7. **CHAPTER-IV**

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

The use of saliva as a diagnostic fluid has been increasingly reported worldwide in the last decade. Technological advancement has taken place during the past few years enabling the use of saliva as a clinical specimen to diagnose disease and predict disease progression (Streckfus and Bigler, 2002).

Initially, saliva was used as a clinical specimen for antibody detection in the diagnosis of infectious diseases. Salivary antibody detection was found to be useful for the diagnosis of bacterial infections caused by *Helicobacter pylori*, *Shigella* and *Borrelia burgdorferi* (Loeb et al., 1997; Schultsz et al., 1992; Schwartz et al., 1991) and various viral infections such as hepatitis A, hepatitis B, hepatitis C, measles, mumps, rubella, rotavirus, dengue, parvovirus B 19 and HIV (Parry et al., 1989; El-Medany et al., 1999; Thieme et al., 1994; Jayashree et al., 1988; Cuzzubbo et al., 1998; Rice and Cohen, 1996; Malamud, 1992). Salivary antibody detection has also been studied for the diagnosis of some parasitic infections caused by *Toxoplasma gondii*, *Schistosoma mansoni*, *Taenia solium* and *Entamoeba histolytica* (Loyola et al., 1997; Garcia et al., 1995; Ramos et al., 1997).

Subsequently, saliva has also been used for antigen detection in the diagnosis of pneumococcal pneumonia (Krook et al., 1986), hepatitis B virus, measles, mumps and rubella (Chaita et al., 1995; Friedman, 1982; Perry et al., 1993; Brown et al., 1994). There is only one report, till date on the detection of salivary lectin antigen
of the parasite *E. histolytica* for the diagnosis of amoebic liver abscess (ALA) with a sensitivity and specificity of 22% and 97.4% respectively (Abd-Alla et al., 2000).

The reports on the use of saliva for DNA detection for the diagnosis of infectious diseases, however, are limited (LaDuca et al., 1998; Miller et al., 2006; Pozo and Tenorio, 1999; Crepin et al., 1998; Li et al., 1995). The polymerase chain reaction (PCR) has been used to facilitate diagnosis of viral infections such as Epstein-Barr, cytomegalovirus, human herpes viruses 6, 7, and 8 and rabies by using saliva (LaDuca et al., 1998; Miller et al., 2006; Pozo and Tenorio, 1999; Crepin et al., 1998). The PCR has also been evaluated for detection of *H. pylori* associated peptic ulcer, by demonstration of *H. pylori* DNA in saliva (Li et al., 1995). However, reports on the detection of parasite DNA in saliva for the diagnosis of a parasitic infection, even, amoebiasis are still lacking. In the present study, therefore, PCR was evaluated for the detection of *E. histolytica* DNA, possibly secreted in the saliva for the diagnosis of ALA, a condition which is the most important and serious extra-intestinal manifestation of the amoebiasis, which is associated with high morbidity and mortality. An early and specific diagnosis of the condition followed by immediate treatment reduces morbidity and mortality due to the disease to a great extent.

**OBJECTIVE**

To evaluate saliva as a specimen for the diagnosis of ALA
MATERIALS AND METHODS

1. Sample details

The present study was conducted in the Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER) hospital, Puducherry, India, during a period from August 2005 to March 2006.

Patients with ALA (n=28)

The study included 28 ALA patients, diagnosis of which was established on the basis of criteria mentioned earlier in chapter-II. In the present study, the 28 ALA patients included 8 patients in which the metronidazole therapy was not initiated and 20 patients in which the metronidazole therapy was already initiated.

Patients with pyogenic liver abscess (PLA) and other diseases of the liver (n=21)

The study included cases of PLA (n=13), 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 *E. histolytica* infection by microscopy and culture.

2. Sample collection

*Saliva*

Saliva specimens were collected from all 28 ALA patients, 21 patients with PLA and other diseases of the liver; and 35 healthy controls. 5ml of saliva specimen was collected from each individual in a sterile container using aseptic techniques and was stored at 4°C until used.

*Liver abscess pus*

The aspiration of liver abscess pus was indicated only under the conditions mentioned earlier in 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 28 ALA patients and 13 PLA patients, and was stored at -20°C in a sterile container until used.

*Blood*
Blood specimens were collected from all 28 ALA patients, 21 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 $\geq 1:128$ was considered positive for ALA (Parija et al., 1988). The detailed protocol of Rapid-IHA test is already mentioned in chapter-I.

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 $\times$ 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 saliva by PCR
**Chapter-IV**

*Extraction of Entamoeba genomic DNA*

**Saliva**

For extracting DNA from saliva specimen 5 ml of the saliva sample was centrifuged at 12,000 g for 8 min at 4 °C. The supernatant was discarded and the pellet was suspended in 250 μl of sterile distilled water. To the suspension of saliva sediment (250 μl) 5 μl of proteinase-K (10 mg/ml) and 40 μl of 10% SDS were mixed and incubated for three hour at 65 °C. Then, 60 μl of 5 M NaCl and 15 μl of 10% CTAB were added to the mixture and incubated for 45 min at 65 °C. The suspension thus obtained was treated with 350 μl of chloroform, mixed by inversion and centrifuged at 12,000 g for eight minutes. The aqueous layer was mixed with 350 μl of phenol: chloroform: isoamyl alcohol mix (25:24:1) in a fresh tube, mixed by inversion and centrifuged at 12,000 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 12,000 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 12,000 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 saliva 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 saliva 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 saliva and liver abscess pus specimen**

DNA quantification in spin column purified DNA extract from saliva 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.
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 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 saliva 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*

*Saliva 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 1.0μl of 25mM MgCl₂ 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 1.0μl 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 saliva 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 saliva (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**

**Primer 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 saliva 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 saliva 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**

*Saliva 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 2μl 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 saliva PCR, except that 2.0μl of 25mM MgCl₂ was added.

The conditions for 16S-like r RNA gene based nested multiplex PCR for saliva 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 saliva and liver abscess pus and specimen with target DNA, the nested multiplex PCR was checked with reference DNA (DNA from standard culture of \textit{E. histolytica}, \textit{E. dispar} and \textit{E. moshkovskii}) spiked with DNA from saliva (negative control group) and liver abscess pus (PLA pus negative for \textit{E. histolytica}) followed by nested multiplex PCR amplification.

\textbf{Cysteine proteinases gene based nested PCR–RFLP}

\textit{Primers design}

The primer design has already been described in details in \textit{chapter-I}. The primer sequences used for cysteine proteinases gene based nested PCR–RFLP is shown in table 1-2 of \textit{chapter-I}.

\textit{Primer validation}

The primer sequences designed for \textit{E. histolytica} and \textit{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 saliva 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/Blast.cgi.). The identity between the sequencing results of PCR product of *E. histolytica* from saliva 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*

*Saliva 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 2.5μl 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].
Liver abscess pus PCR: The PCR mix composition was the same as described above for saliva 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 saliva and liver abscess pus and 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 saliva 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. dispar) spiked with DNA from saliva (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, χ²-squared and Fisher’s exact test were performed. The χ²-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 22 (78.6%) of 28 ALA patients and 2 (5.7%) of 35 healthy controls. The IHA test was negative for anti-amoebic antibodies in the serum of all 21 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 13 (46.4%) of 28 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 13 patients with PLA.

5. Detection of Entamoeba DNA in saliva by PCR

Quantification of DNA in saliva and liver abscess pus specimen

The quantification of DNA in the saliva and liver abscess pus specimen by spectrophotometric analysis showed the DNA yield to be approximately 33 and 85μg/ml respectively. The purity of DNA extract from liver abscess pus and saliva
specimens 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 saliva (Figure IV-1) and liver abscess pus (Figure IV-2) and 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 saliva (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.

**Nested PCR-RFLP**

The nested PCR performed on the representative saliva specimen is shown in figure IV-3. The nested PCR detected *E. histolytica* DNA in 7 (25%) of 28 saliva specimens collected from ALA patients (Table IV-1). The nested PCR was negative for *E. histolytica* DNA in the saliva of all the 8 ALA patients who were tested prior to treatment with metronidazole. In contrast, prior metronidazole
Sequencing result of nested PCR product of 16S-like r RNA gene of *E. histolytica* from saliva specimen with species specific primer Eh-1 and Eh-2

**Figure IV-1**

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 IV-2**

**Figure IV-3**
treatment significantly increased the ability to detect *E. histolytica* DNA in the saliva, with 7 (35%) out of 20 ALA patient specimens positive (Fisher’s Exact test, \( P = 0.074 \)). The test did not detect *E. histolytica* DNA in saliva samples collected from all 21 patients with PLA and other diseases of the liver; and 35 healthy controls (Table IV-1).

### Table IV-1. Detection of *Entamoeba* DNA in liver abscess pus and saliva 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>Saliva specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>E. histolytica</em></td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td>ALA(^a)</td>
<td>28</td>
<td>28 (100)</td>
<td>0</td>
</tr>
<tr>
<td>PLA(^b)</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ODI(^c)</td>
<td>8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HC(^d)</td>
<td>35</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

\(^a\) amoebic liver abscess; \(^b\) pyogenic liver abscess; \(^c\) other diseases of liver; \(^d\) healthy controls

The result of nested PCR-RFLP performed on the liver abscess pus is depicted in figure IV-3. The nested PCR was positive for *E. histolytica* DNA in all (100%) of 28 liver abscess pus specimens from ALA patients (Table IV-1). The test did not detect *E. histolytica* DNA in liver abscess pus from all 13 patients with PLA.

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

**Primer validation**

The sequencing result of PCR product of *E. histolytica* from saliva (Figure IV-4, Figure IV-5 and Figure IV-6) and liver abscess pus (Figure IV-7, Figure IV-8)
and Figure IV-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 saliva (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

The nested multiplex PCR performed on the representative saliva specimen is shown in figure IV-10. The nested multiplex PCR detected *E. histolytica* DNA in 8 (28.6%) of 28 saliva specimens collected from ALA patients (Table IV-2). The nested multiplex PCR was negative for *E. histolytica* DNA in the saliva of all the 8 ALA patients who were tested prior to treatment with metronidazole. In contrast, prior metronidazole treatment significantly increased the ability to detect *E. histolytica* DNA in the saliva, with 8 (40%) out of 20 ALA patient specimens positive ($\chi^2 = 2.73, P = 0.062$). The test did not detect *E. histolytica* DNA in saliva samples collected from all 21 patients with PLA and other diseases of the liver; and 35 healthy controls (Table IV-2).

The result of nested multiplex PCR performed on the liver abscess pus is depicted in figure IV-10. The nested multiplex PCR was positive for *E. histolytica* DNA in
Figure IV-4
Sequencing result of nested multiplex PCR product of 16S r RNA gene of *E. histolytica* from saliva specimen with species specific primer EH-1 and EH-2

Sequence with forward primer EH-1

TTTTCAATGCTGCGCTATAGCTAGATCTGCTAGCTAATTGGAATGCTCTAATCTGCG
GCTGAACTTTAAATCGGATGAACTTGCGCTGCTGGCTGTTATGGCTGTTACTCAAC
GGGAAAACTTACAGGAGCCAGTGCATAGTAAAGATGAAGATCTTATTCTACTG
AGTGGTGGATCGGCGCCTTCCTTAAGTTGATGCTGAGCGATTATTCTCAGGTTAATT
CCGTTAAGCAGAAGCTGCTGTTAGTAGCCACTCTTTAAGGACACATATTCTTCTCT
TTAAAA

99% identities with X59991

Sequence with reverse primer EH-2

TTCTTCTGCTACATGTTAGGCTGCGTCTTTCCTTCTCTAGCTTAAACAACGGGTATTAGGGAACCT
TCAGTTTCTACTACGCGACGAAATACATATAATGATATGCTCAGATCTGCTGCTTTACCCGAM
TTAACCATAGCAATATTACTGACACACTAAGAACGCGATGCGAACCTACATTGAAAGAAC
CTTTAACTGCTACCTGCTACGGTCTCTGCTGTTAAGTTTCCGCGTGGATGCTAATTTAAGCGGAG
GCTCTGACTCTGCTGCGCTTCTGCTTATTCTTATTACTCGCGTCTGAGGATTGCTGCTG
TCCATTAAACTGAGATTCTCTTTAAAGTTGCTGAAACAGGATATTGATAATCTAGATCTG
TTAAAA

99% identities with X59991

Figure IV-5
Sequencing chromatogram of nested multiplex PCR product of 16S r RNA gene of *E. histolytica* from saliva specimen with species specific primer EH-1

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Figure IV-6
Sequencing chromatogram of nested multiplex PCR product of 16S r RNA gene of *E. histolytica* from saliva specimen with species specific primer EH-2

209
Figure IV-7
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

```
CCCCCATGTTCGGTTACATTTGAAGGTTAAGGACAACTTTGCTGGCAACATCGCTGACATCG

99% identities with X56991
```

Sequence with reverse primer EH-2

```
CCCCCATCATCAGAATGGATGTACGTTAACAAGCTGCTGCAACATCGCTGACATCG

99% identities with X56991
```

Figure IV-8
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 IV-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
Chapter-IV

all (100%) of 28 liver abscess pus specimens from ALA patients (Table IV-2).

The test did not detect *E. histolytica* DNA in liver abscess pus from all 13 patients with PLA.

Table IV-2. Detection of *Entamoeba* DNA in liver abscess pus and saliva 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>Saliva specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16S-like r RNA gene based nested multiplex PCR for:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. histolytica</em></td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td>ALA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28</td>
<td>28 (100)</td>
<td>0</td>
</tr>
<tr>
<td>PLA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ODL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>35</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup>amoebic liver abscess; <sup>b</sup>pyogenic liver abscess; <sup>c</sup>other diseases of liver; <sup>d</sup>healthy controls

Cysteine proteinases gene based nested PCR–RFLP

Primer validation

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

Assessment of competition of non target DNA

The result of assessment of competition of other non-target DNA present in saliva (negative control group) and liver abscess pus (PLA pus negative for *E. histolytica*)
Sequencing result of nested PCR product of cysteine proteinase gene of *E. histolytica* with species specific primer HCP-1 and HCP-2

**Figure IV-11**

Sequence with forward HCP-1

```
ATAGGAGAGGAACTATCTATTGTATCTTAAGATCTCAAGATGATGATGCTCTGCTGAGCT
```

100% identities with EHE12000

Sequence with reverse primer HCP-2

```
CCATGATGTCACAGAGTTTCATTAATCCCAATATTTACATTTAACCATTCTATTTAGACATACAAACACGAC
```

100% identities with EHE12000

**Figure IV-12**

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

Lane 1/2-3/4: DNA from liver abscess pus specimen showing *E. histolytica*; Lane 5/6: DNA from saliva specimen showing *E. histolytica*. M: A 100-bp DNA ladder.
specimen with target DNA, showed expected amplification and no non-specific amplification in nested PCR-RFLP.

**Nested PCR-RFLP**

The nested PCR performed on the representative saliva specimen is shown in figure IV-12. The nested PCR detected *E. histolytica* DNA in 7 (25%) of 28 saliva specimens collected from ALA patients (Table IV-3).

The nested PCR was negative for *E. histolytica* DNA in the saliva of all the 8 ALA patients who were tested prior to treatment with metronidazole. In contrast, prior metronidazole treatment significantly increased the ability to detect *E. histolytica* DNA in the saliva, with 7 (35%) out of 20 ALA patient specimens positive (Fisher’s Exact test, P= 0.074).

The test did not detect *E. histolytica* DNA in saliva samples collected from all 21 patients with PLA and other diseases of the liver; and 35 healthy controls (Table IV-3).

The result of nested PCR-RFLP performed on the liver abscess pus is depicted in figure IV-12. The nested PCR test was positive for *E. histolytica* DNA in all (100%) of 28 liver abscess pus specimens (Table IV-3).

The test did not detect *E. histolytica* DNA in liver abscess pus from all 13 patients with PLA.
Table IV-3. Detection of Entamoeba DNA in liver abscess pus and saliva 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>Saliva specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. histolytica</td>
<td>E. dispers</td>
</tr>
<tr>
<td>ALA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28</td>
<td>28 (100)</td>
<td>0</td>
</tr>
<tr>
<td>PLA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ODL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HCl&lt;sup&gt;d&lt;/sup&gt;</td>
<td>35</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup> amoebic liver abscess; <sup>b</sup> pyogenic liver abscess; <sup>c</sup> other diseases of liver; <sup>d</sup> healthy controls

**DISCUSSION**

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 (Smoger et al., 1998; Kasper et al., 2005). Imaging techniques although are highly sensitive to detect abscesses in the liver of varied aetiology, however fail to distinguish specifically ALA from that of PLA.

The demonstration of *E. histolytica* trophozoite in liver pus by microscopy confirms the diagnosis of ALA, but in best of the conditions, the amoebic trophozoites can be demonstrated in only 15% of the liver pus (Parija, 1993). Therefore, the laboratory diagnosis of ALA is usually established by antibody-based serological tests, but the serum antibody levels in individuals living in endemic areas, continues to remain positive even for years after eradication of infection with *E. histolytica* (Gandhi et al., 1987; Jackson et al., 1984; Yang and Kennedy, 1979). A monoclonal antibody-based second generation TechLab *E. histolytica* II enzyme-linked immunosorbent (ELISA) assay has been reported to
be 78% and 40.7% sensitive for the detection of *E. histolytica* Gal/GalNAc lectin antigen in the serum and liver pus of ALA patients respectively (Haque et al., 2000).

Earlier the PCR to detect *Entamoeba* DNA in stool samples has been evaluated as a sensitive and specific method for the diagnosis of intestinal amoebiasis (Khairnar and Parija, 2007; Fotedar et al., 2007; Lebbad and Svard, 2005; Roy et al., 2005; Freitas et al., 2004; Khairnar et al., 2007; Nunez et al., 2001; Ali et al., 2003; Parija and Khairnar, 2005). Studies conducted in various laboratories worldwide has shown that PCR has a varied sensitivity ranging from 33% to 100% for detecting *E. histolytica* DNA in liver abscess pus for the diagnosis of ALA (Haque et al., 2000; Khan et al., 2006; Zengzhu et al., 1999; Zaman et al., 2000; Parija and Khairnar, 2007). Recently, for the first time the excretion of *E. histolytica* DNA in the urine of ALA patients by applying PCR was reported from our laboratory at JIPMER, Puducherry (Parija and Khairnar, 2007).

Like urine, saliva is also an easily accessible and non-invasive body-fluid. Salivary antigen detection has been studied earlier for the diagnosis of a few viral infections such as Epstein-Barr virus and Hepatitis B virus (Crowcroft et al., 1998; Chaita et al., 1995). There is only one report on the detection of *E. histolytica* Gal/GalNAc lectin antigen in the saliva of ALA patients (Abd-Alla et al., 2000). Although, reports are available for the detection of salivary DNA by PCR for the diagnosis of Epstein-Barr virus, cytomegalovirus, human herpes virus 6, 7 and 8 virus, rabies virus (LaDuca et al., 1998; Miller et al., 2006; Pozo and Tenorio, 1999; Crepin et
al., 1998) and *H. pylori* (Li et al., 1995), but to the best of our knowledge till now there is no report available on detection of *E. histolytica* DNA in the saliva.

In the present study, for the first time, an attempt was made to evaluate PCR to detect *Entamoeba* DNA in saliva of ALA patients.

The 16S-like r RNA gene based nested PCR-RFLP, detected *E. histolytica* DNA in 7 (25%) of 28 saliva specimens collected from ALA patients (Table IV-1). The 16S-like r RNA gene based nested PCR-RFLP detected *E. histolytica* DNA in saliva of 7 of 20 cases of ALA which were already treated with metronidazole. The 16S-like r RNA gene based nested PCR-RFLP did not detect *E. histolytica* DNA in saliva of rest of the 8 cases of ALA, which were not treated with metronidazole. The probability of *E. histolytica* DNA detection in saliva by 16S-like r RNA gene based nested PCR-RFLP was 20 times more in ALA patients who had received prior metronidazole therapy (OR = 20.3, 95% CI = 1.62 to 20.3) than in the ALA patients who did not receive prior metronidazole therapy. The odds ratio was statistically significant as the 95% CI of OR was greater than 1.

The 16S-like r RNA gene based nested multiplex PCR, detected *E. histolytica* DNA in 8 (28.6%) of 28 saliva specimens collected from ALA patients (Table IV-2). The 16S-like r RNA gene based nested multiplex PCR detected *E. histolytica* DNA in saliva of 8 of 20 cases of ALA which were already treated with metronidazole. The 16S-like r RNA gene based nested multiplex PCR did not detect *E. histolytica* DNA in saliva of rest of the 8 cases of ALA, which were not treated with metronidazole. The probability of *E. histolytica* DNA detection in
saliva by 16S-like r RNA gene based nested multiplex PCR was 11 times more in ALA patients who had received prior metronidazole therapy [Odds Ratio (OR) = 11.56, 95% Confidence Interval (CI) = 1.59 to 240.2] than in the ALA patients who did not received prior metronidazole therapy. The odds ratio 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 7 (25%) of 28 saliva specimens collected from ALA patients (Table IV-3). The cysteine proteinases gene based nested PCR–RFLP detected *E. histolytica* DNA in saliva of 7 of 20 cases of ALA which were already treated with metronidazole. The cysteine proteinases gene based nested PCR–RFLP did not detect *E. histolytica* DNA in saliva of rest of the 8 cases of ALA, which were not treated with metronidazole. The probability of *E. histolytica* DNA detection in saliva by cysteine proteinases gene based nested PCR–RFLP was 20 times more in ALA patients who had received prior metronidazole therapy (OR = 20.3, 95% CI = 1.62 to 20.3) than in the ALA patients who did not received prior metronidazole therapy. The odds ratio 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 saliva specimen was tested after treatment with metronidazole. This might be due to the release of *E. histolytica* DNA from the dying *E. histolytica* trophozoites when metronidazole therapy was initiated, which might have lead to the secretion of *E. histolytica* DNA in the saliva of ALA patients.
Three different PCR methods for detection of *E. histolytica* DNA in liver abscess pus and saliva 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 saliva was 25%, 28.6%, and 25% 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 (100%), using McNemar’s $\chi^2$ test (p < 0.0001). All saliva specimens from 21 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 (Table IV-1, Table IV-2 and Table IV-3). This represents a specificity of 100%.

In the present study, none of the liver abscess pus and saliva specimens were positive for either *E. dispar* or *E. moshkovskii* by PCR, which confirm the non-invasive nature of these non-pathogenic species (Table IV-1, Table IV-2 and Table IV-3).

In conclusion, the present study for the first time demonstrates that the PCR can be used as a laboratory assay to detect *E. histolytica* DNA secreted in the saliva of patients with ALA, thereby providing a new approach for the diagnosis of the condition. Saliva as a clinical specimen offers distinctive advantages over blood as a diagnostic fluid. First and foremost being it can be collected non-invasively by individuals with minimal training. Second, the use of saliva as a clinical specimen may prove to be a cost-effective approach for the screening of large number of
patients especially in epidemiological studies. Finally, the saliva has the potential to be used for diagnosis of invasive infectious diseases, because it is collected readily and is also known to contain serum constituents. The serum constituents present in saliva are derived from the salivary gland’s local vasculature which ultimately reaches the oral cavity through the flow of gingival fluid (Kaufman and Lamster, 2002). However, we feel that much additional study is needed in the field before the true clinical value of saliva as a diagnostic fluid can be established.

SUMMARY

The use of saliva as a diagnostic fluid and non-invasive clinical specimen alternate to blood has been increasingly reported worldwide in the last decade.

Saliva has been used as a clinical specimen for antibody and antigen detection in the diagnosis of some bacterial, viral and parasitic diseases. Recently, saliva has also been used as a clinical specimen for DNA detection for the diagnosis of some viral and bacterial diseases, however, reports on the detection of parasite DNA in saliva for the diagnosis of a parasitic infection such as amoebiasis are still lacking.

In the present study, three different PCR methods i.e. 16S-like rRNA gene based nested PCR- RFLP and 16S-like rRNA gene based nested multiplex PCR, and cysteine proteinases gene based nested PCR–RFLP were evaluated to detect Entamoeba DNA in saliva of 28 ALA patients, 21 patients with PLA and other diseases of the liver; and 35 healthy controls. The 28 ALA patients included 8
patients in which the metronidazole therapy was not initiated and 20 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 25%, 28.6%, and 25% respectively for detecting *E. histolytica* DNA in saliva of ALA patients. All the three PCRs were negative in saliva of all 21 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 saliva specimen was tested after treatment with metronidazole. This might be due to the release of *E. histolytica* DNA from the dying *E. histolytica* trophozoites when metronidazole therapy was initiated, which might have lead to the secretion of *E. histolytica* DNA in the saliva of ALA patients.

In the present study, none of the liver abscess pus and saliva specimens were positive for either *E. dispar* or *E. moshkovskii* by PCR, which confirm the non-invasive nature of these non-pathogenic species.

The present study for the first time demonstrates that the PCR can be used as a laboratory assay to detect *E. histolytica* DNA secreted in the saliva of patients with ALA, thereby providing a new approach for the diagnosis of the condition. However, we feel that much additional study is needed in the field before the true clinical value of saliva as a diagnostic fluid can be established.