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# Direct amplification of *Entamoeba histolytica* DNA from amoebic liver abscess pus using polymerase chain reaction

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**Abstract** An important and serious complication of intestinal infection with *Entamoeba histolytica* is the involvement of the liver (hepatic amoebiasis). Hepatic amoebiasis is usually diagnosed by the clinical picture (pain in the right upper quadrant and fever), ultrasound examination and positive serology. However, none of these tests are definitive and the picture overlaps with pyogenic liver abscess caused by bacteria. It is for this reason that the feasibility of using polymerase chain reaction (PCR) for the detection of *E. histolytica* DNA in liver abscess pus was investigated. A comparative study was done to verify the sensitivity of ten pairs of primers specific for detecting *E. histolytica* in stools. Samples of liver abscess pus from 22 serology-positive patients were collected under ultrasound guidance; and these were used directly in PCR assays without any prior pre-treatment of the samples. Of the ten pairs of previously published primers tested, two pairs of primers (P1 + P2 and P11 + P12) were found to give 100% sensitivity. Based on these results, we recommend that PCR assay can be successfully used to confirm the diagnosis of amoebic liver abscess with the primers identified.

**Key words** *Entamoeba histolytica* · Liver abscess · Polymerase chain reaction

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## Introduction

The protozoan parasite *Entamoeba histolytica* has a simple life cycle, consisting of an infective cyst stage and a multiplicative trophozoite stage, with transmission occurring via the faecal–oral route through ingestion of the cyst. It is now generally accepted that two morphologically identical species of *Entamoeba* exist – a non-pathogenic *E. dispar* which resides as a commensal in the human intestine, and a pathogenic *E. histolytica* which is capable of invading the tissue and causing both intestinal and extra-intestinal pathology (Jackson 1998).

Amoebiasis, the disease produced by *E. histolytica*, is the third leading cause of morbidity and mortality amongst the parasitic infections, after malaria and schistosomiasis (Walsh 1986). Death from amoebiasis is mainly due to extra-intestinal pathology, in which liver abscess (hepatic amoebiasis) is very common. It is critical to differentiate amoebic liver abscess (ALA) from pyogenic liver abscess (PLA) as the treatment for ALA and PLA are different. Left untreated, ALA can burst into neighbouring tissues and spread via the haematological route to the brain and other organs. Similarly, untreated PLA is usually fatal (Smoger et al. 1998). ALA also needs to be differentiated from space-occupying lesions of the liver, such as hydated cyst and hepatoma. Clinical diagnosis of liver abscess is based on tenderness in the right upper quadrant, fever, leucocytosis, and ultrasound observation, features which are common to both ALA and PLA (Sheen et al. 1989).

Laboratory confirmation of ALA is usually based on serological tests. However, this approach has a drawback in that antibody levels in individuals living in endemic areas could be high because of frequent and/or past exposure to this parasite, rather than acute disease. Microscopic examination of pus from ALA rarely reveals the parasite, as it is located in the periphery of the abscess. For this reason, cultural diagnosis is also unsatisfactory.

Polymerase chain reaction (PCR) has been shown to be a sensitive, reliable, and rapid method for detecting *Entamoeba* cysts in stool samples and for differentiating the morphologically identical *E. histolytica* from *E. dispar* (Tachibana et al. 1991a, b, 1992a; Tannich and Burchard 1991; Acuna-Soto et al. 1993; Clark and Diamond 1993, 1997; Katzwinkel-Wladarsch et al. 1994; Aguirre et al. 1995; Novati et al. 1996; Rivera et al. 1996; Britten et al. 1997; Gomes et al. 1997; Sanuki et al. 1997; Troll et al. 1997; Walderich et al. 1997; Haque et al. 1998). However, there are only two previous reports in which PCR has been used for the detection of *E. histolytica* DNA isolated from liver abscess pus samples (Tachibana et al. 1992b; Zengzhu et al. 1999).

In this paper, we compare various published primers, originally used for the detection of *E. histolytica* in stools, in order to identify the most useful primer for the diagnosis of ALA.

## Materials and methods

### ALA samples

Abscess fluid was obtained under ultrasound guidance from 22 patients with ALA. The presumptive diagnosis of ALA in these patients was based on clinical picture, ultrasound examination, and positive serology using ELISA.

### PCR conditions

Typically, 1–2 μl of ALA fluid was used in all PCR amplification tests. ALA fluid was added to a 100-μl volume containing 1 μl of DyNAzyme II polymerase (2 U/μl), 10 μl of 10× PCR buffer (for DyNAzyme II polymerase), 1 μl of primers (250 μm each), and 2 μl of 10 mM dNTP mix. Cultured trophozoites of *Entamoeba histolytica* and *E. dispar* were used as positive and negative controls respectively. The sequences of the ten primer pairs used are shown in Table 1. The conditions used for each of the ten primer pairs are listed below:

1. The primer pair P1 + P2 amplifies a 125-bp region of the extra-chromosomal circular DNA of *E. histolytica* (Acuna-Soto et al. 1993; Aguirre et al. 1995; Britten et al. 1997). After an initial denaturation step at 94 °C for 2 min, each amplification cycle was performed as follows: denaturation at 94 °C for 30 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s. After 35 cycles, the reactions were incubated at 72 °C for an additional 5 min and stored at −20 °C until needed. The primer pair NP1 + NP2, which amplifies *E. dispar* DNA, was used as a negative control.

2. The primer pair P11 + P12 amplifies a 100-bp sequence of the gene coding the 30-kDa antigen of pathogenic *E. histolytica* (Tachibana et al. 1991b, 1992b; Rivera et al. 1996; Sanuki et al. 1997; Zengzhu et al. 1999). After an initial denaturation step at 94 °C for 2 min, each amplification cycle was performed as follows: denaturation at 94 °C for 60 s, annealing at 55 °C for 90 s, and extension at 72 °C for 90 s. After 45 cycles, the reactions were incubated at 72 °C for an additional 5 min and stored at −20 °C until needed. The primer pair P13 + P14, which amplifies *E. dispar* DNA, was used as a negative control.

3. The primer pair Entf + Entr amplifies a 420-bp sequence of the small subunit rRNA (SSU rRNA) of both *E. histolytica* and *E. dispar* (Novati et al. 1996). The primer pair brackets a polymorphic *Dde*I restriction site, which can be used to distinguish between *E. histolytica* and *E. dispar*. Typically, *Dde*I digestion of PCR products results in three fragments for *E. histolytica* (240, 130, and 50 bp respectively) and two fragments for *E. dispar* (290 and 130 bp respectively). After an initial denaturation step at 94 °C for 2 min, each amplification cycle was performed as follows: denaturation at 94 °C for 30 s, annealing at 60 °C (first 5 cycles), 55 °C (next 5 cycles), 50 °C (last 25 cycles) for 30 s, and extension at 70 °C for 240 s. After 35 cycles, the reactions were incubated at 72 °C for an additional 5 min and stored at −20 °C until needed.

4. The primer pair P1 + P4 amplifies a 531-bp region of the gene encoding the 30-kDa antigen of both the pathogenic *E. histolytica* and the non-pathogenic *E. dispar* (Tachibana et al. 1991a, 1992b). *Eco*T221, *Hin*cII and *Taq*I cut the PCR product from the pathogenic strain but not from the non-pathogenic strain; *Hin*fI cuts both the PCR products from *E. histolytica* and *E. dispar* strains but results in different band patterns. After an initial denaturation step at 94 °C for 2 min, each amplification cycle was

**Table 1** The ten pairs of primers investigated for their ability to detect *Entamoeba histolytica* in liver abscess fluid samples

| Primer sequence | Reference |
|---|---|
| Primer pair 1 P1: 5′-TCAAAATGGTCGTCGTCTAGGC-3′<br>P2: 5′-CAGTTAGAAATTATTGTACTTTGTA-3′ | Acuna-Soto et al. 1993; Aguirre et al. 1995;<br>Britten et al. 1997 |
| Primer pair 2 P11: 5′-GGAGGAGTAGGAAAGTTGAC-3′<br>P12: 5′-TTCTTGCAATTCCTGCTTCGA-3′ | Tachibana et al. 1991b; Tachibana et al. 1992b<br>Rivera et al. 1996; Sanuki et al. 1997; |
| Primer pair 3 Entf: 5′-GGATTGGATGAAATTCAGATGT-3′<br>Entr: 5′-ATGTGTCCCTTTAAGAAGTGGT-3′ | Zengzhu et al. 1999; Novati et al. 1996 |
| Primer pair 4 P1: 5′-TAAAGCACCAGCATATTGTC-3′<br>P4: 5′-TTAATTCCATCTGGTGTTGG-3′ | Tachibana et al. 1991a; Tachibana et al. 1992a |
| Primer pair 5 Eh5: 5′-GTAACTTACTTAACCGGTAAAACATG-3′<br>Eh3: 5′-TCTCTTCGTAACAAAGATCTAGACTC-3′ | Troll et al. 1997 |
| Primer pair 6 SSG5: 5′-GGTCTCAAAAAACCCACGAG-3′<br>SSG3: 5′-CAAACGATAAAATCTAGCAAACTAC-3′ | Clark and Diamond 1993 |
| Primer pair 7 SREHP5: 5′-GCTAGTCCTGAAAAGCTT<br>GAAGAAGCTG-3′ SREHP3:<br>5′-GGACTTGATGCAGCATCAAGGT-3′ | Clark and Diamond 1993 |
| Primer pair 8 RD5: 5′-GGAAGCTTATCTGGTTGATCCTGCCAGTA-3′<br>RD3: 5′-GGGATCCTGATCCTTCCGCAGGTTCACCTAC-3′ | Clark and Diamond 1997 |
| Primer pair 9 EH1: 5′-TTTGTATTAGTACAAA-3′ EH2:<br>5′-GTA(A/G)TATTGATATACT-3′ EHP1:<br>5′-AATGGCCAATTCATTCAATG-3′ EHP2:<br>5′-T(C/T)TAGAAACAATGCTTCTCT-3′ | Katzwinkel-Wladarsch et al. 1994; Haque et al. 1998 |
| Primer pair 10 P1-S17: 5′-GCAACTAGTGTTAGTTA-3′<br>P1-AS20: 5′-CCTCCAAGATATGTTTTAAC-3′ | Tannich and Burchard 1991; Gomes et al. 1997;<br>Walderich et al. 1997 |

performed as follows: denaturation at 94 °C for 60 s, annealing at 55 °C for 120 s, and extension at 72 °C for 120 s. After 35 cycles, the reactions were incubated at 72 °C for an additional 5 min and stored at −20 °C until needed.

5. The primer pair Eh5 + Eh3 amplifies a 880-bp region of the multicopy 16S rRNA gene of *E. histolytica* (Troll et al. 1997). After an initial denaturation step at 94 °C for 2 min, each amplification cycle was performed as follows: denaturation at 94 °C for 60 s, annealing at 60 °C for 60 s, and extension at 72 °C for 120 s. After 40 cycles, the reactions were incubated at 72 °C for an additional 10 min and stored at −20 °C until needed.

6. The primer pair SSG5 + SSG3 amplifies a 400-bp region of the strain-specific gene (SSG) of *E. histolytica* (Clark and Diamond 1993). After an initial denaturation step at 94 °C for 2 min, each amplification cycle was performed as follows: denaturation at 94 °C for 60 s, annealing at 50 °C for 90 s, and extension at 72 °C for 120 s. After 35 cycles, the reactions were incubated at 72 °C for an additional 5 min and stored at −20 °C until needed.

7. The primer pair SREHP5 + SREHP3 amplifies a 550-bp region of the serine-rich antigen gene (SREHP) of *E. histolytica* (Clark and Diamond 1993). After an initial denaturation step at 94 °C for 2 min, each amplification cycle was performed as follows: denaturation at 94 °C for 60 s, annealing at 50 °C for 90 s, and extension at 72 °C for 120 s. After 35 cycles, the reactions were incubated at 72 °C for an additional 5 min and stored at −20 °C until needed.

8. The primer pair RD5 + RD3 amplifies a 2,000-bp region of the small subunit ribosomal RNA genes of both the pathogenic *E. histolytica* and the non-pathogenic *E. dispar* (Clark and Diamond 1997). After PCR amplification, restriction enzyme digestion using either *Xba*I, *Rsa*I or *Taq*I is required to differentiate between the pathogenic and non-pathogenic strains. For example, *Xba*I digestion results in four DNA bands for *E. histolytica* and three DNA bands for *E. dispar*. After an initial denaturation step at 94 °C for 2 min, each amplification cycle was performed as follows: denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s, and extension at 72 °C for 90 s. After 35 cycles, the reactions were incubated at 72 °C for an additional 10 min and stored at −20 °C until needed.

9. The primer pair EH1 + EH2 amplifies a 900-bp region of the small subunit rRNA genes of both *E. histolytica* and *E. dispar*, while the nested primer pair EHP1 + EHP2 is specific to *E. histolytica* (Katzwinkel-Wladarsch 1994; Haque et al. 1998). PCR amplification using primer pair EH1 + EH2 was carried out first, followed by amplification using primer pair EHP1 + EHP2. PCR amplification conditions were as follows for EH1 + EH2: denaturation at 92 °C for 60 s, annealing at 43 °C for 60 s, and extension at 72 °C for 60 s. After 50 cycles, the reactions were incubated at 72 °C for an additional 5 min. PCR amplification conditions were as follows for EHP1 + EHP2: denaturation at 92 °C for 60 s, annealing at 62 °C for 60 s, and extension at 72 °C for 90 s. After 50 cycles, the reactions were incubated at 72 °C for an additional 5 min and stored at −20 °C until needed.

10. The primer sequences of P1-S17 + P1-AS20 flank a 482-bp fragment of the cEh-P1 and cEh-NP1 cDNA clones (Tannich and Burchard 1991; Gomes et al. 1997; Walderich et al. 1997). Restriction fragment analysis is essential for the differentiation between *E. histolytica* and *E. dispar*. For example, *Acc*I cleaves the PCR product from the non-pathogenic strain but not the pathogenic strain, while *Taq*I and *Xmn*I cleave the PCR products from the pathogenic strain but not from the non-pathogenic strain. After an initial denaturation step at 94 °C for 2 min, each amplification cycle was performed as follows: denaturation at 94 °C for 90 s, annealing at 42 °C for 120 s, and extension at 72 °C for 180 s. After 35 cycles, the reactions were incubated at 72 °C for an additional 5 min and stored at −20 °C until needed.

### Agarose gel electrophoresis

Amplified samples (10 µl) were electrophoresed on a 2% agarose gel containing 0.5 µg ethidium bromide/ml. Agarose gels were photographed under ultraviolet light.

## Results and discussion

In total, ten pairs of primers were tested on 22 ALA samples obtained from hospitals in Karachi, Pakistan. Out of these ten pairs of primers investigated, only three pairs (i.e., P1 + P2, P11 + P12, and Entf + Entr) resulted in amplified products when tested directly on the ALA samples (Table 2). It is not clear as to why the other seven pairs of primers did not yield any amplified products when tested on the ALA samples, although it should be noted that these pairs of primers were originally optimised for the detection of *E. histolytica* in stool samples. One possible explanation as to why the other seven pairs of primers did not yield any amplified products could be given by intrinsic differences in the sensitivities of the various PCR assays. Zengzhu et al. (1999) have also reported such differences in PCR sensitivities when attempting to amplify various *E. histolytica* genes from liver abscess samples.

The first pair of primers, P1 + P2, amplifies a 125-bp region specific for pathogenic *Entamoeba histolytica* extra-chromosomal circular DNA containing highly repeated sequences that are different between pathogenic and non-pathogenic *Entamoeba* strains (Acuna-Soto et al. 1993; Aguirre et al. 1995; Britten et al. 1997). Acuna-Soto et al. (1993) reported that PCR analysis of stool samples using this pair of primers identified: (1) 24 out of 25 individuals (previously diagnosed to be infected with *E. histolytica* by microscopy) as being positive (sensitivity = 96%), and (2) three out of 176 "uninfected" individuals as being positive (specificity = 98%) for *E. histolytica* infection, respectively. We found that this pair of primers could also be effectively used for PCR analysis of ALA samples. In our study, the P1 + P2 primer pair produced a distinct 125-bp amplified product in all 22 ALA samples tested. This showed that the P1 + P2 primer pair has a sensitivity of 100% in detecting the presence of *E. histolytica* in the ALA samples, one of the highest sensitivity rating amongst the ten primer pairs investigated. It should be cautioned that the annealing temperature for this pair of primers is extremely critical for its sensitivity. For example, when annealing temperature was increased from 55 °C to 58 °C, there was a decrease in sensitivity from 100% to 77%. This demonstrates the importance of maintaining stringent temperature conditions during the PCR amplification steps.

The second pair of primers studied, P11 + P12, is able to detect and amplify a 100-bp PCR fragment of the gene coding the 30-kDa antigen of pathogenic *E. histolytica* (Tachibana et al. 1991b, 1992b; Rivera et al. 1996; Sanuki et al. 1997). Tachibana et al. (1991b) showed that P11 + P12 amplified a 100-bp product from the genomic DNA of 11 *E. histolytica* isolates with pathogenic zymodeme patterns, while no PCR products were amplified from any of the ten isolates with non-pathogenic zymodemes. Furthermore, Tachibana et al. (1992b) tested 19 liver abscess fluids (obtained from 14

patients with presumptive ALA) and found that, while only tao of the 19 samples were positive microscopically, PCR analysis of DNA extracted from the 19 samples showed that all tested positive. Thus, the 100-bp PCR product was amplified in 17 liver abscess samples that were negative microscopically. No PCR products were detected in any of the five control samples collected from patients with non-ALA. Zengzhu et al. (1999) reported that the gene encoding the 30-kDa protein was detected by PCR in all 42 ALA samples tested. Sanuki et al. (1997) found that out of 12 individuals previously tested positive for the presence of amoebic cysts in stool specimens, seven were found to be positive for *E. histolytica* when stool samples were tested using P11 + P12, while the other five were positive for *E. dispar*. Sanuki et al. (1997) concluded that the sensitivity of P11 + P12 was high enough for an accurate identification of amoeba cysts in stools. In our study, this P11 + P12 primer pair gave a band of 100 bp in all 22 ALA samples tested, giving a sensitivity of 100% in terms of the ability to detect the presence of pathogenic *E. histolytica* in ALA samples. However, one drawback of this pair of primers is that the 100-bp PCR product migrates very closely to the primer dimers, thus making interpretation of results difficult. Therefore, untrained technicians may mistake the primer dimer as a positive PCR product, which may lead to a "false positive" diagnosis. It should be noted that while Tachibana et al. (1992b) and Zengzhu et al. (1999) isolated DNA from the liver abscess fluid samples prior to PCR analysis, we found that PCR could be performed directly on "neat" pus samples without the need for a DNA isolation step, thereby saving time and effort.

The third pair of primers, Entf + Entr, is specific for both pathogenic *E. histolytica* and non-pathogenic *E. dispar*; and it amplifies the small subunit ribosomal RNA (SSU-rRNA; Novati et al. 1996). The amplified 420-bp fragment encompasses a polymorphic *Dde*I restriction site which allows the distinction between pathogenic *E. histolytica* and non-pathogenic *E. dispar* after restriction enzyme digestion. *Dde*I digestion of the 420-bp fragment results in three fragments for *E. histolytica* (240, 130, and 50 bp), and two fragments for *E. dispar* (290 and 130 bp). Novati et al. (1996) showed that the Entf + Entr primers produced the expected concentration of bands from 100, 10, and 5 trophozoites respectively, and also from stool samples to which trophozoites were added. In our study, this pair of primers (Entf + Entr) amplified a 420-bp band in 21 out of 22 ALA samples tested. Thus, this primer pair was 95% accurate in detecting the *E. histolytica* in the ALA samples. However, a disadvantage of this primer pair is that a *Dde*I restriction enzyme digestion of the 420-bp PCR product is essential in order to differentiate between *E. histolytica* and *E. dispar* strains. Thus, unlike the other three primer pairs in Table 2, the Entf + Entr pair requires an additional restriction digestion step in order to differentiate between the two *Entamoeba* strains.

In summary, the primer pair P1 + P2 (specific for the *E. histolytica* extra-chromosomal circular DNA) was found to be the most sensitive primer pair to use, out of the ten pairs of primers tested for detecting *E. histolytica* in liver pus samples. The P1 + P2 and P11 + P12 primers gave a distinct band of 125 bp and 100 bp respectively in all 22 liver pus samples tested, and thus have a sensitivity of 100% in clinically diagnosed ALA cases. This is the first report that PCR can be applied directly to ALA samples, without the need for any pretreatment of the samples, such as protease treatment and DNA isolation steps. In conclusion, PCR assay is useful in confirming the diagnosis of ALA if aspirated pus is available. PCR assay is sensitive (that is, only small amounts of pus sample is required), rapid (that is, results are available within 24 h), and specific (that is, reacting only to pathogenic *E. histolytica*).

**Table 2** Summary of results obtained from polymerase chain reaction (PCR) analysis of 33 amoebic liver abscess (ALA) samples using three primer pairs (P1 + P2, P11 + P12, and Entf + Entr). For PCR assay, + indicates presence of band and – indicates absence of band on agarose gels. All ALA samples were negative when tested with NP1 + NP2 and P13 + P14, both of which are specific for non-pathogenic *E. dispar*. For IgG antibody assay, IgG positivity cut off is OD = 0.308, as determined in controls ($n$ = 22). This cut off represents mean + 2 SD of the controls. *NA* not applicable

| Sample | PCR assay | | | IgG Antibody assay (OD) |
|---|---|---|---|---|
| | P1 + P2 | P11 + P12 | Entf + Entr | |
| ALA1 | + | + | + | + (0.973) |
| ALA2 | + | + | + | + (1.22) |
| ALA4 | + | + | + | + (1.30) |
| ALA5 | + | + | + | + (1.74) |
| ALA6 | + | + | + | + (0.78) |
| ALA7 | + | + | + | + (0.685) |
| ALA8 | + | + | + | + (0.432) |
| ALA9 | + | + | + | + (0.689) |
| ALA10 | + | + | + | + (0.746) |
| ALA11 | + | + | + | + (0.845) |
| ALA12 | + | + | + | + (1.432) |
| ALA13 | + | + | + | + (1.86) |
| ALA14 | + | + | + | + (0.934) |
| ALA15 | + | + | + | + (0.903) |
| ALA16 | + | + | + | + (2.2) |
| ALA17 | + | + | + | + (0.807) |
| ALA18 | + | + | + | + (0.82) |
| ALA19 | + | + | + | + (0.385) |
| ALA20 | + | + | + | + (1.32) |
| ALA21 | + | + | – | + (0.36) |
| ALA2 | + | + | + | + (0.832) |
| ALA10 | + | + | + | + (1.488) |
| Total positive | 22 | 22 | 21 | NA |
| Total negative | 0 | 0 | 1 | NA |
| % Sensitivity | 100% | 100% | 95% | NA |

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