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Molecular diagnosis of *Toxoplasma gondii* in four species of birds in Al-Najaf Province

Haider M. A. Alkaabi

Biology Department, Faculty of Education for Girls, University of Kufa, Iraq

Jasim H. R. Alkhuzai

Altoosi University College

Corresponding author email: Jasim.alkhuzai@uokufa.edu.iq

Abstract---The current study aimed to investigate the *T. gondii* in four species of birds, including Pin-tailed Sandgrouse (*Pterocles alchata*), Chukar partridge (*Alectoris chukar*), Common Moorhen (*Gallinula chloropus*), and Helmeted Guineafowl (*Numida meleagris*) in Al-Najaf Al-Ashraf province in Iraq for the period from April 2021 until the end of April 2022 by using the polymerase chain reaction (PCR) technique. To our knowledge, this study is the first in Iraq to investigate these parasites in these species of birds by using this technique. Gene 18S rRNA with molecular weight 590bp of *T. gondii* was used, 25 samples were collected for each species of target birds except for *P. alchata*, 40 samples. The results of testing 115 tissue samples from the birds included in the study indicated that there were 8 samples (6.95%) of birds that gave a positive test result, It included 4 samples (50%) of *N. meleagris*, 2 samples (25%) of *A. chukar*, 2 (25%) of *G. chloropus*, and no case of the presence of the gene 18S rRNA was recorded in the samples from *P. alchata*. The results of the PCR showed that the highest rate of infection of the *T. gondii* positive samples was recorded in *N. meleagris*, and the lowest was in *P. alchata*. The results of using the PCR test to detect *T. gondii* also showed that the infection rate of males (5 samples with 62.5%) was higher than the infection rate for females (3 samples with 37.5%).

Keywords---*Toxoplasma gondii*, *Gallinula chloropus*, *Numida meleagris*, *Pterocles alchata*, *Alectoris chukar*, birds, PCR.

Introduction

Monitoring and controlling diseases in wild animals is important in the field of health and the environment because they reflect early environmental changes because act as final or intermediate hosts for many parasites (Dorny *et al.*, 2009;

Andrade *et al.*, 2016). Birds are important animals for the environment and humans, they play an important role in biological control and ecological balance by feeding on some species of rodents (Tabur & Ayvaz, 2010), as well as agricultural pests such as insects, so some countries prevent hunting them during the breeding period to preserve them from extinction. (Fernandes *et al.*, 2012). The class of birds includes about 10000 species spread all over the world (Tabur & Ayvaz, 2010), some of them are aquatic and others are wild, feeding on grains, fruits, flower nectar, fish, rodents, dead animals, or insects (Myo *et al.*, 2020). On the other hand, birds have many species of parasites such as protozoa and worms, as well as viruses, bacteria, and fungi (Bush & Clayton, 2018). Therefore, it plays an important role in spreading parasites on a wide global scope, it also transmits the parasites to domestic animals such as cats, dogs, fishes, and sometimes humans (Rahman *et al.*, 2020). Captive birds are more susceptible to parasites compared to wild birds because they are left in an unfavorable environment and suffer from poor and inappropriate conditions and management (Ombugadu *et al.*, 2018), and Sohn *et al.*, (2011) stated that birds transmit some pathogens naturally or accidentally to humans and may cause death to him. The parasites that may be present in or on birds, some internally and some externally, the external parasites include mites, flies, and ticks. In contrast, the internal parasites include cestodes, trematodes, nematodes, acanthocephalans, and protozoans (Ombugadu *et al.*, 2018). *Toxoplasma* sp. and *cryptosporidium* sp. are parasites that spread widely among birds (Shaapan *et al.*, 2011). Birds are also important in the life cycle and epidemiology of *T. gondii* because their tissues represent an essential source of protein in feeding cats and humans (Andrade *et al.*, 2016). *T. gondii* belongs to the class Sporozoa within the group coccidia, it is an obligatory intracellular parasite (Duszynski, 2021). it causes toxoplasmosis, which is a zoonotic disease and infects all species of warm-blooded vertebrates, including cats and humans (Rouatbi *et al.*, 2019). Humans, birds, livestock, and rodents are intermediate hosts (Zhao & Ewald, 2020), while the Felidae family is the only final hosts of *T.gondii* (Seo *et al.*, 2020). This parasite is transmitted to humans by ingesting food and water contaminated with the Oocyst that comes with the feces of infected cats, or through undercooked meat containing bradyzoite (Hill & Dubey 2018). Diagnosis of *T. gondii* is made by several methods, including Polymerase Chain Reaction (PCR) or by using serological techniques, including Indirect Fluorescent Antibody Test (IFAT), Sabin Feldman dye test, Indirect Haemagglutination test (IHT), Complement Fixation test (CFT) and Intra Dermal Test (IDT) (Al-Sray *et al.*, 2019). It is also diagnosed using the Direct Latex Agglutination Test (DLAT), Modified Agglutination Test (MAT), and Enzyme-Linked Immunosorbent Assay (ELISA) (Issa *et al.*, 2020).

Materials and Methods

Collection of Birds samples

115 wild birds were collected, they included four species that were caught live by the net from different areas in Al-Najaf Al-Ashraf province, but 25 of them were domestic birds that were bought from the birds market in this province (25 samples for each species of birds except for *Pterocles alchata* 40 samples). The birds were transported to the place designated for them for the period from the first of April 2021 until the end of April 2022, and the date of collection of the

birds, their weight, and gender were recorded. It was classified according to its basic characteristics, such as the size of the bird, the shape of the beak, its color, the color of the feathers, and the shape and color of the legs and wings.

Polymerase Chain Reaction (PCR)

This test was carried out using its own kits supplied by Promega, and this test is based on the following:

Extraction of DNA from tissue samples

After the birds were dissected, pieces of heart, liver, and intestine tissues were taken, which were used to extract DNA from those tissues according to the method described in the test kit provided by INTRON Biotechnology, and as follows:

1. The liver and heart of the target birds were taken out for the detection of *T. gondii*. Fresh animal tissues were used directly for DNA extraction. And sometimes the tissues were not used immediately, so they were stored in a deep freezer (below - 80 ° C).
2. The prepared sample was cut to a suitable size by scalpel or scissors.
3. The sample material cut into strips was placed in a grinding vessel (slurry). Liquid nitrogen was added to the mortar. The sample was kept immersed in liquid nitrogen and carefully inactivated until the sample was completely homogenized. Liquid nitrogen was left to evaporate. Note: Turbulence and homogenization time depend on the type of tissue samples. It is recommended to cut the samples completely so that no lumps of tissue appear. The tissue sample clumps will be difficult to analyze properly and will result in lower DNA production. It is very important to keep the sample frozen in liquid nitrogen during the inactivation and homogenization step to lower DNA yield and prevent DNA degradation. Caution should be exercised when handling liquid nitrogen.
4. 25 mg of tissue sample was measured, then transferred to a 1.5 ml Eppendorf tube using a spoon.
Note: to prevent a frozen sample from thawing, use a pre-cooled spoon and 1.5 mL tube (when the tube has been pre-cooled, the tube cap should always be open) with liquid nitrogen during transport. Repeated freezing and thawing of a frozen sample will result in DNA degradation. Furthermore, exceeding the recommended optimum amount of starting material will result in inefficient hydrolysis, resulting in lower DNA yield and purity.
5. 200 µL of CL buffer, 20 µL of Proteinase K, and 5 µL of RNase A solution were added to the sample tube and mixed by vortexing vigorously.
NOTE: It should be ensured that the solutions of Proteinase K and RNase A are always kept under freezing (below -10°C).
6. The lysate was incubated at 56°C using a water bath for 10-30 minutes.
Note: To aid lysis of the tissue sample, the tube was mixed by inverting every 2 minutes during the incubation. Decomposition time varies by sample type. However, the G-spin total DNA extraction kit provides a robust lysis mechanism against the tissue sample. After incubation, the lysate may

appear sticky, but it should not be gelatinous as it may clog the spin column.

7. When lysis was complete, 200 μ L of Buffer BL was added into the upper sample tube and mixed well. Then the mixture was incubated at 70 °C for 5 min.

Note: The strong vortex may induce the breaking of genomic DNA. In order to ensure effective lysis, the lysate sample and Buffer BL are mixed well.

8. The sample tube was centrifuged at 13,000 rpm for 5 min to remove non-dissolving tissue particles. Then 350-400 μ L of the supernatant was carefully transferred into a new 1.5 mL tube.

Note: if insoluble tissue clumps remain in a homogeneous mixture, occasional clogging of the spindle shaft will occur. This step helps the sample mix with the buffer during the binding step. Also, it prevents clogging of the shaft from insoluble lumps.

9. The 1.5 mL tube was briefly centrifuged to remove droplets from inside the cap.

10. 200 μ L of absolute ethanol was added to the lysate, and mixed well by vortex cruise. After mixing, we centrifuged a 1.5 mL tube briefly to remove droplets from inside the cap.

NOTE: This step is an equilibration step for binding genomic DNA to the column membrane. It is important to ensure proper mixing after adding the ethanol so that no two unmixed phases appear. Also, this step causes the potency cell analyzer to be passed through a column.

11. The mixture was carefully placed from step 10 to the spin column (in a 2 mL collection tube) without flange wetting, cap closed, and centrifuged at 13,000 rpm for 1 min. The filter was discarded and the spin column was placed in a new 2 mL collection tube (supplementary).

Note: Close each spindle to avoid aerosol formation during centrifugation. In order not to transport any solid materials.

12. 700 μ L of WA buffer was added to the spin column without wetting the tip, and centrifuged for 1 min at 13,000 rpm. Flux through the collector tube has been discarded and reused.

13. 700 μ L of Buffer WB was added to the spin column without tip wetting and centrifuged for 1 min at 13,000 rpm. The efflux was eliminated by placing the column in a new 2.0 mL collection tube (also supplied), then again centrifuged for an additional 1 min to dry the membrane. The flow-through and collector tube was completely ignored.

NOTE: It is very important to dry the shaft membrane because residual ethanol may inhibit subsequent reactions. After centrifugation, carefully remove the spin column from the collection tube without touching the flow-through, as this will migrate the ethanol.

14. The spin column was placed in a new 1.5 mL tube (not supplied), and 30-100 μ L Buffer CE was added directly to the membrane. It was then incubated for 1 min at room temperature and then centrifuged for 1 min at 13,000 rpm for elimination.

NOTE: In general, elution with 30 μ L (instead of 50 μ L) increases the final DNA concentration, but decreases the total DNA production. A new 1.5mL tube can be used for the second trimmings to prevent the first trim from loosening.

15. After, all samples were extracted, the polymerase chain reaction was performed using specific primers in which a pair of primers from the diagnostic gene 18S rRNA (590bp) from the *T. gondii* was used for PCR.

Measurement of the concentration and purity of the extracted DNA

The concentration and purity of the extracted DNA were measured using a Nanodrop spectrophotometer. The DNA was detected by determining its concentration (mg\µl) and measuring its purity by reading the absorbance at a wavelength ranging between 260-280 nm. The device was used as follows:

1. The Nanodrop spectrophotometer was turned on and the DNA measurement software was selected.
2. The scale substrate (optical lens) was wiped twice with device blotting paper by placing 1 µL of ddH₂O using a sterile micropipette on the surface of the scale substrate.
3. Zeroing and cleaning of the substrate for sampling were performed.
4. The DNA concentration was measured using one microliter of each DNA sample and then the device substrate was cleaned again to measure the other sample and so on for several samples.
5. The purity of the extracted DNA was determined by absorbance reading at a wavelength between 260-280 nm, whereby the extracted DNA is considered pure when the absorbance is between 1.8 - 2.1 nm. (Components 1-5 are readily available in the PCR mix tube included with the kit).

Amplification of DNA using special primers *T. gondii*

In the current study, the primers shown in Table (1) were used to detect *T. gondii* in target birds and were designed by using Primer plus 3 software supplied by the Korean Macrogen Corporation.

Table 1: Single-nucleotide sequencing of the nitrogenous bases of the primers used for the detection of *T. gondii* and the size of the product of the Conventional PCR

Product (bp) size	Sequence	Primers
590bp	F GAGACCCCGTAAACGTGCTA	<i>Toxoplasma gondii</i>
	R ACCGTCTATTTTCCGGCCTC	

Preparation of the PCR master mix

The PCR mixture was prepared using the Go Taq® G2 Green Master Mix kit prepared by Promega (USA), the mixture was prepared in PCR tubes equipped with a kit containing PCR components, and other components were added to the reaction mixture according to the company's instructions As in Table (2).

Table 2: Components of The polymerase Chain Reaction Mixture

Components	Volume
PCR Master Mix	12.5 Ml
DNA template	5 Ml
Forward primer, 10Mm	1.5 μ L
Reverse primer, 10 μ M	1.5 μ L
Nuclease-Free water	4.5 μ L
Total	25 μ L

1. After completing the preparation of the polymerase chain reaction mixture, the tubes were closed and carefully mixed with a rotary mixer device for 5 seconds.
2. To perform the thermal cycles, the tubes were transferred to the PCR Thermocycler.

PCR Thermocycler conditions

The device is programmed according to the conditions below. Table (3)

Table 3: PCR Heat Cycle Conditions

PCR Step	Temperature ($^{\circ}$ C)	Time	Repeat cycle
Initial denaturation	95	4 min.	1
Denaturation	95	30 sec.	35
Annealing 60 30sec.	52	30 sec.	35
Extension	72	45 sec.	35
Final extension	72	5 min.	1

Gel electrophoresis

Electrophoresis was performed using 1% agarose gel to read PCR product analysis as follows:

1. One gram of agarose gel was dissolved in 100 ml of TBE buffer at 1X concentration and used a magnetic hot plate stirrer for 15 minutes.
2. The gel was left to cool at room temperature, then 3 μ l of ethidium bromide radioactive dye at a concentration of 0.5 mg/ml was added and mixed well with the gel (the radioactive dye ethidium bromide was prepared by dissolving 50 mg in 100 ml distilled water).
3. The agarose gel was poured into the Tray migration tray containing the comb to locate the samples of the PCR products and then the gel was left to solidify at room temperature for 15 minutes and then the comb was carefully removed from the gel.
4. Comb pits were loaded with 10 μ l of DNA produced from the polymerization process starting from hole 2, while hole 1 was loaded with 8 μ l of Ladder solution, which was equipped with a test kit from Bioneer, then the agarose gel was immersed in 1X TBE Buffer solution and closed the cover of the

transfer plate and then The relay was operated with a current of 100 V and 80 mA for one hour.

5. After the migration process, the gel containing the PCR product was examined using a UV light source to determine the result with a calibrator unit (Ladder), then the migration product was photographed using a digital camera.

Results and Discussion

115 samples of birds collected during the period from the first of April 2021 until the end of April 2022 from Najaf province were examined species of birds. The results showed different infection rates for *T. gondii* according to the PCR method. The results of using the conventional PCR technique to examine 115 samples collected from the birds included in the study (25 samples for each species except *P. alchata* 40 samples), showed that the presence of the diagnostic gene 18S rRNA with a molecular weight of 590 bp of the parasite *T. gondii* was 6.95% in 8 samples of the total samples were distributed among 4 samples of *N. meleagris* as shown in Figure (5) with a percentage of 16% out of 25 samples (Table 4) and a percentage of 50% of the total positive samples (Figure 1), two samples of *A. chukar* as shown in Figure (3) with a percentage of 8% out of 25 samples (Table 4) and a percentage of 25% of the total positive samples (Figure 1), also two samples of *G. chloropus* as shown in Figure (4) with a percentage of 8% out of 25 samples (Table 4) and a percentage of 25% of the total positive samples (Figure 1), while no case of the presence of the 18S rRNA gene was recorded in the samples from *P. alchata* as shown in Figure (2) with a percentage of 0.0% out of 40 samples (Table 4) and a percentage of 0.0% percent of the total positive samples (Figure 1). The results showed that there were significant differences in all species of examined birds except for *P. alchata*. This test showed that the highest infection rate was in *N. meleagris*, which amounted to 16% in 4 samples, and the lowest was in *P. alchata*, which amounted to 0% without any infection. Under the probability level of ($P < 0.05$) as shown in Table (4), Figure (1).

Table 4: Numbers and percentages of presence of the diagnostic gene 18S rRNA (590bp) of *T. gondii* in birds tissue samples using conventional PCR

Sample Species	Total	Positive n (%)	Negative n (%)	Chi-Square	p-value
<i>Pterocles alchata</i>	40	0(0%)	40(100%)	1	N.A
<i>Alectoris chukar</i>	25	2(8%)	23(92%)	17.640	0.0001 *
<i>Gallinula chloropus</i>	25	2(8%)	23(92%)	17.640	0.0001 *
<i>Numida meleagris</i>	25	4(16%)	21(84%)	11.560 ^b	0.001 *
Total	115	8(6.95%)	107(93.05%)	85.226	0.0001 *

*Significant differences at p-value <0.05. Chi-square or Fisher- Exact Test expected count less than 5.

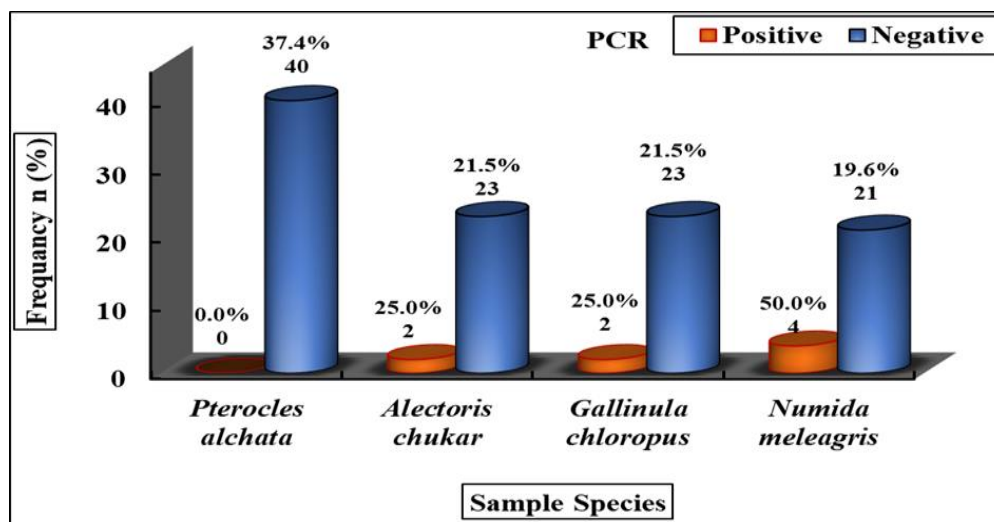


Figure 1: Numbers and percentages of presence of the diagnostic gene 18S rRNA (590bp) of *T. gondii* in avian tissue samples using conventional PCR

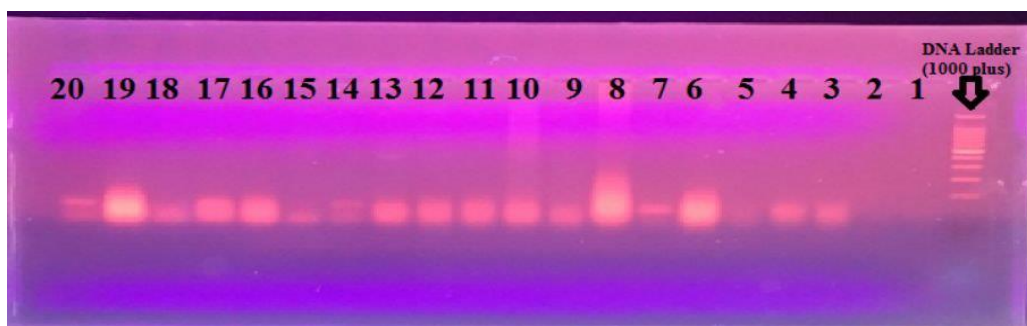


Figure 2 : Columns 1-20 represent tissues samples of *P. alchata* negative for the PCR, where the gene 18S rRNA with a molecular weight 590bp of *T. gondii* did not appear in the samples

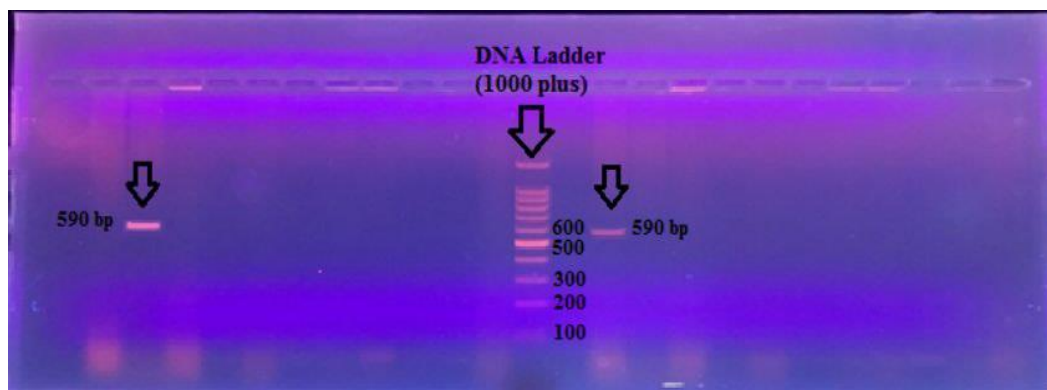


Figure 3: Agarose gel electrophoresis that contained the results of the PCR test for the 18S rRNA gene with a molecular weight 590bp of *T. gondii*, showed two positive samples in *A. chukar*

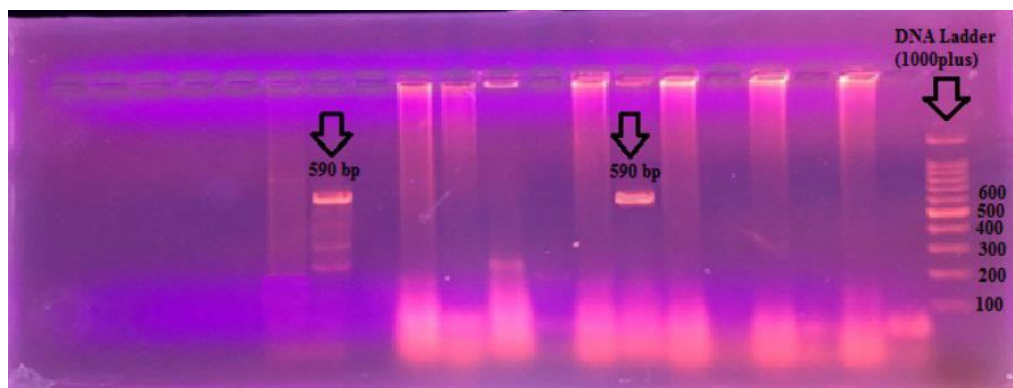


Figure 4: Agarose gel electrophoresis that contained the results of the PCR test for the 18S rRNA gene with a molecular weight 590bp of *T. gondii* , showed two positive samples in *G. chloropus*

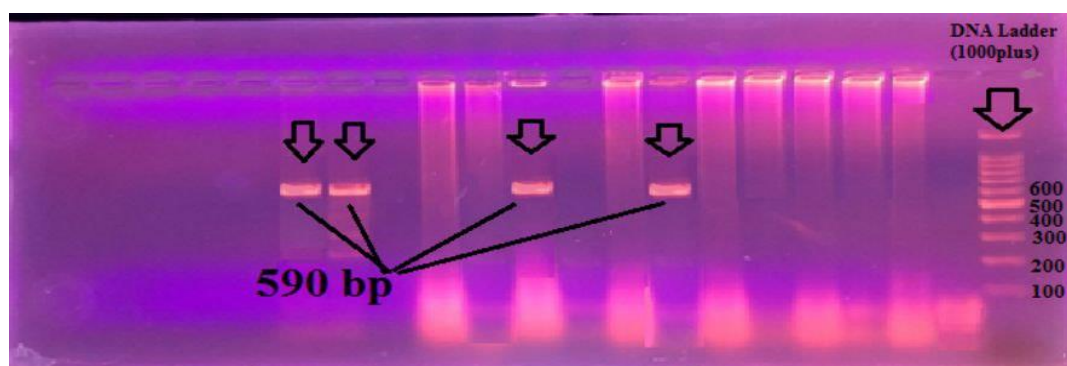


Figure 5: Agarose gel electrophoresis that contained the results of the PCR test for the 18S rRNA gene with a molecular weight 590bp of *T. gondii*, showed four positive samples in *N. meleagris*

Discussion

To our knowledge, this study is the first in Al-Najaf province to detect *T. gondii* in this the four species of birds and by the methods that were mentioned in this study. The researcher did not find any similar study on this species in this province.

Detection by using conventional polymerase chain reaction (PCR)

The results of the PCR test showed that the infection rate of *T. gondii* in *N. meleagris* was 16% (4 positive samples), which is the highest percentage recorded in this study compared with the rest of the birds' species because this species of bird is a domestic animal, therefore, it is close to the source of infection such as sewage and waste, as well as close to other animals in a closed environment, especially cats, which are considered a source of infection with the *T. gondii* (Lass *et al.* 2022, Badparva *et al.* 2015). The results of the PCR test showed that the infection rate of *T. gondii* in *G. chloropus* was 8% (2 positive samples), the reason for infection in this species of bird may be due to its lifestyle, as this bird often

prefers swimming in the water and the accompanying excretion of feces inside the water, especially in the water of ponds and swamps, and therefore this environment helps the transmission of parasitic infection between these birds (Bush & Clayton, 2018). The results of the PCR test showed that the infection rate of *T. gondii* in *A. chukar* was 8% (2 positive samples). The results of the PCR test showed that the infection rate of *T. gondii* in *P. alchata* was 0.0% (Without any positive samples), the reason is that these birds live in the desert environment (Hinsley *et al.*, 1993), so the decrease in the number of hosts has a clear effect on the lack of transmission of pathogens (Khan *et al.* 2019).

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