A single step duplex PCR to distinguish *Entamoeba histolytica* from *Entamoeba dispar*

M. A. R. FREITAS[1], E. N. VIANNA[1], A. S. MARTINS[2], E. F. SILVA[1], J. L. PESQUERO[2,3] *and* M. A. GOMES[1*]

[1] *Departamento de Parasitologia*, [2] *Fisiologia e Biofisica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, CEP: 31270-901 Belo Horizonte, MG, Brasil*
[3] *Universidade de Mogi das Cruzes, São Paulo, SP, Brasil*

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SUMMARY

In this study, a single-step duplex polymerase chain reaction procedure was developed for rapid, specific and sensitive identification of *Entamoeba histolytica* and for its diagnostic differentiation from *E. dispar*. Specific oligonucleotide primers were combined for the amplification of a cysteine proteinase 5 gene target sequence of 242 bp, present only in *E. histolytica*. Additionally, another oligonucleotide primer pair for both the *E. histolytica* and *E. dispar* actin gene target of 300 bp was designed to amplify only from amoebae DNA. The PCR developed was specific and efficiently identified and differentiated these parasites from each other in either cultured parasites or from stool material.

Key words: *Entamoeba histolytica–Entamoeba dispar* diagnosis, PCR, actin, cysteine proteinase 5.

INTRODUCTION

Entamoeba histolytica is a protozoan parasite of the human intestine causing the disease known as amoebiasis. Approximately 100 000 people may die yearly due to this parasite infection world-wide (Walsh, 1986). Morphologically similar to *E. histolytica* and also found in the human intestine is *E. dispar*. However, this parasite does not cause disease. Diagnostic methodology to distinguish these two species from each other is a priority in amoebic infections and a number of approaches have been proposed (Haque *et al*. 1995; Britten *et al*. 1997; Verweij *et al*. 2000; Nunez *et al*. 2001; Blessmann *et al*. 2002). The majority of these methods are costly and are time consuming due to many protocol steps. Recently, we have described (Gomes *et al*. 1999) a simple and rapid PCR protocol to identify *E. histolytica* from *E. dispar*, based on conformational polymorphism of the 482 bp fragment from the M17 gene (Edman *et al*. 1990). In the present study we propose a single-step double PCR protocol with two distinct target fragments. One of them of 242 bp from cysteine proteinase 5 of *E. histolytica* (Bruchhaus *et al*. 1996) and the other with 300 bp from actin of *E. histolytica* (Edman, Meza & Agabian, 1987; Huber *et al*. 1987) and *E. dispar*.

* Corresponding author: Departamento de Parasitologia, ICB-UFMG, Av. Antonio Carlos 6627, 31270-901 Belo Horizonte, Minas Gerais, Brasil. Tel: +55 31 34992845. Fax: +55 31 34992970. E-mail: magomes@mono.icb.ufmg.br

MATERIALS AND METHODS

Entamoeba *strains and cell culture conditions*

Five strains of *E. histolytica* (CSP, 462, 32, RPS and 452) and 4 of *E. dispar* (JCAO, VEJ, ADO and WIL) were used. *E. moshkovskii* strain EMCR was also used. All were isolated at the Laboratory of Amoebiasis, Department of Parasitology of the Institute of Biological Sciences, UFMG (LADP-ICB/UFMG), Brazil. The *E. histolytica* strains have been maintained in TYI-S-33 medium (Diamond, Harlow & Cunnick, 1978) and the *E. dispar* and EMCR in YE polyxenic medium (Silva & Mayrink, 1974). Parasites from culture were identified by zymodeme (Sargeaunt, Williams & Grene, 1978) and their DNA was extracted using 1×10^6 cells by the GenomicPrep System (Amersham, USA) following the manufacturer's instructions.

Stool samples

Human stool samples were collected from the LADP-ICB/UFMG. The *E. histolytica*/*E. dispar* cysts were obtained from microscopically positive faecal samples using a formalin–ether sedimentation procedure (Ridley & Hawgood, 1956). From each of 8 positive and 1 negative faecal samples 0·2 g was used. The specificity of primers was confirmed by using samples containing cysts of *E. coli*, *Endolimax nana*, *Blastocystis hominis*, *Giardia duodenalis* or eggs of *Ascaris lumbricoides*. The DNA from cysts (or eggs) was extracted using the QIAamp DNA stool mini kit (Quiagen, Germany) according to the manufacturer's instructions.

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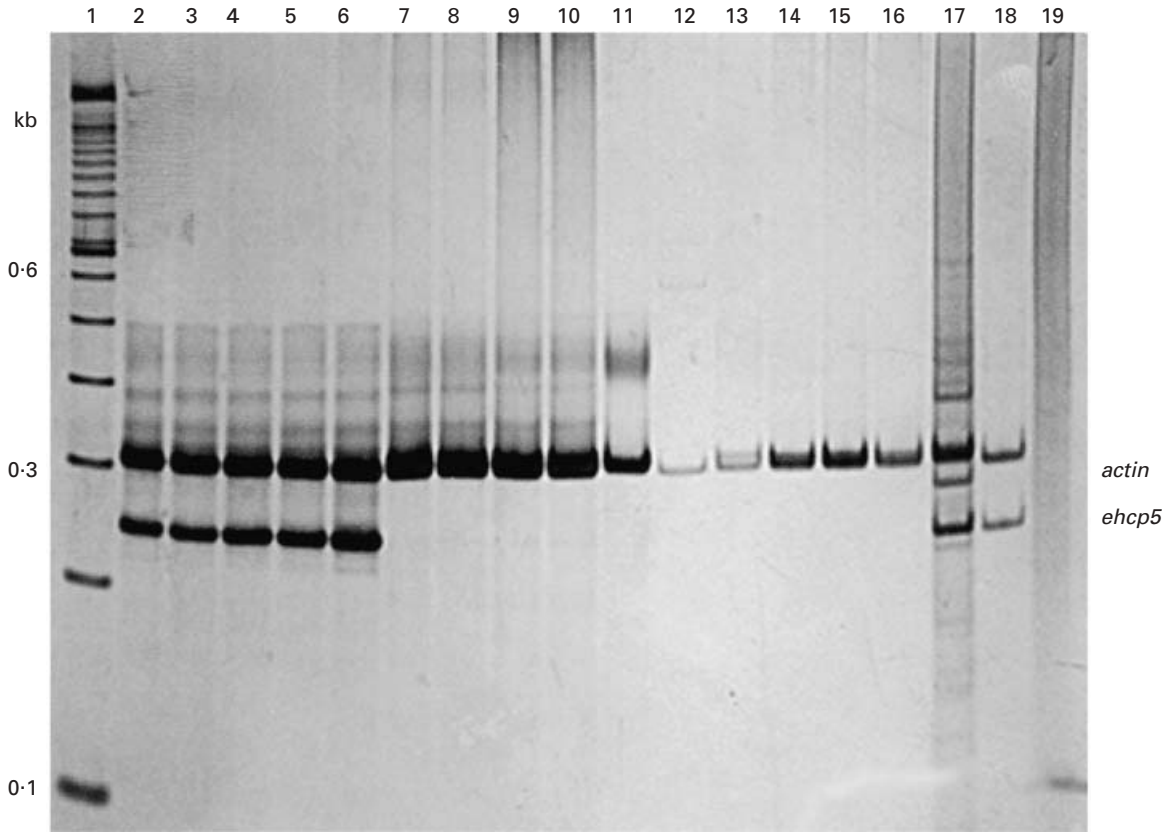

Fig. 1. Typical duplex PCR results showing diagnostic differentiation of *Entamoeba histolytica* from *E. dispar* following silver-stained polyacrylamide gel electrophoresis. The amplicons from the actin gene, common to both amoebae, and the *ehcp5* specific to *E. histolytica*, are identified as 300 bp and 242 bp, respectively. Lane 1 is 100-bp DNA ladder marker. Other lanes are: (2–10) cultivated samples; (11–18) positive fecal samples; (19) negative fecal sample by optical microscopy. *E. histolytica* was identified in cultivated samples 2–6 and fecal samples 17–18 as well. Lanes 12 and 13 from samples containing just 1 cyst.

Duplex PCR

Two gene sequences were targeted simultaneously in this PCR procedure. One comprised an internal segment of 242 bp of the cysteine proteinase 5 (EhCP5) gene, present only in *E. histolytica* (EhCP5), and the oligonucleotide primers designed were EhCP6F forward (5′GTTGCTGCTGAAGAAACTTG3′) and reverse EhCP6R (5′GTACCATAACCAACTACTGC3′). Another PCR target was a 300 bp sequence within the actin gene, and the oligonucleotide primers designed were Act3 forward (5′GGGACGATATGGAAAAGATC3′) and Act5 reverse (5′CAAGTCTAAGAATAGCATGTG3′), common to both *E. histolytica* and *E. dispar*. Five pmols of each primer were used in a mixture containing 10 mM Tris–HCl, pH 9·0, 1·5 mM $MgCl_2$, 0·2 mM dNTPs, 0·1% Triton X–100, 0·1 U of *Taq* DNA polymerase (Promega, USA) and 2 μl of DNA extracted from a stool sample or 50 ng from culture, in a final reaction volume of 10 μl. Thermal cycling was performed as follows: 30 cycles of 95 °C/30 s, 60 °C/30 s and 72 °C/45 s. The PCR products were submitted to electrophoresis in 4% polyacrylamide gel and amplicons were visualized by silver staining.

RESULTS

Duplex PCR amplicons are shown in Fig. 1. Parasites from culture identified as *E. histolytica* or *E. dispar* by zymodeme were confirmed by PCR (wells 2–10). Among positive faecal samples chosen for this study 2 were identified by PCR as being *E. histolytica* (wells 17 and 18) and the other 6 samples as *E. dispar* (wells 11–16). The negative sample by optical microscopy did not yield any PCR amplification (well 19). No amplification product was observed for any of the samples from *E. moshkovskii* and other parasites (data not shown).

DISCUSSION

E. dispar parasitizes the human intestine but does not cause illness. Its morphological similarity to *E. histolytica*, which is responsible for amoebic dysentery, does not allow differentiation of these two organisms from each other by means of optical microscopy. Before the confirmation of *E. dispar* as a distinct species (WHO, 1997), it was estimated that 0·5 billion people were infected around the world by *E. histolytica*. Of these, 50 million were symptomatic and 100 000 died annually (Walsh, 1986). Adapting

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these data to the present, one could say that at least 500 million people might be infected by *E. histolytica*. However, the percentage of infection due to either *E. histolytica* or *E. dispar* among the remaining 450 million cannot be estimated. In this regard, any apparently asymptomatic person may develop the disease and become symptomatic, that is, the parasite may change its behaviour, becoming virulent and start to cause lesions. Therefore, the development of fast and sensitive methods to distinguish the amoebae is urgent as this differentiation is crucial for selecting treatment protocols for these patients.

Recently, differences between *E. histolytica* and *E. dispar* in the expression patterns of proteins thought to be involved in the virulent behaviour of *E. histolytica* have been shown (Reed *et al*. 1995; Jacobs *et al*. 1998; Hellberg *et al*. 2001; Bruchhaus *et al*. 2002). Among them are two cysteine proteinases that are exclusively expressed in *E. histolytica*. EhCP5 is one of these proteins that presents as a good candidate for differentiating the parasites either by protein expression levels or by gene structure since its gene is highly degenerate in *E. dispar* (Willhoeft, Hamann & Tannich, 1999). A 242 bp internal sequence of this gene was chosen since no PCR product should be obtained from *E. dispar* DNA, from other strains of amoebae or from protozoa from the human gut. In addition, we included a segment of actin gene as a second marker that amplifies only from amoebae DNA. Actin is one of the most abundantly expressed proteins in *E. histolytica* (Huber *et al*. 1987) and we also observed it in *E. dispar*. Thus, in the present work, we propose a double PCR of the EhCP5 gene target sequence that specifically detects *E. histolytica*. Combined with the actin gene for both amoebae, this PCR was demonstrated to be a very selective strategy to detect and distinguish *E. histolytica* from *E. dispar* in a rapid single-step procedure that could be completed in a very short time-period (60 min).

In this study polyxenic and axenic cultures of *E. histolytica* and *E. dispar* were used and identified by zymodeme analysis. All samples identified as *E. histolytica* or *E. dispar* by zymodeme were confirmed by PCR. Considering the possibility that *E. moshkovskii* may be infecting the human gut (Ali *et al*. 2003), the specificity of our primers was tested with one *E. moshkovskii* strain. The results showing no amplification product attested this, and additionally, showed that this duplex PCR approach may be applied also to stool samples, confirming either positives or negatives as determined by optical microscopy, with the sensitivity to detect just one cyst in the sample (wells 12 and 13). In conclusion, these results demonstrate the potential (i) to facilitate the separation of these two parasites in positive samples, and (ii) to provide a tool for important diagnostic differentiation.

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