MOLECULAR DETECTION OF *ENTAMOEBA DISPAR* FROM SURFACE WATER SAMPLES IN THAILAND

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Abstract. Accurate identification of *Entamoeba* species is crucial for a better understanding of the nature of these protozoan parasites and obtaining precise epidemiological data. Humans typically become infected with *Entamoeba* through the fecal-oral route, particularly from contaminated water in areas with poor sanitation. Therefore, it becomes important to determine the prevalence of human *Entamoeba* in surface water samples. We extracted DNA from surface 140 water samples collected along locations along canals, Pathum Thani Province, Thailand, for analysis of the presence of *Entamoeba dispar*, *E. histolytica* and *E. moshkovskii* using quantitative PCR, and of *Entamoeba coli* using nested PCR. Samples (14%) were positive for *E. dispar* and negative for the other three species. Although infection with *E. dispar* is generally non-pathogenic, intestinal symptoms in *E. dispar* infected patients have beebn reported in various regions. Routine surveillance of *Entamoeba* spp in surface water samples should help reduce the prevalence of amebiasis in Thailand.

Keywords: *Entamoeba* spp, canal, molecular detection, PCR, surface water

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INTRODUCTION

Amebiasis is a common parasitic disease that significantly contributes to diarrhea, particularly in developing countries with poor sanitation (Costa *et al*, 2018). *Entamoeba* infection affects around 500 million people annually, resulting in over 100,000 deaths each year from amebic dysentery and liver abscess, commonly in developing countries, largely due to poor sanitation and fecal contamination of water sources (WHO, 1997; Fotedar *et al*, 2007). Infections can also occur in developed countries, particularly among travelers, immigrants, men who have sex with men, and individuals with weakened immune systems (Shirley *et al*, 2018).

Entamoeba dispar is approximately ten times more prevalent than *E. histolytica*, with most individuals infected with *E. histolytica* actually carrying *E. dispar* (Huston and Petri, 1999). *Entamoeba* spp is transmitted through the ingestion of amoebic cysts *via* the fecal-oral route, typically through contaminated food or water sources, typically due to inadequate environmental sanitation or poor personal hygiene practices (Pritt and Clark 2008; Stensvold *et al*, 2011; Domazetovska *et al*, 2018; Feng *et al*, 2018; Jones *et al*, 2019).

Generally, *E. dispar* infection in humans is considered commensal and not associated with morbidity (Sargeaunt *et al*, 1978; Sargeaunt *et al*, 1980; Tanyuksel and Petri, 2003). The similar morphology of *Entamoeba* spp among *E. bangladeshi*, *E. dispar*, *E. histolytica*, *E. moshkovskii*, and *E. bangladeshi* makes it challenging to differentiate these four *Entamoeba* spp by microscopy, and often leads to misdiagnosis (Carrero *et al*, 2020). Molecular methods, such as conventional polymerase chain reaction (PCR), nested PCR, quantitative (real-time) (q)PCR, and loop-mediated isothermal amplification (LAMP) have been developed for differential diagnosis of *E. coli*, *E. dispar*, *E. histolytica*, and *E. moshkovskii* in clinical samples (Roy *et al*, 2005; Hamzah *et al*, 2006;

Khairnar and Parija, 2007; Liang *et al*, 2009; Hamzah *et al*, 2010; Rattaprasert *et al*, 2022).

Improper wastewater treatment creates a favorable environment for protozoan pathogens in their cyst form, which can be transmitted when people use such wastewater to bathe, wash household utensils, fish, or consume vegetables grown in such water (Ferrer *et al*, 2012). Few studies have been conducted to detect human *Entamoeba* in water samples. In Thailand, 27% of water samples were positive for *Entamoeba* spp using genus-specific primers (Sukprasert *et al*, 2008) and a TaqMan qPCR assay was used to detect *Giardia lamblia* and *E. histolytica* in wastewater samples (Ferrer *et al*, 2012). In Germany, *E. histolytica* was detected in wastewater samples using LAMP (Ajonina *et al*, 2018).

In this study, molecular detection of human *Entamoeba* spp, *eg*, *E. coli*, *E. dispar*, *E. histolytica*, and *E. moshkovskii*, was performed on surface water samples collected from canals in Pathum Thani Province, Thailand. The existence of these human *Entamoeba* in water may lead to infection in communities and pose a public health risk. The results not only raise awareness among the public but also provide a basis for developing monitoring and pollution mitigation strategies to protect public health.

MATERIALS AND METHODS

Water samples

During May 2022 – July 2023, a total of 140 water samples was collected from Municipal Irrigation Canal One and a parallel Canal Two, which flow through communities and agricultural areas in Pathum Thani Province, Thailand. Five samples (2l aliquot each) were collected from locations distanced 700-900 meters apart upstream and downstream along the waterways (Anceno *et al*, 2007), and were transported to the laboratory at ambient temperature within two hours for analysis.

The 2-l samples were centrifuged at 4,000 g for 10 minutes at 4 °C.

The supernatant was discarded and sediment used for DNA extraction using a 5% guanidine thiocyanate lysis buffer and a freeze-thaw process (5 minutes at -40 °C followed by 5 minutes at 95 °C), followed by proteinase K treatment (55 °C for 1 hour), and six 10-s bursts of sonication at 30% amplitude using a 130-W probe sonicator (Cole-Parmer, Chicago, IL) (Anceno *et al*, 2007). Extracted DNA was precipitated with isopropanol and stored at -20 °C until used (following 4-6 months of storage).

Multiplex qPCR detection of *E. dispar, E. histolytica* and *E. moshkovskii*

Multiplex qPCR was carried out using a LightCycler™ instrument (Roche Molecular Biochemicals, Mannheim, Germany) as previously described (Hamzah *et al*, 2010). The forward primer (EhdmF: 5′ CGAAAGCATTTCACTCAACTG 3′) and the reverse primer (EhdmR: 5′ TCCCCCTGAAGTCCATAAACTC 3′) target the conserved SSU rRNA sequences of *E. dispar*, *E. histolytica* and *E. moshkovskii*. Three hybridization probes were employed: a universal fluorescein-labeled probe (Ehdm-FL: 5′ ACTATAAACGATGT-CAACAAGGATTGGATGAAA-FITC 3′) and two LCRed-labeled probes (Ehd-640: 5′ TCAGATGTA-CAAAGATAGAGAAGCATTGTT TCTA-phosphate 3′ and Em-705: 5′ AAGAAATTCGCGGATGAA-GAAACATTGTTT-phosphate 3′ to detect *E. histolytica*, *E. dispar* and *E. moshkovskii*, respectively). Positive controls were DNA from *E. dispar* SAW 760, *E. histolytica* HM-1:IMSS and *E. moshkovskii* Laredo.

To distinguish between the amplicons of *E. dispar* and *E. histolytica*, a melting curve analysis was employed (LightCycler™; Roche Molecular Biochemicals, Mannheim, Germany). *E. dispar* amplicon exhibited a melting temperature (T_m) of 55.5-57.5 °C while *E. histolytica* amplicon that of 60.5-62.5 °C.

Nested PCR detection of *E. coli*

Two sets of primers were employed: genus-specific primers for *Entamoeba* (Verweij *et al*, 2001) and primers specific for *E. coli* SSU rDNA sequence (Rattaprasert *et al*, 2022). For amplification of *Entamoeba* DNA, the forward and reverse primer is Entam1 (5' GTTGATCCTGCCAGTATTATATG 3') and Entam2 (5' CACTATTGGAGCTGGAATTAC 3') respectively. For amplification of *E. coli* SSU rDNA, the forward and reverse primer is EcoliF (5' CTAAGCACAAAGTCCTAGTATGATG 3') and EcoliR (5' CCTCATCGATTACACTCCCAGAG 3') respectively.

First round of nested PCR was conducted in a 30 µl mixture containing 1X PCR buffer (GE Healthcare, Amersham, UK), 1.5 mM $MgCl_2$, 200 µM each dNTP, 25 pmol of each *Entamoeba* genus-specific primer, 1 U Taq DNA polymerase (GE Healthcare, Piscataway, NJ), and 2 µl of DNA. Positive control DNA was from *E. coli* (strain 07-286). Thermocycling was carried out using a Px2 Thermal Cycler (ThermoHybaid, Middlesex, UK) as follows: 94 °C for 5 minutes; 35 cycles of 94 °C for 60 seconds, 55°C for 60 seconds and 72 °C for 60 seconds; and a final step of 72 °C for 7 minutes. The resulting amplicon (550 bp) could be visualized using 1.5% agarose gel-electrophoresis with ethidium bromide staining.

Second round of nested PCR was conducted in a 20 µl reaction mixture containing 1X PCR buffer (GE Healthcare, Piscataway, NJ), 1.5 mM $MgCl_2$, 50 µM each dNTP, 0.5 µM of each *E. coli*-specific primer, 0.5 U Taq polymerase (GE Healthcare, Piscataway, NJ), and 1 µl of the post-first round PCR solution. Thermocycling (Px2 Thermal Cycler) conditions were as follows: 94 °C for 5 minutes; 25 cycles of 94 °C for 30 seconds, step-down temperature ranging from 68°C to 60°C for 30 seconds and 72 °C for 30 seconds; 10 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds; and a final step of 72°C for 2 minutes. The resulting amplicon (166 bp) was visualized using a 2.0% agarose

gel-electrophoresis with ethidium bromide staining.

RESULTS

Of the 140 water samples collected, 20 were tested positive for *E. dispar*, while *E. histolytica* and *E. moshkovskii* were not detected in any of the water samples. The detection of *E. dispar* was effectively achieved by analysis of the amplicon melting temperature (T_m) (Fig 1). The observed range of T_m values for *E. dispar* amplicons ranged from 55.6 to 57.4 °C (Table 1). Furthermore, no water samples showed positive results for *E. coli* assessed using a nested PCR assay.

DISCUSSION

In general, the diagnosis of *Entamoeba* spp using microscopy techniques, which have a sensitivity of 60%, is not as reliable as isoenzyme analysis or cultures (Fotedar *et al*, 2007). Moreover, microscopic examination cannot differentiate between *E. dispar* and *E. histolytica* due to their morphological similarity (Gonin and Trudel, 2003; Davis *et al*, 2009). Hence, several types of PCR assays have been developed for the specific detection of *E. coli*, *E. dispar*, *E. histolytica*, and *E. moshkovskii* (Hamzah *et al*, 2006; Zebardast *et al*, 2016; Berglund *et al*, 2017; Mohammed *et al*, 2017; Ali and Roy, 2020; Singh *et al*, 2021). As expected, the multiplex qPCR assay is more sensitive and effective than other PCR-based methods (Hamzah *et al*, 2010). We showed that 14% of water samples collected from two canals, Pathum Thani Province, Thailand, were positive for *E. dispar* and not for *E. coli*, *E. histolytica*, and *E. moshkovskii*. This supports the observation that *E. dispar* is perhaps 10 times more common than *E. histolytica* worldwide (Petri *et al*, 2000).

Previous studies have mainly focused on detecting *E. histolytica* in water samples. For example, in 2003, 32% of water samples collected from the Ankara River, Turkey, were positive for *E. histolytica* using a PCR-based assay (Bakir

et al, 2003). In Iran, using Gram staining of water samples, 0.7 and 2.3% were positive for *E. coli* and *E. histolytica* respectively (Yousefi *et al*, 2009); a later study using a PCR method confirmed 2% of surface water samples were positive for *E. histolytica* (Hemmati *et al*, 2015). In Egypt, using a PCR assay, 5.6% of irrigation water samples were positive for *E. histolytica* (Saleh *et al*, 2018). In Yemen, 22.2% of

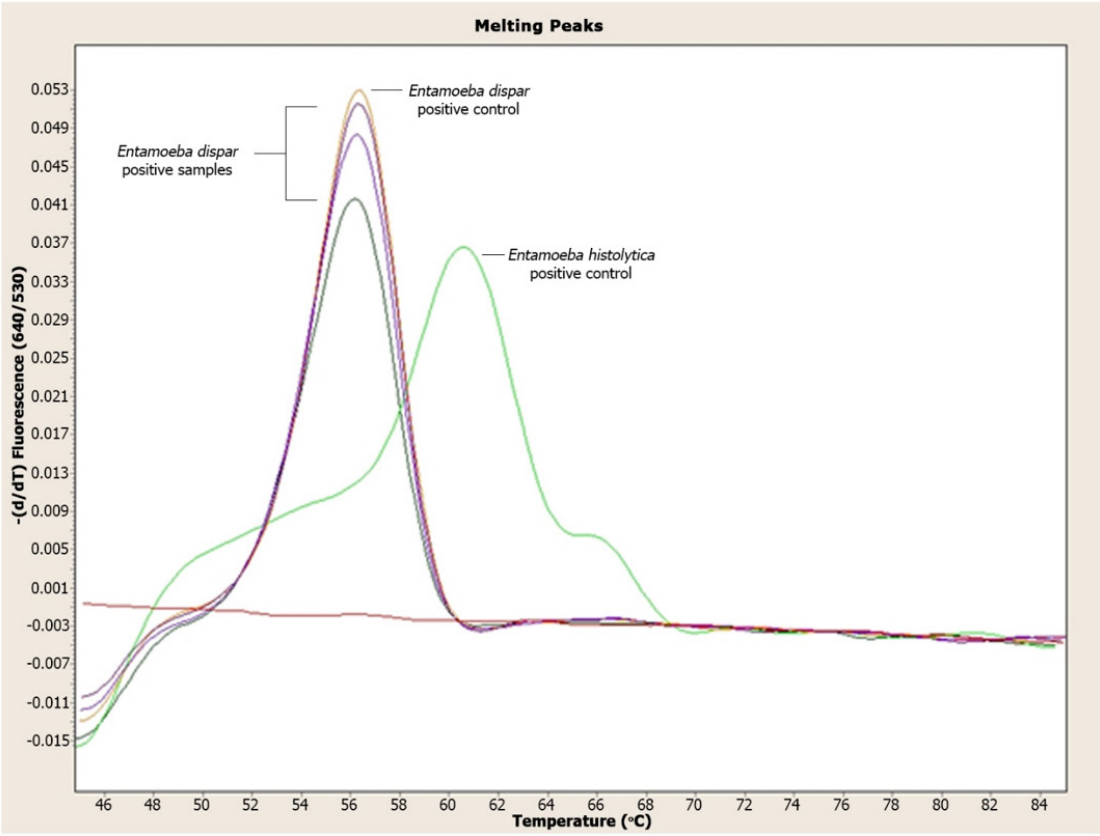

Fig 1 - DNA melting curves of *Entamoeba dispar* and *E. histolytica*

Note: The melting curves were generated using the LightCycler™ software (Roche Molecular Biochemicals, Mannheim, Germany).

°C: degrees Celsius

Table 1

DNA melting temperatures (T_ms) using multiplex real-time PCR of control *Entamoeba dispar* (SAW 60) and *E. dispar* samples (n = 20) from canals, Pathum Thani Province, Thailand (May 2022-July 2023)

Entamoeba spp	T_m (°C)
Control	
E. dispar (SAW 60)	56.4
E. dispar positive sample (n = 20)	
n = 1	55.6
n = 1	55.9
n = 2	56.0
n = 5	56.3
n = 1	56.5
n = 5	56.6
n = 2	56.9
n = 1	57.1
n = 1	57.2
n = 1	57.4

PCR: polymerase chain reaction; °C: degrees Celsius

wastewater samples were positive for *E. histolytica* (Al-Nihmi *et al*, 2020).

Epidemiological surveys have shown that most asymptomatic infections are caused by *E. dispar* (Oliveira *et al*, 2015). Although *E. dispar* is typically considered a commensal parasite of the human intestinal tract, only *E. histolytica* and *E. polecki* are regarded human pathogens. A previous study reported that patients infected with *E. dispar* in association with

E. moshkovskii showed intestinal symptoms (Parija and Khairnar, 2005). However, intestinal symptoms in *E. dispar*-infected patients have been reported in various regions, eg, Bangladesh, India, Brazil, and Italy (Ali *et al*, 2003; Khairnar and Parija, 2007; Oliveira *et al*, 2015), including a rare case of enteritis in Italy (Graffeo *et al*, 2014). Although *E. coli* was not found in our water samples, *E. coli* was detected in 1.6 and 2.7% of fresh vegetable samples in Tunisia and the United Arab Emirates respectively (El Bakri *et al*, 2020; M'Rad *et al*, 2020).

Entamoeba cysts remain alive outside the host for weeks or months, especially in damp conditions or in water (Tanyuksel and Petri, 2003; Chowdhury *et al*, 2022), but are rapidly destroyed at temperatures below -5 °C and above 45 °C (Kahn and Visscher, 1975). *E. dispar* is commonly found in feces released from septic tanks and improper wastewater treatments, particularly in developing countries (Ben Ayed *et al*, 2009). Unlike their cyst stage, trophozoites are quickly eliminated when outside the human body (Chowdhury *et al*, 2022).

Entamoeba spp are resistant to standard chlorine levels, while bromine is often a more effective disinfectant (Stringer *et al*, 1975). Ozone can be effective against *E. dispar* and *E. histolytica* (Newton and Jones, 1949). A more recent study demonstrated ozone is an effective disinfection for waterborne parasites (Hoseinzadeh *et al*, 2021). However, there is a lack of studies on *E. dispar* inactivation. Given the facile fecal-oral transmission of this parasite, it is important to develop effective methods to remove *E. dispar* from environmental water sources. In addition, there is a need for a more sensitive method than the currently used nested PCR assay to detect *E. coli* in water samples.

In conclusion, molecular detection revealed the presence of *E. dispar* in canal water samples from Thailand. *E. dispar* showed a high prevalence among the tested *Entamoeba* species. These

findings indicate a risk of infection through contaminated water or the consumption of contaminated vegetables in the communities. Further investigations and necessary precautions should be applied to prevent contamination.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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