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Microscopic and Molecular Prevalence of *Giardia duodenalis* in diarrheic pet dogs

Enas MM. Al-Eodawee

Department of Parasitology, College of Veterinary Medicine, University of Wasit, Wasit, Iraq

Email: emhihi@uowasit.edu.iq

ORCID: https://orcid.org/0009-0002-4796-5611

Abstract

This study was aimed to microscopically and molecularly identification the prevalence rate of *Giardia duodenalis* in diarrheic pet dogs with evaluation the associated risk factors. Fresh fecal samples were collected from 79 diarrheic dogs, and used to preparation the slide smears stained with Giemsa, and to extraction the DNAs that tested molecularly by the conventional polymerase chain reaction (PCR) assay through targeting the *18S rRNA* gene. Our findings revealed that 2.53% and 29.11% of study dogs were positive to *G. duodenalis* using the light microscopy and PCR assay, respectively. In relation to risk factors, the positive results by microscopy and molecular assay were distributed significantly among the groups of age and sex factors. Regarding age factor, prevalence rate of *G. duodenalis* was increased significantly in dogs aged ≤4 months using of microscopy (7.69%) and molecular assay (53.85%) when compared to results of both assay in other age groups; 5-11 months (0% and 18.42%), and ≥12 months (0% and 13.33%). Concerning sex factor, the results of females and males were showed no significant differences between the values of both microscopy (3.45% and 0%), and molecular assay (29.31% and 28.57%). In conclusion, this study confirmed the effectiveness of molecular PCR assay in diagnosis of *G. duodenalis* infection suggesting that biomolecular methods are of great importance in clinical and epidemiological surveys. In addition, molecular classification tools are important to understand the pathogenesis and host range of *Giardia* isolates derived from a variety of animals. The significance of *Giardia* infection in dogs warrants further investigation, particularly with regard to production loss.

Keywords: Conventional PCR, Light microscopy, Risk factors, *18S rRNA* gene, Iraq

Introduction

Giardia duodenalis is a flagellated unicellular microorganism that can infect different species in the animal kingdom including different mammalians such as birds, reptilian, domestic animals and humans (Argüello-García and Ortega-Pierres, 2021). *Giardia duodenalis* (also known as *Giardia lamblia Giardia intestinalis*) is an intestinal parasite, which can cause gastrointestinal infections ranging from mild to severe as well as chronic disease in human while in domestic animals its show clinical importance and economic significant losses (Zajaczkowski *et al*., 2021). Among the six species identified in the *Giardia* genus, only *G. duodenalis* infects human and numerous other mammals (Sprong *et al*., 2009).

Giardia lifecycle alternates between the cyst and trophozoite forms, and both are found in feces. Cysts are more often found in non-diarrheal feces, and they are the infectious stage of

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parasite because infection begins when a new host ingests the cysts in contamination water, food or fecal orally. After ingestion, each quadrinuclear cyst gives rise to two binuclear trophozoites that multiply asexually by binary fission in small intestine, either as free floating bodies or attached to intestine epithelium. As trophozoites migrate toward the large intestine, they retreat into the cyst form in a process called encystations to be excreted in feces (Danciger and Lopez, 1975; Rajurkar *et al*., 2012; Lagunas-Rangel *et al*., 2021). Therefore, the main route for transmission of the parasite is the fecal-oral method by ingestion of contaminated water and food (Siwila, 2023). Venereal transmission occurs through fecal oral contamination. Additionally, diaper changing and inadequate hand washing are risk factors for transmission from infected children (McNeil *et al*., 2022). Lastly, food borne epidemics of *Giardia* have developed through the contamination of food by infected handlers (Dixon, 2021). Mechanical transmission by flies is one of the most important modes of *G. duodenalis* transmission in which flies carry the cysts from unhygienic sites and deposition the organism on visited surfaces (Moratal *et al*., 2020). Studies showed that these pathogens are viable and infectious while carried by flies, thus, nonbiting flies can cause human or animal's giardiasis, transmission by flies is intensive because it is achieved through defection, regurgitation, or mechanical dislodgment (Rousseau *et al*., 2018; Issa, 2019; Patel *et al*., 2022).

Numerous isolates of *Giardia* collected from different host species in various geographical location have been genotyped, and the occurrence of the same genotype in humans and other animals has been well demonstrated (Colli *et al*., 2015). Clinical signs of giardiasis include acute or chronic diarrhea, dehydration, abdominal pain, weight loss, listlessness, fatigue, mucus in the stool, and anorexia that could be of various extents and could be not manifested in infected individuals (Kostopoulou, 2018; Schertzer and Garmel, 2018; Naeem *et al*., 2023). Although direct smear method remain the gold-standard method of diagnosis, it characterized by low rates of sensitivity and specificity (Barrera *et al*., 2024). In last decades, utilization of molecular techniques has provided a high sensitive, specific and valuable tool for diagnosing of different infection in different samples (Gharban, 2023; Gharban *et al*., 2023). Hence, the current study was aimed to microscopically and molecularly identification the prevalence rate of *Giardia duodenalis* in diarrheic pet dogs with evaluation the associated risk factors (age and sex).

Materials and methods

Ethical approval

This study was licensed, performed, and approved by the Scientific and Ethical Committee of the College of Veterinary Medicine, University of Wasit, Wasit, Iraq.

Study samples

A total of 79 diarrheic pet dogs of various age and sex groups were selected randomly from a number of private veterinarian clinics located in Wasit province-Iraq during November (2023)- April (2024). Using a disposable plastic spoon, diarrheic fresh fecal samples were collected directly from each animal and divided using the safe-lock plastic eppendorf tubes. All fecal samples were kept cooled and transported to the laboratory to preparing the slide

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smears as soon as possible, and then, keeping them frozen until be used for molecular examination.

Microscopy

In this study, the slide smears were prepared from all fresh fecal samples and stained by the Giemsa (BDH, England) stain as described by other studies (Alkefari *et al*., 2017; Gharban *et al*., 2022). After staining, the slides were examined using the light microscope under 40× and 100× objective lenses to detect the cyst and / or trophozoite of *Giardia* (Al-Gharban and Dhahir, 2015).

Molecular assay

After thawing in water bath at 37°C, approximately 1 gram of each fecal sample was used for DNAs extraction following the manufacturer instructions (Geneaid, Korea). The concentration (ng/µl) and purity of extracted DNA samples were checked using the Nanodrop system (Thermo Scientific, USA) at absorbance of A260 / A280nm. To preparing the Mastermix tubes, one set of specific primers [F: (5´-CTC TCC CCA AGG ACA CAA GC-3´) and R: (5´- GAA CCC TGA TTC TCC GCC AG-3´)] was designed based on *18S rRNA* gene of the NCBI-GenBank *G. duodenalis* (ID: LC437364.1) isolate, and provided by the Scientific Researcher. Co. Ltd (Iraq). Following the manufacturer instructions (Promega, USA), the tube of Mastermix was prepared at a final volume of 25 µl, and subjected to the conditions of Thermal Cycler (Bio-Rad, USA) system as following: 1 cycle for initial denaturation (95°C/7 min), 30 cycles for denaturation (95°C/1 min), annealing (58°C/1 min) and extension (72°C/1 min), and 1 cycle for final extension (72°C/7 min). The PCR products was analyzed using the stained 1.5% agarose gel with Ethidium bromide at 100 volt and 80 Am for 1 hour, and visualized under the UV transilluminator to detect positive PCR products at a product size of 258 bp.

Statistical analysis

All data were documented using the Microsoft Office Excel (Microsoft Windows, USA) and analyzed using the GraphPad Prism (GraphPad Software Inc., USA). The *t*-test was applied to evaluate association between microscopic and molecular findings, and to estimate association the results of these diagnostic assays to epidemiological risk factors. Statistically, values represent percentages (%), and differences were considered significant at $P<0.05$ (Gharban, 2022).

Results

Among totally 79 fecal samples, the findings revealed that 2.53% (total no=2) and 29.11% (total no=23) of study dogs were positive to *G. duodenalis* using the light microscopy and PCR assay, respectively (Figures 1, 2).

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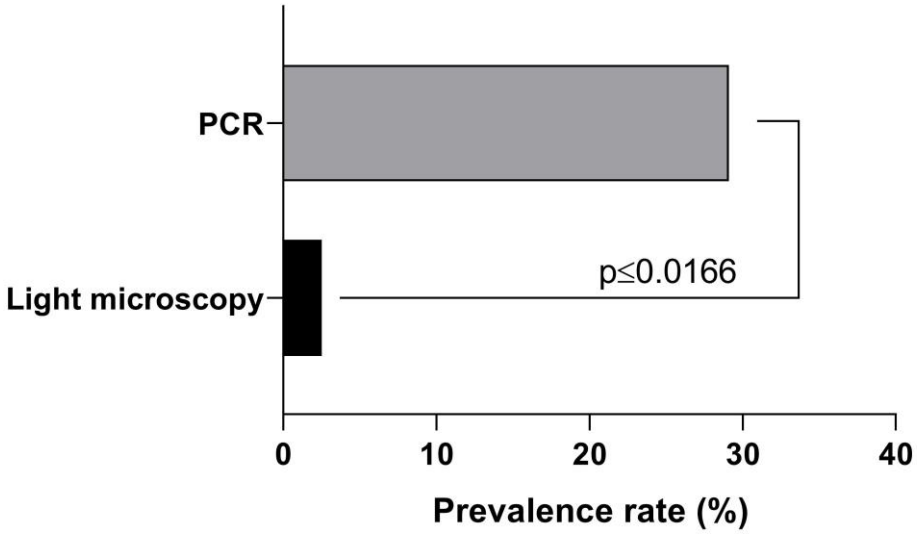

Figure (1): Total results of light microscopy and PCR assay for detection *G. duodenalis*

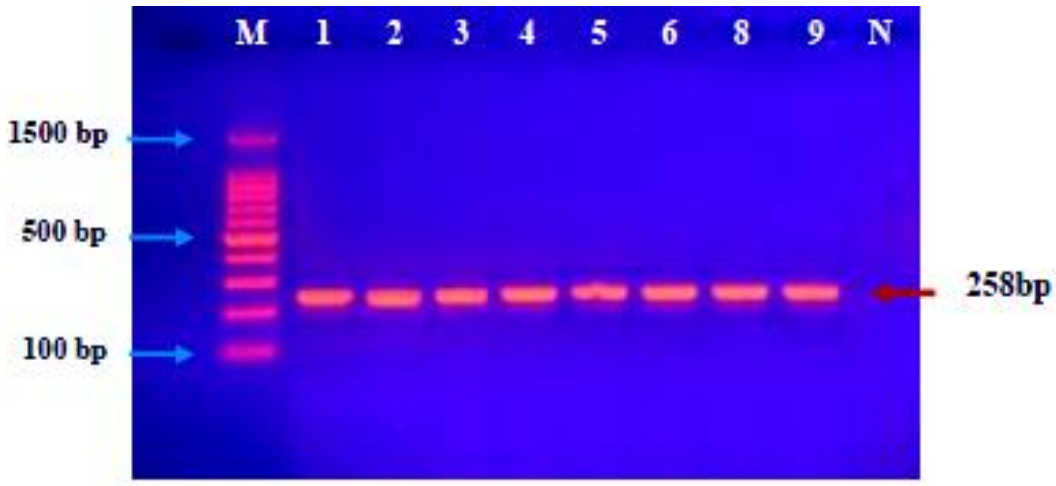

Figure (2): Agarose-gel electrophoresis of some positive PCR products targeting *18S rRNA* gene; in which, Lane (M) represents ladder marker (100-1500), lanes (1-9) represent positive PCR products to *G. duodenalis*, and lane (N) represent negative control

In relation to risk factors, the positive results by microscopy and molecular assay were distributed significantly among the groups of age and sex factors. Regarding age factor, prevalence rate of *G. duodenalis* was increased significantly in dogs aged ≤4 months using of microscopy [7.69% (2/26)] and molecular assay [53.85% (14/26)] when compared to results of both assay in other age groups; 5-11 months [0% (0/38) and 18.42% (7/38)], and ≥12 months [0% (0/15) and 13.33% (2/15)], (Figures 3, 4).

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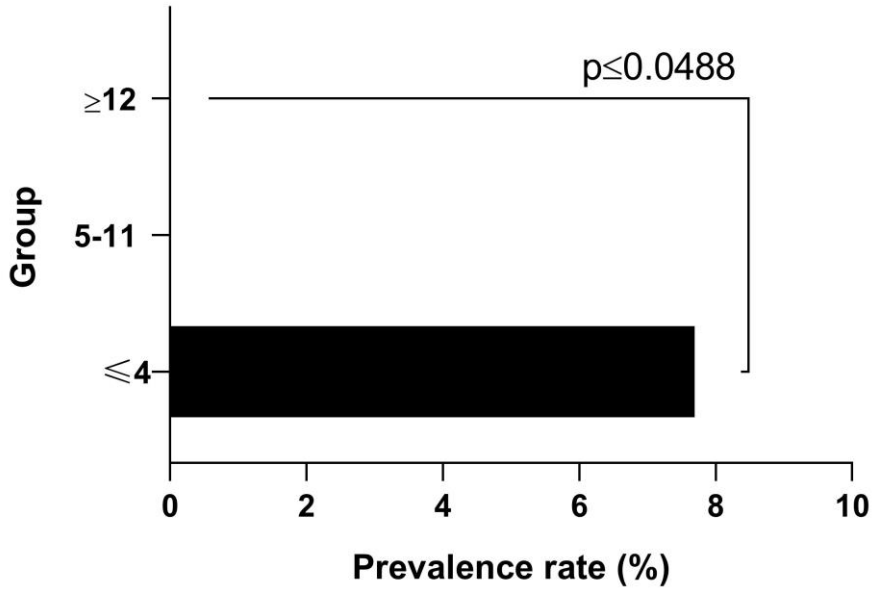

Figure (3): Prevalence rate of positive *G. duodenalis* by light microscopy among different age groups

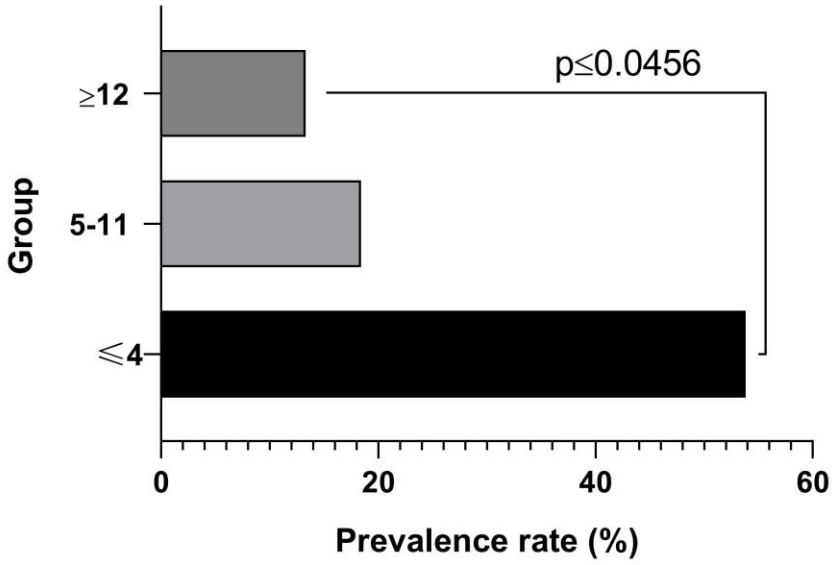

Figure (4): Prevalence rate of positive *G. duodenalis* by molecular PCR assay among different age groups

Concerning sex factor, the results of females and males were showed no significant differences (p≤0.051 and p≤0.081, respectively) between the values of both microscopy [3.45% (2/58) and 0% (0/21)], and molecular assay [29.31% (7/58) and 28.57% (5/21)], (Figures 5, 6).

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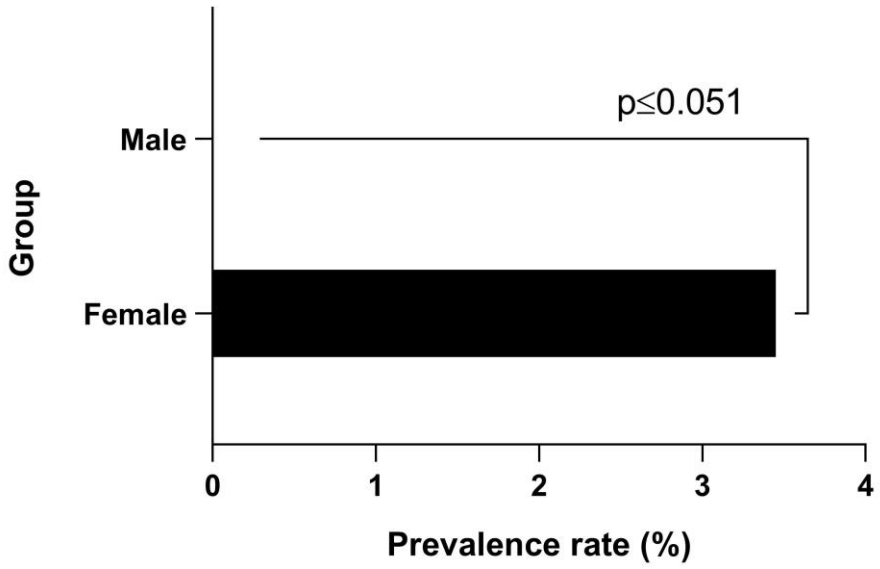

Figure (5): Prevalence rate of positive *G. duodenalis* by light microscopy among different sex groups

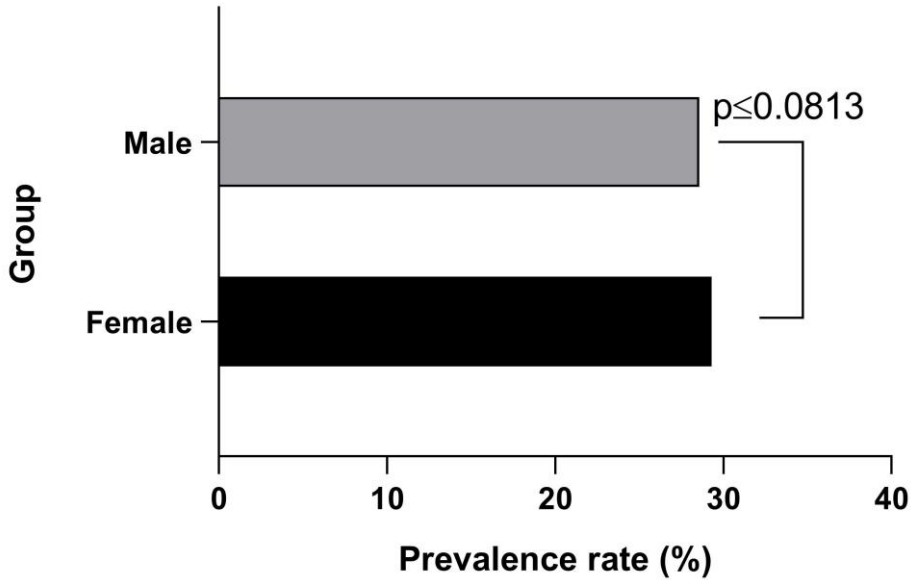

Figure (6): Prevalence rate of positive *G. duodenalis* by molecular PCR assay among different sex groups

Discussion

Giardia duodenalis is one of the most common enteric protozoan organisms in the world. There has been considerable interest in identifying animal hosts that may be reservoirs for *Giardia* species, and play a potential role for transmission the infection to humans (Qays Ibrahim, 2012; AL-Khayat, 2015), through fecal contamination of drinking water sources by such hosts (Robertson *et al*., 2010; Ivanova, 2017). The data of the present study indicate that

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Giardia is common in diarrheic animals which similar to those reported by other researchers in different animals, as *Giardia* infection (Sulaiman *et al*., 2003; Santín *et al*., 2007; Helmy *et al*., 2014). Buret *et al*. (1990) demonstrated occasional occurrence of diarrhea or soft feces in all experimentally infected animals with *Giardia*, most frequently during the first 20 days of the study, and less frequently from day 21 to day 60, and never thereafter; suggesting that, diarrhea did not appear to represent an abnormal clinical feature. However, the importance of giardiasis as a cause of diarrhea in animals is unclear, especially given that diarrhea is often multifactorial with more than one pathogen detected (O'Handley and Olson, 2006). Additionally, gastrointestinal infections may predispose for co-infection in particular with *Cryptosporidium* as reported by several studies (Becher *et al*., 2004; Verweij *et al*., 2004; Rinaldi *et al*., 2007).

The findings of the current study revealed, significantly, higher prevalence of *Giardia* infection by PCR compared to light microscopy. The low results of microscopy might be attributed to low, sporadic and intermittent shedding of cyst/trophozoite, direct smear preparation without applying of concentration methods for cysts such as zinc sulphate method and formalin-ether method which increases the efficiency of the examination. The intermittent excretion of cysts in the feces and the sensitivity of trophozoites to atmospheric conditions suggest that dogs should be tested many times at intervals of 3-4 days after the first negative result. Chakarova (2016) recorded that in a single examination, cyst can be found in a 60-70% of cases, and rises to 97% when tested three times. Over the past decade, molecular typing of pathogens has enabled highly informative insight into the epidemiology of many infections. Implementation of nucleic acid-based detection and typing methods has lead researchers for bettering understand complexity of the *Giardia* genus including the parasite population relevant to veterinary health and human (Cooper *et al*., 2007; Thompson and Monis, 2012). The reported prevalence of *Giardia* tends to vary considerably between studies and is often influenced by the sensitivity of the diagnostic test used and whether only a one-off fecal sample was examined, giving the intermittent nature of cyst excretion (Yang *et al*., 2009; Soares and Tasca, 2016). The PCR-prevalence of *Giardia* infection in dogs with diarrheal symptoms samples here was unexpectedly low. Helmy *et al*. (2014) suggested the protective effect of *Giardia* colonization against unrelated causes of diarrhea; alternatively, it may reflect a lower rate of parasite detection in symptomatic cases if diarrhea is viewed as a response of the host organism to reduce parasite burden.

In this study, the findings showed that the pet puppies (≤4 months) were at higher risk of *Giardia* infection than the young and adult groups (≥5 months). Decreasing age has been implicated as an important risk factor for canine giardiosis (Bugg *et al*., 1999; Capelli *et al*., 2006; Szénási *et al*., 2007; Uiterwijk *et al*., 2019) and is probably related to immunological naivety to the pathogen (Szénási *et al*., 2007). Development of immunological competence may also explain why dogs kennelled for more than 12 months had a lower prevalence of infection (Scaramozzino *et al*., 2009).

As identified in other studies (Coggins *et al*., 1998; Mundim *et al*., 2007; Uiterwijk *et al*., 2019; Šmit *et al*., 2023), no significant effect for *G. duodenalis* and the sex of study animals. Although it is unclear whether sex influences the prevalence of G. duodenalis, Upjohn *et al*.

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(2010) and Meireles *et al*. (2008) discovered a higher incidence of G. duodenalis infection in bitches. Pallant et al. (2015) found assemblage D to be more dominant in male than female dogs. In the current investigation, there was no association between sex and assemblage prevalence. Other risk variables, such as animal housing, living routines, and even breed traits, appear to be more important than animal sex (Mohamed *et al*., 2013; French *et al*., 2023; Šmit *et al*., 2023).

Conclusion

This study emphasized that molecular PCR assay showed an active role in diagnosis of infection, hence, biomolecular methods mainly genotyping of isolates are of great importance in clinical and epidemiological surveys. In addition, molecular classification tools are important for understanding the pathogenesis and host range of *Giardia* isolates derived from a variety of animals. The significance of *Giardia* infection in dogs warrants further investigation, particularly with regard to production loss. Also, this study suggested that fecal samples of diarrheic animals should be tested three to four times to avoid false-negative results due to intermittent excretion of *Giardia* cyst and trophozoite. In this study,

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