



PCR detection of *Giardia lamblia* in stool: targeting intergenic spacer region of multicopy rRNA gene

S. Ghosh,¹ A. Debnath,² A. Sil,¹ S. De,² D. J. Chattopadhyay^{1*} and P. Das²

¹Department of Biochemistry and Dr B. C. Guha Centre for Genetic Engineering and Biotechnology, University of Calcutta, India, ²Department of Microbiology, National Institute of Cholera and Enteric Diseases, ICMR, Calcutta, India

(Received 5 January 2000, Accepted 14 April 2000)

A PCR based detection that amplifies the 552-bp intergenic spacer (IGS) region of multicopy rRNA gene of *Giardia lamblia* and 320-bp internal sequences to first PCR product has been used in diagnosis of giardiasis in stool sample. The primers were found highly specific to *Giardia* spp. only, because no amplification was observed with DNAs from other enteric pathogens like *Escherichia coli*, *Shigella dysenteriae* and *Entamoeba histolytica*. The test could detect even less than 2 pg of genomic DNA from *Giardia* trophozoites. In direct diagnosis of *Giardia lamblia* in stool samples, it was observed that PCR amplification of IGS followed by nested PCR could enhance the sensitivity and specificity of the tests manifold and the system was able to detect as low as 10 parasites in 100 µl of stool. The comparative evaluation of the present system with conventional microscopy, CIEP and ELISA in the diagnosis of giardiasis from diarrhoeic stool samples and control subjects demonstrated a 100% correlation among nested PCR, microscopic examination and ELISA in patients suggestive of giardiasis (Group I) and control subjects (Group II). In Group I cases (patients suffering from other than giardiasis), CIEP, ELISA and nested PCR showed better results than microscopic examination. However, among them, PCR was found most sensitive and specific because 20% positivity was noticed by PCR whereas CIEP and ELISA showed only 7.14% and 12.85%, respectively. Break-up results showed that all the samples which were positive by CIEP or ELISA, also found positive by PCR. The present observation clearly suggests the use of PCR that amplifies the intergenic spacer region of multicopy rRNA gene of *Giardia lamblia* followed by nested PCR for routine, quick and reliable detection of *Giardia lamblia* in stool samples.

© 2000 Academic Press

KEYWORDS: PCR, *Giardia lamblia*, diagnosis, nested PCR, ELISA, rRNA.

INTRODUCTION

Giardia lamblia is an aerotolerant protozoan parasite that is a common cause of human giardiasis.^{1–3} It inhabits the small intestine and causes diarrhoea with a diverge range of symptoms ranging from asymptomatic carrier stage to severe malabsorption, diarrhoea and weight loss.⁴ It is the leading protozoal cause of

diarrhoeal illness, frequently implicated in water-borne outbreaks.⁵ The management of the disease is made difficult by the availability of only insensitive and non-specific diagnostic methods. The diagnosis is made conventionally by microscopic examination of stool samples for the presence of cysts or trophozoites.⁶ *Giardia* cysts are shed sporadically and their number may vary from day to day.⁷ Routine

* Author to whom all correspondence should be addressed at: Department of Biochemistry, Dr B. C. Guha Centre for Genetic Engineering and Biotechnology, University of Calcutta, 35, Ballygunge Circular Road, Calcutta-700 019, India.
E-mail: ratnac@cal2.vsnl.net.in

examination of stool specimens collected on consecutive days or even within the recommended 10-day time frame may not confirm infection with this organism. Only 50% to 70% sensitivity has been reported by this technique.⁸ The low sensitivity is due to the irregular excretion of cysts or trophozoite and examination of stool samples by unskilled personnel.⁹ Other methods such as jejunal biopsy and duodenal aspirate examination give better results but they find little utility as they are invasive. Immunodiagnosis of the parasite based on detection of *Giardia* specific antibody in serum and antigens in the stool proved to be better diagnostic methods.^{10–14} However, the findings suffered from serious drawbacks pertaining to false positive results due to cross reactivity with other microorganisms. In the last few years, DNA based diagnosis of diseases is gaining importance. This system has the advantage over other diagnosis methods for being highly specific and at the same time sensitive. Recently, a number of DNA-based methods for detection of *Giardia* have been described.^{15,16} The polymerase chain reaction has been employed for selective amplification of *Giardia* DNA¹⁶ using giardin gene as the amplification target.

Ribosomal RNA gene has been suggested a very good target for amplification by PCR due to its high copy numbers in all organisms.¹⁷ In *G. lamblia* about 60–130 copies of rRNA gene per nucleus, arranged in tandem repeat have been reported.^{18,19} In this paper, we report the development of a PCR based assay to amplify and detect a segment of the intergenic spacer (IGS) region of multicopy rRNA of *G. lamblia* for diagnosis of giardiasis. The assay was compared with other existing methods for routine diagnosis of *G. lamblia* directly from faecal sample.

MATERIALS AND METHODS

Cultivation of *G. lamblia* and other enteropathogens

Axenic cultures of *G. lamblia* (PD-1, PD-2 local and P1 reference strain)¹⁸ were maintained in filter sterilized TYI-S-33 medium as described elsewhere.²⁰ The isolates were sub-cultured three times a week.

Three common enteric pathogens viz. *Escherichia coli*, *Shigella dysenteriae* and *Entamoeba histolytica* were cultured in different media. In brief, trophozoites of *E. histolytica* were grown in TYI-S-33 medium at 37°C whereas *E. coli* and *S. dysenteriae* were cultured at 37°C in Tryptic Soy Broth.

Oligonucleotide primers

The primers used in this study were designed from the IGS region of rRNA operon of *G. lamblia* which were found *G. lamblia* specific in earlier studies.¹⁸ The first PCR primers, AS1 and AS2 (Table 1) specify a 552 bp fragment of IGS (Fig. 1). The second set of primers, SG3 and SG4, were designed from internal sequences of 552 bp fragment and used for nested PCR (Table 1). This will amplify a 320 bp region within the first PCR product (Fig. 1).

Isolation of genomic DNAs

Genomic DNAs were isolated as described previously.¹⁸ In brief, *Giardia* trophozoites of late log phase were harvested by chilling the culture tube in ice and centrifuged at 600 g for 10 min to pellet the cell. Nuclei from cells were obtained by lysing them in 1% (v/v) NP40 solution followed by centrifugation at 500 g for 3 min. Subsequently, nuclei were lysed by overnight incubation at 50°C in lysis buffer containing 20 mM Tris HCl (pH 8), 50 mM EDTA, 0.5% (w/v) sarcosyl and 500 µg proteinase K. The lysate was mixed with CTAB/NaCl solution and incubated at 65°C for 10 min. DNA was extracted with phenol/chloroform and ethanol precipitated in presence of 0.3 M Na-acetate and suspended in sterile water. The procedure for isolation of genomic DNAs from the other enteric pathogens was identical to that followed for DNA extraction from *Giardia* nuclei.

Polymerase chain reaction

PCR mixture contained final concentration of 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 M MgCl₂, 0.01% (w/v) gelatin, 250 µM deoxyribonucleoside triphosphate mix (dATP, dGTP, dCTP, and dTTP), 3 U of *Taq* Polymerase (Bangalore Genei), and 200 ng of each primers.

The PCR was carried out in 25 µl volume containing 5 µl of DNA extract for first PCR or 1 µl of the first PCR product for nested PCR. All the conditions for conducting both the PCR were identical. Precisely 30 cycles were used for both reactions with denaturation at 95°C for 60 s, annealing at 55°C for 60 s and extension at 72°C for 60 s. Products of first PCR were visualized on a 1.5% (w/v) agarose gel and for nested PCR in 2% agarose gel.

Three sets of experiments were conducted to analyse the sensitivity of the assay method.

Different numbers of *G. lamblia* trophozoites in 100 µl buffer were boiled for 10 min and centrifuged

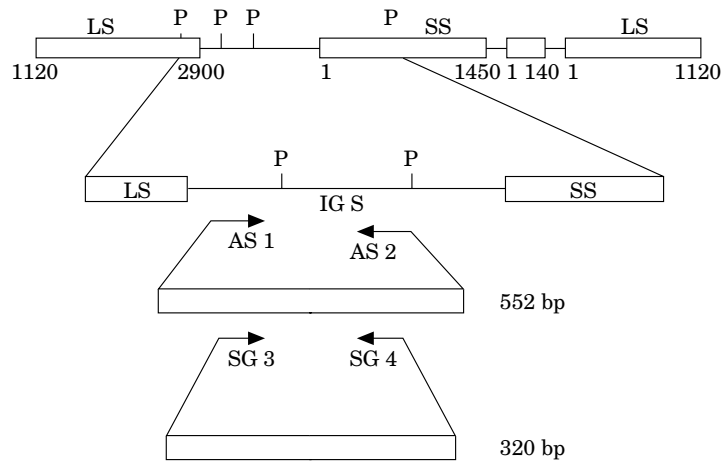


Fig. 1. Amplification strategy from the intergenic spacer region (IGS) of the multicopy rRNA gene of *Giardia lamblia* showing positions of primers. Primers AS1 and AS2 amplify a 552-bp segment of the IGS. Primers SG3 and SG4 amplify a 320-bp fragment within the first PCR product.

Table 1. Primers used and their locations

Function	Name	Nucleotide sequence	Location
Upstream primer (external primer)	AS1	5'CGACCGGGAGACACGCCC3'	4003–20
Downstream primer (external primer)	AS2	5'AGGACTGCATATCACGGC3'	4538–55
Upstream primer (internal primer)	SG3	5'AGAGCAGCCGATCCCCCG3'	4105–22
Downstream primer (internal primer)	SG4	5'AATGGAGGCTGACTGTG3'	4408–25

for 10 min at 12 000 *g*. Five microlitres of supernatant was subjected to PCR amplification.

In the second set of experiment, 100 µl of known *Giardia* negative stool specimen was mixed with known numbers of *Giardia* trophozoites, boiled for 10 min and centrifuged at 12 000 *g* for 5 min. Twenty microlitre of supernatant was diluted to 200 µl and from the diluted sample, 5 µl was used for first PCR using primers AS1 and AS2. However, for second PCR only 1 µl of the first PCR product and SG3 and SG4 primers were used.

In the third set, 100 µl of microscopically negative and positive *Giardia* stool samples were boiled for 10 min followed by centrifugation at 12 000 *g* for 5 min. Twenty microlitres of clear supernatant was diluted to 200 µl and 5 µl was used in first PCR. The second PCR was carried out with SG3 and SG4 primers and 1 µl of first PCR product.

Evaluation of PCR

The efficacy of nested PCR was compared with other existing methods in diagnosis of *G. lamblia* directly in stool samples collected from different categories of diarrhoea cases and control subjects.

Clinical subjects

Based on the clinical symptoms, patients hospitalized or attending the Outpatient Department of the B. C. Roy Children's Hospital, Calcutta, were categorized into three groups (Groups I, II, III). Stool samples collected from 30 patients with history suggestive of giardiasis (pain in abdomen, loose motion of six to eight times, flatulence, mucus in stool, anorexia, nausea and vomiting) were categorized into Group I. Stool examination of these patients showed presence of cyst and/or trophozoites of *G. lamblia*. Group II comprised of 45 healthy control subjects who had no complaints of diarrhoea or dysentery for the preceding one month, and in Group III, 70 subjects were included whose stools were negative for *G. lamblia* by microscopy but positive for other parasites, e.g. *Entamoeba coli*, *E. histolytica*, *Trichuris trichura*, *Ascaris*, *Entamoeba hartmanni* and hook-worm (Table 2).

Stool examination

Stool samples were collected from all the above patients and examined under microscope by wet

Table 2. Evaluation of different diagnostic tests in the detection of *Giardia lamblia* infection in various categories of patients

Groups ^a	Clinical symptoms	No. examined	Microscopy (%)	CIEP (%)	ELISA (%)	Nested (%)
I	Giardiasis	30	100	76.6	100	100
II	Healthy subjects (controls)	45	0	0	0	0
III	Random hospital patients suffering from gastrointestinal problems other than giardiasis	70	0	7.14	12.85	20

^a Basis of characterization of the three groups is explained in the text.

mount (Lugol's iodine, normal saline) and permanent preparation. Doubtful samples were confirmed by formalin ether concentration technique.²¹ For PCR and antigen analysis stool samples were stored at -70° until used.

Enzyme linked immunosorbant assay

Processing of stool samples

1 gm of fresh faecal sample (collected within 2–3 h) was homogenized in 2 ml of 10 mM PBS (pH 7.2) containing 0.05% sodium dodecyl sulphate.²² Coarse debris was eliminated by centrifugation at 500 g for 20 min, the clear supernatant was collected and 1 mM phenyl sulphonyl fluoride (PMSF), a protease inhibitor, was added and kept frozen at -20°C until used.

Multilayer ELISA

The method used was a modification of the double antibody sandwich ELISA described by Ungar *et al.*²³ and Sengupta *et al.*²⁴ In a model system, a polystyrene microtiter plate (Nunc, Denmark) was coated with 3 $\mu\text{g}/\text{well}$ of IgG isolated from anti-*Giardia* guinea pig serum²⁵ in 0.07 M carbonate-bicarbonate buffer (pH 9.6) for 2 h at 37°C . The wells were washed to remove the unbound antibody and the extra binding sites were blocked with 200 $\mu\text{l}/\text{well}$ of 3% (w/v) BSA in 10 mM PBS for overnight at 4°C . Varying concentrations of standard soluble antigen prepared from axenic *G. lamblia* trophozoites or parasitologically negative/positive stool samples (100 $\mu\text{l}/\text{well}$) were incubated overnight at 4°C . Control wells received IgG purified from normal rabbit serum and/or no antigen. Washed wells were treated with 2 $\mu\text{g}/100 \mu\text{l}/\text{well}$ (optimal as determined by checker board titration) of IgG isolated from anti-*Giardia* rabbit serum for 1.5 h at 37°C . The immunoreaction was performed by adding goat anti-rabbit IgG labelled with HRP as conjugate followed by OPD as the substrate in presence of 0.03% H_2O_2 in 0.1 M citrate buffer (pH 4.5). The reaction was stopped with 2 N H_2SO_4 and the optical

density at 492 nm was measured in a Titertek Multi-scan ELISA reader (Flow Laboratories Inc., McLean, USA).

Countercurrent immunoelectrophoresis (CIEP)

The test was performed according to the method described by Chaudhuri *et al.*²⁶ In brief, 3 ml of 0.9% agarose in barbital buffer (pH 8.8, 0.05 M) was layered on a clean grease-free slide and kept at room temperature for solidification. Processed stools for antigen and antibody were placed in respective wells and electrophoresis was carried out in electrophoresis chamber (Shandon, UK) at a constant voltage of 160 V or 10 mA per slide for 20–30 min. Results were recorded immediately after the test and incubating the slides at 4°C for 30 min.

RESULTS

Amplification of rRNA gene locus

Design of the primers

Our earlier results¹⁸ clearly demonstrated that IGS region of rRNA of *G. lamblia* was specific to *Giardia* and results of present study once again strengthen the earlier findings. AS1 and AS2 primers designed from this IGS showed amplification with *Giardia* genomic DNA only. No amplification was observed from genomic DNAs of other enteric organisms like *E. coli*, *S. dysenteriae* and *E. histolytica* (Fig. 2).

Sensitivity of the assay

To determine the lowest possible amount of DNA that could be amplified by this system, PCR was performed using serial dilutions of template DNA. The results revealed that the system is sensitive enough to amplify as little as 2.5 pg of plasmid DNA containing rRNA gene of *Giardia* (Fig. 3).

By the present PCR, a minimum of 10 trophozoites

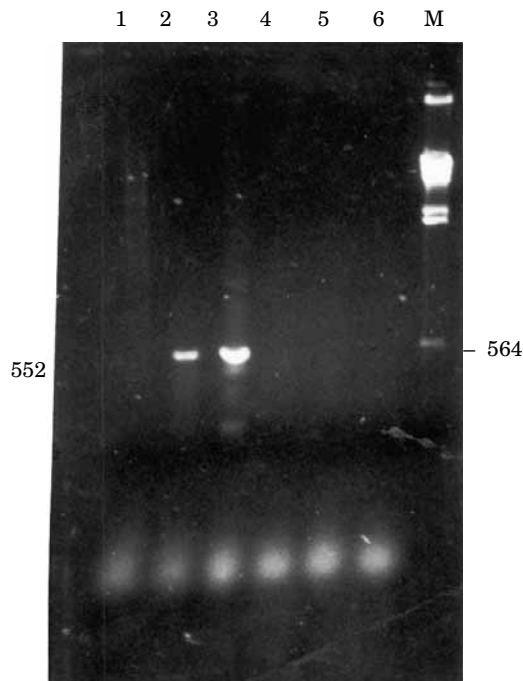


Fig. 2. Detection of specificity of primer pair AS1 and AS2 in recognizing genomic DNAs of different enteropathogens. Figure represents ethidium bromide (EtBr) stained 1.5% agarose gel. The amplified 552-bp product is shown. Lane 1 represents control reaction containing no DNA; lanes 2 and 3, genomic DNAs of two different strains of *Giardia lamblia*, PD1 and PD2; lane 4, *E. coli* DNA; lane 5, *E. histolytica* DNA; lane 6, *S. dysenteriae* DNA and Lane M, λ HindIII marker. The position of 564-bp band is shown.

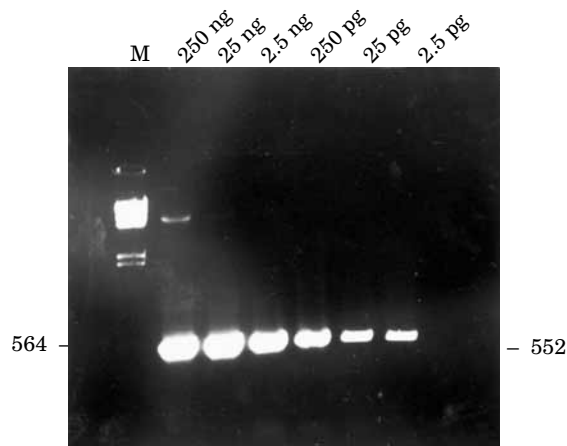


Fig. 3. Detection of sensitivity limit of rDNA plasmid by PCR, using primer pair AS1 and AS2. The 552-bp amplified product is shown. Figure represents EtBr stained 1.5% agarose gel of PCR product obtained by using different amount of plasmid DNA. Lane M represents λ HindIII marker. The 564-bp band is shown.

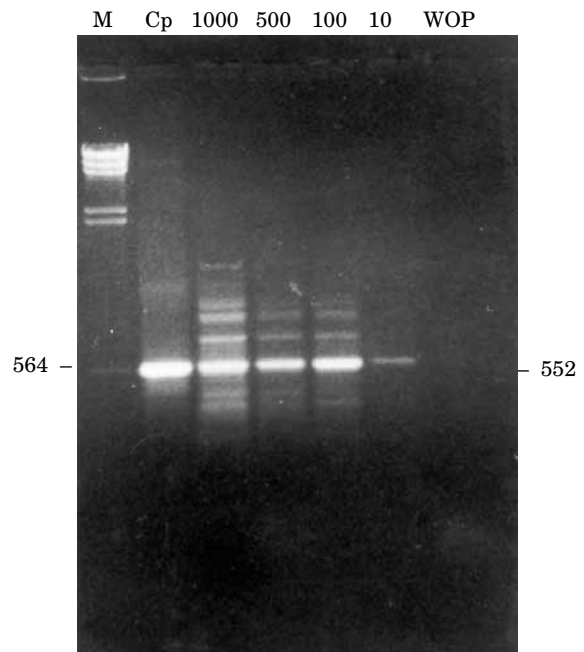


Fig. 4. Limit of detection of *Giardia* cells by PCR using primer pair AS1 and AS2. Figure represents EtBr stained 1.5% agarose gel of PCR product obtained by using different numbers of *Giardia* trophozoites in 25 μ l reaction. Ten microlitres of PCR product was run in each lane. Lane M represents λ HindIII marker with the position of the 564-bp band; Lane Cp represents positive control where PCR reaction was one using rDNA plasmid as template; Lane WOP represents negative control containing no *Giardia* cells.

could be detected (Fig. 4). In other words, taking the DNA content of one trophozoite to be 0.144 pg as reported by Erlandsen *et al.*,²⁷ the present system is sensitive enough to amplify 1.44 pg of *Giardia* genomic DNA.

Based on these results, we wanted to test whether the PCR based diagnosis is possible from stool specimens. So, PCR was carried out where a variable number of *Giardia* trophozoites was added to a giardiasis negative faecal sample. The results of initial standardization of PCR in detection of *G. lamblia* in faecal specimens demonstrated less sensitivity. This was likely due to the presence of inhibitory substances in stool like bilirubin, urobilinogens and the bile salts.²⁸ To minimize the problem, 50 times or more dilution of stool was used and was found optimal in minimizing the inhibition and getting specific amplification. However, a minimum of 2000 trophozoites was found to be required for seeing the product in gel (Fig. 5). To eliminate the possibilities of non-specific amplification and to increase the sensitivity nested PCR was done using genomic DNA and primers SG3 and SG4 which amplified a 320 bp region (Fig. 1) within the first PCR product. The control

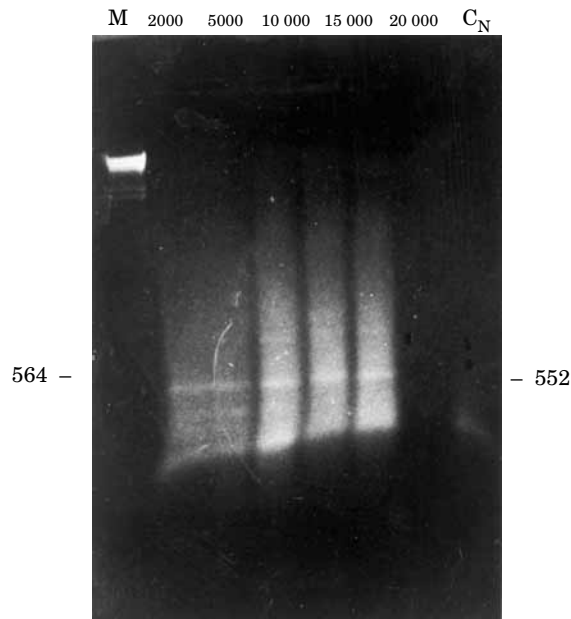


Fig. 5. PCR amplification of model faecal sample mixed with different numbers of *Giardia* cells using primer pair AS1 and AS2. Figure represents EtBr stained 1.5% agarose gel of PCR product. Lane M represents λ HindIII marker. The 564-bp band is shown. Lane C_N represents PCR amplification from negative stool, taken as control.

stool sample gave no amplification in nested PCR (Fig. 6a). The control negative sample was mixed with different numbers of *Giardia* trophozoites, and the nested PCR helps to detect as low as 10 trophozoites (Fig. 6(b)). The added advantage of using the nested PCR step was that it negated the chance of any false positive result in the first PCR which was commonly observed during amplification from faecal specimen. This method was then used in analysis of 145 stool samples. The sensitivity of the nested PCR was clear from Fig. 7, lane C_p, where there is a faint band in Fig. 7a but prominent band in Fig. 7b using plasmid DNA. In the experimental samples, lanes 2 and 8 were found to be negative as the specific band did not appear in the first or nested PCR. In lanes 5 and 6 although no band appeared in the first PCR but nested PCR yielded a band at 320-bp region. This clearly demonstrates the efficiency of the nested PCR. The extra bands obtained in the positive samples in lanes 1, 3, 4, 7 and 9 may be due to mutations in the multiple copies of the gene.

Evaluation of PCR

To evaluate the efficiency, the present PCR based *G. lamblia* detection system was compared with

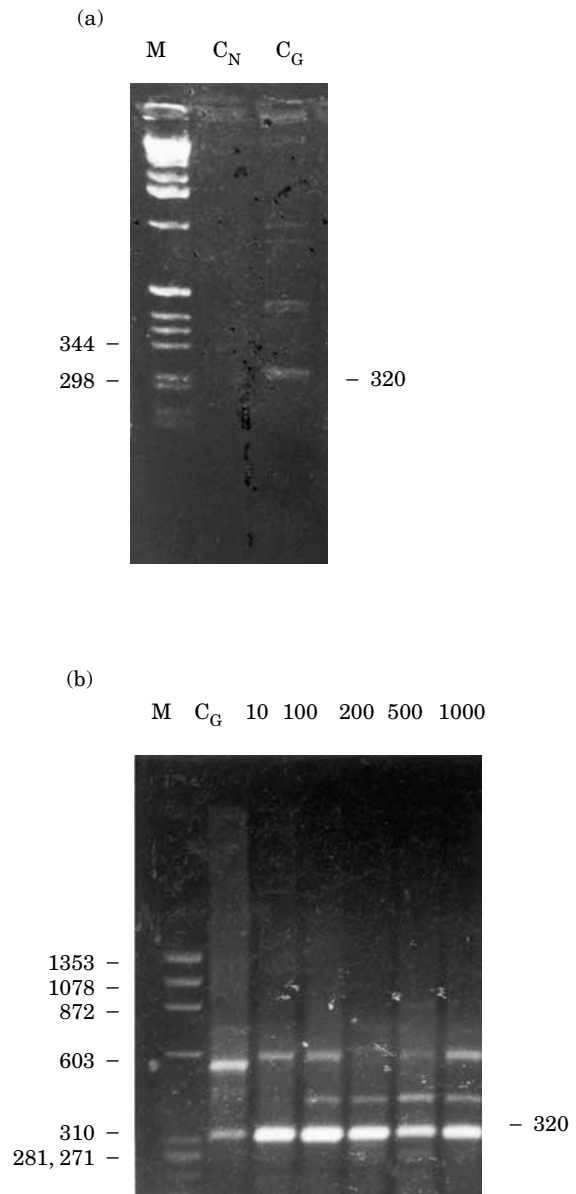


Fig. 6. (a) EtBr stained 2% agarose gel of PCR amplification of control negative stool sample using primer pairs AS1/AS2 and SG3/SG4. Lane C_N represents PCR amplification from negative stool, taken as control; lane C_G represents control positive reaction where PCR was carried out with genomic DNA of *Giardia lamblia*. The position of the 320-bp amplified product is shown; lane M represents 1 kb ladder marker from GIBCO-BRL. The positions of 298-bp and 344-bp bands are shown. (b) PCR amplification of model faecal sample mixed with different numbers of *Giardia* cells using nested primer pair SG3 and SG4. Figure represents EtBr stained 2% agarose gel of nested PCR product. Lane M represents HaeIII-digested ϕ X-174 DNA size markers. The 603-bp and 310-bp fragments are shown; lane C_G Represents control positive reaction where PCR was carried out with genomic DNA of *Giardia lamblia*.

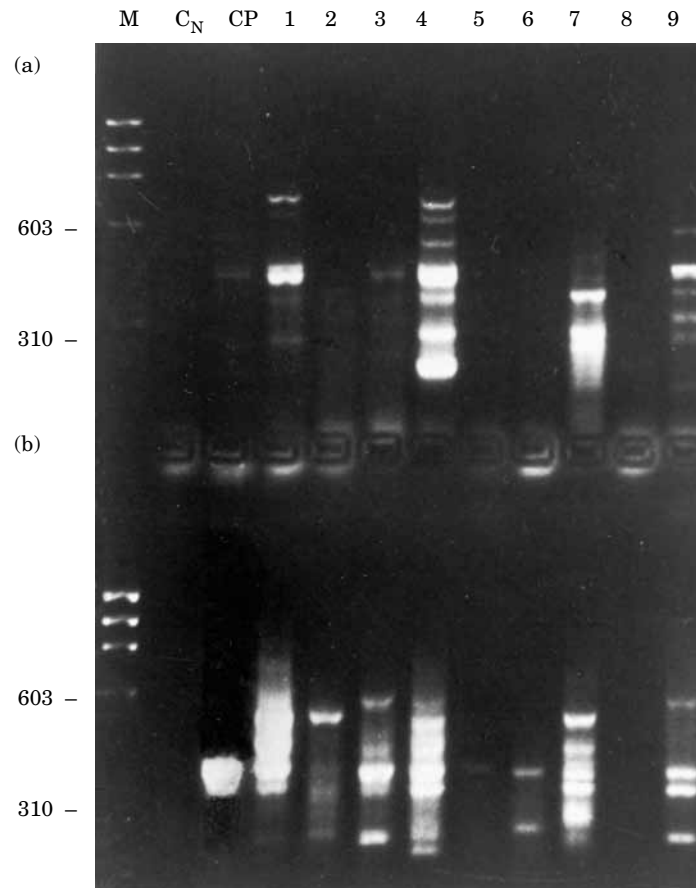


Fig. 7. Detection of rDNA of *Giardia lamblia* in clinical samples by first (a) PCR and then (b) nested PCR. Figure represents EtBr stained 2% agarose gel. Lane M represents *Hae*III-digested ϕ X-174 DNA size markers. The 603-bp and 310-bp fragments are shown. Lane C_N, PCR amplification from negative stool, taken as control; lane C_P, rDNA plasmid; lanes 1–9, clinical samples.

microscopy, CIEP and ELISA in stool samples of different categories of patients and controls. A 100% correlation among nested PCR, microscopy and ELISA in Group I (suggestive giardiasis cases) (Table 2). In Group II (control subjects) all the tests showed similar observations as none of the tests demonstrated positive results. However, in Group III cases (Random hospital patients suffering from gastrointestinal problems other than giardiasis) CIEP, ELISA and nested PCR showed better diagnostic results than microscopic examination but among the three (viz. CIEP, ELISA and PCR), PCR was found most sensitive and specific in the sense that it showed 20% positive reaction whereas CIEP and ELISA showed only 7.14 and 12.85%, respectively (Table 2).

The break-up results of each group are shown in Table 3. Of the 30 stool samples (Group I) which were clinically suggestive of giardiasis and detected positive by microscopy for presence of cysts or trophozoites, 23 were positive by all the four tests, and seven were only positive by microscopy, ELISA and

PCR. These results suggest the sensitivity and specificity of each test. In Group III, out of 70 samples examined, 56 were negative by all the tests. Among the 14 positives, five were by CIEP, ELISA and PCR, four by ELISA and PCR and five by only PCR (Table 3).

DISCUSSION

Timely diagnosis by appropriate technique is a prerequisite for quick and effective therapy of disease. *G. lamblia* is diagnosed conventionally by microscopic examination or by antigen or antibody detection. Both the methods revealed its limitations. In recent years, DNA based diagnosis and especially, amplification of functional genes by polymerase chain reaction²⁹ has revolutionized the fields of biological and medical sciences because of its power to produce many copies of a desired, previously undetectable

Table 3. Break-up of positive and negative samples in each group with different tests

Groups	No. examined	Tests			
		Microscopy	CIEP	ELISA	Nested PCR
I	23	+	+	+	+
	7	+	—	+	+
II	45	—	—	—	—
	56	—	—	—	—
III	5	—	+	+	+
	4	—	—	+	+
	5	—	—	—	+

nucleic acid target. However, before that the technique must be optimized for the extraction and amplification of template DNA from heterogeneous human body fluids and especially from stool. Since there are many factors in stool which inhibit PCR reaction, methods that allow for nucleic acid amplification without substantial levels of inhibition or inconsistency must be standardized. Atlas in 1991¹⁶ used the polymerase chain reaction to selectively amplify the giardin gene for detection of *G. lamblia* from water. However, the test was not found highly sensitive, probably due to low copy number of giardin gene. In the last few years the rRNA gene has been selected as the target for detection by PCR in many parasites because of its high copy number.^{18,19} da Silva *et al.*³⁰ has targeted the SSrRNA gene in detection of coccidian infection (Microsporidia). In a similar fashion, the SSrRNA gene of *Giardia* has been used as the target for detection.³¹ It is now well established that most of the sequences of the SSrRNA are evolutionary conserved and there is every possibility of getting cross reactivity with other organism and species. In contrast, sequence variability at the intergenic regions of rDNA has been demonstrated among the inter and intraspecies.^{32,33} Recently, different groups successfully used the intragenic regions of rRNA gene to distinguish different isolates of a number of prokaryotes and eukaryotes.³⁴ Here, intergenic spacer region of multi-copy (132 copies/nucleus) rRNA gene(s) of a local isolate of *G. lamblia*, which was cloned and sequenced earlier,¹⁸ was used in PCR supported by nested PCR in the direct detection of *G. lamblia* in stool samples without prior DNA purification. In the current assay samples were prepared just by rapid boiling method and the PCR products were analysed by gel electrophoresis.

The AS1 and AS2 primers were found highly specific and sensitive because it didn't amplify the genomic DNAs of other enteric organism and detected as less than 2.5 pg of DNA from plasmid containing full length rRNA genes of *G. lamblia* or DNA from 10 trophozoites. However, the same primers were

not found very sensitive when stool samples were mixed with known number of *Giardia* trophozoites. The system could detect up to 2000 cells mixed to 100 µl of stool sample. One of the reasons could be presence of inhibitory substances such as bilirubin, bile salts and mucus etc. in the stool sample. The sensitivity was increased 200 folds by introducing the nested PCR to the same samples i.e. 10 parasites could be detected.

The results of comparative evaluation clearly demonstrated the superiority of nested PCR based detection of *Giardia lamblia* in stool samples over the other three tests viz. microscopy, antigen detection by CIEP and ELISA because in one time analysis of Group III stool samples (random hospital patients other than giardiasis) the nested PCR showed more positive results than the other three tests. This allows for a distinct advantage of PCR over the sensitive ELISA or other methods. Thus the highly sensitive nested PCR method is expected to contribute significantly in diagnosis of giardiasis and so it may be used routinely. More trials are necessary to optimize the finer tuning of the PCR conditions.

Acknowledgements

Mr S. Ghosh and A. Debnath are grateful to the University Grants Commission, India and Indian Council of Medical Research, India, respectively, for providing the Senior Fellowships.

REFERENCES

1. Nash, T. E. (1989). Antigenic variation in *Giardia lamblia*. *Experimental Parasitology* **68**, 238–41.
2. Wolfe, M. S. (1990). Clinical symptoms and diagnosis by traditional methods. In *Giardiasis* (Meyer, E. D., ed.) Pp. 175–85. Amsterdam: Elsevier.
3. Adam, R. D. (1991). The biology of *Giardia* spp. *Microbiological Review* **55**, 706–32.
4. Farthing, M. J. G. (1990). Immunology of Giardiasis. *Springer Seminars in Immunopathology* **12**, 269–82.

5. Levine, W. C., Stephenson, W. T. & Craun, G. F. (1990). Waterborne disease outbreaks, 1986–1988. *Morbidity Mortality Weekly Report* **39**, 1–13.
6. Goka, A. K. J., Rolston, D. D. K., Mathan, V. I. & Farthing, M. J. G. (1990). The relative merits of faecal and duodenal juice microscopy in the diagnosis of giardiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **84**, 66–7.
7. Garcia, L. S. & Bruckner, D. A. (1997). *Diagnostic Medical Parasitology*, 3rd edn. Washington, DC: ASM Press.
8. Burke, J. A. (1977). The clinical and laboratory diagnosis of giardiasis. *Critical Review of Clinical Laboratory Science* **7**, 373–91.
9. Danciger, M. Lopez, M. (1975). Number of *Giardia* in the faeces of infected children. *American Journal of Tropical Medicine and Hygiene* **7**, 237–42.
10. Sun, T. (1980). The diagnosis of giardiasis. *American Journal of Surgical Pathology* **4**, 265–71.
11. Ungar, B. L. P., Yolken, R. H., Nash, T. E. & Quinn, T. C. (1984). Enzyme linked immunosorbent assay for the detection of *Giardia lamblia*. *Journal of Infectious Diseases* **149**, 90–7.
12. Goldin, A. J., Apt, W., Aguilera, X., Zulantay, I., Warhurst, D. C. & Miles, M. A. (1990). Efficient diagnosis of giardiasis among nursery and primary school children in Santiago, Chile by capture ELISA for the detection of faecal *Giardia* antigens. *American Journal of Tropical Medicine and Hygiene* **42**, 538–45.
13. Chaudhuri, P. P., Das, P., Bhattacharya, S. K. & Pal, S. C. (1991). Significance of IgM antibodies in the serodiagnosis of Giardiasis. *European Journal of Clinical Microbiology and Infectious Diseases* **10**, 7–9.
14. Winiacka-Krusnell, J. & Linder, E. (1995). Detection of *Giardia lamblia* cysts in stool samples by immunofluorescence using monoclonal antibody. *European Journal of Clinical Microbiology and Infectious Diseases* **14**, 218–22.
15. Butcher, P. D. & Farthing, M. J. G. (1989). DNA probes for the fecal diagnosis of *Giardia lamblia* infections in man. *Biochemical Society Transactions* **17**, 363–4.
16. Atlas, R. M. (1991). Environmental applications of the polymerase chain reaction. *ASM News* **57**, 630–2.
17. Waters, A. P. & McCutchan, T. F. (1990). Ribosomal RNA: nature's own polymerase amplified target for diagnosis. *Parasitology Today* **6**, 56–60.
18. Sil, A. K., Das, P., Bhattacharya, S., Ghosh, S. & Chattopadhyay, D. J. (1998). Cloning of ribosomal RNA genes from an Indian isolate of *Giardia lamblia* and the use of intergenic non-transcribing spacer regions in the differentiation of *Giardia* from other enteric pathogens. *Journal of Biosciences* **23**, 557–64.
19. Boothroyd, J. C., Wang, A., Campbell, D. A. & Wang, C. C. (1987). An unusually compact ribosomal DNA repeat in the protozoan *Giardia lamblia*. *Nucleic Acids Research* **15**, 4065–84.
20. Diamond, L. S., Harlow, D. R. & Cunliffe, C. C. (1978). A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **72**, 431–2.
21. Ritchie, L. S. (1948). An ether sedimentation technique for routine stool examinations. *Bulletin of the United States Army Medical Department* **8**, 326.
22. Del Muro, R., Oliva, A., Herion, P., Capin, R. & Ortiz-Ortiz, L. (1987). Diagnosis of *Entamoeba histolytica* in feces by ELISA. *Journal of Clinical and Laboratory Analysis* **1**, 322–5.
23. Ungar, B. L. P., Yolken, R. H. & Quinn, T. C. (1985). Use of a monoclonal antibody in an enzyme immunoassay for the detection of *Entamoeba histolytica* in fecal specimens. *American Journal of Tropical Medicine and Hygiene* **34**, 465–72.
24. Sengupta, K., Das, P., Johnson, T. M., Chaudhuri, P. P., Das, D. & Nair, G. B. (1993). Production and characterization of monoclonal antibodies against a highly immunogenic fraction of *Entamoeba histolytica* (NIH: 200) and their application in the detection of current amoebic infection. *Journal of Eukaryotic Microbiology* **40**, 722–6.
25. Das, P., Sengupta, K., Pal, S., Das, D. & Pal, S. C. (1993). Biochemical and immunological studies on soluble antigens of *Entamoeba histolytica*. *Parasitology Research* **79**, 365–71.
26. Chaudhuri, P. P., Pal, S., Pal, S. C. & Das, P. (1988). Studies on *Giardia lamblia* trophozoite antigens using Sephacryl S-300 column chromatography, polyacrylamide gel electrophoresis and enzyme-linked immunosorbent assay. In *Advances in Giardia Research* (Wallis, P. M. & Hammond, B. R., eds) Pp. 191–4. Canada: The University of Calgary Press.
27. Erlandsen, S. L. & Rasch, E. M. (1994). The DNA content of trophozoites and cysts of *Giardia lamblia* by microdensitometric quantitation of Feulgen staining and examination by laser scanning confocal microscopy. *Journal of Histochemistry and Cytochemistry* **42**, 1413–6.
28. Widjojoatmodjo, M. N., Fluit, A. C., Torensma, R., Verdonk, G. P. H. T. & Verhoef, J. (1992). The magnetic immuno polymerase chain reaction assay for direct detection of salmonellae in fecal samples. *Journal of Clinical Microbiology* **30**, 3195–9.
29. Saiki, R. K., Scharf, S., Faloona, F. et al. (1985). Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis for sickle cell anemia. *Science* **230**, 1350–4.
30. da Silva, A. J., Bornay-Llinaras, F. J., del Augilada Puente, C. del A. et al. (1997). Diagnosis of *Enterocytozoon bieneusi* (microsporidia) infections by polymerase chain reaction in stool samples using primers based on the region coding for small subunit ribosomal RNA. *Archives of Pathology and Laboratory Medicine* **121**, 874–9.
31. Weiss, J. B. (1993). PCR detection of *Giardia lamblia*. In *Diagnostic Molecular Microbiology: Principles and Applications* (Persing, D. H., Smith, T. F., Tenover, F. C. & White, T. J., eds) Pp. 480–5. Washington, DC: American Society for Microbiology.
32. Clark, C. G., Cross, G. A. M. & De Jonckheere, J. F. (1989). Evaluation of evolutionary divergence in the genes of *Naegleria* by analysis of ribosomal DNA plasmid restriction patterns. *Molecular and Biochemical Parasitology* **34**, 281–96.
33. Long, E. O. & David, I. B. (1980). Repeated genes in eukaryotes. *Annual Review of Biochemistry* **49**, 727–64.
34. Clark, C. G. & Diamond, L. S. (1991). Ribosomal RNA genes of 'pathogenic' and 'nonpathogenic' *Entamoeba histolytica* are distinct. *Molecular and Biochemical Parasitology* **49**, 297–302.