TRADITIONAL AND MOLECULAR STUDY OF CRYPTOSOPORIDIUM SPP. IN DOMESTIC DOGS IN BAGHDAD CITY,IRAQ

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ABSTRACT

The aim of this study to investigate the prevalence of *Cryptosoridium* spp. in domestic dogs in Baghdad city, by using microscopic examination(flotation and staining) and molecular techniques .The results revealed that the rate of *Cryptosporidium* infection was 15% by microscopic examination and 28.6% using PCR, our study showed that the infection rate was 8.5% in males and 21.42% in females by microscopic examination , while the infection rate by using PCR in males and females was 21.42%, 35.71% respectively. The percentage of infection in the age groups <1 year and >1 year was 20%, 10% respectively by microscopic examination, while by using molecular assay the rate of infection in the age groups <1 year was 40% and 17.14% in >1 year. This is the first study in Iraq to detect *Cryptosoridium* spp. in domestic dogs using molecular technique.

Key words: Cryptosoridiosis , microscopic examination، PCR, phylogenetic tree, dogs .

الدراسة التقليدية والجزيئية لطفيلي البوغ الخبيئ في الكلاب المنزلية في بغداد , العراق

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المستخلص

هدفت الدراسة الحالية للتحري عن انتشار الابواغ الخبيئة في الكلاب المنزلية في مدينة بغداد ، وذلك باستخدام الفحص المجهري(التطويف والتصبيغ) والتقنيات الجزيئية. أظهرت النتائج أن معدل الإصابة بطفيلي البويغ الخبيئ كان 15٪ بالفحص المجهري و 28.6٪ باستخدام تفاعل تسلسل البلمرة ، اظهرت نتائج الفحص المجهري معدل الاصابة في الذكور 8.5٪ و 21.42٪ في الإناث ، في حين كان معدل الإصابة باستخدام تفاعل تسلسل البلمرة في الذكور والإناث 21.42 ٪ ، 35.71 ٪ على التوالي.اظهرت نسبة الاصابة بالفحص المجهري في الفئات العمرية <1 سنة كانت20٪ و> 1سنة 10٪ ، بينما كان معدل الاصابة باستخدام الفحص الجزيئي في الفئات العمرية <1 سنة 40٪ و 17.14٪ في الاعمار> 1 سنة. تعد هذة الدراسة الاولى في العراق للكشف عن الابواغ الخبيئة في الكلاب المنزلية باستخدام التقنيات الجزيئية.

الكلمات المفتاحية: الابواغ الخبيئة ،الفحص المجهري ،تفاعل تسلسل البلمرة،الشجرة الوراثية، الكلاب .

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INTRODUCTION

Cryptosporidiosis is one of the important a parasitic diseases , caused by *Cryptosporidium*. It is infects wide range of vertebrates, including, humans, livestock, companion, and wildlife animals. Infection is occur via the fecal–oral route after the ingestion of contaminated food or water with oocysts (17, 3), Diarrhea is the most important clinical signs of disease humans and animals (14). and dogs also act as reservoirs for a large number of parasitic zoonoses, including cryptosporidiosis (20,11). Diagnoses *Cryptosporidium* of infection in fecal samples of dogs is based by identifying the oocysts using microscopic techniques, identifying the parasite antigen by ELISA, or by pathogen DNA using PCR and the basic interest of last one compared with other assays is that the amplification products can be analyzed by sequencing analysis to define the species of *the* parasite (13,2). There is little information on the *Cryptosporidium* spp. present in infected dogs in Iraq. This study was conducted to determine the prevalence and molecular diagnosis *Cryptosporidium* species in domestic dogs in Baghdad city, Iraq.

MATERIALS AND METHODES

Detection of *Cryptosporidium* oocysts

One hundred and forty fecal samples (10 g) from domestic dogs, of different sexes and ages referred that a small animal private clinics in Baghdad, Iraq. During the period from 1/1/2018 to 31/5/2018, analyzed by flotation methods and modified Ziehl-Neelsen staining (4,6). Finally the samples were saved at 2.5% potassium dichromate ($K_2Cr_2O_7$) (12), and stored at (-20 C °) until DNA was extracted.

DNA Extraction and PCR

DNA extraction procedure was performed using DNA stool kit (ABM, Canada) based on the manufacturer's guideline. By using for 18S ribosomal gene *Cryptosoridium* . design this primer by the (NCBI)gene-Bank data according to program(Primer3 plus online) (Table1,Figure 1)

Table 1. Oligonucleotide primers used to amplify and sequence parasite 18S rRNA genes

Primers	Primer sequence (5′ to 3′)		Product size (bp)
18S rRNA Gene	F R	GAGGTAGTGACAAGAAATAACAATACACC CTGCTTTAAGCACTCTAATTTTCTCAAAG	300

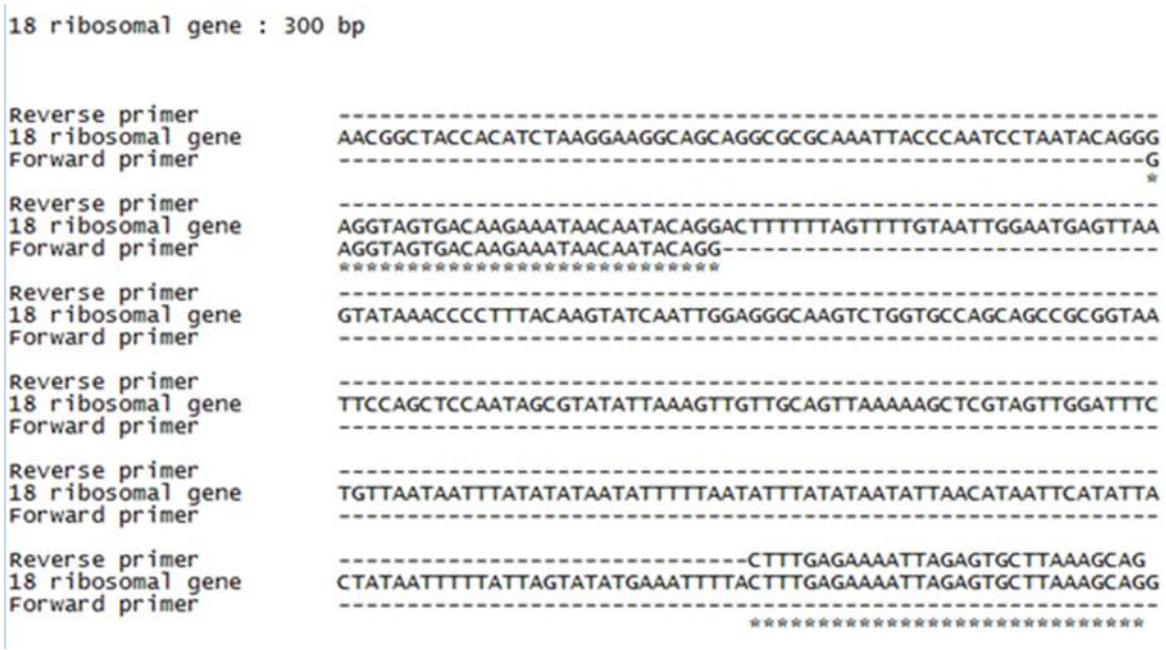

Figure 1.Amplification and sequence analysis of 18s(300bp)of the rRNA gene

Statistical analysis: Statistically analyzed of the data was done by using SAS software(Statistical Analysis System –Version 9.1) (15).

RESULTS AND DISCUSSION

The results showed the infection rate of *Cryptosporidium* spp. of domestic dogs by microscopic examination revealed 21(15.0%) positive sample and 119(85.0%) negative sample and PCR positive samples 40(28.6%) and negative samples 100 (71.4%). Also, PCR was done and showed 300 bp band which confirmed only 40 samples as *Cryptosporidium* spp., the specificity was 94.00 and the low sensitivity was(37.50) which means that not useful for con firm the infection also the value of kappa (0.367) reflects a fair agreement between two test (Table 2, Figure 2,3).

Table 2. Infection rate of *Cryptosporidium spp.*by microscopic and PCR technique.

Observer A	**PCR**		
Observer B	**Microscopic**		
	Observer A		
Observer B	**0**	**1**	
0	**94**	**25**	**119 (85.0%)**
1	**6**	**15**	**21 (15.0%)**
	100 (71.4%)	**40 (28.6%)**	**140**

Weighted Kappa[a]	**0.367**
Standard error	**0.087**
95% CI	**0.197 to 0.538**

Sensitivity	**37.50**
Specificity	**94.00**

- **Poor agreement = Less than 0.20**
- **Fair agreement = 0.20 to 0.40**
- **Moderate agreement = 0.40 to 0.60**
- **Good agreement = 0.60 to 0.80**
- **Very good agreement = 0.80 to 1.00**

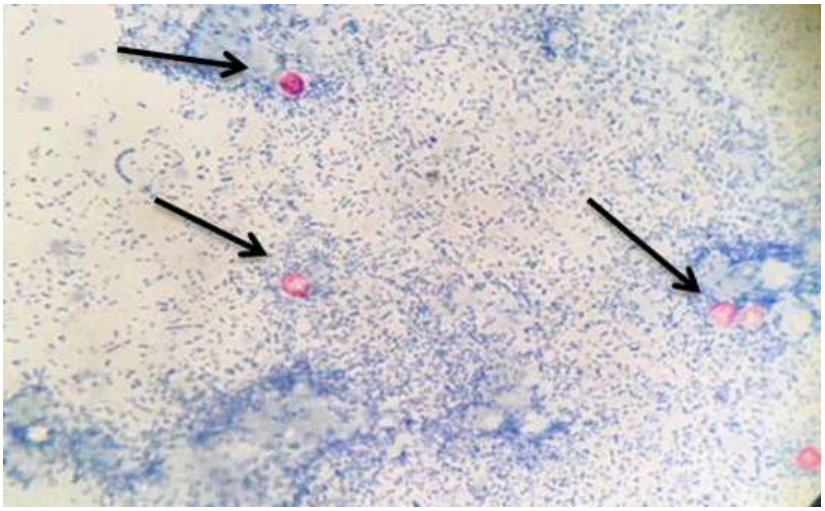

Figure 2.*Cryptosporidium* oocysts stained by Modified Ziehl Nielsen stain (X100).

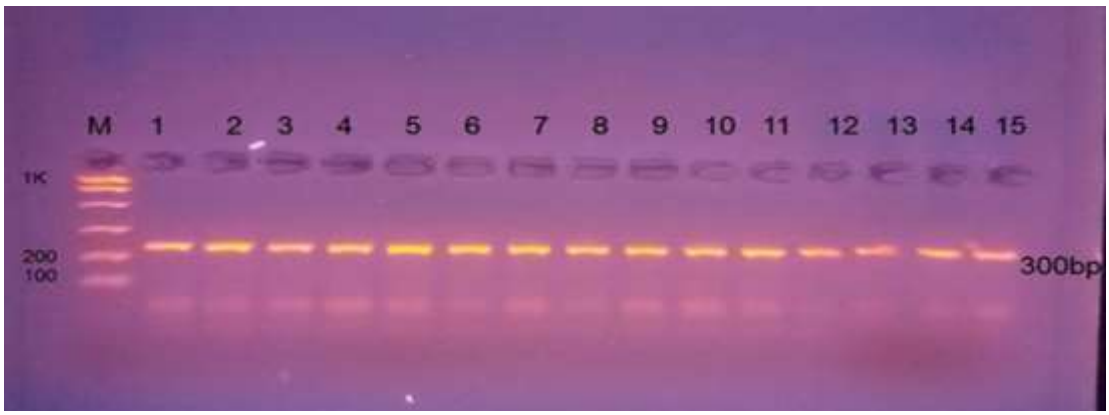

Figure3. Gel electrophoresis of PCR product of 18S rRNA *(*300bp), for *Cryptosporidium parvum* using 2% agarose gel at 60volt for 1 hour. Lane 1- 15: PCR product positive for *18S rRNA* genes

Table 3 shows that the infection rate in males was 8.5% and in females 21.42% by microscopic examination , while the infection rate by using PCR in males and females was 21.42%, 35.71% respectively. The association between males and females was not significant

Table 3. Prevalence of *Cryptosporidium spp*.in domestic dogs by microscopic examination and PCR according to sex

Sex	No. of fecal samples examined	Microscopic Examination No. infected (%)	PCR No. infected (%)
Male	70	6(8.5)	15 (21.42)
Female	70	15(21.42)	25(35.71)
Total	140	21 (15.0)	40(28.6)
Chi square value			3.50
P			0.06

Results revealed that the percentage of infection in the age group <1 year and >1 year was 20%, 10% respectively by microscopically examination, while by using the molecular assay the rate of infection in the age group <1 year was 40% and 17.14% in age group >1year with statistical differences at(<0.01) (Table ,4).

Table 4. Prevalence of *Cryptosporidium spp*.in domestic dogs by microscopic examination and PCR according to age

Age year	No. of fecal Samples examined	Microscopic Examination No. infected (%)	PCR No. infected (%)
<1	70	14(20)	28 (40)
>1	70	7(10)	12(17.14)
Total	140	21 (15.0)	40(28.6)
Chi square value			8.96
P			<0.01

Phylogenetic Analysis

The BLASTIN analysis of the *18S rRNA* gene to the our sample that isolated and sequences with the recorded strains that described in the databases using the MEGA 6.06. program. The evolutionary relationship was designed using molecular sequences in sequences in determining cluster due to variation in 18SrRNA monochromatic sequence. It can be stretched between 0.1.when analysis and compare with the NCBI-Gen bank *Cryptosporidum parvum* isolates that based on offered information with local isolate of *Cryptosporidum parvum* that recorded under accession number (MH716021.1) (Figure 4). Figure 4: Show the phylogenetic tree inferred the degree of relatedness between *18SrRNA* sequence deposited in the international oligonucleotide primers used to amplify and sequence parasite *18S rRNA* genes. Bank sequence database(NCBI) of *Cryptosporidium parvum* as isolated from infected dogs under accession number (MH716021.1), sample based on a partial sequence of *18S rRNA* Gene GenBank *Cryptosporidium* is a coccidian, intracellular protozoan parasite pathogen, which causes diarrheal illness of animals and

humans (18). The present study showed that the rate of infection *Cryptosporidium* spp. oocysts in dogs fecal was 30% and 57.40% by using staining and molecular methods, respectively. This rate is a high than the results of some previous studies, Bahrami *et al.* (1) reported 7.04% infection in stray dogs of Ilam using the Ziehl Neelsen staining method. in Bahía Blanca of Argentina Sala *et al*. (9) was recorded 14.7% by using by ZiehlNeelsen staining. . in Thailand Koompapong *et al*. (8) who recorded 2.1% by using PCR and in Japan Yoshiuchi *et al.* (20) who recorded 3.9% by PCR.. Results revealed that the microscopic test has a low sensitivity %37. This is attributed to the experience of the examiner to observation of oocysts in the feces specimens , also microscopic examination does not distinguish between different species of parasites.; thus, molecular assay like PCR have been used with high sensitivity, specificity, and rapidly features are capable of differentiating between species and genotypes in different samples, although they are expensive. Previously, genotyping of *Cryptosporidium* spp. has been done successfully by PCR method according to (*18S rRNA*) gene (12, 10 ,19). Our results indicate that the age may affect the rates of infection through the significant high prevalence of *Cryptosoridium* in the young dogs than in adult dogs using both methods. Similar research were reported in dogs (5, 20). This could be attributed to lower resistance in the young compared to older animals. In otherwise, no statistical differences relationship was between the infection of *Cryptosporidium* and the sex of the dogs. Agree with two studies conducted in China and Brazil recorded similar findings (16,7). This refers that sex is not a major affecting Cryptosporidiosis infection in dogs. In the present study, a first phylogenetic analysis of *C. parvum* isolates in Iraq from infected domestic dogs, based on the *18S rRNA* gene sequence showed that there were relationships between *C.parvum* local isolate (MH716021.1) and other global isolates the local isolates were closely related to France isolate under accession number EF158462.1 with nucleotide sequence identity 91%. Furthermore, showed gradual similarities with the other isolates from the high grade to the lower indicated on identity between (91-92%).

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