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Detection of cryptosporidium parvum in cat by conventional methods and nested PCR in Al-Diwaniyah province

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Abstract--This study was conducted in Al-Diwaniyah province / Iraq during the period from September 2021 to the end of February 2022 to determine the Infection rate of *Cryptosporidium spp* parasites in cat to study the effect of some epidemiological factors such as age , gender, months and type of cat, as well as molecular determination of *Cryptosporidium spp.* by amplification of 18srRNA gene by N-PCR. fecal samples were collected from 100 domestic cats and stained by Ziehl-Neelsen stain. Microscopic examination results showed that 33% of 100 stool samples were infected by *Cryptosporidium spp.* the highest percentage was in September and October at 40% , and the infection rates was 40% in males compared to females at 30% and in young 42% while in adults 24%, and for domestic and stray cats about 40% compared with pet shop 22.5% where there was no significant difference at $P < 0.05$. Genomic DNA was extracted From 100 fecal samples, the 18srRNA gene for *C. parvum* was amplified by N-PCR . The (N-PCR) technique showed that out of 100 stools 35% of the samples were positive for *C.parvum*. Identification and characterization of *Cryptosporidium spp* in cats in Diwaniyah was the first and very important for avoiding infection with other animals, handlers and for implementing control programmes.

Keywords--cryptosporidium parvum, cat, nected PCR, DNA extraction.

Introduction

Around the world, a variety of animals and people can become infected with the obligatory intracellular (extracytoplasmic) protozoan parasites known as *Cryptosporidium* spp (1,2). A coccidian parasite called *C.parvum* has infected the intestines of both humans and animals. Diarrhea is the most prevalent clinical sign of *C. parvum* infection, and the illness can be fatal and chronic, especially in immunocompromised individuals (3,4). People and animals that frequently come into contact with employees or animals can contract *Cryptosporidium* spp (5,6). In order to prevent infection of handlers and other animals, it was essential to identify and define *Cryptosporidium* species in animals (7). Most cats with *Cryptosporidium* spp. infections are asymptomatic. Young and newborn cats are more likely to get diarrhea (8). For the purpose of preventing the spread of the parasite from cats to their owners and caregivers, epidemiological information on *Cryptosporidium* species infection in household cats and pet store kittens is essential (9). The majority of cases of cryptosporidiosis have been identified using microscopic and molecular techniques (10), and it is presumed to be zoonotic in infected people and animals. Polymerase chain reaction (PCR) technology was employed to recognize *Cryptosporidium* oocysts for the first time in 1991. (11).Nested PCR was used in conjunction with a very dependable DNA extraction technique to determine the amounts of *C. parvum* DNA in old or fresh formalin-fixed cat feces.

Material and Methods

Collection of samples 100 faeces samples were randomly obtained from pet shop kittens and stray cats in Al-Diwaniyah province ,were collected from September 2021 to February 2022.The specimens were collected directly from the animal's excrement in clean plastic containers with labels that included the number, age, gender, type and month of collection, as per medical protocol. To avoid contamination, precautions were followed, such as donning a mask, lab coat, and disposable gloves that were changed with each sample .The samples were subsequently delivered in a cold bag to the University of Al-Veterinary Qadisiyah's Medicine College's parasitology laboratory for the requisite tests. The samples were split into two halves, one maintained cool at 4° C for laboratory investigations and the other deep frozen at -20° C for DNA extraction.

Microscopic Examination

The diagnosis of *Cryptosporidium* Oocysts is made through microscopic inspection of a faecal smear, which commonly involves the use of modified acid fast stain techniques, such as the MZNS (12). One hundred fresh faecal samples were examined on slides either directly or after concentration by flotation to concentrate the quantity of oocytes using a -NaCl flotation solution, and then stained using a modified mZNS procedure. The smears were fixed for 3 minutes in absolute methanol and then stained for 4 minutes with carbolic fuchsine modified acid fast stain. After that, they were cleaned and stained with 30% ethyl alcohol. The slides were cleaned and stained with methylene blue for 1 minute before being washed and dried outside. To see *Cryptosporidium* oocysts, the samples were inspected with a 100X oil immersion lens.

DNA Extraction and molecular analysis

Genomic DNA was extracted from feces using the GeneAid DNA Stool Kit (GeneAid, Korea) following the manufacturer's instruction of Protocol, and DNA was stored at -20°C before it was used in PCR amplification reactions. The PCR protocol was nested PCR and the primers were as follows: outer primers including CF201 (5'-GGGTTGTATTTATTAGATAAA GAAC-3') and CR201 (5'-CTTTAAGCACTCTAATTTTCTC-3) were specific to the genus *Cryptosporidium*. Then the second round (inner primers) were CPF202 (5'-GACTTTTGGTTTTGTAATTGGAATG-3') and CPR202 (5'-TAAATTATTAACAGAAATCCAACACTACGAGC-3') were specific to *C. parvum*. (13). The amplicons from these two reactions were 540 bp and 165 bp, respectively. Amplification reactions were carried out in 20 µl volumes consisting of 10 µl Taq Master Mix (2X), 1 µl of each primer (10X), 6 µl nuclease-free water and 2 µl DNA. Reaction conditions were comprised of a hot start at 95°C for 5 min followed by 35 cycles at 95°C for 35 s, 56°C for 35 s and 72°C for 40 s and a final extension at 72°C for 5 min. The conditions of the second round were performed similar to the first round. The products were tested by electrophoresis and then the sample is stained with ethidium bromide and the UV lighter is used for watching the bands.

Statistical analysis

The Statistical Package of Social Sciences (SPSS), version 23 (Inc., Chicago, IL, USA) computer program was used to do all statistical calculations. The chi-square test was used to examine differences across groups (X²). The statistical significance level was set to alpha = 0.05 (α = 0.05). A statistically significant value of P 0.05 was used.

Result

Diagnostic characterization of *Cryptosporidium* spp by Microscopic Examination

By using a modified acid fast stain (MZNS), the *Cryptosporidium* spp. oocysts were discovered in a regular round form with a reddish-pink tint on a blue background (Figure 1). The findings of the examination revealed that 33% of the 100 samples of cat excrement examined microscopically had positive results.

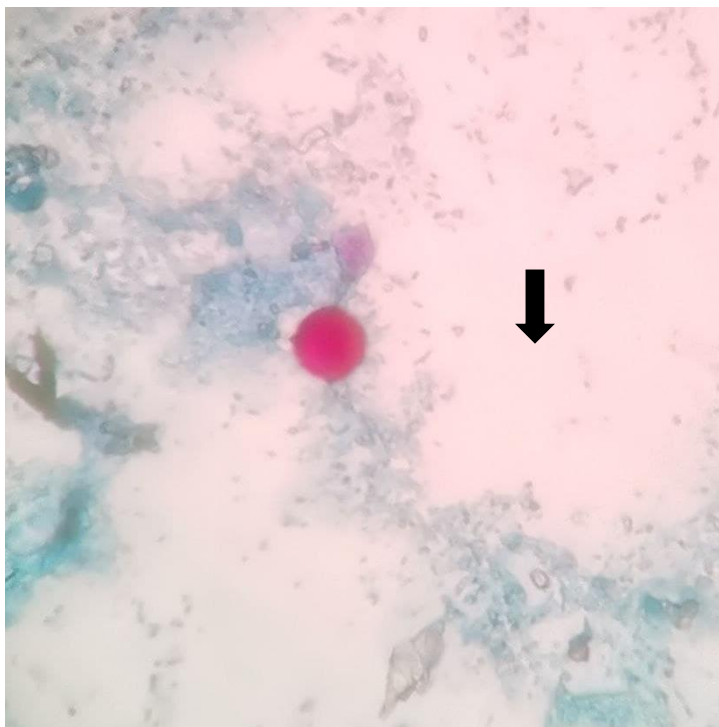


Figure 1. Show *Cryptosporidium spp.* oocyst stained with M.Z.N.S 100 x

Rate of *Cryptosporidium spp.* in cat according to the gender

In this study the males had the greatest infection rate (12 / 100) 40%, while the female had the lowest infection rate (21 / 100) 30%, with no significant differences at level ($p < 0.05$.) as in table (1):

Table 1

Rate of infection by *Cryptosporidium spp.* in cat according to the gender

Gender	Examination No.	Positive No.	Percentage%
Female	70	21	30%
Male	30	12	40%
Total	100	33	33%
Chi-Square (χ^2)	0.950*	P value	0.330

No significant difference $P < 0.05$

Rate of *Cryptosporidium spp.* in cat according to the type .

The study found that the highest infection rate in domestic and stray cats 40% compared to pet shop 22.5%

Table 2
Rate of infection by *Cryptosporidium spp.* in cat according to the type

Type	Examination No.	Positive No.	Percentage %
Pet shop	40	9	22.5%
Household and stray	60	24	40%
Total	100	33	
Chi-Square (x2)	3.32*	P value	0.062

No significant difference $P < 0.05$

Rate of *Cryptosporidium spp.* in cat according to the age.

In this study the greatest rate was found in young 42%, while the lowest rate was found in adults 24%, with no significant differences at level ($p < 0.05$). As in table (3).

Table 3
Rate of infection by *Cryptosporidium spp.* in cat according to the age

Age	Number examined	of	Number infection	of	Percentage %
Adult	50		12		24%
Young	50		21		42%
Totally	100		33		33%
Chi-Square (x2)	3.66*		P value		0.056

No significant difference $P < 0.05$

Rate of *Cryptosporidium spp.* In cat according to the months of study.

The study found that the highest rate of infection was in September and October 40% , and the lowest rate was in February 25% with no significant differences at level ($p < 0.05$) . As in table (4).

Table 4
Rate of infection by *Cryptosporidium spp.* In cat according to the months of study

Month	Examination No.	Positive No.	Percentage %
September	20	8	40%
October	10	4	40%
November	20	7	35%
December	20	6	30%
January	10	3	30%
February	20	5	25%
Total	100	33	
Chi-Square (x2)	1.4*	P value	0.924

No significant difference $P < 0.05$

Results of Molecular Examination

Cryptosporidium parvum in cat depended on N- PCR

According to N-PCR detection of *C. parvum* 18SrRNA gene, the overall prevalence of *C. parvum* was 35% . As shown in the figure (2) below.

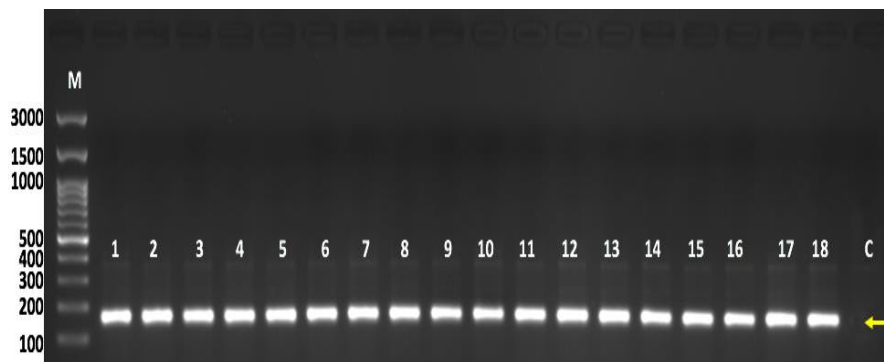


Figure 2. show agarose gel electrophoresis(1.5%) that showed the Nested - PCR product analysis of (18SrRNA gene) in *C. parvum*. positive samples. Where M: marker (3000-100bp) and lane (1-18) positive *C. parvum*. were showed at (165bp) PCR product

Discussion

The global frequency of cryptosporidiosis in cats has been found to range from 0% to 29.4%, with these discrepancies linked to the diagnosis procedures used (14). The infection rate with *Cryptosporidium* in this study was 33% (33/100), which is similar to Hassan and Barzinji (2018) in Kirkuk, who recorded a high rate of infection of 27.08%, but differs from Al-Aredhi (2015), who recorded 6.97% in Iraq's Al-Diwaniyah province, and Spain (2001) in New York State, who recorded a lower rate of 3.8% (10/263). The differences in infection rates recorded by these studies could (15). . Domestic cats had a greater incidence of injuries, with 40% in domestic cats and 22.5% in pet shop, while males had a higher incidence of injuries than females, with 40% in males and 30% in females, according to this study. The presence of *Cryptosporidium* infection in this case could be linked to the cat's living environment and age. According to some authors , the likelihood of infection with *Cryptosporidium* agents is higher in stray/outdoor cats than in indoor/pet cats (16).

Apart from that, cryptosporidiosis has been discovered to be more common in young cats, which has been related to their undeveloped immune system (17). *Cryptosporidium* spp. are increasingly being detected and differentiated using PCR. In the current investigation, PCR testing revealed that 35% (35/100) of cats tested positive for *C. parvum*.. However, the results were higher than those found in Australia utilizing the (18S-rDNA gene), where the infection rate was 10%. (18) and Li et al. (2015) found 3.8% (SSU rRNA gene) in northeast China, while Ito et al. (2017) found 2.3% in Japan (19) . This is due to a variety of factors including geographic location, animal ownership status, and religious differences (20). The prevalence revealed in this study is higher than that reported in other faecal

epidemiological investigations. Infection rates in cats have previously been found to vary by geographical region, ranging from 3.8% in Japan (21) to 8.1% (22) and 12.3% (23) in Scotland. However, the variations could be due to the different diagnostic procedures utilized and the amount of tiny fields seen. (24). The findings of this study show that Cryptosporidiosis is a prevalent infection in cats living in Al-Diwaniyah province, and that it should be regarded an endemic disease in this location.

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Conflict of interest

There are no conflicts of interest between the authors regarding the publication of this work.

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