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Molecular detection of *Entamoeba histolytica* and *Entamoeba dispar* infection among wild rats in Kuala Lumpur, Malaysia

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Abstract. *Entamoeba histolytica* infection is the third-greatest parasitic disease responsible for death in the world. Wild rats harbouring *E. histolytica* can be the possible reservoir hosts for human amoebiasis. There were numerous studies on prevalence of intestinal parasites among wild rats in Malaysia but none has reported *E. histolytica*. Rats were captured from Sentul and Chow Kit areas, Kuala Lumpur, Malaysia. The preserved stool samples were used for microscopy examination and molecular analysis. Out of 137 samples collected, 12 were positive for *E. histolytica / E. dispar / E. moshkovskii* microscopically. Two *E. histolytica* (1.4%), 1 *E. dispar* (0.7%) and 6 mixed infections of *E. histolytica* and *E. dispar* (4.3%) were detected using PCR. This is the first report of molecular detection of *E. histolytica/dispar* infection among wild rats in Malaysia. This study provides useful information about the potential risks of zoonotic agents and the importance of developing control measures to prevent zoonotic transmission.

INTRODUCTION

Rats have adapted to live in close association with human, using our agriculture and waste as their own food resources and our buildings are excellent rat homes. Rats are hosts of more than 60 known diseases. They are able to and play both direct and indirect roles in the transmission of human diseases (Ratzooman, 2010).

Entamoeba histolytica infection is the third-greatest parasitic disease responsible for death in the world after malaria and schistosomiasis (Voigt & Strobel, 1999). It affects approximately 180 million people, of whom 40,000 to 110,000 die each year (Pestehchian *et al.*, 2011). *Entamoeba histolytica* infections are worldwide, more common in the tropics and subtropics. Food and drink contaminated with faeces containing the cysts is a common source of infection. Most cases arise from human carriers, or cyst passers, who pass cysts in formed or semiformed stools. Natural infections with *E. histolytica* occur in monkeys, dogs, and possibly pigs, but these animals constitute, at the most, a minor source of human exposure compared with man himself (Beaver *et al.*, 1984).

The natural infection of wild rats with *E. histolytica* was first reported by Lynch (1915). Epstein & Awakian (1937) reported the discovery of wild rats harbouring *E. histolytica*. These naturally infected rats were restricted to a localized focus, in which cases of human amoebiasis were discovered, thus providing circumstantial evidence of the human origin of the infection, and throwing light on the possible role of rats as reservoir hosts (Neal, 1951).

After 1951, many studies have been done on the prevalence of parasites among wild rats throughout the world including Sudan, Taiwan, USA, Egypt, Thailand, Australia and Korea (Singleton *et al.*, 1991; Namue & Wongsawad, 1997; Easterbrook *et al.*, 2008;

Elshazly *et al.*, 2008; Fagir & El-Rayah el, 2009; Tung *et al.*, 2009), but *E. histolytica* was not detected. There were numerous studies on prevalence of intestinal parasites among wild rats in Malaysia but none has reported *E. histolytica* (Liat *et al.*, 1976; Liat *et al.*, 1977; Sinniah, 1979; Krishnasamy *et al.*, 1980; Paramasvaran *et al.*, 2009).

Entamoeba histolytica infections have primarily been diagnosed based on microscopic examinations of stool for cysts and trophozoites, but this technique is insensitive and cannot differentiate *E. histolytica* cyst from nonpathogenic species, such as *E. dispar* and *E. moshkovskii*, which are morphologically identical (Diamond & Clark, 1993; Ali *et al.*, 2003). Specific and sensitive means to detect *E. histolytica* in stool are now available and include antigen detection and the polymerase chain reaction (PCR) (Haque *et al.*, 2000).

The objective of this study was to establish distribution of *E. histolytica*, *E. dispar* and *E. moshkovskii* in the rats by molecular method. This is the first reported case of *E. histolytica* and *E. dispar* infection among rats in Kuala Lumpur, Malaysia.

MATERIALS AND METHODS

Ethics Statement

Ethics approval for the study was obtained from the University of Malaya Ethics Committee of Animal House (Pusat Haiwan Makmal) (approval no. PAR/20/09/2011/JI [R]). The study was conducted adhering to the Institute for Laboratory Animal Research's guidelines (USA) for animal husbandry.

Sample collection

Rats were captured from Sentul and Chow Kit areas, Kuala Lumpur, Malaysia by Kuala Lumpur City Hall workers. Stool samples were collected in screw-capped stool containers and preserved in 5% potassium dichromate to avoid fungal growth and stored in the cold room at 4ºC before being processed.

Molecular analysis

The preserved samples were used for microscopy examinations involving direct wet mount and concentration technique (Shafiyyah *et al.*, 2012). DNA was extracted from the positive stool samples by Mo Bio Power Soil DNA Isolation Kit (Mo Bio Laboratories California, USA) according to the procedure provided. After extraction, DNA was stored at -20ºC until PCR amplification. For molecular identification, nested polymerase chain reaction (PCR) targeted at small subunit ribosomal RNA (*ssu rRNA*) gene was used to differentiate the DNA of *E. histolytica*, *E. dispar* and *E. moshkovskii* following the procedure of Que & Reed (1991). In primary reaction, the genus specific primers used were E1 (5'-TAA GAT GCA GAG CGA AA-3') and E2 (5'-GTA CAA AGG GCA GGG ACG TA-3'). In secondary reaction the primers used were specific for detection of *E. histolytica* (EH1: 5'-AAG CAT TGT TTC TAG ATC TGA G-3 and EH2: 5'-AAG AGG TCT AAC CGA AAT TAG-3'), *E. dispar* (ED1: 5'-TCT AAT TTC GAT TAG AAC TCT-3' and ED2: 5'-TCC CTA CCT ATT AGA CAT AGC-3') and *E. moshkovskii* (MOS-1: 5'-GAA ACC AAG AGT TTC ACA AC-3' and MOS-2: 5'-CAA TAT AAG GCT TGG ATG AT-3'). All polymerase chain reactions were carried out in 50 µl reaction mixture containing 0.2 µg of DNA, 1.5 µM concentration of each primer, 1.5 mM $MgCl_2$, 0.1 µg of bovine serum albumin per µl, 200 µM concentration of each deoxynucleoside triphosphate (dNTP) and 1.5 U of taq DNA polymerase. PCRs were done in 40 cycles with denaturation (96°C, 1 min), annealing (56°C, 1 min in the primary reaction, and 48°C, 1 min in the secondary reaction) and extension (72°C, 1 min). After the last cycle, a primer extension was continued for 5 min at 72°C. Amplification products from all PCRs were analyzed by electrophoresis through a 1.5% agarose gel stained with SYBR® Safe DNA gel stain (Life Technologies, USA). PCR products were ligated to pGEM-T vector (Promega, USA) prior to sequencing. The cloned nucleotide sequences were confirmed by NHK Bioscience Solution Sdn Bhd

(Malaysia). Nucleotide sequence of the amplified fragment was analysed and compared with ssu rRNA sequences of *Entamoeba* species available in the GenBank. Multiple sequence alignment was carried out using ClustalW and phylogenetic analysis was conducted using the Maximum Parsimony method in the MEGA4 (Tamura *et al.*, 2007).

RESULTS

A total of 137 rats were captured from Sentul and Chow Kit areas, Kuala Lumpur, Malaysia by Kuala Lumpur City Hall workers. Five rat species were captured including 92 *Rattus rattus diardii* (67%), 14 *Rattus norvegicus* (10%), 14 *Rattus argentiventer* (10%), 12 *Rattus tiomanicus* (9%), and 5 *Rattus exulans* (4%). *E. histolytica/E. dispar/E. moshkovskii* cysts were detected in 12 rats microscopically. The cysts were detected in all rat species except *Rattus norvegicus* (Shafiyyah *et al.*, 2012). PCR detected 2 *E. histolytica* (1.4%), 1 *E. dispar* (0.7%) and 6 mixed infections of *E. histolytica* and *E. dispar* (4.3%) (Table 1). PCR products corresponded to the expected size of 437 bp and 153 bp for *E. histolytica* and *E. dispar*, respectively (Fig. 1) were sequenced and analysed phylogenetically. The two fragments are located at different region on the full length ssu rRNA. Fig. 2 is the phylogenetic tree based on ssu rRNA sequences which distinctly shows that *E. histolytica* (accession number: KC763012) and *E. dispar* found from the rats form a cluster with *E. histolytica* and *E. dispar* found in human and monkey, respectively.

DISCUSSION

A total of 12 samples were detected with cysts of *E. histolytica/E. dispar/E. moshkovskii* in the stool samples of *Rattus rattus diardii*, *Rattus argentiventer*, *Rattus tiomanicus*, and *Rattus exulans*. PCR further confirmed that 9 samples were that of *E. histolytica* and *E. dispar* infection. This is the first report of identifying infection of *E. histolytica* and *E. dispar* in rats. In addition, phylogenetic analysis based on sequencing of the *ssu rRNA* gene indicated that the *E. histolytica* and *E. dispar* isolated from the rats were closely related to *E. histolytica* and *E. dispar* discovered in human and monkey.

Rats are known to be a reservoir host of several protozoan parasites, not merely those normally found in rats, but others, like *Trichomonas hominis*, *Giardia lamblia*, *E. histolytica* and *Balantidium coli* (Farris, 1949). Abd el-Wahed *et al.* (1999) investigated 172 rats caught from various areas in Egypt. Among them, 40.7% were infected with protozoa (22.7% *Cryptosporidium parvum*, 20.3% *C. muris*, 8% *G. lamblia* and 12.8% *Entamoeba* cysts). No further study was conducted in determination of the species of *Entamoeba*. *Entamoeba histolytica* is the most pathogenic intestinal protozoa as it can cause amoebiasis with acute and chronic diarrhoea in immunocompromised as well as immunocompetent patients. Mature cyst of *E. histolytica* has 1-4 nuclei which can be easily distinguished from *E. muris*, the most common amoeba of rodents, which has eight nuclei. The microscopic examination of stool alone, however, fails to differentiate *E. histolytica* cyst from those of morphologically similar non-pathogenic species such as *E. dispar* and its infection does not justify treatment (Khairnar & Parija, 2007). On top of that identification of *E. histolytica* in stool sample by microscopy requires well-trained and competent microscopists. False-positive and false-negative results can be found by microscopy due to lack of expertise (Delialioglu *et al.*, 2004). Experts and experienced observers are needed in detecting the presence of *E. histolytica* in fecal sample. Therefore, in this study, microscopic examination of the fecal samples is used only for the initial screening. Molecular method was further used to confirm and differentiate the species. Three false positive samples were detected using microscopy technique and the genus specific primers were not able to detect any *Entamoeba* in any of these samples.

The ever increasing garbage collected and the burgeoning of the slums in big cities

Table 1. Prevalence of *E. histolytica* and *E. dispar* among wild rats in Kuala Lumpur by PCR

Rat species	Number (n)	*E. histolytica*	*E. dispar*
Rattus rattus diardii	1	+	–
Rattus rattus diardii	4	+	+
Rattus rattus diardii	1	–	+
Rattus rattus diardii	1	–	–
Rattus tiomanicus	1	+	+
Rattus tiomanicus	1	–	–
Rattus exulans	1	+	+
Rattus exulans	1	+	–
Rattus argentiventer	1	–	–

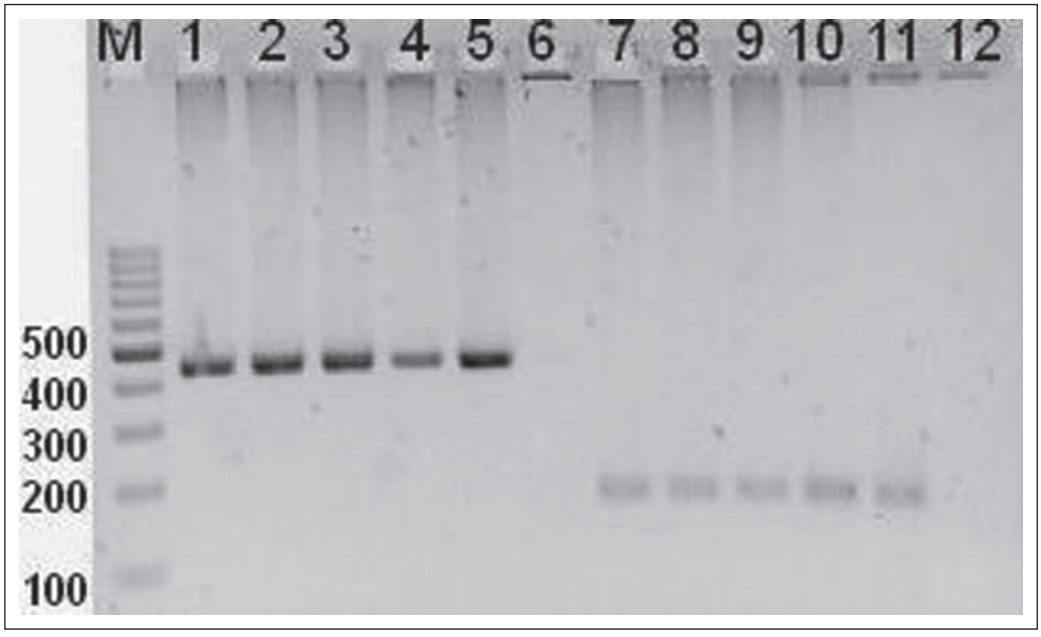

Figure 1. PCR detection of *E. histolytica* and *E. dispar*
Lanes 1-5 were loaded PCR product of *E. histolytica* specific primers. A band was observed in each lane with the expected size of 437 bp. Lanes 7-11 were loaded with PCR product of *E. dispar* specific primers with the expected size of 153 bp. Lanes 6 and 12 were loaded with negative control for *E. histolytica* and *E. dispar*, respectively.

contribute to the increasing prevalence of rats. Rats, being closely associated with human and harbour many different kinds of intestinal and blood parasites, are feared to present a serious risk to public health. This study shows that *E. histolytica* and *E. dispar* are prevalent in rats in urban city in Kuala Lumpur, Malaysia. Further studies on the biological properties of *E. histolytica* and *E. dispar* detected in rats will help explain the zoonotic potential of these genotypes. The control of domestic rats is of prime importance in the prevention of zoonotic infections in humans.

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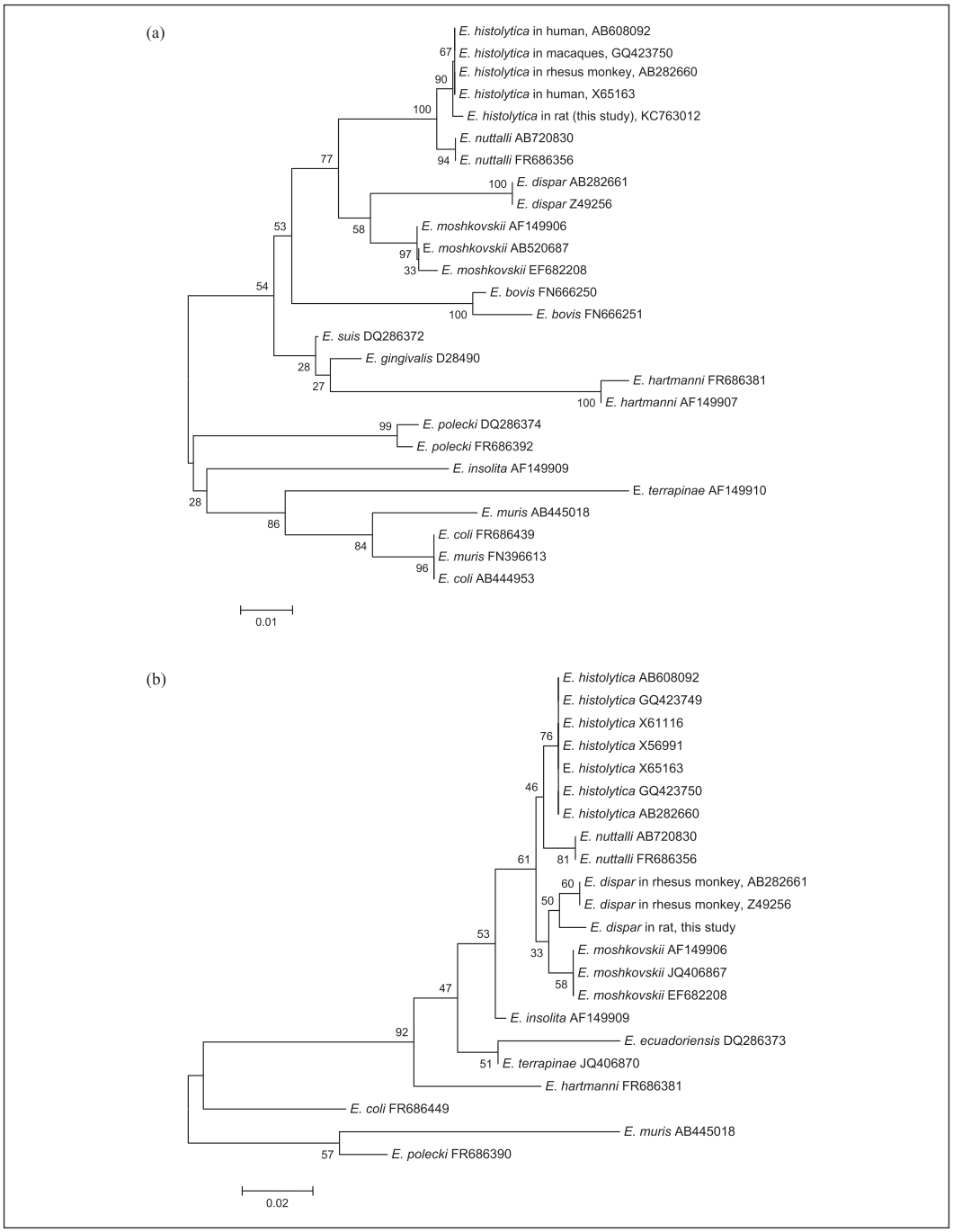

Figure 2. Phylogenetic tree based on ssu rRNA sequences of *E. histolytica* and *E. dispar*
(a) Phylogenetic tree showing *E. histolytica* found in this study (accession number: KC763012) being grouped with *E. histolytica* AB608092, GQ423750, AB282660 and X65163. The tree is based on ssu rRNA sequences (nucleotide positions 1057-1493) of *Entamoeba* spp., and is constructed using the Neighbour Joining method available in MEGA4 (Tamura *et al.*, 2007). The percentage of replicate trees in which the associated isolates cluster together in the bootstrap test (1000 replicates) are shown next to the branches. (b) Phylogenetic tree showing *E. dispar* found in this study being grouped with *E. dispar* AB282661 and Z49256. The tree is based on ssu rRNA sequences (nucleotide positions 1472-1624) of *Entamoeba* spp., and is constructed using the Neighbour Joining method available in MEGA4 (Tamura *et al.*, 2007). The percentage of replicate trees in which the associated isolates cluster together in the bootstrap test (1000 replicates) are shown next to the branches.

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