6. CHAPTER-III

EVALUATION OF URINE AS A SPECIMEN FOR THE DIAGNOSIS OF ALA BY PCR
BACKGROUND

*Entamoeba histolytica* the causative agent of amoebiasis, results in 40 to 100 thousand deaths worldwide each year (WHO, 1997). Mortality from amoebiasis is mainly due to extra-intestinal manifestation, of which amoebic liver abscess (ALA) is the most common. It is difficult to diagnose specifically ALA from other space occupying lesions of liver (Smoger et al., 1998; Kasper et al., 2005). Imaging techniques although have excellent sensitive to detect abscesses in the liver of varied aetiology, but fail to diagnose specifically ALA.

The approaches employed till date for the diagnosis of ALA include; demonstration of *E. histolytica* trophozoite in liver abscess pus by microscopy (Parija, 1993), demonstration of anti-*E. histolytica* antibodies in serum (Gandhi et al., 1987; Jackson et al., 1984; Yang and Kennedy, 1979), demonstration of *E. histolytica* antigen in serum and liver pus (Haque et al., 2000), and recently demonstration of *E. histolytica* DNA in liver pus (Khan et al., 2006; Zengzhu et al., 1999; Zaman et al., 2000; Haque et al., 2000).

Unfortunately, the collection of blood or liver abscess pus involves invasive procedure, and the procedures require technical expertise and disposable syringes (Parija, 1998). The method if not carried out under stringent conditions is associated with the risk of acquiring blood borne infections such as hepatitis B virus and human immunodeficiency virus (HIV).
Therefore, of late much interest has been shown towards the use of urine as a specimen alternate to the blood for the diagnosis of some parasitic infections including malaria, schistisomiasis, kala-azar, cystic echinococcosis and neurocysticercosis (Parija, 1998). Urinary antigen for cystic echinococcosis (CE) and neurocysticercosis has been reported for the first time from our laboratory at Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India (Parija et al., 1997; Parija et al., 2004). Our laboratory has developed for the first time a counter-current immunoelectrophoresis (CIEP) and co-agglutination (Co-A) to detect the hydatid antigen excreted in the urine for the diagnosis of CE (Parija et al., 1997; Ravinder et al., 2000), and Co-A to detect cysticercus antigen in the urine for the diagnosis of neurocysticercosis (Parija et al., 2004).

Detection of DNA in urine by PCR has been employed for the diagnosis of *Toxoplasma gondii, Neisseria gonorrhoeae, Borrelia burgdorferi, Mycobacterium tuberculosis, Mycobacterium leprae* and *Chlamydia trachomatis* infections (Crotchfelt et al., 1997; Pleyer et al., 2001; Fuentes et al., 1996; Aceti et al., 1999; Parkash et al., 2004). Some studies have also shown that the kidney barrier in rodents and humans is permeable to DNA molecules large enough to be analyzed by standard genetic methodologies (Botezatu et al., 2000; Su et al., 2004). To the best of our knowledge till now there is no report available on detection of *Entamoeba* DNA in the urine for the diagnosis of ALA. In the present study, therefore, polymerase chain reaction (PCR) was evaluated for detection of *Entamoeba* DNA possibly excreted in the urine for the diagnosis of ALA.
OBJECTIVE

To evaluate urine as a specimen for the diagnosis of amoebic liver abscess (ALA)

MATERIALS AND METHODS

1. Sample details

The present study was conducted in the JIPMER hospital, Puducherry, India, during a period from March 2005 to March 2006.

Patients with ALA (n=53)

The study included 53 ALA patients, diagnosis of which was established on the basis of criteria mentioned earlier in chapter-II.

In the present study, the 53 ALA patients included 23 patients in which the metronidazole therapy was not initiated and 30 patients in which the metronidazole therapy was already initiated.

Patients with pyogenic liver abscess (PLA) and other diseases of the liver (n=23)

The study included cases of PLA (n=15), hydatid cyst in liver (n=2), liver hepatoma (n=1), liver cirrhosis (n=3), and viral hepatitis (n=2).

Healthy control (n=35)
The study included 35 healthy controls who had no history of recent dysentery or diarrhea and whose stool samples were negative for \textit{E. histolytica} infection by microscopy and culture.

2. Sample collection

\textit{Urine}

Urine specimens were collected from all 53 ALA patients, 23 patients with PLA and other diseases of the liver; and 35 healthy controls. 10ml of urine specimen was collected in a sterile container using aseptic techniques; urine sample was stored at -20°C until use.

\textit{Liver abscess pus}

The aspiration of liver abscess pus was indicated only under the conditions mentioned earlier in \textit{chapter-II}. 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 53 ALA patients and 15 PLA patients, and was stored at -20°C in a sterile container until used.

\textit{Blood}
Blood specimens were collected from all 53 ALA patients, 23 patients with PLA and other diseases of the liver; and 35 healthy controls. 5ml of venous blood was collected in a sterile container; sera was separated and stored at -20°C until used.

3. 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 ≥ 1: 128 was considered positive for ALA (Parija et al., 1988). The detailed protocol of Rapid-IHA test is already mentioned in chapter-I.

4. 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 × g for 10 min, and 100 μl 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.

5. Detection of Entamoeba DNA in urine by PCR
Extraction of Entamoeba genomic DNA

Urine

For extracting DNA from urine specimen 10 ml of the urine sample was centrifuged at 12,074 g for 15 min at 4°C. The supernatant was discarded and the pellet was suspended in 500 μl of sterile distilled water. The suspension of urine sediment (500 μl) in sterile distilled water was boiled for 10 minutes followed by sudden cooling. Next, 5 μl of proteinase-K (10 mg/ml) and 60 μl of 10% SDS were mixed and incubated for three hour at 65 °C. Then, 80 μl of 5 M NaCl and 20 μl of 10% CTAB were added to the mixture and incubated for 45 min at 65 °C. The suspension thus obtained was treated with 500μl of chloroform, mixed by inversion and centrifuged at 12,074 g for eight minutes. The aqueous layer was mixed with 500μl of Phenol: Chloroform: Isoamyl alcohol mix (25:24:1) in a fresh tube, mixed by inversion and centrifuged at 12,074 g for eight minutes. The aqueous layer was taken in a fresh tube and excess of cold absolute ethanol (kept at -20°C) was added. The contents of the tube were kept at -20 °C overnight. Later on, the tube was centrifuged at 12,074 g for 15 min; the supernatant was discarded leaving the pellet in tube. The pellet was washed with 70% ethanol (200 μl) and the tube was centrifuged at 12,074 g for 8 min, the supernatant was discarded. The pellet was kept for drying in incubator at 37°C for 30 min. Finally, the dried pellet was dissolved in 50μl sterile distilled water.

Liver abscess pus
The protocol for extraction of *Entamoeba* genomic DNA from liver abscess pus has been described earlier in chapter-II.

The protocol for extraction of DNA from urine and 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 urine and liver abscess pus samples were 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 urine and liver abscess pus specimen**

DNA quantification in spin column purified DNA extract from urine and 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 × dilution. The purity of the nucleic acid in the samples was estimated by the ratio of readings at 260nm and 280nm (OD$_{260}$/OD$_{280}$).

**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.
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 shown in table I-1 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 urine and 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**

**Urine PCR:** The protocol for PCR mix composition and PCR conditions were the same as described earlier for *E. histolytica*, *E. dispar* and *E. moshkovskii* stool PCR in chapter-I, except that 1.0 μl of 25mM MgCl\textsubscript{2} was added.

**Liver abscess pus PCR:** The protocol for PCR mix composition and PCR conditions were the same as described earlier for *E. histolytica*, *E. dispar* and *E. moshkovskii* stool PCR in chapter-I, except that 1.0 μl of 25mM MgCl\textsubscript{2} and 2.0 μl 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 urine and 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 urine (negative control group) and liver abscess pus (PLA pus negative for *E. histolytica*) followed by nested PCR amplification.

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

**Primers design**

The primer design has already been described earlier in chapter-II. The primer sequences used for 16S-like r RNA gene based nested multiplex PCR are shown in table II-1 of chapter-II.

**Primer validation**

The primer sequences designed for *E. moshkovskii*, *E. histolytica*, *E. dispar* 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 urine and 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 urine and 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/wblast).

_Nested multiplex PCR protocol_

_Urine PCR:_ For a reaction volume of 25μl, comprising 2.5μl of 10X PCR buffer (Biogene), 1.0μl 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 2.5μl for both genus and species specific PCR. The PCR tubes were finally placed in an Eppendorf Thermal cycler [Master cycler gradient].

_Liver abscess pus PCR:_ The PCR mix composition was the same as described above for urine PCR, except that 2.0μl of 25mM MgCl₂ and 2μl of template DNA was added.
The conditions for 16S-like r RNA gene based nested multiplex PCR for urine and 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 urine and liver abscess pus 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 urine (negative control group) and liver abscess pus (PLA pus negative for *E. histolytica*) followed by nested multiplex PCR amplification.

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

**Primers design**

The primer design has already been described in details in chapter-I. The primer sequences used for cysteine proteinases gene based nested PCR–RFLP is shown in table I-2 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 urine and 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/). The identity between the sequencing results of PCR product of *E. histolytica* from urine and 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**

**Urine PCR:** For a reaction volume of 25μl, comprising 2.5μl of 10X PCR buffer (Biogene), 1.0μl 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 3.0μl 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].

_Liver abscess pus PCR:_ The PCR mix composition was the same as described above for urine PCR, except that 2.0μl of 25mM MgCl₂ and 3.5μl of template DNA was added.

The conditions for cysteine proteinases gene based nested PCR-RFLP for urine and 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 urine and liver abscess pus specimen with target DNA, the nested PCR was checked with reference DNA (DNA from standard culture of _E. histolytica_ and _E. dispers_) spiked with DNA from urine (negative control group) and liver abscess pus (PLA pus negative for _E. histolytica_) followed by nested PCR amplification.

6. Statistical data analysis

Sensitivity and specificity was calculated as per the formula already mentioned in chapter-I. To determine the statistical significance of differences between the proportions, χ-squared test was performed. The χ-squared test was performed and the odds ratio determined with Epi Info Version 6 software. 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. Detection of anti-amoebic antibodies in serum by IHA

The IHA test was positive for anti-amoebic antibodies in the serum of 38 (71.7%) of 53 ALA patients and 2 (5.7%) of 35 healthy controls. The IHA test was negative for anti-amoebic antibodies in the serum of all 23 patients with PLA and other diseases of the liver.

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

The TechLab E. histolytica II ELISA test was positive for E. histolytica Gal/GalNAc lectin antigen in the liver abscess pus of 29 (54.7%) of 53 ALA patients. The TechLab E. histolytica II ELISA test was negative for E. histolytica Gal/GalNAc lectin antigen in the liver abscess pus of all 15 patients with PLA.

5. Detection of Entamoeba DNA in urine by PCR

Quantification of DNA in urine and liver abscess pus specimen
The quantification of DNA in the urine and liver abscess pus specimen by spectrophotometric analysis showed the DNA yield to be approximately 3 and 85μg/ml respectively. The purity of DNA extract from urine and liver abscess pus specimens was found to be satisfactory as the value of ratio of readings at 260nm and 280nm (OD\textsubscript{260}/OD\textsubscript{280}) was approximately 1.8.

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

Primer validation

The sequencing result of PCR product of \textit{E. histolytica} from urine (Figure III-1) and liver abscess pus (Figure III-2) specimen were showing reasonable 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 urine (negative control group) and liver abscess pus (PLA pus negative for \textit{E. histolytica}) specimen with target DNA, showed expected amplification and no nonspecific amplification in nested PCR.

Nested PCR-RFLP

The result of nested PCR-RFLP performed on the urine specimen is shown in figure III-3. The nested PCR test detected \textit{E. histolytica} DNA in 19 (35.8%) of 53
urine samples collected from ALA patients (Table III-1). The test did not detect *E. histolytica* DNA in urine samples collected from all 23 patients with PLA and other diseases of the liver; and 35 healthy controls.

The nested PCR test detected *E. histolytica* DNA in the urine specimens of 3 (13%) of 23 ALA patients who were tested prior to treatment with metronidazole and in 16 (53.3%) of 30 ALA patients who were tested after treatment with metronidazole ($\chi^2 = 7.52, P = 0.006$).

All of the 3 ALA patients, who did not receive prior treatment with metronidazole and whose urine specimens were positive for *E. histolytica* DNA, were available for follow-up study.

Urine specimens were collected from these patients every week for 4 weeks after starting of the therapy with metronidazole; and were tested for *E. histolytica* DNA by PCR. It was observed that 2 weeks after treatment with metronidazole, 2 (66.7%) out of 3 urine specimens became negative for *E. histolytica* DNA. One urine specimen became negative for *E. histolytica* DNA after 4 weeks of treatment with metronidazole.

The result of nested PCR-RFLP performed on the liver abscess pus is depicted in figure III-3. The nested PCR was positive for *E. histolytica* DNA in 51 (96.2%) of 53 liver abscess pus specimens from ALA patients (Table III-1). The test did not detect *E. histolytica* DNA in liver abscess pus from all 15 patients with PLA.
Chapter III

Table III-1. Detection of *Entamoeba* DNA in liver abscess pus and urine specimen of ALA patients by applying 16S-like r RNA gene based nested PCR- RFLP

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No of patients</th>
<th>Liver abscess pus specimen</th>
<th>Urine specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. histolytica</td>
<td>E. dispar</td>
</tr>
<tr>
<td>ALA*</td>
<td>53</td>
<td>51 (96.2)</td>
<td>0</td>
</tr>
<tr>
<td>PLA*</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ODLc</td>
<td>8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HCd</td>
<td>35</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*amoebic liver abscess; *pyogenic liver abscess; *other diseases of liver; *healthy controls

16S-like r RNA gene based nested multiplex PCR

**Primer validation**

The sequencing result of PCR product of *E. histolytica* from urine (Figure III-4, Figure III-5 and Figure III-6) and liver abscess pus (Figure III-7, Figure III-8 and Figure III-9) 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 urine (negative control group) and 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.

**Nested multiplex PCR**

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The nested multiplex PCR result on urine specimen is shown in figure III-10. The nested multiplex PCR test detected *E. histolytica* DNA in 21 (39.6%) of 53 urine samples collected from patients with ALA (Table III-2). The test did not detect *E. histolytica* DNA in urine samples collected from all 23 patients with PLA and other diseases of the liver; and 35 healthy controls.

The nested multiplex PCR test detected *E. histolytica* DNA in the urine specimens of 4 (17.4%) of 23 ALA patients who were tested prior to treatment with metronidazole and in 17 (56.7%) of 30 ALA patients who were tested after treatment with metronidazole ($\chi^2 = 6.83$, $P= 0.009$). All of the 4 ALA patients, who did not receive prior treatment with metronidazole and whose urine specimens were positive for *E. histolytica* DNA, were available for follow-up study. Urine specimens were collected from these patients every week for 4 weeks after starting of the therapy with metronidazole; and were tested for *E. histolytica* DNA by PCR. It was observed that 2 weeks after treatment with metronidazole, 3 (75%) out of 4 urine specimens became negative for *E. histolytica* DNA. One urine specimen became negative for *E. histolytica* DNA after 4 weeks of treatment with metronidazole.

The result of nested multiplex PCR performed on the liver abscess pus is depicted in figure III-10. The nested multiplex PCR was positive for *E. histolytica* DNA in 51 (96.2%) of 53 liver abscess pus specimens from ALA patients (Table III-2). The test did not detect *E. histolytica* DNA in liver abscess pus from all 15 patients with PLA.
Sequencing result of nested PCR product of 16S-like rRNA gene of *E. histolytica* from urine specimen with species specific primer Eh-1 and Eh-2

**Figure iii-1**

Sequence with forward primer Eh-1

ACATTCTAAAGTGAATTAGGTGACACACCTCTGTTTGAGGAGGAGACATGGTACGATCAGAATGATTACCCCTTCACTCCTTCTCGGTCTACTTCAAGAATGAGGAATAC

99% identities with X59891

Sequence with reverse primer Eh-2

CACAGTTGAGTAAATGGCTCTCGCTCAGATCTTTAAATTGAACATGGTACGATCAGAATGAGGAATAC

100% identities with X59891

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

**Figure iii-2**

Sequence with forward primer Eh-1

AGGAAATCGGACGATCGGACGACAGGAGGACTTTACAGATGCTACACCTCTCAAAGGAAGGCAGCAGGGAACGTAATTAGCAGAATGAGGAATAC

100% identities with X59891

Sequence with reverse primer Eh-2

TTCTTGAGTACGACTTTGCCATTGATGCTGACATCTTACAGAATGAGGAATAC

99% identities with X59891
Sequencing result of nested multiplex PCR product of 16S rRNA gene of *E. histolytica* from urine specimen with species specific primer EH-1 and EH-2

**Sequence with forward primer EH-1**

```
GGATCTGCTCTAATATAGCTTTAGGCTGTAAGGCGTTACAGCTTCATAGGCGATAGTTCCTGATGCTGTCTCAGTCAGCTCTGCT
```

99% identities with X56991

**Sequence with reverse primer EH-2**

```
CATGAAATGCTCTGTTACTGTTAGTGGCTCCCTTAGGAAAGTCTGACTGACCCCTGCTTGGGAACACTTTTCTGCTATGCTATAGGCAG
```

99% identities with X56991
Figure III-5

Sequencing chromatogram of nested multiplex PCR product of 16S r RNA gene of *E. histolytica* from urine specimen with species specific primer EH-1

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Figure III-6

Sequencing chromatogram of nested multiplex PCR product of 16S r RNA gene of *E. histolytica* from urine specimen with species specific primer EH-2
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

```
CCCGCATGTTCGTTCAGCTTAACTAACAGTTGTTATGGCATGATGCAAGCTGAACTTAAAGG
AAATTGAGGAAAGGCCACACCGAGGTGAGCTGCGCTTTATGTCAACAGCGGAAAACCTAACAGCGAACA
GTAGAAGGAAATTGACAGATTAAAGGTTTTACCTTATGATTATTTGAGGATGAGTCTG
TTGTCAGGTATATTCCGGTAACGAACGAGACTGAAACCTATTAAATATTGTTTTCTGCTATAAGACAGAAAATGTTGCGAAGA
ACAGGTTCGTAAAGTACCACCTCTTAAAGGGACACATTCCAATTGTCTATTTTAATTGTAATTGCTAATTTCCGCTAGNACC
TCTAAGAA
```

99% identities with X56991

Sequence with reverse primer EH-2

```
CCACATCAGAAAATGATGTTCCTAACTAACAGTTGTTATGGCATGATGCAAGCTGAACTTAAAGG
AAATTGAGGAAAGGCCACACCGAGGTGAGCTGCGCTTTATGTCAACAGCGGAAAACCTAACAGCGAACA
GTAGAAGGAAATTGACAGATTAAAGGTTTTACCTTATGATTATTTGAGGATGAGTCTG
TTGTCAGGTATATTCCGGTAACGAACGAGACTGAAACCTATTAAATATTGTTTTCTGCTATAAGACAGAAAATGTTGCGAAGA
ACAGGTTCGTAAAGTACCACCTCTTAAAGGGACACATTCCAATTGTCTATTTTAATTGTAATTGCTAATTTCCGCTAGNACC
TCTAAGAA
```

99% identities with X56991

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 III-9

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 III-10

Result of nested multiplex PCR on representative urine and liver abscess pus specimen. The E. histolytica (EH) and internal amplification control (IAC) bands are 439 bp and 305 bp respectively. Lane 1 and 4 are positive for E. histolytica DNA in liver abscess pus and urine specimen respectively; Lane 2, 3 and 5 are negative for E. histolytica DNA; Lane 8, 100 bp DNA ladder (Bangalore gene, Bangalore).
Table III-2 Detection of Entamoeba DNA in liver abscess pus and urine specimen of ALA patients by applying 16S-like r RNA gene based nested multiplex PCR

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No of patients</th>
<th>Liver abscess pus specimen</th>
<th>Urine specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA*</td>
<td>53</td>
<td>E. histolytica 51 (96.2)</td>
<td>E. histolytica 21 (39.6)</td>
</tr>
<tr>
<td>PLA*</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ODL*</td>
<td>8</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>HCD*</td>
<td>35</td>
<td>NA</td>
<td>0</td>
</tr>
</tbody>
</table>

*amoebic liver abscess; *pyogenic liver abscess; *other diseases of liver; *healthy controls

Cysteine proteinases gene based nested PCR-RFLP

Primer validation

The sequencing result of PCR product of E. histolytica from urine and liver abscess pus specimen showed 99% identities to the sequence deposited in GenBank, [accession number: S58669] (Figure III-11).

Assessment of competition of non target DNA

The result of assessment of competition of other non-target DNA present in urine (negative control group) and 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.
Figure III-11

Sequencing result of nested PCR product of cysteine proteinase gene of *E. histolytica* with species specific primer HCP-1 and HCP-2

**Sequence with forward HCP-1**

ATAGAAAGAACTATCTATTCTGATACTAATATGATGATCAAGAATGATGCACCTGTTACTGCT
GTTGTTATGTACAAATAGTAATGTTAATATATTGATTATTAGAAACTCATGCGGAA

100% identities with SE8889

**Sequence with reverse primer HCP-2**

CCATGATGTCCCCCACTAGATTTCTAAATACCAATATTTACCATTAGTTGAAACCAAACGAG
ATGACAGCTGATCTCATTCTGTATCTACATTATTGATCGAAATGATAGTT

100% identities with SE8889

Figure III-12

*E. histolytica*-specific nested cysteine proteinase PCR products. Odd and even numbered lanes represent undigested and *Hinf*Ⅳ-digested PCR products respectively.

Lane 1/2: DNA from liver abscess pus specimen showing *E. histolytica*. Lane 3/4: DNA from urine specimen showing *E. histolytica*. M: A 100bp DNA ladder
**Nested PCR-RFLP**

The nested PCR-RFLP result on urine specimen is shown in figure III-12. The nested PCR-RFLP test detected *E. histolytica* DNA in 17 (32.1%) of 53 urine samples collected from patients with ALA (Table III-3). The test did not detect *E. histolytica* DNA in urine samples collected from all 23 patients with PLA and other diseases of the liver; and 35 healthy controls.

The nested PCR test detected *E. histolytica* DNA in the urine specimens of 3 (13%) of 23 ALA patients who were tested prior to treatment with metronidazole and in 14 (46.7%) of 30 ALA patients who were tested after treatment with metronidazole ($\chi^2 = 5.3, P = 0.021$). All of the 3 ALA patients, who did not receive prior treatment with metronidazole and whose urine specimens were positive for *E. histolytica* DNA, were available for follow-up study. Urine specimens were collected from these patients every week for 4 weeks after starting of the therapy with metronidazole; and were tested for *E. histolytica* DNA by PCR. It was observed that 2 weeks after treatment with metronidazole, 2 (66.7%) out of 3 urine specimens became negative for *E. histolytica* DNA. One urine specimen became negative for *E. histolytica* DNA after 4 weeks of treatment with metronidazole.

The result of nested PCR-RFLP performed on the liver abscess pus is depicted in figure III-12. The nested PCR test was positive for *E. histolytica* DNA in 51 (96.2%) of 53 liver abscess pus specimens (Table III-3). The test did not detect *E. histolytica* DNA in liver abscess pus from all 15 patients with PLA.
Table III-3 Detection of Entamoeba DNA in liver abscess pus and urine specimen of ALA patients by applying cysteine proteinases gene based nested PCR–RFLP

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No of patients</th>
<th>Liver abscess pus specimen</th>
<th>Urine specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALA⁴</td>
<td>53</td>
<td>E. histolytica 51 (96.2)</td>
<td>E. histolytica 17 (32.1)</td>
</tr>
<tr>
<td>PLA⁵</td>
<td>15</td>
<td>E. dispar 0</td>
<td>E. dispar 0</td>
</tr>
<tr>
<td>ODL⁶</td>
<td>8</td>
<td>E. moshkovskii 0</td>
<td>E. moshkovskii 0</td>
</tr>
<tr>
<td>HC⁷</td>
<td>35</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

⁴ amoebic liver abscess; ⁵ pyogenic liver abscess; ⁶ other diseases of liver; ⁷ healthy controls

DISCUSSION

In this study, an attempt was made to detect *E. histolytica* DNA in urine of ALA patients by applying three different PCR methods. Also the diagnostic potential of the PCR methods was assessed for detection of *E. histolytica* DNA in urine for the diagnosis of ALA.

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.

In the present study, the 16S-like r RNA gene based nested PCR- RFLP detected *E. histolytica* DNA in the urine specimen of 3 (13%) of 23 ALA patients, who were tested prior to treatment with metronidazole and in 16 (53.3%) of 30 ALA patients, who were tested after treatment with metronidazole by PCR. The probability of *E. histolytica* DNA detection in urine by 16S-like r RNA gene based
nested PCR- RFLP was 7 times more in ALA patients who had received prior metronidazole therapy (OR = 7.62, 95% CI = 1.62 to 40.8) than in the ALA patients who did not receive prior metronidazole therapy. The OR was statistically significant as the 95% CI of OR was greater than 1.

The 16S-like rRNA gene based nested multiplex PCR detected *E. histolytica* DNA in the urine specimen of 4 (17.4%) of 23 ALA patients, who were tested prior to treatment with metronidazole and in 17 (56.7%) of 30 ALA patients, who were tested after treatment with metronidazole by PCR. The probability of *E. histolytica* DNA detection in urine by 16S-like rRNA gene based nested multiplex PCR was 6 times more in ALA patients who had received prior metronidazole therapy (OR = 6.2, 95% CI = 1.47 to 28.37) than in the ALA patients who did not receive prior metronidazole therapy. The OR was statistically significant as the 95% CI of OR was greater than 1.

The cysteine proteinases gene based nested PCR-RFLP detected *E. histolytica* DNA in the urine specimen of 3 (13%) of 23 ALA patients, who were tested prior to treatment with metronidazole and in 14 (46.7%) of 30 ALA patients, who were tested after treatment with metronidazole by PCR. The probability of *E. histolytica* DNA detection in urine by cysteine proteinases gene based nested PCR-RFLP was 6 times more in ALA patients who had received prior metronidazole therapy (OR = 5.83, 95% CI = 1.24 to 31.13). The OR was statistically significant as the 95% CI of OR was greater than 1.
The sensitivities of all the three PCR methods were significantly higher when the urine specimen was tested after treatment with metronidazole. This might be due to release of increased *E. histolytica* DNA from the dying *E. histolytica* trophozoites when metronidazole therapy was initiated, leading to excretion of *E. histolytica* DNA in the urine. One study has demonstrated that the DNA from dying cells can cross the kidney barrier in rodents and humans and can get excreted with urine, which can be used for genetic analysis (Botezatu et al., 2000).

Three different PCR methods for detection of *E. histolytica* DNA in urine and liver abscess pus specimen 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 for urine was 35.8%, 39.6%, and 32.1% respectively. This was found to be significantly lower than 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 for liver abscess pus (96.2%), using McNemar's χ² test (*p* < 0.0001). All urine specimens from 23 patients with PLA and other diseases of the liver; and 35 healthy controls were negative for *E. histolytica* DNA by all the three PCR methods. This represents a specificity of 100%.

*E. histolytica* DNA in urine did not persist longer in ALA patients after treatment with metronidazole as observed in the present study. Majority of urine specimens positive for *E. histolytica* DNA became negative for *E. histolytica* DNA within 2 weeks of treatment with metronidazole. This might be attributed to the reduced excretion of *E. histolytica* DNA in the urine as a result of reduction of *E.
*histolytica* infection load following treatment with metronidazole. The effect of metronidazole in killing of *E. histolytica* and clearing of antigenemia in hamsters suffering from hepatic amoebiasis has been well documented in a study reported by Thammapalerd et al. (Thammapalerd et al., 1996). Results of the present study therefore indicate that the PCR can be used to monitor excretion of *E. histolytica* DNA in urine as a prognostic marker during therapy of ALA with specific anti-amoebic drugs.

In the present study, none of the urine and 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 (Table III-1, Table III-2 and Table III-3).

In conclusion, the present study for the first time shows the excretion of *E. histolytica* DNA in urine of ALA patients. The study also shows that the PCR for detection of *E. histolytica* DNA in urine of ALA patients can also be used as a prognostic marker to assess the course of the diseases following therapy by metronidazole. The detection of *E. histolytica* DNA in urine specimen of ALA patients provides a new approach for the diagnosis of ALA.

**SUMMARY**

Mortality from amoebiasis is mainly due to extra-intestinal manifestation, of which ALA is the most common. It is difficult to diagnose specifically ALA from other
space occupying lesions of liver. Imaging techniques although have excellent
sensitive to detect abscesses in the liver of varied aetiology, but fail to diagnose
specifically ALA. The approaches employed till date for the diagnosis of ALA
include; demonstration of *E. histolytica* trophozoite in liver abscess pus by
microscopy, demonstration of anti-*E. histolytica* antibodies in serum,
demonstration of *E. histolytica* antigen in serum and liver pus, and recently
demonstration of *E. histolytica* DNA in liver pus. Unfortunately, the collection of
blood or liver abscess pus involves invasive procedure. Therefore, of late much
interest has been shown towards the use of urine as a noninvasive alternate
specimen to the blood for the diagnosis of some infectious diseases. To the best of
our knowledge till now there is no report available on detection of *Entamoeba*
DNA in the urine for the diagnosis of ALA.

In the present study, therefore, three different PCR methods i.e. 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 evaluated to
detect *Entamoeba* DNA in urine of 53 ALA patients, 23 patients with PLA and
other diseases of the liver; and 35 healthy controls. The 53 ALA patients included
23 patients in which the metronidazole therapy was not initiated and 30 patients in
which the metronidazole therapy was already initiated.

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 35.8%, 39.6%, and 32.1% respectively for detecting *E.
histolytica* DNA in urine of ALA patients. All the three PCRs were negative in
urine of all 23 patients with PLA and other infections of the liver; and 35 healthy controls thus showing and specificity of 100%.

The sensitivities of all the three PCR methods were significantly higher when the urine specimen was tested after treatment with metronidazole. This might be due to release of increased *E. histolytica* DNA from the dying *E. histolytica* trophozoites when metronidazole therapy was initiated, leading to excretion of *E. histolytica* DNA in the urine. One study has demonstrated that the DNA from dying cells can cross the kidney barrier in rodents and humans and can get excreted with urine, which can be used for genetic analysis.

*E. histolytica* DNA in urine did not persist longer in ALA patients after treatment with metronidazole as observed in the present study. Majority of urine specimens positive for *E. histolytica* DNA became negative for *E. histolytica* DNA within 2 weeks of treatment with metronidazole. This might be attributed to the reduced excretion of *E. histolytica* DNA in the urine as a result of reduction of *E. histolytica* infection load following treatment with metronidazole. The effect of metronidazole in killing of *E. histolytica* and clearing of antigenemia in hamsters suffering from hepatic amoebiasis has been well documented in a study. Results of the present study therefore indicate that the PCR can be used to monitor excretion of *E. histolytica* DNA in urine as a prognostic marker during therapy of ALA with specific anti-amoebic drugs.
In the present study, none of the urine and 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 present study for the first time shows the excretion of *E. histolytica* DNA in urine of ALA patients. The study also shows that the PCR for detection of *E. histolytica* DNA in urine of ALA patients can also be used as a prognostic marker to assess the course of the diseases following therapy by metronidazole. The detection of *E. histolytica* DNA in urine specimen of ALA patients provides a new approach for the diagnosis of ALA.