Research Article

Traditional and Molecular Detection of *Cryptosporidium* Parasite in Quails (*Coturnix coturnix*) in Baghdad Province, Iraq

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Abstract | Cryptosporidiosis is a wildword disease among birds**.** Detection of cryptosporidiosis in quails required to be more sensitivity and specificity using molecular analysis and sequences. A total of 100 fecal samples were collected from quails in various parts of Baghdad/ Iraq from January to September, and they were then tested for the presence of *Cryptosporidium* spp. using molecular methods (PCR), followed by DNA sequencing. The overall infection rate was 37% (37/100) of the samples were *Cryptosporidium*-positive by using PCR. Three of five quails whose *18S gene* was amplified by nested PCR and sequenced were found to belong to *C. meleagridis* in the sequence of this gene deposit on the website of the National Center for Biotechnology Information (NCBI) under accession number (HQ917077.1), while the others were found to be closely related to *C. baileyi* and had the accession number (MN410723.1). The finding suggests that *Cryptosporidium* parasites, which cause zoonotic disease transmission, are widespread among Baghdad quails.

Keywords | *Cryptosporidium*, Quail; nested PCR; Detection; Baghdad.

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INTRODUCTION

Among the most common enteric protozoan parasite that affects a variety of host animals is *Cryptosporidium*. Many *Cryptosporidium* spp. that have been linked to zoonotic illnesses across the globe include *C. andersoni C. baileyi, C. bovis, C. canis, C. cuniculus, C. erinacei, C. fayeri, C. felis, C. hominis, C. meleagridis, C. muris, C. parvum, C. scrofarum, C. suis, C. tyzzeri, C. ubiquitum, and C. xiaoi,* etc. (Ryan et al., 2021).

Tyzzer, (1929) initial description of Cryptosporidiosis in birds involved the caeca of a chicken. Numerous domestic, pet, commercial, and wild birds, including chicken, turkey, quail, duck, ostrich, pheasant, seagull, peafowl, psittacine, finches, cranes, falcon, sparrow, pigeon, flamingo, vulture, crow, dove, geese, and waterfowl, have been isolated and reported to harbor *Cryptosporidium* spp. (Kabir et al., 2020; Hamza and Hameed, 2020a). Avian species *C. andersoni, C. baileyi, C. muris, C. parvum,* and *C. meleagridis* which was the most significant zoonotic protozoan that can infect humans and responsible for 10% of zoonotic transmission (Faraj, 2014; Lu et al., 2022). Clinical sign involves intestinal Cryptosporidiosis characterized by brown foamy diarrhea, and respiratory Cryptosporidiosis show several signs, the most important of which are difficulty breathing, sneezing, and mucous respiratory secretions from nostrils (Wang et al., 2021). The diagnosis of Cryptosporidiosis in birds is depend generally on the traditional and more sensitive molecular test. Because of the widespread prevalence of this parasite and its veterinary and economic importance to it, the study was designed to explore the spread of the *Cryptosporidium* parasite in quail (*Coturnix coturnix*) and identified the species by sequences analysis of some samples.

Table 1: *Cryptosporidium* spp. primers of the nested PCR.

Round		Primer sequence	Produce size
First round	Crypto18S1F	TTCTAGAGCTAATACATGCG	900 bp
	Crypto18S1R	CCCATTTCCTTCGAAACAGGA	
Second round	Crypto18S2F	GGAAGGGTTGTATTTATTAGATAAAG	830bp
	Crypto18S2R	CTCATAAGGTGCTGAAGGAGTA	

Table 2: the NCBI-BLAST Homology Sequence identity percentage between *Cryptosporidium* species quails isolates and NCBI-BLAST closed genetically related *Cryptosporidium* species isolate

***Cryptosporidium* Species isolate**	**Accession number**	**Homology sequence identity (%)**			
		Identical *Cryptosporidium* spp.	**Accession number**	**Related country**	**Identity (%)**
Cryptosporidium sp. Quails No. 1 isolate	OP420778.1	*Cryptosporidium baileyi*	MN410723.1	China	99.72%
Cryptosporidium sp. Quail No. 2 isolate	OP420779.1	*Cryptosporidium meleagridis*	HQ917077.1	China	99.74%
Cryptosporidium sp. Quails No.3 isolate	OP420780.1	*Cryptosporidium meleagridis*	HQ917077.1	China	99.74%
Cryptosporidium sp. Quails No.4 isolate	OP420781.1	*Cryptosporidium meleagridis*	HQ917077.1	China	99.74%
Cryptosporidium sp. Quail No. 5 isolate	OP420782.1	*Cryptosporidium baileyi*	MN410723.1	China	99.86%

MATERIALS AND METHODS

Field Study

Fresh dropping samples were collected from 100 quails in Baghdad at different ages from January 2022 to September 2022. Samples were collected and transferred to the parasitology laboratory of the Veterinary College University of Baghdad,

Samples collection

Each sample was divided into two parts, the first for direct examination for parasite investigation by both direct examination and confirmed by modified Ziehl-Neelson stain and the second was stored at -20 °C for molecular techniques examination which was done in the internal and preventive medicine department of the veterinary college. All samples were examined by nested PCR targeting the *18S rRNA* gene as described below according to the method described by (Yu et al., 2009).

Extraction of Genomic DNA

Dropping-sample-based extraction was conducted by AccuPrep® stool DNA Extraction Kit and according to the company protocol (Bioneer from Korea).

Primers of PCR

The lyophilized primers for the PCR were provided, and the final primer stock concentration of 100 Pico moles/µl was achieved by dissolving the primers in De-ionized water. Before usage, these were stored at -20°C. The working concentration of the primers in the entire PCR experiment was (0.5 Pico mole/ 20µl in the total PCR reaction). All these primers were supplied from Micro gen / Korea (Table 1) and all primer sequences used here were taken from (Xiao et al., 1999).

PCR reaction component preparation: PCR construction was prepared by using (Maxime PCR PreMix Kit) 10µl, 3µl of DNA template, 1µl of *18SrRNA* forward primer 1(10 p mol), 1µl of *18SrRNA* reverse primer1 (10 p mol), and 5µl of PCR water) for first round and (10µl master mix, 1µl DNA of PCR product, 1µl of *18SrRNA* forward primer 2 (10p mol), 1µl of *18SrRNA* reverse primer2 (10 pmol), and 7µl of PCR water). The PCR master mix component was placed in standard Maxime (PCR PreMix) which contains (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$, stabilizer, and tracking dye). Then, all the PCR tubes were transferred into an Exispin vortex centrifuge at (3000 rpm for 3 min.). Then placed in a PCR thermocycler. The cycling conditions consist of a hot start at 94°C for 5 min followed by 30 cycles with denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, and at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes then holding at 4°C forever (Ruecker et al., 2013).

Amplicon sequencing and analysis

Five amplicons were selected randomly for DNA sequenc-

ing and phylogenetic analysis after registering them in the gene bank (NCBI) in Macro Gen Company (Korea).

RESULTS

The oocysts of *Cryptosporidium* spp. under lenses by traditional methods appear oval to spherical shape by direct examination and red cycle body, blue background, and a clear hallow around it by modified Ziehl-Neelson stain Figure (1).

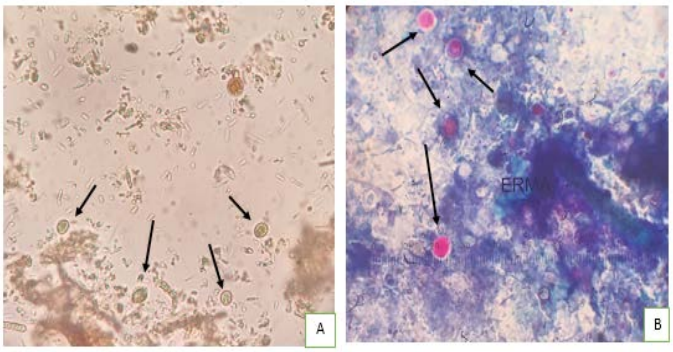

Figure 1: A: a direct examination of *Cryptosporidium* oocysts; B: an examination of *Cryptosporidium* oocysts by Modified Ziehl- Neelsen stain.

The infection rate of *Cryptosporidium* spp. in quails by using nested PCR technique

The molecular diagnosis showed that the infection rate in quails was 37% (37/100), the samples were collected randomly from 100 quails in Baghdad city. All drop samples showed a unique 830 bp on agarose gel for *Cryptosporidium* spp. as shown in Figure (2).

Figure 2: Agarose gel (1.5g/100 ml) electrophoreses of *Cryptosporidium spp*. genomic DNA image showed the PCR product analysis of a small subunit ribosomal RNA gene in *Cryptosporidium spp*. from a quails drop sample. Where M: marker (2000-100bp), lanes showed some positive *Cryptosporidium spp*. at (830 bp) PCR product.

Phylogenetic analysis of *Cryptosporidium* spp. in quails.

The *18S rRNA* gene was used for genetic species typing analyses in local *Cryptosporidium* species from quail-isolated *Cryptosporidium* species isolates from NCBI-Genbank. The phylogenetic tree relationship analysis revealed that the *Cryptosporidium* species quails isolates (No.2, No.3, and No.4) showed closed relationships to NCBI-BLAST *Cryptosporidium meleagridis* (HQ917077.1) and the *Cryptosporidium* species quails isolates (No.1 and No.5) showed closed related to NCBI-BLAST *Cryptosporidium baileyi* (MN410723.1) at total genetic change (0.01-0.04%) as showed in Figure (3).

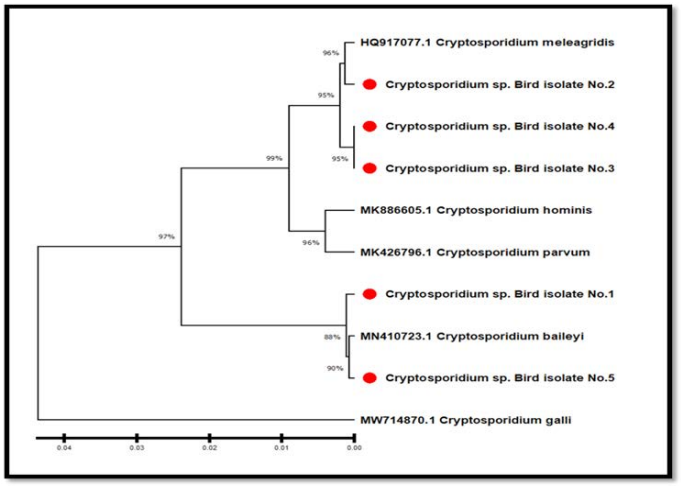

Figure 3: Phylogenetic tree analysis based on 18S ribosomal RNA gene partial sequence in local *Cryptosporidium* species from quail's isolates that were used for genetic species typing analysis. The phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree) in the (MEGA 6.0 version). The *Cryptosporidium* species quails isolates (No.2, No.3, and No.4) showed closed related to NCBI-BLAST *Cryptosporidium meleagridis* (HQ917077.1), and The *Cryptosporidium* species quails isolates (No.1 and No.5) showed closed related to NCBI-BLAST *Cryptosporidium baileyi* (MN410723.1) at total genetic changes (0.04-0.01%).

The genetic homology sequence identity from (88-98%) according to the phylogenetic tree similarity study between local *Cryptosporidium spp*. isolates and NCBI-Genbank-related *Cryptosporidium spp.* according to NCBI-BLAST results, the genetic homology sequence identity between local *Cryptosporidium* species quails closely related to *C. meleagridis* (HQ917077.1) and *C. baileyi* (MN410723.1) varied from (99.72-99.74%) Table (2).

DISCUSSION

Numerous spp. of *Cryptosporidium* have been identified

using a nested PCR technique that amplified DNA from just one oocyst and targeted the *18S rRNA* gene, which has been found to be very sensitive (Karanis et al., 2010; Thigeel, 2016) Compared to the modified Ziehl-Neelsen (mZN) staining method. Therefore, in the current work, *Cryptosporidium* spp. detection was focused on the *18S rRNA* gene fragment due to their repeating organization inside the genome even in the tiniest species that offers an abundance of template DNA for PCR. All living beings are subject to similar selective pressures on the *18S rRNA gene*, which is composed of the ribosomal functional core (Lambert et al., 2019). Nested PCR results showed that 37% of quails had an overall infection rate with *Cryptosporidium* spp.

This finding concurs with that of Al-Zubaidi et al. (2018) who discovered a 35% infection rate in broiler chickens. Al-Ghezey and Al-Zubaidi, (2020) found that the infection rate in layers was 36% which agrees with our result and disagrees with them the same research which recorded the infection rate of broilers as 64% and agreement with Al-Tamimi and Al-Zubaidi, (2021) in Babylon, who discovered that 13/21 (61.90%) of pigeons had been infected with *C. meleagridis*, followed by 7/21 (33.33%) with *C. baileyi*, and only one (1/21) (4.76%) with *C. hominis*. According to Hamza and Hameed, (2020b), ostriches had a 26.5% infection rate. In contrast, Al- Taei, (2015) discovered a 4.4% infection rate in broiler chickens in Babylon and also disagree with Jasim and Marhoon, (2015) in Al-Diwania discovered that the quail infection rate was 76.7%. This result is in conflict with Saeed et al. (2021) indicated that 31/160 (19.4%) of the sample were positive for *Cryptosporidium* a total of 26.6% (8/30) of turkeys, 17% (17/100) of the domestic chicken, and 23.3% (7/30) of wild ducks tested positive for *Cryptosporidium*. The lowest infection rate was 1.9% (5/263) recorded in Beijing Zoo and Harbin North Forest Zoo by Lu et al. (2022). Quails infected with coccidian have the highest infection rate (64.54%) recorded by (AL-Zarkoushi and AL-Zubaidi, 2022).

Birds are thought to spread a range of infections over the world, with the genus *Cryptosporidium* being among the most significant due to its widespread diagnosis in birds. There have been few studies that have attempted to identify *Cryptosporidium* in quails. The high rate of infection in various types of birds may be due to poor management of the birds in the same cage, stressing conditions, area of sampling (farming/ captive), the sampling methods and samples size the presence of small mammals in pet stores that may be infected with *Cryptosporidium* spp., and possibly other factors could have caused the spread of oocyst in the surrounding and birds may be considered as mechanical transporters of *Cryptosporidium* spp. (Al-khayat and Al-Zubaidi, 2015; Lin et al., 2022).

DNA sequencing and phylogenetic analysis have emerged as the most popular methods for identifying *Cryptosporidium* species isolated from various geographic and host origins. Based on the *rRNA* analysis used in this work, DNA sequences are identical to those used in several earlier investigations (Al-Amery and Al-Amery, 2022; Liao et al., 2018).

According to this study, quail *C. meleagridis* and *C. baileyi* sequences are present in the *18S rRNA gene* sequences which support the finding of Hamza and Hameed (2020 b), who discovered that ostriches in the central and southern districts of Iraq four different species of *Cryptosporidium*, including *C. parvum, C. meleagridis, C. baileyi,* and *C. galli.* The nested PCR test in Al- Diwaniya which was done by Saeed et al. (2020) found *C. meleagridis* in 50% of domestic chickens, 27% of turkeys, and 19% of wild ducks and show that *C. meleagridis* is widely distributed among domestic bird species. Our findings resembled those of Kabir et al. (2020), who recovered three cases of *Cryptosporidium spp*. from chicken in Bangladesh. These cases included *C. baileyi, C. meleagridis*, and *C. parvum*. Eight of 48 chicken samples from Hubei province tested positive for *C. baileyi*, and one had *C. meleagridis* infection (Liao et al., 2018). The result of the sequence analysis of the *Cryptosporidium spp*. in Algerian farm chicken broilers matched those of our study (Holubova et al., 2017). Lin et al. (2022) in China determined cryptosporidiosis in birds by 13.2% with two species *C. meleagridis* (7.8%) and *C. baileyi* (4.8%), Hotan Black Chinese Chicken have an infection with *C. meleagridis* and *C. baileyi* with infection rate 11.5% (Feng et al., 2022), according to Yao et al. (2017), another kind of birds in China called Java sparrows infected with *C. baileyi* about 13.42%. sequencing supported the existence of *C. parvum* and *C.baileyi* which is in contrast to Jian et al., (2021) findings, who investigated *Cryptosporidium spp*. in wild birds near Qinghai Lake in China with an infection rate of 8.98%.

By analyzing the *18S rRNA gene* at the molecular level, *C. meleagridis* and *C. baileyi* were confirmed. This finding was in line with earlier research on several bird species, which are key *Cryptosporidium* reservoirs. Both *C. meleagridis* and *C. baileyi* have regularly been found in humans all around the world, particularly in immunocompromised people like newborns and people with HIV/AIDS (Qamar et al., 2021). After *C. hominins* and *C. parvum*. *C. meleagridis* is the third pathogen that affects humans. Additionally, it was found in multiple investigations that humans and birds shared the same species, indicating that there may be cross-species transmission between birds and humans. Birds may act as a source of infection as well as a vector by shedding oocysts into the environment. Foreign travel is a key element in the spread of sickness, and the export

of birds from countries where the disease is endemic also helps (Zaheer et al., 2021).

CONCLUSIONS

Cryptosporidium spp. was widespread in quails and therefore the result obtained in the present study suggests that birds may be considered mechanical transporters of *Cryptosporidium* oocysts. The ducks, chicks, and quails could be purchased for these children and kept as pets inside households, becoming a risk for infection to humans of all ages.

RECOMMENDATIONS

Future studies might focus on investigating the prevalence of 'zoonotic' subtypes of *Cryptosporidium meleagridis* in various species of wild and domesticated birds, and on comparing them with those found in humans in Iraq and other countries.

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CONFLICTS OF INTEREST

There were no conflicts of interest during this investigation.

NOVELTY STATEMENT

There is no data available in Iraq about cryptosporidiosis in quails and due to the extensive prevalence of this parasite and its veterinary and economic importance to it, the study was conducted to explore the spread of cryptosporidium parasite in quail *(Coturnix coturnix)* and identified the species by sequencing analysis of selected samples.

AUTHOR'S CONTRIBUTIONS

Khitam J. Yahya made contributions to the idea and design of the study, data collection, analysis, and interpretation, as well as paper drafting and revision.

Mohammed T.S. Al-Zubaidi was involved in the collection of data, data analysis and interpretation, and paper revision. All authors agree on the journal to which the article has been submitted, granted their final approval of the version to be published, and pledged to take responsibility for every part of the work.

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