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Molecular detection of *Cryptosporidium* spp. in stray cats in Al-Qadisiyah governorate, Iraq

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Abstract

Cryptosporidiosis is an opportunistic zoonotic disease considered a global public health concern. Cats are a potential host for transmitting zoonotic diseases to humans as *Cryptosporidium* spp. infection. The present study aimed to determine the *Cryptosporidium* spp in stray cats using molecular methods in Al-Qadisiyah governorate, Iraq, from September 2021 to February 2022. A total of 100 fecal samples were collected from different geographical areas. All the samples were investigated using Nested-PCR and sequencing methods to identify and characterize *Cryptosporidium* spp. in stray cats. The results of the molecular examination showed that 35% of the fecal samples were infected with *Cryptosporidium* spp. Two species have been identified, *C. parvum* and *C. canis*. In conclusion, the results showed that cats are a reservoir host for *Cryptosporidium* spp for human infection. For the prevention and control of *Cryptosporidium* spp. Cat infection should get more attention from health officials, especially when the prevalence rate is high.

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Introduction

Cryptosporidium spp. infected humans and a wide range of animals, including cats, and dogs, producing Cryptosporidiosis (1,2). Cryptosporidiosis is one of the most severe diseases resulting in intestinal infection in humans and animals (3,4). Stray cats are a public health risk source of human zoonotic diseases such as cryptosporidiosis (5,6). Fever, abdominal pain, weight loss, and diarrhea are symptoms of this disease in neonatal animals, children, and immunocompromised patients (7,8). Since then, there have been asymptomatic and symptomatic cases in cats, with the latter presenting clinical indications of chronic diarrhea, anorexia, and weight loss (9-11). However, this parasite can infect animals and humans through direct and indirect transmission (12). The fecal-oral route for direct transmission is represented by accidental ingestion of oocytes from feces, and another source for direct transmission could be represented by direct interaction and contact with infected animals (13). Cross-contamination of water, food (both foodborne and waterborne), food utensils, and other farm materials exposed to feces containing *Cryptosporidium* spp. oocysts could result in indirect transmission (14,15). Furthermore, these oocysts are a possible contributor to their global spread due to their strong tolerance for environmental conditions. As a result, Cryptosporidiosis in live animals has become a primary concern for animal health, resulting in significant global financial losses. Control methods of Cryptosporidiosis in humans and animals need early detection of parasail but are still critical. Early diagnosis of *Cryptosporidium* could help for the limitation of spread across hosts. *Cryptosporidium*-infected cats can easily infect humans, and the dynamics of the transmission and patterns of infection remain unknown (4). Although *Cryptosporidium* oocysts in feces can be recognized using various techniques, such as microscopic staining and surface antigen detection, these approaches are insufficient for species identification and genotyping because of the oocysts' morphological and antigenic similarities (16,17). Molecular methods like conventional PCR, Nested-PCR, and sequencing identify and characterize the parasite at species and subspecies levels using 18S rRNA

and *gp60* genes (18-20). Several researchers have used the 18S rRNA gene to identify the genotype of parasites (21-24)

In the present study, the Nested-PCR method was used for molecular detection of *Cryptosporidium* spp. in cat feces using the 18S r RNA gene. Subsequently, the sequencing method was used for the characterization of *Cryptosporidium* species.

Materials and methods

Ethical Approval

Ethical approval was obtained from a guidance of Research, Publication and Ethics of the College of Veterinary Medicine, University of, Al-Qadisiyah, Iraq, which complies with all relevant Iraq legislations.

Fecal sample collection

One hundred fecal samples (n = 100) were collected randomly from stray cats from September 2021 to February 2022. The samples were collected from five geographical regions of Al-Qadisiyah governorate, Iraq: Al-Qadisiyah Center, Al-Sannia, Al-Daghara, Al-Sadeir, and Al-Shafeia. The sample was directly collected after defecation from animals in sterile disposable containers. Samples were subsequently delivered in ice bags to the parasitology laboratory of the Veterinary Medicine college; write the name of the University, city, and country for further analysis. The sample was divided into two parts: the first was kept at 4°C for microscopic examination, and the second was kept at -20°C for DNA extraction.

DNA extraction

The genomic DNA (gDNA) of *Cryptosporidium* was extracted from a 250 mg fecal sample using Geneaid DNA Stool Kit (Geneaid, Korea) and based upon the manufacturer's specifications of the protocol. The purified gDNA was kept at -20°C for further molecular investigations.

Molecular analysis

The molecular method was used for the detection of *Cryptosporidium* spp. DNA was achieved by amplifying a partial sequence of 18S rRNA gene by conventional PCR using forward primers CF201 (5'-GGGTTGTATTTATTAGATAAAGAAC-3') and reverse primer CR201 (5'-CTTTAAGCACTCTAATTTTCTC-3'). Subsequently, the PCR product as template was used for nested-PCR using forward primer CPF202 (5'-GACTTTTTGGTTTTGTAATTGGAATG -3') and reverse primer CPR202 (5'-TAAATTATTAACAGAAATCCAACTACGAGC -3') (25). The Initial PCR yielded 540 bp of 18S rRNA gene, and the nested-PCR product yielded 165 bp. The master mix of the conventional PCR reaction was carried out in 20 µl consisting of 10 µL Hot-start Taq Master Mix (2X, Promega), 0.2 µM of each primer (10X), 6 µL nuclease-free water, and 30 ng/µL of the gDNA. Thermocycler conditions were started with an initialization cycle 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 master mix of nested-PCR reaction was performed similarly to the conventional PCR using 5 µL of PCR product template. The amplified PCR products were electrophoresed on a 1.5 agarose gel matrix (Cleaver scientific, UK), stained in ethidium bromide (0.5 µg /mL), and visualized at 302 nm on a UV transilluminator using the gel documentation system (Cleaver scientific, UK).

Sequencing and phylogenetic analysis

The remaining amplicon (ca. 15 µl) from nested-PCR was used for sequencing from Macrogen (Seoul, South Korea). The sequences were analyzed using Megax software (version 10), and the alignment and dendrogram analysis was achieved by the Clustal W method.

Results

The results of gradient- PCR revealed that 60 °C is the optimal temperature for both conventional and nested PCRs, and figure 1A illustrates the results of conventional PCR of the 18 S rRNA gene with a fragment size was 540 bp. Figure 1B illustrates the results of nested-PCR of the gene with a fragment size was 165 bp.

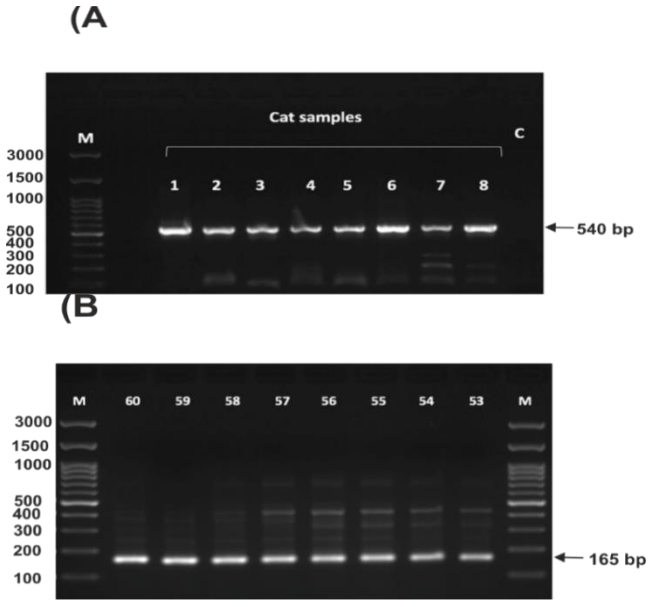

Figure 1: [A] Agarose gel electrophoresis of amplicons of 18S rRNA gene of *Cryptosporidium* spp. with an approximate size of 450 bp. Lanes 3 -8; Lane M, DNA ladder (Genetrix, Korea). [B] Nested-PCR products of 18 S rRNA gene of *Cryptosporidium* spp. with an approximate size of 165 bp. Lanes 54 -60. Lane; M, DNA ladder (Genetrix).

Cryptosporidium spp. was identified in 35 out of 100 samples from stray cats using the Nested-PCR technique targeting the 18S rRNA gene. Ten 10 -positive samples were sequenced, and phylogenic analysis was conducted with reference strains of *C. parvum* and *C. canis* for the 18S rRNA gene. The sequencing results showed two *Cryptosporidium* spp., *C. parvum*, and *C.canis*. the sequence of *C. parvum* showed 100% similarity compared with *C. parvum* reference strains isolated in China and Japan (Table 1 and Figure 2). However, *C. canis* was compared with reference strains from China, Czech, Iraq, and USA and showed similarities ranging from 99.34% to 99.56%. In this study, sex *C. parvum* was detected (6/10), and four *C. canis* (4/ 10) (Table 1 and Figure 2). All sequences were deposited in the NCBI GenBank with accession numbers (ON156760 to ON156769).

Table 1: Homology Sequence identity among the local and reference *Cryptosporidium spp* isolates

Samples	Accession No.	BLAST Homology Sequence identity percentage			
		Identical to	NCBI Accession No.	Country	% Homology
1	ON156760	*C. canis*	MN696800	China	99.56
2	ON156761	*C. canis*	MN036522	Czech	99.34
3	ON156762	*C. canis*	MT329018	Iraq	99.34
4	ON156763	*C. canis*	DQ385546	USA	99.56
5	ON156764	*C. parvum*	MT002720	China	100
6	ON156765	*C. parvum*	MT002720	China	100
7	ON156766	*C. parvum*	MT043865	India	100
8	ON156767	*C. parvum*	MN918253	Japan	100
9	ON156768	*C. parvum*	MN918253	Japan	100
10	ON156769	*C. parvum*	MT648442	China	100

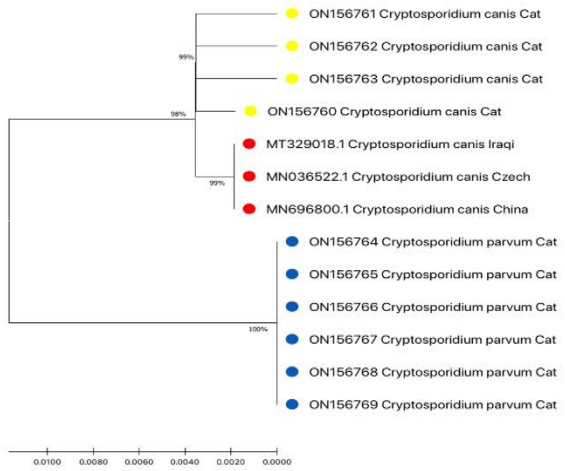

Figure 2: Phylogenetic tree analysis of the identified *Cryptosporidium sp*. targeting 18S rRNA gene in cat isolates. These have been deposited in the gene bank with the accession numbers followed by the identified *Cryptosporidium* sp. and the host from which were identified (Yellow circles referred to *C. canis*, blue circles referred to *C. parvum*, while red circles referred to global isolates).

Discussion

Over the years, zoonotic transmission of various species of *Cryptosporidium* spp. and the role of animals as a reservoir for human infection have been important issues in medical and veterinary practices (26-28). Pets provide several important human benefits but are also associated with health hazards. Besides the risk of bites, scratches, and allergies as common health hazards, cats may harbor various zoonotic parasitic infections. Thus, close contact with these pets is considered a risk factor (29-34). Nearly 300 publications related to *Cryptosporidium* spp. in dogs and cats have been published. Most of these studies were epidemiological surveys of Cryptosporidiosis around the world. In early studies, microscope-based morphological methods and antigen detection assays were commonly used to detect *Cryptosporidium* oocytes (35-42). The use of molecular tools has led to the identification of several zoonotic *Cryptosporidium* spp. in dogs and cats. *Cryptosporidium canis* and *Cryptosporidium felis* are dominant species causing canine and feline Cryptosporidiosis, respectively. Some *Cryptosporidium parvum* infections have also been identified in both groups of animals. The identification of *C. canis*, *C. felis*, and *C. parvum* in pets and owners suggests the possible occurrence of the zoonotic transmission of *Cryptosporidium* spp. between humans and pets (43). However, in this investigation, we found out that the prevalence rate of *Cryptosporidium* spp in cats in Al-Qadisiyah municipality is still unclear, and this study attempts to provide an early assessment of its spread. In this study and by utilizing Nested-PCR and DNA sequencing for the 18S rRNA gene, a relatively small amount of parasite DNA was identified. PCR testing revealed that 35% (35/100) of cats tested positive for

Cryptosporidium sp. The infection rate in this study was higher compared with study (44) where it recorded a rate of 20% (20/100) of cats that were positive with *Cryptosporidium* spp. in Baghdad city by using specific primers for *Cryptosporidium parvum* derived from 18S ribosome gene this was more accurate and species-specific (GenBank). However, the results differed from those found in Australia utilizing the (18S-rDNA gene), where the infection rate was 10%. (45) and Li *et al*. (46) found a 3.8% (SSU rRNA gene) in northeast China. This is due to various factors, including geographic location, animal ownership status, and religious differences (42). This study recorded for the first time that the molecular detection of *Cryptosporidium* in Al-Qadisiyah was 40% and 60% for *C. canis* and *C. parvum*, respectively. Even though *C. canis* prevalence is low, it has never been detected in cats in Iraq and especially in Al-Qadisiyah municipality, and given that *C. parvum* prevalence was high, this might indicate a potential threat to humans and animals' health in Al-Qadisiyah, Iraq. Interestingly,

phylogenetic analysis of *C. parvum* isolates from infected domestic cats in Al-Qadisiyah province, based on the 18S rRNA gene sequence, showed that there were identity 100% relationships between *C. parvum* local isolate (from ON156764 to ON156769) and other global isolates accession number MT002720 China and MT043865 India and MN918253 Japan and MT648442 China. While the detected isolates from genotype *C. canis*, the percentage of concordance with the globally recorded isolates with accession number was 99.56%, identical with MN696800 China, MN036522 Czech 99.34%, MT329018 Iraq99.34%, and DQ385546 USA 99.56%. The findings of this study show that Cryptosporidiosis is a prevalent infection in cats living in Al-Qadisiyah municipality and that it should be regarded as an endemic disease in this location.

Conclusion

These results indicate a common occurrence of two species of *Cryptosporidium* in stray cat (*C. parvum* and *C. canis*), the results showed that cats are a reservoir host for *Cryptosporidium* spp for human infection.

Acknowledgments

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Conflicts of interest

The authors declare that no conflicts of interest are associated with this work.

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الكشف الجزيئي عن طفيلي الأبواغ الخبيئة في القطط الضالة في محافظة القادسية، العراق

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الخلاصة

داء الأبواغ الخبيئة هو مرض حيواني المنشأ انتهازي يعتبر مصدر قلق عالمي للصحة العامة. القطط هي أحد المضيفين المحتملين لنقل الأمراض الحيوانية المنشأ إلى البشر مثل عدوى خفية الأبواغ. هدفت الدراسة الحالية إلى تحديد طفيلي الأبواغ الخبيئة في القطط الضالة باستخدام الطرق الجزيئية في محافظة القادسية، العراق من أيلول ٢٠٢١ إلى شباط ٢٠٢٢. تم جمع ١٠٠ عينة براز من مناطق جغرافية مختلفة. تم فحص جميع العينات باستخدام تقنية تفاعل السلسلة المتلمرة المتداخل وطرق التسلسل قواعد الحمض النووي الريبوز منقوص الأوكسجين (الدنا) لتحديد ووصف أنواع الأبواغ الخبيئة في القطط الضالة. أظهرت نتائج الفحص الجزيئي بان ٣٥٪ من عينات البراز كانت مصابة بطفيلي الأبواغ الخبيئة وتم التعرف على نوعين، خفية الأبواغ الصغيرة وخفية الأبواغ القطية. في الختام، أظهرت النتائج أن القطط هي مضيف مستودعات لطفيلي الأبواغ الخبيئة لإصابة الإنسان. وللوقاية والسيطرة على طفيلي الأبواغ الخبيئة يجب أن تحظى العدوى في القطط باهتمام أكبر من قبل مسؤولي الصحة خاصة عندما يكون معدل الانتشار مرتفعا.