ORIGINAL ARTICLE

Entamoeba histolytica, Identification In Asymptomatic Infection

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ABSTRACT

Background: Reliable detection the etiological agent of amoebic dysentery and extra-intestinal amoebiasis have Public health importance specially in asymptomatic human and animals, Since the acquisition of pet dogs in the recent period has become widespread in our city.
Aim: To give correct perception of infection rate in asymptomatic individuals (human and domestic dogs) for the first aspect and about detection and diagnosis of the pathogenic species of *Entamoeba histolytica* from another morphologically similar and commensal one using the molecular technique in stool samples of asymptomatic individuals the second aspect.
Methods: During the study period from the beginning of September 2020 to the end of February 2021, a total of 95 stool sample was collected from asymptomatic 71 male and 24 female ages between 20-45 years old also 50 fecal samples of asymptomatic domestic dogs (male) belongs to some patients regardless of the breed were in counter.
Results: Microscopic and molecular diagnosis for *E.histolytica* was done using traditional wet mount method and Real Time polymerase chain reaction (RT-PCR) employing phospholipase gene respectively. The result highlighted the microscopic diagnosis of cyst stage in 33 asymptomatic patient out of 95 (34.73%) with significant differences $P<0.05$ between males 22/71(66.66%) and females 11/24 (33.33%). For domestic dogs, 9 out of 50 samples (18%) were detected as positive. On the other hand the molecular diagnosis results showed presence of *E.histolytica* in 10 sample out of 33 (30.3%) with non significant differences $P>0.05$ between males 7/22 (31.81%) and females 3/11 (27.27%) while for domestic dogs no positive results were recorded.
Conclusion: The presence of the pathogen *E.histolytica* in asymptomatic patients. The microscopic examination of stool sample show a possibility of error to differentiate pathogenic *E. histolytica* from those of morphologically similar non-pathogenic species lead to inaccurate results therefore, the molecular methods must be adopted for diagnosis. Domestic dogs do not pose a risk of transmitting *E.histolytica* infection but it can transmit other types of pathogens.
Key words: *Entamoeba histolytica*, Microscopic diagnosis, RT-PCR, Asymptomatic, Domestic dogs.

INTRODUCTION

The human intestinal lumen reside six species of the genus *Entamoeba* including: *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba coli*, *Entamoeba hartmanni*, and *Entamoeba polecki*[1]. The only species associated with pathological infections, found throughout the world and mainly common in the tropics is *Entamoeba histolytica* that cause amoebiasis ,is a parasitic infection caused by unicellular protozoa[2], major cause of morbidity and mortality in tropical and sub-tropical countries, also considered the third cause of death worldwide after Malaria and Schistosomiasis as the parasite dwelling the intestine or other organs such as liver, lungs, or skin of human[3,4].

Dogs are among the most common domesticated pets, regardless of their species, genders and ages. Studies have indicated that dogs are considered reservoirs, carriers and transmitters of several pathogens to human or to other animals thus are of health importance in transmitting infection to humans through direct or indirect contact[5,6].

Infection with *E.histolytica* classified into three categories: asymptomatic or non-invasive disease, invasive disease and Extra-intestinal disease. Although asymptomatic infection can be defined as the presence of protozoa in stool without colitis or extraintestinal infection, the health importance of Individuals who are carriers is shown in two ways: the first ,they consider to be source of infection as these organisms pass the infective stage (cyst) in stool which may be contaminate water or food additionally, if these Individuals left untreated, amebic dysentery and a wide range of other invasive diseases can occur [(7)] the most common manifestation of *E. histolytica* based on the microscopic examination of fecal samples as cysts are usually detected while trophozoites are rarely seen[8,9]. Individuals harboring *E. histolytica* (asymptomatic carriers) can develop antibody titers in the absence of invasive disease[10]. Asymptomatic colonization with *E. histolytica* more often resolves spontaneously without the development of diseases[7,11].

Diagnosis done by fecal examination using light microscope (40X) to observe trophozoites or cysts stage of life cycle, stains are often used to increase their visibility, also biopsies of the colon obtained by colonoscopy conceder to be another method[1]. Because of the great morphologically similarity with other non-pathological species like *Entamoeba dispar, E. moshkovskii*, it was necessary to use alternative technique for differentiation[12].

MATERIALS AND METHODS

Area of the study: This study was conducted during the time period of the month September 2020 to the end of February**/**2021 on patients who attended health care

centers and on domestic dogs belong to some patients at Al-Karkh sector of Baghdad city.

Collection of stool samples: Human samples: a total of **95** stool sample (10 g) was collected in sterile cups from adult (20-45years old) patients (24female and 71male) who do not show clinical signs for acute dysentery and make sure they do not take any antibiotic.

Dog samples: On the other hand, 50 fresh fecal sample (10g) were collected in sterile cups from adult domestic male dogs regardless of the breed by owners with recording information about the sample, such as the date, type of food for the dog and whether or not he is allowed to leave the house for a period of time .Laboratory tests were performed to detect the cystic stage of *E.histolytica*.

Macroscopic and microscopic examination: All samples were subjected to macroscopic examination including color, texture, presence of blood and mucous material. The diagnosis was made in a fecal sample by observing of *E. histolytica* cysts under microscope (40X) by using a direct saline (wet) mount method[13] and for dogs samples the concentration technique (Zinc sulfate flotation) for recovery and identification cysts and the use of iodine solution for facilitate detection [14, 15, 16]. Parasite was identified on the basis of cysts measurement, shape and contents[17].

Molecular analysis:

DNA extraction: All microscopy positive stool samples (n = 33for human and 9 for dogs) underwent direct DNA extraction using the QIAamp DNA stool minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol [18].

Real-Time PCR amplification: PCR were performed by employing phospholipase gene. Under aseptic conditions in laminar air flow**,** the amplification of the phospholipase gene(420bp) Was done using Forward primer (TGCTGATTTGGCTCTTGGGA)
and reverse primer (CCAAGCCCTCTTTCCCCAAA) with Use of the KAPA SYBR FAST qPCR Master Mix (2X)

Table 1: Required volume of each component for RT-PCR

Component	20 µL (Final volume)	Final concentration
KAPA SYBR FAST qPCR Master Mix (2X) Universal	10 µL	2x
Forward primer	0.4 µL	0.2µM
Reverse primer	0.4 µL	0.2µM
Nuclease-free water	Up to 10 µL	
Template DNA Sample Volume	-	1pg-100ng

Statistical Analysis: SPSS version 21 for windows, was used for data entry and data analysis. www.SPSS.com. Using chi_squre _test to compare between variables with p-value

Table 2: Cycling conditions of RT-PCR

	Enzyme activation	Cycling Stage			Final extension
		Denaturation	Annealing	Extension	
Temperature	95 ºC	95 ºC	56 ºC	72 ºC	72 ºC
Time	5 min	20 sec	20 sec	20 sec	2min
No Cycles	Hold	40 Cycles			1

RESULTS AND DISCUSSION

During the period of the study, microscopic diagnosis revealed that 33 asymptomatic patients out of 95 showed positive presence for cysts stage in stool samples including 22 out of 71 for male and 11 out of 24 for female with total infection rate 34.73%. The higher infection rate was recorded for male 66.66% and 33.33% for female. Significant differences were observed. For domestic dogs, only 9 samples out of 50 showed the presence of cyst stage as positive. (table 3) (Figure 1)

Table 3: Infection rate for *E.histolytica* in patient and dogs microscopically

Patients	Total	Positive	%age	P-value
Male	71	22	66.66	0.0131
Female	24	11	33.33	P<0.05
Total	95	33	34.73	S
Dog	50	9	18%	

S: significant

All samples recorded as positive result of the microscopy diagnosis (33 sample for human and 9 for dogs) were subjected to molecular diagnosis method RT-PCR . Amplification plots of positive samples for phospholipase gene (420bp) demonstrated in Figue 2. Only 10 samples are infected with *E.histolytica* including 7 out of 22 for asymptomatic male with infection rate 31.81% and 3 out of 11 for female with rate of 27.27%. Non significant differences were observed (table 4, 5, figure 2). No positive results were observed for dogs samples.

Figure 1: Show cyst stage (10 to 16 µm) of *E.histolytica* in stool sample

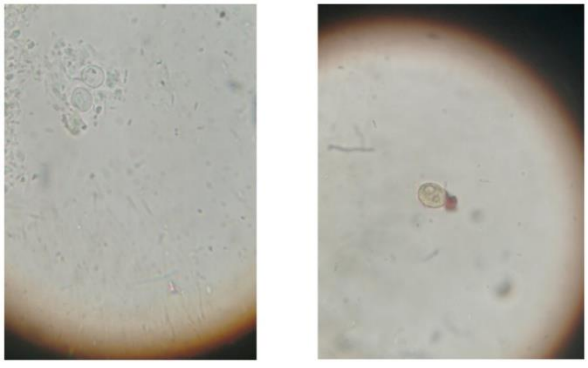

Table 4: Infection rate for *E.histolytica* in patients and domestic dogs diagnosed by RT-PCR

Diagnosis	Microscopic	RT-PCR	Rate %	P-value
Male	22	7	31.81	0.199
Female	11	3	27.27	P>0.05
Total	33	10	30.3	NS
Dog	9	-	-	

NS: non significant

Figure 3: Amplification of the target gene during the PCR cycles

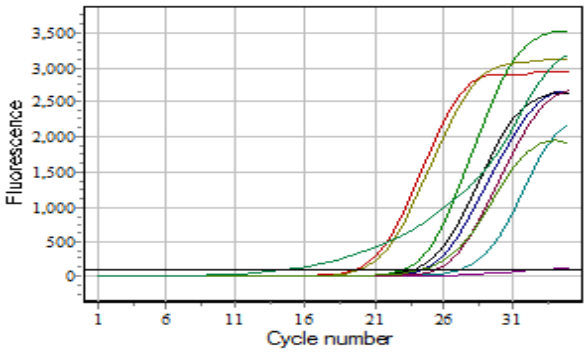

Table 5: Detection of *E.histolytica* by RT-PCR

No of hole	Identificator of the tube	Ct, Fam
A1	Sample_1	19.3
A2	Sample_2	23.0
A3	Sample_3	24.2
A4	Sample_4	19.8
A5	Sample_5	27.0
A6	Sample_6	33.1
A7	Sample_7	23.5
A8	Sample_8	25.3
A9	Sample_9	24.2
A10	Sample_10	14.4

The concept of asymptomatic infection is the presence of *E.histolytica* in stool sample with absence of clinical signs of colitis or extra intestinal infection. The current study included this class of asymptomatic individual. As documented in studies, *E.histolytica* has public health importance in terms of being the causative agent of human amoebiasis and responsible for the morbidity and mortality in development countries[19,20]. The results of current study showed higher incidence rate in asymptomatic patients diagnosed by RT-PCR compared to a study done in Erbil City, northern Iraq which showed infection rate 6%[21]. Another study done in Al- Dewaniyah city revealed that the infection rate was 10.78[22]. In Egypt, the results of a study indicated that 21% was the infection rate[23]. Mail, et al. 2011 recorded that rate of infection for *E.histolytica* was 17.1%[24]. Another study in Kenya revealed 21% the prevalence rate of asymptomatic nature[25]. Differences in infection rate can be explained according to the crowded population index, the economic and social conditions and health behavior of the general population, in addition to personal hygiene, level of immunity and the availability of safe drinking water[26].

According to the gender, non significant differences were observed in this study. Males showed the higher prevalence rate due to the physiological and hormonal nature of man, the nature of work outside the home and some practices such as swimming and eating from street vendors. On the other hand, higher levels of natural killer T cell and gamma interferon in females might increase their resistance to liver abscess[27,28]. Some studies in Turkey and in Japan have shown similarity in results [(29)] while opposite recorded by Malaysian studies[30,31]. Generally the reasons that play a role in that some persons have asymptomatic disease while others are symptomatic are depend on the strain virulence of causative agent, environment and the host's genetic susceptibility, level of immunity, interaction between the normal intestinal flora and pathogen, gender and age[32,33].

Recently, in our city the phenomenon of keeping dogs in homes has spread largely for protection or other purposes such as trading in dogs or entertainment and since dogs play a major role as host or reservoir for many zoonotic parasites and because of direct contact between humans and dogs, the risk of transmitting of zoonotic diseases to human increases[34,35,36].

This study indicated that the presence of the parasite was not diagnosed in the fifty samples that were examined for domestic dogs by means of molecular diagnosis. The reason for these results may be due to the care of dog owners, the maintenance of medical examination appointments, vaccinations and medical drugs and quality of the food provided to the dog. On the other hand, epidemiological studies have shown that the geographical location, season, and breeding methods are all important factors for the infection of the dog with parasites[37,38]. Similarity recorded to what was mentioned in a study conducted on 3099 dogs in Brazil to find out the types of intestinal parasites present in their droppings .the total infection rate was 20.5% and 16.1% infected with more than one type with no evidence of any infection of *E.histolytica*[39]. Also our results were similar to what was recorded in Ethiopia when 340 samples of domestic dogs were examined[40]. A study in Australia showed that there was a positive result for only one sample out of a total of 300[41]. The epidemiology of *E. histolytica*, remains uncertain, because most of the recorded data were obtained using convention methods which are incapable of distinguishing among the morphologically identical species like *E. dispar*, and *E. moshkovskii*. Most people infected with *E. histolytica/E. dispar* carrying *E. histolytica*[(42)].

Determining the prevalence of infection depends on the accuracy of the diagnosis. Based on the results of this study we note that there is a difference in the rate of infection using microscopic diagnosis and molecular method.

In northern Ghana 39.8% was the *E. histolytica/E. dispar* complex diagnosed by microscope, while, the actual prevalence of *E.histolytica* was low using PCR method. Other studies recorded differences in the detection method of *E.histolytic*a through PCR compared with microscope[43].

CONCLUSION

The present study revealed three main findings. First, *E.histolytica* was detected in asymptomatic and their important role in transmitting the infection .Second, Although domestic dogs did not report any positive infection with *E.histolytica*, but they were carriers of other types of pathogenic parasites such as *Giardia SP.* and *Cryptosporidium SP*. , which requires an extensive study on the types of parasites that may be found in domestic dogs. Third, in terms of the methods used for diagnosis, molecular methods are the best compared to traditional methods as Microscopic examination incompatible in the diagnosis of *E.histolytica* cyst stage from other similar morphological species in stool sample

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