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A Simple Method for Cloning *Giardia duodenalis* from Cultures and Fecal Samples

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ABSTRACT: Using a novel method for cloning *Giardia duodenalis* from cultures and fecal samples, 47 clones from 7 isolates were established in vitro. Average colony-forming efficiency in established cultures was 43.2% compared to 11.2% when cloning directly from excystation. The highest success rate of cloning was found with the Portland (P1, ATCC No. 30888) isolate, with a colony-forming efficiency of 92.7%. Cloned and parent populations were compared over a range of 13 enzymes using starch gel electrophoresis. No genetic difference was found between any of the clones and the parent isolates.

Giardia duodenalis is the most commonly reported intestinal protozoan pathogen in Australia. Isolates of *G. duodenalis*, derived from fecal samples of infected hosts and maintained in axenic culture, have been shown to differ in virulence, infectivity, antigenicity, and susceptibility to drugs (Boreham et al., 1984; Gordts et al., 1985; Gasser et al., 1987; Aggarwal and Nash, 1988; Nash et al., 1988; Capon et al., 1989). Genetic comparisons, using isoenzyme electrophoresis and DNA hybridization, also have shown differences among isolates, but the results are complicated by the possibility of genetic heterogeneity within an isolate (Bertram et al., 1983; Nash et al., 1985; Baveja et al., 1986; Meloni et al., 1988). Genetic homogeneity can be assured only by cloning from a single organism, which is essential for meaningful comparative studies on different isolates. This has been emphasized for *Plasmodium* (Thaithong et al., 1988) and *Trypanosoma* (Goldberg and Pereira, 1983; Tanuri et al., 1985) as well as *Giardia* (Boreham et al., 1987). Furthermore, the availability of a sim-

TABLE I. Origin, observed growth, number of vials inoculated, number of positive vials during the experiment, number of clones established, and colony-forming efficiencies for all isolates.

Isolate	Nature of sample*	Observed growth	Number of vials inoculated	Number of positive vials†	Final number of clones established†	CFE (%)‡
BAH7	T	Slow	10	5	3	50
BAH12	T	Very slow	43	26	14	60.5
BAH15	T	Slow	17	2	1	11.8
BAH24	T	Fast	24	4	0	16.7
BAH31	T	Very fast	4	2	0	50
BAH46	C	Very fast	15	2	2	13.3
BAH50	C	Very fast	20	2	1	10
BAH52	C	Fast	9	0	0	0
BAH53	C	Fast	10	0	0	0
BAH55	C	Slow	10	2	0	20
CH-D1	T	Fast	10	1	0	10
CH-S1	T	Fast	26	14	13	53.9
P1	T	Very fast	14	13	13	92.7
CAR/A§	C	—	29	7	0	24.1

* T, trophozoites cloned from established cultures; C, cysts cloned from cysts and excysting trophozoites collected from fecal samples.

† Where there is a discrepancy between the number of positive vials and the number of clones established, clones were lost due to contamination or sudden unexplained death of the trophozoites.

‡ CFE, colony-forming efficiency. Determined using number of positive vials/total number of vials inoculated × 100; CFEs are expressed as percentages.

§ Sample not established in vitro.

ple yet reliable and efficient cloning technique is important for the analysis of naturally occurring strain differences as a single isolate may contain only the faster growing subpopulation from an initially heterogeneous mixture, and this will lead to an underestimation of naturally occurring heterogeneity within hosts (Thompson, 1988). According to Finley and Dvorak (1987), the major problem with any in vitro work is the unintended laboratory-induced selection when isolating and culturing parasites. Analysis of the population dynamics of heterogeneous cloned mixtures of *Trypanosoma cruzi* by these authors showed that the faster growing clone will always prevail in vitro.

Several methods for isolating single trophozoites of *Giardia* have been published. Gillin and Diamond (1980), Boreham et al. (1987), and Baum et al. (1988) all described methods based on limiting dilution, which requires a statistical consideration of the possibility of obtaining a true clone. In addition, the method of Baum et al. (1988) is complex technically and requires an anaerobic incubator to minimize oxygen tension for trophozoites in the microtiter plates. Colony-forming efficiency (CFE; a measure of the success rate of establishing cloned populations from a single organism) of these cloning methods varied from between 20 and 40% (Gillin and Diamond, 1980) to 72.1 ± 10.05% to 95.8 ± 6.63% (Baum et al., 1988).

The method reported here is simple, inexpensive, and reproducible. Its major advantage over previously described procedures is the fact that it allows cloning directly from excystation of fecal samples and thus the examination of the genetic composition of naturally occurring populations of *G. duodenalis*. The only limitation is that the procedure has to be carried out quickly and efficiently to minimize the exposure of trophozoites to oxygen.

Details of *Giardia* isolates used in this study are summarized in Table I. The isolates used during this study are, with the exception of the Swiss dog isolate, all of human origin, including the cyst samples. There is still some confusion concerning the use of species names in *Giardia*. As recently recommended (Meyer, 1990; Thompson et al., 1990), we recognize all the isolates and cyst samples used in this study as belonging to the species *G. duodenalis* as defined by Filice (1952). For routine isolation and excystation of cysts and the culture of trophozoites, the techniques of Meloni and Thompson (1987) were used, modified from Keister (1983), Rice and Schaefer (1981), and Roberts-Thompson et al. (1976). Cultures of trophozoites were grown to mid-to-late log phase and the culture medium (BS-I-33) was changed, replacing new born calf serum (NBCS) with fetal calf serum (FCS), 24–48 hr prior to cloning, as FCS was found to enhance division of trophozoites. To prevent tro-

phozoites from adhering to the walls of the tube, cell samples were kept on ice to maintain all cells in suspension. Fecal samples from natural human infections containing cysts of *G. duodenalis* were between 3 and 4 wk old on arrival at our laboratory. Samples were processed within 2 days and those showing high cyst concentrations (5,000–50,000 cysts/ml) and excystation rates between approximately 50 and 90% were chosen for cloning purposes.

Single microdrops (<1 μl) of the diluted cell suspension (approximately 6–8 trophozoites/μl) from cultures of trophozoites or excysted fecal samples were placed on 5 × 8-mm coverslips using micropipettes and examined under an Olympus CK inverted microscope. Microdrops containing only a single organism were placed immediately into 4-ml cloning vials with prewarmed medium by inserting the coverslip into the vial. These manipulations were performed using aseptic techniques. Four to 10 days after inoculation, if vials showed dividing trophozoites, half the medium was replaced with fresh medium. When sufficient numbers of trophozoites were present, 16-ml culture tubes were inoculated. Established cloned populations subsequently were cryopreserved (Lyman and Marchin, 1984; Phillips et al., 1984), and trophozoites were collected for isoenzyme analysis. For fecal samples, micropipettes obtained from Pasteur pipettes were used to isolate single excysting trophozoites that were placed immediately into 4-ml cloning vials.

Two controls were used. Firstly, in a separate experiment, 3 observers counted the number of trophozoites present in 25 microdrops of a cell suspension. All observers reported the same number of trophozoites per microdrop. Secondly, within each cloning experiment, 3 vials were inoculated with drops of the cell suspension believed to be devoid of trophozoites. None of these was found to contain cells.

A total of 23 cloning experiments was performed, using 15 isolates and cyst samples. The larger number of clones was obtained for BAH12 (14), Portland1 (13), and CH-S1 (13). The overall CFE varied from 0% (cyst samples of BAH52 and BAH53) to 92.7% for the Portland1 (P1) isolate. Even though no clones could be established from the cyst sample of BAH55 and trophozoites of BAH24, BAH31, and CH-D1, single trophozoites did initiate dividing populations that died during the 10-day observation period either through contamination or for unexplained reasons. Average CFE for all cloning experiments using trophozoites from established cultures was 92.7%, compared to 11.2% for cloning from excystation (for details on individual CFEs see Table I).

The genetic homogeneity of cloned populations was established using isoenzyme electrophoresis. This technique has been used previously to characterize all isolates available in our laboratory and a considerable amount of genetic heterogeneity between different isolates has been demonstrated (Meloni et al., 1988, 1989). Cloned populations derived from both established cultures, as well as cyst samples, were compared to their respective parent isolates, using the techniques described by Meloni et al. (1988) for the following 13 enzymes: acid phosphatase, esterase, fructose-1,6-diphosphatase, glutamate dehydrogenase, glutamate–oxaloacetate transaminase, glucose-6-phosphate dehydrogenase, glucose phosphate isomerase, hexokinase, malate dehydrogenase, malic enzyme, purine nucleoside phosphorylase, 6-phosphogluconate dehydrogenase, and phosphoglucomutase.

Isoenzyme electrophoresis revealed no difference between the cloned populations and their parent isolates when comparing zymograms. This included isolate BAH12, which exhibited multiple-banded patterns for 60% of the enzymes examined. Previous enzyme electrophoresis studies (Bertram et al., 1983; Baveja et al., 1986; Korman et al., 1986) have shown predominantly single-banded patterns for *G. duodenalis.* This suggests that *G. duodenalis* is an asexual haploid organism with variant enzymes being the result of occasional mutations (Meloni et al., 1988). Meloni et al. (1988) suggested that complex multiple-banded patterns observed in some isolates may be due to the presence of genetically heterogeneous subpopulations of haploid organisms. The present study has shown that this is not the case, at least for isolate BAH12.

This study is the first time that the genetic homogeneity usually assumed for cloned populations has been investigated and confirmed using isoenzyme electrophoresis in *Giardia.* Determining the genetic homogeneity of clones when they first are established is essential, and isoenzyme electrophoresis also can be used as a means to monitor changes taking place in cloned populations (Andrews et al., 1989).

Isolate P1 was obtained from Dr. P. F. L. Boreham (Queensland Institute of Medical Research) and originated from the American Type Culture

Collection (ATCC No. 30888). CH-D1 and CH-S1 of Swiss dog and sheep origin, respectively, were kindly supplied by Professor J. Eckert (Institut für Parasitologie der Universität Zürich). The National Health and Medical Research Council of Australia provided financial support through a project grant to R. C. A. Thompson.

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Altered Adult Worm Location in Young Male Jirds Infected with *Brugia pahangi*

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ABSTRACT: Male jirds (*Meriones unguiculatus*) were inoculated subcutaneously with 100 *Brugia pahangi* L_3 each at 2, 6, 10, and 15 wk of age to compare their susceptibility and pathologic reactivity to infection. Adult worm recoveries (mean ± SD) ranged from 24.1 ± 15.1 to 36.4 ± 13.9 at 60 days postinfection. No significant difference in susceptibility was measured among the 4 age groups. Jirds infected at 2 wk of age had significantly fewer ($\alpha \leq 0.025$) testicular and intralymphatic worms than all other age groups. Numbers of intralymphatic thrombi were significantly lower ($\alpha \leq 0.01$) in jirds infected at 2 wk of age. Lymphatic lesion severity, expressed as the number of intralymphatic thrombi per intralymphatic worm, was similar between age groups. These data indicate no differences in susceptibility or lymphatic lesion formation following *B. pahangi* infection in 2-wk-old male jirds, despite altered adult worm location.

Differences in age-related susceptibility have unknown effects on the severity of subsequent immunopathological reactions of the host to filarial parasites. Potentially variable immunological and/or pathological responses by the infected host partially could explain the broad spectrum of clinical manifestations observed in human lymphatic filariasis (Otteson, 1980, 1984). The purpose of this study was to use the jird–*Brugia pahangi* model to test age-related susceptibility to infection and subsequent intralymphatic lesion formation.

Groups of 10 male jirds were inoculated individually subcutaneously at 2, 6, 10, or 15 wk of age with 100 *B. pahangi* infective larvae (L_3) each as previously described (Klei et al., 1987). Preinfection levels of serum testosterone were measured by previously described radioimmunoassay procedures (Gay and Kerlan, 1978). Necropsies were performed 60 days after infection by previously described methods (Klei et al., 1982). Data collected at necropsy included adult worm burdens and location, microfilaremias determined by Knott's test (Knott, 1939) using 1.0 ml of blood drawn via the retroorbital plexus, and the enumeration of intralymphatic lesions within infected jirds. Statistical analyses of adult worm recoveries, numbers of intralymphatic thrombi, and testosterone levels were performed using the nonparametric Kruskal–Wallis test. Group mean ranks were compared using a nonparametric equivalent of the Student–Newman–Keuls test (Zar, 1984). Data are expressed as the mean ± SD. Differences between group means were considered significant at $\alpha \leq 0.05$. Percent patency of infections was compared by chi-squared analysis.

Adult *B. pahangi* recoveries and percent patent infections did not differ significantly among the 4 age groups of jirds at 60 days postinfection (Table I). Susceptibility to infection was 100% in all age groups of jirds. Jirds infected at 2 wk of age, however, displayed altered adult worm distribution compared to all other age groups (Table II). Significantly fewer worms were recovered from intralymphatic sites (including testes) in jirds infected at 2 wk of age compared to infections in older jirds.

Jirds infected when 2 wk old developed significantly fewer intralymphatic thrombi (9.0 ±