

Detection of giardine gene in local isolates of *Giardia duodenalis* by polymerase chain reaction (PCR)

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Abstract

Giardia duodenalis is an intestinal parasite that causes diarrhoea and malabsorption in children. The parasite also infects AIDS patients with a weak immune system. A study was carried out on six local isolates of *Giardia duodenalis* (110, 7304, 6304, M007, 2002 and 6307) from faeces of Orang Asli patients admitted to the Gombak Hospital. WB, a reference pathogenic strain from human and *G. muris* from a wild mouse, were commercially obtained from the American Type Culture Collection (ATCC). All the isolates were cultured axenically in TYI-S-33 medium. Two sets of primers were used for the techniques: primers LP1 and RP1 and primers LP2 and RP2. The sets of primers amplified giardine gene of 171 bp and 218 bp in sizes respectively. The study showed that the two sets of primers could detect *G. duodenalis* to the genus and species level specifically.

Key words: Giardiasis, *Giardia duodenalis*, giardine gene, polymerase chain reaction

INTRODUCTION

Giardia organisms are anaerobic, binucleate flagellate protozoa that infect the intestine of human and other vertebrate hosts.¹ *Giardia* trophozoites are mostly found at the mucosal surface but may also be found in the crypts in the upper part of the small intestine. Occasionally, they are found in the gall bladder and in the biliary tract. The adhesive disk at the anterior part of the ventral surface of the organism may produce a considerable degree of mechanical irritation to the tissues. Attachment of *Giardia* to the duodenal mucosa may also be facilitated by a lectin produced by the parasite and activated by duodenal secretion.²

It is not fully known why some infected persons are asymptomatic whereas others suffer from fulminant diarrhoea and malabsorption. Intrinsic differences between different strains of the parasite may be a factor and recent studies have shown major surface-antigenic differences between two isolates of *Giardia*.³ However, only a limited number of axenized isolates have been compared and there are no standard methods to determine differences between *Giardia* isolates. Major epidemiological questions also remain unresolved. Previously it was felt that there were

many species of *Giardia*, each associated with its own mammalian host.⁴

A survey conducted to establish the prevalence of giardiasis in Malaysia reported an overall prevalence of 15.1%.⁵ The highest prevalence was in the KESEDAR Schemes (19.6%), followed by the Orang Asli Settlements (18.1%). Adults were more frequently infected than children with *G. duodenalis*. Majority of the infected persons were asymptomatic (18.4%). The high prevalence was observed in the areas using river water for consumption. This factor may contribute to the transmission of infection in the KESEDAR scheme, Orang Asli settlements and urban slums in Peninsular Malaysia. Out of 729 stool samples (614 from survey and 115 from paediatric wards in the University Hospital in Kuala Lumpur), 18.1% of them were found to be positive for one or more intestinal protozoan cyst. The commonest parasite encountered was *Giardia* (20.4%).⁶

Timing of stool collection for detection of *Giardia* is important. A single negative stool specimen cannot rule out infection and at least three stool specimens should be examined by appropriate methods by a well-trained parasitology technician before moving on to

other diagnostic procedures. Indeed, some of those infected may be low excretors and it has been suggested that examination of three stools a week for 4 or 5 weeks might be required to find *G. duodenalis* in these individuals.⁷ It has also been shown that symptoms may be present before parasites become detectable in the stool, and repeated examinations on weekly basis may be necessary until the infection becomes patent.⁸ These prolonged periods of examination, however, are often impractical, tedious, and expensive for the patient. Many antibiotics, antacids, Kaolin products, paregoric, most enema preparations, and oily laxatives may cause a temporary masking of or disappearance from the stool of parasites. Examination of stool and intestinal content should therefore be deferred for approximately 5-10 days after use of these products.

Polymerase chain reaction (PCR) has been used to amplify a 0.52 kb segment of *Giardia duodenalis* DNA, using primers specific for nucleotide sequences conserved within two genes (tsp 11 and tsa 417) that encodes homologous, cysteine rich trophozoites surface proteins.⁹ Mahbubani reported a method for detection of *Giardia* cyst by using the polymerase chain reaction (PCR) and the giardin gene as target.¹⁰ He reported that discrimination between live and dead cyst can be made by measuring the amounts of RNA or PCR amplified product from the giardin mRNA before and after inductions of excystation. The assay was at least as sensitive as microscopic examination in diagnosing *Giardia* infections and was easier to perform. The objective of this study was to establish a PCR system for detection of *G. duodenalis* in humans.

MATERIALS AND METHODS

Origin of G. duodenalis isolates

Six local isolates were used in the study, namely 110, 7304, 6304, M007, 2002 and 6307. WB, a pathogenic human strain and *G. muris* of wild mouse origin, were obtained commercially from American Type Culture Collection (ATCC). All local isolates were obtained from six Aborigines with giardiasis admitted to the Hospital for Aborigines at Gombak, Selangor. All the isolates were maintained in *in vitro* culture.

In vitro culture of G. duodenalis trophozoites

Cysts of *G. duodenalis* obtained from fresh faecal specimens were repeatedly washed, sedimented

in water and purified by source gradient centrifugation.² The trophozoites were inoculated into each of a series of borosilicate glass culture tubes (125mm x 16mm) previously filled with modified TYI-S-33 medium and incubated at 37°C at a 5 to 7 degree angle and examined daily with an inverted microscope.⁷ One third of culture liquid in each tube was replaced with fresh medium daily for the first 3 days and every 2 or 3 days thereafter until trophozoites formed a compact monolayer on the inner wall of the tubes. In early stock cultures, multiplication of the organisms was slow. Thereafter, the organisms grew luxuriantly and a monolayer of cells was formed by day 7 to 20 of culture. For passage, the culture tube was cooled on ice followed by vortexing to release the trophozoites from the sides. A sample of the mixture was then used to inoculate a new tube with fresh medium.

Harvesting of parasites

Trophozoites cultivated in TYI-S-33 medium were harvested in late log phase after incubating on ice. The tube was cooled on ice for 10 minutes, vortexed and spun at 15G for 10 minutes. After removal of the supernatant an equal volume of phosphate buffered saline (PBS) was added to the pellet and the contents centrifuged again for 10 minutes at 15G. The supernatant was removed and the parasites transferred to an appropriate container for further work.

Polymerase chain reaction (PCR)

The cells were washed 3 times, centrifuged at 3000 rpm (10 minutes each time) with cold phosphate buffered saline, pH7 and processed immediately for DNA extraction. The DNA was extracted using Qiagen DNA Extraction Kit (USA). All the six local isolates were subjected to PCR with specific primers (Table 1). The PCR was performed with 2.5 units of the Taq DNA polymerase (Fermentas AB, Lithuania) in a reaction mixture (100µl) containing dNTPs (200µM) and 2.5mM MgCl₂, subjected to 10 cycles of 1 minute at 95°C (denaturation), 30 seconds at 58°C (annealing) and 1 minute at 72°C (extension). The amplification product was purified using High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany).

RESULTS

Figure 1 shows the electrophoresis of the genomic DNA of the six local isolates. WB strain

TABLE 1: Primers which were used to detect *G. duodenalis*

Primer	DNA Sequence 5' to 3'
LP1	AAGTGC GTCAACGAGCAGCT'
RP1	TAGTGCTTTTGTGACCATCGA
LP2	CATAACGACGCCATCGCGGCTCTCAGGAA
RP2	TTTGTGAGCGCTCTGTCGTGGCAGCGCTAA

TABLE 2: Purity and concentration of DNA extracted using Qiagen kit .

Sample	A ₂₆₀	A ₂₈₀	A ₂₆₀ /A ₂₈₀	Concentration (µg/ml)
WB	0.020	0.012	1.667	0.100
110	0.022	0.013	1.692	0.110
7304	0.020	0.012	1.667	0.100
6304	0.022	0.012	1.883	0.110
M007	0.020	0.011	1.818	0.100
2002	0.020	0.014	1.429	0.110
6307	0.021	0.014	1.500	0.105

A₂₆₀ = absorbance at 260 nm

A₂₈₀ = absorbance at 280 nm

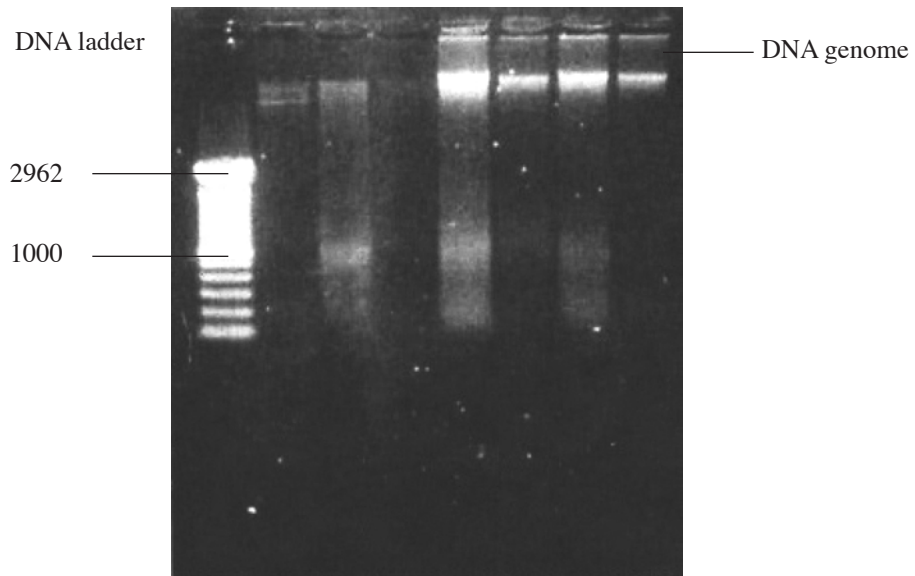


FIG.1: Agarose gel electrophoresis of genomic DNA of all the six local isolates. Lane 1: 1 kb 'ladder'. Lane 2-7: Genomic DNA for local isolates 110, 7304, 6304, 2002, 6307 and M007 respectively. Lane 8: Genomic DNA for WB (reference pathogenic strain from human)

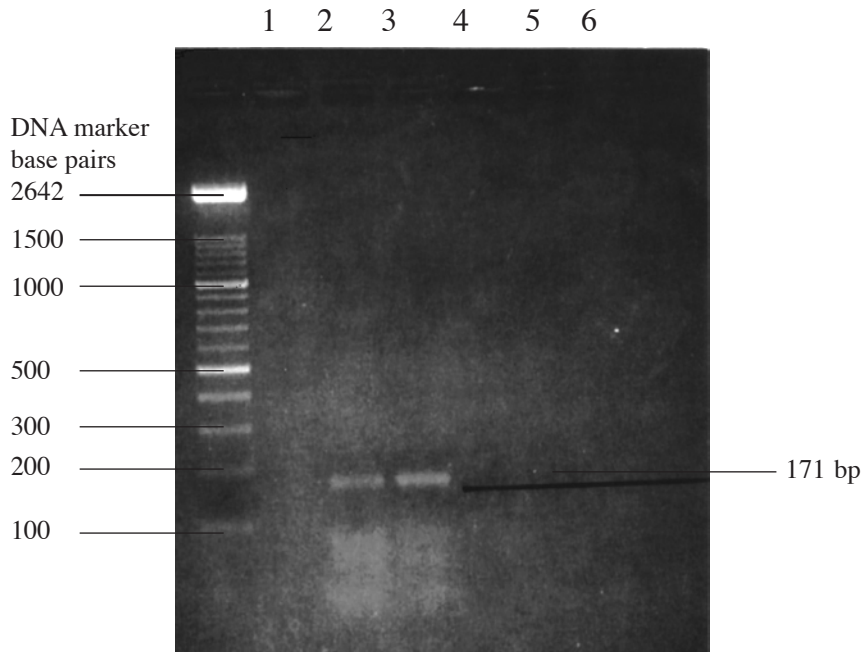


FIG. 2a: Ethidium bromide stained agarose gel electrophoresis of PCR amplified DNA using primer LP1 and RP1. Lane 1: 1 kb ladder; Lane 2: Negative control, Lane 3: WB Strain; Lane 4: *G. muris*, Lane 5: *T. gondii*, Lane 6: *P. falciparum*

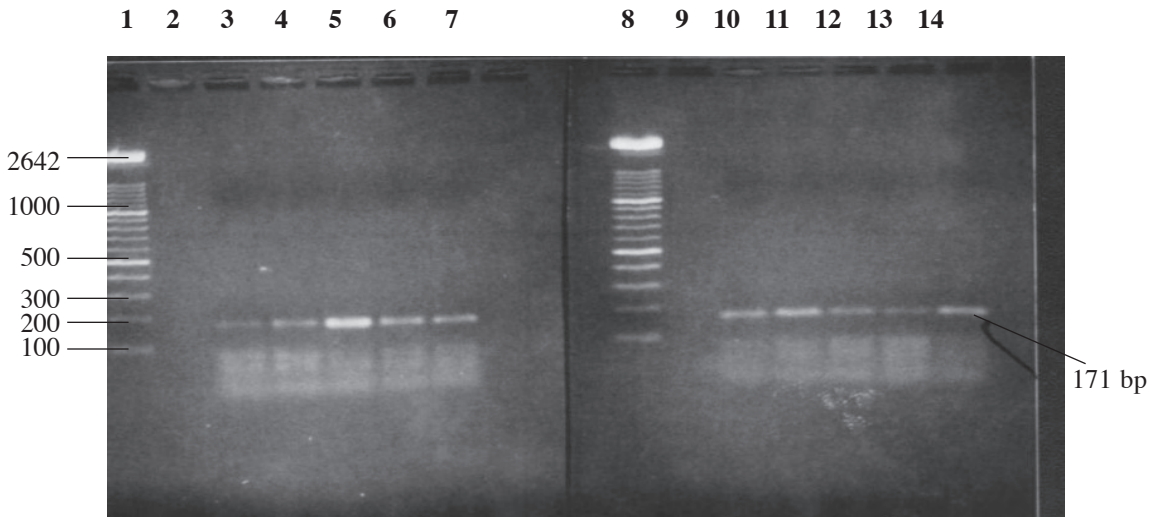


FIG. 2b: Ethidium bromide stained agarose gel electrophoresis of PCR amplified DNA using primer LP1 and RP1. Lane 1: 1 kb ladder. Lane 2: negative control. Lane 3-7: isolates WB, 2002, 6307, 6304 and *G. muris* respectively. Lane 8: 1 kb ladder. Lane 9: negative control. Lane 10 -14: isolates WB, M007, 7304, 110 and *G. muris* respectively.

was used as standard. DNA extracted from all the isolates have good quality because they were of high molecular weight, that was more than 2642 bp compared with the 1kb ladder marker (Boehringer Mannheim, Germany). However the purity of the DNA was not that high (Table 2). The presence of high protein was probably due to contamination from fetal calf serum in the TYI-S-33 media used in the culture of the trophozoite .

Figure 2a shows that the primer LP1 and RP1 amplified *Giardia duodenalis* and *G. muris* DNA but not *T. gondii* and *P. falciparum*. The size of the positive PCR product was 171 bp. Figure 2b shows that all the 6 isolates from Hospital Orang Asli, Gombak gave positive results (171 bp) with primer LP1 and RP1. Figure 3a lane shows that the primer LP2 and RP2 amplified *Giardia duodenalis* DNA (WB strain) but not *G.*

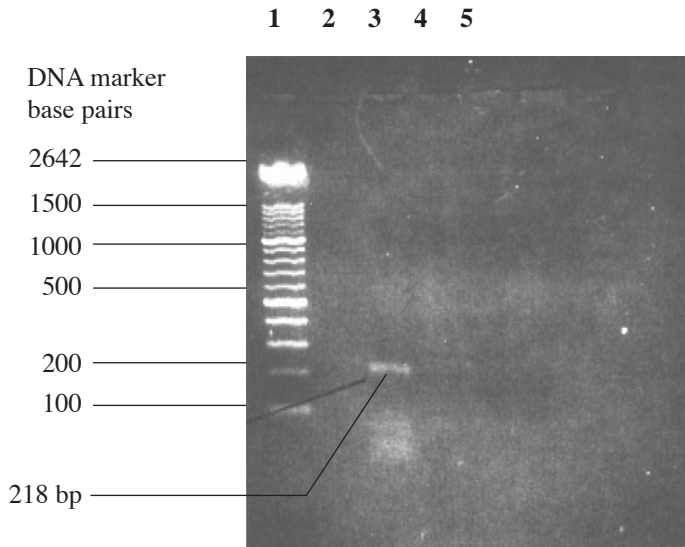


FIG. 3a: Ethidium bromide stained agarose gel electrophoresis of PCR amplified DNA using primer LP2 and RP2. Lane 1: 1 kb ladder; Lane 2: *Toxoplasma gondii*; Lane 3: WB Strain; Lane 4: *G. muris*; Lane 5: *P. falciparum*.

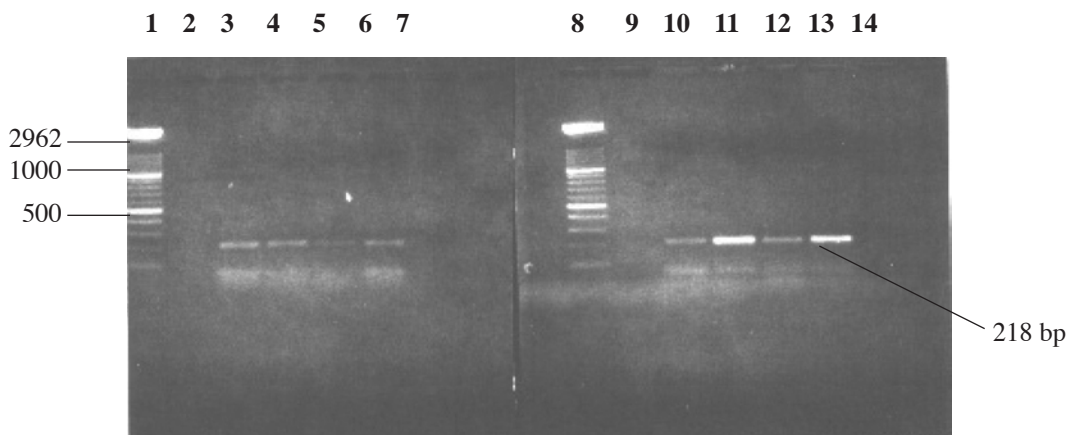


FIG.3b: Ethidium bromide stained agarose gel electrophoresis of PCR amplified DNA using primer LP2 and RP2. Lane 1: 1 kb ladder. Lane 2: negative control. Lane 3-7: isolates WB, 2002, 6307, 6304 and *G. muris* respectively. Lane 8: 1 kb ladder. Lane 9: negative control, Lane 10 -14: isolates WB, M007, 7304, 110 and *G. muris* respectively.

TABLE 3: Protein matches of PCR fragments of *Giardia duodenalis* (isolate 2002) using primer LP2 and RP2 with the GeneBank database (BLAST programme).

Identification	Organism	length	% ID	Score	E Value
Giardin beta chain	<i>G. duodenalis</i>	272	76	106	8x10 ⁻²³
Giardin beta subunit	<i>G. intestinalis</i>	259	76	106	8x10 ⁻²³
Beta giardin	<i>G. intestinalis</i>	229	76	106	8x10 ⁻²³

ID: identity

muris, *T. gondii* and *P. falciparum*. The size of the PCR product was 218 bp. Figure 3b shows that all the 6 isolates of *Giardia* were positive with primer LP2 and RP2.

DISCUSSION

Primer sets LP1/RP1 and LP2/RP2 could detect *Giardia* specifically and could distinguish *G. duodenalis* from other *Giardia* species.¹⁰ Both sets of primers were derived from Giardine gene. The primer set of LP1/RP1 gave 171 bp product with all *Giardia* sp tested. The study also showed that the primer set LP2/RP2 gave 218 bp product with all *G. duodenalis* strains. There was no 218-bp PCR amplified product obtained with *G. muris* and non-*Giardia* species. All the 6 isolates of *Giardia* from the Aborigines showed positive results with both sets of primer LP1/RP1 and LP2/RP2. Multiplex PCR using both primer sets gave two bands with *G. duodenalis* and one band with other species of *Giardia*. This enables the detection of both the genus and the species in a single reaction. In this experiment, all the 6 local isolates of *Giardia* were confirmed as *G. duodenalis*.

In this study, the PCR product of local isolate 2002 was also sequenced. The sequence of the 218 bp product was compared against the European Molecular Biology Laboratory (EMBL) and GenBank nucleotide databases using the BLAST programme¹¹, from the 'National Centre for Biotechnology Information'. The website address is (<http://www.ncbi.nlm.nih.gov/>). The results of BLAST analysis of the sequences are shown in Table 3. The results showed that the open reading frame (ORF) matched with the Giardin gene from *G. duodenalis*. Four genes showed E value of <10e⁻⁵ (E<10e⁻⁵ showed significant value). The highest E value is 8e⁻⁵ with score value of 106 and 76 percent identity.

CONCLUSION

The primers LP1/RP1 and LP2/RP2 which were derived from Giardine gene were specific and sensitive for use in the detection of *Giardia duodenalis* to the genus and species levels. Using these sets of primer, this study showed that all the local isolates tested were genetically genuine *Giardia duodenalis* species.

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