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***Entamoeba* spp. occurrence in domesticated dogs determine by traditional and molecular methods in Kerbala Iraq**

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Abstract

Entamoeba a parasitic protozoan species infects a variety of mammals, including dogs. This study's goal is to use microscopic and genetic (PCR) analysis to ascertain the prevalence of *Entamoeba* species in dogs and the impact of age, sex, and month on disease incidence. The results of present study. In the current investigation, *Entamoeba* spp. were found and identified in domestic dogs in the Iraqi province of Karbala using conventional methods. This was verified by molecular diagnostics using the polymerase chain reaction (PCR) technique with primer 18s (900bp). This study was carried out between early January and June of 2024. 150 samples of dog feces were collected from adults and puppies of both sexes. The results of microscopic examination of the morphological appearance and of cyst showed *Entamoeba* spp by using iodine stain, the cyst has four nuclei and appears round to oval in form, encircled by a thin membrane. When using conventional techniques, the overall infection rate of *Entamoeba* spp. in dogs was recorded at 42% (63/150). Young dogs had a higher infection rate of 48% (36/75) than adult dogs, who had a rate of 36% (27/75). The sex-specific infection rate was higher in females than in males, at 44% (33/75) and 40% (30/75), respectively. Infection rates varied throughout the study period, with the lowest incidence of 28% (6/25) in February and the highest rate of 60% (15/25) in April. PCR overall infection rates in dogs in DNA samples, which were randomly selected from 150 dog fecal samples in Karbala city, revealed 52% (52/100). In conclusion despite the conventional use of microscopic analysis for diagnosing *Entamoeba* species, there is may be of misdiagnosis, and molecular diagnostics is considered an accurate method, with young dogs and females more affected.

Keywords: *Entamoeba* spp, Kerbala Iraq, domesticated dogs, molecular methods, techniques

Introduction

Dogs are linked to several parasitic zoonotic pathogens, despite their significant contribution to human existence, according to Ceylan *et al.* (2024) [5], helminths and gastrointestinal protozoa are the most significant of them. Worldwide, humans and other primates are afflicted by the protozoan parasite *Entamoeba histolytica*. It is a prevalent aquatic pathogen with an oral fecal transmission cycle in endemic places (Alam *et al.*, 2015) [2]. Amoebiasis is an intestinal protozoan disease that is caused by a variety of species of the genus *Entamoeba*, such as *E. histolytica*, *E. poleki*, *E. moshkovskii*, *E. coli*, *E. dispar*, and *E. hartmanni* (Alam *et al.*, 2015; Russul, and Noor, 2023) [2, 17]. Many different types of hosts are infected by different species *Entamoeba*. *Entamoeba histolytica* is the pathogen that causes amoebiasis in humans. Additionally, reports of *Entamoeba* species in canines have surfaced from all over the world, including Iraq (Nguia *et al.*, 2020; Fadhil *et al.*, 2021) [14, 8]. *Entamoeba* is a parasitic protozoan that can infect a variety of vertebrates and invertebrates host. (Matsubayashi *et al.*, 2015; Kawano *et al.*, 2017) [13, 12]. Infections with *Entamoeba* can cause either benign intestine colonization or colonic wall invasion that harms other organs such the brain, liver, and lungs (Petri *et al.*, 2000) [16]. Amoebiasis has been regarded as one of the main public health issues in many nations. It is brought on by the genus *Entamoeba*, which has a number of species, including *E. dispar*, *E. moshkovskii*, and *E. histolytica*.

The absence of sanitary measures and safe drinking water supplies is one of the primary causes of this disease's widespread prevalence in poor nations. Furthermore, the prevalence of parasitic disease rises in these nations due to their disdain (Watanabe and Petri, 2015) ^[22]. All dogs, including owned and stray, contribute to that transmission, even if it's unclear exactly what each population means (Eguia-Aguilar *et al.*, 2005) ^[7]. Due to the fact that dogs are a common companion among farmers and that intimate contact with them is a part of their everyday lives, the companion animals may operate as a mechanical means of spreading the *Entamoeba* infection. Cysts may, for instance, appear on the animal's hair after intimate contact with an infected individual or from the polluted surroundings, where they can spread to other people. Alternatively, the excrement of the diseased animals may pollute the water when they come into touch with the well or riverbank while drinking. There is an urgent need to carry out a more thorough investigation to examine the hypothesis, particularly in rural communities where people frequently have a close relationship with these companion animals, given the increased risks to human health and the potential zoonotic transmission of parasitic diseases (Nguia *et al.*, 2020) ^[14]. Dogs of all kinds, sexes, and ages are among the most popular domesticated pets. According to studies, dogs are important for human health since they may spread infections to people through direct or indirect contact because they are reservoirs, carriers, and transmitters of many diseases to humans or other animals (Gracenea *et al.*, 2009; Adesoye *et al.*, 2024) ^[9, 1]. Numerous studies about *Entamoeba* species in Domestic dogs were conducted in Iraq (Russul, and Noor, 2023) ^[17]. This study aims to estimate the prevalence of *Entamoeba* species in domesticated dogs in Kerbala provenance, Iraq. By using microscopic and molecular examination.

Materials and Methods

Samples collection: The current study was conducted in 2024 from January to June. In various areas of Kerbala, from Domestic dogs of various ages and genders taken 150 fecal samples (10 g). Laboratory tests were carried out to identify the cystic stage while documenting details about the sample, including age, sex date, dog food type, and whether or not the dog is permitted to leave the house for a certain amount of time. The samples were stored in 5 ml tubes with 70% ethanol as a preservative and 2% potassium dichromate for the purpose of detecting *Entamoeba* species (Fadhil *et al.*, 2021) ^[8]. The samples were brought to the Parasitological Laboratory at the College of Veterinary Medicine at the University of Kerbala. Every fecal sample was divided into two parts, one of which was kept at -20°C for molecular identification (PCR) and the other was utilized for direct microscopic examination using iodine staining. A droplet of the specimen was positioned on a glass slide and the coverslip, and *Entamoeba* cysts observed under a 40X microscope using a direct saline or iodine staining (wet) mount method. All samples were analyzed macroscopically, including color, texture, presence of blood, and mucous material (Coles, 1986; WHO, 1991; Samie *et al.*, 2014) ^[6, 23, 18]. Parasites were identified depended on the shape, size and contents of the cysts (Zajac and Conboy, 2012) ^[24].

Extraction of DNA

The whole genomic DNA of 100 fecal specimens was extracted using a commercial DNA extraction kit (ADDBIO, South Korea) in accordance with the manufacturer's

instructions. To lyse around 50 mg of fecal material, 200 µl of lysis buffer was mixed with 20 µl of proteinase k (20 mg/ml). Following spin column purification, the resulting lysate underwent multiple washing steps before being eluted using the supplied elution buffer. To determine the purity of the isolated genomic DNA, it was exposed to a Nanodrop spectrophotometer, which measures absorbance at (260/280 nm). Before undergoing further PCR analysis, the isolated DNA was stored in a deep freezer.

Primers of PCR

After being lyophilized, the primers were dissolved in pure water to create primer stocks with a final concentration of 100 Pico mole/µl. Until further usage, they were stored at -20°C in a concentration of 0.5 Pico mole/20 µl in the whole PCR experiment. Macrogen/Korea supplied all of these primers.

Polymerase chain reaction (PCR)

The 18S rRNA gene was amplified using the PCR method, which was used to directly detect *Entamoeba* species (Table. 1). Amplification of the 900bp 18S1 (F. TAAGATGCACGAGAGCGAAA) and 18S1 (R. GTACAAAGGGCAGGGACGTA) (Johnson and Adejinni. 2010) ^[11]. In order to amplify the targeted gene under the following temperature conditions, the PCR components were then transferred into a thermal cyclor.

Table 1: Shaw PCR reaction mixture

PCR Master mix	Volume
Master mix	10µl
Forward primers1 (10pmol)	1µl
Reverse primers1 (10pmol)	1µl
DNA template	3 µl
PCR water	5µl
Total volume	20µl

Table 2: The thermocycler conditions of PCR

PCR step	Temp.	Time	Repeat
Initial denaturation	94°C	5min.	1
Denaturation	94°C	30 sec.	35cycle
Annealing	60 °C	30 sec.	
Extension	72°C	60 sec	
Final extension	72°C	5min	1

Agarose gel electrophoresis was used to examine the PCR result in the following steps: One gram of powdered agarose was dissolved in TBE buffer and then microwaved for two minutes at 95 degrees Celsius. After cooling, 7 µl of gel dye stain was added to the melted agarose, which was then poured into a gel-tray and solidified for 15 minutes. The comb was removed, and the gel tray was placed in an electrophoresis tank filled with TBE buffer. 5 µl of PCR products were added to each well, and the electric current was set at 100 volt and 80 AM for 30 minutes. The PCR products were visualized using the gel documentation system.

Statistical Analysis

The impact of the different parameters on the study percentages was ascertained using the Statistical Analysis System-SAS (2012) ^[19] tool. In this study, percentages (0.05 and 0.01 probability) were compared in a significant manner using the chi-square test.

Results

Under a microscope, the cyst's morphological appearance revealed *Entamoeba* spp. using the direct saline or iodine staining (wet) mount method. The cyst has four nuclei and appears spherical to oval in shape, encased in a thick membrane (Figure 1). Dogs with a total infection rate of 42% (63/150) tested positive for *Entamoeba* spp. cysts and *Entamoeba* spp. Prevalence.

Molecular results the current investigation demonstrated that the amplification conditions were adjusted for the PCR assay, employing certain primer sequences of the 18S rRNA gene (900 bp) (Figure 2). Samples were submitted to molecular analysis in accordance with conventional-PCR evaluation to determine prevalence of *Entamoeba* spp. The DNA samples of dogs revealed that 52% (52/100) of the 100 investigated fecal samples, which were randomly selected from a total of 150 dog fecal samples in Karbala city, were positive for infection.



Fig 1: Morphology *Entamoeba* spp. by using direct saline method

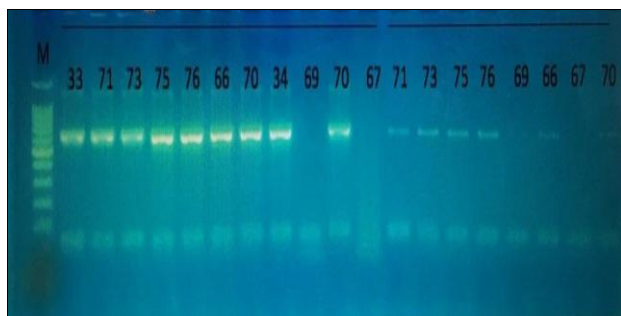


Fig 2: Gel electrophoresis of the 18S rRNA gene PCR product, with an amplicon size of around 900 bp, for *Entamoeba* species using a 1.5% agarose gel at 6 volts per centimeter for one hour

By using microscopic examination, ascertain the prevalence of *Entamoeba* species in dogs

In young dogs, the prevalence of *Entamoeba* spp. was 48% (36/75), while in older dogs, it was 36% (27/75), depending on the age of the animals, as shown in (Table 1). The rate of infection and the prevalence of *Entamoeba* spp. by sex showed that the females had the highest proportion of cases (44%, 33/75), while the males had the lowest percentage (30/75) (Table 2). February had the lowest 28% (6/25) infection rate with *Entamoeba* spp., while April had the highest 60% (15/25) infection rate with *Entamoeba* spp. in dogs (Table 3).

Table 1: Total number of dogs infected with *Entamoeba* species according to age

Age stage	Total number	Positive	Percentage (%)
Young's	75	36	48
Adults	75	27	36
Total	150	63	42%
Chi-Square (χ^2)	---	---	2.217 **

** ($p \leq 0.05$).

Table 2: The total rate of dog infections with *Entamoeba* spp. according to sex

Sex	Total number	Positive	Percentage (%)
Males	75	30	40
Females	75	33	44
Total	1500	63	42%
Chi-Square (χ^2)	---	---	0.246 **

** ($p \leq 0.05$).

Table 3: Total number of dogs infected with *Entamoeba* species according to month

Months	Number of samples	Positive	Percentage (%)
January	25	7	28
February	25	6	24
March	25	11	44
April	25	15	60
May	25	13	52
June	25	11	44
Total	150	63	63
Chi-Square (χ^2)	---	---	11.03 **

** ($p \leq 0.05$).

Discussion

The current study's findings demonstrated that dogs were infected with *Entamoeba* spp. through microscopic and molecular analysis. These findings suggest that dogs may act as reservoir hosts for *Entamoeba* spp. and could potentially be a source of human infection. Several studies in Iraq have documented the parasite in dogs (Russul, and Noor, 2023) [17], also in and other countries such as Malaysia (Nguia *et al.*, 2020) [14], Brazil (Souza *et al.*, 2023), in Nigeria (Adesoye *et al.*, 2024) [1]. According to morphological appearance of detected cysts in fecal samples and that lead to explanation the dogs infects with *Entamoeba histolytica* agree with agree with (Fadhil *et al.*, 2021) [8]. When *Entamoeba* spp. were examined under a microscope, the prevalence was 42%, and when PCR was performed, the infection rates in dogs were 52% agree with previous study conducted in Iraq detected the high 42.85% with Entamoba species Dogs reported by (Russul, and Noor, 2023) [17], the current study dose match with other studies due to recorded low infection rat, in Iraq Baghdad reported infection (15.55%) (Alfadhal *et al.*, 2021), and other countries, in Turkey the Prevalence of *Entamoeba* spp. in Domestic dogs was (0.7%) done by (Ceylan *et al.*, 2024) [5], and in domestic dogs from Argentin the prevalence was (7.9%) this study conducted by (Verónica *et al.*, 2017), while in Brazil the infection rate was (4.8%) (Arruda *et al.*, 2023). This variation infection rate doe to the difference in the number of diagnosed samples, as well as the methods used during these studies, as well as the difference in the months of the study, agree with (Souza *et al.*, 2023). Prevalence of *Entamoeba* spp. in dogs according to age of animals, which recorded the high infection rate was 48% in young dogs, while the lower infection rate 36% was among the adult dogs, agree with (Russul, and Noor, 2023) [17], revealed a lower infection incidence (10%) in the age range of 2–3 years,

whereas a greater infection rate (60%) was seen in the age group older than 1 year. is in line with a research done in Iraq that found that young dogs had a high infection rate of 22%, whereas mature dogs had a reduced infection rate of 6%. (Fadhil *et al.*, 2021) ^[8]. The noteworthy correlation between age and *Entamoeba* infection. The high incidence of *Entamoeba* infections in young dogs might be caused by a lack of immunity at such an early age or most likely by one or more exposures agree with (Hadi and Faraj. 2016). Prevalence of *Entamoeba* spp. according to Sex, the higher infection rate was reported in female (44%), while the lower percentage 40% was presented in the males, in agreement with previous study done by (Fadhil *et al.*, 2021) ^[8], he investigated higher prevalence in females than males were (15.5 and 14.54) respectively and disagreement with (Russul, and Noor, 2023) ^[17], recorded higher infection rate in males (58.33%), while the lower infection rate (10%) was recorded in females (22.22%). The higher infection rates in females than males may be due to many factors such as female hormones, pregnancy and lactation (Pereira *et al.*, 2019). prevalence of *Entamoeba* spp. in dogs according to months, higher infection rate with *Entamoeba* spp 60% (15/25) in April and the lowest 28% (6/25) infection rate in February, this differentiation in prevalence through the months may be due to activation of insects and rain play a role in spreading cyst *Entamoeba* spp. (Bagshaw *et al.*, 2014).

Conclusion

Even though microscopic analysis is a conventional technique for diagnosing *Entamoeba* species, there is a significant chance that these species may be misdiagnosed. The molecular diagnostics approach is regarded as an accurate diagnostic technique. Young dogs and females effected by *Entamoeba* spp more than adults and males.

Recommendation

1. Study the genotyping of *Entamoeba* spp. infect dogs and detect the most speices effect on pet and domesticated dogs.
2. Estimated the zoonotic species of *Entamoeba* and controlled it.

Design program to control the roaming of dogs because dogs consider reservoir host and source of infection for several parasite.

Conflict of Interest

Not available

Financial Support

Not available

Reference

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