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Entamoeba histolytica and *Entamoeba dispar*: comparison of two PCR assays for diagnosis in a non-endemic setting

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Summary Detection of *Entamoeba histolytica*, the causative agent of amoebiasis, is an important goal of the clinical parasitology laboratory. The identification of *Entamoeba dispar* as a morphologically identical but non-pathogenic species has highlighted the need for non-microscopic detection methods able to differentiate between the two organisms. In this study we evaluated the utility of conventional PCR and real-time PCR as methods for identification and differentiation of *E. histolytica* and *E. dispar*. The second aim of this study was to determine the relative proportions of infections caused by *E. histolytica* and the non-pathogenic *E. dispar*, allowing a picture of the epidemiological situation in a non-endemic setting to be obtained. One hundred and sixty-six clinical samples (faecal and liver abscess samples and one intestinal biopsy) belonging to 108 patients were analysed. More patients with *E. dispar* infection (8.3%) than patients with *E. histolytica* infection (5.6%) were found by both PCR assays. It is concluded that routine diagnosis of invasive amoebiasis performed by a combination of microscopy, culture and serology should be complemented with a PCR assay such as real-time PCR that offers a practical and clinically acceptable alternative for rapid and accurate diagnosis of amoebic infection in patients presenting with symptoms indicative of this disease.

1. Introduction

Detection of *Entamoeba histolytica*, the causative agent of amoebiasis, is an important goal of the clinical parasitology laboratory. Amoebiasis is one of the most common causes of death from protozoan parasitic disease, second only to malaria, with approximately 50 million cases and 100 000

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deaths annually, as reported by the WHO (Tanyuksel and Petri, 2003; WHO, 1997). *Entamoeba histolytica* infection may be asymptomatic or may result in dysentery, or extra-intestinal disease involving the liver, lung or brain (Haque et al., 2003). Traditionally, laboratory detection of the potentially invasive *E. histolytica* in human samples has relied upon microscopy (Blessmann et al., 2002), although numerous studies have demonstrated the inadequacies of microscopic examination for the diagnosis both of amoebic colitis and liver abscess (Tanyuksel and Petri, 2003). Amoeba cultivation followed by zymodeme analysis has a low sensitivity and is labour intensive. It also has the problem of possible overgrowth of the culture by other parasites that obscure the presence of *E. histolytica* (Tanyuksel and Petri, 2003).

Detection of antibodies to the amoeba in patient sera by passive haemagglutination has been reported to indicate *E. histolytica* infection. However, with serological testing it may be difficult to distinguish past from present infection (Tanyuksel and Petri, 2003).

The theory formulated by Brumpt in 1925 that the difference between many asymptomatic amoebic infections and those with amoebic disease could be correlated with the existence of two distinct but morphologically identical species, i.e. *E. histolytica* and *Entamoeba dispar*, has been confirmed by extensive genetic, immunological, biochemical and biomolecular data (Diamond and Clark, 1993; Tanyuksel and Petri, 2003). The identification of *E. dispar* as a separate but non-pathogenic species that does not require treatment has highlighted the need for alternative detection methods able to differentiate between the two organisms to replace microscopy and culture (Nunez et al., 2001), for example PCR.

Amplification of amoeba DNA fragments by PCR has been proven to be a sensitive and specific method for the diagnosis of amoebiasis, circumventing the problems of microscopic or culture-based diagnosis. Many investigators have reported successful application of PCR (Blessmann et al., 2002; Gonin and Trudel, 2003; Kebede et al., 2004; Nunez et al., 2001; Verweij et al., 2004). Moreover, this PCR-based approach is suitable for molecular epidemiological studies, which have been strongly encouraged by the WHO. Accurate prevalence data for *E. histolytica* and *E. dispar* are not available even in non-endemic developed countries such as Italy and obtaining them should be a high priority (WHO, 1997).

In this study we evaluated the utility of conventional PCR (Gonin and Trudel, 2003) and real-time fluorescence resonance energy transfer (FRET) PCR (Blessmann et al., 2002) as methods for identification and differentiation of *E. histolytica* and *E. dispar* compared with microscopy and culture. The second aim of this study was to determine the relative proportions of infections caused by the pathogenic *E. histolytica* and the non-pathogenic *E. dispar*, allowing us to obtain a picture of the epidemiological situation in a non-endemic setting.

2. Materials and methods

2.1. Patients and clinical samples

Although our laboratory (Department of Pathology and Laboratory Medicine, University of Parma, Italy) is not in an endemic area for amoebiasis, it receives samples from individuals who immigrate from or travel to developing countries. One hundred and sixty-six samples belonging to 108 patients (64 males, 44 females) sent to our laboratory for identification of intestinal parasites over a period of approximately 17 months were analysed: 158 faeces (from 108 patients, 16 of whom provided more than one faecal sample), 7 liver abscess samples (obtained from four patients: for three of these patients both faeces and liver abscess samples were available; from one patient there was a liver abscess sample only) and 1 intestinal biopsy (both faeces and a colonic biopsy were available from this patient). Patients whose samples were analysed in this study presented with gastrointestinal symptoms (diarrhoea, abdominal pain, weight loss, bloody stool) or liver abscess and/or risk factors for *E. histolytica*/*E. dispar* infection (immigration or adoption from developing countries, travel, poor hygienic conditions, male homosexuality, HIV infection). In particular, most of the patients (95 (88.0%), including 4 with liver abscess) were symptomatic, whereas the samples of the remaining patients (ten adopted children and one adult from developing countries, the wife of an Italian patient with amoebiasis, and one additional Italian patient occasionally drinking spring water) were sent to the laboratory as controls. Fifty-three of the 108 patients (49.1%) presented the following risk factors: 60.4% immigration from developing countries; 18.9% adoption from developing countries; 7.5% travel through developing countries; 9.4% low hygienic dietary habits; 1.9% declared male homosexuality and HIV infection; and 1.9% HIV infection.

2.2. Microscopy and culture

The 166 samples were subjected to microscopic examination (wet mounts) for intestinal parasites (including formalin-ethyl acetate sedimentation technique according to Ritchie (1948)) and cultivation in Robinson's medium for protozoa.

2.3. DNA extraction

DNA was extracted from the 166 specimens using the High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. Extracted DNA was used immediately for PCR assays (conventional and real-time FRET PCR) or frozen at −20 °C until analysed.

2.4. Conventional PCR

A conventional PCR (Gonin and Trudel, 2003) was used with minor modifications. The target for PCR amplification was a small region (135 bp) of the small subunit (SSU) rDNA. This sequence is located on an episomal plasmid, which is present at approximately 200 copies per cell (Blessmann et al., 2002).

Forward primers ED1 (5′-TACAAAGTGGCCAATTTATGTAAGTA-3′) and EH1 (5′-GTACAAAATGGCCAATTCATTCAATG-3′) were used for *E. dispar* and *E. histolytica* detection, respectively, with a shared reverse primer EHD2 (5′-ACTACCAACTGATTGATAGATCAG-3′). PCR amplification was carried out