Am. J. Trop. Med. Hyg., 40(6), 1989, pp. 629–637 (8-237)

CHARACTERIZATION OF *GIARDIA* ISOLATES USING A NON-RADIOLABELED DNA PROBE, AND CORRELATION WITH THE RESULTS OF ISOENZYME ANALYSIS

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Abstract. Forty-seven isolates, identified morphologically as *Giardia duodenalis*, were compared by restriction endonuclease analysis of DNA with hybridization to a non-radiolabeled probe. Seven schizodemes were distinguished, compared to 15 zymodemes identified by isoenzyme electrophoresis. Despite the greater sensitivity of isoenzyme electrophoresis, DNA analysis did detect previously unsuspected variations between isolates in 1 zymodeme. Eighteen different genetic groups were detected among the 49 isolates by isoenzyme and DNA analyses. Genetic differences between groups, calculated from DNA restriction fragment variation, were significantly correlated with differences calculated from allozyme variation. This correlation between the 2 techniques suggests that *G. duodenalis* consists of a complex of genetically diverse clones. Such a genetic structure has important implications for the taxonomy of *Giardia* and the epidemiology of giardiasis.

Giardia is a flagellated protozoan parasite found in the intestine of virtually all classes of vertebrates.[1] The genus has a world-wide distribution and consists of 4 morphological groups which are considered to represent distinct species.[2–4] Members of the *G. duodenalis* (syn. *G. lamblia* and *G. intestinalis*) morphological group infect humans and other animals.

Isolates of *G. duodenalis* differ in a number of extrinsic characteristics, including growth in vitro, drug susceptibility, infectivity, virulence, and host specificity.[5–11] The recent application of intrinsic criteria, such as antigenic, isoenzyme, and DNA analyses, have confirmed that *G. duodenalis* is genetically heterogeneous.[12–21] This genetic heterogeneity has important implications for the taxonomy of *Giardia*.[18] In addition, although no clear correlations have yet been determined between genetic variation and differences in characters of clinical significance,[6, 18, 21] genetic characterization is a desirable prelude to epidemiological or pathophysiological studies.

Most studies on genetic variation in *Giardia* have used isoenzyme electrophoresis.[15–18] This has the ability to provide a large number of genetic markers relatively simply and cheaply. However, it has the disadvantage of being unable to detect nucleotide substitutions which do not alter the net charge on the polypeptide product of a structural gene. Direct analysis of DNA, by sequencing, hybridization, or treatment with restriction enzymes, provides a potentially more sensitive measure of genetic variation. Restriction endonuclease analysis and DNA hybridization are proving to be of increasing value in taxonomic and characterization studies on parasitic protozoa.[22–26] Nash[19] used these techniques to discriminate and suggest relationships between isolates of *Giardia*.

In a previous study, we used isoenzyme electrophoresis to compare isolates of *G. duodenalis* from 25 humans, 4 cats, and a rat.[18] The aim of the present study was to assess the potential of restriction endonuclease analysis, with hybridization to DNA probes, to distinguish these same isolates plus 19 more, including *G. duodenalis* isolated from a dog and 3 more cats.

MATERIALS AND METHODS

Giardia duodenalis *isolates*

Thirty isolates have already been characterized using isoenzyme electrophoresis and are described elsewhere.[18] Isolate BAH12 has subsequently been cloned and both the clone (BAH12C1) and the original isolate were used in this study. Nineteen new isolates, all from Western Australia, are shown in Table 1. With the exception of a dog isolate (BAD1), these were established and grown as axenic cultures from cysts, using methods described previously.[5, 18]

TABLE 1
Isolates of G. duodenalis

Code	Host	Geographic origin	Source*
BAH23	Human	Esperance	SHL
BAH24	Human	Derby	SHL
BAH25	Human	Bunbury	SHL
BAH26	Human	Rockingham	SHL
BAH27	Human	Fitzroy Crossing	PMH
BAH28	Human	Fitzroy Crossing	PMH
BAH29	Human	Fitzroy Crossing	PMH
BAH30	Human	Derby	SHL
BAH31	Human	Broome	SHL
BAH32	Human	Port Hedland	SHL
BAH33	Human	Broome	SHL
BAH34	Human	Perth	PMH
BAH35	Human	Perth	PMH
BAH36	Human	Kununurra	SHL
BAH37	Human	Mundaring	SHL
BAC4	Cat	Murdoch	MU
BAC5	Cat	Murdoch	MU
BAC6	Cat	Murdoch	MU
BAD1	Dog	Murdoch	MU

* SHL = State Health Laboratories of Western Australia, PMH = Princess Margaret Hospital, and MU = Murdoch University.

Isolates from dogs in Western Australia fail to establish in culture.[5] Therefore, cysts were collected from a fecal specimen on a 0.85% sucrose gradient. Human isolates BAH34 and BAH35 are from 2 brothers infected with *Giardia*.

Isoenzyme electrophoresis

Isolate BAH12, its clone, and the nineteen new isolates were analyzed by isoenzyme electrophoresis using 10 enzyme systems as described by Meloni and others.[18]

DNA analysis

Isolation of DNA from Giardia. A reproducible technique for isolating chromosomal DNA from *Giardia* was developed using the method of Yap and Thompson.[27] The procedure is easy to perform, rapid, inexpensive, and yields DNA suitable for restriction endonuclease digestion and cloning techniques. Trophozoite pellets (0.1–0.5 ml) were resuspended in 200 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), 0.5 ml of lysis buffer (8% Triton X-100, 0.25 M sucrose, 50 mM Tris-HCl, 50 mM EDTA, pH 7.5), and 1 mg of Proteinase K (Boehringer Mannheim, West Germany) and incubated at 37°C for 90 min. An equal volume of 2% cetyltrimethylammonium bromide (CTAB: British Drug House) was added and the DNA/CTAB precipitate centrifuged at 1, 900 × *g* for 2 min. Pellets were dissolved in 400 μl NE buffer (2.5% NaCl, 10 mM EDTA, pH 7.7) and diluted with 800 μl E buffer (40 mM Tris-HCl, 2 mM EDTA, pH 7.7). Proteins were removed by extracting with an equal volume of chloroform and centrifuging at 9,000 × *g* for 5 min. The aqueous phase was removed and the DNA precipitated with 2 volumes of ethanol (−20°C) overnight.

DNA probes. DNA probes were constructed by ligating partially digested DNA from *Giardia* isolate BAH1 into the *Bam* HI insertion site of pBR322. Ligation was carried out in a 20 μl reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM ATP, and 1 U T4 DNA ligase. The ligation mix was incubated at 4°C overnight and used to transform competent *E. coli* HB101. Transformants were plated onto ampicillin and tetracycline plates and characterized further by restriction analysis.

Recombinant plasmids to be used as DNA probes were purified by ultracentrifugation through a cesium chloride density gradient as described by Maniatis and others.[28] Probes were labeled with 2 different non-radioactive labeling systems under optimal conditions described by the manufacturers: photobiotin (Biotechnology Research Enterprises South Australia [BRESA]) or sulphonated (Chemiprobe Kit; Orgenics Ltd.). Five probes were chosen for preliminary screening and 1 subsequently selected for the present analysis. This probe (pBM9) contains a 3.7 kb fragment from a repetitive sequence of genomic DNA (B. Meloni, personal communication).

Agarose gel electrophoresis and southern transfer. DNA was extracted from each isolate and 10 μg digested with *Hind* III (Toyobo; Japan). Following digestion, restriction fragments were separated by horizontal agarose gel electrophoresis using 1% gels in 40 mM Tris-HCl, 20 mM acetate, 2 mM EDTA, pH 9.0. DNA was then transferred to nitrocellulose (Schleicher and Schuell; West Germany) or nylon (Biodyne A; Pall Ultrafine Filtration Corp. or Zeta Probe; Bio-Rad Laboratories) membranes using a modification of the technique of Southern.[29] Nitrocellulose membranes were used with both detection systems, but nylon membranes could not be used with photobiotin, due to excessive background.

For southern transfer, gels were soaked at room temperature with agitation for 15 min in 0.25 M HCl; 45 min in 0.6 M NaCl, 0.4 M NaOH; and

60 min in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.0. Membranes were cut to the same size as the gel and soaked in distilled water for 10 min and 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 15 min. The gel was placed on 5 sheets of Whatman type 1 filter paper that had been saturated in 20 × SSC. Wetted membranes were positioned on top of the gel, 5 sheets of dry filter paper were layered onto the membranes followed by a stack of dry paper towels, and blotting was carried out overnight at room temperature. Membranes were removed, rinsed in 2 × SSC, air-dried, and baked at 80°C for 1 hr.

Hybridization. Membranes were prehybridized for 4–24 hr at 55°C in hybridization solution containing 6 × SSC, 1% SDS, 10% skim milk, and 10 mg/ml denatured herring sperm DNA. The DNA probe was denatured by heating at 100°C for 5 min and added to fresh hybridization solution. Probes were used at a concentration of 0.1–1 μg/ml and hybridized overnight at 55°C. Following hybridization, membranes were washed twice, vigorously at room temperature for 5 min in 2 × SSC/0.1% SDS, 0.5 × SSC/0.1% SDS, and 0.2 × SSC/0.1% SDS followed by 15 min at 55°C in 0.1 × SSC/0.1% SDS. Colorimetric detection of the hybridized probe was performed to manufacturer's specifications using materials supplied with the BRESA and Chemiprobe kits.

Interpretation of isoenzyme and DNA data

Isolates with the same banding pattern for the 10 enzymes used in isoenzyme analysis are referred to as a zymodeme.[18] Following the terminology of Morel and others,[30] isolates with the same banding pattern after DNA restriction and hybridization analysis are referred to as a schizodeme.

Enzyme bands of different mobility were interpreted as allozymes. Multilocus genotypes were assigned to zymodemes on the assumption that all isolates were diploid, with multiple-banded enzyme patterns indicating heterozygosity at that locus. Nei's genetic distance coefficient (D)[31] was used as a measure of the number of electrophoretically detectable base substitutions per enzyme locus separating zymodemes.

DNA fragment patterns generated by restriction analysis were used to estimate Upholt's coefficient (p),[32] the number of base substitutions per nucleotide separating schizodemes. The formulae used to calculate p assume that all fragment changes arise from base substitutions. This assumption may not be valid for the DNA sequence recognized by our probe; detectable length mutations would inflate the estimate of sequence differences.

RESULTS

Isoenzyme analysis

Using 10 enzyme systems, 17 different zymodemes were found among the 49 isolates of *Giardia*. The enzyme profile of BAH12C1 was identical to that of the parent isolate, BAH12. Of the 19 new isolates examined, 14 had enzyme profiles identical with previously described zymodemes M1, M3, M12, M4, and M5 (Table 2). Four additional zymodemes were identified; M14 contained the dog isolate (BAD1), while M15, M16, and M17 contained human isolates (Table 2). The enzyme profiles of each zymodeme are shown in Figure 1.

DNA analysis

The hybridization results for the 48 isolates of *Giardia* probed with pBM9 are summarized diagrammatically in Figure 2. Representative blots are shown in Figures 3–5. The probe distinguished 7 schizodemes among the 48 isolates (Table 2). The dog isolate (BAD1) was not examined due to lack of material. The rat isolate (BAR1) did not produce any bands, and a number of other isolates consistently produced faint bands after hybridization to pBM9 (Fig. 4). This probably indicates extensive divergence between these isolates and BAH1 in the sequence of DNA used as a probe.

Correlation between isoenzyme and DNA results

Table 2 compares the groupings of isolates with identical enzyme or DNA profiles. Schizodemes S1, S3, and S7 were divisible into ≥2 zymodemes. Only zymodeme M1 contained isolates belonging to >1 schizodeme.

A total of 18 genetic groups were distinguished by enzyme and DNA analyses. Table 3 shows genetic distances between these groups, estimated from allelic differences at enzyme loci (Nei's D) and restriction site differences for probe pBM9

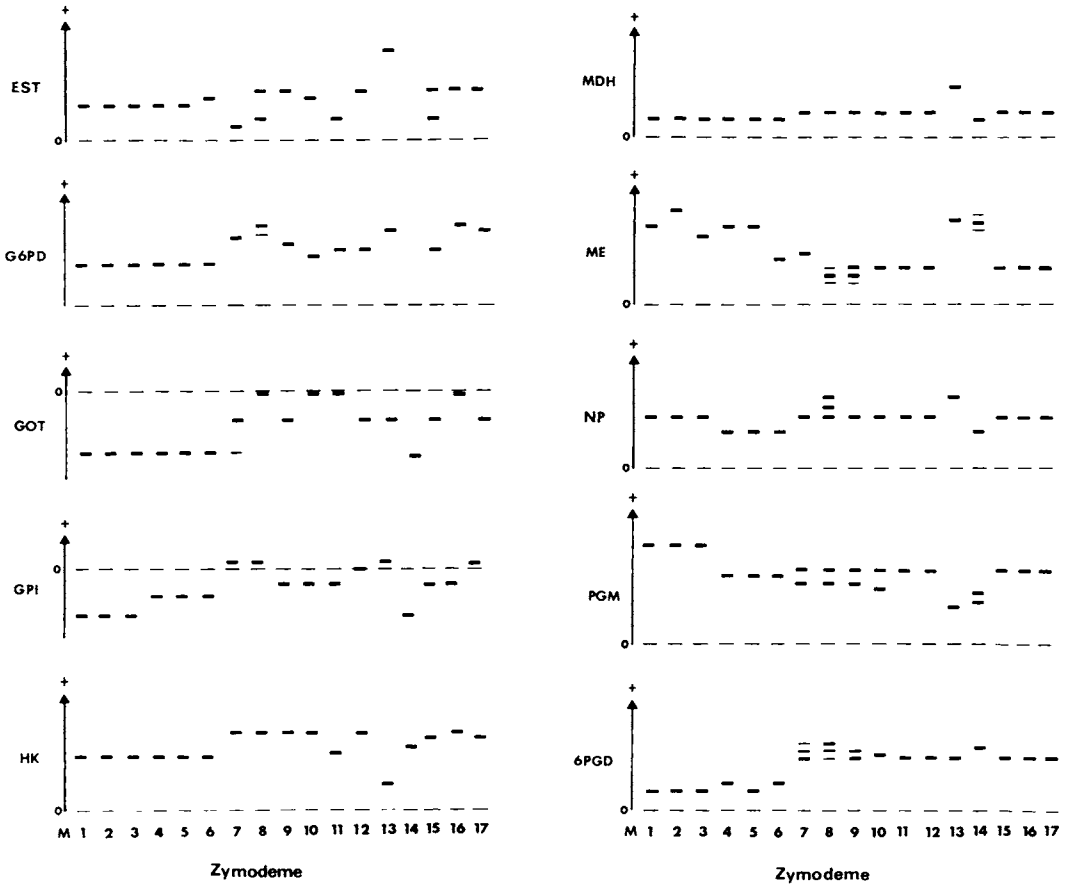

FIGURE 1. Diagrammatic representation of the enzyme profiles of the 17 zymodemes obtained for *Giardia duodenalis* using 10 enzyme systems: esterase (EST), glucose-6-phosphate dehydrogenase (G6PD), glutamate-oxaloacetate transaminase (GOT), glucose phosphate isomerase (GPI), hexokinase (HK), malate dehydrogenase (MDH), malic enzyme (ME), nucleoside phosphorylase (NP), phosphoglucomutase (PGM), and 6-phosphogluconate dehydrogenase (6PGD). Thickness of bands indicates relative staining intensity.

(Upholt's p). The 2 measures are significantly correlated (Spearman's r = 0.54, $P < 0.001$).

DISCUSSION

The 47 isolates of *Giardia* from different animals and geographical areas, examined by restriction analysis with *Hind* III and hybridization to probe pBM9, produced 7 different DNA banding profiles (Fig. 2). Isoenzyme analysis of the same, and 2 additional isolates (BAR1, BAD1), distinguished 17 genetic variants using 10 enzyme systems (Fig. 1). The extensive genetic heterogeneity among isolates of *Giardia* identified by both isoenzyme electrophoresis and DNA hybridization analysis confirms the suspicion from previous clinical, biochemical, and behavioral studies that the morphologically defined species, *G. duodenalis*, encompasses a wide spectrum of genetic variation.[5-21]

We detected more variation using isoenzyme analysis than DNA analysis of the same isolates. This may indicate less rapid evolution for the sequence of DNA we probed than for enzyme loci. It should be noted, however, that we used only 1 endonuclease to generate restriction fragments. The application of a number of restriction endonucleases in conjunction with the probe may have detected more variation, although Tibayrenc and Ayala[33] found that the information obtained from different digests of *Trypanosoma cruzi* kinetoplast DNA was largely redundant. Despite the apparent conservatism of our DNA probe, it did detect differences between isolate

TABLE 2

Grouping of isolates of G. duodenalis *using isoenzyme (zymodemes) and DNA (shizodemes) banding profiles*

Isoenzyme profile (Zymodeme)*	DNA profile (Schizodeme)
1. BAH3,4,5,6,8, 9,10,11,13,17,22, 24,26,27,28,31,37	1. BAH3,4,5,6,8,9, 10,11,13,14,17,18, 21,22,23,24,25,26, 27,28,29,36,37
2. BAH21	2. BAH31
3. BAH14,18,23,25, 29,36	3. BAH1,2 BRIS/83/HEPU/ 106,120,141 BAC2,3,4,5,6 P1
4. BAH1,2 BRIS/83/HEPU/ 106,120,141 BAC3,4,5 P1	4. BAC1
5. BAC2,6	5. BAH7
6. BAC1	6. BAH12
7. BAH7	7. BAH15,16,19,20,30, 32,33,34,35
8. BAH12	
9. BAH16	
10. BAH20	
11. BAH19	
12. BAH15,32	
15. BAH30	
16. BAH33	
17. BAH34,35	

* Zymodemes M13 (BAR1) and M14 (BAD1) not shown.

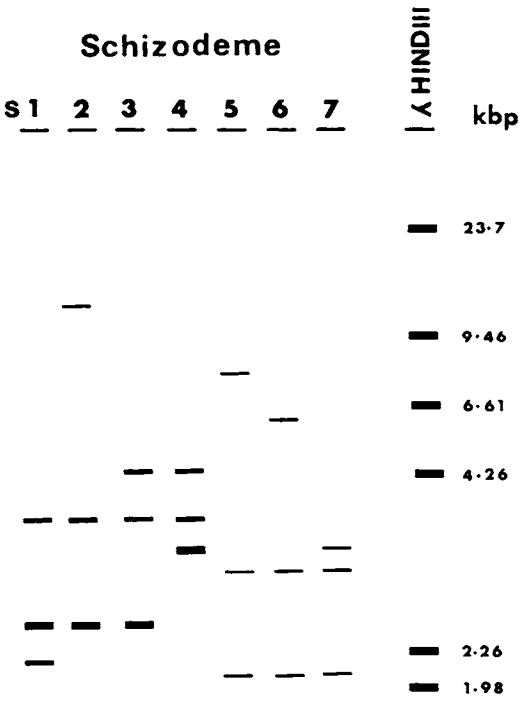

FIGURE 2. Diagrammatic representation of the DNA banding profiles of the 7 schizodemes obtained for *G. duodenalis* after restriction with *Hind* III and hybridization to pBM9. Thickness of bands indicates relative staining intensity.

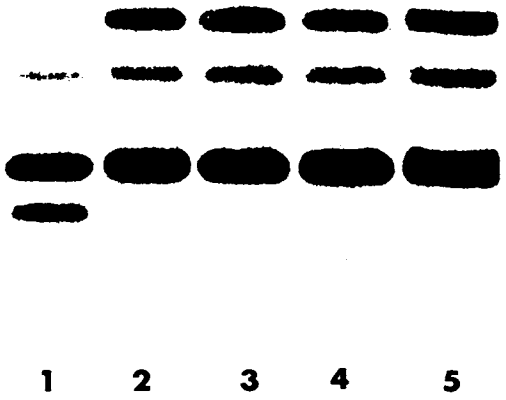

FIGURE 3. Southern blot hybridization of DNA from *G. duodenalis* restricted with *Hind* III and hybridized to pBM9, showing banding patterns for schizodemes S1 (lane 1) and S3 (lanes 2–5).

BAH31 and the other isolates in zymodeme M1, which we had found to be identical at 10 enzyme loci. This illustrates the potential of DNA analysis to detect previously unsuspected genetic variation.

The DNA probe used in this study was not radioactively labeled. Both labeling systems we used are stable at −20°C and are not associated with the problems of radioactively labeled probes, such as expense, hazards in handling and disposal, short shelf lives, and the requirement for specially equipped laboratories.[34] Although non-radioactive labels are attractive candidates for use in clinical or field situations,[35, 36] the high sensitivity of radioactive labeling is usually preferred for comparative studies in ideal conditions. However, for most isolates, we obtained satisfactory results using both the photobiotin and sulphonated chemiprobe systems with nitrocellulose membranes. Improved sensitivity was achieved when nylon membranes, Biodyne-A (Pall) or Zeta-Probe (Bio-Rad), were used with the sulphonated probe and detection system.

Despite differences in the amount of genetic variation detected, isoenzyme and DNA analy-

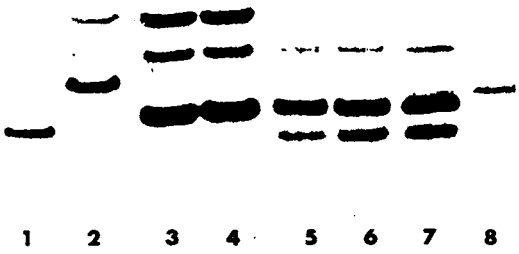

FIGURE 4. Southern blot hybridization of DNA from *G. duodenalis* restricted with *Hind* III and hybridized to pBM9, showing banding patterns for schizodemes S3, (lanes 1, 3, 4), S4 (lane 2), S1 (lanes 5–7), and S7 (lane 8); note difference in staining intensity between S7 and other schizodemes.

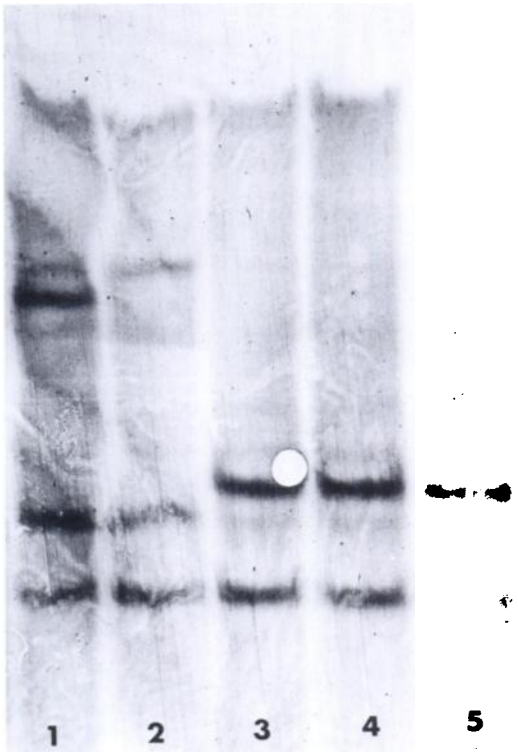

FIGURE 5. Southern blot hybridization of DNA from *G. duodenalis* restricted with *Hind* III and hybridized to pBM9, showing banding patterns for schizodemes S6 (lane 1), S5 (lane 2) and S7 (lanes 3–5).

ses produced very similar groupings of those isolates with identical banding patterns. This correlation between the 2 techniques suggests that the groups represent natural clones of genetically identical organisms. The range of genetic distances between groups, estimated from both allozyme and DNA fragment differences, indicates that a number of species may be present. However, our ability to make realistic taxonomic decisions is restricted by a lack of knowledge about the genetic system of *Giardia*. Recent electrophoretic evidence of complex banding patterns in a number of isolates of *G. duodenalis* has suggested the possibility of diploidy.[18] In the present study, we found that these banding patterns were retained in a clone of 1 of the original isolates (BAH12). This indicates that the isolate does not consist of genetically different haploid organisms and provides further support for diploidy, although alternative explanations cannot be ruled out.[18] A diploid state in the life cycle opens the possibility of sexual reproduction. We believe that the questions of ploidy and mode of reproduction in *Giardia* need to be properly explored before appropriate species concepts can be applied and natural[37] taxa delimited.

It is often possible to relate genetic variation to behavioral characteristics of parasites and so predict the outcome, control, and dissemination of parasitic infection.[6,38] We have previously suggested that the lack of any distinct correlation between genetic variation and host origin provides evidence for cross-transmission between humans and other animals.[18,21] The present study supports this proposal; genetic differences between isolates from different humans were often as great, or greater than, differences between isolates from different host species. Isolates from 7 cats, especially, were very similar to a large number of human isolates, supporting our previous contention that cats are a likely reservoir of infection for humans.[6,18,21] Both dog and rat isolates appeared to be genetically distinct, but further isolates from these hosts must be examined before any conclusions can be drawn.

In addition to providing evidence on possible modes of transmission, the extensive genetic variation detected between isolates of *Giardia* may have clinical and therapeutic implications. Following the results of this and other studies,

TABLE 3

*Matrix of genetic distance measures, estimated from allozyme variation (Nei's D, above diagonal) and DNA restriction fragment differences (Upholt's p, below diagonal), between genetic groups**

1		0	0.10	0.10	0.51	0.37	0.92	1.82	2.82	2.22	2.28	2.30	2.30	∞	1.32	2.28	2.30	2.30
2	0.02		0.10	0.10	0.51	0.37	0.92	1.82	2.82	2.22	2.28	2.30	2.30	∞	1.32	2.28	2.30	2.30
3	0	0.02		0.10	0.69	0.51	0.92	1.82	2.82	2.22	2.28	2.30	2.30	∞	1.32	2.28	2.30	2.30
4	0	0.02	0		0.69	0.51	0.92	1.82	2.82	2.22	2.28	2.30	2.30	∞	1.32	2.28	2.30	2.30
5	0.02	0.02	0.02	0.02		0.10	0.22	2.91	∞	∞	∞	∞	∞	∞	1.32	∞	∞	∞
6	0.02	0.02	0.02	0.02	0		0.37	2.91	∞	∞	∞	∞	∞	∞	1.32	∞	∞	∞
7	0.06	0.06	0.06	0.06	0.02	0.02		2.91	∞	∞	2.28	∞	∞	∞	1.32	∞	∞	∞
8	1	1	1	1	1	1	1		0.54	0.69	1.02	1.12	0.71	1.53	∞	0.94	0.84	0.72
9	1	1	1	1	1	1	1	0.02		0.60	0.65	0.62	0.62	1.43	∞	0.84	0.33	0.62
10	1	1	1	1	1	1	0.06	0.02	0.02		0.64	0.72	0.35	1.82	∞	0.40	0.35	0.52
11	1	1	1	1	1	1	0.06	0.02	0.02	0		0.57	0.77	∞	∞	0.75	0.40	1.02
12	1	1	1	1	1	1	0.06	0.02	0.02	0	0		0.51	2.30	∞	0.26	0.37	0.69
13	1	1	1	1	1	1	0.06	0.02	0.02	0	0	0		1.61	∞	0.26	0.37	0.37
14	1	1	1	1	1	1	0.06	0.02	0.02	0	0	0	0		∞	1.58	2.30	1.20
15	1	1	1	1	1	1	0.06	0.02	0.02	0	0	0	0	0		∞	∞	∞
16	1	1	1	1	1	1	0.06	0.02	0.02	0	0	0	0	0	0		0.40	0.26
17	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		0.51
18	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Zymodeme																		
	M1	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M15	M16	M17	M13	M14
Schizodeme																		
	S1	S2	S1	S1	S3	S3	S4	S5	S6	S7	S7	S7	S7	S7	S7	S7	—	—

* DNA analyses were not performed on group 17, and no banding patterns were visible following hybridization for group 18. For Upholt's p, 1 indicates that there were no restriction fragments shared between groups, while for Nei's D, ∞ indicates no allozymes in common.

future work should concentrate on attempting to correlate genetic variation to characteristics such as pathogenicity, virulence, and susceptibility to drugs.

Acknowledgments: The authors wish to thank Helen Sullivan, Michael Gracey, Vic Bamford, and Val Wymer for supplying the majority of human *Giardia* samples, and Nicolette Binz for providing the cloned isolate used in this study.

Financial support: The National Health and Medical Research Council of Australia.

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