PCR DETECTION OF *ENTAMOEBA* SPP FROM SURFACE AND WASTE WATER SAMPLES USING GENUS-SPECIFIC PRIMERS

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Abstract. In this study DNA of 137 surface and waste water samples collected from Pathum Thani Province, Thailand were examined for *Entamoeba* spp using PCR and genus-specific primers that amplify DNA of *E. polecki, E. chattoni, E. dispar, E. histolytica, E. hartmani, E. coli* and *E. moshkovskii*. The results showed that 27% of the samples were positive for *Entamoeba* spp. When the positive samples were further examined by a single-round PCR assay specific for *E. histolytica*, *E. dispar* and *E. moshkovskii*, all were negative. Their species should also be further investigated using a more sensitive method such as real-time PCR and specific primers for *E. polecki, E. chattoni, E. hartmani,* and *E. coli*.

INTRODUCTION

There are six species of *Entamoeba*, namely, *E. histolytica*, *E. dispar, E. coli*, *E. hartmanni*, *E. moshkovskii* and *E. polecki*, found in humans. Infections with *Entamoeba* spp can result in either a harmless colonization of the intestine or invasion of the colon wall and damage of other host tissues, such as the liver, lung, and brain (amebiasis). Most of the *Entamoeba* species are commensal parasites in intestinal lumen and do not cause human diseases.

E. histolytica is a causative agent of amebic dysentery and invasive extraintestinal amebiasis, which manifest most commonly in the form of liver abscess. *E. polecki* is an intestinal ameba of pigs and monkeys, and is occasionally seen in human and may also cause diarrhea. *E. dispar* and *E. moshkovskii* are morphologically indistinguishable from *E. histolytica* in both cyst and trophozoite forms. *E. moshkovskii* is considered to be primarily a free-living ameba but has been identified and isolated from humans. It is estimated that *E. histolytica* may infect half a billion people worldwide annually. With an annual mortality estimated to be 100,000 worldwide, amebiasis is the third most common cause of death due to parasitic infection after malaria and schistosomiasis (Walsh, 1986).

Although previous studies showed *E. dispar* to be a nonpathogenic parasite, intestinal symptoms in patients infected with this species have been reported (Jetter *et al*, 1997; Ali *et al*, 2003; Parija and Khairnar, 2005). Patients in India who were infected with *E. dispar* in association with *E. moshkovskii* had gastrointestinal symptoms (Parija and Khairnar, 2005). A high prevalence of *E. moshkovskii* (21%) was observed among children 2 to 5 years of age in Bangladesh (Ali *et al*, 2003). In Australia all patients with *E. moshkovskii* infection are also symptomatic (Fotedar *et al*, 2007). This may indicate that perhaps humans are a true host for this putatively free-living ameba and are not just transiently infected. Therefore, the prevalence of *E. moshkovskii* and *E. dispar* infections in other parts of the world need to be investigated in order to

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determine its significant pathogenic potential in humans.

Diagnosis of *Entamoeba* cyst is mostly based on morphological investigation under a light microscope. However, this technique is incapable of distinguishing between *E. histolytica*, *E. dispar* and *E. moshkovskii*, which have identical morphology. PCR-based assays have been developed and widely used to detect *Entamoeba* species because of their high sensitivity (Heckendorn *et al*, 2002; Morran *et al*, 2005; Hamzah *et al*, 2006; Fotedar *et al*, 2007), being 100 times more sensitive than ELISA (Mirelman *et al*, 1997).

As *Entamoeba* species infect humans through fecal-oral route, water transmission is common in developing countries where much of the water supply for drinking is untreated and contaminated with feces. Use of human feces for fertilizer is also an important source of infection. Cysts in external environment can survive days, weeks or month especially under damp conditions and are responsible for transmission (Markell *et al*, 1999). Studies of *Entamoeba* species have used specimens from stool and liver abscess samples. Investigations of the occurrence of *Entamoeba* species in surface and waste water in Thailand have not been hitherto conducted.

MATERIALS AND METHODS

DNA samples

A total of 137 DNA samples from surface and waste water in Pathum Thani Province, Thailand were kindly provided by Asian Institute of Technology, Thailand. Genomic DNA of *Entamoeba histolytica* (HM-1: IMSS), *Entamoeba dispar* (SAW 760) and *Entamoeba moshkovskii* (Laredo strain) were kindly provided by Dr Graham Clark, London School of Hygiene and Tropical Medicine, London, England.

Genus-specific PCR assay

PCR assays for detection of *Entamoeba* species were performed using genus-specific PCR primers based on small-subunit rRNA

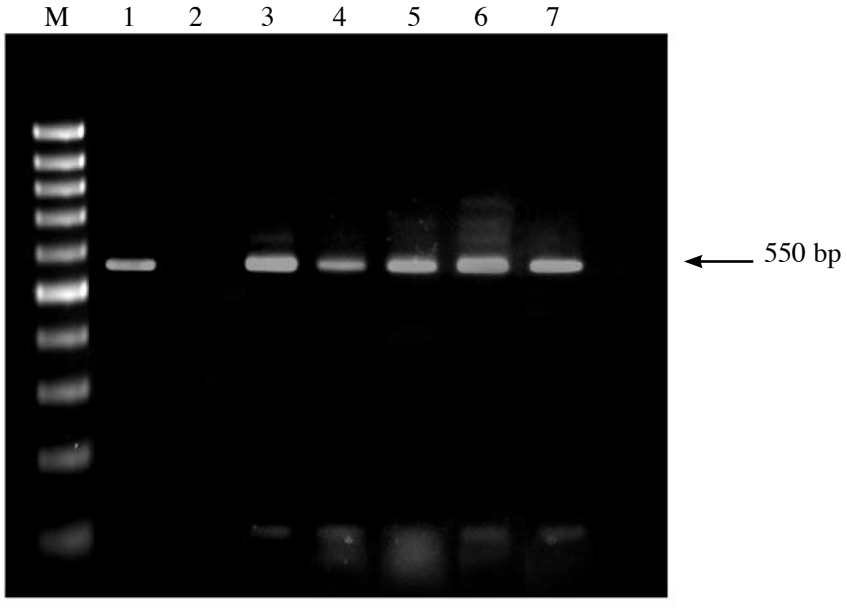

Fig 1- PCR amplification of *Entamoeba* DNA using genus-specific primers. Lane M = molecular marker (100-bp ladders), lane1 = positive control (*E. histolytica* DNA), lane 2 = negative control (H_2O), lanes 3 to 7 = amplified products (550 bp) indicating positive specimens.

gene sequences for *E. polecki*, *E. chattoni*, *E. dispar*, *E. histolytica*, *E. hartmanni* and *E. coli* (Verweij *et al*, 2001). Primers sequences were as follows: forward primer Entam1, 5´GTT GAT CCT GCC AGT ATT ATA TG 3´ and reverse primer Entam2, 5´CAC TAT TGG AGC TGG AAT TAC 3´. Genus-specific PCR amplifications were performed in a final volume of 40 μl containing 1X PCR buffer, 1.5 mM MgCl2, 200 μM of each dNTP, 25 pmol of each genus specific primer, 1 unit of *Taq* DNA polymerase (Amersham) and 2 μl of DNA samples. Reactions were carried out in a thermal cycler (Px2 Thermal Cycler, thermoHybaid, UK) PCR System. Samples were denatured at 94 ˚C for 5 minutes, and then subjected to 35 cycles of 94 ˚C for 1 minute, 55 ˚C for 1 minute and 72 ˚C for 1 minute, following by a final extension at 72 ˚C for 7 minutes. PCR generates 550 bp amplicon (Fig 1).

Single-round PCR assay

Positive samples from *Entamoeba* genus-specific PCR assay were further examined using a single-round PCR assay employing species-specific primers developed for detection and differential diagnosis of *E. histolytica*, *E. dispar* and *E. moshkovskii* (Hamzah *et al*, 2006). The forward primer sequence (EntaF) is the same for all three *Entamoeba* species, whereas the reverse primer is EhR, EdR, and EmR, specific for *E. histolytica*, *E. dispar*, and *E. moshkovskii*, respectively. Primer sequences are as follows: EntaF, 5´ATG CAC GAG AGC GAA AGC AT 3´; EhR, 5´GAT CTA GAA ACA ATG CTT CTC T 3´; EdR, 5´CAC CAC TTA CTA TCC CTA CC 3´; EmR, 5´TGA CCG GAG CCA GAG ACA T 3´. Amplification were performed in a final volume of 40 μl containing 1X PCR buffer, 6 mM MgCl2, 200 μM of each dNTPs, 0.1 μM of each species specific primer, 0.5 unit of *Taq* DNA polymerase (Amersham), and 5 μl of DNA samples. Reactions were carried out in a thermal cycler PCR System. Samples were denatured at 94 ˚C for 3 minutes, and then subjected to 30 cycles of 94 ˚C for 1 minute, 58 ˚C for 1 minute and 72 ˚C for 1 minute, following by a final extension at 72 ˚C for 7 minutes. PCR generates amplicon of a 166-bp for *E. histolytica*, 752-bp for *E. dispar* and 580-bp for *E. moshkovski*. Amplicons from genus-specific and single-round PCR were separated by electrophoresis in 1.5% agarose gel and visualized in a UV transilluminator following staining with 0.5 μg/ml of ethidium bromide.

RESULTS

PCR assays performed using *Entamoeba* genus-specific PCR primers that generate 550-bp amplicon showed that 37 (27%) samples were positive for *Entamoeba* (Fig 1). Using species-specific primers in the single-round PCR assay, these *Entamoeba*-positive samples were negative for *E. histolytica*, *E. dispar* and *E. moshkovskii* (data not shown). This assay could detect as little as 9.5 pg of *E. histolytica* and *E. moshkovskii* DNA, and 19 pg of *E. dispar* DNA (data not shown).

DISCUSSION

This is the first study using molecular techniques to determine the prevalence of *Entamoeba* species of surface and waste water samples in Thailand.

Findings in this study indicate that water is a possible source for transmission of *Entamoeba* to human host. Cysts can survive for prolonged periods in the external environment and are responsible for transmission because of the protection by their cell wall (Markell *et al*, 1999). It is possible that negative results may be due to the small amounts of *Entamoeba* DNA that are lower than the detectable level of the assay or they belonged to other *Entamoeba* species. In contrast to our study, two out of six water samples (32%) collected from Ankara river in Turkey were positive for *E. histolytca* by PCR (Bakir *et al*, 2003).

Since this is the first investigation of the three human *Entamoeba* species in surface and waste water from Thailand, it is not easy to estimate the sample size needed without knowledge of their prevalence. It is also possible that these 37 positive samples may belong to other *Entamoeba* species, such as *E. polecki*, *E. chattoni*, *E. hartmanni*, and *E. coli*. Therefore, detection of the specific species of *Entamoeba* in these water samples should be further examined by using a more sensitive method, such as real-time PCR, and employing primers for *E. polecki*, *E. chattoni*, *E. hartmanni*, and *E. coli*.

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