5. CHAPTER-II

DIAGNOSIS OF AMOEBIC LIVER ABSCESS
Infection with *Entamoeba histolytica*, results in 34 million to 50 million symptomatic cases of amoebiasis worldwide each year, causing 40 to 100 thousand deaths annually (WHO, 1997). Mortality from amoebiasis is mainly due to extra-intestinal pathology, of which amoebic liver abscess (ALA) is the most common. If left untreated, ALA can rupture into neighboring tissue and spread to the brain and other organs via circulation producing serious morbidity and mortality. It is difficult to differentiate clinically the ALA from pyogenic liver abscess (PLA) as well as from other space occupying lesions of liver such as hydatid cyst and liver hepatoma (Smoger et al., 1998; Kasper et al., 2005).

Imaging techniques like ultrasound, computed tomography, and magnetic resonance although are highly sensitive to detect abscesses in the liver of varied aetiology, however fail to distinguish specifically ALA from that of PLA. Less than one third of patients with ALA have active diarrhea (Kasper et al., 2005). Hence, stool microscopy and stool antigen detection is not very helpful for diagnosis of ALA. In fact less than only 10% of ALA patients have identifiable *E. histolytica* in stool specimens (Katzenstein et al., 1982).

Laboratory diagnosis of ALA is usually established by conventional antibody-based serological tests. Nevertheless, the main disadvantage with antibody detection is that serum antibody levels in individuals living in endemic areas, continues to remain positive even for years after infection with *E. histolytica* (Gandhi et al., 1987; Jackson et al., 1984; Yang and Kennedy, 1979). The
demonstration of amoebic antibodies in the serum, therefore, fails to denote the amoebic infection whether it is recent or old. Furthermore, serum amoebic antibodies are not demonstrated in up to 10% of the patients with acute ALA (Kasper et al., 2005).

The demonstration of *E. histolytica* trophozoite in liver abscess pus aspirates by microscopy confirms the diagnosis of ALA, but in best of the laboratories, the amoebic trophozoites can be demonstrated in only 15% of the liver pus (Parija, 1993). Since the trophozoites of *E. histolytica* are found mainly in the periphery of the abscess diagnosis of ALA by culture of liver pus for *E. histolytica* is also unsatisfactory (Zaman et al., 2000).

Demonstration of amoebic antigen and DNA in the clinical specimens is a recent approach for specific diagnosis of the ALA. A monoclonal antibody-based second generation TechLab enzyme-linked immunosorbent assay (ELISA) kit (Blacksburg, Va.) has been reported to be 78% and 40.7% sensitive for the detection of *E. histolytica* lectin antigen in the serum and liver pus respectively for the diagnosis of ALA (Haque et al., 2000). Studies conducted in various laboratories worldwide including ours have shown that polymerase chain reaction (PCR) is a sensitive and specific method for detecting *Entamoeba* DNA in stool samples and for differentiating the morphologically similar *E. histolytica* from *Entamoeba dispar* and *Entamoeba moshkovskii* (Hamzah et al., 2006; Fotedar et al., 2007; Lebbad and Svard, 2005; Roy et al., 2005; Freitas et al., 2004; Verweij et al., 2004; Nunez et al., 2001; Ali et al., 2003; Parija and Khairnar, 2005; Khairnar and Parija, 2007). However, only few studies using the PCR have been reported for
the detection of *E. histolytica* DNA in liver abscess pus for the diagnosis of the ALA (Khan et al., 2006; Zengzhu et al., 1999; Zaman et al., 2000; Haque et al., 2000).

In the present study, therefore, three different methods of PCR were evaluated for the detection of *Entamoeba* DNA in the liver abscess pus for the diagnosis of ALA.

**OBJECTIVE**

To develop and evaluate polymerase chain reaction (PCR) for demonstration of *Entamoeba* DNA in the liver abscess pus for the diagnosis of amoebic liver abscess (ALA).

**MATERIALS AND METHODS**

1. **Sample details**

The subjects in the present study included 139 patients provisionally diagnosed as ALA, who were admitted to JIPMER hospital, Puducherry, as well as 43 controls during a period from September 2004 to March 2006. The provisional diagnosis of ALA was made by the physicians on the basis of patient’s history and clinical features, unfortunately these features are often nonspecific to confirm the diagnosis.
of ALA. Of the 139 provisionally diagnosed ALA patients, 102 had received prior treatment and 37 did not receive prior treatment with metronidazole. The patients and controls were residing in neighboring area of Puducherry.

The control group included 43 individuals who had no history of recent dysentery or diarrhea and whose stool samples were negative for *E. histolytica* infection by microscopy and culture. Thirty five of the controls were healthy asymtomatic volunteers, and the other 8 patients included, hydatid cyst in liver (n=2), liver hepatoma (n=1), liver cirrhosis (n=3), and viral hepatitis (n=2).

The diagnosis of ALA was established on the basis of radiological, symptomatological and laboratory criteria as follows (Kasper et al., 2005; Haque et al., 2000): (i) a space-occupying lesion in the liver diagnosed by ultrasound and suggestive of abscess, (ii) clinical symptoms (fever, pain in the right hypochondrium (often referred to the epigastrium), lower chest, back, or tip of the right shoulder), (iii) enlarged and/or tender liver, usually without jaundice, (iv) raised right dome of the diaphragm on chest radiograph, (v) improvement after treatment with anti-amoebic drugs (e.g., metronidazole), (vi) positive IHA of serum antibody showing a titer (≥ 1:128) against *E. histolytica*; and (vii) liver aspirate appeared like anchovy sauce but was bacteriologically sterile.

2. Sample collection

*Liver abscess pus*
The aspiration of liver abscess pus was indicated only under the following conditions (Kasper et al., 2005). (i) to rule out a pyogenic abscess; (ii) the failure to respond clinically in 3 to 5 days; (iii) the threat of imminent rupture; and (iv) the prevention of rupture of left-lobe abscess into the pericardium. The liver abscess pus aspirates were performed, only for clinical purposes as judged by the clinicians for the patient care and not for the purpose of this study. Liver abscess pus was obtained under ultrasound guidance from all 139 provisionally diagnosed ALA patients and was stored at -20°C in a sterile container until used.

**Blood**

Blood specimen was collected from all 139 provisionally diagnosed ALA patients and 43 control group individuals included in the study. Venous blood (5 ml) was collected in a sterile container; sera were separated and stored at -20°C until used.

**3. Microscopy for Entamoeba**

The liver abscess pus specimens were examined immediately after the aspiration of abscess. The liver abscess pus was first centrifuged at 2,500 g for 5 mins and a loopful (usually inoculating needle loop) of sediment was mixed with a drop of warm saline on a microscope slide. Microscopic examination of an amoebic abscess aspirate from liver may reveal haematophagous trophozoites.

**4. Culture for Entamoeba**
The liver abscess pus specimens were cultured for *Entamoeba* species in Locke-egg (LE) medium (NIH modification of Boeck and Drbohlav's medium) as previously described (Parija and Rao, 1995). The liver abscess pus was first centrifuged at 2,500g for 5 mins and a loopful of sediment was inoculated into the LE medium. It is to be noted that in case of culturing *Entamoeba* from liver abscess aspirates, since the abscess is sterile, bacterial flora (*ATCC Escherichia coli*) was added before inoculation of amoebae into xenic culture. The detailed protocol for preparation of NIH modification of Boeck and Drbohlav's medium is already mentioned in chapter-I. Briefly, a small quantity of liver abscess pus was inoculated in the biphasic egg slope medium and was incubated at 37°C for 48 hours. After 48 hours of incubation, the culture fluid in the tube was properly mixed and a drop of culture fluid was placed on a glass slide by a Pasteur pipette for microscopic examination.

5. Gram stain and bacterial culture for liver abscess pus aspirates

Gram staining of the direct smear and bacterial culture was done for all liver abscess pus aspirates. The liver abscess pus specimens were inoculated on to sheep blood agar, MacConkey agar and chocolate agar plates. The MacConkey agar plates were incubated at 37°C for 18–24 hours whereas the blood agar and chocolate agar plates were incubated in a candle jar at 37°C for 48 hours. Based on the colony morphology and result of culture smears, necessary biochemical tests were done to identify bacteria to the species level.
The protocol for Gram stain and bacterial culture for liver abscess pus sample is mentioned in annexure 3.

6. Detection of anti-amoebic antibodies in serum by IHA

The Rapid-IHA was performed on serum specimen as per the method described earlier (Parija et al., 1989). A titer of $\geq 1: 128$ was considered positive for ALA (Parija et al., 1988). The detailed protocol of Rapid-IHA test is already mentioned in chapter-I.

7. Detection of Gal/GalNAc lectin antigen in liver pus by TechLab *E. histolytica* II ELISA

The TechLab *E. histolytica* II test was performed on liver abscess pus specimens to detect *E. histolytica* specific Gal/GalNAc lectin antigen as per the method described earlier (Haque et al., 2000). Briefly, the liver abscess pus specimen was vortexed and centrifuged at $10,000 \times g$ for 10 min, and 100 $\mu l$ of the resulting undiluted supernatant was added to the micro titer well. The rest of the steps in ELISA for antigen detection in liver abscess pus specimen were similar to the protocol already described in details for antigen detection in stool specimen in chapter-I.

8. Detection of *Entamoeba* DNA in liver pus by PCR

*Extraction of Entamoeba genomic DNA*
The DNA was isolated from liver abscess pus specimens as follows.

1. 1ml of liver abscess fluid or pus (depending on the consistency of liver abscess aspirates) was taken in 1.5ml centrifuge tube and was centrifuged at 12,000 g for 8 minutes. After the centrifugation only 50μl of pellet was retained in the 1.5 ml centrifuge tube.

2. To the liver abscess pus specimen (50μl), 250 μl of lysis buffer (0.25% sodium dodecyl sulphate (SDS) in 0.1M EDTA pH 8.0) followed by of 100μg/ml of proteinase K was added and incubated at 55°C for 2-3 hour.

3. 75 μl of 3.5 M sodium chloride (NaCl), was added and mixed gently then 42 μl of 10% cetyltrimethylammonium bromide (CTAB)/0.7 M NaCl (heated to 55°C) was added, mixed and incubated at 65°C for 30 minutes.

   **Note:** CTAB is a cationic detergent that binds polysaccharides under these salt conditions. It is important to maintain the NaCl concentration above 0.5 M or a CTAB-DNA precipitate will form. Heating at 65°C will be necessary to dissolve the 10% CTAB and the stock should be reheated each time before use to reduce viscosity.

4. At room temperature 400 μl of chloroform was added, mixed well by inversion and centrifuged at 12,000 g in a micro centrifuge for 8 minutes.

   **Note:** This step precipitates the CTAB-Polysaccharide complex.

5. The supernatant was transferred to a fresh tube and 400 μl of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well by inversion and centrifuged as above.
6. The supernatant was transferred to a fresh tube and excess volumes of Ice cold 100% ethanol was added, mixed by inversion, and stored at room temperature for at least 10 minutes and then centrifuged for 15 minutes as above.

7. The supernatant was carefully discarded from the pellet and the pellet was washed in 200μl of 70% ethanol by spinning for 8 minutes as above.

8. The pellet was air dried and re-suspended in up to 50 μl of sterile distilled water.

9. The re-suspended DNA was passed through a DNA clean up spin column (Bangalore genei KT-62) (Optional step).

10. The extracted DNA was stored at -20°C.

The protocol for extraction of DNA from liver abscess pus specimen has been modified in our laboratory from CTAB DNA extraction protocol originally described for DNA extraction from amoebic culture (Clark and Diamond, 1991b).

The extracted DNA from liver abscess pus sample was passed through DNA clean-up spin columns (Bangalore Genei KT-62, Bangalore); to minimize PCR inhibitors as it improved the performance of amplification. The DNA was stored at -20°C until used.

**Quantification of DNA in liver abscess pus specimen**

DNA quantification in spin column purified DNA extract from liver abscess pus specimen was determined by UV absorbance using a Cintra 5 double beam spectrophotometer. DNA yields were calculated on the basis of UV absorbance x
dilution. The purity of the nucleic acid in the samples was estimated by the ratio of readings at 260nm and 280nm (OD\textsubscript{260}/OD\textsubscript{280}).

**Standard strains used**

*E. histolytica* HM-1: IMSS, *E. dispar* SAW760, and *E. moshkovskii* Laredo these were the standard strains used as positive control in the present study. The lyophilized DNA of these strains was generously gifted by Dr. C. Graham Clark from London School of Hygiene & Tropical Medicine, London, UK.

**16S-like r RNA gene based nested PCR- RFLP**

*Primers used*

Based on the sequences of the 16S-like r RNA gene of *E. histolytica* and *E. dispar*, nested set of primers (designated E-1/E-2, Eh-1/Eh-2, and Ed-1/Ed-2) were used, as previously described in 1998, Haque et al., (Haque et al., 1998a) for detecting *E. histolytica* and *E. dispar* in stool specimens.

In addition, based on the sequence of the 16S-like r RNA gene of *E. moshkovskii* Laredo, a nested set of primers (designated Em-1/Em-2 and nEm-1/nEm-2) were used, as previously described in 2003, Ali et al., (Ali et al., 2003) for detecting *E. moshkovskii* in stool specimens. The primer sequences used for 16S-like r RNA gene based nested PCR- RFLP are already shown in table I-I of chapter-I.
Primer validation

The sequence of primers E-1/E-2, Eh-1/Eh-2, Ed-1/Ed-2, Em-1/Em-2 and nEm-1/nEm-2 to be used for identification of *E. histolytica*, *E. dispar* and *E. moshkovskii* were first subjected to Basic Local Alignment Search Tool (BLAST) in the genome database of all organisms in the web site (http://www.ncbi.nlm.nih.gov/blast) and were found to be specific for the study. The amplified PCR products of *E. histolytica* species in liver abscess pus sample was confirmed by getting both the strands of DNA sequenced on ABI 377 sequencer (Indian Institute of Science, Bangalore, India). The sequencing was done using species specific primer. For example, the PCR product of *E. histolytica* DNA was sequenced using the species specific primer Eh-1/Eh-2. The sequences were analyzed for homology by using the nucleotide-nucleotide “BLAST” search feature located on the NCBI web site (http://www.ncbi.nlm.nih.gov/blast/Blast).

The identities between the sequencing result of PCR product of *E. histolytica* with the sequence deposited in GenBank accession number: X56991 was analyzed by using the “Align two sequences (bl2seq)” feature located on the NCBI web site (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

Nested PCR-RFLP protocol

The protocol for liver abscess pus PCR mix composition and PCR conditions were the same as described earlier for stool 16S-like r RNA gene based nested PCR-
RFLP for *E. histolytica*, *E. dispar* and *E. moshkovskii* in chapter-I, except that 1.0μl of 25mM MgCl₂ and 2.0μl of template DNA was added.

**Assessment of competition of non target DNA**

During the standardization to assess the competition of other non-target DNA present in liver abscess pus specimen with target DNA, the nested PCR was checked with reference DNA (DNA from standard culture of *E. histolytica*, *E. dispar* & *E. moshkovskii*) spiked with DNA from liver abscess pus (PLA pus negative for *E. histolytica*) followed by nested PCR amplification.

**Estimation of minimum number of Entamoeba cells detectable by nested PCR**

This was performed for *E. histolytica* with Locke-egg (LE) medium (NIH modification of Boeck and Drbohlav’s medium) liver abscess pus cultures; the amoebae were counted using a standard haemocytometer. The detailed protocol for counting of *Entamoeba* cells in Neubauer’s chamber (standard hemocytometer) is already mentioned in chapter-I.

A cell pellet containing $10^6$ cells was preferred for determining the detection limit of nested PCR for *E. histolytica*. The cell pellet containing $10^6$ cells of *E. histolytica* was diluted 10 folds in phosphate buffer saline (PBS) to obtain different concentration of cells, such as $10^5$, $10^4$, $10^3$, $10^2$ and 10 cells/ml. The different dilutions of cells ranging from $10^6$ to 10 cells/ml were centrifuged and the remaining pellet of each dilution was added to 0.05μl of liver abscess pus (PLA
pus negative for *E. histolytica* followed by DNA extraction and PCR as per the aforementioned protocol.

16S-like *r* RNA gene based nested multiplex PCR

**Primers design**

The genus and species specific primers were designed using nucleotide sequences of 16S-like rRNA gene of *E. dispar*, *E. histolytica* and *E. moshkovskii* Laredo deposited in GenBank [accession number: Z49256], [accession number: X56991] and [accession number: AF149906] respectively (already shown in Figure I-5, Figure I-6 and Figure I-7 of chapter-1). In this study, an internal amplification control (IAC) targeting human 18S ribosomal RNA gene was used to rule out false-negative results in clinical specimens (Figure II-1). All the primers were designed using Prime3 online software available at the website (http://frodo.wi.mit.edu/). The primers used in PCR are shown in table II-1.
Figure II-1

Primer design for human 18Sr RNA gene as IAC

<table>
<thead>
<tr>
<th>Size</th>
<th>Fwd IAC-1 5' GGCTTTGCTGTAGCTAGA 3'</th>
<th>3' 305 bp</th>
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<tbody>
<tr>
<td></td>
<td>Rwd IAC-2 5' CGTTAAGGATTTAAAGTGG 3'</td>
<td></td>
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</table>
Table II-1. Primer sequence used for 16S-like r RNA gene based nested multiplex PCR

<table>
<thead>
<tr>
<th>Genus specific primers (First PCR)</th>
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<tbody>
<tr>
<td><strong>Entamoeba genus</strong></td>
<td></td>
</tr>
<tr>
<td>E-1 5' TAAGATGCACCGAGAGCGAAA 3’ (forward primer)</td>
<td></td>
</tr>
<tr>
<td>E-2 5' GTACAAAGGGCAGGGACGTA 3’ (reverse primer)</td>
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<tr>
<th>Species specific primers (Second nested multiplex PCR)</th>
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</thead>
<tbody>
<tr>
<td><strong>E. histolytica species</strong></td>
<td></td>
</tr>
<tr>
<td>EH-1 5' AAGCATTGTTTCTAGATCTGAG 3’ (forward primer)</td>
<td></td>
</tr>
<tr>
<td>EH-2 5' AAGAGGTCTAAACGGAAATTAG 3’ (reverse primer)</td>
<td></td>
</tr>
</tbody>
</table>

| **E. moshkovskii species**                             |  |
| Mos-1 5' GAAACCAAGAGTTTCAACAAC 3’ (forward primer)   |  |
| Mos-2 5' CAATATAAGGCTTGAGTATG 3’ (reverse primer)     |  |

| **E. dispers species**                                 |  |
| ED-1 5' TCTAATTTGAGATTAGAATCTTCTGAGAAGAGGATTAG 3’ (forward primer) |  |
| ED-2 5' TCCCTACCTATTAGACATAGC 3’ (reverse primer)       |  |

<table>
<thead>
<tr>
<th>Internal amplification control (IAC) primer for PCR</th>
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<tbody>
<tr>
<td><strong>Human 18S ribosomal RNA gene</strong></td>
<td></td>
</tr>
<tr>
<td>IAC-1 5' GGCTTTTGGTGACTCTAGATA 3’ (forward primer)</td>
<td></td>
</tr>
<tr>
<td>IAC-2 5' CGTTAAGGATTAAAGTGG 3’ (reverse primer)</td>
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</tbody>
</table>

**Primer validation**

The primer sequences designed for *E. moshkovskii*, *E. histolytica*, *E. dispers* and IAC were subjected to Basic Local Alignment Search Tool (BLAST) in the genome database of all organisms available at the website (http://www.ncbi.nlm.nih.gov/blast/) and were found to be specific for the study. The amplified PCR products of *E. histolytica* species in liver abscess pus samples was confirmed by getting both the strands of DNA sequenced on ABI3730XL sequencer (Macrogen, Seoul, South Korea). The sequencing was done using species specific primers i.e. EH-1/EH-2 for *E. histolytica*. All sequences were analyzed for homology by using the nucleotide-nucleotide BLAST search feature available at the website (http://www.ncbi.nlm.nih.gov/blast/).
The identity between the sequencing results of PCR product of *E. histolytica* from liver abscess pus with the sequence deposited in GenBank [accession number: X56991] were analyzed by using the “Align two sequences (bl2seq)” feature available at the website (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

**Nested multiplex PCR protocol**

For a reaction volume of 25μl, comprising 2.5μl of 10X PCR buffer (Biogene), 2.0μl of 25mM MgCl₂ (Bangalore genei), 0.75μl of deoxyribo-nucleotide triphosphate mix (10 mM each dNTP, Biogene), 0.3μl (5 IU/μl) of *Taq* polymerase (Biogene), 10 picomoles of target DNA primers (IDT) and 5 picomoles of IAC primers (IDT) were added in genus and species specific PCR. The template DNA volume was 2μl for both genus and species specific PCR. The PCR tubes were finally placed in an Eppendorf Thermal cycler [Master cycler gradient].

The conditions for 16S-like r RNA gene based nested multiplex PCR for liver abscess pus were the same as described earlier for 16S-like r RNA gene based nested multiplex PCR for stool in chapter-I.

**Assessment of competition of non target DNA**

During the standardization to assess the competition of other non-target DNA present in liver abscess pus, urine and saliva specimen with target DNA, the nested multiplex PCR was checked with reference DNA (DNA from standard culture of *E. histolytica*, *E. dispar* and *E. moshkovskii*) spiked with DNA from liver abscess
pus (PLA pus negative for *E. histolytica*), urine (negative control group) and saliva (negative control group) followed by nested multiplex PCR amplification.

*Estimation of minimum number of Entamoeba cells detectable by nested multiplex PCR*

This was performed for *E. histolytica* with Locke-egg (LE) medium (NIH modification of Boeck and Drbohlav’s medium) liver abscess pus cultures; the amoebae were counted using a standard haemocytometer. The detailed protocol for counting of *Entamoeba* cells in Neubauer’s chamber (standard hemocytometer) is already mentioned in chapter-I.

A cell pellet containing $10^6$ cells was preferred for determining the detection limit of nested multiplex PCR for *E. histolytica*. The cell pellet containing $10^6$ cells of *E. histolytica* was diluted 10 folds in phosphate buffer saline (PBS) to obtain different concentration of cells, such as $10^5$, $10^4$, $10^3$, $10^2$ and 10 cells/ml. The different dilutions of cells ranging from $10^6$ to 10 cells/ml were centrifuged and the remaining pellet of each dilution was added to 0.05μl of liver abscess pus (PLA pus negative for *E. histolytica*) followed by DNA extraction and PCR as per the aforementioned protocol.

*Cysteine proteinases gene based nested PCR–RFLP*

*Primers design*
The primer design for cysteine proteinases gene based nested PCR–RFLP has already been described in chapter-I. The primer sequence has already been shown in table 1-3 of chapter-I.

Primer validation

The primer sequences designed for *E. histolytica* and *E. dispar* were subjected to a Basic Local Alignment Search Tool (BLAST) in the genome database of all organisms available at the website (http://www.ncbi.nlm.nih.gov/blast/) and were found to be specific for the study.

The amplified PCR products of *E. histolytica* species in liver abscess pus samples was confirmed by getting both the strands of DNA sequenced on ABI3730XL sequencer (Macrogen, Seoul, South Korea). The sequencing was done using species specific primers. For example, the PCR product of *E. histolytica* DNA was sequenced using the species specific primer HCP-1/HCP-2.

All the sequences were analyzed for homology by using the nucleotide-nucleotide "BLAST" search feature available at the website (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi.). The identity between the sequencing results of PCR product of *E. histolytica* from liver abscess pus with the sequence deposited in GenBank [GenBank: S5866] were analyzed by using the "Align two sequences (bl2seq)" feature available at the website (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).
Nested PCR-RFLP protocol

For a reaction volume of 25µl, comprising 2.5µl of 10X PCR buffer (Biogene), 2.0µl of 25mM MgCl₂ (Bangalore genei), 0.75µl of deoxyribo-nucleotide triphosphate mix (10 mM each dNTP, Biogene), 0.25µl (5 IU/µl) of Taq polymerase (Biogene), 0.3µM of target DNA primers (IDT) and template DNA of 3.5µl was added in first (genus specific) and second (nested species specific) PCR. The PCR tubes were finally placed in an Eppendorf Thermal cycler [Master cycler gradient].

The conditions for cysteine proteinases gene based nested PCR-RFLP for liver abscess pus were the same as described earlier for cysteine proteinases gene based nested PCR-RFLP for stool in chapter-I.

Assessment of competition of non-target DNA

During the standardization to assess the competition of other non-target DNA present in liver abscess pus specimen with target DNA, the nested PCR was checked with reference DNA (DNA from standard culture of E. histolytica and E. dispar) spiked with DNA from liver abscess pus (PLA pus negative for E. histolytica) followed by nested PCR amplification.

Estimation of minimum number of Entamoeba cells detectable by nested PCR
This was performed for *E. histolytica* with Locke-egg (LE) medium (NIH modification of Boeck and Drbohlav’s medium) liver abscess pus cultures; the amoebae were counted using a standard haemocytometer. The detailed protocol for counting of *Entamoeba* cells in Neubauer’s chamber (standard hemocytometer) is already mentioned in chapter-I.

A cell pellet containing $10^6$ cells was preferred for determining the detection limit of nested PCR for *E. histolytica*. The cell pellet containing $10^6$ cells of *E. histolytica* was diluted 10 folds in phosphate buffer saline (PBS) to obtain different concentration of cells, such as $10^5$, $10^4$, $10^3$, $10^2$ and 10 cells/ml. The different dilutions of cells ranging from $10^6$ to 10 cells/ml were centrifuged and the remaining pellet of each dilution was added to 0.05μl of liver abscess pus (PLA pus negative for *E. histolytica*) followed by DNA extraction and PCR as per the aforementioned protocol.

9. **Statistical analysis**

Sensitivity and specificity was calculated as per the formula already mentioned in chapter-I. The negative predictive value was calculated as follows: number of true negatives / (number of true negatives + number of false negatives) $\times$ 100. The agreement between the tests was calculated using the Kappa statistics. To determine the statistical significance of differences between the proportions, Chi-square ($\chi^2$) test and Fisher’s exact test were used. The $\chi^2$ test and odds ratio were found using “Epi Info Version 6”. To calculate the significance of the difference in
sensitivities, McNemar’s Chi-square test was applied. The McNemar's test was performed using “Graph Pad Software”.

RESULTS

3. Microscopy and culture for Entamoeba

Microscopy of the liver pus demonstrated *E. histolytica* trophozoites in 10 of 139 (7.2 %) liver abscess specimens, but only 2 (1.4%) pus specimens were positive by culture for *E. histolytica*.

5. Gram stain and bacterial culture for liver abscess pus aspirates

A total of 102 out of 139 (73.4%) liver abscess pus were negative for aerobic bacteria by Gram’s staining and bacterial culture. Twenty seven liver abscess pus specimens were positive for aerobic bacteria by Gram’s staining and bacterial culture. These included *Klebsiella pneumoniae* (n=9), *Proteus* species (n=5), *Enterobacter* (n=5), *Escherichia coli* (n=3) and *Pseudomonas* (n=5). Ten liver abscess pus specimens showed secondary infection of ALA with aerobic bacteria by Gram’s staining and bacterial culture. These included *K. pneumoniae* (n=3), *Enterobacter* (n=2), *E. coli* (n=1), group B *Salmonella* species (n=1), *Enterococcus* (n=1) and Coagulase negative *Staphylococci* (n=2). Such secondary infection of ALA with bacteria has been reported previously in the literature (Gathiram et al., 1984; Sharma et al., 1997).
6. Detection of anti-amoebic antibodies in serum by IHA

The IHA test was positive for serum antibodies in the serum of 86 (61.9%) of 139 patients provisionally diagnosed as ALA. The test was positive in a higher number of serum (71 of 102 [69.6%]) samples of patients who had received prior treatment with metronidazole than those who had not received any prior treatment with metronidazole (15 of 37 [40.5%]) and this difference was statistically significant ($\chi^2 = 8.53$, $P = 0.003$). Metronidazole treatment was initiated from a few days to several weeks before collection of the blood samples in these patients. Two (4.6%) out of 43 sera from control cases were positive for anti-amoebic antibody by IHA.

7. Detection of Gal/GalNAc lectin antigen in liver pus by TechLab E. histolytica II ELISA

The TechLab E. histolytica II test was positive for E. histolytica Gal/GalNAc lectin antigen in the liver abscess pus of 56 (40.3%) of 139 provisionally diagnosed ALA patients. The TechLab E. histolytica II test detected lectin antigen in 30 (81%) of 37 liver abscess pus of patients which were collected prior to treatment with metronidazole. In contrast, the TechLab E. histolytica test detected the lectin antigen in only 26 (25.5%) of 102 liver pus ($\chi^2 = 32.61$, $P<0.001$), collected after initiation of therapy with metronidazole. The probability of E. histolytica antigen detection in liver abscess pus by ELISA was found to be 12 times more in patients who had not received prior treatment with metronidazole (Odds Ratio (OR) = 12.53, 95% Confidence Interval (CI) = 4.55 to 35.86) than in the patients who
received prior metronidazole therapy. The OR was statistically significant as the 95% CI of OR was more than 1.

In the present study, a total of 112 out of 139 (80.6%) provisionally diagnosed ALA patients were diagnosed as ALA and remaining 27 patients were diagnosed as PLA, by following the criteria mentioned in this study elsewhere.

8. Detection of *Entamoeba* DNA in liver pus by PCR

*Quantification of DNA in liver abscess pus specimen*

The quantification of DNA in the liver abscess pus specimen by spectrophotometric analysis showed the DNA yield to be approximately 85μg/ml. The purity of DNA extract from liver abscess pus specimen was found to be satisfactory as the value of ratio of readings at 260nm and 280nm (OD$_{260}$/OD$_{280}$) was approximately 1.8.

*16S-like r RNA gene based nested PCR- RFLP*

*Primer validation*

The sequencing result of PCR product of *E. histolytica* from liver abscess pus specimen (Figure II-2) was showing reasonable identities to the sequence deposited in GenBank, [accession number: X56991].
Sequencing result of nested PCR product of 16S-like r RNA gene of *E. histolytica* from liver abscess pus specimen with species specific primer Eh-1 and Eh-2

**Figure II-2**

Sequencing result of nested PCR product of 16S-like r RNA gene of *E. histolytica* from liver abscess pus specimen with species specific primer Eh-1 and Eh-2

<table>
<thead>
<tr>
<th>Sequence with forward primer Eh-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGGAATTGCGGTTCAATCGAGAGAGGAACTTATAGATGCTACTCTGTAAGGCAAGCAAGG</td>
</tr>
<tr>
<td>GCCGTTAATTACCCACTTTCGAATTGAGTAGTGGACAGCAACATAACTCTGAGGTGGAAT</td>
</tr>
<tr>
<td>CAATTTCCGAAAGGATGATGAGAGGTATAATTCTCCTACGAATATTTGGGAGGCAAGTCG</td>
</tr>
<tr>
<td>AGCAAGCCGCGGTAATTTCCAGCTCCAATAGGTATATA</td>
</tr>
</tbody>
</table>

100% identities with X56991

<table>
<thead>
<tr>
<th>Sequence with reverse primer Eh-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCGTTGCGGATACTGTTTATAATGAGACTACGGGTACGATCGTCTGCTCTCACTAACTTTCA</td>
</tr>
<tr>
<td>TCTTTGATATGAGAACAGCTGTTGAAATGTTCTCTGCTCTGCTCTGCTCTGCTCTGCTG</td>
</tr>
<tr>
<td>CATCCTCCGTATTTTCTGTAATCCACCCCCCAATTTCTTAAATCCTTTTCACTTTTCTG</td>
</tr>
<tr>
<td>ACGATACTGAGTTGGATACGCCCTTTCTGTTTAAACACCTTTATTTATT</td>
</tr>
<tr>
<td>CAAAGTTAATAAACACTCTAGAGATGTTCTCCTCTTTGATTCAAATTTATTTGAGGAAATTT</td>
</tr>
<tr>
<td>ACTTACAAAGTCTTCAAATTTTAATCAGCTGAGC6TGAATTTTATACAGAGCAAA</td>
</tr>
<tr>
<td>CTTTAAATTTCACTTGGAGTTGAAATCCCGCAATCTGCTGAGCACAGCTTGGAT</td>
</tr>
<tr>
<td>CAGTACGAAATAATCTCTATCTGTCCTGCAAAATCTGATTACTCAACCTATGATGCTG</td>
</tr>
<tr>
<td>CAGCTCTTCTGAAATTTGAGGAATACTGCTG</td>
</tr>
</tbody>
</table>

90% identities with X56991
Assessment of competition of non-target DNA

The result of assessment of competition of other non-target DNA present in liver abscess pus (PLA pus negative for *E. histolytica*) specimen with target DNA, showed expected amplification and no nonspecific amplification in nested PCR.

Estimation of minimum number of Entamoeba cells detectable by nested PCR

It was found to be approximately 30 *E. histolytica* cells as even 3.0µl of template DNA from 1000 *E. histolytica* cells / 100µl of Tris- ethylenediamine tetraacetic acid (EDTA) (TE) buffer produced a positive signal (Figure II-3).

Nested PCR-RFLP

The result of nested PCR-RFLP performed on the liver abscess pus is depicted in figure II-4. The nested PCR was positive for *E. histolytica* DNA in 86 (76.8%) of 112 liver abscess pus specimens (Table II-2). The nested PCR could detect *E. histolytica* DNA in the liver abscess pus of 35 (94.6%) of 37 ALA patients, who were tested prior to treatment with metronidazole. In contrast, prior metronidazole treatment significantly decreased the ability of the PCR to detect *E. histolytica* DNA in the liver abscess pus, with only 51 (68%) of 75 liver pus samples positive ($\chi^2 = 8.4$, $P= 0.004$). The probability of *E. histolytica* DNA detection in liver abscess pus by nested PCR-RFLP was 8 times more in patients who had not received prior metronidazole therapy (OR = 8.24, 95% CI = 1.71 to 53.94) than in
Figure II-3

Sensitivity of PCR for detection of minimum number of *E. histolytica* (EH) cells. 0.85μl of liver abscess pus (PLA pus negative for *E. histolytica*) specimen seeded with serially diluted *E. histolytica* cells corresponding to 10^6 cells (lane 1), 10^5 cells (lane 2), 10^4 cells (lane 3), 10^3 cells (lane 4), 10^2 cells (lane 5), and 10 cells (lane 6) were subjected to DNA extraction followed by PCR amplification. Amplified products were analyzed by agarose gel electrophoresis. The sizes of the amplification products are indicated on the left (in base pairs).

Figure II-4

*E. histolytica* specific nested SSU-rDNA PCR products. Odd and even numbered lanes represent undigested and Dra-I and Sau964-digested PCR products respectively.

Lane 1/2/5/6: DNA from liver abscess pus showing *E. histolytica*. III: A 100-bp DNA ladder
the patients who received prior metronidazole therapy. The OR was statistically significant as the 95% CI of OR was greater than 1.

The comparison of results of 16S-like r RNA gene based nested PCR-RFLP and TechLab *E. histolytica* II ELISA test on liver abscess pus from ALA patients is summarised in the **Table II-2**.

**Table II-2. Comparison of result of 16S-like r RNA gene based nested PCR-RFLP and antigen detection in liver abscess pus specimen of ALA patients**

<table>
<thead>
<tr>
<th>PCR results</th>
<th>Antigen detection result (no. of specimens positive)</th>
<th>Total no. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. histolytica</em></td>
<td>52a</td>
<td>34</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>56</td>
<td>56</td>
</tr>
</tbody>
</table>

*a* *E. histolytica* was detected by microscopy and/or culture in 10 of these 52 ELISA and PCR positive liver abscess pus specimens.

_16S-like r RNA gene based nested multiplex PCR_

**Primer validation**

The sequencing result of PCR product of *E. histolytica* from liver abscess pus (Figure II-5, Figure II-6, Figure II-7) specimen showed 99% identities to the sequence deposited in GenBank, [accession number: X56991].

**Assessment of competition of non target DNA**
The result of assessment of competition of other non-target DNA present in liver abscess pus (PLA pus negative for *E. histolytica*) specimen with target DNA, showed expected amplification and no non-specific amplification in nested multiplex PCR.

*Estimation of minimum number of Entamoeba cells detectable by nested PCR*

It was found to be approximately 15 *E. histolytica* cells as even 1.5μl of template DNA from 1000 *E. histolytica* cells / 100μl of TE buffer produced a positive signal (Figure II-8).

*Nested multiplex PCR*

The result of nested multiplex PCR performed on the liver abscess pus is depicted in figure II-9. The nested multiplex PCR test was positive for *E. histolytica* DNA in 90 (80.4%) of 112 liver abscess pus specimens (Table II-3). The nested multiplex PCR could detect *E. histolytica* DNA in the liver abscess pus of all 37 ALA patients (100%), who were tested prior to treatment with metronidazole. In contrast, prior metronidazole treatment significantly decreased the ability of the PCR to detect *E. histolytica* DNA in the liver abscess pus, with only 53 (70.6%) of 75 liver pus samples positive (Fisher's Exact test, P= 0.0006). The probability of *E. histolytica* DNA detection in liver abscess pus by nested multiplex PCR was 31 times more in patients who had not received prior metronidazole therapy (OR = 31.54, 95% CI = 1.879 to 624.2) than in the patients who received prior metronidazole therapy.
Figure II-5
Sequencing result of nested multiplex PCR product of 16S r RNA gene of *E. histolytica* from liver abscess pus specimen with species specific primer EH-1 and EH-2

Sequence with forward primer EH-1

```
CCCTCATGCTCTGTTACCTTTAAGAATTTGAGGATTATACTTCAGCTAGATGCTGAACTCTATTTTGACTCAACACGATGCTGAACTCTATTTTGAC
```

99% identities with XS6991

Sequence with reverse primer EH-2

```
CCACATCAGAATGAAAAATATGTGCTGTTACTCAGCACCCTGTTGACTGATCGAGATTTTGACTTACACACGAAATTTGCCTATTTCTACTGCTATTACTG
```

99% identities with XS6991

Figure II-6
Sequencing chromatogram of nested multiplex PCR product of 16S r RNA gene of *E. histolytica* from liver abscess pus specimen with species specific primer EH-1

Figure II-7
Sequencing chromatogram of nested multiplex PCR product of 16S r RNA gene of *E. histolytica* from liver abscess pus specimen with species specific primer EH-2
Figure II-8

Sensitivity of PCR for detection of minimum number of *E. histolytica* (EH) cells. 0.06 µl of liver abscess pus (PLA) was negative for *E. histolytica* specimen counted with serially diluted *E. histolytica* cells corresponding to $10^6$ cells (lane 1), $10^5$ cells (lane 2), $10^4$ cells (lane 3), $10^3$ cells (lane 4), $10^2$ cells (lane 5), and 10 cells (lane 6) were subjected to DNA extraction followed by PCR amplification. Amplified products were analyzed by agarose gel electrophoresis. The sizes of the amplification products are indicated on the left (in lane 1). 

Figure II-9

Result of nested multiplex PCR on representative liver abscess pus. The *E. histolytica* (EH) and internal amplification control (IAC) bands are 438 and 305 bp respectively. Lane-1 and 4 are positive for *E. histolytica* DNA in liver abscess pus specimen; Lane-2, 3 and 5 are negative for *E. histolytica* DNA; Lane-6, 100bp DNA ladder (Bangalore genei, Bangalore).
The OR was statistically significant as the 95% CI of OR was greater than 1.

Table II-3. Comparison of result of 16S-like r RNA gene based nested multiplex PCR and antigen detection in liver abscess pus specimen of ALA patients

<table>
<thead>
<tr>
<th>PCR results</th>
<th>Antigen detection result (no. of specimens positive)</th>
<th>E. histolytica</th>
<th>Antigen negative</th>
<th>Total no. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. histolytica</td>
<td>55&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>21</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>56</td>
<td>112</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> *E. histolytica* was detected by microscopy and/or culture in 10 of these 55 ELISA and PCR positive liver abscess pus specimens.

The nested multiplex PCR did not detect *E. histolytica* DNA in a total of 49 liver abscess pus specimens, which included 27 PLA and 22 ALA pus specimens. The probability of negative nested multiplex PCR results, in these 49 liver abscess pus specimens due to PCR inhibitors was ruled out by the inclusion of an internal amplification control (IAC) in the nested PCR reaction.

The comparison of results of 16S-like r RNA gene based nested multiplex PCR and TechLab *E. histolytica* II ELISA test on liver abscess pus from ALA patients is summarised in the table II-3.

**Cysteine proteinases gene based nested PCR–RFLP**

*Primer validation*

156
The sequencing result of PCR product of *E. histolytica* from liver abscess pus specimen showed 99% identities to the sequence deposited in GenBank, [accession number: S586691] (Figure II-10).

**Assessment of competition of non target DNA**

The result of assessment of competition of other non-target DNA present in liver abscess pus (PLA pus negative for *E. histolytica*) specimen with target DNA, showed expected amplification and no non-specific amplification in nested PCR-RFLP.

**Estimation of minimum number of Entamoeba cells detectable by nested PCR**

It was found to be approximately 35 *E. histolytica* cells as even 3.5 µl of template DNA from 1000 *E. histolytica* cells / 100µl of TE buffer produced a positive signal (Figure II-11).

**Nested PCR-RFLP**

The result of nested PCR-RFLP performed on the liver abscess pus is depicted in figure II-12. The nested PCR test was positive for *E. histolytica* DNA in 82 (73.2%) of 112 liver abscess pus specimens (Table II-4). The nested PCR could detect *E. histolytica* DNA in the liver abscess pus of 34 (91.9%) of 37 ALA patients, who were tested prior to treatment with metronidazole. In contrast, prior metronidazole treatment significantly decreased the ability of the PCR to detect
Sequencing result of nested PCR product of cysteine proteinase gene of E. histolytica with species specific primer HCP-1 and HCP-2

**Figure II-10**

<table>
<thead>
<tr>
<th>Sequence with forward HCP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATAGAAGAAGGGAACTATCTATATCTGATATACTAAATGATACAAAAAGAATGATATGATAGATGTTTCTGCT</td>
</tr>
<tr>
<td>GATGTCTATAGTGGGTCAATATATATATGATTTATTGATATTAGAAAAACACTCATGCGAAA</td>
</tr>
</tbody>
</table>

**Figure II-11**

Figure II-11: Sensitivity of PCR for detection of minimum number of E. histolytica (E) cells. 10^3 of four human DNA (PLA) were reacted with a variety of E. histolytica cells corresponding to 10^4 cells (lane 1), 10^3 cells (lane 2), 10^2 cells (lane 3), 10^1 cells (lane 4), 10^0 cells (lane 5), and 0 cells (lane 6) were subjected to DNA extraction followed by PCR amplification. Amplified products were analyzed by agarose gel electrophoresis. The sizes of the amplification products are indicated on the left (in base pairs).

**Figure II-12**

Figure II-12: E. histolytica-specific nested cysteine proteinase PCR products. Odd and even numbered lanes represent unligated and HindIII-digested PCR products, respectively.
E. histolytica DNA in the liver abscess pus, with only 48 (64%) of 75 liver pus samples positive ($\chi^2 = 8.5$, $P = 0.004$). The probability of E. histolytica DNA detection in liver abscess pus by nested PCR-RFLP was 6 times more in patients who had not received prior metronidazole therapy (OR = 6.38, 95% CI = 1.65 to 28.79). The OR was statistically significant as the 95% CI of OR was greater than 1.

The comparison of results of cysteine proteinases gene based nested PCR-RFLP and TechLab E. histolytica II ELISA test on liver abscess pus from ALA patients is summarised in the table II-4.

<table>
<thead>
<tr>
<th>PCR results</th>
<th>Antigen detection result (no. of specimens positive)</th>
<th>E. histolytica</th>
<th>Antigen negative</th>
<th>Total no. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. histolytica</td>
<td>51*</td>
<td>31</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>25</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>56</td>
<td>112</td>
<td></td>
</tr>
</tbody>
</table>

* E. histolytica was detected by microscopy and/or culture in 10 of these 51 ELISA and PCR positive liver abscess pus specimens.

DISCUSSION

In this study, the E. histolytica DNA was detected in liver abscess pus of ALA patients by applying three different PCR methods. Also the diagnostic potential of all the three PCR methods for detection of E. histolytica DNA in liver abscess pus for the diagnosis of ALA was assessed.
In the present study, 76.8% (86 of 112) of ALA patients were positive for anti-amoebic antibody in serum by the IHA. This result was similar to that reported from Bangladesh where serum anti-amoebic antibodies were found in 78% of ALA patients (Haque et al., 2000), but differed from that of the study reported from South Africa, where serum anti-amoebic antibodies were found in a higher 99% of ALA patients (Gathiram and Jackson, 1987).

Only two out of 10 ALA pus samples which were positive for *E. histolytica* trophozoite by microscopy were positive for the amoebae by culture and the rest were negative, this may be due to the inhibition of growth by culture itself. In majority of patients, *K. pneumoniae* was the major bacterial pathogen responsible for PLA and as secondary bacterial infection of ALA. One ALA pus specimen was positive for Group B *Salmonella* species, this patient had liver abscess with perihepatic collection, with severe sepsis and disseminated intravascular coagulation, finally the patient died. In this study, the anaerobic culture of liver abscess pus aspirate was not done. Therefore, the possible etiology of liver abscess due to anaerobic organisms such as *Bacteroides* remained undetermined.

In the present study, 50% (56 of 112) of liver abscess pus were positive for *E. histolytica* lectin antigen. The sensitivity of the test in our study was observed to be slightly higher than that of the study using the same TechLab *E. histolytica* II kit (40.7 % sensitivity) on liver pus reported from Bangladesh (Haque et al., 2000). However, results of other studies using polyclonal antibody based ELISA showed a very high sensitivity for the detection of amoebic antigen in the liver pus.
Amoebic antigen was detected in liver abscess pus in 97.6% (41/42) of ALA cases by ELISA as reported from China (Zengzhu et al., 1999) and in 92% and 96% of liver pus by using immunoelectrophoresis and ELISA respectively, from India (Bhave et al., 1985).

In developing countries like India where amoebiasis is endemic, anti-amoebic drugs and antibiotics are used indiscriminately, making it difficult to obtain an accurate treatment history. Most of the patients in the present study had already been treated with metronidazole at the time of collection of clinical specimens. The serum amoebic antibodies were detected in higher percentage (94.7%) of ALA patients treated earlier with metronidazole, but were detected in only 40.5% of patients who did not receive any prior treatment with metronidazole. This might be due to the late antibody response during the course of the disease.

Unlike serum amoebic antibody detection, *E. histolytica* lectin antigen was detected in liver pus by TechLab ELISA in a higher percentage (81%) of ALA patients, who were tested prior to treatment with metronidazole, but was detected in only 34.6% of ALA patients, who were tested after the initiation of therapy with metronidazole. This might be due to the rapid clearing of amoebic antigen from the liver pus due to killing of *E. histolytica* trophozoites on treatment with metronidazole.

All the three PCR methods for the detection of *E. histolytica* DNA in liver abscess pus had a much higher sensitivity when tested prior to treatment with metronidazole, but had a lower sensitivity when tested after the initiation of
treatment with metronidazole. This might be attributed to the clearing of *E. histolytica* DNA from the liver abscess due to the death and lysis of *E. histolytica* trophozoites on treatment with metronidazole.

The PCR for *E. histolytica* DNA detection and ELISA for *E. histolytica* antigen detection in liver abscess were showing a fair agreement between the two tests by Kappa statistic.

PCR for detection of *E. histolytica* DNA and ELISA for detection of *E. histolytica* lectin antigen in liver abscess pus were evaluated for the diagnosis of ALA (*p* < 0.0001). The sensitivity of 16S-like r RNA gene based nested PCR-RFLP, 16S-like r RNA gene based nested multiplex PCR, and cysteine proteinases gene based nested PCR-RFLP was 76.8%, 80.4%, and 73.2% respectively. This was found to be significantly higher than that of ELISA (50% sensitivity) using McNemar’s *χ²* test (*p* < 0.0001). All 27 liver abscess pus aspirates diagnosed as PLA were negative for *E. histolytica* DNA by all three PCR methods and for *E. histolytica* lectin antigen by TechLab ELISA, which represents a specificity of 100%.

ELISA for detection of liver abscess pus *E. histolytica* lectin antigen demonstrated a 100% positive predictive value and a 32.5% negative predictive value.

The 16S-like r RNA gene based nested PCR-RFLP, 16S-like r RNA gene based nested multiplex PCR, and cysteine proteinases gene based nested PCR-RFLP for the detection of liver abscess pus *E. histolytica* DNA demonstrated a 50.9%,
55.1%, and 47.4% negative predictive value, respectively. All the three PCR methods had a 100% positive predictive value.

In the present study, none of the liver abscess pus PCR results were positive for either *E. dispar* or *E. moshkovskii* specific PCR products, which confirm the non-invasive nature of these species.

The detection of *E. histolytica* DNA and *E. histolytica* specific lectin antigen in the serum specimen of ALA patients was not carried out in this study. A controlled prospective study to evaluate the detection of *E. histolytica* DNA and *E. histolytica* specific lectin antigen in the serum specimen of ALA patients has been intended to be carried out in future in our laboratory.

In conclusion, the PCR for the detection of liver abscess pus *E. histolytica* DNA was found to be useful for the diagnosis of ALA when liver abscess pus aspirate was available. The 16S-like r RNA gene based nested PCR-RFLP and cysteine proteinases gene based nested PCR–RFLP strategies were found to be useful for the specific detection of *E. histolytica* species in liver abscess pus samples, but were found to be more labour intensive and time consuming method because after the PCR amplification the RFLP was mandatory to confirm the species. However, the 16S-like r RNA gene based nested multiplex PCR strategy for specific detection of *E. histolytica* species in liver abscess pus specimens was found to be highly specific, sensitive and also rapid; results of the test were available within 12 hours of receipt of liver abscess pus specimens.
SUMMARY

Infection with *E. histolytica*, results in 34 million to 50 million symptomatic cases of amoebiasis worldwide each year, causing 40 to 100 thousand deaths annually. Mortality from amoebiasis is mainly due to extra-intestinal pathology, of which ALA is the most common. If left untreated, ALA can rupture into neighboring tissue and spread to the brain and other organs via hematological route producing serious morbidity and mortality. It is difficult to differentiate clinically the ALA from PLA as well as from other space occupying lesions of liver such as hydatid cyst and liver hepatoma.

In the present study, three different PCR methods i.e. 16S-like r RNA gene based nested PCR- RFLP, 16S-like r RNA gene based nested multiplex PCR, and cysteine proteinases gene based nested PCR-RFLP were evaluated for the detection of *Entamoeba* DNA in the liver abscess pus for the diagnosis of ALA.

The 16S-like r RNA gene based nested PCR- RFLP and 16S-like r RNA gene based nested multiplex PCR, and cysteine proteinases gene based nested PCR-RFLP were carried out to detect *Entamoeba* DNA in liver abscess pus of 139 patients provisionally diagnosed as ALA. TechLab *E. histolytica* II ELISA test was performed to detect Gal/GalNAc lectin in liver abscess pus of 139 patients provisionally diagnosed as ALA. Rapid-IHA was performed to detect serum anti-amoebic antibodies in 139 patients provisionally diagnosed as ALA and in 43 negative controls.
The 16S-like r RNA gene based nested PCR-RFLP, 16S-like r RNA gene based nested multiplex PCR, and cysteine proteinases gene based nested PCR-RFLP showed a sensitivity of 76.8%, 80.4%, and 73.2% respectively and specificity of 100%.

All the three PCR methods for the detection of *E. histolytica* DNA in liver abscess pus had a much higher sensitivity when tested prior to treatment with metronidazole, but had a lower sensitivity when tested after the initiation of treatment with metronidazole. This might be attributed to the clearing of *E. histolytica* DNA from the liver abscess due to the death and lysis of *E. histolytica* trophozoites on treatment with metronidazole.

In the present study, none of the liver abscess pus PCR results were positive for either *E. dispar* or *E. moshkovskii* specific PCR products, which confirm the non-invasive nature of these species.

The ELISA for the detection of lectin *E. histolytica* antigen in the liver abscess pus showed a sensitivity of 50% and the IHA test for detection of amoebic antibodies in the serum showed a sensitivity of 76.8% for the diagnosis of the ALA.

The PCR for the detection of liver abscess pus *E. histolytica* DNA was found to be useful for the diagnosis of ALA when liver abscess pus aspirate was available. The 16S-like r RNA gene based nested PCR-RFLP and cysteine proteinases gene based nested PCR-RFLP strategies were found to be useful for the specific detection of *E. histolytica* species in liver abscess pus samples, but were found to be more
labour intensive and time consuming method because after the PCR amplification the RFLP was mandatory to confirm the species. However, the 16S-like r RNA gene based nested multiplex PCR strategy for specific detection of *E. histolytica* species in liver abscess pus specimens was found to be highly specific, sensitive and also rapid; results of the test were available within 12 hours of receipt of liver abscess pus specimens.