IDENTIFICATION OF *ENTAMOEBA HISTOLYTICA* AND *ENTAMOEBA DISPAR* BY PCR ASSAY OF FECAL SPECIMENS OBTAINED FROM THAI/MYANMAR BORDER REGION

Apiradee Intarapuk[1], Thareerat Kalambaheti[2], Nitaya Thammapalerd[2], Pakpimol Mahannop[1], Pradit Kaewsatien[3], Adisak Bhumiratana[1] and Dechavudh Nityasuddhi[4]

[1]Department of Parasitology, [4]Department of Statistics, Faculty of Public Health, [2]Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok; [3]Department of Epidemiology, Armed Forces Research Institute of Medical Science, Bangkok, Thailand

Abstract. Due to the indistinguishable morphology between *Entamoeba histolytica* (pathogenic) and *Entamoeba dispar* (non pathogenic), PCR-based assays were conducted. Based on microscopy, suspected *Entamoeba* cells were detected in 30 out of 455 fecal samples obtained from individuals residing at Thai/Myanmar border region. The target genes for PCR amplification included genes encoding small subunit rRNA (SSU-rRNA), chitinase and serine rich *Entamoeba* protein. PCR primers derived from SSU-rRNA gene amplified both *E. histolytica* and *E. dispar* genes producing an amplicon of 1,080 bp, and detected 3 out of 30 samples. PCR primers derived from chitinase gene of *E. histolytica* generating amplicons of 500 and 1,260 bp, samples were positive in 12 out of 30 samples. Due the large difference of gene encoding serine rich protein between *E. histolytica* and *E. dispar*, two specific sets of primers were designed. SREH-primer set, specific for *E. histolytica*, generated amplicons of 550 and 700 bp and detected 22 out of 30 samples. SED-primer set, specific to *E. dispar*, produced an amplicon of 550 bp, and together with a nested primer pair generating an amplicon of 477 bp, detected 16 out of 30 samples. Thus, detection of single and mixed infections of the two *Entamoeba* species could be effectively achieved directly from DNA extracted from feces without the need to culture the parasites.

INTRODUCTION

Amebiasis is one of the important health problems in Thailand, with clinical manifestations ranging from asymptomatic to colitis with bloody diarrhea. Amebiasis is epidemic along the Thai-Myanmar border where water supply is inadequate and there is poor sanitation (Wongstitwilairoong *et al*, 2007). It is now generally accepted that there are two genetically distinct but morphologically indistinguishable species of *Entamoeba*, namely, *Entamoeba histolytica* and *E. dispar* (WHO/PAHO/UNESCO report, 1997). *E. histolytica* has the potential to cause dysentery and extra-intestinal disease, while *E. dispar* is considered to be a harmless commensal protozoa. If *E. histolytica* is present

Correspondence: Apiradee Intarapuk Department of Parasitology, Faculty of Public Health, Mahidol University, 420/2 Ratchawithi Road, Bangkok 10400, Thailand.
Tel: +66 (0) 89 1045213
E-mail: intarapuk@hotmail.com

in a clinical sample, the patient needs to be treated, while if only *E. dispar* is identified, treatment is unnecessary. More than 90% of amebic infections were reported to be colonized by *E. dispar* (Jackson, 1998), and only 10% of *E. histolytica* can progress to obvious clinical symptoms (Gathiram and Jackson, 1985). Although many individuals with *E. histolytica* infection have no symptom, the infections have an impact on health in the long term, and their cysts can be spread within the population.

The traditional diagnosis of *Entamoeba* infection is based on microscopic examination of fecal sample, but differentiation between these two *Entamoeba* species is impossible by this method and sensitivity is only 60% even under optimal conditions (Haque *et al*, 1995). Ameba cultivation followed by zymodeme analysis was the traditional method for identifying and differentiating between *E. histolytica* and *E. dispar* (Haque *et al*, 1990), but culturing procedure has low sensitivity and is labor-intensive. Overgrowth of other parasites can obscure the presence of *E. histolytica* in the culture (Tanyuksel and Petri Jr, 2003).

Detection of antibodies to ameba in patient sera by immunoassay has been used to indicate *E. histolytica* infection (Nesbitt *et al*, 2004; Haghighi and Rezaeian, 2005). However, serological testing is not able to distinguish past from present infection. Newer approaches to identify *E. histolytica* infection are based on detection of *E. histolytica* DNA in fecal sample. Polymerase chain reaction (PCR) assay has been utilized to increase sensitivity and specificity of *Entamoeba* diagnosis in a variety of clinical specimens including fecal and liver abscess pus samples (Anane and Khaled, 2005; Paul *et al*, 2007; Kurt *et al*, 2008). PCR based on amplification of the small subunit rRNA gene (SSU-rDNA) was reported to be 100 times more sensitive than ELISA for *E. histolytica* detection (Mirelman *et al*, 1997; Fotedar *et al*, 2007). SSU-rDNA is widely used as target for detection and differentiation of *Entamoeba* species, as this target is present in multicopies, present on extrachromosomal plasmids (Bhattacharya *et al*, 1989), making the SSU-rDNA more easily detected than a DNA target present in a single-copy gene. Other gene targets used in PCR detection include genes encoding chitinase and serine-rich *E. histolytica* protein (SREHP) (Stanley Jr *et al*, 1990; Ramos *et al*, 2005). Both genes have tandem repeats. *E. histolytica* chitinase gene repeats range from 84-252 nucleotides corresponding to 4 heptapeptide repeats (28 amino acids) to 12 hepapeptide repeats (84 amino acids). The SREHP gene contains tandem repeats of 24 and 36 bases in length, encoding 8 and 12 amino acid repeats, respectively (Haghighi *et al*, 2002; Ramos *et al*, 2005). However, as the repeat-containing region of the chitinase gene appears to be less polymorphic as compared to SREHP gene, this gene has been used for species identification more frequently than strain differentiation (Acuna-Soto *et al*, 1993).

In this study fecal specimens containing *Entamoeba* cysts or trophozoites were collected from individuals residing at the Thai-Myanmar border area, and were subjected to molecular diagnosis of *Entamoeba* infection. PCR assays based on the SSU-rDNA, chitinase and SREHP genes were performed.

MATERIALS AND METHODS

Entamoeba control strains

E. histolytica HM1:IMSS strain was provided by Prof Gordon B Bailey, Morehouse School of Medicine, Atlanta, Georgia, USA, and axenic strain of *E. dispar* by Keio University, School of Medicine, Japan. These axenic *Entamoeba* strains were propagated in

TYI-S-33 as described by Diamond (1968). *E. histolytica* strain S was isolated from a patient at Chulalongkorn Hospital, Bangkok, Thailand and was maintained as xenic culture in Locke egg media as described by Clark and Diamond (2002).

Other protozoa and bacterial strains

Fecal specimens containing *Giardia lamblia, Endolimax nana* and *Entamoeba coli* were collected and subjected to DNA extraction. In addition, enteric bacterial strains that can cause enteric infection, as *Escherichia coli, Enterobacter* spp, *Salmonella weltevreden, Shigella sonnei,* and *Shigella flexneri* were included. These bacterial strains were from stock cultures provided by Microbiology Unit, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Fecal samples

Fecal samples (455) were collected from individuals with a high risk of *E. histolytica*/*E. dispar* infection. Participating individuals included 231 Myanmar migrants and 224 Thai residents in Phang-Nga Province, Thailand during September to October 2006. Myanmar migrants lived in area with poor sanitation, and lacking good hygienic in food and water supplies. All fecal samples were screened for amebic cells by microscopic examination. Samples collected in containers without preservation were concentrated using a formalin-ether sedimentation technique for identification of cysts and trophozoites (Allen and Radley, 1953). Fecal samples containing amebic cells were stored at -20ºC until DNA extraction was performed.

DNA isolation procedure

Entamoeba cells or bacterial cell pellets were subjected to DNA extraction using a commercial genomic DNA extraction kit. Fecal samples of approximately 200 μg were subjected to QIAamp DNA Stool Mini Kit, (QIAGEN, Hilden, Germany) according to manufacturer's instructions. To increase the efficiency of cell lysis, the fecal samples were frozen with liquid nitrogen and ground to powder before the lysis step. DNA samples were kept frozen at -20ºC until required for PCR.

PCR conditions

The primers used in this study are tabulated in Table 1. PCR in 25 μl reaction mixture contained 200 μM each of dNTP, 10 pmole of each primer, 1.0 U *Taq* DNA polymerase (RBC; Korea), 1.5 mM $MgCl_2$ and approximately 100 ng of genomic DNA. Thermal cycling was as follows: initial denaturation at 94ºC for 5 minutes followed by 35 thermal cycles of 94ºC for 1 minute, 46-60ºC for 1 minute, and 72ºC for 1 minute, followed by final step at 72ºC for 5 minutes. The optimized annealing temperature of each primer set was as follows: 60ºC for E1/E2, 48ºC for CEH1/CEH2, 48ºC for SREH1/SREH2, 53ºC for SED1/SED2 and 50ºC for nSED1/nSED2. Each primer set was verified for specificity to either *E. histotytica* or *E. dispar* DNA. Specificity of each PCR amplification was also determined among the DNA of parasites and bacteria that could also cause diarrhea, as *Giardia lamblia, Endolimax nana, Escherichia coli, Salmonella weltevreden, Shigella flexneri, Shigella sonnei* and *Enterobacter* spp. The PCR amplicons were separated electrophoretically in 1% agarose gel stained with ethidium bromide. The gels were visualized by UV light and photographed.

Ethical clearance

This study was approved by Committee on Human Rights Related to Human Experimentation, Mahidol University, Bangkok, Thailand before collecting the fecal samples. Written consent was obtained from each individual who provided personal information and clinical sample.

Table 1
Entamoeba gene-targeted primers used in the study.

Gene target	Primer	Primer sequence (5'-3')	Tm (ºC)	Length (bp)	Reference
SSU-rDNA	E1	5' TAG GAT GAA ACT GCG GAC GGT 3'	60	1,080	This study
	E2	5'AGC CTT GTG ACC ATA CTC CC 3'			
Chitinase for *E. histolytica*	CEH1	5' GGA ACA CCA GGT AAA TGT ATA 3'	48	500, 1,260	(Haghighi *et al*, 2002)
	CEH2	5' GGT ATC ATT TGG TCA TCA TTC C 3'			
Serine rich *E. histolytica* protein (SREHP)	SREH1	5'ACG AAG CTA GTC CTG AAA AGC 3'	48	550, 700	(Ramos *et al*, 2005)
	SREH2	5' CAA TGA ATG GAC TTG ATG CAG 3'			
Serine rich *E. diaper* protein (SREDP)	SED1	5' GAA CGA AGC TAG TCC TGA AAA A 3'	53	550	(Ramos *et al*, 2005)
	SED2	5'GCT TGC TTC TGG TTT ATT ATC TGG 3'			
	nSED1	5'CTT GAA GAA AAT AAA GAA GAT GAA3'	50	477	This study
	nSED2	5' GGT TTA TCA CTT GAA CTT GCT TCT 3'			

RESULTS

Microscopic examination

Investigations of formalin-ether sedimentations of fecal samples by microscopy showed 30 samples containing either *E. histolytica* or *E. dispar*. Cysts were found in most fecal samples, while trophozoites were presented in only 7 samples. Ingested red blood cells in the *Entamoeba*'s cytoplasm, which usually is found in *E. histolytica*, were not visible in this study, thus distinguishing *E. histolytica* from *E. dispar* by microscopic examination was not possible.

E. dispar is not recognized as a cause of diarrhea, dysentery or amebic liver abscess. This study found some cases of *E. dispar* infections with dysentery or diarrhea-like symptom with feces containing mucus. From the microscopic detection, we found co-infection of *E. dispar* with other intestinal parasites, especially hookworm or *Trichuris trichiura*, which could cause diarrhea with mucus.

PCR profiles of *Entamoeba* detection

All primer pairs selected for this study, namely, those derived from SSU-rDNA, chitinase, SREHP, and SREDP genes, were quite specific, and did not amplify DNA derived from the selected enteric bacteria and protozoa used in this study.

The E1 and E2 primers were designed to amplify SSU-rDNA in both *E. histolytica* and *E. dispar*, when an optimum annealing temperature of 60ºC was employed, and to yield amplicon of approximately 1,080 bp (Fig 1). This E1/E2 primer pair was positive in just 3 out of 30 of microscopically positive fecal samples.

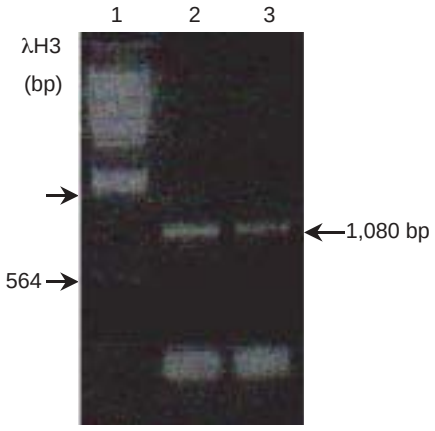

Fig 1–Agarose gel analysis of PCR products amplified by E1 and E2 primers. PCR was conducted using annealing temperature of 60ºC and PCR amplicon is 1,080 bp. Lane 1, Lambda DNA/*Hin*dIII standard size markers; lane 2, genomic DNA of *E. histolytica* HM1: IMSS strain; lane 3, genomic DNA of *E. dispar.*

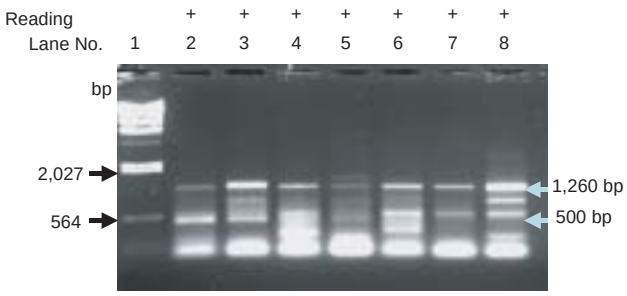

Fig 2–Agarose gel analysis of PCR products amplified by CEH1 and CEH2 primers. PCR was conducted using annealing temperature of 48ºC and template DNA was extracted from feces. Lane 1, Lambda DNA/*Hin*dIII standard size markers; lanes 2-7, template DNA from M149, M294, M290, M37, T46, and T113, respectively; lane 8, genomic DNA from *E. histolytica* strain S representing as positive control. The predicted amplicons are 500 and 1,260 bp, with additional amplicons of approximately 200 and 700 bp.

Using an annealing temperature of 48ºC, the CEH1 and CEH2 primers could specifically amplify the chitinase gene of *E. histolytica*, with amplicons of 500 and 1,260 bp. As shown in Fig 2, *E. histolytica* strain S, positive control, produced additional amplicons of 700 and 200 bp, indicating additional repeat regions of chitinase gene present in the genome. Fecal sample was regarded as positive when either amplicon of 500 or 1,260 bp was present. In this study, CEH1/CEH2 primer pair of chitinase gene was positive in 12 out of 30 of microscopically positive fecal samples.

The SREH1 and SREH2 primers specifically amplified the gene encoding serine rich *E. histolytica* protein and the predicted PCR amplicons were 550 and 700 bp. In Fig 3, apart from the predicted amplicons, *E. histolytica* strain S displayed amplicons of approximately 2 kb and 200 bp. Any prominent DNA bands derived from this primer

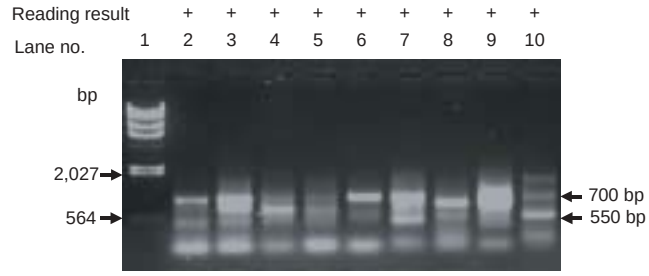

Fig 3–Agarose gel analysis of PCR products amplified by SREH-1 and SREH-2 primers. DNA was extracted from fecal specimens and PCR was conducted using annealing temperature of 48°C. Lane 1, Lambda DNA/*Hin*dIII standard size markers; lanes 2-9, DNA from M130, M141, M149, M168, M176, M294, M290, and T46, respectively; lane 10, genomic DNA from *E. histolytica* strain S representing positive control. The predicted PCR amplicons are 550 bp and 700 bp, with addition amplicons of approximately 2 kb.

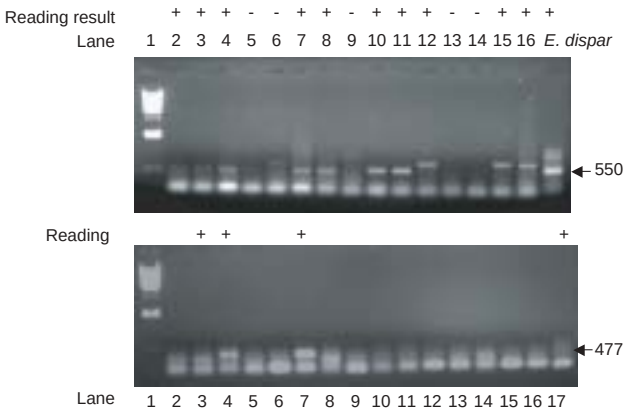

Fig 4–Agarose gel analysis for detection of *E. dispar* from the 15 fecal samples using nested primers derived from serine rich *E. dispar* protein gene. Upper panel, lanes 2-16 represent amplicons derived from 1st round PCR using SED1/2 primers and lane 17 of genomic DNA of *E. dispar* as positive control. Lower panel, lanes 2-16 repeated amplicons derived from 2nd round PCR using nSED1/2 primers. Lane 1 is lambda DNA/*Hin*dIII standard size markers.

pair were thus regarded as positive. In this study, SREH1/SREH2 primer pair of SREHP gene was positive in 22 out of 30 of microscopically positive fecal samples.

The SED1 and SED2 primers are specific for the serine rich *E. dispar* protein and generate an amplicon of 550 bp. The product was also internally amplified using nSED1 and nSED2 primers to generate an amplicon of 477 bp. For amplification of genes encoding serine rich *E. dispar* protein, the positive results were considered, when expected DNA band was observed in the 1st round or 2nd round PCR. Ten fecal samples were positive using SED1/2 primer pair, while an additional 6 samples were positive with nSED1/2 primer pair. Thus, primer pairs of serine-rich *E. dispar* protein were positive in 16 out of 30 of microscopically positive fecal samples (Fig 4).

The results of PCR detection of *Entamoeba* infection of the 30 fecal samples are summarized in Table 2. Two samples could not be detected by PCR which may be due to loss of DNA during the extraction process, as only 200 µg of fecal samples were used. From PCR analysis of 30 fecal samples, only *E. histolytica* was identified in 12 out of 30 (40%) samples, only *E. dispar* in 6 of 30 (20%) and both in 10 of 30 (33%).

DISCUSSION

Microscopic examination cannot distinguish between *E. histolytica* and *E. dispar* species as both possess morphologically identical cysts and small trophozoite. Molecular detection of DNA extracted from clinical specimens is now widely used in clinical research laboratories. PCR amplification of fecal DNA has more benefit, as diagnostic results can be obtained without *in vitro* culturing of parasites. Several studies have revealed that PCR analysis is a sensitive and specific tool to differentiate between infection with pathogenic *E. histolytica* and non-pathogenic *E. dispar* (Verweij *et al*, 2000; Blessmann *et al*, 2002; Gonin and Trudel, 2003).

By microscopy amebic cysts and trophozoites were observed from fecal specimens. In both *E. histolytica* and *E. dispar* infections, trophozoites could be observed in fresh fecal specimens, but the trophozoites generally degenerate rapidly in unfixed feces (Proctor, 1991). *E. histolytica* trophozoites can ingest red blood cells but they do not frequently appear in chronic amebic infections. Trophozoites containing ingested red blood cells are not present in patients who do not have acute dysentery (Gonzalez-Ruiz *et al*, 1994).

This study employed PCR assays based on three genes, which have been shown previously to specifically detect *Entamoeba* spe-

Table 2
Results of PCR assays obtained from *Entamoeba* SSU-rRNA, chitinase and serine-rich protein genes.

Reference gene	SSU-rDNA	Chitinase		SREHP		SREDP	
Specificity	EH or ED	EH		EH		ED	Identified *Entamoeba* species
Sample	1,080 bp	500 bp	1,260 bp	550 bp	700 bp	557 or 477 bp	
M66	-	-	-	-	-	+	ED
M82	-	-	-	-	-	+	ED
M130	-	-	-	-	+	+	Mixed
M137	-	-	-	-	+	-	EH
M141	-	-	-	+	+	-	EH
M149	-	+	+	-	+	+	Mixed
M154	-	-	-	-	-	+	ED
M168	-	-	+	-	+	-	EH
M175	-	-	+	-	+	+	Mixed
M176	-	-	-	-	+	+	Mixed
M249	-	-	+	-	+	-	EH
M278	-	-	-	-	-	-	Negative
M280	-	-	-	-	-	-	Negative
M281	-	-	-	-	+	-	EH
M282	-	-	-	-	+	-	EH
M294	+	+	+	+	+	+	Mixed
M266	-	-	-	-	+	+	Mixed
M153	-	-	+	-	+	+	Mixed
M290	+	+	+	+	+	-	EH
M14	-	-	+	-	+	-	EH
M37	-	-	+	-	+	+	Mixed
T48	-	-	-	-	-	+	ED
T29	-	-	-	-	+	-	EH
T35	-	-	-	-	+	+	Mixed
T46	+	+	+	+	+	-	EH
T113	-	+	+	-	+	-	EH
T228	-	-	+	-	+	+	Mixed
T230	-	-	-	-	+	-	EH
T231	-	-	-	-	-	+	ED
T233	-	-	-	-	-	+	ED

EH, *Entamoeba histolytica*; ED, *Entamoeba dispar*

cies. The non-protein coding region, SSU-rDNA, was detected by designing primers from shared conserved region between *E. histolytica* HM1:IMSS strain and *E. dispar*. Although SSU-rDNA is the most ubiquitous, gene sequences of *Entamoeba* strains found in nature could be changed due to selective pressure. The low sensitivity of this primer

pair, E1/E2, in *Entamoeba* detection is probably due to DNA variation among strains. In contrast, the genes of chitinase and serine rich protein are composed of repeating unit, thus allowing more chance for these primer pairs to amplify fecal DNA. The CEH1/2 primer pair was specific to *E. histolytica* and negative for *E. dispar*, other protozoa and enteric bacteria. As the serine rich *E. dispar* protein, SREDP, shares about 40% identity to SREHP, specific primers designed for each protein should be reliable for differentiation. To increase sensitivity of SED primer pair, nested primers were also designed. The outer primer of SED could detect *E. dispar* in 10 specimens, while an additional of 6 specimens were detected with the nested primer pair.

Polymorphism in the chitinase and SREHP genes was demonstrated among ameba DNA in this study. Haghighi *et al* (2002) described polymorphism in the type, location and number of repeat unit among those two genes in Japanese isolates. Based on the gene encoding chitenase protein of *E. histolytica* (XM647113), CEH1/2 primer pairs were expected to produce two amplicons of 500 and 1,260 bp, but additional bands were demonstrated in Thai *Entamoeba* DNA, indicating variation in number of repeat unit and their location. Using the same primer pairs (Haghighi *et al*, 2002), polymorphic bands derived from SREHP loci were revealed in both Japanese and Thai isolates. PCR product length polymorphism derived from size variations within the SREHP gene results in mostly single but also multiple bands. A previous study by Ayeh-Kumi *et al* (2001) showed 34 distinct patterns among 54 *E. histolytica* isolates from Bangladesh.

The sensitivity of the PCR detection was only 93% when compared to microscopic detection. The negative PCR results in 2 samples with positive microscopic detection may have resulted from the low number of parasites in the sample. The 200 μg of fecal sample used for PCR also limited the yield of extracted DNA.

Based on molecular identification, the prevalence of *E. histolytica* infection was greater than *E. dispar* infection in the studied population of Thailand. A similar trend of *E. histolytica* infection was reported in a highly endemic region in Mexico (Acuna-Soto *et al*, 1993; Ramos *et al*, 2005). A previous study in Thailand revealed 13.3% of amebiasis patients are infected with *E. histolytica* and 20% with *E. dispar* (Hamzah *et al*, 2006). In contrast in developed countries where *E. histolytica* infection is not endemic, prevalence of *E. dispar* among patients is greater than *E. histolytica*. For instance, in Australia 3.4% of patients' specimens contained only *E. histolytica*, while 33.7% of samples contained only *E. dispar*, and 24.7% of samples contained only *E. moshkovskii* (Fotedar *et al*, 2007). Mixed infection with *E. dispar* and *E. moshkovskii* was found in 36% of samples, and 1.1% of sample contained both *E. histolytica* and *E. moshkovskii* (Fotedar *et al*, 2007). *E. moshkovskii* is a free-living ameba, and lives usually commensally in human intestine. In the Netherlands, 6.7% of microscopic positive fecal samples were identified as *E. histolytica*, while 91.2% of microscopic-positive fecal samples are *E. dispar* (Visser *et al*, 2006). In Canada, 2.9% of samples contain *E. histolytica* and 97.1% of samples contain *E. dispar* (Gonin and Trudel, 2003).

In this study molecular methods were applied for identifying amebiasis directly from fecal samples. PCR for the *Entamoeba* DNA detection was employed using SSU-rDNA. However this assay had low sensitivity to detect the infection. The chitinase and serine rich protein genes were selected as targets of PCR for differentiating between *E. histolytica* and *E. dispar*. The sensitivity and specificity of the tests were high. We have demonstrated the usefulness of PCR as a tool

for detecting intestinal parasitic infection without the need for an intervening step of ameba cultivation.

ACKNOWLEDGEMENTS

This work was supported by Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Department of Parasitology, Faculty of Public Health, Mahidol University, and Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok, Thailand.

REFERENCES

Acuna-Soto R, Samuelson J, Girolami D, *et al.* Application of the polymerase chain reaction to the epidemiology of pathogenic and nonpathogenic *Entamoeba histolytica*. *Am J Trop Med Hyg* 1993; 48: 58-70.

Allen AVH, Radley DS. Further observation on the formalin-ether concentration technique for faecal parasites. *J Trop Med Hyg* 1953; 23: 545-6.

Anane S, Khaled S. *Entamoeba histolytica* and *Entamoeba dispar:* differentiation methods and implications. *Ann Biol Clin* 2005; 63: 7-13.

Ayeh-Kumi PE, Ali IM, Lockhart LA, Gilchrist CA, Petri Jr WA, Haque R. *Entamoeba histolytica*: genetic diversity of clinical isolates from Bangladesh as demonstrated by polymorphisms in the serine-rich gene. *Exp Parasitol* 2001; 99: 80-8.

Bhattacharya S, Bhattacharya A, Diamond LS. Circular DNA of *Entamoeba histolytica* encodes ribosomal RNA. *J Protozoa* 1989; 36: 455-85.

Blessmann J. Buss H, Nu PAT, *et al.* Real-time PCR for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in fecal samples. *J Clin Microbiol* 2002; 40: 4413–7.

Clark CG, Diamond LS. Methods for cultivation of luminal parasitic protists of clinical importance. *Clin Microbiol Rev* 2002; 15: 329-41.

Diamond LS. Techniques of axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-like amebae. *J Parasitol* 1968; 54: 1047-56.

Fotedar R, Stark D, Beebe N, Marriott D, Ellisand J, Harkness J. PCR detection of *Entamoeba histolytica, Entamoeba dispar,* and *Entamoeba moshkovskii* in stool samples from Sydney, Australia. *J Clin Microbiol* 2007; 45: 1035-7.

Gathiram V, Jackson TFHG. Frequency distribution of *Entamoeba histolytica* zymodemes in a rural South African population. *Lancet* 1985; i: 719-21.

Gonin P, Trudel L. Detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* isolates in clinical samples by PCR and enzyme-linked immunosorbent assay. *J Clin Microbiol* 2003; 41: 237-41.

Gonzalez-Ruiz A, Haque R, Aguirre A, *et al.* Value of microscopy in the diagnosis of dysentery associated with invasive *Entamoeba histolytica*. *J Clin Pathol* 1994; 47: 236-9.

Haghighi A, Kobayashi S, Takeuchi T, Masudaand G, Nozaki T. Remarkable genetic polymorphism among *Entamoeba histolytica* isolates from a limited geographic area. *J Clin Microbiol* 2002; 40: 4081-90.

Haghighi A, Rezaeian M. Detection of serum antibody to *Entameba histolytica* in various population samples of amebic infection using an enzyme-linked immunosorbent assay. *Parasitol Res* 2005; 97: 209-12.

Hamzah Z, Petmitr S, Mungthin M, Leelayoova S, Chavalitshewinkoon-Petmitr P. Differential detection of *Entamoeba histolytica, Entamoeba dispar* and *Entamoeba moshkovskii* by a single-round PCR assay. *J Clin Microbiol* 2006; 44: 3196-200.

Haque R, Hall A, Tzipori S. Zymodemes of *Entamoeba histolytica* in Dhaka, Bangladesh. *Ann Trop Med Parasitol* 1990; 84: 629-32.

Haque R, Neville LM, Hahn P, Petri Jr WA. Rapid diagnosis of *Entamoeba* infection by using *Entamoeba* and *Entamoeba histolytica* stool antigen detection kits. *J Clin Microbiol* 1995; 33: 2558-61.

Jackson TFHG. *Entamoeba histolytica* and *Entam-*

oeba dispar are distinct species; epidemiological and serological evidence. *Int J Parasitol* 1998; 28: 181-6.

Kurt O, Demirel M, Ostan I, *et al*. Investigation of the prevalence of amoebiasis in Izmir province and determination of *Entamoeba* spp. using PCR and enzyme immunoassay. *New Microbiol* 2008; 31: 393-400.

Mirelman D, Nuchamowitz Y, Stolarsky T. Comparison of use of enzyme-linked immunosorbent assay-based kits and PCR amplification of rRNA genes for simultaneous detection of *Entamoeba histolytica* and *E. dispar*. *J Clin Microbiol* 1997; 35: 2405-7.

Nesbitt RA, Mosha FW, Katki HA, Ashraf M, Assenga C, Lee CM. Amebiasis and comparison of microscopy to ELISA technique in detection of *Entamoeba histolytica* and *Entamoeba dispar*. *J Natl Med Assoc* 2004; 96: 671-7.

Paul J, Srivastava S, Bhattacharya S. Molecular methods for diagnosis of *Entamoeba histolytica* in a clinical setting: an overview. *Exp Parasitol* 2007; 116: 35-43.

Proctor EM. Laboratory diagnosis of amebiasis. *Clin Lab Med* 1991; 11: 829-59.

Ramos F, Moran P, Gonzalez E, *et al*. *Entamoeba histolytica* and *Entamoeba dispar*: prevalence infection in a rural Mexican community. *Exp Parasitol* 2005; 110: 327-30.

Stanley Jr SL, Becker A, Kunz-Jenkins C, Foster L, Li E. Cloning and expression of a membrane antigen of *Entamoeba histolytica* possessing multiple tandem repeats. *Proc Natl Acad Sci USA* 1990; 87: 4976-80.

Tanyuksel M, Petri Jr WA. Labolatory diagnosis of amoebiasis. *Clin Microbiol Rev* 2003; 16: 713-29.

Verweij JJ, Blotkamp J, Brienen EA, Aguirre A, Polderman AM. Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* cysts using polymerase chain reaction on DNA isolated from faeces with spin columns. *Eur J Clin Microbiol Infect Dis* 2000; 19: 358-61.

Visser LG, Verweij JJ, Van Esbroeck M, Edeling WM, Clerinx J, Polderman AM. Diagnostic methods for differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in carriers: performance and clinical implications in a non-endemic setting. *Int J Med Microbiol* 2006; 296: 397-403.

WHO/PAHO/UNESCO report. A consolation with experts on amoebiasis. *Epidemiol Bull* 1997; 18: 13-4.

Wongstitwilairoong B, Srijan A, Serichantalergs O, *et al*. Intestinal parasitic infections among pre-school children in Sangkhlaburi, Thailand. *Am J Trop Med Hyg* 2007; 76: 345-50.