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Using Nested-PCR in the Study of *Cryptosporidium spp* Parasite Spread in Domestic Birds

Saafa Ressan Abdullah Al-Kaeebi

*Corresponding author. College of Nursing College, University of Al-Qadisiyah, Iraq. E-mail address: safaa.abdullah@qu.edu.iq

Rana Saleh Al-Difaie

Biotechnology College, University of Al-Qadisiyah, Iraq. E-mail address: rana.rana@qu.edu.iq

Fatima Ibrahim Mohammad

College of science, University of Al-Qadisiyah, Iraq. E-mail address: fatima.mohammad@qu.edu.iq

Abstract

Objectives

This study examined the number of cases of Cryptosporidium spp. infection in domestic birds in Diwaniyah Governorate, as well as the consequences of this illness and the methods used to diagnose it.

Materials and Methods

In this study, 205 samples from different bird species were analyzed without gender being taken into account. Some of the methods used for diagnosis were the Acid Fast Stain method and the DNA nested Polymerization Technique (PCR). Nested - PCR proved out to be better than AFS because it could find Cryptosporidium faster, with highest accuracy, and sensitivity.

Results

The results showed that 90 of the samples had Cryptosporidium infections. Among the bird species that were studied, falseco eleonorae had the lowest incidence of cryptosporidiosis at 16%, while gallus gallus domesticus had the highest incidence at 53.3%. Out of the 60 samples taken for each species, 48.3% of Meleagris gallopova and 41.6% of Anas platyrhynchos were infected. The microscope examination showed a 43.9% infection rate (90 out of 205 samples) when

compared to the other method. To use nested-PCR for molecular identification, DNA had to be taken from positive infected samples using Proteinase K method. This produced positive bands that were usually amplified at around 587 bp. The nested-PCR test showed that 47.5% of the samples (19 out of 40) were infected.

Conclusions

Finally, this study highlights the critical need for efficient diagnostic tools and intervention plans by demonstrating the very high incidence of Cryptosporidium infection in domestic birds in Diwaniyah Governorate. In order to prevent the spread of Cryptosporidiosis in birds, the results stress the use of nested polymerase chain reaction (PCR) methods for infection diagnosis.

Key words: Nested-PCR, Microscopic Examination, Infection Rate, Birds, and *Cryptosporidium.*
Paper Type: Research Paper.

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Introduction

Livestock production in general and domestic chicken production in particular plays a vital socio economic role for people living in low income countries of Africa and Asia (Mohammadifar et al. 2014; Moazeni et al. 2016a). Domestic chickens are widely distributed avian species around the world, due to their short generation interval and adaptability in a wide range of agro ecologies (Mohammadifar and Mohammadabadi, 2018; Moazeni et al. 2016b; Khabiri et al. 2022). The domestic chickens provide high quality protein and income for the poor rural households and are the most widely kept livestock species in the world (Mohammadabadi et al. 2010; Mohammadifar

and Mohammadabadi, 2018). This is due to the presence of the valuable traits of chicken like disease resistance, adaptation to harsh environments and ability to utilize poor quality feeds (Shahdadnejad et al. 2016; Khabiri et al. 2023). Further research into the epidemiology and transmission dynamics of Cryptosporidium spp. infections in domestic bird populations is paramount for devising targeted control and prevention strategies. Understanding the implications of Cryptosporidium spp. infections in domestic bird populations not only safeguards animal welfare but also protects public health in the region (Shukla et al. 2013; Craddock et al. 2019). The identification of Cryptosporidium spp (ACAR & Yüksekdağ, 2023). as a significant pathogen in avian species necessitates ongoing surveillance and research to develop effective management strategies. Additionally, public awareness campaigns regarding the potential risks associated with Cryptosporidium transmission from birds to humans are essential for preventing zoonotic infections. By addressing these challenges collaboratively, stakeholders can work towards minimizing the impact of Cryptosporidium spp. infections on both animal and human health within Diwaniyah Governorate and beyond (Cadé & Blanchet 2013; Wang et al. 2013).

The disease cryptosporidiosis is prevalent in both humans and animals. Zoonotic infections are ubiquitous throughout the world and are brought on by the parasite *Cryptosporidium spp* (Laberge and Griffiths 2000) that belongs to the coccidia class, is a highly contagious parasite, and is categorized into several species depending on the host it infects. This has an impact on a variety of vertebral hosts, including different types of fish, reptiles, birds, rodents, and mammals (Casemore 2000). Additionally, some of its types are identified by their lack of host-specificity, and the parasite is distinguished by its capacity to infect many tissues (Guyot et al. 2001; Alseady and Kawan 2019). One of the earliest avian parasites discovered was *Cryptosporidium spp*. (Xiao et al. 2004). This parasite affects the respiratory system and the digestive system (Valigurova et al. 2008; Suljić 2021; Al-dolaimy et al. 2024). The direct life cycle of the parasite results in the infection of new hosts when they consume oocysts in food or water (Barta & Thompson 2006). Particularly in the United States of America and South Korea, the parasite causes high rates of infection up to 50% and death rates that can approach 25% in flocks of chickens, especially in the United States of America and South Korea. The parasite infects chickens, turkeys, pigeons and other wild birds (Rhee et al. 1991). In cases of intestinal infection, the parasite is present in the cloca and fabric pod in severe cases in addition to the primary site which is the intestine (Santin 2013). The infection with this parasite is one of the diseases that are described as Asymptomatic disease, but it causes tissue changes in the organs it affects because it affects the epithelial layer of villi, which leads to its necrosis, shatters, and haemorrhage in it (Blagburn et al. 2003). This infection results in a reduction in the surface area of absorption as a result of villi atrophy and cell death (Nadham et al. 1996). The parasite affects the small intestine of its host, causing Intestinal

Cryptosporidiosis disease, which is characterized by severe diarrhea, especially in young hosts (Hunter & Nichole 2003; Abdullah 2020). Sometimes the diarrhea is accompanied by blood when severe infection, which causes difficulty in diagnosis (Jeon et al. 2023). The injury may lead to lethargy in feed consumption and an increase in water consumption (Voelz & May 2010) .Cases were also recorded in the lymph nodes and in the uterus in various types of hosts (Lazo et al. 1986; Karthikeyan et al. 2019; Margiana et al. 2022).

Given the importance of this disease and its losses in livestock in Diwaniya Governorate, after the existence of special projects for poultry and their economic importance in society for this, the aim of this study was to study of Cryptosporidium spp parasite spread in domestic birds using Nested-PCR.

Materials and methods

Samples were collected from 60 local chickens, 60 from turkeys, 25 from *Falco eleonorae*, and 60 from the *Anas platyrhynchos* and the parasite ovarian cysts were isolated.

Acid fast stain method: The stool samples collected in a clean and sterile with tight lid plastic containers to maintain sample moisture has been the samples examination by the dye steadfast acid AFS examination and, as shown in (Helmy 2014) where this method was carried out in several stages using the centrifuge, sedimentation, heat stabilization and methylene blue staining in the last step.

DNA extraction and PCR: The stool lysis technique method with Proteinase K was used to extract the DNA using the Stool DNA extraction Kit (Bioneer. Korea). The extraction was completed in accordance with the company's instructions. Subsequently, a NanoDrop spectrophotometer was used to examine the isolated DNA. Then until employed in PCR amplification, stored at -20 °C in the refrigerator. To diagonose the *Cryptosporidium* parasite, nested PCR technique was used to amplify 18S rRNA gene. PCR primers were chosen from Tahseen and Najlaa (2018).

First round primers (Gautam and Kumar et al. 2020; Thantip et al. 2016) "5’-GACATATCATTCAAGTTTCTGACC-3’" and "CTGAAGGAGTAAGGAACAACC" were used to amplify a product size of 763 bp, while nested primers (Gautam and Kumar et al. 2020) "CCTATCAGCTTTAGACGGTAGG" and "TCTAAGAATTTCACCTCTGACTG" were applied to amplify a product size of 587 bp. These primers synthesized by Bioneer Company (Korea). The first round's PCR positive samples have been used in nested amplification at the same amplification condition to amplified (587bp) product size.

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Statistical means: Averaging, standardizing, and determining relative standard deviation will be the first steps in this process. In this way, you may compare your results to the mean of all the results. By adding up all the individual outcomes and then dividing that total by the total number of values (n), we can get the average result ($\bar{X}$):

$$\bar{X} = \frac{X_1 + X_2 + X_3}{n}$$

As a measure of the degree to which the individual numbers agree with one another, the standard deviation assesses the accuracy of the average. It is a metric for the sort of error that individuals aren't particularly good at controlling: random error. Here is the formula:

$$Standard\ Deviation = \sqrt{\frac{(X_1 - \bar{X})^2 + (X_2 - \bar{X})^2 + (X_3 - \bar{X})^2 + \ldots.}{n-1}}$$

Results

The findings of the present investigation revealed that 90 infections of the *Cryptosporidium* parasite were present overall in the 205 samples collected from the birds under examination, with no mention of sex. Table 1. After examination and diagnosis, the findings of our study, which covered four different types of study birds, are displayed in Table 2. It was discovered that *Gallus gallus domesticus* had the highest incidence of Cryptosporidiosis (53.3%) and *Falco eleonorae* had the lowest incidence (16%). *Meleagris gallopova* had a parasitic infection rate of 48.3% of the whole 60 samples, according to the results, whereas *Anas platyrhynchos* had a parasitic infection rate of 41.6% of the total 60 samples. Microscopic inspection has a 43.9% (90/205) infection rate in birds, according to the comparison between nested PCR and microscopic examination in this study. While 10 samples were taken from each bird being researched prior to completing a direct examination, the total infection rate was 47.5% (19/40) in the nested-PCR test, as indicated in Table 3. Molecular detection of the *Cryptosporidium* oocysts, After DNA was extracted from 40 feces samples, the DNA positive sample was amplified using nested-PCR; most of the samples yielded positive bands and were amplified at the size of nearly 587 bp. One of the recorded images was then labeled (Figure 1) according to Figure 2.

After examination of 60 samples of domestic chickens and turkeys, the diagnosis revealed 32 infections and with rate of 53.3% of domestic chickens and of turkeys 29 infections and with an infection rate of 48.3%, which is upmost than the infection rates recorded (Lengmei et al. 2014; Yosra et al. 2017; Jarad 2020) in Algeria, which showed that the infection rates for chickens and turkeys with *Cryptosporidium* were 34% and 44%, respectively, with all positive turkeys having 25 and the majority of positive chickens (26/31) having cryptosporidiosis

Table 1. The prevalence of *Cryptosporidium* oocysts infection in the studied birds

Poultry	Number of samples	Infection number	Percentage of infection%
Total	205	90	43.9

Table 2. The percentages and types of infected birds of *Cryptosporidium* and their numbers

species	Number of samples	Infection number	Percentage of infection%	Mean of infection	Std. Deviation of infection
Gallus gallus domesticus	60	32	53.3		
Meleagris gallopova	60	29	48.3	22.5000	12.66228
Falco eleonorae	25	4	16		
Anas platyrhynchos	60	25	41.6		
total	205	90	43.9		

Discussion

This study examined 205 samples of domestic birds using simple methods as well as using modern and specialized methods for the *Cryptosporidium spp* parasite type. The results of the study proved that birds infected with Cryptosporidiosis at a rate of (43.9%). Several existing tectiques found that low infection arte in several bird species (Majewska et al. 2009; Adejinmi & Oke 2011; Qi et al. 2011; Baroudi et al. 2013).

Table 3. A Comparison between Nested Polymerase Chain Reaction and Microscopic Examination to Diagnose *Cryptosporidium*

Method	Number of samples	Positive No	Percentage%	Mean of Positive No	Std. Deviation of Positive No
Microscopic Examination	205	90	43.9		
Nested Polymerase Chain Reaction	40	19	47.5	54.5000	50.20458

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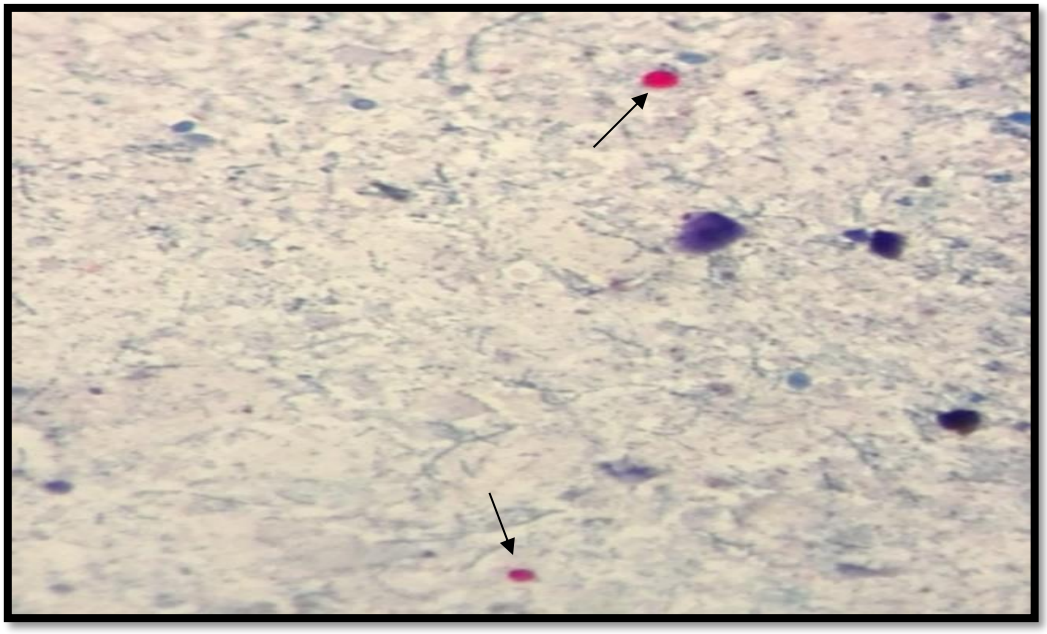

Figure 1 *Cryptosporidium* oocysts in Acid fast stain method X[40]

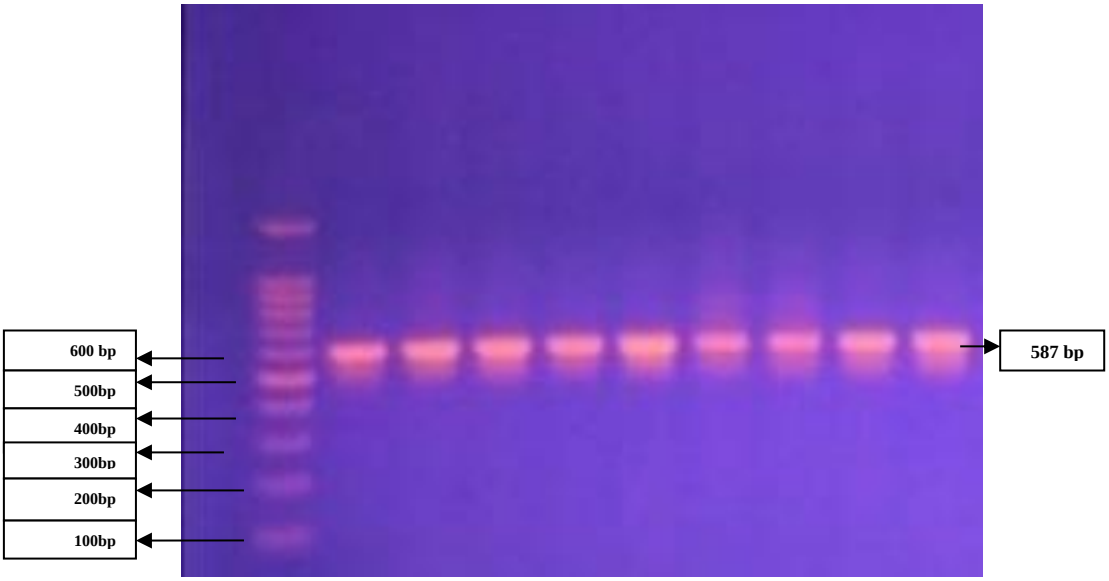

Figure 2. Nested PCR product of the 18S rRNA gene used in the detection of *Cryptosporidium spp.* in fecal samples is seen on a garose gel electrophoresis image. In Lane (M) ladder (100-2000bp), Lanes 1, 2, 4, 5, 6, 7, 8, and 10 are positive for *Cryptosporidium spp*. at a 587bp PCR product size. Where (1, 2, 3) *Gallus gallus domesticus* samples, (4, 5, 6) *Meleagrisa gallopov* samples, (7-8) *Anas platyrhynchos* samples, (9) *Falco eleonorae* samples

The reason may be due to appearance of such differences in the rates of infection can be attributed to several factors, including the different locations for collecting samples, the ages of examined hosts, feeding conditions, the degree of contamination of the food provided to them and

drinking water by parasite cysts. Out of the 205 faecal samples examined 60 samples were collected from ducks (*Anas platyrhynchos*), (41.6%) were positive for *Cryptosporidium* parasites, this result is not consistent with (Abbas et al. 2022; Bashar et al. 2022; Lei et al. 2022; Arif et al. 2023; Lafta et al. 2023) in Ibadan Southwestern Nigeria, also with Cadé and Blanchet (2013) in China, which showed that the infection rate was (15.4%) and (16.3%) respectively. This ratio compared to the results of our research is low. The results of the study showed that the diagnosis of 25 specimens of feces of ornamental birds belonging to the type *Falco eleonorae* infection rate was 16%, and this is not consistent with (Hussein et al. 2002; Al Nazari et al. 2023; Althomali et al. 2023; Hjazi et al. 2023; 31-35). These variation in infection rates can be attributed to the different regions and environments from which the samples were collected. When comparing the two main methods for diagnosing the *Cryptosporidium* parasite, the ratios were 43.9%, 47.5%, for each of methods diagnosing which are acid fast stain method, and DNA Polymerization Technique reaction method Respectively, This is not consistent with some studies that have compared of the methods of examination (Suljić 2021; Al-Jassani et al. 2022; Gupta et al. 2023; Sane et al. 2023; Ze et al. 2023). We were able to detect the *Cryptosporidium* parasite in the feces samples of several birds in our investigation by using specific primers created in accordance with the gene of 18S rRNA, as in more recent research. In Henan, China, the prevalence of *Cryptosporidium spp*. in quails, chickens, Pekin ducks, pet birds, and ostriches has been thoroughly described (Al-Hawary et al. 2023). Furthermore, 256 fecal specimens from farmed poultry in Germany were selected at random and tested for the presence of *Cryptosporidium spp*. The results of nested PCR amplification show that *Cryptosporidium* parasites infect broilers and turkeys in Germany on a regular basis. Given the size of the chicken industry and the widespread consumption of poultry meat (Al-Safi & Qasim 2023; Khursheed et al. 2023; Zaman et al. 2023; 42-44). The parasite has been identified using the PCR method in numerous molecular studies in Iraq for various species of birds, including broiler chicken in the Al-Qadisiyah Province.

Conclusion: This study confirmed that the nested polymerase chain reaction (PCR) method is the most effective and specialized tool for detecting the Cryptosporidium parasite, and it also demonstrated that birds undergoing diagnosis had a high prevalence of Cryptosporium infections compared to the acid fast stain method, the conventional method of examination.

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مجله بیوتکنولوژی کشاورزی
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استفاده از Nested-PCR در مطالعه انتشار انگل Cryptosporidium spp در پرندگان اهلی

صفا رسن عبدالله الکیبی

*نویسنده مسئول. دانشکده پرستاری، دانشگاه القادسیه، عراق. ایمیل: safaa.abdullah@qu.edu.iq

رعنا صالح الضیفایی

دانشکده بیوتکنولوژی، دانشگاه القادسیه، عراق. ایمیل: rana.rana@qu.edu.iq

فاطمه ابراهیم محمد

دانشکده علوم، دانشگاه القادسیه، عراق. ایمیل: fatima.mohammad@qu.edu.iq

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چکیده

هدف: ین مطالعه به بررسی تعداد موارد عفونت .Cryptosporidium spp در پرندگان اهلی در استان دیوانیه و همچنین پیامدهای این بیماری و روش های تشخیص آن پرداخت.

مواد و روش‌ها: در این مطالعه، ۲۰۵ نمونه از گونه های مختلف پرنده بدون در نظر گرفتن جنسیت مورد تجزیه و تحلیل قرار گرفت. برخی از روش‌های مورد ا ستفاده برای ت شخیص، روش رنگ آمیزی ا سید ف ست و تکنیک ن ستد PCR بود. ثابت شد که نستد PCR بهتر از رنگ آمیزی ا سید فست AFS ا ست. زیرا می‌تواند کریپتو سپوریدیوم را سریعتر، با بالاترین دقت و حسا سیت شناسایی کند.

نتایج: نتایج نشان داد که ۹۰ نمونه از کل نمونه‌ها دارای عفونت کریپتوسپوریدیوم بودند. در بین گونه های پرنده مورد مطالعه، falseco eleonorae با ۱۶٪ کمترین بروز کریپتوسپوریدیوزیس را داشت، در حالی که گالوس گالوس داخلی با ۵۳/۳ درصد بیشترین بروز را دا شت. از ۶۰ نمونه گرفته شده برای هر گونه، ۴۸/۳ در صد از گونه Meleagris gallopova و ۴۱/۶ در صد از گونه Anas platyrhynchos آلوده بودند. بررسی میکروسکوپی میزان عفونت ۴۳/۹ درصد (۹۰ نمونه از ۲۰۵ نمونه) را در

مقایسه با روش دیگر نشان داد. برای استفاده از Nested-PCR برای شناسایی مولکولی، DNA باید از نمونه های آلوده مثبت با استفاده از روش پروتئیناز K استخراج شود. این کار باندهای مثبتی را تولید کرد که معمولاً در محدوده ۵۸۷ جفت بازی تکثیر می‌شدند. تست Nested-PCR نشان داد که ۴۷/۵ درصد نمونه ها (۱۹ از ۴۰ نمونه) آلوده بودند.

نتیجه‌گیری: در نهایت، این مطالعه با نشان دادن شیوع بسیار بالای عفونت کریپتوسپوریدیوم در پرندگان اهلی در استان دیوانیه، نیاز حیاتی به ابزارهای تشخیصی کارآمد و طرح‌های مداخله‌ای را برجسته کرد. به منظور جلوگیری از گسترش کریپتوسپوریدیوزیس در پرندگان، نتایج بر استفاده از روش‌های واکنش زنجیره‌ای پلیمراز نستد (nested-PCR) برای تشخیص عفونت تاکید دارد.

کلیدواژه‌ها: بررسی میکروسکوپی، پرندگان، کریپتوسپوریدیوم، میزان آلودگی، Nested-PCR

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