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Differential detection and phylogenic study of *Entamoeba spp* in Rats

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Abstract

Rats are found worldwide and live close to human inhabitation. Rats are reservoir hosts for human amoebiasis. The present study microscopic examination show that 14% of fecal samples in rats , the highest rate of infection (15.5%) in Alshaeb , whereas the lowest rate (12.5) was recorded in AbuGharib , the study showed no significant differences .The study recorded a significant differences ($p<0.01$) in percentage of infected rats during different months ,there was no significant differences recorded between infected males and females . The study revealed (16.4%) in males and (9.09%) in females of infection. The study recorded a total infection with *Entamoeba spp* (26%) by PCR method and result nested multiplex polymerase chain reaction using three primers ,that show *Entamoeba histolytica* (26%)only .This is the first report of molecular detection of *E. histolytica, E. dispar and E. moshkovskii* 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

Entamoebaisis is a free-living protozoan parasitic species that can infect a variety of vertebrate and invertebrate hosts (Matsubayashi *et al*., 2015; Kawano *et al*., 2017; Matsubayashi *et al.*, 2018). There are up to 24 species of Entamoeba described worldwide, but only several species such as *Entamoeba histolytica*, *E. coli, E. dispar*, *E. moshkovskii*, *E. hartmanni* and *E. polecki* reside in the human intestinal lumen (DiMiceli 2004; Ali, 2015).

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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; Al-Areeqi et al., 2017). *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 semi formed stools (Terraube, 2015). 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).

Rodents are the most frequent and important mammals on the Earth, because they can adapt themselves to the different locations and environmental changes. These animals live on almost every continent except Antarctica(Meerburg *et al.*,2009). Rodents are considered as reservoirs of various zoonotic diseases including *toxoplasmosis* , *babesiosis*, and *leishmaniasis* (Davami *et al*.,2014). Never the less, rodents cannot directly cause disease in humans and disease is mainly transmitted to humans if human is in contact with rodents' feces and secretory materials (Sharma *et al.*,2013). Transmission of the zoonotic pathogens to humans can occur via rodent's urine ,feces ,hair ,and saliva (Ratzooman, 2010).

E. moshkovskii, *E. histolytica* and *E. dispar* are morphologically in distinguishable; it is not possible to differentiate the three species on the basis of traditional microscopic examination. In the identification of *E. histolytica*, new approaches are used, based on detection of *E. histolytica* specific antigen and DNA in stool and other clinical samples Molecular diagnostic tests, including nested PCR, have been developed for the detection and differentiation of *E. histolytica*, *E.dispar*, and *E. moshkovskii* in clinical samples. (Haque *et al.*,2003).

Recently though, a multiplex nested PCR method targeting the 18S-like rRNA gene for the instant detection and differentiation of *E. histolytica*, *E. dispar* and *E. moshkovskii* directly in fecal samples has been developed by (Bahrami *et al.*,2019).

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Because of lack data about prevalence and differentiated between *Entamoeba* species in Iraq specially in rat the present work will contacted to cover the followings

Materials and method

One hundred black rats were trapped using cage trap baited with cheese, bread and date from four districts (Alsheab, Abu Gharib, Haifa street and Alamerya) in Baghdad city during the same period , the rats trapped were trapped at night from different places such as old buildings and garbage. Trapped rat were immediately transferred to the laboratory of parasitology in college of veterinary /Baghdad university, thereafter ,the rats were caught from tail injected with 0.1 ml of the anesthesia (9:1 ketamin and xylazine) per 100gm/Bw as described by (struck et al., 2011), the rats were handled according to the standardized international animals care and use. Rats were dissected according to the protocol previously described. Briefly, animals were dorsoventrally placed in a dissecting tray, limbs fixed with dissecting pins and the skin incised, pinched and raised with dissecting forceps, while scissors were used to extend the cut from the posterior through to anterior regions, exposing the diaphragm. The diaphragm was later slit through the midline from throat to anus thereby, after that , the small and large intestine were removed, their intestines longitudinally cut the fecal content were taken from different location (Al-Bajalan,2018).

Fecal samples will collected in screw- capped stool containers and preserved in 70% alcohol and stored in the cold room at 4c before being processed, the information about (sex, area, month of the study) were record.

molecular study include

DNA Extraction

The kits were used throughout the study for genomic DNA extraction from stool samples. These are Presto™ Stool DNA Extraction Kit according to the manufacturers instruction. The DNA were stored at -20 C until PCR amplification. For molecular identification , nested multiplex PCR targeted (18S rRNA gene) was used to differentiate

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the DNA of *E.histolytica, E. dispar and E.moshkovskii* (Bahrami et al.,2019). In primary reaction, the genus specific primers used were E-1 (5′-TAAGATGCACGAGAGCGAAA-3′) and E-2 (5′-GTA CAAAGGGCAGGGACGTA-3′), was used to amplify about 900 bp of 18S rRNA gene. In secondary reaction of nested multiplex PCR, three pairs of primers: EH-1 (5′-AAGCATTGTTTCTAGATCTGAG-3′) and EH-2 (5′-AAGAGGTCTAACCGAAATTAG-3′); Mos-1 (5′-GAAACC AAGAGTTTCACAAC-3′) and Mos-2 (5′CAATATAAGGC TTGGATGAT-3′); and ED-1 (5′-TCTAATTTCGATTAGAAC TCT-3′) and ED-2 (5′-TCCCTACCTATTAGACATAGC-3′). the reaction conditions were optimized for amplifying species-specific product sizes (439, 553 and 174 bp for *E. histolytica, E. moshkovskii and E. dispar,* respectively).

The first PCR reaction was performed in a final volume of 20nl contain 5nl DNA template and 1nl of Forward primers(10pmol) , 1nl of reveres primers (10pmol) and 13nl PCR water . the reaction were performed in an automatic DNA thermo cycler (THECHNER,USA) for 35 cycles .Each cycle consisted of a denaturing step of 30 sec . at 95 c, and annealing step of 30 sec. at 58 c and 1 min of extension step at 72 c with the final extension step of 72c for 10 min .

The second PCR reaction was performed in a final volume of 20 µl, contain 2.5 µl of the first PCR product , 1 µl for each primer and 11.5 µl PCR water . The reaction conditions for the second PCR were optimised to combine the primers of E. histolytica (EH-1 and EH-2) with E. dispar (ED-1 and ED-2) and E. moshkovskii (Mos-1 and Mos-2) primers in a single reaction mixture under the same conditions.the PCR products were electrophoresed on 1% Agarose gel stained ethidium bromide and visualized by UV transilluminator .

Sequencing analysis of *Entamoeba spp*

DNA sequencing analysis was performed for confirmative detection of local *Entamoeba spp* and study of phylogenetic relationship tree analysis between local *Entamoeba spp* isolates and NCBI –Blast submission of *Entamoeba spp* as well as submission of local isolates in NCBI-GenBank . thirty PCR positive products of local

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Entamoeba spp isolates were sent to Bioneer Company in Korea in ice bag for performed the DNA sequencing by Applied Biosystem (AB) DNA sequencing system.The NCBI-GenBank submission was carried out using Bankit submission tool.

Statistical Analysis:

The Statistical Analysis System- SAS (2012) program was used to detect the effect of difference factors in study percentage. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study.

Results

The results of the microscopic examination of 100 fecal samples from rats showed 14(14 %) was infected with *Entamoeba spp* , The present study also showed that the rate of infection with *Entamobea spp in Rats* was higher in males (16.4) than females (9.09) without significant differences Table(1). The study revealed that nearly same rate showed the presence of infection with *Entamoeba spp*. They includes 19 samples from Al-sheab 3 (15.7) samples were infected, 32 samples from Abu Ghraib 4 (12.5%) were infected, 21 samples from Haifa street 3(14.2%) infected and 28 samples from Alamerya 4 (14.2) . The total number of infected cases were 14 (14%) The infection was demonstrated along the months of the study with variable rates significantly($p<0.05$) , the highest rates of Entamoebiasis was 15.38% seen in August , while the lowest rate was 11.11% in February month. Whereas 26 of 100 (26%) rat were positive by PCR method (Fig 1), From 26 samples were positive by PCR examined by Nested multiplex PCR this study recorded total proportion of E. histolytica infection estimated (26%) only (Fig. 2)

Table (1): Infected rate with *Entamoeba spp* in rat according to sex by microscopic examination

Sex	**Number of examined rats**	**Number of positive cases**	**%**	***P* values**
Males	67	11	16.4	0.0682 NS
Females	33	3	9.09	
Total	100	14	14	
NS: Non-Significant.				

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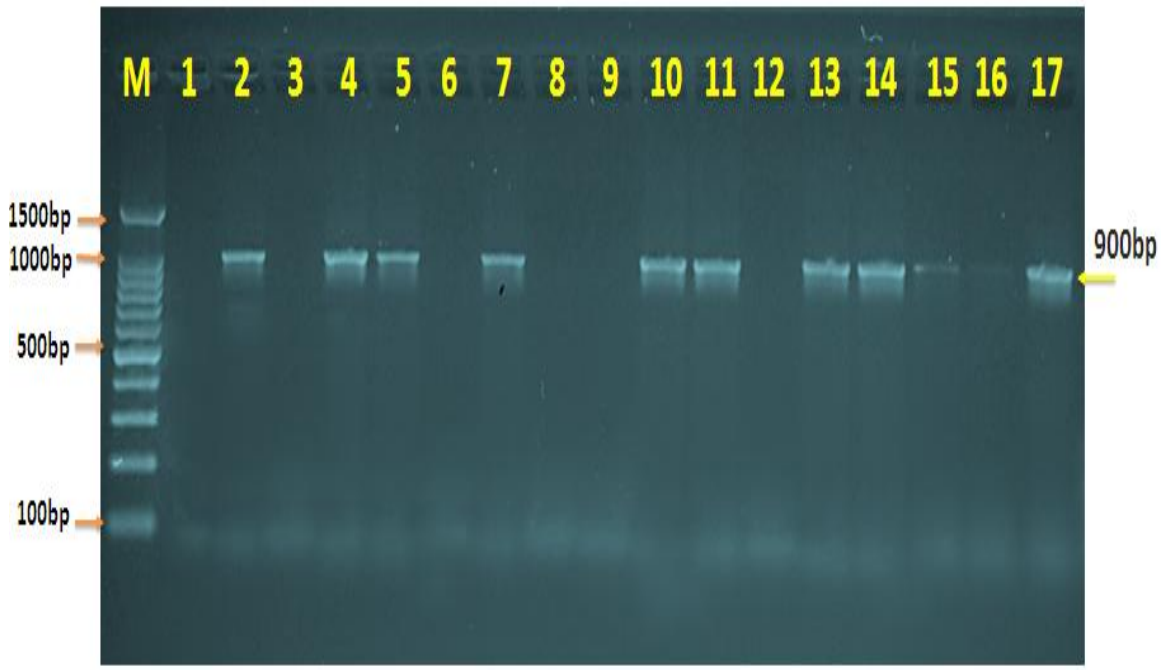

Figure (1): Agarose gel electrophoresis image that showed the PCR product analysis of 18S rRNA gene in Entamoeba sp. from Rats feces samples. Where , the Lane (M): DNA marker ladder (1500-100bp) and the Lane (1-17) were showed some positive PCR amplification of 18S rRNA gene in Entamoeba sp. at 900bp PCR product size.

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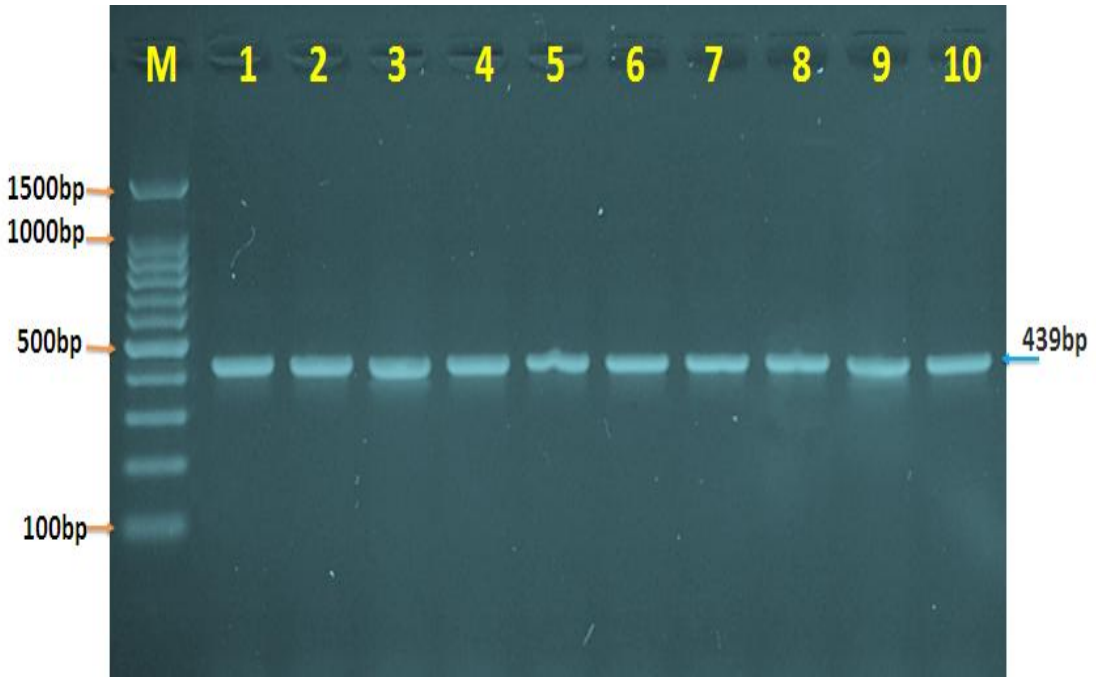

Figure (2): Agarose gel electrophoresis image that showed the Multiplex Nested PCR product analysis of 18S rRNA gene in Entamoeba species from Rat positive samples. Where , the Lane (M): DNA marker ladder (1500-100bp) and the Lane (1-10) were showed positive Entamoeba histolytica at 439bp PCR product size.

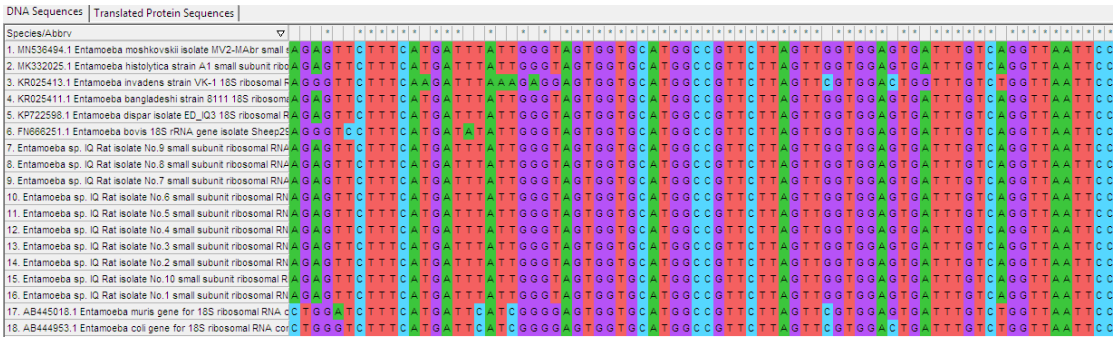

Figure (1): Multiple sequence alignment analysis of small subunit ribosomal RNA gene in local Entamoeba sp. IQ Rat isolates and NCBI-Genbank Entamoeba species isolates. The multiple alignment analysis was constructed using ClustalW alignment tool in (MEGA X version). That alignment analysis was showed the nucleotide alignment similarity as (*) and substitution mutations in small subunit ribosomal RNA gene between different Entamoeba species.

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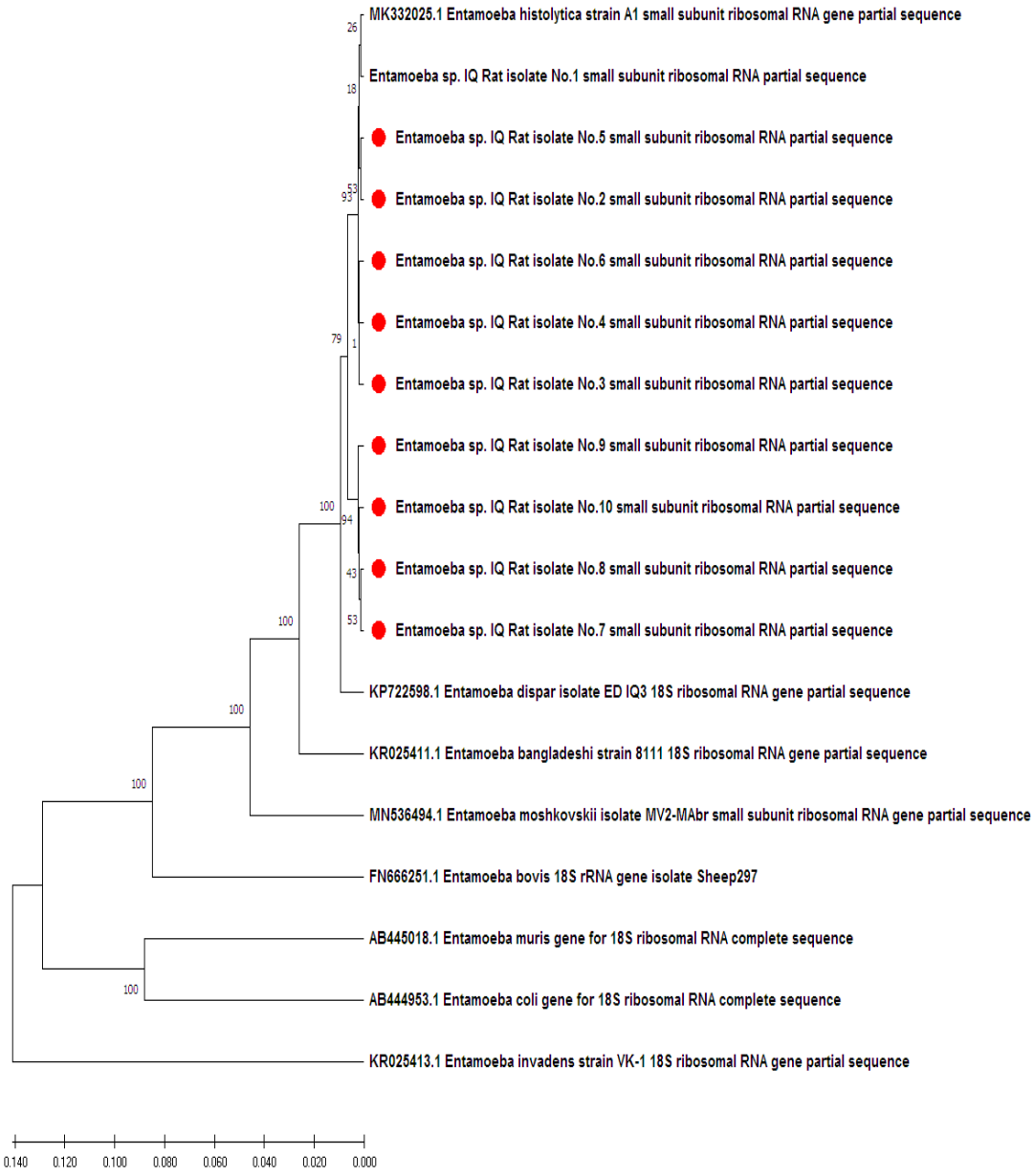

Figure (2): Phylogenetic tree analysis based on small subunit ribosomal RNA gene partial sequence in local Entamoeba sp. Rat isolates that used for genetic Entamoeba species identification. The phylogenetic tree was constructed using Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local Entamoeba isolates (No.1 into No.10) were showed closed related to NCBI-BLAST Entamoeba histolytica (MK332025.1). Whereas, other NCBI Entamoeba species were showed different at total genetic changes (0.140-0.020%)

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Discussion

Rats play an important role as reservoir host for zoonotic disease .The present study showed that ,the infection rate with *Entamoeba spp* in rat was relatively 14%(14/100) by microscopic examination , This results agreed with the results of Amin (2019) who recorded 9.1% infection rate in *Entamoeba spp* in Kurdistan region , in addition the results agreed with Tijjani et al.,(2020) in Malaysia and Al-Bashan.,(2012) in Saudi Arabic , who recorded that the infection rates were 17.9 %, 10,9, respectively with Entamoeba spp in Rat. However this results disagreed with results of Seifollahi et al.(2016) they recorded infection rate 21.2% in Iran . The ever increasing garbage collected and the burgeoning of the slums in big cities contribute to the increasing prevalence of rats. Rats, being closely associated with human and harbor many different kinds of intestinal and blood parasites, are feared to present a serious risk to public health. In our study mle rats were highly infected with Entamoebaisis than females .These finding were also in agreement with some researches (Tijjani et al.,2020; Amin.,2019; Chaisiri et al., 2010 and Majeed., 2016 who reported that male rats were significantly more infected with parasites than female in Malaysia , Kurdistan region, Thailand and Baghdad respectively. mentions that female mammals in general are more resistant to parasitic infections than male due to the gender associated differences in exposure and immunosuppressive properties of testosterone.

This result related to an effect of some environmental conditions like humidity and temperature , which are considered a success factor for increasing resistant of cysts .The highest infection rate was recorded in December , as well as reports that indicate that during cold seasons and *Entamoeba spp* cysts are infective for longer period than during seasons(Al-Azawi ,2009) . The result of first round for humans DNA samples showed 26 (26%) positive among 100 DNA samples, This study was disagree with Lau et al.,(2014) in Malaysia found the infection rate 6.5% (9/137). The ever increasing garbage collected and the burgeoning of the slums in big cities 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. The result of second round nested multiplex PCR show three species of *Entamoeba histolytica*

was recorded at 26(26) only. This study was disagree with Lau et al.,(2014) in Malaysia found the infection rate 2 Entamoeba histolytica (1.4%), 1 Entamoeba dispar (0.7) and 6 mixed infections of Entamoeba histolytica and Entamoeba dispar (4.3%) were detected using PCR.

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