Comparison of PCR and antigen detection methods for diagnosis of *Entamoeba histolytica* infection

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**ORIGINAL ARTICLE**

Aims: To assess different laboratory methods for the identification of *Entamoeba histolytica* in clinical samples.

Methods: Antigen detection enzyme linked immunosorbent assay, polymerase chain reaction solution hybridisation enzyme linked immunosassay (PCR-SHELA), and a commercial Lightcycler PCR were compared using 101 stool and pus samples.

Results: Fifteen of the 101 samples were positive for *E histolytica* by one or more method. There were discrepancies between the results in five of these 15 samples when the assays were compared.

Conclusions: All three methods performed adequately, so that the choice of assay will depend on each individual laboratory’s budget and projected turnaround time.

**METHODS**

We studied 101 samples sent to the department of clinical parasitology at the Hospital for Tropical Diseases, London, UK, for amoebic investigations in the year up to May 2003. These comprised 94 microscopy positive stools (two had trophozoites, 92 had cysts) and seven pus samples from liver or lung abscesses of unknown aetiology.

The light microscopy was performed on formol-ether concentrates of stool. Pus samples were not routinely investigated microscopically for parasites, because this method is known to be insensitive.

Antigen detection was performed on all stool samples and on two of the pus samples, using the Techlab *E histolytica* II kit (Techlab, Blacksburg, Virginia, USA), an enzyme linked immunosorbent assay (ELISA) method based upon detection of the galactose adhesin of *E histolytica*. Samples of fresh stool or pus were placed in buffer on the day of receipt and processed within one week. Only two of the seven pus samples were tested, because the remainder had been frozen and therefore were not considered suitable for assay. The assay was performed according to the manufacturer’s instructions and read on a plate reader.

All samples underwent DNA extraction before PCR. Some samples were stored for a period of time before extraction. Of the 94 stools, 13 were extracted from fresh stool using the Qiagen (Hilden, Germany) stool kit according to the manufacturer’s instructions. Ten stool samples were frozen at −20°C before extraction using the Qiagen stool kit according to the manufacturer’s instructions. Seventy one stool samples were stored as formol-ether concentrates at 4°C before extraction, which was performed using a modification of the Qiagen blood mini kit protocol as follows. The formol-ether concentrate was centrifuged at 6500 rpm for two minutes in a benchtop mini centrifuge. The supernatant was discarded and the deposit was frozen at −80°C for 10 minutes, after which 180 μl of ATL buffer (Qiagen kit) and 20 μl of proteinase K were added to the deposit, which was incubated at 56°C for two hours. Next, 200 μl of AL buffer (Qiagen kit) was added and the mixture was incubated at 70°C for 10 minutes. The mixture was then centrifuged at 13000 rpm for one minute, the supernatant was retained and transferred to a fresh 1.5 ml microcentrifuge tube, and 200 μl of ethanol (96–100%) was added. The subsequent purification was carried out using Qiagen spin columns according to the manufacturer’s instructions.

Of the seven pus samples, one was extracted at the time of sample receipt and the remainder were frozen at −20°C before extraction using the Qiagen blood mini kit according to the manufacturer’s instructions.

Two separate PCR methods were used. The PCR solution hybridisation enzyme linked immunosassay (SHELA) was developed by the London School of Hygiene and Tropical Medicine, London, UK, for the detection and differentiation of *E histolytica* and *E dispar*, using separate primers specific for each species. It uses a colorimetric ELISA based method for the detection of the PCR products, so that the final results can be read on a plate reader or by eye. PCR-SHELA was performed on all 101 samples. The Artus RealArt PCR (LC-PCR; Artus, Hamburg, Germany) is a commercially available kit run on a Lightcycler (Roche, Mannheim, Germany), which amplifies a 230 bp region of the *E histolytica* genome: it is a quantitative PCR with fluorometric detection, and incorporates an internal control. At present, the manufacturers

**Abbreviations:** ELISA, enzyme linked immunosorbent assay; LC, Lightcycler; PCR, polymerase chain reaction; SHELA, solution hybridisation enzyme linked immunoassay
advise that it is used only on extracts from fresh or frozen stool. For financial reasons, use of LC-PCR was restricted to 34 samples, including all samples that were positive for *E histolytica* in another assay.

**RESULTS**

Stool microscopy was positive in all cases as an entry criterion to our study. Tables 1 and 2 provide further details of the results.

Fifteen of the 101 samples were positive for *E histolytica* by one or more method. These positives comprised five liver pus and 10 stool samples. In general, there was good concordance between the methods, but five stool samples gave discrepant results. One was Techlab ELISA negative but positive for *E histolytica* by PCR-SHELA and by LC-PCR. The second was Techlab ELISA positive, but identified as *E dispar* by PCR-SHELA, and LC-PCR was negative. The third was positive by Techlab ELISA and LC-PCR, but negative by PCR-SHELA. The fourth and fifth samples were positive by Techlab ELISA and PCR-SHELA, but negative by LC-PCR.

**DISCUSSION**

Ten of the 96 samples tested were positive by the Techlab antigen detection assay. One of these positives was a sample of liver pus from a patient with suspected amoebic liver abscess, confirming that the assay can sometimes work on non-faecal samples, despite the manufacturer’s recommendation that this assay should be performed only on fresh buffered stool. The remaining nine positives were stool samples from patients with suspected intestinal amoebic infection. All but one were confirmed as *E histolytica* by at least one other method. The Techlab *E histolytica* II assay is technically simple, relatively inexpensive, and takes approximately two hours to perform. Previous studies have found its sensitivity to be 85% compared with culture and isoenzyme analysis, therefore approaching that of PCR. The manufacturer states that the assay does not crossreact with other faecal pathogens such as *Giardia lamblia* or *Entamoeba coli*.

Twelve of 34 samples were positive by LC-PCR. All of these were also positive for *E histolytica* by at least one other method. The advantages of this assay are its simplicity and speed: the LC-PCR takes minutes to set up and less than an hour to run, following which the results are immediately available without the need to run a gel. Therefore, the entire process, including DNA extraction, can be performed within a day. The inclusion of an internal control is another advantage, allowing the user to detect inhibition within an individual reaction tube. The fluorescent detection system means that tubes need not be opened after amplification, which reduces the risk of contamination of the laboratory with PCR products. Results are archived on the computer system for ease of retrieval. The major disadvantage of this assay is the requirement for a Lightcycler PCR machine. Another issue is the lack of information about the assay, which works as a “black box”, with little information about the target DNA sequence.

Using PCR-SHELA, 10 samples were positive for *E histolytica* alone, three were positive for both *E histolytica* and *E dispar*, 64 were positive for *E dispar* alone, and 24 samples were negative. This assay is highly sensitive, because the primers are based on the sequences of repetitive elements in the abundant (several hundred/trophozoite) 25 kb ribosomal DNA episomes: Britten et al suggest a detection limit of $10^{-3}$ trophozoites for each gram of faeces for *E histolytica*, and one to 10 trophozoites for each gram of faeces for *E dispar*. The assay is more sensitive for *E histolytica* than for *E dispar* because the sequence of the repetitive element of *E dispar* does not allow for optimal primer design. The assay is robust and can be managed with basic PCR equipment. The main disadvantage of this assay is the length of time required: the whole process, including DNA extraction, takes a day and a half. However, this may not be an issue for laboratories that “batch” samples and process them at intervals.

Five of the 101 samples gave discrepant results when assayed by different methods (table 2). There are several possible explanations for these differences.

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Techlab ELISA</th>
<th>Artus PCR-PCR</th>
<th>PCR-SHELA</th>
<th>Storage before DNA extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Stool</td>
<td>Negative</td>
<td>Positive</td>
<td>Mixed <em>E histolytica</em> and <em>E dispar</em></td>
<td>Formol ether concentrate 4°C</td>
</tr>
<tr>
<td>2 Stool</td>
<td>Positive</td>
<td>Negative</td>
<td><em>E dispar</em></td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>3 Stool</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>4 Stool</td>
<td>Positive</td>
<td>Negative</td>
<td>Mixed <em>E histolytica</em> and <em>E dispar</em></td>
<td>Formol ether concentrate 4°C</td>
</tr>
<tr>
<td>5 Stool</td>
<td>Positive</td>
<td>Negative</td>
<td><em>E histolytica</em></td>
<td>Formol ether concentrate 4°C</td>
</tr>
<tr>
<td>6 Liver pus</td>
<td>N/A</td>
<td>Positive</td>
<td><em>E histolytica</em></td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>7 Liver pus</td>
<td>N/A</td>
<td>Positive</td>
<td><em>E histolytica</em></td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>8 Liver pus</td>
<td>N/A</td>
<td>Positive</td>
<td>Mixed <em>E histolytica</em> and <em>E dispar</em></td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>9 Liver pus</td>
<td>N/A</td>
<td>Positive</td>
<td><em>E histolytica</em></td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>10 Liver pus</td>
<td>N/A</td>
<td>Positive</td>
<td><em>E histolytica</em></td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>11 Stool</td>
<td>Positive</td>
<td>Positive</td>
<td><em>E histolytica</em></td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>12 Stool</td>
<td>Positive</td>
<td>Positive</td>
<td><em>E histolytica</em></td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>13 Stool</td>
<td>Positive</td>
<td>Positive</td>
<td><em>E histolytica</em></td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>14 Stool</td>
<td>Positive</td>
<td>Positive</td>
<td><em>E histolytica</em></td>
<td>Frozen – 20°C</td>
</tr>
<tr>
<td>15 Stool</td>
<td>Positive</td>
<td>Positive</td>
<td><em>E histolytica</em></td>
<td>Frozen – 20°C</td>
</tr>
</tbody>
</table>

ELISA, enzyme linked immunosorbent assay; LC, Lightcycler; N/A, not available; PCR, polymerase chain reaction; SHELA, solution hybridisation enzyme linked immunoassay.
The choice of assay will probably depend upon the available laboratory facilities, the required ‘‘turnaround time’’ for the laboratory, and the number of samples likely to be processed. It is clear that the Techlab ELISA was the simplest and most rapid assay, and therefore the most appropriate for field laboratories in the developing world. The PCR-SHELA may also be a feasible option for the developing world, because the molecular equipment required is relatively basic and the reagents, although expensive to buy initially, are sufficient to test hundreds of samples. The PCR-SHELA also has the advantages of validation for samples other than stool and the ability to identify E. dispar.

Many laboratories in the UK rarely come across entamoeba cysts in stool samples, so that their preferred option is to refer these samples to a reference laboratory. The reference laboratory must have an assay that is reliable, reproducible, and ideally inexpensive. PCR has the advantage of reproducibility in that DNA extracts can be stored, so the extract can be re-tested at a later date if required; in addition, the PCR product can be stored indefinitely and can even be sequenced if the result is in doubt. Both the PCR-SHELA and the Artus LC-PCR are suitable assays for the reference laboratory.

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