# Investigation of Human Giardiasis by Karyotype Analysis

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## Abstract

**The patterns of transmission of *Giardia lamblia* and the potential contribution of strain differences to pathogenicity of infection is poorly understood. We used pulsed field gradient gel electrophoresis (PFGE) to separate chromosome-sized DNA molecules of 22 stocks of *G. lamblia* isolated from 13 individuals (6 symptomatic, 7 asymptomatic) living in Jerusalem. PFGE gels run under a variety of conditions revealed up to nine ethidium bromide–stained bands per isolate ranging in size from 0.7 to > 3 megabasepairs. Relative staining intensities indicated that some bands contained multiple chromosomes. Major differences in the number, size, and intensity of bands allowed a clear differentiation of the karyotypes of isolates from each of the different individuals. This is in contrast to previous studies where the karyotype of different isolates have been strikingly homogeneous. Hybridization of Southern blots with surface antigen, β-tubulin, and ribosomal RNA genes revealed that these gene families were distributed to different sized chromosomes amongst the different isolates. PFGE thus revealed major differences in the karyotypes of different *G. lamblia* isolates that were obtained over a short period of time from a relatively confined geographic area. In contrast, karyotypes of isolates established either by direct cultivation of duodenal trophozoites or by excystation of stool cysts from the same individuals were almost identical. Also, isolates from the same individuals obtained over a prolonged period of time revealed only minor differences in their karyotype, suggesting that recurrent infection can be caused by genetically similar organisms. We conclude that chronic giardiasis can result from recurrence of occult infection or reinfection from a common source. (*J. Clin. Invest.* 1992. 89:1725–1733.) Key words: *Giardia lamblia* • chromosomes • genome heterogeneity • pulsed field gradient electrophoresis**

## Introduction

*Giardia lamblia* (*G. lamblia*, also known as *G. duodenalis* and *G. intestinalis*) is a flagellated protozoan parasite of worldwide distribution. Its life cycle consists of two stages: the binucleated trophozoites adhere to the mucosa of the upper small intestine and replicate by binary fission; a proportion of trophozoites undergo encystation, producing the quadrinucleated cyst which is excreted in the stools and is resistant to a range of environmental conditions. The majority of *G. lamblia* infections in man are asymptomatic, but some cases may be associated with acute or persistent diarrhea with or without malabsorption. The factors responsible for development of symptomatic versus asymptomatic infection are poorly understood, but the existence of pathogenic and nonpathogenic strains, akin to the situation for *Entamoeba histolytica* (1), remains an intriguing possibility.

The development and application of methods for the in vitro axenic cultivation of *G. lamblia* have been instrumental in promoting basic investigation of the biology of this parasite. Cultures of *G. lamblia* trophozoites may be established either directly from trophozoites in aspirated duodenal fluid (2, 3), or indirectly using cysts isolated from stool samples and excysted in vitro (4) or in laboratory animals (5). A large number of human and nonhuman isolates from diverse geographic regions have thus been established and characterized. Different isolates were shown to generate clinically variable experimental infections in humans (6) and in laboratory animals (7, 8). Isolates have also been shown to vary in their susceptibility to infection with the *Giardia*-specific double-stranded RNA virus (9). Evidence for genetic heterogeneity among isolates that could explain the variable biological behaviour of different isolates has been observed by a variety of techniques. These include variability in isoenzyme profiles (10, 11), protein electrophoresis (12), analysis of surface antigens and excretory–secretory products (13), drug sensitivity assays (14–16), restriction fragment length polymorphisms (17–19) and karyotype analysis (20–23). However, the genetic markers responsible have not been identified. A further elucidation of the genetic heterogeneity could be of value (*a*) in clarification of the currently confused taxonomic classification of *Giardia* (24); (*b*) in identification of potential markers of infectivity, virulence, and drug resistance; and (*c*) as an epidemiological tool in studying transmission of *Giardia* among individuals or within a community.

Previous *Giardia* characterization studies have usually been based on analysis of unrelated isolates with little information available regarding clinical and epidemiological correlates. In this report, we describe a karyotype analysis of *G. lamblia* isolated from symptomatic patients as well as asymptomatic carriers, all from within a single geographic region. The isolates chosen for analysis in this study were collected to enable an epidemiological comparison of: (*a*) repeated isolates obtained from the same individual over a period of time; (*b*) isolates established simultaneously from both trophozoites and cysts from the same individual; (*c*) isolates from subjects having close contact with each other.

To characterize and compare these isolates, we used pulsed field gradient gel electrophoresis (PFGE),[1] a technique which

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*Received for publication 4 November 1991 and in revised form 16 January 1992.*

J. Clin. Invest.

0021-9738/92/06/1725/09 $2.00
Volume 89, June 1992, 1725-1733

1. *Abbreviations used in this paper:* Mb, megabasepairs; PFGE, pulsed field gradient gel electrophoresis; TSE, Tris saline EDTA.

separates chromosome-sized DNA molecules of up to ~ 10 megabasepairs (Mb) in size. In other protozoa, a pattern of distribution of chromosome size-classes (the karyotype) which is specific for the species in question can frequently be identified; nevertheless, heterogeneity in chromosome size can be detected among different isolates of that species. Previous investigations of *Giardia* karyotype have identified no more than five or six chromosome size classes, and only limited karyotype heterogeneity has been apparent (20–23). In the present study, we demonstrate that the *Giardia* genome can be separated into at least nine discrete size classes and that extensive karyotype heterogeneity exists among isolates from within a single geographic area. These findings are discussed in light of the recently identified high rearrangement rates that can occur at some *Giardia* chromosomes (25) and in terms of their clinical and epidemiological implications.

## Methods

*Source of isolates.* Specimens for *G. lamblia* isolation were obtained from patients attending Hadassah University Hospital in Jerusalem, Israel, as well as from residents of Jerusalem and surrounding neighborhoods. From a total of more than 50 isolates established, 22 isolates from 13 subjects were selected for analysis according to the criteria defined in the Introduction. Table I summarizes the age, sex, and clinical features of the subjects, and the date and source (cysts or trophozoites) of the original specimens. Symptomatic patients were those in whom giardiasis was diagnosed during a work-up for diarrhea or other symptoms related to the gastrointestinal tract. Asymptomatic carriers were healthy individuals who had *G. lamblia* identified in stool specimens submitted for routine medical examinations. In addition, two *G. lamblia* strains obtained from the American Type Culture Collection (ATCC), Rockville, MD, were used for comparison: Portland 1 (ATCC 30888), originally isolated from a patient in Oregon but thought to have been contaminated by a cat isolate, and WB (ATCC 30957), isolated in Maryland from a patient presumed to have been infected in Afghanistan (26).

*Isolation and cultivation of G. lamblia.* For isolation of *G. lamblia* from stool specimens, cysts were purified by centrifugation on a 0.8 M sucrose gradient (27), and stored in water at 4°C for 5–10 d after the specimen had been submitted. Excystation was then induced by incubation of the cysts in hydrochloric acid, pH 2, for 30 min at 37°C (4). Cysts were then precipitated by centrifugation and transferred to a sterile, screw-capped glass borosilicate tube filled with 12 ml of Trypticase, yeast extract, iron-serum (TYI-S-33) medium modified by the addition of bovine bile (28) and antibiotics: penicillin 200 IU/ml and streptomycin 200 μg/ml. For cultivation of *G. lamblia* directly from trophozoites, a small volume of intestinal fluid was obtained using the string test (Entero-Test®; HDC Corp., San José, CA) as previously described (3), or by aspiration during a small intestine biopsy procedure (isolate $I_2$, Table I) or fiber-optic endoscopy (isolate $G_1$). One or two drops of this fluid were immediately transferred to a culture tube with medium as above except for the addition of vancomycin 20 μg/ml and clindamycin 20 μg/ml. These antibiotics were required to eliminate contamination with oropharyngeal flora and were omitted after the initial isolation phase.

Culture tubes were maintained vertically in a 37°C incubator and the medium was periodically aspirated and replaced with fresh medium until the trophozoites attached to the inner glass surface approached a monolayer. Cultures were then cryopreserved in fresh medium containing 10% dimethyl sulfoxide as previously described (3) and maintained in liquid nitrogen until required for karyotype analysis.

*Cloning of G. lamblia.* Two isolates, $A_2$ and $J_2$ (Table I), were cloned by limiting dilution (29). To remove any possible aggregates of trophozoites, the culture medium was aspirated and replaced with fresh medium. The tubes were chilled to detach the adherent trophozoites which were then diluted in fresh medium to a concentration of 0.1

*Table I. 22 Giardia lamblia Isolates from 13 Jerusalem Subjects*

| Study subject | Sex | Age | Clinical features | Comments | Isolate no. | Lab stock no. | Origin | Sample date |
|---|---|---|---|---|---|---|---|---|
| | | *yr* | | | | | | |
| A | f | 34 | Asymptomatic | | $A_1$ | MS 88/3 | Cyst | 1.4.88 |
| | | | | | $A_2$ | MS 88/1 | Troph | 1.7.88 |
| B | m | 22 | Asymptomatic | | $B_1$ | MS 89/3 | Cyst | 1.26.89 |
| | | | | | $B_2$ | MS 89/1 | Troph | 1.30.89 |
| C | f | 3 | Asymptomatic | Day-care center | $C_1$ | MS 89/31 | Cyst | 12.20.89 |
| D | m | 3 | Asymptomatic | Day-care center | $D_1$ | MS 89/32 | Cyst | 12.20.89 |
| E | m | 3 | Asymptomatic | Day-care center | $E_1$ | MS 89/33 | Cyst | 12.20.89 |
| F | m | 3 | Asymptomatic | Day-care center | $F_1$ | MS 89/34 | Cyst | 12.20.89 |
| G | f | 21 | Symptomatic, recurrent | Sister of patient H | $G_1$ | MS 89/18 | Troph | 4.11.89 |
| H | f | 4 | Asymptomatic | Sister of patient G | $H_1$ | MS 90/3 | Troph | 1.14.90 |
| I | m | 1–2 | Symptomatic, recurrent | Day-care center | $I_1$ | MS 88/39 | Troph | 9.9.88 |
| | | | | | $I_2$ | MS 89/23 | Troph | 5.28.89 |
| | | | | | $I_3$ | MS 89/24 | Cyst | 5.29.89 |
| | | | | | $I_4$ | MS 89/30 | Cyst | 10.18.89 |
| J | m | 1 | Symptomatic | | $J_1$ | MS 89/5 | Cyst | 1.28.89 |
| | | | | | $J_2$ | MS 89/2 | Troph | 1.30.89 |
| K | m | 17 | Symptomatic, recurrent | | $K_1$ | MS 89/15 | Cyst | 3.7.89 |
| | | | | | $K_2$ | MS 89/25 | Cyst | 5.21.89 |
| L | f | 21 | Symptomatic | | $L_1$ | MS 89/21 | Cyst | 5.17.89 |
| | | | | | $L_2$ | MS 89/22 | Troph | 5.22.89 |
| M | m | 2 | Symptomatic | | $M_1$ | MS 90/5 | Cyst | 1.24.90 |
| | | | | | $M_2$ | MS 90/6 | Cyst | 1.24.90 |

cells/ml. Aliquots of 0.2 ml were transferred to 96-well microtiter plates which were incubated at 37°C in an anaerobic environment (Bio-Bag Environment Chamber type A; Becton Dickinson Microbiology Systems, Cockeysville, MD). By this method, the probability that clones were derived from a single organism is 96.8% (29). The clone of isolate $J_2$ was recloned to generate subclones.

*Preparation of chromosome-sized G. lamblia DNA.* Late logarithmic phase cultures were chilled and harvested by centrifugation at 4°C followed by three washes in ice-cold Tris saline EDTA (TSE) (10 mM Tris/HCl pH 8, 100 mM NaCl, 50 mM EDTA). After being counted, trophozoites were resuspended in TSE to a concentration of $5 \times 10^8$ cells/ml, mixed with an equal volume of molten 1% low melting point agarose in TSE at 42°C and allowed to gel briefly in a chilled Perspex mold to form 75-$\mu$l blocks. The blocks were put into 10 ml of lysis mix (0.5 M EDTA pH 9, 1% sarkosyl, 2 mg/ml proteinase K), incubated at 51°C with gentle shaking for 2 d and thereafter stored at 4°C until required.

*Pulsed field gradient gel-electrophoresis.* PFGE was performed using an LKB 2015 Pulsaphor apparatus (2015; LKB Instruments, Inc., Gaithersburg, MD) as previously described (30). In general, one-sixth of a block was loaded per well and sealed with molten 1% low melting point agarose. *Trypanosoma brucei* variant 118 clone 1 chromosomes were used as size markers (31). Conditions of field strength (V/cm), pulse time (s) and run duration (d) were selected as appropriate to the size class of the chromosomes to be resolved, and specific conditions are listed in each of the figure legends. After electrophoresis, gels were stained in ethidium bromide in distilled water (0.25 $\mu$g/ml) for 30 min, destained in distilled water for 60 min, and photographed under ultraviolet transillumination. Before Southern transfer, DNA was partially hydrolyzed by acid depurination in 0.25 M HCl for 10 min, denatured in 0.5 M NaOH and 1.5 M NaCl for 60 min, and neutralized in 1 M Tris/HCl pH 8 and 1.5 M NaCl for 60 min.

*DNA probes.* The following DNA probes were used: ribosomal RNA (rRNA): a 1.2-kb Bam HI/Eco RI fragment of pGRPI (32), a complete repeat unit of the *G. lamblia* rRNA gene cloned into the Sma I site of pUC 8, kindly provided by Dr. C. C. Wang (University of California, San Francisco, CA). Telomere repeat: a 530-bp Sma I/Pst I fragment from a subclone (pGCIΔN) of a cloned *G. lamblia* telomere (pGCI, cloned into the Sma I/Xba I site of Bluescript), consisting of the telomere-specific pentanucleotide repeat $[TAGGG]_n$ which comprises 0.5- to 1-kb tandem arrays on *G. lamblia* chromosome ends (33). $\beta$-Tubulin: 1.35-kb Eco RI insert of 2S-4, a $\beta$-tubulin cDNA cloned into pUC 18, kindly provided by Dr. P. Chakraborty (Merck Research Laboratories, Rahway, NJ) (34). Surface antigen: D2dS, a 1.4-kb Sma I/Kpn I insert in Bluescript from the coding sequence of TSA 417, a cloned gene encoding two major *G. lamblia* trophozoite surface antigen species (35), kindly provided by Dr. F. D. Gillin (University of California, San Diego, CA). Surface antigen: M2-1, a 1-kb Eco RI insert in M13 mp 18, encoding part of a 170-kD cysteine-rich surface protein gene (36), kindly provided by Dr. R. Adam (University of Arizona, Tucson, AZ).

Plasmid manipulations were performed using standard protocols. *Escherichia coli* strains DH5$\alpha$, HB101, and XL1 (Stratagene Inc., La Jolla, CA) were used. Inserts were excised using the appropriate restriction enzymes and separated from vector DNA by low melting point agarose gel electrophoresis. Probes were labeled with either [$\alpha^{32}$P]dCTP or [$\alpha^{32}$P]dGTP by the random priming method (Random Prime Kit; Boehringer Mannheim Corp., Indianapolis, IN). Unincorporated isotope was removed using a Sephadex G-50 (Sigma Chemical Co., St. Louis, MO), spin column.

*Hybridizations.* Chromosome-sized DNA separated on PFGE gels was transferred to nylon filters (Hybond N; Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions. After transfer, the filters were exposed to ultraviolet transillumination for 3 min. Southern blots were prehybridized for 2–4 h at 65°C in 50 ml of 10% dextran sulfate, 3 × standard saline citrate (SSC), 10× Denhardt's reagent, and 0.1% SDS with 100 $\mu$g/ml of sheared and denatured salmon sperm DNA. Labeled DNA probes were denatured by boiling for 5 min and added to the prehybridization solution. Hybridization was carried out for 16–20 h at 65°C. Blots were washed once in 2×SSC for 15 min at 65°C, twice in 2 × SSC/0.1% SDS for 15 min at 65°C, and finally at 0.1 × SSC/0.1% SDS for 20 min at 65°C. The filters were wrapped in plastic film (Saran Wrap) and exposed for autoradiography with an intensifying screen at −80°C for the appropriate period of time. Before subsequent rehybridization, filters were stripped in 0.4 M NaOH for 30 min at 45°C, washed in 0.2 M Tris/HCl pH 7.5, 0.1×SSC and 0.1% SDS for 30 min at 45°C, and exposed for autoradiography to evaluate any residual signal.

## Results

*Karyotype heterogeneity among trophozoites of human G. lamblia isolates.* Ethidium bromide–stained chromosome-sized DNA molecules of two different *G. lamblia* isolates, $B_1$ and $J_1$ (see Table I), are visualized in panels *a* and *b* respectively, of Fig. 1. According to the PFGE conditions chosen, specific size-classes of chromosomes can be maximally separated; hence, several lanes are shown in each panel to illustrate optimal separation of the different size-classes of chromosomes for each isolate. From left to right, ethidium-stained bands containing chromosomes that range in size from ~ 700 kb to 3 Mb are shown with bands of identical molecular weight connected by dark or dotted lines. The compression zone (*c.z.*) contains chromosomes of different molecular weight that could not be size separated under the conditions chosen. Both isolates $B_1$ and $J_1$ can each be separated into nine bands (numbered 1–9 in each panel). Since these bands stain nonstoichiometrically, they are likely to contain different numbers of chromosomes and the total number of chromosomes thus exceeds nine. It cannot yet be determined whether the more intensely staining bands contain multiple homologous chromosomes, or different chromosomes belonging to the same size class.

To determine the extent of heterogeneity among *G. lamblia* isolates from within a single geographic region, we compared 13 isolates, one from each of the 13 individuals listed in Table I, in a series of PFGE gels presented in Fig. 2. In panels *a–d,* ethidium bromide–stained PFGE gels are shown in which the same series of isolates is run under different separation conditions. Optimal size separation of the smaller chromosomes is illustrated in panel *a;* as the pulse time is lengthened in panels *b* and *c,* the size of the optimally separated chromosomes increases. A comparison of the different ethidium-stained bands in the lanes clearly reveals that chromosome-sized DNA molecules of the different isolates are of drastically different sizes (compare for example isolates $F_1$ through $L_1$ in panel *b*). This heterogeneity comprises differences in (*a*) the number of bands, e.g., in panel *b,* isolate $J_1$ has seven bands whereas $K_2$ has three bands below the compression zone; (*b*) the size distribution of bands, e.g., in panel *b,* isolate $L_1$ has all three bands > 1.5 Mb compared to $K_2$ which has all three bands ≤ 1.5 Mb; (*c*) the relative intensity of bands, e.g., for isolates $J_1$ and $K_2$ which in panel *b* both have a pair of bands sized just > 1.1 Mb, the larger (i.e., slower migrating) band (labeled with an asterisk) is more intensely staining in $J_1$, whereas in $K_2$ the converse is true.

The importance of using a range of electrophoresis conditions is illustrated by comparing isolates $D_1$ and $M_1$ which appear similar at the 650-s pulse time (panel *a*) but are clearly differentiated at the 950-s pulse time (panel *c*) by the presence of an ~ 3-Mb band (labeled with an asterisk) in $M_1$ but not $D_1$ (note arrows indicating lower bands of isolates $D_1$ and $M_1$).

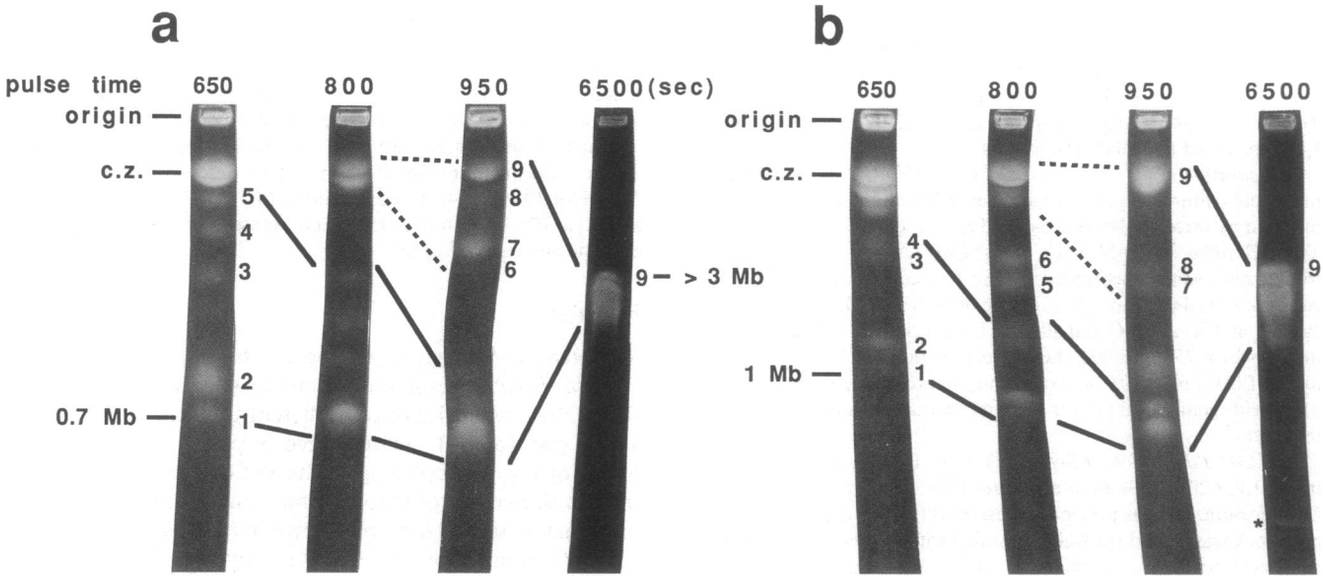


*Figure 1.* PFGE analysis of *G. lamblia* karyotypes using different electrophoresis conditions. (*a*) Composite of individual lanes from four separate ethidium bromide–stained PFGE gels, each containing chromosome-sized DNA from isolate $B_1$ (see Table I) separated at (from left to right) pulse times of 650 s, 800 s and 950 s, all at 7.5 V/cm for 6 d, and 6,500 s at 5 V/cm for 6 d. (*b*) Same as *a*, for isolate $J_1$.c.z. indicates the compression zone. Corresponding regions which are compressed or expanded between adjacent lanes as pulse time increases are indicated by pairs of solid or dashed lines, respectively. Discrete identifiable bands are numbered on their right side. The identity of bands in adjacent lanes was established by analysis of hybridizations with the probes listed in Methods (data not shown). *Potential ds RNA virus.

Conversely, $G_1$ and $I_4$ closely resemble each other in panel *c*, but are obviously different in panel *a*.

In Fig. 2 *d*, we have separated the largest chromosome-sized DNA molecules to determine the upper size limit of the chromosomes. From this comparison, it is clear that chromosome-sized DNA molecules larger than ~ 3 Mb are rare or absent from each of the *G. lamblia* isolates. Also, the overall size-distribution of chromosomes is confined to between 700 kb and ~ 3 Mb, independent of the extensive size heterogeneity that can be observed among the chromosomes confined to this size class. Finally, a potential double-strand RNA virus (9) was seen in only one of the isolates (band marked with an asterisk in panel *d*).

Results of hybridizations with telomere, rRNA, and tubulin probes (Fig. 2, *right side*) reinforce the karyotype heterogeneity. The banding pattern obtained with the *Giardia* telomere probe (panel $a_1$) is identical to the ethidium bromide staining pattern, indicating that the chromosomes in these bands contain telomeres and are therefore linear. Both the telomere (panel $a_1$) and rRNA (panel $a_2$) hybridizations demonstrate the extent of heterogeneity already seen in the ethidium bromide staining; for example, isolates $E_1$ and $F_1$ which appear almost identical in panel *a* apart from some faint, poorly visualized bands, are clearly differentiated in panels $a_1$ and $a_2$. These findings are consistent with our previous observations revealing a high rearrangement rate (estimated to be 3% per division) of rRNA encoding chromosomes in *G. lamblia* (25). Finally, the hybridization to chromosomes encoding tubulin genes reveals that their location is relatively conserved but some heterogeneity is still apparent at the higher pulse times (compare panels $a_3$ and $c_1$).

*Analysis of cloned G. lamblia isolates.* In view of the extreme diversity demonstrated amongst our *G. lamblia* isolates and the relatively large number of bands in individual karyotypes, we needed to exclude the possibility that the karyotypes of our uncloned isolates represented composites of simpler karyotypes resulting from a mixed population. We therefore prepared clones by limiting dilution from two of the isolates ($A_2$ and $J_2$). These were chosen because they contained a larger number of bands in their karyotypes than most other isolates. Of the three clones of isolate $A_2$ illustrated in Fig. 3, only the center one lacks a single ~ 3-Mb band but is otherwise identical to the uncloned parental $A_2$ population, as are the two other clones. The clone and first two subclones of isolate $J_2$ shown in the right side of Fig. 3 as well as 10 other subclones not shown in this gel are all identical to the uncloned parent $J_2$ population; the third subclone, however, lacks one of the pair of bands just below the compression zone and has an additional, fast migrating band (marked with an asterisk).

The limited karyotype heterogeneity, affecting only one band out of an otherwise identical separation pattern, indicates that the samples that we have isolated and propagated in the laboratory do not consist of mixed *G. lamblia* populations. Furthermore, each karyotype shown in Fig. 2 shows no evidence of being composed of a mixture of basic karyotypes that would suggest the presence of mixed populations. We therefore conclude that each *Giardia* isolate presented in Fig. 2 is a single population and that each isolate contains chromosomes of varying size. Hybridization of these clones and subclones with an rRNA probe (Fig. 3, *bottom panel*) shows that one of the new chromosomes in a subclone of isolate $J_2$ encodes rRNA genes. Since rRNA genes have been shown to be located on hypervariable chromosomes (25), this minor karyotype heterogeneity is likely to result from a new mutation that occurred while propagating *Giardia* in the laboratory.

*Karyotype comparison of G. lamblia isolated from cysts and trophozoites.* In vitro cultures of *G. lamblia* trophozoites may be established either by direct cultivation of trophozoites from

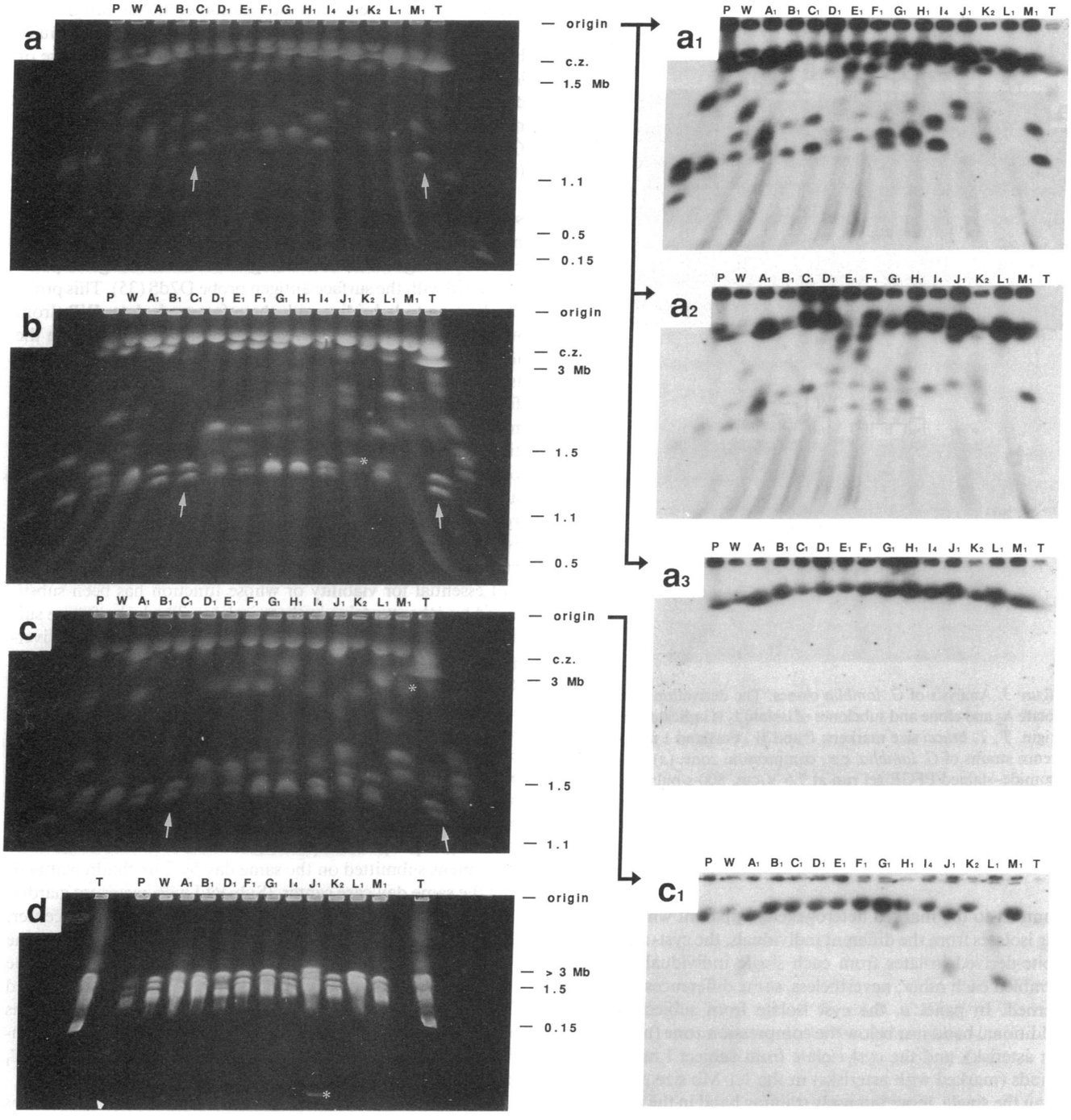


*Figure 2.* Comparison of *G. lamblia* karyotypes by PFGE electrophoresis. The identity of each isolate is indicated above the origin of its lane. *P,* reference strain Portland 1; *W,* reference strain WB; $A_1$–$M_1$, one isolate from each of the 13 study subjects (see Table I). *Trypanosoma brucei* chromosomes (lane *T*) were used as size markers (31). c.z. is the compression zone. Electrophoresis conditions used were: 650-s pulse time, 7.5 V/cm, 5 d (*a*); 800 s, 7.5 V/cm, 6 d (*b*); 950 s, 7.5 V/cm, 6 d (*c*), and 6,500 s, 5 V/cm, 6 d (*d*). Specific bands referred to in the text are marked with an asterisk at their right end. The right side of the figure shows a Southern blot of the gel in panel *a* hybridized with each of the following probes: telomere ($a_1$), rRNA ($a_2$), tubulin ($a_3$), and a Southern blot of the gel in panel *c* hybridized with the tubulin probe ($c_1$). For derivation of the probes and hybridization conditions, see Methods.

duodenal aspirates, or by excystation of stool-derived cysts. Previously reported characterizations of *G. lamblia* have used isolates established by one or other of these approaches, but a direct comparison of isolates established from both life cycle stages in the same individual has not been described. We therefore wished to investigate whether the karyotype heterogeneity observed might be the result of differences obtained as a result of the trophozoite to cyst, or cyst to trophozoite differentiation. Five of the subjects in this study (A, B, I, J, and L; see Table I) had *G. lamblia* cultured both from trophozoites as well as from cysts excysted and subsequently propagated as trophozoites. A PFGE analysis of these paired isolates is presented in Fig. 4. In

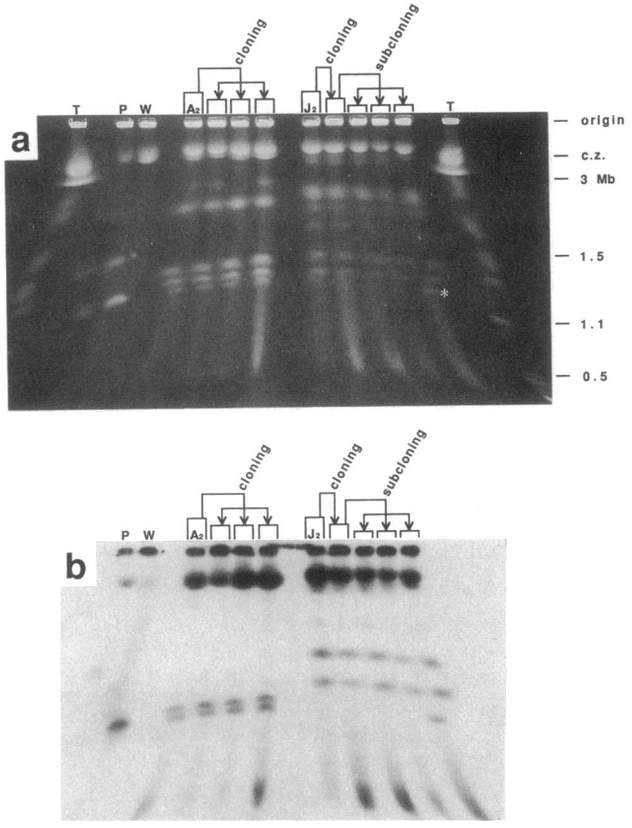


*Figure 3.* Analysis of *G. lamblia* clones. The derivation of clones of isolate $A_2$ and clone and subclones of isolate $J_2$ is indicated above the origin. *T*, *T. brucei* size markers; *P* and *W*, Portland 1 and WB reference strains of *G. lamblia; c.z.*, compression zone. (*a*) Ethidium bromide–stained PFGE gel run at 7.5 V/cm, 800-s pulse for 6 d. (*b*) Autoradiogram of Southern blot of the gel in panel *a*, hybridized with the rRNA probe. A specific band referred to in the text is marked with an asterisk at its right end.

contrast to the marked heterogeneity apparent when comparing isolates from the different individuals, the cyst- and trophozoite-derived isolates from each single individual closely resembled each other; nevertheless, some differences can be discerned. In panel *a*, the cyst isolate from subject B has an additional band just below the compression zone (marked with an asterisk), and the cyst isolate from subject I has two faint bands (marked with asterisks) in the 1.1-Mb size range rather than the single, more intensely staining band in the trophozoite isolate. The importance of using a range of PFGE conditions is again emphasized by the isolates from subject J which appear identical in panel *b* (950-s pulse) but exhibit several size polymorphisms in panel *a* (650-s pulse). The isolates from subject L are indistinguishable in panel *a*, but in panel *b* the cyst isolate has an obvious, additional band between the 3 Mb and 1.5 Mb size ranges (marked with an asterisk). Despite these differences, however, the overall similarity within the cyst and trophozoite pairs allows them to be identified as being from the same individual.

Hybridization of the filter from panel b of Fig. 4 with a β-tubulin coding sequence probe (panel $b_1$) further confirmed the overall conservation of the location of this gene family among the different isolates. In isolates B, J, and L, some differences can be observed when the location of the tubulin genes is compared in cysts and trophozoites from the same individual. However, although the majority of their chromosomes are of identical size in these paired isolates, this heterogeneity indicates that changes in the size of these chromosomes may accumulate, for instance during differentiation or propagation of *Giardia* in the laboratory, depending on the starting material (cysts or trophozoites) chosen.

Finally, we also determined the chromosomal location of several of the surface antigen genes which have been shown to be differentially expressed among different *Giardia* isolates. Panel $b_2$ in Fig. 4 is an autoradiogram of the PFGE gel in panel b, probed with the surface antigen probe D2dS (35). This probe can be seen to hybridize with the reference isolates WB (from which the probe is derived) and Portland 1 and also with the isolates from study subjects I and B; however, no hybridization is apparent with either the cyst- or trophozoite-derived isolates from subjects A, J, and L. (Successful transfer of DNA to the nylon filter in these lanes is confirmed by the positive hybridization with the tubulin probe in panel $b_1$.) The isolates from the other eight study subjects which are not included in Fig. 4 were all recognized by the D2dS probe (data not shown). Identical results were obtained with a probe for the surface antigen gene isolated by Adam and co-workers (36) revealing that these two surface antigen gene families encode proteins that are either not essential for viability or whose function has been substituted by other proteins in these isolates. The lack of these surface antigen genes also indicates the degree of genetic divergence between some of the Israeli isolates and the WB strain, and is consistent with previous findings (10).

*Karyotype conservation among individuals that may be cross-infected.* Having demonstrated significant karyotype heterogeneity amongst unrelated *G. lamblia* isolates from within a single geographic region, we were interested to determine whether there may be homogeneity amongst a subgroup of isolates from individuals in close contact with each other. Isolates $C_1$, $D_1$, $E_1$, and $F_1$ in Fig. 5 were obtained from stool specimens submitted on the same day by four children attending the same day-care center. (Subject I with recurrent giardiasis, see below and Fig. 6, also attended this day-care center; however, isolate $I_4$ was obtained two months earlier). The karyotypes of isolates $C_1$ and $D_1$ are almost indistinguishable (although not completely identical), in contrast to the marked hetergeneity apparent when comparing "unrelated" isolates as seen in Fig. 2. Similarly, isolates $E_1$ and $F_1$ very closely resemble each other; however, they are again quite different from $C_1$ and $D_1$.

Patient G had recurrent symptomatic *G. lamblia* infections documented over more than a year, despite repeated courses of appropriate therapy. Her sister (subject H) was investigated during a search for possible sources of repeated infection and was found to have asymptomatic giardiasis. Isolates $G_1$ and $H_1$ compared in Fig. 5 were obtained nine months apart. Again, although not identical, the degree of similarity between these isolates is striking in the context of the variability observed in the other unrelated isolates. We conclude that isolates from individuals living in close contact are more similar than those of totally unrelated individuals. This may be due to cross-infection.

*Chronic or recurrent giardiasis.* A minority of patients with giardiasis suffer from recurrent, symptomatic infection; it is not known whether these recurrent infections are caused by

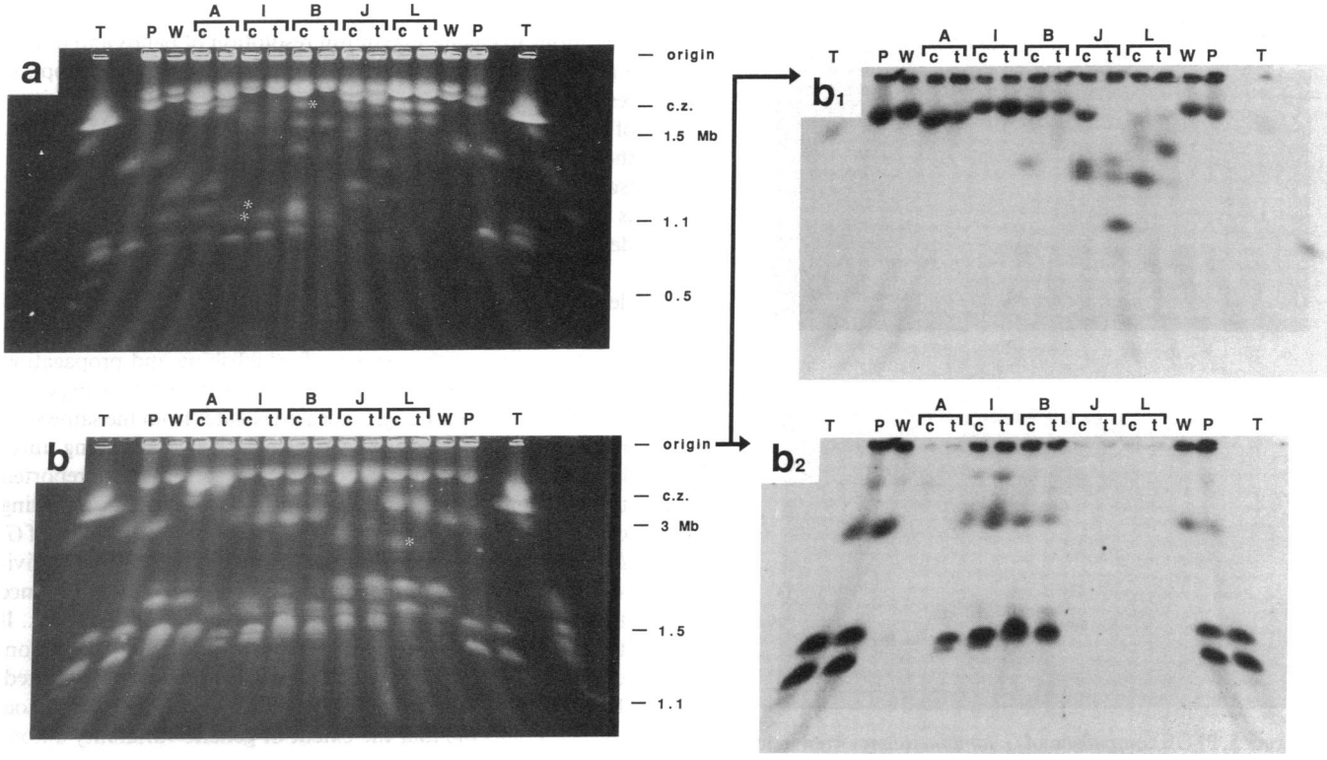


*Figure 4.* PFGE comparison of *G. lamblia* isolates established simultaneously from cysts and trophozoites from the same individuals. For each of the subjects A, I, B, J, and L (see Table I), axenic cultures were established both by excystation of stool-derived cysts as well as by direct cultivation from duodenal trophozoites. Cyst (*c*) and trophozoite (*t*) cultures are compared in pairs of adjacent lanes for each individual; (the cyst and trophozoite-derived isolates for subject I are $I_3$ and $I_2$, respectively, in Table I). *T*, *T. brucei* size markers; *P* and *W*, Portland 1 and WB reference strains of *G. lamblia; c.z.,* compression zone. (*a*) ethidium bromide–stained PFGE gel run at 7.5 V/cm, 650-s pulse time for 6 d; (*b*) 7.5 V/cm, 900-s pulse time for 6 d. Specific bands referred to in the text are marked with an asterisk at their right end. A Southern blot of the gel in panel *b* was hybridized with the β-tubulin probe ($b_1$) and the surface antigen (D2dS) probe ($b_2$).

genetically similar or dissimilar organisms. The application of PFGE to the investigation of this question is illustrated in two such cases analyzed in Fig. 6. Six positive stool samples were taken from Subject I during the first two years of life despite repeated courses of appropriate therapy. Subject I was otherwise healthy. Extensive evaluation failed to demonstrate any defect of humoral or cell-mediated immune function. Four *G. lamblia* isolates ($I_{1-4}$) were obtained over a 13-mo period. Drug resistance was excluded by determining in vitro susceptibility of the isolates to metronidazole by a method similar to that of Gordts et al. (14, data not shown). Comparison of the PFGE karyotypes of $I_{1-4}$ reveals that they are similar overall. Subject K had recurrent symptomatic giardiasis two months after treatment for a previous episode. Again, the isolates from these two episodes ($K_1$ and $K_2$) are similar. Minor karyotype heterogeneity was also seen in two different isolates obtained on the same day from subject M: these differences are likely to be due to changes that accumulate during differentiation or propagation of *Giardia* in the laboratory (see above). We conclude that recurrent giardiasis in these patients involved related isolates.

## Discussion

The degree of karyotype heterogeneity described in this group of *G. lamblia* isolates from a single geographic region is in stark contrast to previously reported comparative studies. Campbell et al. compared the electrophoretic karyotypes of 12 North American isolates using orthagonal field alternating gel electrophoresis and transverse alternating field electrophoresis and reported that all isolates were strikingly similar to each other (21). Adam et al. in a PFGE analysis of five human *G. lamblia* isolates and one cat isolate, described some size polymorphisms predominantly among minor (lightly staining) bands; the major bands were similar in most of the isolates (20). Based on a karyotype analysis of 54 *Giardia* stocks from human as well as animal hosts using field inversion gel electrophoresis, Upcroft et al. concluded that all *Giardia* stocks from North American hosts were of basically one karyotype, while those from Australia belonged to a second karyotype (23). Only minor, or no differences at all were apparent when comparing stocks from within either group (23). In a comparison of human and animal isolates from a single geographic region (southeastern Alberta), Uji et al. reported that all isolates were remarkably similar (37).

In attempting to explain these contrasting findings, it could be argued that the genetic diversity of *G. lamblia* observed in Israel but not elsewhere results from the high rate of tourism and immigration into the country from throughout the world, in addition to the extensive international travel undertaken by Israeli nationals; however, similar influences operate in North American and Australian populations, even if to a lesser degree. Alternatively, technical factors related to the methodological approaches used by different investigators may be responsi-

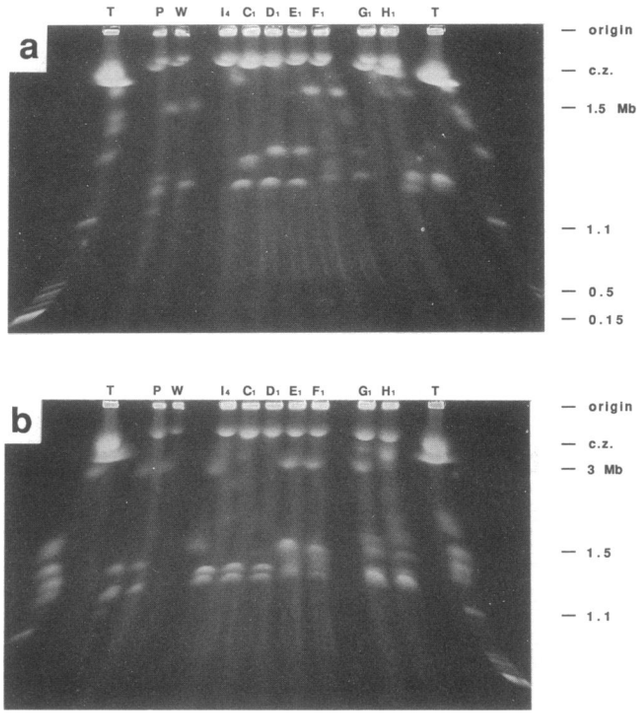


*Figure 5.* PFGE comparison of *G. lamblia* isolates from individuals in close contact with each other. Subjects I, C, D, E, and F attended the same day-care center. Subjects G and H are sisters. *T*, *T. brucei* size markers; *P* and *W*, Portland 1 and WB reference strains of *G. lamblia; c.z.*, compression zone. Electrophoresis conditions: 7.5 V/cm, 6-d run, 650-s pulse (*a*), 950-s pulse (*b*).

ble. Although newer techniques such as field inversion gel electrophoresis, orthagonal field alternating gel electrophoresis, and transverse alternating field electrophoresis used in other reported studies may have the advantage of producing straight lanes in gels, the extensive experience with the PFGE technique

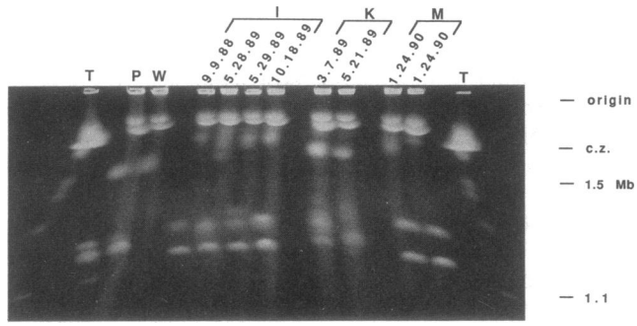


*Figure 6.* PFGE comparison of repeated isolations of *G. lamblia* from the same individuals. The dates (mo · d · yr) on which repeated specimens were obtained from each individual are listed above the origin. Subjects I and K had recurrent giardiasis after therapy. Subject M submitted two separate specimens on the same day. The two M isolates differ in that $M_1$ (*lefthand lane*) contains an extra band, running just below the compression zone, that is absent in $M_2$ (*righthand lane*). *T*, *T. brucei* size markers; *P* and *W*, Portland 1 and WB reference strains of *G. lamblia; c.z.*, compression zone. PFGE conditions: 7.5 V/cm, 650 s, 6 d.

in our laboratory (30, 31, 38) enabled selection of appropriate conditions to ensure maximum resolution of bands and differentiation of isolates. The critical importance of the electrophoresis conditions chosen is illustrated by the PFGE comparison of the isolates in panel *d* of Fig. 1 (6,500-s pulse); had this been the only set of conditions used, few differences between the isolates would have been recognized. With such an approach, it is likely that an equivalent degree of genetic diversity could be demonstrated in isolates from other regions.

Several mechanisms could be postulated to account for the development of heterogeneity among *G. lamblia* isolates from a single geographic region. We believe that these findings are not an artifact of the process of establishing and propagating cultures in vitro. If this were the case, we would have expected the heterogeneity amongst repeated isolates from the same subject or related subjects to be as extensive as that among unrelated isolates, yet this was not the case. Recently, we reported that frequent rearrangement events involving rRNA-encoding chromosomes can be observed during culture and cloning of *G. lamblia,* suggesting an estimated mutation rate of 3% per division (25). Such rearrangements are indeed mutations since they involve changes in the primary sequence of the DNA. If there is an in vivo equivalent to this in vitro phenomenon, extensive heterogeneity as observed would indeed be expected. Karyotype heterogeneity is also common in other protozoa, though it appears that the extent of genetic variability among the different *G. lamblia* isolates is more extreme than that described for any other protozoan thus far. The biological significance of this variability is still obscure; we have speculated that it may play a role in antigenic variability of *Giardia* (25).

The technique of karyotype analysis described in this report may be of limited value for purposes of clarifying the confused taxonomy of *Giardia* by virtue of the very extent of heterogeneity which it reveals; clearly, not every isolate with a unique PFGE karyotype can be classified as a new strain. On the other hand, the capacity of karyotype analysis to differentiate between isolates from a single geographic region may render it invaluable as a "fingerprinting" tool in analyzing patterns of *G. lamblia* infection and transmission.

Some preliminary experience in using such approaches is presented in this report. Recurrent symptomatic giardiasis is a not uncommon clinical problem, as illustrated by subjects I, G, and K in this study. Analysis of repeated isolates from subjects I and K (Fig. 6) suggests that recurrent infection in these cases was caused by a genetically similar organism, rather than by any of the genetically diverse isolates demonstrated to exist in the same geographic area. This could indicate occult persistence and relapse of the original infection, or perhaps, as suggested by the findings for sisters G and H in Fig. 5, reinfection from a common source.

Giardiasis is endemic in many day-care centers in North America (39) and elsewhere (40); although commonly asymptomatic, it constitutes a significant public health concern. Some children can excrete *G. lamblia* in their stools for a year or longer (41); it is not clear whether this represents a single, persistent infection, or repeated reinfections with genetically heterogeneous organisms. Application of the karyotype analysis described herein will help to resolve this issue and also lead to improved understanding of the patterns of transmission of *G. lamblia* in different geographic settings.

## Acknowledgments

We would like to thank Ms. E. Hais for her invaluable technical assistance in establishing and propagating *G. lamblia* isolates. We thank Dr. C. C. Wang, Dr. P. Chakraborty, Dr. R. Adam, and Dr. F. Gillin for kindly providing us with cloned *Giardia* genes, and all colleagues from the laboratory for critical reading of the manuscript.

This work was supported by a National Institutes of Health grant A126497 in the International Collaborative Infectious Disease Research Program to L. H. T. Van der Ploeg, by a World Health Organization grant to S. H. Korman, and by a grant from the John D. and Catherine T. MacArthur Foundation to L. H. T. Van der Ploeg, who is a Burroughs Wellcome Scholar in Molecular Parasitology.

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