Chapter 5

Ribosomal RNA gene based diagnostic of
Giardia lamblia
5.1 Introduction

*Giardia lamblia*, the protozoan parasite is the cause of giardiasis in humans (Nash, 1989; Wolfe, 1990; Adam, 1991). It inhabits the small intestine and cause diarrhoea with a diverge range of symptoms ranging from asymptomatic carrier stage to severe malabsorption, diarrhoea and weight loss (Farthing, 1990). It is the most common cause of diarrhoeal illness sometimes leading to epidemics (Levine et al., 1990). The management of the disease is made difficult by the availability of only insensitive and nonspecific diagnostic methods. Conventionally the diagnosis is made by microscopic examination of stool samples for the presence of cysts or trophozoites (Goka et al., 1990). *Giardia* cysts are shed sporadically and their number may vary from day to day (Garcia and Bruckner, 1997). Moreover despite symptoms some patients may not excrete cysts for several weeks. So routine examinations of stool specimens collected on consecutive days or even within the recommended ten days time frame may not confirm infection with this organism. Sampling over long periods of time may therefore be necessary (Danciger and Lopez, 1975; Hiatt et al., 1995). These problems reduce the sensitivity of the method. Only 50% to 70% sensitivity has been reported by this technique (Bruke, 1997). Another reason for low sensitivity is the examination of stool samples by unskilled personnel (Danciger and Lopez, 1975). Other methods such as jejunal biopsy and duodenal aspirate examination gave better results but they find little utility, as they are invasive (Bown, et al., 1996).

Immunological method of detection of the parasite based on detection of *Giardia* specific antibody in serum and antigens in the stool shows better result over conventional microscopy (Sun, 1980; Ungar et al., 1984; Goldin et al., 1990; Chaudhuri et al., 1991; Zimmerman and Needham, 1995; Dedcova et al., 1999). Diagnosis based on indirect immunofluorescence to detect *Giardia* cysts in stool samples using cyst-specific anti *Giardia lamblia* monoclonal antibody has also been reported (Winiecka-krusnell and Linder, 1995). Infact a number of immunoassay kits (enzyme immunoassay and direct fluoreescence) are available in the market for detection of *Giardia* in fecal specimens (Garcia and Shimizu, 1997). Though above 90% specificity and sensitivity has been claimed for detection of the parasite by these methods, determination of serum anti-*Giardia* antibodies has not been found satisfactory for the demonstration of giardiasis (Jokipii et al., 1988; Shetty et al., 1990). There is always chance of getting false 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 diagnostic methods for being highly specific and at the same time sensitive as it relies on the sequence of the DNA. Moseley et al., first reported the use of DNA probes to detect organisms harboring the genes encoding LT enterotoxins in enterotoxigenic *E. coli* in 1980 (Moseley et al., 1980). Thereafter lots of DNA based diagnostic probes for detection of different pathogens have been reported (Totten et al., 1983; Boileau et al., 1984; Ambinder et al., 1985). Wirth and Pratt, were the first to demonstrate the utility of DNA probe for direct detection of parasitic agents in clinical lesions (Wirth and Pratt, 1982). A number of DNA-probe based diagnosis for giardiasis has also been reported. In 1989 Butcher reported a DNA probe for diagnosis of *Giardia* (Butcher and Farthing, 1989). In 1990 Lewis et al. reported total genomic DNA as probe which can detect as much as 10μg of *G. lamblia* DNA, 10⁴ trophozoites and 10⁴ cysts (Lewis et al., 1990). But it shows cross reactivity with high amount (5μg) of *T. cruzi*. Ribosomal DNA based probe for diagnosis of giardiasis has also been reported from our laboratory (Sil et al., 1998).

The technique of gene amplification by polymerase chain reaction (PCR) (Saiki and Amhein, 1985) has revolutionized the field of molecular diagnosis because of its power to produce many copies of a desired, previously undetectable nucleic acid target. PCR allows detection of organisms present in very low numbers by amplifying specific DNA segments in a few hours. This is a definite advantage over DNA-probe based diagnosis and people rapidly shifted over to PCR-based diagnosis. Within a span of few years number of reports came on PCR based diagnosis and epidemiological studies of different organisms. PCR has been used for the detection of bacterial (Varela et al., 1994; Li et al., 1996) viral (Sacramento et al., 1991; Wilde et al., 1990) and protozoal (da Silva et al., 1997; Ombrouck et al., 1997; Sanuki et al., 1997) pathogens. The first utilization of PCR in detection of *Giardia* from wastewater was done by Atlas who employed the giardin gene as the amplification target (Atlas, 1991). A 163bp region of heat shock protein (HSP) gene region of *Giardia* has also been employed in the detection of the parasite (Abbasszadegan et al., 1993). Weiss was the first to report the PCR detection of the parasite from fecal specimen targeting a 183bp region of the SSU rRNA (Weiss, 1993). But all these methods required rigorous processing of water or clinical samples which often takes a day.

Ribosomal RNA gene is a very good target for amplification by PCR as it is present in higher copy numbers (Waters, and McCutchan, 1990). In *G. lamblia* there are about 63-132 copies of rRNA gene per nucleus arranged in tandem repeat (Boothroyd et al., 1987; Sil et al., 1998). The present work is aimed at developing a PCR based assay to amplify and detect a
segment of the IGS region of rRNA of *Giardia lamblia* which were found to be *Giardia* specific (Sil, et al., 1998). The assay was compared with other existing methods for routine diagnosis of *Giardia* directly from fecal sample.

5.2. Methods

5.2.1. Cultivation of enteropathogens

Three different enteropathogens namely *Escherichia coli, Shigella dysentrie* and *Entamoeba histolytica* along with *Giardia lamblia* were cultured in different media. *G. lamblia* and *E. histolytica* were grown in TYI-S-33 medium (section 3.3.1 and 4.1) at 37°C. *E. coli* and *S. dysenteriae* were cultured at 37°C in Tryptic Soy Broth (section 3.3.2 and 4.2).

5.2.2. DNA isolation

The procedure for isolation of DNA from the different enteropathogens was as mentioned in section 4.3.1. Plasmid DNA was isolated as mentioned in section 4.4.

5.2.3. Designing of oligonucleotide primers

Primers were designed from the intergenic spacer region (IGS) region of rRNA operon of *G. lamblia* that were found to be *G. lamblia* specific (Sil et al., 1998). The IGS region was sequenced and two pairs of primers were designed from the sequence. For the first PCR, primers pair AS1 and AS2 (Table 5.1) which specifies a 552-bp fragment was used (Fig. 5.1). For the second (nested) PCR, the nested primer pair used was SG3 and SG4 that will amplify a 320-bp region within the first PCR product.

5.2.4. Polymerase chain reaction

The PCR was carried out in 25μl volume containing 5μl of DNA extract or 1μl of the first PCR product. Reaction mixture contained 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 250 μM deoxynucleotide triphosphate mix (dATP, dGTP, dCTP, and dTTP), 3U of Taq Polymerase (Bangalore Genei) and 200ng of each primers. 30 cycles were performed for both reactions with denaturation at 95°C for 60 seconds, annealing at 55°C for 60 seconds and extension at 72°C for 60 seconds. The reaction mixture was incubated initially at
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95°C for 5 min for complete denaturation of the template and at 72°C for 5 min for final extension at the end of the reaction. Products of first PCR were visualized on a 1.5 % agarose gel and for nested PCR in 2% agarose gel.

![Amplification strategy](image)

**Fig. 5.1.** Amplification strategy from the intergenic spacer region (IGS) of the multicopy rRNA gene of *Giardia lamblia* showing positions of primers. AS1 and AS2 amplifies a 552 bp segment of the IGS. Primers SG3 and SG4 amplifies a 320 bp fragment within the first PCR product. SS: small subunit ribosomal RNA; LS: large subunit ribosomal RNA; P: PstI.

<table>
<thead>
<tr>
<th>Function (external)</th>
<th>Name</th>
<th>Nucleotide sequence, 5' to 3'</th>
<th>Location</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upstream primer</td>
<td>AS1</td>
<td>5' - CGACCCGGAGACACGCCC-3'</td>
<td>4003-20</td>
<td>69°C</td>
</tr>
<tr>
<td>Downstream primer</td>
<td>AS2</td>
<td>5' - AGGACTGCATATCACGGC-3'</td>
<td>4539-55</td>
<td>61°C</td>
</tr>
<tr>
<td>Upstream primer (internal)</td>
<td>SG3</td>
<td>5' - AGAGCAGGCAGATCCCCCG-3'</td>
<td>4105-22</td>
<td>67°C</td>
</tr>
<tr>
<td>Downstream primer (internal)</td>
<td>SG4</td>
<td>5' - AATTGGAGGCTGACTGTG-3'</td>
<td>4408-25</td>
<td>59°C</td>
</tr>
</tbody>
</table>

Table 5.1. PCR primers for detection of *G. lamblia*.
5. 2. 5. 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 6 to 8 times, flatulence, mucus in stool, anorexia, nausea and vomiting) were categorized into Group I. Stool examination of this group showed presence of cyst and/or trophozoites of *Giardia lamblia*. Group II comprised of 45 healthy control subjects who had no complaints of diarrhoea or dysentry for the preceding one month. In Group III, 70 subjects were included whose stools were negative for *Giardia lamblia* by microscopy but positive for other parasites, e.g. *Entamoeba coli*, *Entamoeba histolytica*, *Trichuris trichura*, *Ascaris*, *Entamoeba hartmanni* and hook-worm (Table 5.2).

5. 2. 6. Stool collection and examination

Stool samples were collected from patients with various gastrointestinal disorders and diarrhoea. Each stool sample was examined under microscope by wet mount (Lugol’s iodine, normal saline) and permanent preparation. Doubtful samples were rechecked after formalin ether concentration technique (Ritchie, 1948). For PCR and antigen analysis stool samples were stored at -70°C until used.

5. 2. 7. Sample Preparation for PCR

100 µl of stool samples from clinical subjects was boiled for 10 min followed by centrifugation at 12,000g for 5 min. 20µl of the supernatant was diluted to 200µl. From this diluted sample 5µl was used for PCR using primers AS1 and AS2. 1µl of the first PCR product was used for nested PCR using primers SG3 and SG4.

5. 2. 8. Antigen preparation

Antigen was prepared from 48 to 72 hours old cultures of *Giardia lamblia*. Briefly, *Giardia* trophozoites were dislodged from the walls of culture tubes by chilling in an ice bath for 10 min followed by centrifugation at 800g for 5min. Pooled trophozoites were washed thrice in sterile phosphate buffered saline (PBS, pH 7.4) and finally the counted cells were resuspended in measured volume of the same buffer. The suspension was then sonicated with eight 30 seconds
bursts (MSE sonicator, U.K). The sonicated material was centrifuged at 10,000g for 20 min at 4°C and the supernatant collected was termed as crude soluble antigen (CSA). Protein contents were estimated by the method of Bradford (Bradford, 1976).

5.2.9. Antisera production

Production of antibodies in albino rabbits and guinea pigs against the whole Giardia trophozoites was essentially the same as described earlier (Das et al., 1993).

5.2.10. Immunological assays

5.2.10.1. Enzyme linked immunoabsorbent assay (ELISA)

5.2.10.1.1. Processing of stool samples

1 gm of fresh fecal sample (collected within 2 to 3 hrs) was homogenized in 2 ml of 1X PBS (pH 7.2) containing 0.05 % sodium dodecyl sulphate (Muro et al., 1987). Coarse debris were eliminated by centrifugation at 500g for 20 min, the clear supernatant was collected and 1mM phenylmethylsulphonylfluoride (PMSF), a protease inhibitor was added and kept frozen at -20°C until used.

5.2.10.1.2. Purification of Immunoglobulin G

IgG from hyperimmune serum of rabbit and guinea pig raised against the whole Giardia trophozoites was purified by affinity chromatography on a Protein A Sepharose-CL-4B column as described elsewhere (Chaudhuri et al., 1991).

5.2.10.1.3. Multilayer ELISA

The method used was a modification of the double antibody sandwich ELISA described by Ungar (Ungar et al., 1985) and Sengupta (Sengupta et al., 1993). In a model system, a polystyrene microtiter plate (Nunc, Denmark) was coated with 3µg/well of IgG isolated from anti-Giardia guinea pig serum in 0.05 M carbonate-bicarbonate buffer (pH 9.6) (section 3.4.13.2) for two hrs at 37°C. The wells were washed to remove the unbound antibody and the extra binding sites were blocked with 200 µl/well of 3% BSA (w/v) in 10mM PBS for overnight at 4°C. Varying concentrations of standard soluble antigen of axenic G. lamblia trophozoites either in PBS or with parasitologically negative stool samples (100µl/well) were incubated overnight at
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4°C. Wells receiving IgG purified from normal rabbit serum and no antigen were used as controls. The washed wells were incubated with 2 µg/100µl well (optimal concentration determined by checker board titration) of purified IgG from anti-*Giardia* rabbit serum for 1.5 hr at 37°C. The immune reaction was performed by adding goat anti-rabbit IgG labelled with HRP as conjugate followed by OPD as the substrate in presence of 0.03% H2O2 in 0.1 M citrate buffer (pH 3.7) (section 3.1.13.3). The reaction was stopped with 2N H2SO4 and the optical density at 492 nm was measured in a TiterTek Multiscan ELISA reader (Flow Laboratories Inc., McLean, USA).

5.2.10.2. Countercurrent Immunoelectrophoresis (CIEP)

The test was performed according to the method described by Chaudhuri et al (Chaudhuri et al., 1988). Briefly, 3 ml of 0.9% agarose in barbital buffer (pH 8.6, 0.12 M) (section 3.4.13.4) was layered on a clean grease-free slide and kept at room temperature for solidification. Processed stool antigen and antibody were placed in respective wells and electrophoresis was carried out in electrophoresis chamber (Shandon, U. K) at a constant voltage of 160 V or 10mA per slide for 20-30 min. Results were recorded immediately after the test and incubating the slides at 4°C for 30 min.

5.3. Results

5.3.1. Amplification of rRNA gene locus from cultivated *Giardia* trophozoites

5.3.1.1. Specificity of Primers

PCR was done to test the specificity of the primers AS1 and AS2. Reactions were carried out using the primers AS1 and AS2 and 200ng genomic DNAs of *Giardia lamblia* and other enteropathogens (Fig 5.2). The 552 bp amplified product was obtained only for *G. lamblia*. The primers could not differentiate between two strains of *Giardia* PD1 and PD2 (Fig. 5.2, lanes 2 and 3) but could differentiate between *Giardia* and three other enteropathogens. This was expected since the primers were derived from the intergenic regions of the rRNA gene of *Giardia lamblia* that is *Giardia* specific.
5.3.1.2. Sensitivity of the assay

The PCR was initially done for the detection of *Giardia* rRNA gene sequence present in a plasmid. Serial dilution of the plasmid DNA was used as template in 25μl reaction. 10μl of the PCR product was run in 1.5% agarose gel. It was seen that the 552bp amplified product could be visualised in gel after PCR amplification from as little as 2.5pg of plasmid (Fig. 5.3).

We next evaluated the sensitivity of the assay. Different number of cultured *Giardia* trophozoites were taken in 20μl water and boiled for 10 min. It was then centrifuged for 10 min and the supernatant was subjected to PCR amplification in a total volume of 25μl. The product was analysed in 1.5% agarose gel. A minimum of 10 trophozoites could be detected (Fig. 5.4). In other words taking the DNA content of one trophozoite to be 0.144pg (Erlandsen and Rasch, 1994) the reaction was sensitive enough to detect 1.44 pg of *Giardia* genomic DNA.
5. 3. 2. Amplification of rRNA gene locus from cultivated *Giardia* trophozoites mixed with stool samples

To assess the utility of the method for diagnosis, the assay system was employed on stool samples. This was initially investigated by using a model system similar to the one above, in which different dilution of cultivated *Giardia* trophozoites were added to a fecal specimen obtained from an adult with diarrhoea but microscopically negative of *Giardia*. Direct PCR of feces was known to be less sensitive than PCR of purified DNA because of the presence of inhibitory compound in stool like bilirubin, urobilinogens and the bile salts (Widjojoatmodjo et al., 1992). One way of removal of inhibitory substances was to purify the sample by treatment with a combination of detergent, protease and heat treatment (Weiss, 1993). But this procedure takes a lot of time and also adds expense to sample preparation that is disadvantageous when handling a lot of samples. Another way of overcoming the inhibitory effect was to do PCR in presence of bovine serum albumin (BSA) which has wide-spread use for relieving interference in PCR as well as in a variety of enzymatic reactions (Hoss, et al., 1992; Loomis, 1974). We have used high concentration of BSA (400ng/μl) but that could not overcome the inhibitory effect of stool. The easiest and cheapest way of removing the inhibition was by diluting the stool samples. This had been successfully utilised for detection of *Vibrio cholera* in stool samples (Varela, et al., 1994). They had found that 100 times dilution of stool samples was sufficient to prevent inhibition of PCR reaction by contaminants. In our case the disadvantage of diluting the fecal specimen was that the number of *Giardia* parasite present in clinical fecal sample was too low and there was every chance of diluting out the parasite or the DNA.

We tried different dilutions and found that fifty times dilution could relieve the inhibition. Different numbers of *Giardia* trophozoites were taken in 100μl of control fecal sample that was found negative of *Giardia* and boiled for 10 min. It was then centrifuged at 12,000g. 20μl of the supernatant was taken and diluted to 200μl. From the diluted supernatant 5μl was used for PCR amplification in 25μl reaction. The entire PCR product was run in 1.5% agarose gel. It was observed that a minimum of 2000 trophozoites could be detected against a smeary background.
(Fig 5. 5). In other word fifty times or more dilution of stool was optimal in minimizing the inhibition and specific amplification from 2000 trophozoites.

Fig. 5. 3. Sensitivity limit PCR using rDNA plasmid and primer pair AS1 and AS2. Figure represents ethidium bromide stained 1.5% agarose gel of PCR product obtained by using different amounts of plasmid DNA. Lane 1: λ Hind III marker. The 564bp band is shown; Lane 2 to 7: amplification using 250ng, 25ng, 2.5ng, 250pg, 25pg and 2.5pg plasmid DNA respectively; The 552bp amplified product is shown. Lane 8: negative control where no template was added.

Fig. 5. 4. Limit of detection of Giardia cells by PCR using primer pair AS1 and AS2. Figure represents 1.5% EtBr stained agarose gel of PCR product obtained by using different numbers of Giardia trophozoites in 25µl reaction. 10µl of PCR product was run in each lane. Lane 1: λ Hind III marker. The 564bp band is shown by arrowhead. Lane 2: positive control where PCR was done using rDNA plasmid as template; Lane 3- 6: PCR with 1000, 500, 100 and 10 trophozoites. Lane 7: negative control where reaction was done without any trophozoites. The 552bp amplified product was shown by arrowhead.
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**Fig. 5.** PCR amplification from control negative fecal sample mixed with different numbers of *Giardia* cells using primers AS1 and AS2. Figure represents EtBr stained 1.5% agarose gel of PCR product. Lane 1: λ HindIII marker. The position of the 564bp band is shown by arrowhead. Lane 2 to 6: amplification from stool samples mixed with 2000, 5000, 10000, 15000 and 20000 cells. The 552bp amplified product is shown by arrowhead. Lane 7: amplification from negative control stool where no cells were mixed.

**Fig. 5.6.** PCR amplification of negative control fecal samples 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. In fig A, lane 1: 1Kb DNA ladder with the 344bp and 298bp marker band shown by arrowheads. Lane 2: PCR amplification from negative control fecal sample lane 3: positive control reaction where amplification was done with *Giardia* genomic DNA and nested primers. In fig B, lane 1: φX 174 HaeIII marker with the position of 310bp marker band shown by arrowhead. Lanes 2: a positive control where PCR amplification was obtained from *Giardia* genomic DNA. Lanes 3-7: amplification from 100μl fecal samples mixed with 10, 100, 200, 500 and 1000 trophozoites respectively. The position of the 320bp amplified PCR product is shown by arrowhead.
5. 3. 3. Amplification of rRNA gene locus from cultivated *Giardia* trophozoites mixed with stool samples by nested PCR

In reality it was not possible to get so many trophozoites in 100μl stool. To increase the sensitivity nested PCR protocol was included in the assay. Nested primers SG3 and SG4 were designed that would amplify a 320bp region within the first PCR product (Fig. 5. 1). Different number of *Giardia* trophozoites were mixed with 100μl of control stool specimen boiled for 10 min and centrifuged at 12000g for 10 min. 20μl of the supernatant was diluted to 200μl and 5μl of the diluted sample was subjected to PCR using primers AS1 and AS2 in a 25μl reaction. 1μl of the PCR product was subjected to nested PCR using primers SG3 and SG4. Inclusion of the nested PCR protocol increased the detection limit to 10 trophozoites (Fig. 5. 6) if not less. The authenticity of the 320bp nested PCR product was further checked by digesting the PCR product with PstI which yielded a 245bp fragment due to the presence of one PstI site in the amplified region of the rRNA (figure not shown).

5. 3. 4. Amplification of rRNA gene locus from clinical samples

Having tested the utility of our method we evaluated whether this method could be used to detect *Giardia* from clinical samples. 30 clinical samples reported to be positive for *Giardia* both by microscopy and immunological assays were subjected to PCR analysis followed by nested PCR. A 100% correlation was obtained with the other methods (Table 5.2, Fig 5. 7). 45 fecal specimen from healthy subjects found to be negative of *Giardia* infection with other assays were also analysed. Here also 100% correlation was observed (Table 5.2).

5. 3. 5. Comparative Evaluation

In this study, we evaluated the utility of different methodologies, like microscopy and immunological assays viz. CIEP and ELISA along with the PCR method to determine the presence of *Giardia* in stool samples of different categories of patients and controls. The comparative evaluation of four tests in different categories of patients showed a 100% correlation among nested PCR, microscopy and ELISA in Group I (suggestive giardiasis cases) (Table 5. 2).
However CIEP showed 23.4% less sensitivity than microscopy and PCR. In Group II (control subjects) all the tests showed similar observation as none of the tests demonstrated positive results (Table 4.2). However, in Group III cases (random hospital patients suffering from gastrointestinal problems other than giardiasis as found by microscopy) CIEP, ELISA and nested PCR showed better results than microscopic examination. Among the three tests (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 7.14% and 12.85% respectively (Table 5.2).

In further analysis, it was observed that out of 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 7 were only positive by microscopy, ELISA and PCR. These results probably suggest the sensitivity and specificity of each test, which was again reflected in the randomly selected cases (Group III) where PCR showed more positivity than CIEP and ELISA. Here out of 70 samples examined, 56 were negative by all the tests. Among the 14 positives, 5 were by CIEP, ELISA and PCR, 4 by ELISA and PCR and 5 only by PCR (Table 5.3).

Table 5.2. Evaluation of different diagnostic tests in the detection of *G. lamblia* infection in various categories of patients.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Clinical symptoms</th>
<th>No. examined</th>
<th>Microscopy (%)</th>
<th>CIEP (%)</th>
<th>ELISA (%)</th>
<th>Nested PCR (%)</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Giardiasis</td>
<td>30</td>
<td>100</td>
<td>76.6</td>
<td>100</td>
<td>100</td>
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<td>II</td>
<td>Healthy subjects (controls)</td>
<td>45</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>III</td>
<td>Random hospital patients suffering from gastrointestinal problems other than giardiasis</td>
<td>70</td>
<td>Nil</td>
<td>7.14</td>
<td>12.85</td>
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</table>

*Basis of characterization of the three groups is explained in the text.*
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Fig. 5. Detection of *Giardia lamblia* in clinical sample by nested PCR. Figure represents EtBr stained 2% agarose gel of 320bp nested PCR product. Lane 1: φX174 HaeIII marker. The position of the 310bp product shown by arrowhead. Lane 2: positive control where amplification is obtained from plasmid containing rDNA. Lane 3 to 8: amplification from different clinical samples that were tested positive of *Giardia* both by microscopy and immunological techniques.

Table 5.3. Break-up of positive and negative samples in each group with different tests.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. Examined</th>
<th>Microscopy</th>
<th>CIEP</th>
<th>ELISA</th>
<th>Nested PCR</th>
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<tr>
<td>I</td>
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5.4. Discussion

Timely diagnosis of a disease is a prerequisite for quick and effective therapy. The diagnostic method should be highly sensitive, specific, reproducible, cost effective and also should not be time consuming. Conventionally, *Giardia lamblia* is diagnosed by microscopic examination of stool or by different immunoassays. Both the methods have their own limitations. Detection system based on DNA analysis successfully meets all the requirements for good diagnostic tools. Sensitive methods of nucleic acid detection have been devised for the specific detection of small amount of nucleic acid (Moseley et al., 1982; Totten et al., 1983; Ambinder et al. 1985). The technique of gene amplification by polymerase chain reaction (Saiki and Arnheim, 1985) has revolutionized the fields of molecular biology because of its power to produce many copies of a desired, previously undetectable nucleic acid target. But before that, techniques must be devised for the amplification of microbial nucleic acid extracted from heterogenous human body fluids especially stool. Since there are many factors in stool that inhibits PCR reaction, methods that allow for nucleic acid amplification without substantial levels of inhibition or inconsistency must be devised. Also for obtaining best results a careful selection of primers for amplification of target molecule and extraction of template DNA has been suggested.

We have previously shown that the intergenic spacer region (IGS) of the rRNA gene of *G. lamblia* can be used as probe for identification of *G. lamblia* from other enteric pathogens (Sil et al., 1998). In the present investigation primers designed from this region had been used for PCR detection of giardiasis. The primer pair AS1 and AS2 were found highly specific and sensitive in amplification of a 552bp region of the IGS because it didn't show any amplification from genomic DNAs of other enteropathogens and detected as little as 10 *Giardia* trophozoites. However the assay was not found so effective while amplification from stool samples. Different inhibitory substances present in stool hampered the PCR reaction. The inhibitory effect could be removed by extensive purification to generate PCR-compatible material. However, because purification adds to the time and expense of sample preparation, as well as to the loss of target nucleic acids, a more satisfying approach to the problem of PCR inhibition was to relieve the interference rather than attempt to remove all the offending substances. We chose to relieve the inhibition by dilution of the fecal sample. But this resulted in decreased sensitivity of the method. The sensitivity was restored by inclusion of the nested PCR protocol that amplified a 320bp region within the first PCR product. An added advantage of using the nested PCR step was that it negated the chance of any false positive results in the first PCR that is commonly observed during amplification from fecal samples. As a double check a PstI site was included in the
amplified nested PCR product that indicate the uniqueness of the amplified DNA sequence. This type of follow up of the PCR product of specific size is needed when some controversy arises. Comparative study of 145 clinical samples examined by microscopy, CIEP, ELISA and nested PCR showed the better efficiency of nested PCR detection system over other diagnostic method. A 100% correlation among nested PCR, microscopy and ELISA were found in both Group I (suggestive giardiasis cases) and Group II (control subjects). In Group III cases nested PCR showed better diagnostic results where out of the 14 cases that were found to be positive by PCR, ELISA and CIEP, 9 were negative by only CIEP and 5 were negative by both CIEP and ELISA. None of these could be detected by microscopy. Further all the clinical samples that showed positive results by microscopy, CIEP and ELISA were also found to be positive by PCR. No PCR-negative cases were found to be positive by the other detection system. Thus this new assay procedure appears to be a simple, rapid, accurate and sensitive method for the diagnosis of giardiasis. The PCR positive and ELISA negative results shows the greater sensitivity and specificity of PCR over ELISA. It is important to note that the nested PCR step act as a double check whereby if there is any false amplification in the first PCR then there would be no amplification in the second PCR.

PCR-based diagnosis of giardiasis has been reported earlier targeting different genes of *G. lamblia*. Primers specific for *Giardia* targeted a 183-bp product from the small subunit (SSU) rRNA gene (Weiss, 1993); 218-and 171-bp amplicon from a giardin gene (Mahbubani et al., 1991; Mahbubani et al., 1992); and a 163-bp product from a heat shock protein (Abbaszadegan et al., 1993) gene. An effective comparative study of these primers for detection of *G. lamblia* in water had been done (Rochelle et al., 1997). The MAH433F-MAH592R primer pair that amplify 218-bp DNA from the giardin gene can distinguish between *G. lamblia* and *G. muris* but the sensitivity was very low and so a second round of PCR of 40 cycles with 5µl of the product from the first reaction mixture was required. The ABB97F-ABB220R primer pair that amplifies a 163-bp DNA from heat shock protein gene gave amplification from both *G. lamblia* and *G. muris* though the amplification products differ considerably in size on the basis of which the two species could be distinguished. The WEI primer pair that amplifies a 183-bp product from the small subunit rRNA was found to give inefficient amplification of the correct-size product (183-bp) from purified cyst and no amplification from cysts seeded into environmental water samples. Moreover in addition to *G. lamblia* and *G. muris*, these primers also produced amplification products of various sizes from *Entamoeba histolytica*, *Leishmania major* and *Trypanosoma brucei*. This may be because the primer targets the SSU rRNA gene that is known to have
conserved sequences all along the evolutionary tree. Thus it was seen that none of the primer pair had the potential to meet all the requirements of a successful diagnostic tool.

In this respect our nested PCR method that target the IGS region of high copy number rDNA and is very specific for *Giardia lamblia* proved to be better than the previously mentioned PCR-based diagnostics. The nested PCR step helped in increasing the sensitivity and specificity of the method. The simplicity and cost effectiveness added to its advantage. Thus the highly sensitive nested PCR method is expected to contribute significantly in diagnosis of giardiasis.