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Molecular recognition and phylogenetic tree analysis of *Cystoisospora canis* in stray dogs in Diwaniyah city, Iraq

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Abstract

Cystoisospora spp. are Apicomplexa, protozoan parasites with a global prevalence of 1-5% in dogs. The current study aimed to find out about the presence and molecular features of *Cystoisospora* among homeless dogs in Al-Diwaniyah province in Iraq. The 200 faecal samples from street dogs from various regions were floated, sedimented and then tested by direct smear. We identified molecularly using PCR from the 18S rRNA gene and sequenced and phylogenetic. A 19% prevalence of *Cystoisospora* spp. was observed under a microscope, the province's first known case of the parasite. In our infection dogs, the infection rates were higher in females, 22.34%, than in males, 16.03%. Younger dogs (puppies) were much more familiar, with prevalence of 38.02%, compared with 8.52% for older dogs. *Cystoisospora* DNA was found in 56% (17/30) of the specimens by PCR. Sequence alignment of the 511 bp region of the 18S rRNA gene identified 100% identity with *Cystoisospora canis* strains already in GenBank from Canada. The strains in the locality were identified as *Cystoisospora canis* by phylogenetic recognition, including the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The molecular phylogenetic data from this research on *Cystoisospora canis* in Iraqi street dogs represents the first such molecular phylogenetic data on this parasite in Iraq and adds to the epidemiology of this parasite in Iraq.

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Introduction

Dogs on the loose are a significant reservoir for disease, particularly in Asian countries where the populations are notoriously free of management and represent serious public health risks (1,2). These are the animals who are vectors of various disease viruses for people and other animals, thereby contributing to outbreaks of zoonotic diseases. Out of all these organisms, intestinal parasites are especially worrisome, being very transmissible and capable of causing severe gastrointestinal damage in infected patients. The scourge of this public health problem is made worse by the stray dog populations in both cities and rural communities, where low levels of veterinary care and saline provide ideal conditions for the growth of these parasites (3). These are parasites that cause all kinds of gastrointestinal disorders, some of which are life-threatening (4). Coccidiosis is a protozoal disease of the family Eimeriidae that is endemic to wild animals and domesticated ones, with a high contagiousness rate and spread throughout the animal kingdom (5). Vomiting, abdominal pain, inappetence, anorexia, thirst and diarrhoea (sometimes bloody faeces) are the symptoms of coccidiosis (6,7). Usually, coccidiosis is diagnosed using these clinical symptoms and the detection of Isospora oocysts in stools (8). It's a severe illness, particularly in younger or immunocompromised animals, where infection can result in serious complications or death (6). A few coccidian parasites infect dogs, *and Isospora spp. is* the most frequent (9). Within this genus, *Cystoisospora* spp. are apicomplexan protozoan parasites that cause disease

in dogs with a global prevalence of 1-5% (10,11). *Cystoisospora* infections, while usually ruled nonpathogenic, are sometimes clinically relevant, especially in diarrhoea, where they are particularly prevalent (12). Also, new *Isospora* species have kept our knowledge of the genus growing; more than 248 species have been named since 1986 (13). That means new diagnostic and epidemiological research is urgently needed to detect and describe new species (14). At the molecular level, the ribosomal DNA (rDNA) molecule is an essential genetic tag since it is involved in the formation and function of ribosomes. rDNA primary and secondary structure is so well conserved in many taxa that it's a goldmine for phylogenetic analysis. These preserved structures help to align rDNA sequences precisely so scientists can make strong phylogenetic inferences. By looking at regions of the rDNA molecule, scientists can check for evolutionary correspondences and better map parasite diversity and category. This molecular technique is now a standard tool in *Cystoisospora* spp. Research allows strains to be more precisely identified and categorized, understanding their evolutionary history (15).

The current study aimed to discover the presence and molecular features of *Cystoisospora* among homeless dogs in Al-Diwaniyah province in Iraq.

Materials and methods

Ethical approve

The study was ethically approved by the Committee of Research Ethics in the College of Veterinary Medicine, University of Al-Qadisiyah, issued 1890, dated August 28, 2023.

Sample collection

They used 200 faecal samples from stray dogs of both sexes and ages. Samples were collected from dogs directly according to the protocols (16). All samples were in labelled plastic boxes for identification and contamination. The samples were kept in a cooler after they had been collected so that they would be preserved during transport to the research laboratory at the College of Veterinary Medicine. Once in port, the samples were thawed for analysis.

Coprological examination

Both faecal specimens were coprologically tested using flotation and sedimentation for *Cystoisospora* spp. The samples were floated for *Cystoisospora* oocysts. A beaker filled with about 4-5 g of excrement filled with tap water. It was then passed through a sieve to remove large pieces of debris, and the filtrate was transferred to new 15 ml test tubes. The tubes were centrifuged for 3 minutes at 1000 rpm, and the supernatant was discarded. Sheather's sugar solution was added to the sediment, muddled and centrifuged at the same speed for 5 minutes more. A drop of the floating film was tapped down with a pipette, applied to a microscope slide, and covered with a cover slip. The slides were inspected at 10x and 40x magnification with a microscope for *Cystoisospora* Oocysts (12).

Molecular study

Molecular analysis confirmed the *Cystoisospora canis* in the faecal samples, and the phylogenetic tree was built. This was genomic DNA extraction, DNA quantification, PCR and primer construction (17).

Genomic DNA extraction

Genomic DNA was taken from the faecal fluid using the AccuPrep® Stool DNA Extraction Kit (Bioneer, Korea). It was run as the manufacturer instructed to obtain quality DNA for downstream molecular analyses (17).

Genomic DNA estimation

We measured the concentration and purity of the isolated DNA with a Nanodrop spectrophotometer (THERMO, USA). Pure DNA was measured by 260 nm and 280 nm absorbance for good quality to amplify by PCR (17).

Polymerase chain reaction

The PCR was done on the faecal samples to find *Cystoisospora canis* DNA. We amplified it with a thermal cycler per the kit manufacturer's instruction manual. This process involves denaturation, annealing, and extension to amplify target sequences unique to *Cystoisospora canis* (17).

Primers

The primers (F: CCTCTGGAAGGGCAGTGTTT and R: TGCCGGAATTTCACCACGTA; 511bp) for the *Cystoisospora* 18S ribosomal RNA gene (badger isolate 02/04964) were constructed using NCBI Gene database. They were created by Bioneer Company (Korea). The primers were tuned for sensitivity and efficiency for *Cystoisospora canis* detection from faecal DNA.

Results

By Flotation of 200 faecal samples, 19% of the faecal samples were positive for *Cystoisospora oocysts*. The other 81% of samples were *Cystoisospor*a Oocyst-free, indicating no infection in most of the studied population; they suggest the prevalence of *Cystoisospora* in the sample (Figure 1 and Table 1). Table 2 shows the prevalence of *Cystoisospora* parasite among 106 males examined, recorded a low infection rate of 16.03%. In contrast, the prevalence among 94 female dogs examined recorded a high infection rate of 22.34%, and the total infection rate was 19%. Table 3 shows a higher prevalence of Cystoisospora sp. was recorded in Puppies 71(38.02%) compared to adult group 129 (8.52%).

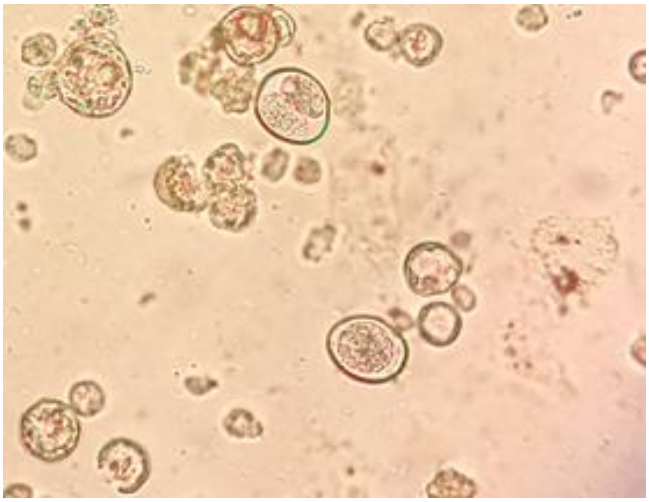

Figure 1: Oocyst of *Cystoisospora* spp.

Table 1: Overall prevalence of *Cystoisospora* in dogs

Status	Number (%)
Infected	38 (19%)
Not Infected	162 (81%)
P value	0.001*

* Significant difference.

Table 2: *Cystoisospora* sp. prevalence with sex

Sex	Examined n	Infected n(%)
Male	106	17 (16.03)
Female	94	21 (22.34)
Total	200	38 (19.00)
P value	0.307	

* Significant difference.

Table 3: *Cystoisospora* sp. prevalence with sex with age

Sex	Examined n	Infected n(%)
Puppies	71	27 (38.02)
Adults	129	11 (8.52)
Total	200	38 (19.00)
P value	0.00001*	

* Significant difference.

PCR results

Using PCR analysis, *Cystoisospora* spp. This was detected in 56% (17 out of 30) of the fecal material from dogs. The probes were detected with specific primers against the *18S rRNA* gene that amplified a 511 bp segment. The PCR products were electrophoresed on an agarose gel, and *Cystoisospora* DNA was found in the positive samples (Figure 2).

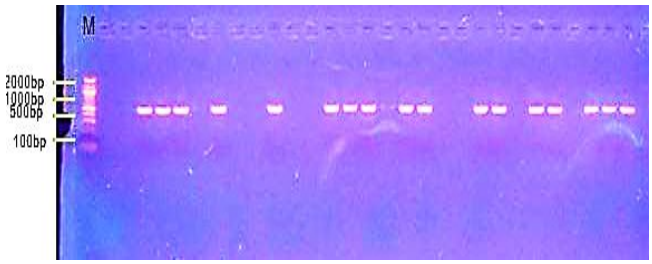

Figure 2: Agarose gel electrophoresis image shows the PCR product analysis of DNA extracted from faecal samples of stray dogs, where Lane M: ladder (2000-100bp), lanes 3, 4, 5, 7, 10, 13, 14, 15, 17, 18, 21, 22, 24, 25, 27, 28, and 29 show positive *Cystoisospora* spp, at 511bp PCR product size.

DNA sequencing and phylogenetic tree construction

The local *Cystoisospora* spp. Isolates collected during this research were submitted to the NCBI GenBank database, with accession numbers assigned to each sequence (Table 4). The entire sequence reads of the 18S rRNA gene was used to confirm all four isolates as *Cystoisospora canis*. This genetic identification robustly confirms the species-level classification, enabling confidence in the molecular results. Phylogenetic analysis of the evolutionary relationships was done using MEGA version 6.0, a popular tool for molecular evolutionary analysis. The tree generated from this study showed that all the isolates of *Cystoisospora canis* reported here were phylogenetically related to an earlier identified *Cystoisospora canis* isolate from Canada (entirely known from NCBI GenBank accession number KT184362.1). The local isolates shared 100% sequence identity with this Canadian reference strain, which is high genetic homology and implies the potential for this gene to be conserved in different geographical locations (Figures 3 and 4).

Table 4: NCBI-BLAST Homology Sequence identity (%) between local *Cystoisospora canis* isolates and NCBI-BLAST submitted Cystoisospora *canis* isolates

Cystoisospora canis isolate	GenBank Accession number	GenBank Accession number	County	Identity (%)
Isolate number 1	MK967970	KT184362.1	Canada	100%
Isolate number 2	MK967971	KT184362.1	Canada	100%
Isolate number 3	MK967972	KT184362.1	Canada	100%
Isolate number 4	MK967973	KT184362.1	Canada	100%

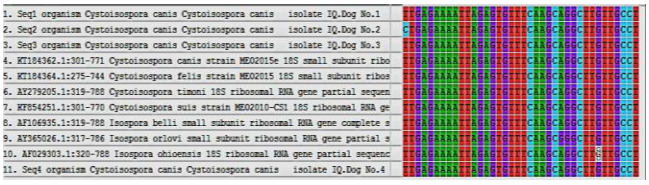

Figure 3: Multiple sequence alignment between local *Cystoisospora canis* isolates obtained here and *Cystoisospora canis* small subunit ribosomal RNA (18S rRNA) gene sequences of these local isolates and *Cystoisospora canis* were also performed. NCBI GenBank database). Alignment was done using the ClustalW alignment utility that is part of MEGA version 6.0, an internationally accepted molecular sequence analysis software. Alignment yielded high levels of nucleotide agreement across the sequences, as you can see, with conserved areas displaying an asterisk (*).

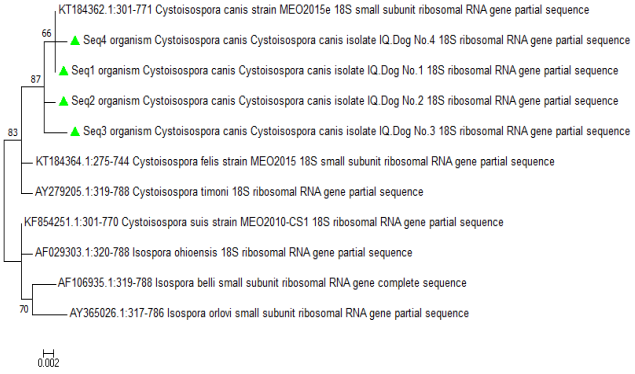

Figure 4: Phylogeny tree from the partial sequence of the small subunit ribosomal RNA (18S rRNA) gene to genetically classify *Cystoisospora* species. The tree was drawn using the unweighted pair group method with an arithmetic mean (UPGMA) of MEGA 6.0. It was found that the local isolates of *Cystoisospora* (No. 1 to No. 4) closely matched an NCBI-BLAST isolate of *Cystoisospora canis* (accession number KT184362.1).

Discussion

Dogs worldwide are infected with *Cystoisospora*, and the only way to diagnose this parasitic disease is to find oocysts in their faeces. Genus *Cystoisospora* species have long been identified by their oocyst shapes. However, such morphological species-level identifications are limited, and molecular methods now permit species-level identification (17).

In this work, by Flotation, 19% (38/200) of faecal samples taken from abandoned dogs tested positive for Isosporiosis. It is not more prevalent than in other geographical areas. In disagreement with the current study finding, in the Hantana district in Sri Lanka, for example, the prevalence was 76.7%, compared to 21.4% in Baquba City in Iraq (18,19). These geographical differences in prevalence might be explained by differences in climate, weather conditions (temperature and humidity), diet, immune function and sanitation. Hot and humid areas, for instance, are more likely to support and sporulate Cystoisospora oocysts, potentially increasing infection (17).

The present study showed more Isosporiosis in female dogs, 22.34%, than in male dogs, 16.03%. This matched a survey from the Hantana district, which found 100% female stray dogs (18). This higher incidence in women could be explained by hormonal and physiological differences and even possible exposure in a reproductive cycle, when the immune system may temporarily be turned off.

Old age was the primary determinant of infection rates; puppies had much higher rates, 38.02%, than adults, 8.52%. Other researchers have seen similar findings with up to a 100 per cent rate in puppies under 1 year old (18). The puppy rate is high because the mother's poop may contain infective oocysts, and dogs often ingest it. Puppies are usually low in the immune system and, therefore, more vulnerable to infection. It's one reason that proper sanitation and control are so essential to avoid spreading in young animals (12).

This was the first molecular analysis of *Cystoisospora canis* performed in Iraq, and it adds information to the parasite's worldview. The species identification was carried out by microbial methods, including PCR using the 18S rRNA gene. This method corresponds with the findings in Japan, where Cystoisospora detection using the 18S rRNA gene has proven successful (20). Molecular phylogenetics can be particularly useful in understanding evolutionary relationships among taxa and identifying host-parasite relationships that are hard to see from morphology alone (21).

When the phylogenetic data for this research was presented, local *Cystoisospora canis* isolates were identified as part of a unique clade of canine hosts. We BLASTed the 18S rRNA gene sequences to detect a 100% match between the local isolates and an earlier reported *Cystoisospora canis* isolate from Canada (GenBank accession no. KT184362.1). This striking genetic homology also suggests slight genetic variation among *Cystoisospora canis* populations (regardless of location). These isolates grouped in the same node may result from the parasite being carried over regions by paratenic hosts (rodents, birds, etc) (22-30).

The microbial techniques can now be used to obtain accurate species-level identification and provide better information on the diversity of these parasites. Those variations in prevalence will probably depend on environmental factors: climate and weather conditions, such as temperature and humidity, diet, immune status and sanitation (31-36). The warmer, moister climates, for example, could favor the sporulation of Cystoisospora oocysts and thus the chances of infection. In addition, this

current study found that isosporiosis was more common in female dogs at 22.34% than in males at 16.03%. This higher incidence in females might be due to hormonal and physiological variations, especially in reproductive years when the immune system can be temporarily shut down. Other factors that influenced infection were age. This is also the first molecular examination of Cystoisospora canis performed in Iraq and reveals something new about the parasite's genetic variation and evolutionary history (37-44).

Conclusion

This research demonstrates the value of molecular methods such as PCR and phylogenetics in determining the right Cystoisospora species and their evolutionary relationships. The minimal genetic difference among *Cystoisospora canis* isolates in different regions implies that the 18S rRNA gene is highly conserved and a sound species-hunting candidate. Phylogenetic analysis can tell us about parasite migrations and inter-regional connections made by wildlife. All these findings make integrating molecular techniques into parasitological studies and surveillance imperative to further improve parasite ecology and epidemiology.

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Conflict of interest

There is no conflict of interest for the current work.

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التمييز الجزيئي وتحليل شجرة النشوء والتطور لمتماثلة الأبواغ الكلبية في الكلاب السائبة في مدينة الديوانية، العراق

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الخلاصة

تعد الأبواغ من طفيليات الأوالي التابعة لصنف الابيكومبليكسا، وتبلغ نسبة انتشارها عالميا بين الكلاب ١-٥%. هدفت هذه الدراسة إلى الكشف عن وجود السيستوايزوسبورا ودراسة خصائصها الجزيئية بين الكلاب الضالة في محافظة الديوانية في العراق. تم جمع ٢٠٠ عينة براز من كلاب الشوارع من مناطق مختلفة، وتم فحصها باستخدام تقنيات الطفو والترسيب والفحص المباشر. تم تحديد الطفيليات جزيئيا باستخدام تقنية تفاعل البلمرة المتسلسل واستهداف القطعة الصغيرة للجين الريبوسومي، كما تم تحليل التسلسل وتحديد شجرة النشوء والتطور .أظهرت الفحوصات المجهرية وجود نسبة انتشار ١٩% لطفيلي الأبواغ، وهي أول حالة مسجلة لهذا الطفيلي في المحافظة. وُجد أن معدل الخمج كان أعلى بين الإناث (٢٢,٣٤%) مقارنة بالذكور (١٦,٠٣%). كما كان الخمج أكثر شيوعا بين الكلاب الصغيرة (الجراء)، حيث بلغت نسبة الإصابة ٣٨,٠٢% مقارنة بـ ٨,٥٢% بين الكلاب الأكبر سنا .تم الكشف عن الحمض النووي لطفيليات الأبواغ في ٥٦% (٣٠/١٧) من العينات باستخدام تقنية تفاعل البلمرة المتسلسل. وأظهر تحليل محاذاة التسلسل لمنطقة ٥١١ زوجا قاعديا من القطعة الصغيرة للجين الريبوسومي تطابقا بنسبة ١٠٠% مع سلالات الأبواغ الكلبية المسجلة سابقا في قاعدة بيانات بنك الجينات / كندا. تم تحديد السلالات المحلية على أنها الأبواغ الكلبية باستخدام تمييز شجرة النشوء والتطور، بما في ذلك طريقة المجموعة الزوجية غير المرجحة مع المتوسط الحسابي تمثل البيانات الجزيئية والنشوء والتطور التي تم الحصول عليها من هذه الدراسة حول الأبواغ الكلبية في الكلاب الضالة في العراق أول تحليل من نوعه لهذا الطفيلي في العراق، مما يضيف إلى فهم وبائيات هذا الطفيلي في البلاد.