating that they can only resolve relatively large genetic differences (i.e. relatively large phylogenetic distances). PCR analyses, using primers specific for different parts of the β -giardin gene, similarly distinguished *G. muris* and *G. ardeae* from various samples of *G. duodenalis* (Mahbubani *et al.*, 1992). The latter included both human-derived (*G. intestinalis*) and animal-derived isolates, but the technique apparently failed to detect any difference between them.

Other studies have used restriction analysis and Southern blotting with a variety of DNA probes to detect differences between *G. duodenalis* isolates from animals and humans (Meloni *et al.*, 1989) and between isolates of *G. intestinalis* (Nash *et al.*, 1985; de Jonckheere *et al.*, 1989; Meloni *et al.*, 1989). Because each study analysed only a few genetic characters (i.e. restriction sites), the

results can demonstrate similarities or differences for these characters between isolates but they cannot be used for the purpose of quantitative systematics or to construct phylogenies. The same considerations also apply to studies on *Giardia* chromosomes by pulsed-field electrophoresis, which in some cases have failed to resolve differences between isolates of *G. intestinalis* (Adam *et al.*, 1988; de Jonckheere *et al.*, 1990), although allowing correlation of karyotype with Australian or North American origin in another study (Upcroft *et al.*, 1989).

In the present study, we have also compared restriction fragments from a number of *in vitro* cultured isolates of *G. intestinalis* on Southern blots (Southern, 1975), using two independent DNA probes. However, we have combined the study with allozyme electrophoresis and have therefore been able to correlate restriction fragment patterns with genetic groups defined allozymically. The results indicate that some restriction sites represent fixed genetic differences between the allozymically defined genetic groups I and II of *G. intestinalis* and they can therefore be used in the systematics of the species.

Methods

Materials. Proteinase K, restriction endonucleases, digoxigenin-labelled dUTP, alkaline-phosphatase-labelled anti-digoxigenin antibodies and other digoxigenin labelling and detection reagents (random hexanucleotides, Klenow enzyme, etc.) were purchased from Boehringer Mannheim. RNAase A, herring sperm DNA and 5-bromo-4-chloro-3-indolyl phosphate were obtained from Sigma, and positively charged nylon membranes (Hybond N⁺) were from Amersham. All other reagents were of analytical grade.

Source of *G. intestinalis* isolates and cultivation. The Ad-1 and Ad-2 isolates have been described elsewhere (Andrews *et al.*, 1989). Isolates Ad-3, Ad-6 and Ad-10 were obtained initially by inoculating newborn mice with cysts derived from human stool samples and they were later established in axenic culture (Mayrhofer *et al.*, 1992) in TY1-S-33 medium (Keister, 1983) supplemented with ox bile. The Ad-113 isolate was similarly established from trophozoites in a duodenal aspirate sample (Mayrhofer *et al.*, 1992). Clonal populations of these isolates were established from single trophozoites collected under direct microscopic examination (Andrews *et al.*, 1992). Axenic cultures of BRIS/83/HEPU/136, BRIS/87/HEPU/768, BRIS/83/HEPU/99 (clone 2) and BRIS/87/HEPU/694 were provided by Dr P. F. L. Boreham (Queensland Institute of Medical Research, Brisbane, Australia). Two clonal cultures were established from BRIS/83/HEPU/136 (Bris-136/C1 and Bris-136/C2), each giving identical allozyme and DNA profiles. Axenic cultures of the isolates BAH-1 (Meloni *et al.*, 1988) and BAH-12 (Meloni *et al.*, 1989) were obtained from Dr R. C. A. Thompson (Murdoch University, Perth, Australia). The Portland-1 isolate (Meyer, 1976) was obtained from Professor E. A. Meyer (Portland, Oregon, USA). Analyses were performed on clonal populations of trophozoites, except in the cases of BRIS/87/HEPU/768, BAH-1, Ad-113 and Portland-1.

Characterization of isolates by allozyme electrophoresis. Sonicates of trophozoites were analysed by allozyme electrophoresis on cellulose acetate ('Cellolog', Chemetron, Milan) as described previously

Fig. 1. Restriction map of Ad-1 chromosomal DNA (A) in the region of the *tsp11* gene cloned in pKK11, and predicted map of group II *G. intestinalis* DNA (B) over the corresponding chromosomal region. The genomic fragment (hatched box) of Ad-1 DNA cloned in plasmid pKK11, the location of the restriction fragments used to prepare hybridization probes 1A, 1B and 1C, and the open reading frame (ORF, thick arrow) corresponding to *tsp11*, are indicated. The single *Eco*RI, *Pst*I (P_2), *Xba*I and *Cla*I sites, and the two *Hind*III sites (H_2 , H_3), located within the pKK11 insert are shown (vertical arrows) in (A). The positions of two additional *Hind*III sites, one (H_1) upstream and the other (H_4) downstream of the cloned segment, as well as the two adjacent *Pst*I sites (P_1 , P_3), are also shown in (A). The predicted locations of the three *Hind*III sites (H' , H_2 , H'') and the three *Pst*I sites (P_1 , P_2 , P_3') detected in group II isolates are depicted in (B). The sizes of the predominant *Hind*III and *Pst*I fragments detected in group I isolates (a , b , c ; d , e) and in group II isolates (f , g ; d , h) are shown.

(Andrews *et al.*, 1989). Two clones derived from the Ad-1 and Ad-2 isolates were used as representatives of genetic groups I and II respectively (Andrews *et al.*, 1989). BAH-12 (group III) and BRIS/87/HEPU/694 (group IV) were also used as type standards.

Genomic DNA isolation. Chromosomal DNA was isolated from trophozoites by digestion at 37 °C for 2 h with proteinase K (0.2 mg ml⁻¹) in 0.1 M-Tris/HCl, 0.2 M-NaCl, 0.1 M-EDTA, 1% (w/v) SDS, pH 8.0. The digests were extracted twice with phenol/chloroform/isopentanol (50:48:2, by vol.), once with chloroform and then treated with boiled RNAase A (50 µg ml⁻¹; 3 h, 37 °C). The DNA was precipitated in 70% (v/v) ethanol, washed, and redissolved in 10 mM-Tris/HCl, 1 mM-EDTA, pH 8.0 (TE).

Electrophoresis and hybridizations. Samples of DNA were incubated at 37 °C with *Hind*III or *Pst*I and the reactions monitored to ensure complete digestion. Samples containing 5 µg of digested plasmid DNA or between 5 and 15 µg of digested *Giardia* DNA were subjected to electrophoresis in 0.8% agarose gels in Tris/borate/EDTA (TBE). Gels were stained briefly with ethidium bromide, photographed, then immersed in 0.25 M-HCl (10 min) and 0.5 M-NaOH, 1.5 M-NaCl (25 min) before being blotted onto positively charged nylon membranes by transfer in 0.4 M-NaOH for 6–12 h. Each membrane was subsequently washed twice with 150 ml aliquots of 2 × PE (1 × PE is 0.133 M-sodium phosphate, pH 6.9, plus 1 mM-EDTA), 0.1% SDS (10 min each) and then with 100 ml of 5 × PE, 1% SDS (3 h, 65 °C). Prehybridization was carried out for 6–12 h at 42 °C in 20 ml of 50% (v/v) formamide, 5 × SSC (1 × SSC is 0.15 M-NaCl plus 0.015 M-

sodium citrate), 5 × Denhardt's solution, 50 mM-sodium phosphate, pH 6.4, and 0.1 mg herring sperm DNA ml⁻¹. Digoxigenin-labelled probe (0.1–0.5 µg in 25 µl of TE) was denatured at 95 °C for 5 min and mixed immediately with 20 ml of fresh hybridization solution, in which the filter was incubated for a further 12–18 h at 42 °C. Blots were washed at moderate stringency (twice at 25 °C with 2 × SSC + 0.1% SDS, then twice with 0.2 × SSC + 0.1% SDS) and incubated with alkaline-phosphatase-conjugated anti-digoxigenin antibodies before staining with 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim manual). For re-use, blots were destained in dimethyl-formamide, stripped of probe in 100 ml 0.1% SDS + 0.2 M-NaOH (37 °C, 30–60 min), rinsed in 2 × SSC and finally in water.

DNA probes.

Four probes were used. These were prepared from two genomic DNA fragments (from the Ad-1 *G. intestinalis* isolate) that had been cloned by ligation into the *Bam*HI cloning site of plasmid pUC8. Probes 1A, 1B and 1C were derived from the recombinant plasmid pKK11, which contains a gene, *tsp11*, that encodes a predicted M_r 69000 cysteine-rich trophozoite surface protein, TSP11 (Ey *et al.*, 1992), related to the TSA417 antigen described by Gillin *et al.* (1990). Probe 2 was prepared from an unrelated genomic fragment isolated from plasmid pKK40 (K. Khanna, P. Ey & G. Mayrhofer, unpublished results).

Probe 1A. *Eco*RI fragment of pKK11. This 0.43 kb fragment constitutes the 5' non-coding end of the pKK11 insert, commencing from the *Eco*RI site within the polylinker (see Fig. 1A). It lies upstream from *Hind*III site H_2 (located 0.691 kb from the 5' cloning site) and the

Fig. 2. Analysis of *Hind*III-digested genomic DNA from different *G. intestinalis* isolates by hybridization with probe 1B, specific for part of the *tsp11* gene. The loading order was: Ad-1, Ad-3, Ad-6, Ad-10, BAH-1, Bris-768 and Portland-1 (lanes 2-8, allozyme group I); Ad-2, Ad-113, Bris-99 and Bris-136 (lanes 9-12, allozyme group II). Fragments produced by digestion of the plasmid pKK11 with *Sma*I or *Hind*III are evident in lane 1 (2.9 kb) and lane 13 (2.1 kb) respectively. Washing was done at moderate stringency.

single open reading frame of *tsp11*, which starts at 1.26 kb (Fig. 1A). The fragment was purified from an *Eco*RI digest of pKK11 by electrophoresis in TBE on a 6% (w/v) polyacrylamide gel. Probe 1A was prepared by random hexamer priming (Feinberg & Vogelstein, 1984) using the purified fragment as a template for labelling with digoxigenin-labelled dUTP (Boehringer Mannheim manual).

Probe 1B. *Xba*I-*Cla*I fragment of pKK11. This 0.56 kb fragment lies within the *tsp11* gene (Fig. 1A). pKK11 was digested with *Xba*I and *Cla*I, the fragment was purified (see probe 1A above) and a digoxigenin-labelled probe was prepared by random priming.

Probe 1C. *Hind*III fragment of pKK11. A digoxigenin-labelled probe was similarly prepared from the 0.38 kb *Hind*III fragment of pKK11. This fragment lies immediately downstream from *Hind*III site H₃ (Fig. 1A) and extends to the *Hind*III site located within the polylinker at the 3' end of the pKK11 insert. It comprises the promoter-distal portion of the *tsp11* gene.

Probe 2. *Eco*RI-*Hind*III fragment of pKK40. This 0.83 kb fragment has been fully sequenced (K. Khanna, P. Ey, & G. Mayrhofer, unpublished results). It shows no homology with the pKK11 insert and is devoid of potential reading frames. The insert was purified electrophoretically from an *Eco*RI-*Hind*III digest of pKK40 and used to prepare a digoxigenin-labelled probe by random priming.

Results

Detection of restriction fragments hybridizing with probes 1A-C.

Probe 1B is derived from a highly conserved region within *tsp11*, a gene that encodes a *M_r* 69000 cysteine-rich trophozoite surface protein (Fig. 1A). The 3.186 kb fragment containing *tsp11* was cloned in pKK11 from a genomic library of Ad-1 DNA and it has been sequenced completely (Ey *et al.*, 1992; GenBank accession no. M95814). The promoter distal part of *tsp11* exhibits substantial sequence homology, both at the nucleotide

and amino acid level, with the *tsa417* gene (Gillin *et al.*, 1990) that encodes a *M_r* 72500 surface antigen on trophozoites of *G. intestinalis* isolate WB.

We have constructed a detailed restriction map of the regions flanking the pKK11 fragment in the Ad-1 genome and determined by high stringency blotting that there is only one copy of *tsp11*. The locations of the chromosomal *Hind*III and *Pst*I sites are depicted in Fig. 1A. Digestion of the pKK11 insert with *Hind*III yields a 2.124 kb fragment (by cleavage at sites H₂ and H₃) that contains the entire *Xba*I-*Cla*I segment corresponding to probe 1B (cf. Fig. 2, lane 13). This probe hybridized with an equivalent 2.1 kb fragment in *Hind*III digests of genomic DNA from Ad-1 (Fig. 2, lane 2) and six other *G. intestinalis* isolates (Ad-3, Ad-6, Ad-10, BAH-1, Bris-768, Portland-1; Fig. 2). The 2.1 kb fragment hybridized strongly in each of these seven isolates. Three additional fragments of approximately 4.8, 5.3 and 7.2 kb hybridized only weakly. Because the washings were done at high stringency, it is likely that these faint bands represent either residual incompletely digested fragments or independent sequences with significant homology. The remaining four isolates (Ad-2, Ad-113, Bris-99, Bris-136) showed no evidence of a 2.1 kb band, but instead a less intense 6.9 kb fragment was detected, with fainter bands of approximately 8 kb and 9 kb. The eleven isolates could therefore be categorized into two groups, one (seven isolates) possessing the 2.1 kb fragment and the other (four isolates) possessing a predominant, but less pronounced, 6.9 kb fragment. Allozyme analyses on replicates of the samples used for the Southern hybridization confirmed that all of the isolates of the former group also belonged to genetic group I whereas Ad-2, Ad-113, Bris-99 and Bris-136

Table 1. *Allozyme data on group I and group II isolates used in this study*

The loci shown (see Andrews *et al.*, 1989) are diagnostic for groups I and II (Gpt, Np1, Np2); groups I/II versus III/IV (Got, Gpt, Mdh, Me, Np1); and groups III versus IV (Got, Np1). Allozymes are designated alphabetically in order of increasing electrophoretic mobility.

Isolate	Enzyme locus					
	Got	Gpt	Mdh	Me	Np1	Np2
Ad-1/C1	a	b	a	c	a	a
Ad-3/C2	a	b	a	c	a	a
Ad-6/C1	a	b	a	c	a	a
Ad-10/C1	a	b	a	c	a	a
BAH-1	a	b	a	c	a	a
Bris-768	a	b	a	c	a	a
Portland-1	a	b	a	c	a	a
Ad-2/C2	a	c	a	c	b	b
Ad-113	a	c	a	c	b	b
Bris-99/C2	a	c	a	c	b	b
Bris-136/C1	a	c	a	c	b	b
Group III*	c	a	b	ab	d	b
Group IV*	bd	a	b	b	c	b

* Reference standards (Andrews *et al.*, 1989) were isolates BAH-12 (group III) and BRIS/87/HEPU/694 (group IV).

(which possessed the 6.9 kb fragment) belonged to group II. The data on six enzyme loci (diagnostic for groups I-IV) are presented in Table 1.

To investigate further the differences between group I and group II isolates in this genomic region, separate blots (not shown) containing DNA cleaved with *Hind*III or *Pst*I were hybridized with probe 1A (representing the 5' non-coding end of the pKK11 insert, between *Hind*III sites H₁ and H₂ of Ad-1 DNA) and subsequently with probe 1C (representing the 3' coding end, between sites H₃ and H₄). The results, like those of Fig. 2, revealed two distinct groups. Using probe 1A, a single 3.9 kb *Pst*I fragment was identified in all eleven isolates, confirming that the same chromosomal region was being analysed in cells from both genetic groups (Fig. 1, fragment *d*). However, whereas all of the group I isolates yielded a single 4.1 kb *Hind*III fragment (Fig. 1A; fragment *a*) that hybridized to the probe, the only band detected in *Hind*III-restricted DNA from the group II isolates corresponded to a 1.4 kb fragment (Fig. 1B, fragment *f*). The group II isolates therefore possess a unique *Hind*III site located only slightly upstream from the segment corresponding to probe 1A.

Hybridization with probe 1C, comprising the conserved promoter-distal portion of the *tspl1* gene, was more complex. Two fragments, one hybridizing approximately twice as strongly as the other, were identified in both *Hind*III- and *Pst*I-digested DNA from all group I isolates (data not shown). The *Hind*III fragments were

3.6 kb (major band) and 4.8 kb (minor band), whilst the *Pst*I fragments were 4.7 kb (major) and 3.5 kb (minor). Probe 1C also hybridized (but only weakly) to a single *Hind*III and *Pst*I fragment (6.9 kb and 3.5 kb, respectively) from the group II isolates. The detection by probe 1C of two prominent restriction fragments in both *Hind*III- and *Pst*I-digested DNA from group I isolates can be explained if these isolates possess the *tspl1* gene (or its equivalent) and also a second, related gene whose homology with *tspl1* is higher in the region represented by probe 1C than in the other (upstream) regions detected with probes 1A and 1B. The more readily detected bands would thus represent the *tspl1* gene, the 3.6 kb *Hind*III fragment resulting from cleavage at sites H₃ and H₄ (fragment *c*) and the 4.7 kb *Pst*I fragment from cleavage at sites P₂ and P₃ (fragment *e*) as depicted in Fig. 1A. If the fainter 4.8 kb *Hind*III fragment of group I isolates does represent an homologous second gene, it is unlikely to be a coincidence that the combined size of this fragment and the 2.1 kb fragment *b* of *tspl1* (Fig. 1A) is 6.9 kb, equivalent to the major *Hind*III fragment detected in group II isolates with probe 1B (Fig. 1, fragment *g*). Indeed, the data are wholly consistent with the presence in group I isolates of two related genes, one of which has restriction characteristics similar (but not identical) to the homologous gene detected in group II isolates. The latter gene and its homologue in group II isolates appears to lack *Hind*III site H₄ and has a new *Pst*I site (P₃', Fig. 1). The gene in group II isolates also lacks the *Hind*III site H₃ and has an additional *Hind*III site H' (Fig. 1B). The detection of the 6.9 kb *Hind*III fragment *g* (Fig. 1B) in group II isolates and the 4.8 kb *Hind*III fragment (not shown in Fig. 1) as a minor band in group I isolates suggests that the *tspl1*-like gene detected in group II organisms contains *Hind*III site H₂. The 1.4 kb *Hind*III fragment detected in group II isolates with probe 1A would therefore be formed by cleavage at sites H' and H₂ as depicted in Fig. 1B (fragment *f*). Accordingly, it appears that *Hind*III site H₂ and the two upstream *Pst*I sites P₁ and P₂ are common to isolates from both genetic groups; two *Hind*III sites (H₃ and H₄) and one downstream *Pst*I site (P₃) are unique to group I organisms; and *Hind*III site H' (Fig. 1B) is unique to group II organisms. Sites H₃ and H' therefore represent fixed genetic differences that appear to define these groups.

Detection of restriction fragments hybridizing with probe 2.

Probe 2 is unrelated to probes 1A-C and it hybridizes with many fragments in digests with various restriction enzymes of Ad-1 DNA (K. Khanna, P. Ey & G. Mayrhofer, unpublished results). It appears, therefore, to

Fig. 3. Analysis of *Pst*I-digested genomic DNA from different *G. intestinalis* isolates using probe 2. The 0.83 kb insert of pKK40 (released by digestion with *Eco*RI + *Hind*III), is evident in lane 1 and the entire 3.5 kb plasmid (linearized with *Eco*RI) is stained in lane 13. The *Giardia* DNA samples were: Ad-1, Ad-3, Ad-6, Ad-10, BAH-1, Bris-768 and Portland-1 (lanes 2–8); Ad-2, Ad-113, Bris-99, Bris-136 (lanes 9–12). Washing was done at moderate stringency.

represent a sequence that exists in multiple copies within the genome and this contrasts with the unique or low copy gene detected with probes 1A–C. Probe 2 identified numerous fragments in *Pst*I digests of all the isolates tested (Fig. 3). Two banding patterns were evident, corresponding to the allozyme-defined groups I and II. The first pattern (group I; lanes 2–8) was invariant, both in the sizes (0.8 kb to $\approx$ 12 kb) and relative strength of hybridization of the fragments. At least 17 common bands were identified in every sample, with several very faint additional bands also evident. The most prominent bands corresponded to fragments of approximately 1.2 kb, 1.7 kb, 2.1 kb (strongly stained), 2.7 and 2.8 kb (doublet), 3.1 and 3.3 kb (doublet), 3.7 and 3.9 kb (doublet), 4.9 kb, 5.4 kb (the major band), 6.2 kb, 8.4 kb and 10.8 kb.

The second pattern (group II; lanes 9–12) comprised fragments ranging in size from 0.7 kb to 10.4 kb and it was quite distinct from the group I pattern. Only two fragments (the 3.1 and 3.3 kb doublet band) could be considered common to all isolates from both groups (I and II). Four additional fragments (approximately 0.8 kb, 2.0 kb, 2.4 kb and 5.1 kb respectively) appear to be common to the four group II isolates and therefore specific for this group. However, these isolates were distinguished from each other by the presence (or absence) of a small number of additional bands, i.e. each isolate possessed a unique 'fingerprint'. The common 2.4 kb fragment hybridized most strongly to probe 2, but overall the fragments from the group II isolates hybridized less well than did those of the group I isolates.

Discussion

This study describes the occurrence of restriction fragment length polymorphisms (RFLP) in isolates of *G. intestinalis*, detected by two DNA probes cloned from the genomic DNA of the Ad-1 isolate. In itself, this is not a novel finding because RFLPs have been described in *G. intestinalis* previously, using different DNA probes (Nash *et al.*, 1985; de Jonckheere *et al.*, 1989; Meloni *et al.*, 1989). However, while of use in identifying similarities or differences between isolates, these studies have been of limited taxonomic value because only a few genetic characters (i.e. restriction sites) were examined in each isolate and the sequences detected by hybridization were otherwise uncharacterized. The unique value of the present study is that firstly, it has utilized a conserved gene whose sequence is known (Ey *et al.*, 1992) and secondly, it was done concurrently with an electrophoretic analysis of 26 enzyme loci in the same strains, a technique which has been shown (Andrews *et al.*, 1989) to distinguish genetic groups within *G. intestinalis* that exhibit fixed allelic differences at between 23% and 77% of the loci examined. In an earlier study, Meloni *et al.* (1989) also compared RFLP patterns (obtained with a different DNA probe) with electrophoretic analysis of allozymes in 47 axenic isolates of *G. duodenalis*. However, they presented their electrophoretic data as zymodemes, while we have used an allozymic interpretation and have quantified genetic relationships between isolates in terms of fixed allelic differences. We have therefore been able to relate RFLP patterns to

Table 2. Grouping of *G. intestinalis* isolates according to restriction fragment length polymorphisms and allozyme patterns

Isolate code	<i>tsp11</i> gene <i>Hind</i> III fragments (kb)*			Repetitive DNA pattern† (probe 2)	Allozyme grouping‡
	Probe 1A	Probe 1B	Probe 1C		
Ad-1/C1	4.1§	2.1§	3.6§	A	I
Ad-3/C2	4.1	2.1	3.6	A	I
Ad-6/C1	4.1	2.1	3.6	A	I
Ad-10/C1	4.1	2.1	3.6	A	I
BAH-1¶	4.1	2.1	3.6	A	I
Bris-768¶	4.1	2.1	3.6	A	I
Portland-1¶	4.1	2.1	3.6	A	I
Ad-2/C2	1.4§	6.9§	6.9§	B1	IIa
Ad-113¶	1.4	6.9	6.9	B2	IIb
Bris-99/C2	1.4	6.9	6.9	B4	II
Bris-136/C1	1.4	6.9	6.9	B3	II

* Based on RFLPs identified in *Hind*III-digested DNA using the probes indicated (Fig. 2) and data from other blots.

† From *Pst*I-digested DNA (Fig. 3).

‡ From Table 1.

§ Derived by cleavage with *Hind*III at sites (See Fig. 1) H₁→H₂ (4.1 kb); H₂→H₃ (2.1 kb); H₃→H₄ (3.6 kb); H'→H₂ (1.4 kb); H₂→H' (6.9 kb).

|| A 4.8 kb fragment, less heavily stained than the 3.6 kb fragment, was also evident in all group I isolates.

¶ Uncoloned isolates.

established genetic groups and thus define restriction fragments that are unambiguously diagnostic for those groups. Conversely, the correlation between RFLP data and electrophoretic data adds weight to the genetic groupings defined by the latter technique.

The grouping of the eleven isolates examined in the present study is summarized in Table 2. It is clear that the major RFLPs defined by probes 1B and 1C (identifying sequences within the *M*_r 69000 surface antigen gene cloned in pKK11) and by probe 1A (specific for a region approximately 0.8 kb upstream from the start of this gene) correlate with the electrophoretic groups I and II defined by Andrews *et al.* (1989). The distinction of these genetic groups by these probes is not surprising, since it appears to be based on the simple loss or gain of restriction sites. We have not yet identified the precise nature of these mutations.

The repetitive sequence detected with probe 2 represents a more complex situation, but this probe also revealed group-specific differences. Furthermore, the detection of repetitive sequences enables sampling of a much larger portion of the genome than is possible using probes specific for unique genes, and this should increase the probability of detecting genetic differences between closely related isolates. This certainly appears to be the

case for the group II isolates. Although there seems to be a substantial commonality of banding patterns within this group (Fig. 3; Table 2), the detection of minor differences between each of four group II isolates (but not between seven group I isolates, despite these being derived from diverse geographical areas) may indicate that group II is less homogeneous than group I. The number of samples examined so far in each group is very small, however, and many more isolates need to be compared before a definitive conclusion can be reached on this point.

We showed previously that group I and II isolates have fixed allelic differences at 31% of the 26 enzyme loci examined electrophoretically (Andrews *et al.*, 1989). This level of genetic dissimilarity is typical of species differences in genera of sexual vertebrates (Richardson *et al.*, 1986) and invertebrates (Chilton *et al.*, 1992), and between species of protozoa with clear differences in morphology and geographic distribution (Andrews *et al.*, 1988). The mutations underlying the restriction site differences (detected by the diagnostic RFLPs) that distinguish group I from group II organisms must have occurred subsequent to the divergence of the groups from their common ancestor. These mutations may be relatively ancient but analysis of further samples is necessary to show whether the RFLP differences that they cause are indeed group-specific, or whether there is evidence of intragroup heterogeneity.

Electrophoretic groups III and IV have fixed allelic differences from groups I and II at 65–77% of the 26 enzyme loci examined by Andrews *et al.* (1989). We have not yet assessed the suitability of the DNA probes described herein for the study of these more distantly related *G. intestinalis* isolates or for morphologically-distant species such as *G. muris*. The relatively weak hybridization of the pKK11 probes with DNA from group II isolates (which are less distantly related to group I isolates than are isolates of groups III/IV) makes this prospect unlikely. Furthermore, studies in progress (using oligodeoxyribonucleotides complementary to highly conserved sequences within the *tsp11* gene as primers for PCR amplification) indicate that the genetic divergence between groups I/II and groups III/IV is too great to allow probes based on this gene to hybridize at high stringency (P. Ey, unpublished results). DNA probes specific for highly conserved structural genes (e.g. tubulin or giardin; reviewed by Adam, 1991) or sequence analysis of these genes may be more suitable adjuncts to allozyme electrophoresis (upper range, 60–70% fixed allelic differences ≈ 20 million years; Richardson *et al.*, 1986) for studying the phylogenetic relationships between these more distantly related organisms.

It is now important to test the diagnostic grouping by RFLP analysis on a wider range of *G. intestinalis* from

genetic groups I and II and to extend the studies to examine isolates from genetic groups III and IV and also *G. duodenalis* from animal sources. Furthermore, it is also important to recognize that analysis by Southern hybridization has severe limitations when applied to organisms such as *Giardia*. As was pointed out by Nash *et al.* (1985), this technique requires large amounts of DNA, essentially making *in vitro* culture a requisite. Unfortunately, many *Giardia* isolates cannot be grown *in vitro* and are available in very limited quantities (Mayrhofer *et al.*, 1992). Application of PCR methodology to *Giardia* should circumvent these technical problems, because the technique has been applied successfully to single cysts (Mahbubani *et al.*, 1992). However, the apparent failure of the latter study to detect differences between different members of the *G. duodenalis* group using primers specific for the β -giardin gene indicates that the choice of the target sequence may be critical. We are encouraged by the results presented herein to believe that judicious selection of diagnostic sequences (such as those described in the present study) will lead eventually to a systematics for the genus *Giardia* based principally on genetic rather than on morphological and other phenotypic characters.

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