Use of Random Amplified Polymorphic DNA (RAPD) Analysis for the Identification of *Giardia intestinalis* Subtypes and Phylogenetic Tree Construction

**JITKA ŠEDINOVÁ,† JAROSLAV FLEGR, PETER L. EW AND JAROSLAV KULDA**

†Department of Parasitology, Faculty of Science, Charles University, Prague, 128 44, Czech Republic, and
∗Department of Molecular Biosciences, Adelaide University, Adelaide SA 5005, Australia

**ABSTRACT.** A comparison of random amplified polymorphic DNA (RAPD) was used to investigate genetic polymorphisms among 25 isolates of *Giardia intestinalis* and to assess the utility of RAPD for subtype detection and genealogical analysis. Using data obtained for six human and 19 animal-derived isolates in polymerase chain reactions using 13 different primers, phylogenetic trees were constructed and bootstrap values computed with the program FreeTree. Three major clades were distinguished, corresponding to previously defined genetic assemblages A, B, and C. The purported specificity of assemblage E genotypes for artiodactyl hosts was supported. Assemblages A and B showed wide host spectra, including human and animal hosts. No correlation was found between the genotype of analyzed isolates and the presence or absence of the double-stranded RNA Giardivirus. The results indicate that RAPD data provide reliable genetic information that can be used for both "fingerprinting" and genealogical purposes.

**Key Words.** dsRNA virus, fingerprinting, genetic polymorphism, genotyping, polymerase chain reaction.

*Giardia* are ubiquitous gastrointestinal protozoan parasites that cause diarrheal disease in a wide range of host species. The taxonomy of the genus is based on morphological characters, particularly the shape and position of the median bodies, the shape of the cell, and the size of the ventral adhesive disc. Several morphological groups have been recognized and classified as species (Filice 1952; Kulda and Novák 1996). *G. intestinalis* (Lamb, 1859) [syn. *G. duodenalis* (Davaine, 1875), and *G. lamblia* (Stiles, 1915), *G. muri* (Grassi, 1879), *G. agilis* Künstler, 1882 (Feely and Erlandsen 1985) and *G. ardeae* Näveler, 1920 (Erlandsen et al. 1990)]. However, some of these morphotypes appear to represent higher taxonomic units that include multiple species. Within the *G. intestinalis* group, two additional species, *G. psittaci* Erlandsen and Benrick, 1987, and *G. microti* Kofoid and Christiansen, 1915 (Feely 1988), have been discerned on the basis of ultrastructural, developmental, and 18S rDNA data (van Keulen et al. 1998). Moreover, there is evidence of considerable genetic diversity among other members of this group.

Using phylogenetic analysis of nucleotide sequence and allozyme data, Monis et al. (1999) divided isolates of *G. intestinalis* into seven different lineages, designating assemblages A–G (review, Thompson et al. 2000). All characterized isolates from humans fall within two major genetic assemblages, A or B, which also include many isolates from animals. Assemblages A and B each encompass a range of genotypes, with two distinct subgroups, Group I (subtype A-I) and Group II (subtype A-II), prominent within assemblage A (Andrews et al. 1989; Ely et al. 1992–97; Mayrhofer et al. 1995; Monis et al. 1996). Monis et al. (1996) have shown that assemblages A and B correspond respectively to the "Polish" and "Belgian" genotypes of Homan et al. (1992) and to groups 1 and 2 of Nash (Nash et al. 1985; Nash and Mowatt 1992). The other assemblages are defined by unique genotypes found only in animals: assemblages C and D (dogs; Monis et al. 1998), assemblage E (hooved livestock; Ely et al. 1997), assemblage F (cats) and assemblage G (rats; Monis et al. 1999). The definition of genetic lineages that correlate with distinct phenotypic characters (Binz et al. 1992; Karasin and Ely 1998; Meloni and Thompson 1987; Monis et al. 1998; Weiss et al. 1992) supports the hypothesis that the *G. intestinalis* is a species complex consisting of several distinct species (Andrews et al. 1989). The restricted host range of isolates belonging to assemblages C-G contrasts with the broad host spectra observed for genotypes belonging to assemblages A and B. The A-I subtype represents a zoontic genotype, as it is ubiquitous in human and animal hosts worldwide (Thompson 2000).

Techniques that have been used to compare *G. intestinalis* isolates genetically include analysis of allozymes (Andrews et al. 1989; Mayrhofer et al. 1995; Meloni et al. 1995; Strandén et al. 1990), karyotypes (Sarris and Isaac-Renton 1993; Upcroft et al. 1989), restriction fragment length polymorphisms (RFLP; Ely et al. 1992; Homan et al. 1992; Nash et al. 1983), nucleotide sequences (Baruch et al. 1996; Ely et al. 1993b, 1996, 1997; Monis et al. 1996, 1999; van Keulen et al. 1995, 1998; Weiss et al. 1992) and random amplified polymorphic DNA (RAPD; Morgan et al. 1993; Paintlia et al. 1999; van Belmonte et al. 1993). RAPD is a DNA fingerprinting technique based on the amplification of undefined segments of the genome by PCR using single arbitrary primers (Williams et al. 1990). The multi-locus character of RAPD allows sensitive detection of polymorphisms in different parts of the genome. If performed with a sufficient number of primers, the method has the potential to provide reliable information about the genetic identity of isolates (Tibayrenc 1998). A drawback of the method is a requirement for DNA samples free of any contaminating DNA. Consequently, applicability of RAPD analysis is limited to axenic in vitro isolates which need not represent full spectrum of *G. intestinalis* diversity.

The aim of the present study was to evaluate the suitability of RAPD analysis for determining the genetic heterogeneity of *G. intestinalis* isolated mainly from animal sources and its utility for assessing the zoontic potential of field isolates. Bootstrap analysis was used for the first time to estimate the robustness of a RAPD-based tree for *G. intestinalis*.

**MATERIALS AND METHODS**

*Giardia* isolates. Data on the *G. intestinalis* isolates examined in this study are listed in Table 1. Assemblage A and B "type" references (Mayrhofer et al. 1995) included human-derived axenic isolates Ad-1a/7 (A, Group I), B1-136/d1 (A, Group II) and Ad-28 (B) (Andrews et al. 1989; Ely et al. 1993a), as well as sheep isolate CH-03 (A, Group III-Iike; Ely et al. 1996; Strandén et al. 1990). The axenic *C. intestinalis* isolate P15, from a pig (Koudelka et al. 1991), served as a type reference for genetic assemblage E (Ely et al. 1997; Monis et al. 1999).

Three other isolates were of human origin; HP-1 [= Portland 1 (Meyer 1976), ATCC 20888] obtained from Prof. A. E. Meyer (Oregon Health Science University, Portland, OR); H1, isolated in Prague from a Czech visitor to India; and Ber-1, isolated from a patient with treatment-refractory chronic diarrhea.
Two rodent isolates, P 114 (= GG PRP-114) from a Gambian giant pouched rat, *Cricetomys gambianus* and P 117 (= CP-117 from a South American cuscus, *Galea musteloides*) established in Poland from animals maintained in the Poznan Zoological Garden (De Jongheere et al. 1990), had been tested previously (Monis et al. 1996). A culture of beaver *Giardia* (Beaver ORI) provided by Prof. Meyer was originally established from fecal samples of kill-trapped beaver by Erlandsen et al. (1990b). Other isolates listed in Table 1 were established in axenic culture from domestic or captive animals in the Czech Republic. All these isolates are deposited in the culture collection of the Department of Parasitology, Charles University, Prague.

**Cultivation in vitro.** *Giardia intestinalis* isolates used for DNA isolation were initiated from cryopreserved samples maintained in liquid nitrogen. Cultures were grown axenically in TYI-S-33 medium modified according to Keister (1983) containing 10% heat-inactivated bovine serum, bovine bile, and antibiotics (penicillin 1,000 IU/ml, amikacin 250 μg/ml). Cultures were incubated at 37 °C in 7.5-ml screw-cap tubes in a horizontal position and transferred (or harvested) after the trophozoites had formed an adherent monolayer (4–8 days).

**Clonal lines.** Clonal lines of isolate P15 were established from micro-drops of a cell suspension that were placed on fragments of sterilized coverslips and determined by microscopy to contain a single trophozoite (Binz et al. 1991).

**DNA isolation.** Adherent trophozoites were detached from the tubes in cold phosphate-buffered saline, pH 7.4 (PBS) and after chilling the tubes on ice for 15 min, the cells were harvested by centrifugation (1,000 g, 10 min). The cell pellet was resuspended in 200 μl of lysis buffer (0.5% Sarkosyl/0.15 M NaCl/50 mM Tris-HCl/0.5 mM EDTA, pH 8.0) and shaken for 2 min. The lysis was extracted twice, first with phenol/chloroform/isoamylalcohol (25:24:1), then with chloroform/isoamylalcohol (24:1). DNA was precipitated at −20 °C (overnight) by addition of 2.5 vol. of 96% ethanol. The pellet was washed in 70% ethanol, air-dried and dissolved in 100 μl of sterile distilled water. After treatment with RNase A (Sigma, St. Louis, MO; 20 μg/ml, 30 min, 36 °C), the phenol extraction and ethanol precipitation were repeated. The DNA pellet was dissolved in 50 μl of sterile distilled water and stored at −20 °C.

**Detection of dsRNA virus.** For detection of the double-stranded RNA (dsRNA) Giardiavirus, total nucleic acid extracts (not treated with RNase A) were subjected to electrophoresis in 1% agarose gels. The dsRNA was recognized as a distinct satellite band (below the wide band of genomic DNA) with a mobility corresponding to a 7.0-kb DNA molecule.

**RAPD analysis.** Polymerase chain reactions (PCR) were performed in 20-μl reaction vol. containing: PCR buffer without MgCl2 (0.1% Triton X-100, 50 mM KCl, 10 mM Tris-HCl, pH 9.0; Promega Corporation, Madison, WI), 2.5 mM MgCl2 (Promega), 200 μM of each dNTP (Promega), 10 μM of a single oligodeoxynucleotide primer (University of British Columbia, NC-PS Unit, BC), 2 units of Taq DNA polymerase (Promega), and 10 ng of template DNA. In preliminary analyses, 30 random primers were tested with DNA samples of one *G. intestinalis* isolate (HP-1) and only those, that produced rich banding patterns were chosen for testing other isolates. Samples from the 25 *G. intestinalis* isolates were analyzed by RAPD using 13 primers, whereas clones derived from isolate P15 were analyzed using 15 primers (Table 2). PCR were performed using a PT C-200 thermocycler (MJ Research, Inc., Watertown,

MA) programmed for an initial denaturation step at 92 °C (100 s) followed by 35 reaction cycles (94 °C/40 s, 52 °C/40 s, 72 °C/90 s) and a final extension step at 72 °C (10 min). RAPD products were identified after electrophoresis in 2% agarose gels (TAE buffer) and staining with ethidium bromide. For each primer, DNA samples from all isolates (or the 14 clones) were tested simultaneously in a single reaction run. The complete reaction mixture (minus template DNA) was prepared as a single batch and aliquots were dispensed into 0.2-ml PCR tubes containing test DNA samples or water (negative control).

**Data analysis.** The electrophoretograms were captured by digital camera (model ST-7) using PC program SkyPro. The gels were displayed on a computer screen and the bands obtained were transformed manually into a binary data matrix, i.e. individual bands obtained using a given primer were scored as present (1) or absent (0) for each isolate. All visible electrophoretic bands were included in the RAPD analysis. Genetic distances [d] of samples were computed from Nei-Li’s coefficient of similarity [x] (Nei and Li 1979), where d = (1 - x). Phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei 1987) as well as by the unweighted pair-grouping method (UPGMA) (Sneath and Sokal 1973). The robustness of trees was assessed by bootstrap analysis (250 replications). Computations of genetic distances, construction of trees, and bootstrap analyses were performed by the program FreeTree (Hampl et al. 2001; Pavlíček et al. 1999).

**Statistical testing of tree concordance.** Permutation tail probability testing was used to estimate the correspondence of the presence of dsRNA virus (Giardiaivirus) in examined isolates with their position in the tree (Vanhucová et al. 1997). The presence of Giardiaivirus was coded as 1 and its absence as 0. The test was done with the program Treept (Flegel et al. 1998).

**RESULTS**

**RAPD analysis of *Giardia intestinalis* strains.** Samples of DNA from 25 G. intestinalis isolates, including “type” references for genetic assemblages A (A-I, A-II, A-III), B and E, were tested in single-primer PCR using 13 different oligonucleotides (Table 2). Each primer yielded numerous amplified DNA fragments, as exemplified in Fig. 1 for primer UBC 180. The overall banding patterns differed markedly, a unique banding pattern being obtained for each “type” isolate (Fig. 1). Comparisons with the test isolates indicated that the banding patterns were related to the genotype of the organisms, the differences between isolates that yielded a similar overall banding profile being, in general, only minor (c.f. lanes 9-11, 16 and 23-25). Similar results were obtained using the other primers, the banding patterns being largely distinctive for one or other of the three defined genetic assemblages (data not shown).

In order to compare the banding data rigorously, all of the bands evident in each data (primer) set were allotted binary status (1 = present, 0 = absent) and used to build a data matrix. The final matrix containing 407 binary RAPD characters (bands) from all 25 isolates was used for construction of phylogenetic trees (Fig. 2). Both NJ (Fig. 2) and UPGMA methods (data not shown) produced similar results. The isolates formed three distinct clades, supported by high bootstrap values (≥ 99%). The distribution of “type” isolates within the tree indicated that these clades represented the three genetic assemblages A, B, and E defined by Monis et al. (1999). The mean intraclade Nei-Li distances were similar (A:B = 0.75, A:E = 0.76, B:E = 0.72). These were approximately twice the mean intraclade distances (0.36, 0.39, and 0.25 for assemblage A, B, and E respectively), consistent with the high bootstrap support for each clade.
Fig. 2. Phylogenetic tree for 25 isolates of Giardia intestinalis constructed from pooled transformed RAPD data by the Neighbor-Joining method and separated in Assemblages A, B, and E. Branch lengths reflect relative genetic distances between isolates. Bootstrap values for particular branches are indicated. Isolate names printed in bold designate type references for particular genetic assemblages. Asterisks designate isolates in which Giardiaivirus dsRNA was detected.

The assemblage A clade comprised 19 isolates: three "type" references (Ad-1/c7, Bris-136/c1 from humans, and CH-03 from sheep), together with two human (HP-1, Ber-1) and five animal isolates from cats, horse, pig, and sheep. Within this assemblage, the Group I and Group II "type" isolates were resolved within separate clades, with bootstrap values of 98% and 96%, respectively (Fig. 2). Seven isolates co-clustered with the Group I reference (Ad-1/c7), but none showed identity with this isolate. Bootstrap support for the branching order within this clade was extremely weak (mean, 38.3%). Only one isolate, Ber-1 (human-derived), co-clustered with the Group II reference, Bris-136/c1, but the two isolates were not identical.

The assemblage B clade included two human isolates, HH and the Ad-28 "type" reference, plus seven animal isolates—from dog, chinchillas, porcupine, and exotic rodents (C. gambianus, G. musieloides) (Fig. 2). The internal branching order was not well supported (mean bootstrap value, 65%).

The third major clade, corresponding to assemblage E (comprising "Hoofed livestock" genotypes), contained six isolates—all from hosts within the Order Artiodactyla (pig, sheep, bearded argali, mouflon). Clonal lines derived from isolate P15 were genetically homogenous and stable (data not shown).

Giardiaivirus detection. Giardiaivirus dsRNA was detected in the nucleic acid samples from seven of 21 isolates examined (Fig. 2). These included representatives of assemblage A (four isolates), assemblage B (one isolate), and assemblage E (two isolates). No statistically significant concordance was detected between the presence or absence of Giardiaivirus RNA and the position of isolates within the phylogenetic tree (p = 0.152).

DISCUSSION

The findings of this study show that RAPD can be used to reliably identify distinct subtypes of G. intestinalis and that banding data, if transformed to binary form for incorporation into data matrices, are suitable for phylogenetic reconstructions. Our analysis of 25 isolates distinguished three highly distinct clades, corresponding to genetic assemblages A, B, and E, which had been defined previously on the basis of sequence data and other data (Ey et al. 1997; Mayrhofer et al. 1995; Monis et al. 1996, 1999, Thompson et al. 2000). The three clades representing assemblages A, B, and E were supported by very high bootstrap values (≥ 99%). All of the "type" isolates were placed correctly into separate clades or, for the multiple assemblage A "type" samples, into the same major clade. The distinctive, stable RAPD banding patterns observed in this study contrast with previous RAPD data reported for G. intestinalis. However, the isolates examined in these earlier studies were either less diverse genetically than those comprising the current panel (e.g., Le Blanc et al. 1991; Painthilia et al. 1999), or else the patterns were interpreted as "fingerprints" (with definition of 10 "rapidemesis"; Morgan et al. 1993) or as evidence of genetic "variation" among isolates or clones. These differences can be attributed in part to high frequency chromosome rearrangements occurring within telomeric repeats and rDNA tandem arrays (Le Blanc et al. 1991; van Bellum et al. 1993). Such alterations in chromosome structure do not reflect long-term (mutational) evolutionary changes and they are not observed in genetic assays that detect nucleotide substitutions within protein-encoding or rRNA genes. Similarly, they would not be detected by RAPD analysis, unless the RAPD primers targeted telomeric segments that span chromosomal breakpoints.

The differentiation of isolates within each assemblage by RAPD analysis has a less certain genetic basis than the resolution of the three major clades, as the distinctions rest on more subtle, undefined differences. Nevertheless, the resolution of Group I, Group II, and Group I/II-like "type" isolates, which are known to be genetically distinct members of assemblage A (Monis et al. 1996), indicates that the differentiation observed between all of the isolates is likely to be correct, even if the branching order is uncertain. In this context, use of conventional assays (Karnans and Ey 1998) to type several of these isolates on the basis of RFLP indicated that isolate Ber-1 was identical to Bris-136/c1 and that isolates P2-MER, Ko-1, and Ko-188 were identical to Ad-1/c7. On this basis, RAPD analysis is more discriminatory but the significance of the fine differences observed by this technique between nominally identical isolates is unclear. In terms of biological significance, the detection of Giardiaivirus dsRNA in isolates belonging to genetic assemblages A, B, and C suggests that susceptibility to viral infection is not genotype-dependent.

The recovery of Group I (subtype A-I) organisms and closely related genotypes from many different animals (e.g., cats, dogs, beavers, cattle, horses, and sheep) as well as humans, as demonstrated in this and previous studies (Ey et al. 1996; Homan et al. 1992; Karnans and Ey 1998; Monis et al. 1996; Nash and Mowatt 1992), indicates that the A-I subtype has a broad host spectrum and high transmissibility and that it thus represents a threat of zoonotic infection (Thompson et al. 1990). Members of this group have been identified by different analytical techniques worldwide and interpreted as descendants of a successful clone that has become dispersed in relatively recent time by human migration (Monis et al. 1996). Group II isolates (subtype...
A-II) have so far been recovered almost exclusively from humans (Karamis and Ey, 1998; Ponce-Maceta et al. 2002).

The clade identified by RAPD as assemblage B consisted of two human isolates (Ad-28 and HH), one dog isolate (D47), three isolates from chimpanzees and others from animals in zoos—a porcupine and two exotic rodents, a guinea pig, and a Gambia giant pouched rat. One assemblage B isolate (P14), derived from a giant pouched rat, caused giardiasis in an experimentally infected human volunteer (Majewska 1994). Our axenic G. intestinalis isolate D47 from a dog, was shown previously to belong to assemblage B (Monis et al. 1998), unlike Australian canine isolates (established by propagation in suckling mice), which were found to be specific 'dog' genotypes that define assemblages C and D (Monis et al. 1998). The low success rate in establishing Giardia from dogs in vitro is a notorious problem (Kulda and Novýková 1996), probably due to differences in the metabolic capacity of assemblage C and D subtypes.

Six isolates, including the reference strain from pig (P-15), formed the clade recognized as genetic assemblage E. This assemblage has been found, in this study and previously (Ey et al. 1997; Thompson et al. 2000), to consist only of Giardia recovered from the mammalian order Artiodactyla. In the present study using RAPD, isolates derived from a pig, sheep, a bearded ass, and a mouna formed the assemblage E clade. These genotypes defined originally by Ey et al. (1997) seem to have a narrow host spectrum confined to cloven-hoofed animals.

In conclusion, our results confirm the utility of RAPD analysis for genealogical studies with axenic isolates of G. intestinalis and possibly other organisms. The multi-locus character of RAPD, the capacity to undertake phylogenetic reconstructions on the basis of binary transformed RAPD banding data, and the means to test the resulting phylogenetic trees by bootstrap analysis make this technique a valuable supplement to other methods used in epidemiological studies.

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