

ORIGINAL ARTICLE

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

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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 immunoassay (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.

The protozoan parasite *Entamoeba histolytica* is the causative agent of amoebic dysentery and amoebic liver abscess.^{1,2} It is a major cause of morbidity and mortality in the developing world, and with increasing travel, amoebic infections are now seen more frequently in the UK. Light microscopy of stool, the traditional diagnostic method, is unable to differentiate between cysts of *E histolytica* and the non-pathogenic amoeba *E dispar*.^{3,4} Therefore, newer methods, including antigen detection and polymerase chain reaction (PCR), are undergoing evaluation as diagnostic tools. We aimed to evaluate antigen detection and two different PCR methods for use in the diagnostic laboratory.

"With increasing travel, amoebic infections are now seen more frequently in the UK"

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.⁵

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 immunoassay (SHELA)^{6,7} 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

Table 1 Summary of results

| Method | Number of samples tested | Number of positives |
|-----------------|--------------------------|---------------------|
| Techlab ELISA | 96 (94 stool+2 pus) | 10 (9 stool+1 pus) |
| Lightcycler PCR | 34 (29 stool+5 pus) | 12 (7 stool+5 pus) |
| PCR-SHELA | 101 (94 stool+7 pus) | 13 (8 stool+5 pus) |

ELISA, enzyme linked immunosorbent assay; 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,⁸ 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 studies⁹ 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 10⁻¹ trophozoites for each gram of faeces for *E histolytica*, and one to 10 trophozoites for each gram of faeces for *E dispar*.⁷ 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 2 Summary of results positive for *Entamoeba histolytica*

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

ELISA, enzyme linked immunosorbent assay; LC, Lightcycler; N/A, not available; PCR, polymerase chain reaction; SHELA, solution hybridisation enzyme linked immunoassay.

- (1) The time between sample receipt and assay. The Techlab ELISA was performed under "ideal" conditions, in that the stool sample was placed into buffer on the day of receipt and assayed within one week. However, many of the DNA extracts used for PCR were made from stool samples stored for up to a year: of the five stool samples giving discrepant results, three had been stored as formol-ether concentrates at 4°C (for two, five, and nine months), and two had been frozen at -20°C (for four months and 12 months) before DNA extraction.
- (2) The nature of the sample. Some stool samples were stored at -20°C, whereas some were stored as formol-ether concentrates at 4°C. Qiagen recommends that fresh or frozen stool should be used for extraction, and does not recommend extraction from formol-ether concentrates: however, most such samples extracted and amplified successfully. Of the 71 extracts from formol-ether concentrates, 56 amplified in the PCR-SHELA. The remaining 15 failed to amplify, but the lack of an internal extraction control makes it impossible to say whether this was a failure of extraction or simply a negative result.
- (3) The presence of inhibitory substances. Stool samples often contain PCR inhibitors and DNA damaging substances, but their effect should be minimised by the use of InhibitEX tablets (which adsorb DNA damaging substances and PCR inhibitors) in the Qiagen procedure for DNA extraction from fresh or frozen stool. Inhibition of PCR can be identified in the LC-PCR, which incorporates an internal control, but not in the PCR-SHELA. To reduce the possible impact of inhibitors on the PCR-SHELA, all DNA extracts were added to the PCR mix both neat and at a 1/10 dilution, which should dilute the concentration of inhibitors in the final PCR mix, but at the cost of reducing the amount of target DNA in the PCR mix. This had no impact on the detection of pure *E histolytica* infection, but of the 64 pure *E dispar* infections, nine were detected only with neat extract, 15 only with a 1/10 dilution, and 40 were positive with both the neat samples and the 1/10 dilutions. Both the presence of inhibitors and the reduction in target DNA may have a more adverse effect on the *E dispar* PCR because the primer design for *E dispar* is suboptimal.
- (4) Crossreactivity between *Entamoeba* species. It is important that crossreactivity should not occur, because if other *Entamoeba* species are wrongly identified as *E histolytica* then false positive results will be issued. Although most research has concentrated on distinguishing *E histolytica* from *E dispar*, other species such as *E moshkovskii* also resemble *E histolytica*.¹⁰ The Techlab ELISA is designed to be specific for *E histolytica*, but there is occasional crossreactivity between *E histolytica* and *E dispar* in practice.⁹ This was demonstrated by case 2, where the Techlab ELISA was positive, LC-PCR was negative, and PCR-SHELA detected *E dispar*. The PCR-SHELA uses separate, species specific primers and probes for *E histolytica* and *E dispar*. Therefore crossreactivity should not occur. Cross contamination errors must be avoided by the use of good laboratory practice.¹¹ The LC-PCR primers are designed to be specific for the *E histolytica* genome (S Patel, Artus, personal communication, 2003), but further information on primer design is not available.

In conclusion, there was a good overall correlation between the methods. The three assays for *E histolytica* used in our study all proved comparable in sensitivity and specificity. The time required for the assays ranged from two hours to 1.5 days. The equipment required ranged from basic pipettes, vortex, and plate reader for the Techlab ELISA, to full molecular laboratory equipment incorporating a Lightcycler.

Take home messages

- We compared the sensitivity and specificity of three assays for the detection of *Entamoeba histolytica* infection—antigen detection enzyme linked immunosorbent assay, polymerase chain reaction (PCR)-SHELA, and a commercial Lightcycler PCR—using 101 stool and pus samples
- All three methods performed comparably, so that the choice of assay will probably depend upon the available laboratory facilities, the required "turn-around time" for the laboratory, and the number of samples likely to be processed

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 PCR assays for the reference laboratory.

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