

Species Differentiation of *Giardia* by PCR

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Introduction

There are several species of *Giardia* but two of these, *G.intestinalis* and *G.muris*, are the most common. *G.intestinalis* infects humans and a range of animals, whereas *G.muris* is believed to infect mainly rodents and does not infect humans.

The standard method for detecting cysts in water; namely staining concentrated cysts using fluorescein-labelled monoclonal antibodies directed against a group-specific antigen, does not distinguish these species. Hence, if a water supply became contaminated with *G.muris*, even if *G.intestinalis* is not present, it would be wrongly assumed to be infectious for humans.

Molecular genetics, and in particular the development of the polymerase chain reaction (PCR), has provided a way to distinguish these organisms. One primer pair specific for the genus *Giardia* is available (Mahbubani *et al.* 1991) as is a primer pair specific for *G.intestinalis*, (Mahbubani *et al.* 1992). During experiments in this laboratory, designed to compare strains and species of *Giardia* using random amplification of polymorphic DNA (RAPD) (Williams *et al.* 1990 and Welsh *et al.* 1991), we detected a prominent amplified band with *G.muris* but not with *G.intestinalis* DNA. The base sequence of this band is reported here and using this information a primer pair was selected to amplify most of the sequence.

We report here that this primer pair is specific for *G.muris* and may be useful for identification of *Giardia* species from various sources including water supplies.

Materials and methods

Source of cysts.

G.muris cysts were obtained from three sources: the New Zealand isolate from a mouse at the Small Animal Production Unit (SAPU), Massey University, Palmerston North, New Zealand; the USA Isolate from Dr. W. Jakubowski, United States Environmental Protection Agency, Environmental Monitoring Systems, Cincinnati, OH 45268, USA; and the Roberts-Thomson (R/T) isolate from Dr. H. Stibbs, Waterborne Inc., 6047 Hurst St., New Orleans, LA 70118, USA.

G.intestinalis cysts (S1, from a sheep; VANC/85/UBC/7, from a human; and BE-1, from a Beaver) were obtained from Dr. J. Isaac-Renton, Medical Microbiology, Heather Pavilion, 2733 Heather Street, Vancouver, Bc., Canada V5Z 1M9. Inoculation doses containing 4×10^3 *Giardia* cysts were orally administered to five 15-day-old *Giardia* free mice, over a period of three days and approximately 10^6 to 10^7 cysts were recovered from the faeces of each mouse, 7 days following the first inoculation.

Collection of trophozoites from infected mice.

Giardia infected mice were sacrificed and their gut (from the stomach to the appendix) removed. The gut was sliced longitudinally and placed into a 50 ml FalconTM centrifuge tube containing ice cold phosphate buffered saline (0.15 M NaCl, 7.5 mM Na₂HPO₄.12H₂O, 2.5 mM

NaH₂PO₄·2H₂O, pH 7.0; PBS), shaken vigorously and stored on ice for 20 min allowing the trophozoites to detach from the gut. The supernatant containing the gut contents was decanted into a 50 ml Falcon™ tissue culture flask and topped up with TSY-1-S33 medium (Keister, 1983) pre-warmed at 37°C. The tissue culture flask was incubated at 37°C for 45 min to allow the trophozoites to adhere to the plastic surface. Unattached trophozoites and other gut microorganisms were decanted and the attached trophozoites were washed 6-times using pre-warmed sterile PBS. The trophozoites were detached by chilling the flask at 4°C for 20 min, collected by centrifugation at 500 g for 10 min and resuspended in 1 ml Tris EDTA buffer (0.01 M Tris-HCl, pH 7.5; 0.01 M EDTA, disodium salt, pH 7.2; TEB).

Nucleic acid release from the trophozoites for RAPD analysis.

1.0 ml of the trophozoite suspension in TEB was incubated at 37°C for 15 min and 0.1 ml of a 10% solution of sodium dodecyl sulphate and 0.1 ml of Pronase (10 mg ml⁻¹) was added. Incubation was continued overnight at 50°C. Sodium perchlorate was added to a final concentration of 1 M. The mixture was incubated for a further hour at 50°C and diluted to 5.0 ml with STE buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.5 and 1 mM EDTA); it was then extracted three times with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1). The upper layer was removed following each extraction. A 1/20 volume 5 M NaCl followed by 2 volumes of ethanol was added and the mixture stored at -20°C for 30 min to precipitate the DNA. The DNA was collected by centrifugation at 15 500 g for 20 min and the pellet washed with cold 70% ethanol and dried at 37°C. The nucleic acid was dissolved in 0.1 ml TEB and stored at 4°C.

Identification and purification of G.muris specific RAPID analysis product.

G.muris and *G.intestinalis* DNA were amplified by PCR using a single oligonucleotide RC09 (5'-GAT AAC GCA C-3'). PCR amplification was achieved using a DNA thermal cycler (9600 Perkin-Elmer Cetus Corp., Norwalk, Conn). PCR was performed in a 200 ul thin wall microtube (Perkin-Elmar) by adding 2 ul of a 10x concentration of PCR amplification buffer (10x buffer contained; 200 mM Tris-HCl [pH 8.4], 500 mM KCl), 0.6 ul MgCl₂ (50 mM), 4 ul deoxynucleoside triphosphates (1.25 uM each), 1 ul of each primer (0.02 ng), 2.0 ul template DNA (100 pg ul⁻¹), 0.5 ul Taq polymerase (5 U ul⁻¹) and sterile water to make a final volume of 20 ul.

The thermal cycler was programmed for 1 cycle of 2 min at 95°C, 30 s at 35°C and 30 s at 72°C; 30 cycles of 30 s at 94°C, 30 s at 35°C and 30 s at 72°C; and 1 cycle of 30 s at 94°C, 30 s at 35°C and 5 min at 72°C.

The amplified PCR products were electrophoresed through a 1.6% agarose gel in Tris Acetate EDTA buffer (0.04mM Tris-acetate and 0.001mM EDTA disodium salt, pH 8.0; TAE) and stained with ethidium bromide (0.5 ug ml⁻¹). A 367 base pair fragment specific for *G.muris* was excised from the gel and eluted (Heery *et al.* 1990). The fragment was precipitated from the eluent by the addition of 1/10th volume sodium acetate (3M) and 3 volumes of 70% ethanol, centrifuged at 15 500 g for 10 min, the supernatant removed and the pellet resuspended in TE buffer.

Purification and cloning of the 367bp G.muris- specific fragment.

The PCR product was purified from an agarose gel according to the method of Heery *et al.*, 1990 and cloned into the Sma I site of the vector pUC118. Transformants were selected by blue white colour selection and checked for the presence of inserts. One recombinant was selected and plasmid DNA was prepared according to the Alkaline Lysis method (Birboim and Doly, 1979).

PCR amplification using Genus and species-specific oligonucleotides.

PCR primer sequences used to amplify the genus *Giardia* and the species *G.intestinalis* are those reported by Mahbubani et al., 1991 and 1992 respectively. Primers GspL (5'-AAG TGC GTC AAC GAG CAG CT-3') and GspR (5'-TTA GTG CTT TGT GAC CAT CGA-3') amplify a 171-bp region of the *Giardia* giardin gene (bp 639-809, Baker *et al.* 1988). While primers GIL (5'-CAT AAG GAC GCC ATC GCG GCT CTC AGG AA-3') and GIR (5'-TTT GTG AGC GCT TCT GTC GTG GCA GCG CTA A-3') amplify a 218bp region of the giardin gene (bp 405-622, Baker *et al.* 1988) present only in *G. intestinalis*. Primers GMR (5'-CAT AAA TCA GTG CAG AGT GTT TC-3') and GML (5'-GAG GAA TCA TCA GAA CCT CGC-3'), amplifies a 306bp fragment present only in isolates of *G.muris*. The thermal cycler was programmed for the *Genus* and species-specific primers using the following parameters: 1 cycle of 2 min at 95°C, 30 s at 60°C and 30 s at 72°C; 30 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C; and 1 cycle of 30 s at 94°C, 30 s at 60°C and 5 min at 72°C.

Detection of amplified products.

PCR-amplified DNA was detected by agarose gel electrophoresis. The DNA was separated using 1.6% agarose gel run in TAE buffer (0.04 mM Tris-acetate, 0.001 mM EDTA, pH 8.0). The gels were run at 6 V cm⁻¹ for 3.5 - 4.0 h, stained in a solution of ethidium bromide (0.5 µg ml⁻¹) and visualised with a UV transilluminator. The gels were photographed through a OG509 glass filter (Glass Fab Inc., Rochester, NY) on T-MAX 12.5 x 10.0 cm format film (Eastman Kodak Co., Rochester, N.Y.).

Results

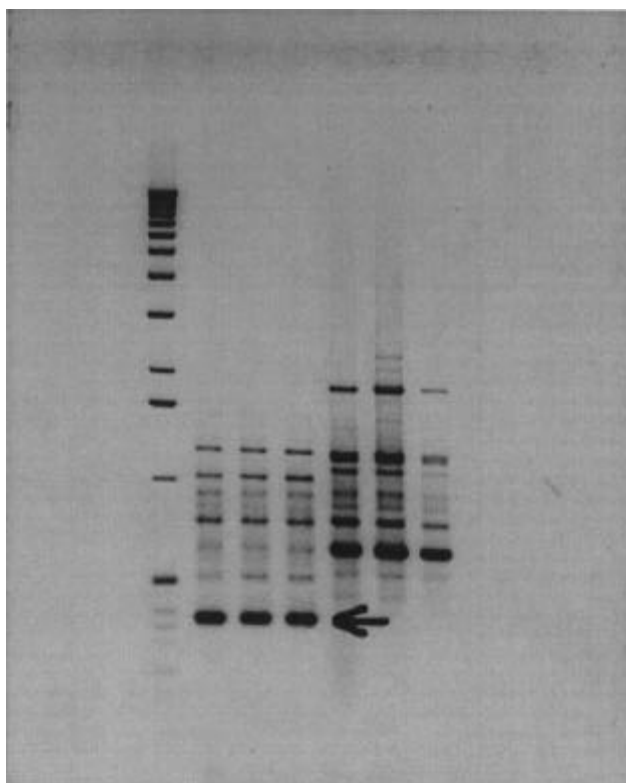


Figure 1: A comparison of PCR amplified DNA between three *G. muris* and three *G. intestinalis* isolates using a single arbitrary chosen primer RCO9 (5'-GAT AAC GCA C-3'). Lane 1 1kb molecular weight ladder; Lanes 2-4: *G. muris* (SAPU, USA and R/T isolates respectively); Lanes 5-7: *G. intestinalis* isolates (S1, VANC/85/UBC/7 and BE-1). Note: a common amplified product is present in all three *G. muris* isolates (arrowed) but absent in *G. intestinalis* isolates.

Comparison of *G.muris* and *G.intestinalis* isolates using RAPD.

DNA was extracted from trophozoites from three isolates of *G.intestinalis* isolates (S1, Vanc/85/UBC/7 and BE-1) and three isolates of *G.muris* (SAPU, USA and R/T). The isolates were compared using a variety of 10-mer primers, where a number of bands were detected. The 10-mer primer RCO9 (5'-GAT AAC GCA C-5'), produced a well defined band of 367bp with DNA from *G.muris* (fig.1) and this band was absent in DNA from *G.intestinalis* isolates. This fragment was excised from the gel and cloned into a plasmid vector (pUC118).

Sequencing of DNA amplified from *G.muris* and selection of a specific primer pair.

The 367bp band produced with the RCO9 primer was excised, cloned into pUC118 and sequenced using Sequenase Version 2.0 (UBS). The sequence is shown in fig. 2. From the sequence a primer pair consisting of 21-mer (GML) and a 23-mer (GMR) were selected to amplify a 306bp fragment. The primer pair sequences are indicated in fig. 2.

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                PRIMER RCO9                                PRIMER GML
1   GATCCCCTGA TACGCACTA AGGATTGCGG AGGTGTGTCA GAGGAATCAT

51  CAGAACCTCG CGCCCGTTTT CATGAAGTGT GGGATCTCCA TCTCAGAGAC

101 TGACGATCAT GTCAGTGAGG AAGTCAATGG GGAAGTCAGT GGGAAAGAAA

151 GGTTCCTATTC AGTGATTTCG GATAGTCCCT CGAAACTTCC TGGTAGTACC

201 TTGGAAAGCG TCGAGCAAGT TAGTGAGGTG GGAAGGACAA GGCTCATGGA
    Sac II SITE
251 CGCCGCGGCT GATGGAGACG TGGATAAGGT CTNTCCACGT GCGCTTCTCG
                PRIMER GMR
301 GAGTTTGGAC AGGTAGACAA TAAGAAACAC TCTGCACTGA TTTATCTTT
                PRIMER RCO9
351 AGAGCTGCTA GCCCTGGGTG CGTTATCAGG G

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Figure 2: Nucleotide sequence of a cloned fragment of *G.muris* DNA. The binding sites of the single arbitrary chosen primer RCO9 are shown underlined. The binding sites of the *G.muris*-specific primers GML and GMR are shown in bold and underlined.

Amplification of *G.muris* and *G.intestinalis* DNA using the selected primer pair.

DNA was extracted from three isolates of *G.intestinalis* and from three isolate of *G.muris* and was amplified using the GML and GMR primer pair. The result is shown in fig. 3. Note that a band consistent with the expected size (306bp) was amplified from *G.muris* DNA. However, no band was detected with *G.intestinalis* DNA.

Confirmation of the identity of the amplified DNA sequence.

Cysts from a New Zealand strain of *G.muris*, the Roberts-Thomson and the USA strain were used to extract DNA. The new primer pair GML and GMR was then used to amplify DNA from these strains. A single band of the expected size (306bp) was again detected. This band was excised from the electrophoresis gel and the sequence was determined by the cycle-sequencing technique.

The sequence was identical to that determined for the New Zealand, Roberts-Thomson and the USA strain USA strain of *G.muris* recorded (within the inner primer pairs) in fig. 2.

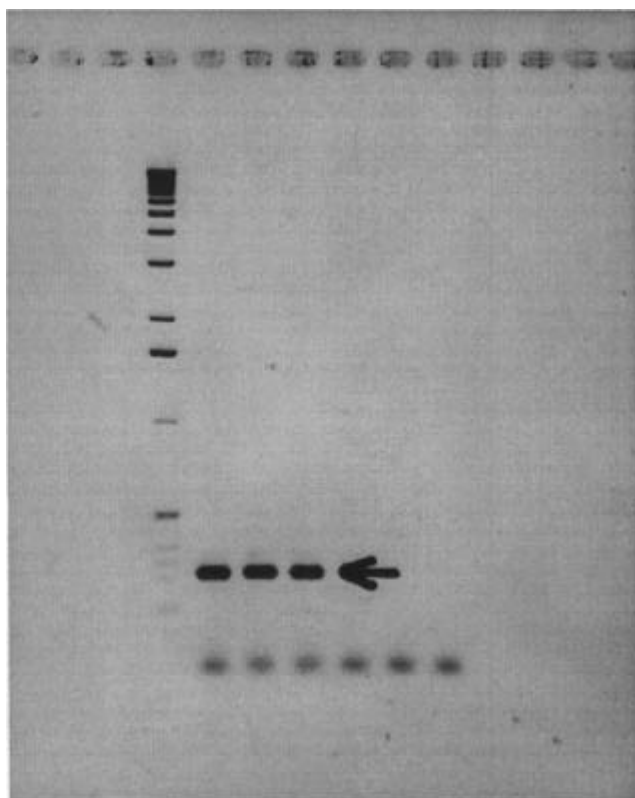


Figure 3: PCR amplification using the *G. muris*-specific primer pair GMR (5'-CAT AAT TCA GTG CAG AGT GTT TC-3') and GML (5'- GAG GAA TCA GAA CCT CGC-3'). An amplified product (306 bp) is observed only with DNA obtained from the three *G. muris* isolates (arrowed) and not from the three *G. intestinalis* isolates. Lane 1: 1 kb molecular weight ladder; Lanes 2-4: DNA from *G. muris* isolates (SAPU, USA and R/T, respectively); Lanes 5-7: *G. intestinalis* isolates (S1, VANC/85/UBC/7 and BE-1, respectively).

Discussion

Since not all species of the *Giardia* are pathogenic for humans it is desirable to have a technique available to distinguish cysts of pathogenic and non-pathogenic species which may contaminate water supplies and in particular to distinguish *G.muris* and *G.intestinalis*.

The morphology of *G.muris* and *G.intestinalis* cysts is not identical (Filice, 1952) but the differences, especially the differences in size, is small. The approximate length and width of *G.muris* cysts is 9-12 x 5-7 μ m and that of *G.intestinalis* is 12-15 x 6-8 μ m (Feely *et al.* 1984). Although this difference is adequate to distinguish between populations of the two organisms, some variations occurs in individual cysts, so it is not possible to unequivocally identify the species of individual cysts detected in water supplies by light microscopy alone.

Giardia cysts are routinely detected in water, following concentration by filtration and centrifugation and subsequent staining with fluorescein-labelled monoclonal antibody. This allows identification of the organism even if, as not frequently occurs, only one or a few cysts are seen. PCR can also be used to detect *Giardia* cysts in both water and in faeces and it has been recently shown that it is possible to amplify and detect DNA from a single cyst (Mahbubani *et al.* 1992).

A potential advantage of the use of PCR is that, with the design of appropriate primers, it is possible to identify the species of any *Giardia* cyst detected in water. The present study has developed a primer pair that is specific for *G.muris* and in particular did not amplify DNA from *G.intestinalis*. The primers are of sufficient length (21 and 23-mer) to make it improbable that a DNA from unrelated organism, e.g. bacteria, would be amplified.

Our experiments to develop a primer pair specific for *G.muris* were done using DNA derived from purified trophozoites. For the primers to be useful for routine purposes, it was necessary to show that they were specific for *G.muris*, even in the presence of other contaminating DNA (such as may be found in a faecal sample). This requirement was satisfied when a single band of the expected size (306bp) was amplified directly from mouse faeces (using the primer pair). The identity of this band (as *G.muris* DNA) was confirmed by sequencing.

It would be of interest to use the primers described here in conjunction with *G.intestinalis*/*G.muris*-specific primers and genus-specific primers to examine *Giardia* cysts in natural waters of New Zealand. Since this laboratory has a program to test such waters using standard methods, positive samples will be retested using PCR to compare the sensitivity of genetic techniques with the standard approach and to gain information concerning the relative prevalence of *G.intestinalis* and *G.muris* in New Zealand waters.

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