An improved method to distinguish *Entamoeba histolytica* and *Entamoeba dispar*

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SUMMARY

A 482 base pair gene fragment from samples of amoebae *E. histolytica* and *E. dispar* was amplified by PCR. The amplification products of fragments from the 2 species of amoebae presented differences in mobility in non-denaturing polyacrylamide gel, probably due to sequence-dependent conformational alterations in the DNA fragments. The method described here permits *E. histolytica* and *E. dispar* to be distinguished with greater sensitivity and rapidity.

Key words: *Entamoeba histolytica–Entamoeba dispar* differentiation, DNA electrophoresis, DNA conformation, diagnosis, PCR.

INTRODUCTION

Amoebiasis is an important public health problem that kills approximately 100000 people a year world-wide (Martinez-Palomo, 1987). Following the discovery of *E. dispar*, distinguishing it from *E. histolytica* became extremely important. Identification techniques include determination of the isoenzymatic profile (zymodeme) of certain enzymes of the glycolytic pathway (Sargeaunt, Williams & Grene, 1978), analysis by restriction fragment length polymorphism (RFLP) of certain genes (Clark & Diamond, 1991; Tannich & Burchard, 1991; Tachibana *et al*. 1992; Novati *et al*. 1996) and use of monoclonal antibodies directed against specific antigens of *E. histolytica* and *E. dispar* (Haque *et al*. 1995; Mirelman, Nuchamowitz & Stolarsky, 1997; Moody *et al*. 1997).

Each of these techniques presents difficulties in its execution. Zymodeme determination requires culture of samples, restriction fragment analysis depends on the use of restriction enzymes for specific diagnosis and use of specific antigens/antibodies for immunological tests has low sensitivity. In this study we show that the 2 *Entamoeba* species can be distinguished by electrophoresis of PCR products from DNA samples.

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MATERIALS AND METHODS

Data corresponding to the 19 strains of amoeba used in the study are presented in Table 1. All the strains were maintained in TYI-S-33 medium (Diamond, Harlow & Cunnick, 1978). The samples were identified by zymodeme and by RFLP of a DNA fragment of 482 bp (Tannich & Burchard, 1991) obtained by PCR. Because of difficulty in exact determination of the molecular mass of the fragments in the gel, it is necessary to compare the PCR products of the samples with those from DNA of standard strains of *E. histolytica* or *E. dispar*, such as the HM1 strain of the former used in this study.

Identification of the samples

Zymodeme. This was determined by analysing the mobility of the enzymes HK, GPI and PGM in amide gel (Sargeaunt, Williams & Grene, 1978; Farri *et al*. 1979).

PCR. The DNA of each sample was obtained by the phenol–chloroform method (Sambrook, Fritsch & Maniatis, 1989) using cultures of *E. histolytica* or *E. dispar* in the exponential growth phase. A 482 bp fragment derived from the M17 gene was amplified using the primers P1-S17 (5′-GCAACTAGTGTTAGTTA) and P1-AS20 (5′-CCTCCAAGATATGTTTTAAC) designed by Tannich & Burchard (1991). PCR was carried out as described by Gomes *et al*. (1997) and the product was analysed in 5% polyacrylamide gel (9·67% acrylamide, 0·33%

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Table 1. Isolates of *Entamoeba histolytica* and *E. dispar* identified by zymodeme and RFLP of the 482 bp DNA fragment

Sample no.	Strains	Zymodeme	RFLP*			Origin	Identification conclusion
			*Taq*I	*Xmn*I	*Acc*I		
1	RPSM	I	−	−	+	Brazil	*E. dispar*
2	EEM	I	−	−	+	Brazil	*E. dispar*
3	MCR	I	−	−	+	Brazil	*E. dispar*
4	VEJ	I	−	−	+	Brazil	*E. dispar*
5	SC	I	−	−	+	Brazil	*E. dispar*
6	JCAO	I	−	−	+	Brazil	*E. dispar*
7	W1	I	−	−	+	Brazil	*E. dispar*
8	W2	I	−	−	+	Brazil	*E. dispar*
9	ICS	I	−	−	+	Colombia	*E. dispar*
10	HM1	II	+	+	−	Mexico	*E. histolytica*
11	200:NIH	II	+	+	−	USA	*E. histolytica*
12	HK-9	II	+	+	−	Korea	*E. histolytica*
13	C6	II	+	+	−	Colombia	*E. histolytica*
14	CSP	II	+	+	−	Brazil	*E. histolytica*
15	DRP	II	+	+	−	Brazil	*E. histolytica*
16	EGG	XIX	+	+	−	Brazil	*E. histolytica*
17	462	II	+	+	−	Brazil	*E. histolytica*
18	452	II	+	+	−	Brazil	*E. histolytica*
19	32	II	+	+	−	Brazil	*E. histolytica*

* RFLP of the 482 bp DNA fragment obtained with enzymes *Taq*I, *Xmn*I and *Acc*I: (+) positive cut to enzyme and (−) negative cut to enzyme.

*bis*acrylamide) in TBE buffer (0·09 M Tris–borate, 0·002 M EDTA, pH 8·3) for 2 h at 100 V. The fragments were visualized by silver staining (Santos, Pena & Epplen, 1993) or by ethidium bromide staining in 1% agarose gels. Species identification was confirmed by submitting the 482 bp fragment to digestion with the enzymes *Acc*I, *Taq*I and *Xmn*I under the conditions recommended by the manufacturers (Life Technologies/BRL, USA).

Polymorphism analysis

Sequencing. The amplification products of the 482 bp fragment from *E. histolytica* and *E. dispar* samples were cloned in pGEM-T plasmid (Promega) and submitted to automatic sequencing using the Thermo Sequenase Fluorescent Labelled Primer reaction kit (Amersham) and the products separated by electrophoresis in polyacrylamide gel in a Vistra DNA Sequencer 725.

Curvature and bendability. The most likely conformation of the DNA sequences of the amplified fragments of samples of *E. histolytica* and *E. dispar* were analysed using the prediction method based on curvature propensity and consensus bendability values described by Gabrielian, Vlahovicek & Pongor (1997). The program used, 'bend.it®', is located on website http://www2.icgeb.trieste.it/~dna/bend-it.html.

RESULTS

The PCR products of the 19 samples studied were analysed both in agarose gels stained with ethidium bromide (data not shown) and in silver-stained polyacrylamide gels (Fig. 1). As can be observed from Fig. 1, the amplified fragments of *E. dispar* presented a greater mobility than those of *E. histolytica*. This difference in mobility was not observed when the products were analysed in agarose gels, a single product of 482 bp being seen for all the samples.

Sequencing of the PCR products of *E. histolytica* and *E. dispar* confirmed the findings of Tannich & Burchard (1991). Taking into account that the results of sequencing showed some differences in the composition of bases but not in the size of the fragments obtained from the 2 strains, we subjected the sequences obtained to conformational analysis by the bend.it® program. From the graphs curvature versus bendability obtained by the bend.it® program (not shown), we observed significant differences in the conformations of the 2 DNA strands that were more accentuated in the regions involving bases 60–90, 240–260 and 280–370 for curvature propensity and 60–80, 180–370 for bendability. The DNA fragment amplified from *E. dispar* showed higher curvature and lower bendability degrees than that amplified from *E. histolytica*.

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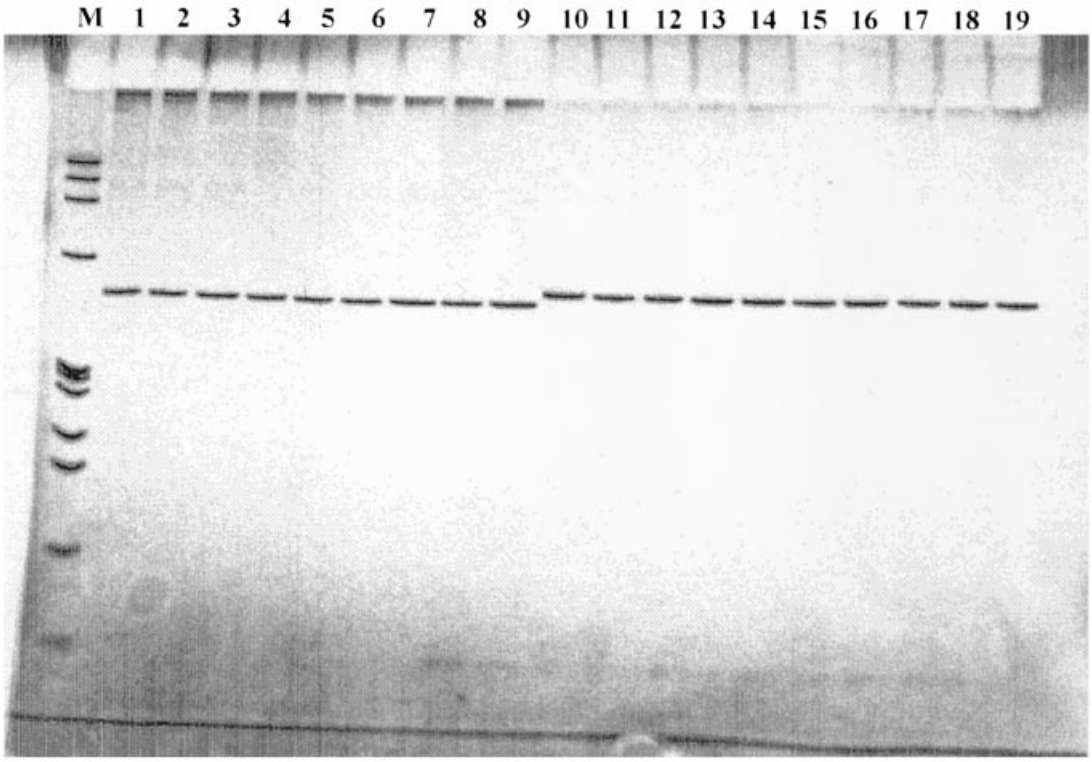

Fig. 1. A 5% silver-stained polyacrylamide gel showing the amplification products using the primers P1-S17 and P1-AS20 with different *Entamoeba dispar* (lanes 1–9) and *E. histolytica* (lanes 10–19) samples.

DISCUSSION

The morphological similarity between *E. histolytica* and *E. dispar* and their medical and epidemiological consequences make accurate differentiation between the 2 species important for diagnostic purposes. Various techniques have been described with this in mind (Sargeaunt *et al.* 1978; Clark & Diamond, 1991; Tannich & Burchard, 1991; Tachibana *et al.* 1992; Haque *et al.* 1995; Novati *et al.* 1996; Mirelman *et al.* 1997; Moody *et al.* 1997). One of these techniques enabled Tannich & Burchard (1991) to distinguish between the 2 species by RFLP of a 482 bp gene fragment. This fragment can be amplified using DNA samples both of *E. histolytica* and *E. dispar* using a single pair of primers. Despite having the same size in the 2 species, these fragments present differences with respect to their nucleotide compositions. These differences can be recognized by 3 restriction enzymes, of which 2 (*Taq*I and *Xmn*I) are specific for *E. histolytica* and the third (*Acc*I) for *E. dispar*. In addition to the modification in the restriction profile, sequence differences can modify the conformation of double-stranded DNA fragments. This sequence-dependent conformation difference may produce a variation in electrophoretic mobility in polyacrylamide gels of greater than 10% which can be used to distinguish variants (Saad *et al.* 1994). Some DNA fragments migrate anomalously slowly in non-denaturing polyacrylamide gels, this effect probably being caused by the inherent curvature of the DNA fragment (Englund & Marini, 1980). It might be explained by higher friction in the polyacrylamide pores that results in a slow rate of migration of curved molecules. As the DNA fragment amplified from *E. dispar* showed higher curvature and lower bendability degrees than that amplified from *E. histolytica*, lower mobility for the *E. dispar* fragment would be expected. The fall in the friction of *E. dispar* DNA fragment may, however, be explained by the formation of transient kinks at certain sequence elements like CA/TG and AC/TG (Bolshoy *et al.* 1991) that could lead to a more strong structural change in the *E. dispar* DNA fragment. Indeed, if we compare both we can see a slightly higher number of such elements in the sequence of the *E. dispar* DNA fragment. The differences in the conformation of the amplified DNA fragments of *E. histolytica* and *E. dispar*, as analysed by the bend.it® program, may not totally explain the difference observed in the mobility of the fragments. However, the possibility that transient kinks are responsible for the higher mobility of the *E. dispar* DNA fragment is suggestive that there is correlation between curvature propensity and kinks generation.

The electrophoretic patterns of the amplification products of the 482 bp fragments of *E. histolytica* and *E. dispar* were found to be identical in all of the 19 samples examined, demonstrating the reproducibility of the technique.

These results show that the use of polyacrylamide for the characterization of polymorphism may im-

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prove not only differential diagnosis between *E. histolytica* and *E. dispar*, but also identification of infections caused by these amoebae, using a DNA sample from *E. histolytica* or *E. dispar* as a standard for comparison with test samples. This modification could signify even greater speed and sensitivity in the identification of these amoebae, given that the approach employs a single PCR and electrophoresis in polyacrylamide gels, this being much more efficient in the separation of the fragments than traditional analysis in agarose gels. This method can also be used to identify mixed infections with *E. histolytica* and *E. dispar*. The Tannich & Burchard (1991) method has already proved to be sensitive and could represent an important tool for the routine diagnosis of *E. histolytica* and *E. dispar* infections with the incorporation of the modifications presented here.

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