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Molecular detection and phylogenetic of *Cryptosporidium* spp. of birds in the city of Al-Diwaniyah, Iraq

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Abstract Many vertebrate species, including birds, are susceptible to the important protozoan Cryptosporidium. Evaluating the public health risk associated with Cryptosporidium requires a thorough understanding of species diversity and their distribution across populations. Poultry feces samples were obtained from Al-Diwaniyah, Iraq, and the aim of this study was to investigate their molecular identity and phylogenetic patterns for Cryptosporidium species identification, 70 feces samples were collected from birds and molecular identification by Nested PCR Using phylogenetic analysis and sequencing of positive PCR results was used to identify Cryptosporidium spp. The molecular method had a much higher detection rate than microscopic observation, 8 samples (11.42% of total) showed Cryptosporidium oocysts, while 18 samples (25.71% of total) showed positive results from nested PCR in Phylogenetic analysis showed Three Cryptosporidium species: Cryptosporidium baileyi (61.11%), . Cryptosporidium gallii (26.77%), and Cryptosporidium melegridis (11.11%) comparing local Cryptosporidium isolates from the NCBI GenBank database with expression sequences ranging from 97.80% to 99.45%. Up to the genetic homogeneity known Iraq The birds of Al-Diwaniyah have been infected with various Cryptosporidium species, and this study provides the first molecular data on this topic. these results demonstrate the importance of accurate identification of Cryptosporidium species and how this virus can be transmitted from birds to humans in the field using simple genetic methods to date.

Keywords: Birds, Cryptosporidium, nested PCR, phylogenetic analysis

Introduction

The zoonotic protozoan parasite Cryptosporidium infects a wide variety of vertebrate groups, including humans, and cattle [1,2 Diarrhea, abdominal pain, diarrhea, and vomiting are gastrointestinal disorders, a major cause of infection have some symptoms [3] The disease usually resolves spontaneously in healthy individuals, but Serious complications or even death can occur in humans of their immune systems [4] It is well documented that birds can be infected with several species of Cryptosporidium such as C. baileyi, C. meleagridis, C. galli [5,6] humans harbor these Cryptosporidium species, which prefer birds, and contact with diseased birds or contaminated droppings, or will be exposed to contaminated food or water by ingestion [7,8] Since As Cryptosporidium is a pathogen, it is important to understand the distribution and diversity of species in avian populations to assess risks to public health Lack of information on frequency and genetic analysis on Cryptosporidium species infecting birds in Iraq was previous research in Al-Diwaniyah area using microanalysis found Cryptosporidium oocysts in bird feces samples [9] . Molecular-based methods are needed to better identify Cryptosporidium species and their genetic relatives. This study set out to investigate the possibility of detection and identification of Cryptosporidium species in bird feces samples taken in Al-Diwaniyah, Iraq This study will help fill the gap in our knowledge of cryptosporidiosis epidemiology in the region addressed to shed light on the possibility that parasites can be vectors of parasites transmitted from birds to humans will expand on the subject.

Materials and methods

Ethical approval

The study was approved (1890) in 28/8/2023 issued by the College of Veterinary Medicine, University of Al-Qadisiyah, Iraq.

Study design and sample collection

The cross-sectional study was conducted from March to June 2024 in Al-Diwaniyah, Iraq. In total, 70 specimens of bird were obtained from wild birds. After collection, samples were placed in sterile

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containers and brought to the laboratory in a freezer box for analysis. Backyards, farms and public parks in Al-Diwanyiah were among the places from which the birds were randomly selected. The ease and availability of specimens determined the birds used in the study. No specific criteria were used to select the species to ensure that the sample was truly representative of the local bird population. A label was used to record the date of collection. Samples were kept at 4 °C until ready for analysis. The Institutional Review Committee, Al Qadisiyah University, Iraq, gave the stamp of approval for this study.

Microscopic examination

Following the guidelines of the World Health Organization, we used the modified Ziehl-Neelsen (ZN) staining method to detect Cryptosporidium oocysts in fecal samples [11] . In summary, in this method the smears were air dried, fixed with methanol, stained with carbol fuchsin, decolorized with 1% acid alcohol and counterstained with methylene blue for small, spherical, Cryptosporidium oocysts. bright red was present At 1000x magnification for detection Used with a light microscope used for slide analysis

Molecular detection and phylogenetic analysis:

DNA extraction: Genomic DNA was extracted from the stool samples using a commercial DNA extraction kit (Geneaid DNA Stool Mini Kit, Taiwan) according to the manufacturer's instructions.

Nested PCR: A nested PCR targeting the 18S rRNA gene of Cryptosporidium was performed as described by Rafiei et al. [12]. The first round of PCR used the outer primers 18SFor (5'-TTCTAGAGCTAATACATGCG-3') and 18SRev (5'-CCCTAATCCTTCGAAACAGGA-3'), which amplify a 1325 bp fragment. The second round of PCR used the inner primers 18SnestedFor (5'-GGAAGGGTTGTATTTATTAGATAAAG-3') and 18SnestedRev (5'-AAGGAGTAAGGAACAACCTCCA-3'), which amplify a 830 bp fragment. The amplified products were visualized on a 1.5% agarose gel stained with ethidium bromide.

Sequencing and phylogenetic analysis: Positive Nested PCR results were purified and sequenced by Sanger sequencing. The obtained sequences were analyzed using Basic Local Alignment Search Tool (BLAST) to identify Cryptosporidium species. To determine the genetic relatedness of Cryptosporidium species, we ran a phylogenetic tree using the Neighbor-Joining method of MEGA X (Kumar et al., 2018) and reference sequences from the NCBI GenBank database.

Statistical analysis

The data were analyzed using the chi-square (χ2) test to determine the statistical significance of the differences in the prevalence of Cryptosporidium species between age groups and gender. A p-value of less than 0.05 was considered statistically significant by using SPSS.

Results

Microscopic examination and molecular detection:

The present study used Ziehl-Neelsen (ZN) staining to detect Cryptosporidium in poultry fecal samples. The uterus was found to be small, round, smooth, and single-walled. Bright red could be seen on a blue background, as shown in Fig. (1). Cryptosporidium was distinguished from other bacteria by analyzing the results for loss of spore sac, oocyte size and wall structure. Cryptosporidium sp. are shown in Table (1). Based on microscopic examination, 8 out of 70 samples (11.42% of the total) were positive for Cryptosporidium sp. The PCR test yielded a high number of positive samples, as shown in Figure (2). 24.71 percent was the result of sample 18. The findings of the microscopic and PCR analyzes were significantly different (p-value = 0.030), the latter being associated with Cryptosporidium sp. In a higher proportion of samples.

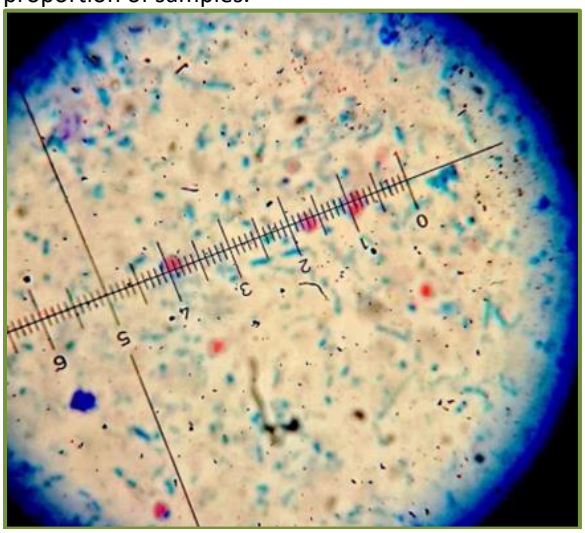

Figure 1: shows the oocyst in fecal samples (Birds) with Ziehl-Neelsen stain under a microscope (objective lens.40X).

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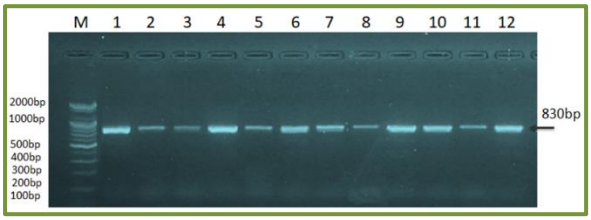

Figure 2: The Nested PCR product electrophoresis of bird DNA samples. M: (DNA marker 2000-100bp. The lane 1-12 show some positive Cryptosporidium sp. at (830bp) product size.

Table 1: Microscopic and PCR results/ birds.

Total examined samples	Microscopic exam. positive samples		RCR exam. positive samples	
	No.	%	No.	%
70 samples	8	11.42	18	25.71
X^2	4.72			
P value	0.030 (S)			

S: Significant difference at $p<0.05$

Distribution of infection according to the sex in bird

Positive samples are distributed for detection of Cryptosporidium sp. The infection was determined by the sex of the birds as shown in Table (2). A total of 70 birds were examined. They included 32 male and 38 female birds. Microscopic examination revealed eight high-quality specimens. Males constituted 9.37% and females 13.15% of the normal sample. With a total of 18 positives, the PCR analysis yielded a good number of positive samples. Feathered birds made up 21.05 percent of the samples obtained, with 10 samples from males and 8 from females. Microscopic examination (p=0.620) and polymerase chain reaction (PCR) analysis (p=0.331) showed no statistically significant changes in the frequency of positive samples obtained in molluscs.

Table 2: Distribution of positive samples according to the sex /birds.

Gender	Total No.	Microscopic Positive No. and %	PCR Positive No. and %
Male	32	3 (9.37)	10 (31.25)
Female	38	5 (13.15)	8 (21.05)
X^2		0.246	0.946
P value		0.620 (NS)	0.331 (NS)

NS: No significant difference at $p<0$.

DNA sequencing and phylogenetic relationship among DNA samples in bird

Figure 3 shows that, of the total genetic variation (0.02-0.01%), local Cryptosporidium sp. IQB-frames containing Cryptosporidium baileyi (DQ898161.1) and local Cryptosporidium sp. NCBI-BLAST of IQB feathers showed a strong association with Cryptosporidium galli (MG516766.1). Only 2 local Cryptosporidium sp. NCBI-BLAST isolates from IQH birds showed strong relatedness to Cryptosporidium meleagridis (KT151537.1), as shown in the phylogenetic tree.

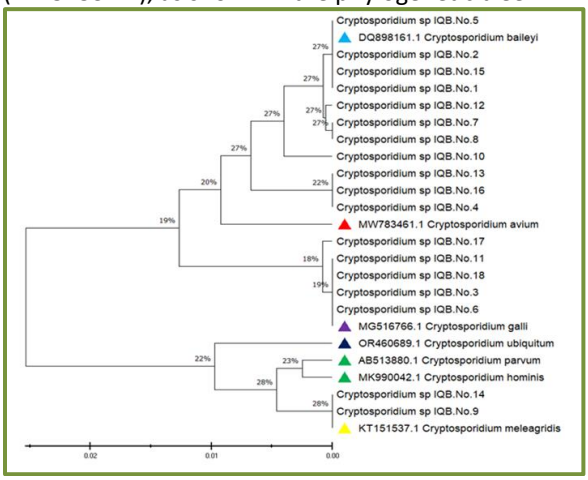

Figure 3: Phylogenetic tree analysis based 18S ribosomal RNA gene partial sequence in 18 local Cryptosporidium species from Birds that used for genetic species typing analysis. The phylogenetic tree was constructed using the Neighbor-Joining method in (MEGA 6.0 version).

The homology sequence identity between local Cryptosporidium spp. in Birds closed related to NCBI-BLAST Cryptosporidium species were showed genetic homology sequence identity ranged from (97.80-99.45%) as show in table (3)

Cryptosporidium sp. isolate	Accession number	Homology sequence identity (%)		
		Identical Cryptosporidium sp.	**Accession number**	**Identity (%)**
IQB.No.1	PQ192188	*Cryptosporidium baileyi*	DQ898161.1	99.15%
IQB.No.2	PQ192189	*Cryptosporidium baileyi*	DQ898161.1	98.18%

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IQB.No.3	PQ192190	*Cryptosporidium galli*	MG516766.1	99.40%
IQB.No.4	PQ192191	*Cryptosporidium baileyi*	DQ898161.1	98.50%
IQB.No.5	PQ192192	*Cryptosporidium baileyi*	DQ898161.1	98.18%
IQB.No.6	PQ192193	*Cryptosporidium galli*	MG516766.1	99.40%
IQB.No.7	PQ192194	*Cryptosporidium baileyi*	DQ898161.1	98.75%
IQB.No.8	PQ192195	*Cryptosporidium baileyi*	DQ898161.1	99.40%
IQB.No.9	PQ192196	*Cryptosporidium meleagridis*	KT151537.1	99.45%
IQB.No.10	PQ192197	*Cryptosporidium baileyi*	DQ898161.1	98.75%
IQB.No.11	PQ192198	*Cryptosporidium galli*	MG516766.1	99.40%
IQB.No.12	PQ192199	*Cryptosporidium baileyi*	DQ898161.1	99.3A5%
IQB.No.13	PQ192200	*Cryptosporidium baileyi*	DQ898161.1	98.50%
IQB.No.14	PQ192201	*Cryptosporidium meleagridis*	KT151537.1	99.45%
IQB.No.15	PQ192202	*Cryptosporidium baileyi*	DQ898161.1	99.18%
IQB.No.16	PQ192203	*Cryptosporidium baileyi*	DQ898161.1	98.50%
IQB.No.17	PQ192204	*Cryptosporidium galli*	MG516766.1	98.55%
IQB.No.18	PQ192205	*Cryptosporidium galli*	MG516766.1	99.40%

Genotypes of cryptosporidium samples in bird

Of the 18 samples tested positive for Cryptosporidium in bird samples, 11 were found to be Cryptosporidium baileyi (61.11%), as shown in Table (4), Cryptosporidium galli was the most frequently detected, with five associated samples to (27.77%). Two samples or 11.11% were Cryptosporidium melegridis, which has some statistical results but is not associated with the distribution of Cryptosporidium species in avian samples.

Table 4: DNA sequence species typing/ birds

Species	No.	%
Cryptosporidium baileyi	11	61.11
Cryptosporidium galli	5	27.77
Cryptosporidium meleagridis	2	11.11
Total	18	100
X^2	10.5	
P value	0.005 (HS)	

HS: Highly significant difference at $p<0.05$

Discussion

Molecular data on Cryptosporidium species infecting birds in Al-Diwaniyah, Iraq, have never been collected before the present study The results identified three Cryptosporidium species: C. baileyi, C. galli, and C. meleagridis. C. baileyi was shown to be the most common. The nested PCR had a significantly higher detection rate of Cryptosporidium (25.71%) than the microscopic examination (11.42%), indicating the sensitivity of molecular methods for the detection of parasites. This is consistent with other studies indicating that methods a based on PCR for Cryptosporidium in birds and other animals than conventionally meets microscopic approaches It has been well studied [13,14. The results of this study support others who sampled birds from different parts of the world and found that C. baileyi is the most common species when it comes to domesticated wild

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birds, C. baileyi is the most common species of Cryptosporidium far more than [15-16]. Chickens, turkeys, ducks, and various wild birds are among the many groups of birds known to be infected by this species, suggesting a suitable host [17,18]. Also of note is the presence of Cryptosporidium gallii and Cryptosporidium melegridis in avian samples. C. galli, a new species, is present in both wild and domestic birds [19,20] Birds are susceptible to respiratory and gastrointestinal diseases from these species [21,22] . In contrast, C. meleagridis is an endemic Cryptosporidium that can infect humans and a wide range of avian species [23,24] . There is a reason for public concern about possible cross-species transmission between birds and humans as the genetic similarity of local Cryptosporidium isolates varies from 97.80% to 99.45% compared to reference sequences in the NCBI GenBank database of the bird samples containing the Tight genetic of this species ho which relationships between described Cryptosporidium species showed Previous studies revealed a great deal of genetic similarity among Cryptosporidium isolates from different regions [25,26], which is consistent with our present findings Birds can be reservoirs and bacterial vectors for Cryptosporidium species, e.g. Cryptosporidium species can occur in birds as evidenced by the presence of these organisms in experimental bird specimens in close contact between birds and humans or when bird droppings contaminate water and food and play an important role in cryptosporidiosis epidemiology [27-28]. The results of this study highlight the need to monitor bird populations in the Al-Diwaniyah area of Iraq for public health and epidemiological purposes. Cryptosporidium infection rates should be closely monitored. Further research is needed in terms of both the susceptibility of the local population to cryptosporidiosis and possible routes of avian transmission.

Conclusion

Finally, molecular identification of birds infected with Cryptosporidium species in Al-Diwaniyah, Iraq, molecular identification and phylogenetic characterization is supported by current work on the spatial potential of animal vectors this and the importance of birds in the epidemiology of cryptosporidiosis. as of the zoonotic C. melegridis, the discovery highlights These results add to what is already known about Cryptosporidium species and prevalence in Iraq and highlight the need for continued surveillance and public health measures to Cryptosporidium species the availability has decreased.

Acknowledgments

Not applicable

Conflicts of Interest

The authors declare there is no conflict of interest.

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