

## Use of molecular tools to distinguish *Entamoeba histolytica* and *Entamoeba dispar* infection among the aborigines in Cameron Highlands

Noor Azian, M.Y<sup>1</sup>., Lokman Hakim, S<sup>2</sup>. and Maslawaty, M.N<sup>1</sup>.

<sup>1</sup> Parasitology Unit, Infectious Diseases Research Centre, Institute for Medical Research, 50588, Kuala Lumpur.

<sup>2</sup> Environmental Health Research Centre, Institute for Medical Research, 50588, Kuala Lumpur.

**Abstract.** Amoebiasis is an infectious disease caused by parasitic one-celled protozoan called *Entamoeba histolytica*. Numerous protozoa also can inhabit the gastro-intestinal tract of human. Majority of these protozoa are non-pathogenic commensals or only causes disease under certain circumstances. Morphologically, *E. histolytica*, the invasive form, share the same characteristic with the nonpathogenic form, *E. dispar*. Both strains can be distinguished by using DNA identification. Many previous researches in Malaysia only reported infection with *E. histolytica* infection. Therefore in this study we tried to classify infection among the aborigines in Cameron Highland as true *E. histolytica* or *E. dispar* by Nested Polymerase Chain Reaction (Nested PCR) and Restriction enzyme (RE) digestion. Results showed that 31 samples were positive by microscopic examination, however of these 28 (13.2%) samples were positive for *E. histolytica* and 12 (5.6%) samples were positive for *E. dispar* by molecular tools.

### INTRODUCTION

Amoebiasis is defined as an intestinal or extraintestinal infection with the protozoan parasite *Entamoeba histolytica*. Persons with amoebiasis may experience a wide range of symptoms, including diarrhoea, fever, and cramps. The disease may also affect the liver or other parts of the body. The parasite is found in all parts of the world but most frequently in tropical and subtropical regions where the socio-economic status and environmental sanitation are poor (Hooshyar *et al.*, 2004).

More than 500 million people worldwide are infected, and up to 110,000 of those infected die every year (Troll *et al.*, 1997). Based on biochemical, immunological and genetic data, *E. histolytica* was redefined / reclassified in 1993 and the existence of two morphologically identical but genetically distinct protozoan parasite was recognised: *E. histolytica*, the aetiological agent of invasive intestinal and

extraintestinal amoebiasis and *E. dispar*, the non-pathogenic intestinal parasite. Together, *E. histolytica* and *E. dispar* infect about 10% of the world's population. Infection with the commensal *E. dispar* is much more common, and the true prevalence of invasive *E. histolytica* is perhaps closer to 1% worldwide (Que & Reed, 2000).

The concept of the existence of the two morphologically identical amoeba species was put forward as early as 1925. However, it was not until 1991 that Clark & Diamond gave the ultimate redescription of these two species. Now, both serological and molecular tools are available for distinguishing these two species (Wonsit *et al.*, 1992; Acuna-Soto *et al.*, 1993; Katzwinkel-Wladarsch *et al.*, 1994; Troll *et al.*, 1997; Haque *et al.*, 1998; Yvonne *et al.*, 2001; Gonin & Louise, 2003).

Sensitive and specific serological and molecular techniques that are able to distinguish *E. histolytica* from *E. dispar*

have been developed recently. These include, the detection of *E. histolytica* antigen using an enzyme-linked immunosorbent assay (ELISA) (Gonin & Louise, 2003), the detection of *E. histolytica* and *E. dispar* antigen by monoclonal antibodies against a recombinant 170kDa subunit of the Gal or GalNac lectin of *E. histolytica* that specifically recognized *E. histolytica* (Wonsit *et al.*, 1992 Yvonne *et al.*, 2001) and the use of the polymerase chain reaction (PCR) to amplify amoebic DNA (Acuna-soto *et al.*, 1993; Katzwinkel-Wladarsch *et al.*, 1994; Troll *et al.*, 1997; Haque *et al.*, 1998).

In Malaysia, a study by Rajeswary *et al.* (1994) on children living in Gombak, Malaysia, showed that 9.9% of the children were infected with *E. histolytica*. Noor Aza *et al.* (2003) showed that the infection rate of this species among rural communities in Sabah was 21%. However, these prevalences rates were based on laboratory methods which could not differentiate between *E. histolytica* and *E. dispar*. During a recent outbreak of diarrhoea in an aboriginal community in Cameron Highlands, we found that more than 15% of the stool samples were positive for *E. histolytica/E. dispar* by microscopic examination. The aim of this study was to determine the true *E. histolytica* infection among the diarrhoeic cases by molecular tools.

## MATERIALS AND METHODS

### Collection of stool samples

During a diarrhoeal outbreak in July 2004, stool samples were collected from four villages (Kg Kuala Boh, Kg Panggen, Kg Rantau and Pos Mensun) in Ulu Jelai, Cameron Highlands. The samples were preserved in polyvinyl alcohol (PVA). Smears were made from the preserved stools and stained with Trichrome stain and examined under microscope. Samples which were positive for *E. histolytica/E. dispar* were selected for molecular identification.

### DNA extraction from PVA preserved stool

Two-hundred microliters (200 µl) of PVA-preserved stool was pipetted into a 2 ml microcentrifuge tube. The QIAamp® DNA Stool Mini Kit (Qiagen) was used to extract DNA from the stool and no purification of the DNA product was carried out because according to Troll *et al.* (1997), these methods does not require any further purification. The extracted DNA was kept at -20°C until used. Positive control was obtained from the *E. histolytica*, HK-9 strain (ATCC® 30015) *in vitro* culture. Extraction of the DNA followed the procedure of the DNA extraction as mentioned above.

### Nested polymerase chain reaction (Nested-PCR) and restriction endonuclease (RE) digestion

The Nested PCR was performed to differentiate the pathogenic and non-pathogenic *Entamoeba* spp. The primers for the primary PCR were EH-1 (5' TTT GTA TTA GTA CAA A 3') and EH-2 (5' GTA (A, G)TA TTG ATA TAC T 3'). The nucleotide sequence of these primers were based on the study by specific Katzwinkel-Wladarsch *et al.* (1994). For the Secondary PCR, two different pairs of primers were used. The primers specific for *E. dispar* were EHN-1 (5' AGT GGC CAA TTT ATG TAA GT 3') and EHN-2 (5' TTT AGA AAC AAT GTT TCT TC 3') and for *E. histolytica*, the primers were EHP-1 (5' AAT GGC CAA TTC ATT CAA TG 3') and EHP-2 (5' TCT AGA AAC AAT GCT TCT CT 3').

PCR amplification was performed in a total volume of 50 µl. For the primary PCR, the PCR mixture contained 25.5 µl sterile distilled water, 10 µl DNA, 2.5 mM MgCl<sub>2</sub>, 5 µl 10X PCR buffer, 0.2 mM dNTPs (Fermentas®), 18 pmole of each primers (EH-1 and EH-2) and 0.5 µl *Taq* DNA polymerase (Fermentas®). Amplification was carried out in a T-Gradient thermal cycler (Biometra®) according to the following parameters: initial denaturation for 2 minutes at 96°C, followed by 40 cycles of denaturation for 1 minute at

92°C, 1 minute of annealing at 43°C, extension for 1 minute at 62°C and final extension step for 10 minutes at 72°C, followed by 4°C to hold the amplification.

The PCR mixture for the secondary PCR contained 33.5 µl of sterile distilled water, 2 µl of primary product, 2.5 mM MgCl<sub>2</sub>, 5 µl 10X PCR buffer, 0.2 mM dNTPs, 18 pmole of each primer EHN-1 and EHN-2 for *E. dispar* or EHP-1 and EHP-2 for *E. histolytica* and 0.5 µl *Taq* DNA polymerase (Fermentas®). The PCR cycle consisted of initial denaturation at 96°C for 2 minutes followed by 40 cycles of denaturation (92°C, 1 minute), annealing (60°C, 1 minute) and extension (72°C, 1 minute) and final extension at 72°C for 10 minutes. Amplified products were analysed on a 2% biotechnology grade agarose gel (BST Techlab®) which was stained with 0.2 µg/ml ethidium bromide (Sigma®) for visual analysis. Ten microliters of the secondary product for EHP and EHN were digested with 1.0 µl (10 U/µl) *Dra*I (Promega®) and *Sau* 96I (Biolabs®) respectively, for 2 hours at 37°C. Digested DNA was separated on a 2% agarose gel containing 0.2 µg/ml ethidium bromide (Sigma®).

### Analysis

Determination of the *Entamoeba* species was based on the digested PCR fragments by the restriction enzymes. The target regions for PCR and RE analysis was the 16SrRNA gene. The expected sizes of the PCR digestion were 0.55 kbp and 0.35 kbp for *E. histolytica* when digested with *Dra*I. The products of *Sau*96I for *E. dispar* were 0.68 kbp and 0.2 kbp. Data was then entered and analyzed using in SPSS®

software. Stools sample were divided into 8 age groups. Proportion of the positive cases for age group and gender were calculated and analysed using the Chi-square analysis.

## RESULTS

A total of 212 stool samples from the four villages (111 from Kg Kuala Boh, 22 from Kg Panggen, 43 from Kg Rantau and 36 from Pos Mensun) were collected and examined for *E. histolytica/E. dispar*. Of these 31 samples were positive for *E. histolytica/E. dispar* by microscopic examination and were subjected to Nested PCR and RE digestion. Of the 31 positive samples, 28 were positive for *E. histolytica* and 12 were positive for *E. dispar* by the molecular assay (Table 1).

Of the 28 positive samples of *E. histolytica*, only 10 produced both the 0.55 kbp and 0.35 kbp bands and 18 unequivocal samples produced only one (0.55 kbp) band for *E. histolytica*. For *E. dispar*, 2 samples showed only 0.68 kbp band, 10 others produced only between 0.2 kbp. Three samples which were positive by microscopic examination were negative for *E. histolytica* and 19 samples were negative for *E. dispar*. Of the 12 unequivocal samples of *E. dispar*, 8 were also unequivocal for *E. histolytica*, and 4 positive with *E. histolytica*. Therefore there was a possibility of mixed infection in these 12 samples (Table 2).

Among the 4 villages, the highest prevalence was from Kg. Panggen (9.1%), followed by Kg Rantau (6.8%), Kg Kuala

Table 1. Prevalence of true *E. histolytica* and *E. dispar* by Nested PCR and RE Digestion

Samples tested by microscopy	E.h/E.d by microscopy (%)	Nested PCR and RE digestion					
		<i>E. histolytica</i>			<i>E. dispar</i>		
		Positive (%)	Negative (%)	Unequivocal (%)	Positive (%)	Negative (%)	Unequivocal (%)
212	31 (14.6)	10 (4.7)	3	18	0 (0.0)	19	12

Table 2. Digestion fragment sites of the unequivocal *E. histolytica* and *E. dispar* results as detected in agarose gel electrophoresis

Total unequivocal samples for <i>E. h</i>	<i>DraI</i> fragment: <i>E. histolytica</i>		Total unequivocal samples for <i>E. d</i>	<i>Sau96I</i> fragment: <i>E. dispar</i>	
	0.55 kbp only	0.35 kbp only		0.68 kbp only	0.20 kbp only
18	18	0	12	2	10

Boh (3.6%) and Pos Mensun (2.8%) but the difference between the villages was not significant ( $\chi^2=2.03$ ,  $p=0.566$ ) (Table 3). There was also no significant difference in the prevalence of *E. histolytica* between age groups ( $\chi^2=9.07$ ,  $p=0.248$ ) although the highest prevalence was among the 5-10 years old age group (Table 4). The prevalence rate by gender was also not significantly different ( $\chi^2=1.164$ ,  $p=0.686$ ) with 5.3% in female and 4.1% in male.

## DISCUSSION

Amoebiasis is one of the major health concern in many developing countries. The World Health Organisation estimated that amoebiasis is the third most common cause of death due to parasitic infections after malaria and shistosomiasis. Approximately 10% of the world population is infected with *E. histolytica*/*E. dispar*, but most infections are due to the noninvasive species. Epidemiological studies have shown that low socioeconomic status and unsanitary conditions are significant risk factors for infection in the developing countries (Hooshiyar *et al.*, 2004).

The realization that *E. histolytica* and *E. dispar* were two distinct but morphologically identical species had a major impact on all aspects of amoebiasis diagnosis and research. Tools that allow accurate differentiation based on DNA amplification have been a research focus (Zaki *et al.*, 2002). According to Troll *et al.* (1997), the chosen target for the PCR amplification in the 16SrRNA gene allowed for amplification of a 0.9 kbp gene fragment which is revealed as a single

Table 3. Prevalence of *E. histolytica* by locality

Locality	No. of samples	No. of positive by microscopy	No. positive with Nested PCR and RE (%)
Kg Kuala Boh	111	14	4 (3.6)
Kg Panggen	22	2	2 (9.1)
Kg Rantau	43	7	3 (6.8)
Pos Mensun	36	8	1 (2.8)

Table 4. Prevalence of *E. histolytica* by age group

Age Group (Year)	Total	Nested PCR and RE digestion
0-4	44	0 (0.0)
5-10	38	4 (10.5)
11-19	24	0 (0.0)
20-29	31	1 (3.2)
30-39	22	2 (9.1)
40-49	15	0 (0.0)
50-59	22	2 (9.1)
60 and above	16	1 (6.25)

band by agarose gel electrophoresis. Both PCR products vary only one nucleotide in length but they differ in the *Sau96I* restriction site. In addition, the gene fragment contained a *DraI* restriction site which distinguishes both species from other protozoan parasites. The amplified gene fragment could be cut with *DraI*, resulting in two fragments of 0.55 kbp and 0.35 kbp, as predicted from the nucleic

acid sequence. When the amplified DNA of *E. dispar* was digested with *Sau96I*, two fragments of the expected length (0.68 kbp and 0.2 kbp) were detected, whereas the DNA derived from *E. histolytica* was not cleaved by this enzyme.

Studies by Katzwinkel-Wladarsch *et al.* (1994) showed that the common restriction site of *DraI* and *Sau96I* confirmed the presence of *E. histolytica* or *E. dispar* DNA by producing a 0.55 kbp band. The remaining 0.35 kbp fragment contained a *Sau96I* restriction site of 0.2 kbp from the 3' end of the nonpathogenic sequence with a base mutation in the pathogenic form. Therefore, the pathogenic DNA exhibit the characteristic bands of 0.55 kbp and 0.35 kbp, usually with some of the undigested DNA yield a band of 0.9 kbp. The nonpathogenic strain amplified DNA yield a band of 0.68 kbp and a confluent double band of 0.2 kbp, often with a partial digestion product of 0.7 kbp.

From the 31 samples positive by microscopic for *E. histolytica* /*E. dispar*, our results showed various DNA fragments after restriction enzymes digestion which was detected in the agarose gel electrophoresis. However, only 10 samples showed the typical 2- bands pattern of 0.55 kbp and 0.35 kbp after *DraI* digestion for *E. histolytica*. Although 18 other samples showed the 0.55 kbp, it lacked the other diagnostic band at 0.35 kbp. Similar unequivocal results were also noted with *Sau96I* digestion for *E. dispar*. Although those single-band samples could probably be *E. histolytica* or *E. dispar*, the diagnosis remain uncertain because of the lack of the double band characteristic as described by Troll *et al.* (1997) and Katzwinkel-Wladarsch *et al.* (1994).

There are several factors that may be account for the detection of a single band of either 0.55 kbp, 0.35 kbp, 0.68 kbp and 0.20 kbp. The first factor may be because of the geographic diversity. Using high resolution genotyping based on the nucleotide sequences of four polymorphic loci of *E. histolytica*, Haghghi *et al.* (2003) were able to demonstrate that this parasite

from endemic areas in Southeast Asia has an extremely polymorphic genetic structure.

Secondly, the results were also unlikely to be due to low numbers of parasite. The fact that it was positive by microscopy showed that the density was high enough to be detected. Previous studies have also concluded that the extraction of DNA and the amplification and restriction enzyme digestion were very sensitive and specific. These PCR method was very sensitive at detecting one copy of the target gene of one species (Troll *et al.*, 1997).

Thirdly, the chemical used (PVA) to preserve the stool might have also affected the results. Fresh stool or Sodium acetate-acetic acid-formalin (SAF) fixative stool will produce better results (Troll *et al.*, 1997). Due to unforeseen circumstances SAF fixed specimens were not available for this study.

Nevertheless, this study shows the potential use of molecular tools in the detection of *E. histolytica* and *E. dispar*. It offers promising tools for epidemiological studies of the infections, particularly in differentiating the pathogenic and non-pathogenic species of the parasites. Further studies should be carried out to sequence the DNA of the isolates so that more specific primers can be designed for a multiplex PCR.

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