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MOLECULAR CHARACTERIZATION OF *CRYPTOSPORIDIUM* ISOLATES FROM HUMANS AND OTHER ANIMALS USING RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS

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Abstract. Genetic variation in 25 *Cryptosporidium* isolates was analyzed using the random amplified polymorphic DNA (RAPD) technique. Simple reproducible polymorphisms were generated (using five primers) from *Cryptosporidium* DNA that was free of contaminating bacterial DNA. The results generated by four of the five primers were statistically correlated ($P < 0.001$). The combined data from three primers were used to construct a phenogram using Jaccard's distance. Four groupings could be distinguished. Two *C. serpentis* isolates from snakes formed a distinct group of their own, whereas *C. parvum* isolates were divided into two main groups: one containing most human isolates and the other containing mostly domestic animals plus two remaining human isolates. Due to the sensitivity of the RAPD technique, isolates can now be analyzed genetically, directly from fecal samples without further biological amplification. This represents a significant advance on current techniques.

The sporozoan parasites of the genus *Cryptosporidium* have recently been recognized as significant pathogens of vertebrate hosts, including humans and many other mammals, birds, reptiles, and fish.[1] Most clinical infections have been associated with acute transient diarrhea (particularly in neonates), but persistent infections may develop causing severe chronic disease that may become life-threatening (particularly in reptiles and immunocompromised mammals). The parasite is considered to be the most commonly detected enteropathogen affecting patients with acquired immunodeficiency syndrome and there is currently no effective treatment available.[2–4]

Numerous species of *Cryptosporidium* have been recorded since it was first thought to be host specific,[1] but cross-transmission studies have shown that infections may be transmitted between different host species belonging to the same vertebrate class but not different classes.[1,5] At the present time, only six species are regarded as valid on the basis of differences in oocyst morphology, site of infection, and vertebrate class specificity: *C. muris* and *C. parvum,* which infect mammals; *C. meleagridis and C. baileyi* in birds; *C. serpentis* in reptiles; and *C. nasorum* in fish. Recently, a strong argument has been presented that *C. wrairi* from guinea pigs is a valid and distinct species.[6] The species *C. parvum* appears to be the most widely distributed and is the major cause of infections in humans and livestock.[1]

There is an urgent need to determine the extent and nature of genetic diversity within *Cryptosporidium* isolates infecting humans and other mammals. Appropriate molecular characterization procedures for doing so need to be applied so that sources of transmission can be identified and correlated with clinically important parameters such as severity of infection and drug sensitivity. A major research need is to determine levels of genetic and antigenic heterogeneity within *Cryptosporidium* and to examine its zoonotic potential and the clinical significance of parasite heterogeneity.[7]

While few differences have been found in the morphologic characteristics and developmental cycles of *C. parvum* isolates from mammalian hosts,[8] some evidence of isolate (or strain) variation has been observed for several other biological and molecular characters. *Cryptosporidium parvum* isolates from both calves and human patients have been found to differ in their virulence for susceptible calves.[9,10] Some variation has been detected in the protein profiles of different mammalian, avian, and reptilian isolates examined by polyacrylamide gel electrophoresis, two-dimensional gel electrophoresis, and oocyst surface iodination.[11–13] Antigenic differences have also been detected between different mammalian isolates in Western blot studies using host immune sera, rabbit antisera, and mouse monoclonal antibodies.[14–16] Restriction fragment length polymorphisms have been detected between human and calf isolates from different geographic locations,[17] and isoenzyme electrophoretic studies have demonstrated genetic diversity both within and between different *Cryptosporidium* spp.[18,19]

A major limitation of all these characterization techniques, however, is their requirement for large amounts of parasite material that are generally not available from clinical samples. Recourse is often made to passaging isolates through experimental animals that may inadvertently select for specific genotypes present in the original isolate. While parasites can be established and maintained in vitro,[20,21] routine amplification of sufficient organisms for characterization studies is not possible.

The advent of polymerase chain reaction (PCR)–based technologies has revolutionized the molecular characterization of isolates where material has been limiting.[22,23] We have applied random amplified polymorphic DNA (RAPD) analysis to the detection of genetic variation between isolates of *Cryptosporidium.* This technique uses primers of arbitrary sequence in the PCR.[24–26] Amplification using a particular RAPD primer can reveal polymorphisms in a broad range of species, and single base changes in the primer can generate completely different reproducible patterns of polymorphisms. No prior sequence information is required and since the technique is PCR-based, it is very sensitive and therefore useful when only small amounts of material are available. Due to the nonspecificity of this technique, however, (i.e.,

TABLE 1
Isolates of Cryptosporidium *used in this study*

Code	Host	Geographic origin	Source*
H1	Human	Perth, Western Australia	PMH
H2	Human	Narrogin, Western Australia	SHL
H3	Human	Nannup, Western Australia	PMH
H4	Human	Perth, Western Australia	PMH
H5	Human	Perth, Western Australia	PMH
H6	Human	Perth, Western Australia	PMH
H7	Human	Perth, Western Australia	PMH
H8	Human	Perth, Western Australia	SHL
H9	Human	Perth, Western Australia	SHL
H10	Human	Perth, Western Australia	PMH
H11	Human	Perth, Western Australia	SHL
H12	Human	Perth, Western Australia	SHL
H13	Human	Horsham, Victoria, Australia	CVL
H14	Human	Port Lincon, South Australia	CVL
L1	Lamb/deer	Edinburgh, Scotland	MAH
C1	Calf	Millicent, South Australia	CVL
C2	Calf	Lucindale, South Australia	CVL
C3	Calf	Meadows, South Australia	CVL
C4	Calf	Lucindale, South Australia	CVL
C5	Calf	Penola, South Australia	CVL
C6	Calf	Willunga, South Australia	CVL
C7	Calf	Penola, South Australia	CVL
C8	Calf	Penola, South Australia	CVL
S1	Snake	Tanunda, South Australia	CVL
S2	Snake	Tanunda, South Australia	CVL

* PMH = Princess Margaret Hospital, Perth, Western Australia; SHL = State Health Laboratories of Western Australia; CVL = Central Veterinary Laboratories, , South Australian Department of Agriculture, South Australia; MAH = Moredun Animal Health Ltd., Edinburgh, Scotland.

any contaminating DNA will also be amplified), analysis cannot be performed directly on fecal samples and it requires extensive purification of parasite material prior to analysis.

The RAPD analysis has been increasingly applied to parasite populations and has been used to differentiate isolates of *Trichinella spiralis,*[27] schistosomes,[28] *Echinococcus granulosus,*[29] and more recently, isolates of the coccidian parasite *Eimeria.*[30,31] This type of analysis has been compared with isoenzyme characterization studies for some seven parasitic protozoan species and RAPD markers have proven to be reliable and suitable for constructing species phylogenies and strain differentiation.[32,33] The application of the RAPD technique to *Cryptosporidium* is, therefore, particularly suitable because the parasite is available only in small quantities, it is refractory to routine amplification, and there is little sequence information available.

MATERIALS AND METHODS

Collection and purification of parasite material. A total of 25 *Cryptosporidium* isolates were obtained from different mammals and reptiles (Table 1). All isolates from human patients and calves originated from natural clinical infections diagnosed by local laboratories. A purified isolate, originally from a deer, that had been passaged in lambs was obtained from the Moredun Research Institute (Edinburgh, Scotland). The snake isolates were collected from a chronically infected taipan (*Oxyuranus scutellatus*) and a common death adder (*Acanthophis antarcticus*). Fecal samples from infected hosts were stored at 4°C in an equal volume of 2% potassium dichromate solution prior to oocyst harvest.

Oocysts were partially purified from fecal material by filtering through sterile gauze followed by phosphate-buffered saline (PBS)-ether sedimentation (ether was omitted for snake isolates due to disruption of oocysts). All isolates were further purified by Ficoll-density centrifugation[11] and washed three times in PBS. The bacterial pellets resulting from the Ficoll purification step were also collected and washed for use as contamination controls. Samples were incubated in 10% sodium hypochlorite on ice for 10 min and then washed again three times in PBS. The purity of each preparation was confirmed by microscopic analysis prior to DNA isolation.

TABLE 2
Primers tested for random amplified polymorphic DNA analysis of Cryptosporidium

Code	Size	Sequence (5' to 3')
RH3	25 mer	GCCTTGCACAGAGAGACGCCCGTGT
RH5	25 mer	GCGCGGAAGCGTCGGATTATTCACC
GIAR 1	24 mer	ATGTACGACCAGCTCAACGAGAAG
IR-1367	20 mer	CCTCCGTCAATGCCTTCAAG
R-2936	20 mer	CCGTATGGCTAATAGGTGGA*
R-2817	20 mer	GCTTGGTCTGCTCAATGTGG*
T7	19 mer	GTAATACGACTCACTATAG
USP	17 mer	GTAAAACGACGGCCAGT*
[GACA]^4	16 mer	GACAGACAGACAGACA*
[GAA]^5	15 mer	GAAGAAGAAGAAGAA*

* Primers sequences that gave reproducible profiles for *Cryptosporidium.*

Isolation of DNA. Oocysts were resuspended in 300 μl of lysis buffer (containing 50 mM EDTA, 10 mM Tris, 250 mM sucrose, and 8% Triton-X 100), subjected to three freeze-thaw cycles, and then incubated at 56°C for 3 hr with 50 μl of proteinase K (10 mg/ml). The DNA was isolated from the lysate using the Prep-A-Gene DNA purification kit (Bio-Rad, Richmond, VA). The DNA was similarly isolated from the bacterial pellets and included as contamination controls in all amplifications.

Primer selection and amplification conditions. A total of 10 primers were tested for reproducibility and reliability, five of which gave reproducible, clear profiles (Table 2). These were two *Giardia*-specific 20mers that were synthesized for other purposes (R-2936 and R-2817), two microsatellite primers chosen at random ([GAA]^5 and [GACA]^4), and the universal sequencing primer (USP). The PCRs were set up as previously described,[33] with some modifications. Briefly, 10–20 ng of DNA was amplified in 20 mM Tris-HCl (pH 7.6), 30 mM KCl, 4 mM $MgCl_2$ 200 μM of each deoxynucleotide triphosphate, 25 pmol of primer, 2 units of *Tth* Plus (Biotech International, Perth, Western Australia), and sterile distilled water. Reactions were performed on a thermal reactor (Hybaid, Teddington, United Kingdom). Cycles for the 17–25 mers were as follows: two cycles at 94°C for 5 min, 48°C for 5 min, and 72°C for 5 min, followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, and a final cycle at 72°C for 10 min. For the shorter microsatellite primers, stringency was decreased to one cycle at 94°C for 2 min, 35°C for 1 min, and 72°C for 2 min, followed by 35 cycles at 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min, and a final cycle at 72°C for 10 min. In all amplifications, a bacterial lysate was included as a control. Negative controls (no DNA) were also included in every reaction. Amplification products were subject to electropho-

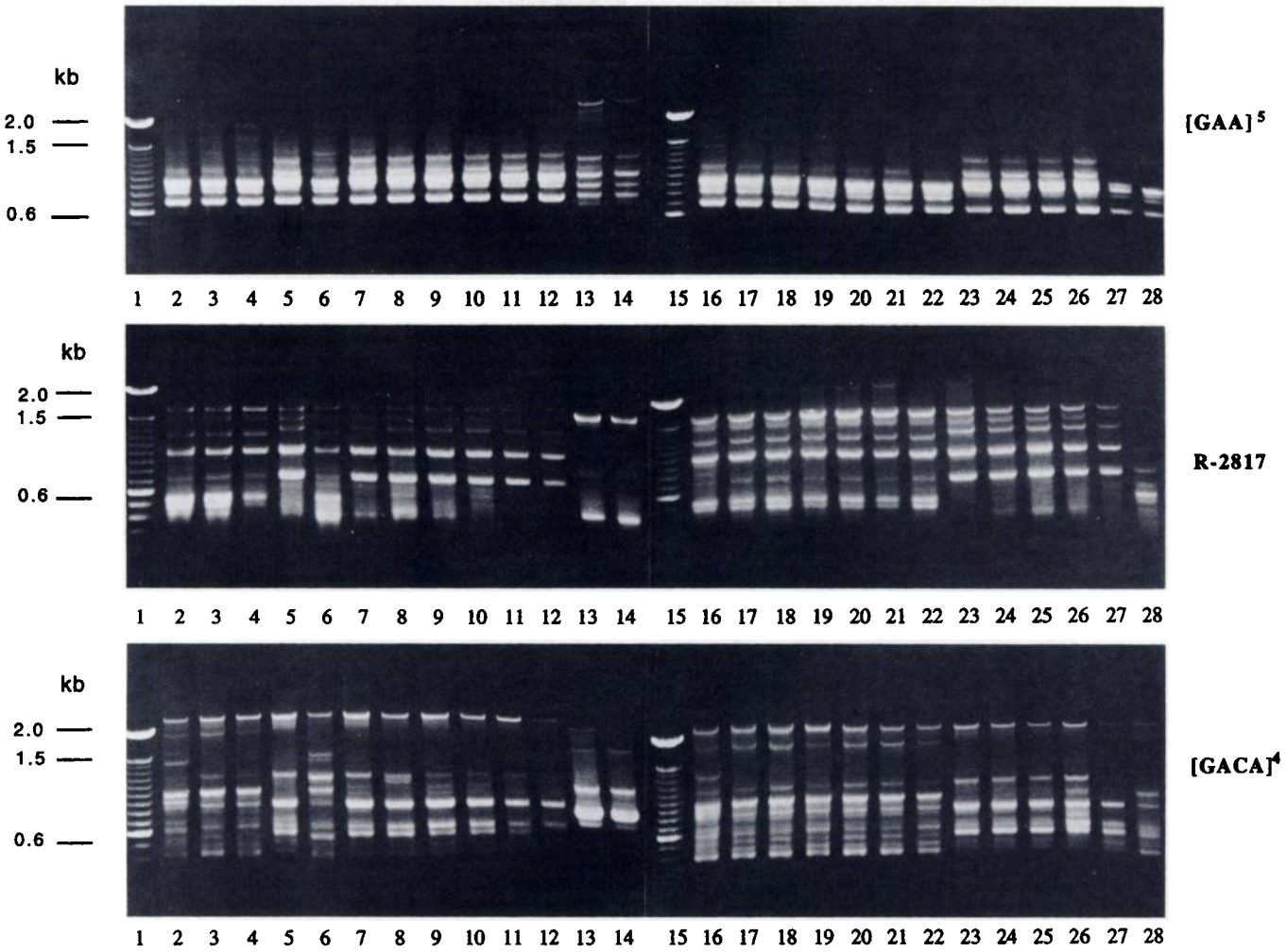

FIGURE 1. Agarose gels stained with ethidium bromide, showing random amplified polymorphic DNA profiles generated by three different primers ([GAA]5, R-2817, and [GACA]4, respectively,) of 25 isolates of *Cryptosporidium*. Lane 1 = molecular weight marker (100-basepair ladder; Gibco-BRL, Gaithersburg, MD); 2 = L1; 3 = C1; 4 = C2; 5 = H1; 6 = H2; 7 = H3; 8 = H4; 9 = H5; 10 = H6; 11 = H7; 12 = H8; 13 = S1; 14 = S2; 15 = molecular weight marker; 16 = L1; 17 = C3; 18 = C4; 19 = C5; 20 = C6; 21 = C7; 22 = C8; 23 = H9; 24 = H10; 25 = H11; 26 = H12; 27 = H13; 28= H14. kb = kilobases.

retic separation in 1.5% agarose gels stained with ethidium bromide.

Analysis of data. Individual bands were scored as present or absent for each isolate and the inverse of Jaccard's coefficient was calculated from the resulting data matrices.[33] Data matrices generated for each primer were compared statistically.[34] Phylogenetic relationships were determined by the group-average clustering strategy or the unweighted pair group method of analysis (UPGMA) using a modified Fortran computer program (UPGMA2).[35] Phenograms were constructed based on data from three primers (R-2817, [GAA]5, and [GACA]4).

RESULTS

The DNA profiles were generated using five primers, (USP, R-2936, R-2817, [GAA]5, and [GACA]4). Distance matrices between isolates for four of the primers (R-2817, R-2936, [GAA]5, and [GACA]4) were highly correlated ($P < 0.001$).[34] Profiles generated using the USP primer were not significantly correlated with any of the other four primers. Data from this primer were not included in the analysis because it was considered at variance with the other four primers; for example, USP was unable to differentiate between snake and human isolates. Data from R-2936 was also excluded from the analysis because a complete data set was not available for this primer due to a shortage of material. Profiles from three primers (R-2817, [GAA]5, and [GACA]4) are shown in Figure 1. Combined data from these three primers grouped the 25 isolates into four rapdemes (isolates grouped genetically using RAPD patterns),[33] respectively.

A phenogram based on Jaccard's distance was constructed from the RAPD data to indicate phylogenetic relationships (Figure 2). Four major groups were evident. The reptilian isolates were grouped separately from the mammalian isolates. Isolates from domestic animals were found to belong to one group and humans to another with the exception of two human isolates. One human isolate was grouped within the domestic animal isolates while the other formed a dis-

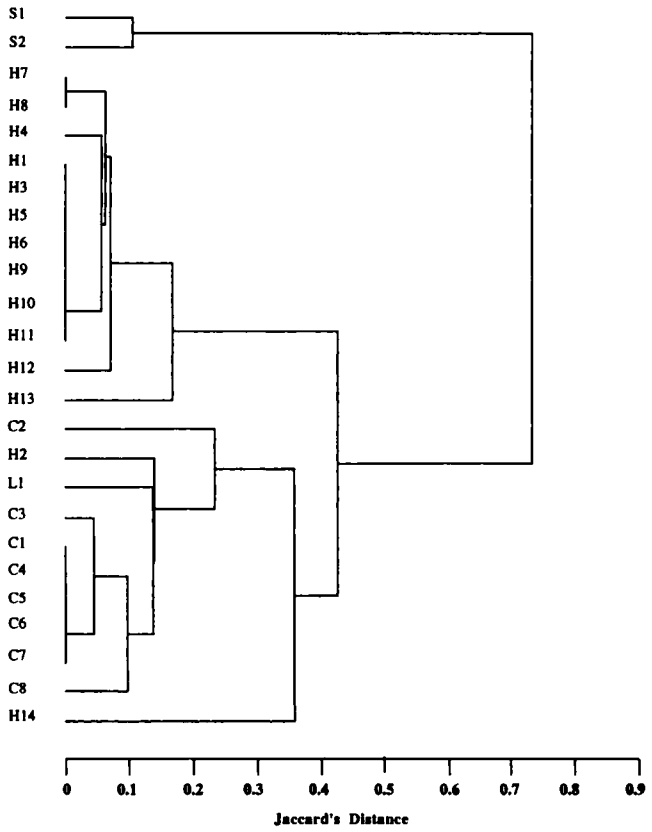

FIGURE 2. Phenogram of Jaccard's distance among rapdemes of *Cryptosporidium*, clustered by the group average (unweighted pair group method of analysis) strategy.

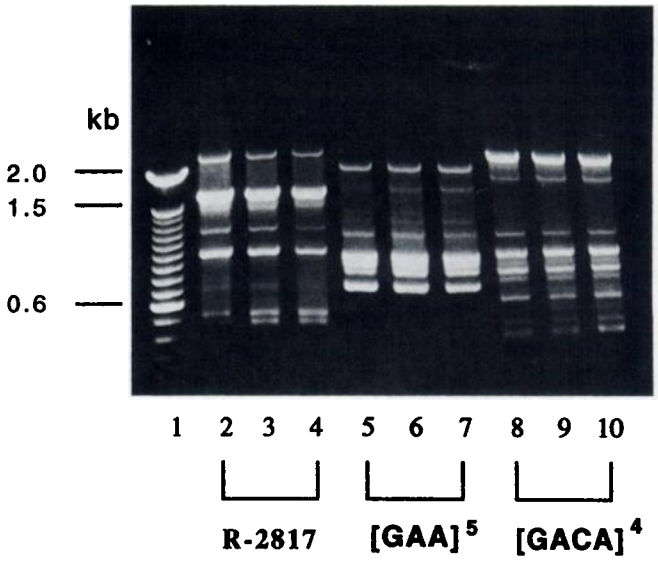

FIGURE 3. Agarose gel stained with ethidium bromide, showing random amplified polymorphic DNA profiles generated by three different primers (see Figure 1) on individual passages of a calf isolate (C1) in mice. Lane 1 = molecular weight marker (same as in Figure 1); lanes 2, 5, and 8 = CI, original isolate; lanes 3, 6, and 9 = C1, first passage; lanes 4, 7 and 10 = C1, second passage. kb = kilobases.

tinct group closer to the domestic group than to the human group.

DISCUSSION

The RAPD analysis revealed at least four distinct genetic groups among the 25 *Cryptosporidium* isolates from mammals and snakes. The snake isolates grouped together but differed from the mammalian isolates, which supports their classification as a separate species based on host specificity, site of infection, parasite pathogenicity, and morphology. The *C. parvum* isolates from domestic animals were grouped together, with minor differences between the lamb and calf isolates. Human *C. parvum* isolates were found to be remarkably similar to each other with the exception of three isolates. One isolate (H2) displayed a profile very similar to calf isolates with most primers. The isolate was from a child living in a rural area of Western Australia. Another isolate (H13) from an 18-year-old woman from Victoria, Australia showed some differences from the other human isolates, but was similar enough to be grouped overall with humans. The third human isolate (H14), which was from a six-month-old boy from South Australia, while sharing some similarities with the other human isolates, was genetically distinct enough to be grouped separately.

Previous studies have shown that RAPD profiles correlate very well with isoenzyme analysis, at least for clones of parasitic protozoa.[32,33] Isoenzyme characterization studies have previously found four distinct genetic groups among mammalian isolates; two groups confined to human patients, one to calves and one to goats.[18] Another study also reported differences between a single human isolate and several domestic animal isolates.[19]

The genetic differences found between human and domestic isolates using RAPD and isoenzyme analysis raises serious questions about their taxonomic status and whether they should all be classified as the single species *C. parvum*. In addition, these results suggest that isolates from domestic animals may vary in their zoonotic potential. However, little can be presently inferred about the epidemiologic, zoonotic, or clinical significance of parasite genetic variation. Most *C. parvum* isolates originated from diverse geographic locations, including one isolate from overseas, and all were associated with acute or chronic clinical disease in their respective hosts. Further characterization studies are now being performed on a broader range of subclinical and environmental isolates from the same and different geographic locations with a view to finding genetic markers for parasite pathogenicity and zoonotic potential, particularly since oocysts are now frequently being detected in both treated and untreated water supplies.[36,37]

Reproducible RAPD profiles were generated using between 0.1 and 1 ng of DNA, which constitutes approximately 4×10^4 oocysts. Isoenzyme analysis required soluble extracts of at least 10^7–10^8 oocysts for each enzyme[19] and necessitated the in vivo amplification of the oocysts prior to commencement of the studies. Due to the sensitivity of the RAPD assay, genetic analysis can be performed directly on *Cryptosporidium* oocysts purified from fecal samples without any further biological amplification. This represents a significant advance on current techniques for analyzing genetic variation in this organism.

The RAPD analysis is not without problems. Due to the nonspecificity of the technique, great care must be taken to avoid contamination. This is particularly true when amplifying oocyst DNA isolated from fecal material. Standard protocols for avoiding contamination[38] are strictly observed along with other protocols. A bacterial lysate was included as a negative control in each reaction but in every case it did not share bands with the *Cryptosporidium* DNA. Amplification of DNA in the PCR is significantly affected by temperature and Mg^{++} concentration. Recently, it has been reported that significant qualitative differences in RAPD profiles were observed when comparing conditions that differed in primer and template concentrations.[39] These results, however, were obtained using short 10mer primers; we routinely use longer primers in our studies (15–24mers),[33] because we find them to be much more reliable, and therefore have not detected this lack of reproducibility due to varying template and primer concentrations in our research.

Reproducibility of RAPD profiles was determined by amplifying the DNA of the various isolates with each primer repeatedly. The intensity of some bands changed and occasionally faint bands disappeared or appeared but the basic profile remained unaltered. As to the stability of RAPD profiles over time, we are at present passaging different isolates repeatedly through mice and analyzing each passage to determine if the genetic profiles remain constant over time. Initial results from one isolate (C1) indicate that after two passages, the genetic profiles have remained unaltered (Figure 3), and further passages are ongoing.

The need to purify oocysts from fecal material is a drawback of the RAPD technique because extensive purification is required to obtain preparations free of bacterial contamination. A method of analyzing genetic variation directly on fecal samples without the need for such extensive purification would therefore be very useful. In this respect, a series of RAPD fragments common to most or all isolates will be subcloned and sequenced and specific primers constructed. The RFLP analysis can then be conducted on the amplified products.

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