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Diagnostic Study Of Cryptosporidium Parvum In Some Birds Species

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ABSTRACT

The current study included the detection of Cryptosporidium parvum 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 molecular tests, Polymerase chain reaction (PCR) for the detection of the gene 18S rRNA with a molecular weight of 730bp in the intestines. A total of 115 birds, including 25 samples, were collected for each species of birds that were included in the study, except for P. alchata, 40 samples. The results of the molecular examination for the presence of the diagnostic gene 18S rRNA with a molecular weight of 730bp of the parasite showed the presence of 7 samples were infected with a percentage of 6.10%, which included 2 samples (28.6%) of N. meleagris, one sample (14.3%) of A. chukar, one sample (14.3%) of G. chloropus, and 3 samples (42.9%) of P. alchata. The results of using PCR test, showed that the highest infection rate of positive samples of C. parvum was recorded in N. meleagris, and the lowest was in P. alchata.

Keywords: Cryptosporidium parvum, Gallinula chloropus, Numida meleagris, Pterocles alchata, Alectoris chukar, Birds, PCR.

INTRODUCTION

Monitoring and controlling diseases in wild animals is important in the field of the health and the environment because they reflect early environmental changes because they 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 types 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 transmit 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, as the external parasites include mites, flies and ticks, while 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). Cryptosporidium spp. is also an important parasite, it is an intracellular, obligate, highly virulent, protozoan parasite belonging to class coccidian of phylum Apicomplexa (Beier, 2000 ; Chukwuma, 2019), it causes Cryptosporidiosis disease in a wide range of vertebrates such as fishes, reptiles, mammals and birds (Anah & Al-Mayali, 2018). This parasite is inhabits mainly the digestive tract, especially in the small intestine, Bursa of Fabricius, causing severe diarrhea, and characterized by its ability to infect other organs, such as the stomach, bile duct, liver, pancreas, respiratory system, and kidney (Tasar et al., 2019). It is classified according to the species of host into several species of hosts, it is a zoonotic disease common between humans and animals, and spread widely throughout the world (Zahedi et al., 2016). This parasite is characterized by multiple ways of infection, as well as its ability to cause self-infection in its host, innate resistance to disinfectants (Hassan et al., 2021) , and also the absence of symptoms when infected with it, but it causes histological changes in the organs that it infects, which leads to necrosis, damage, and bleeding in them, so the interest in it increased because of its danger and widespread around the world. When infecting the epithelial layer in the villi, it leads to a reduction in the absorption area in the intestine due to the death of epithelial cells (Ahmed et al., 2020). The severity of the disease caused by the parasite varies according to several factors, including those related to the parasite, such as the virulence of the strain, and the place in which it resides (Schmidt et al., 2000). In addition to is related to the host such as age, immune status, healthy nutrition conditions, and the extent of its exposure to sources Infection, as yeast with this parasite is more dangerous in patients with weak immunity Immunosuppressed patients as AIDS and Leukaemia Patients treated with immunosuppressive drugs and children suffering from malnutrition (Ashigbie et al., 2021).

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 intestines of the target birds were taken out for the detection of C. parvum. 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 μl 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 μl 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 μL 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 μL 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 μL 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 μL 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 μL (instead of 50 μL) 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.
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 (730bp) from the C. parvum 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 C. parvum

In the current study, the primers shown in Table (1) were used to detect C. parvum 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 C. parvum and the size of the product of the Conventional PCR.

Product (bp) size	Sequence	Primers
730bp	F GTTCGATTCCGGAGAGGGAG	Cryptosporidium parvum
	R TTTCAGCCTTGCGACCATCT	

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 µL
Reverse primer, 10µM	1.5 µL
Nuclease-Free water	4.5 µL
Total	25 µL

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 (°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 µl 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 µl of DNA produced from the polymerization process starting from hole 2, while hole 1 was loaded with 8 µl 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.

Statistical Analysis
All data were analyzed by the SPSS software (V.28 Inc., Chicago, USA). Nominal variables were expressed as frequency and percentage (%) were compared between studied groups using the Chi-square test or Fisher-Exact Test expected count of less than five. Nominal regression test for identified the independent technique or test of diagnostic were expressed as odds ratios (OR), 95% confidence intervals (CI), and p values; the Significance of differences were detected at $p < 0.05$.

RESULTS AND DISCUSSION

Detection by using conventional polymerase chain reaction (PCR)
The PCR technique was used as a diagnostic method to confirm the results of the microscopic test. 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 there were significant differences under the probability level of ($P < 0.05$) for the presence of the diagnostic gene 18S rRNA with a molecular weight of 730bp of C. parvum was 6.10% in 7

samples of the total samples were distributed among 2 samples of N. meleagris as shown in Figure (5) with a percentage of 8% out of 25 samples (Table 4) and a percentage of 28.6% of the total positive samples (Figure 1) , one sample each from A. chukar and G. chloropus as shown in Figure (3) and (4) with a percentage of 4% out of 25 samples of each (Table 4) and a percentage of 14.3% of the total positive samples of each (Figure 1) , and 3 samples of P. alchata as shown in Figure (2) with a percentage of 7.50% out of 40 samples (Table 4) and a percentage of 42.9% of the total positive samples (Figure 1). The results showed that there were significant differences in all species of examined birds. This test showed that the highest infection rate was in N. meleagris, which amounted to 8% in 2 samples, and the lowest in A. chukar and G. chloropus, which amounted to 4% each has one sample. 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 (730 bp) of C. parvum in birds tissue samples by using conventional PCR.

Sample Species	Total	Positive	Negative	Chi-Square	p-value
Pterocles alchata	40	3(7.50%)	37(92.50%)	28.900	0.0001 *
Alectoris chukar	25	1(4.00%)	24(96.00%)	21.160	0.0001 *
Gallinula chloropus	25	1(4.00%)	24(96.00%)	21.160	0.0001 *
Numida meleagris	25	2(8.00%)	23(92.00%)	17.640	0.0001 *
Total	115	7(6.10%)	108(93.90%)	88.704	0.0001 *
*Significant differences at p-value <0.05. Chi-square or Fisher- Exact Test expected count less than 5.					

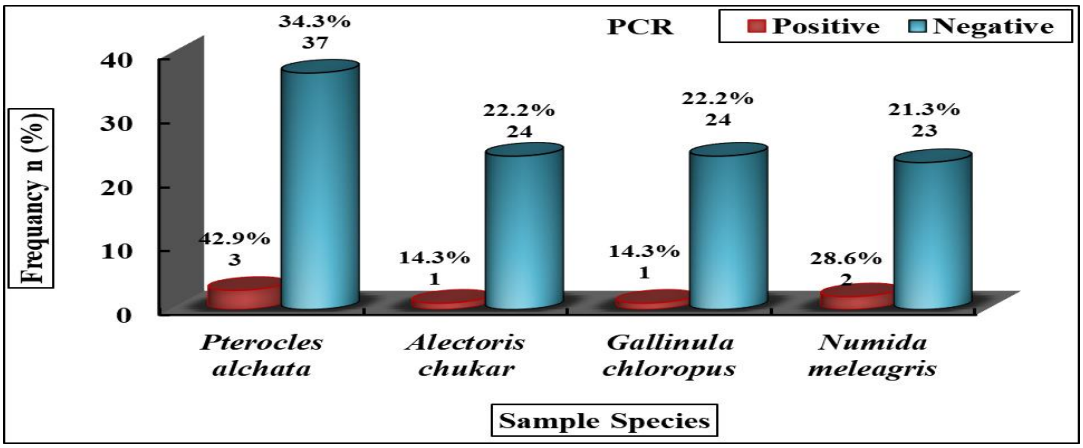

Figure 1: Numbers and percentages of presence of the diagnostic gene 18S rRNA (730 bp) of C. parvum in avian tissue samples using conventional PCR.

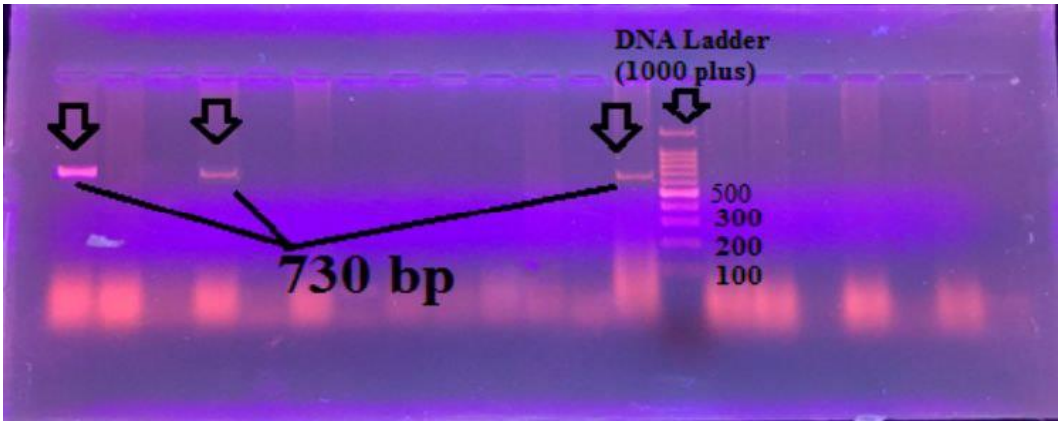

Figure 2: Agarose gel electrophoresis that contained the results of the PCR test for the 18S rRNA gene with a molecular weight 730bp of C. parvum, showed three positive samples in P. alchata

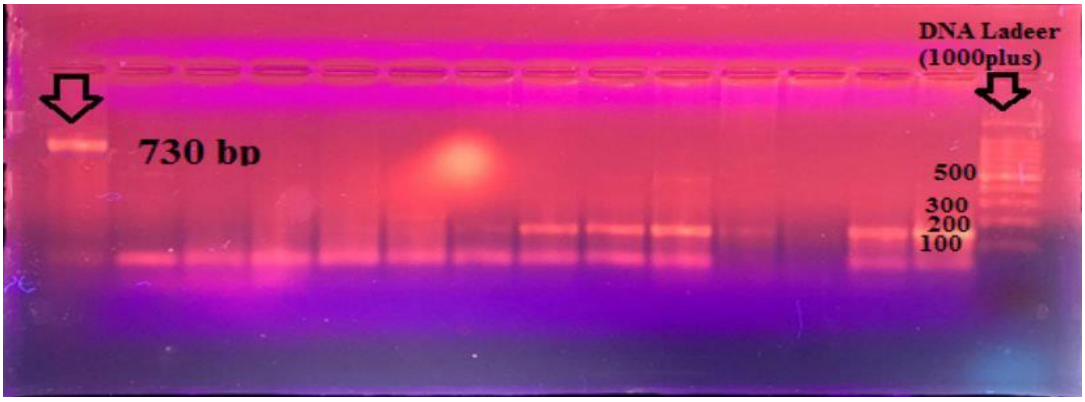

Figure 3: Agarose gel electrophoresis that contained the results of the PCR test for the 18S rRNA gene with a molecular weight 730bp of C. parvum, showed one positive samples in A. chukar

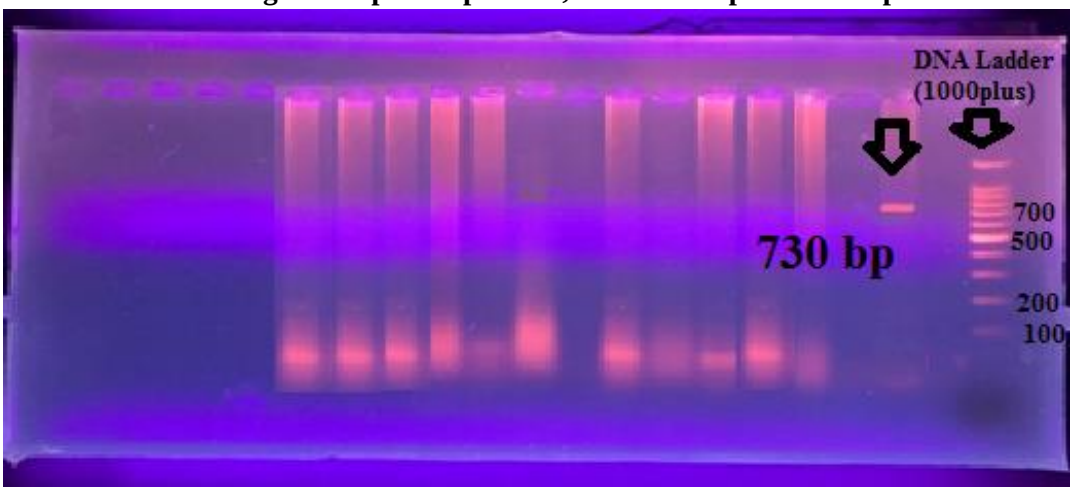

Figure 4: Agarose gel electrophoresis that contained the results of the PCR test for the 18S rRNA gene with a molecular weight 730bp of C. parvum, showed one positive samples in G. chloropus

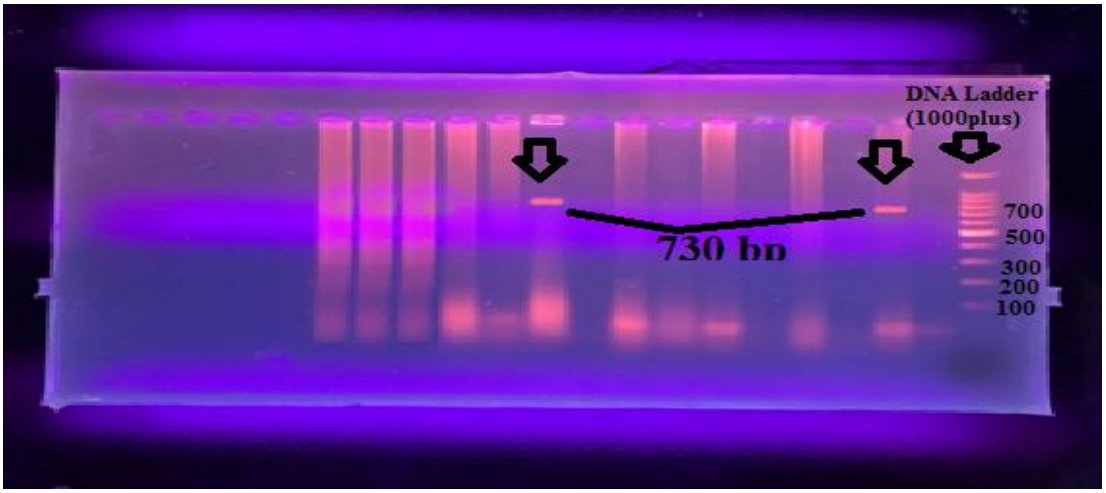

Figure 5: Agarose gel electrophoresis that contained the results of the PCR test for the 18S rRNA gene with a molecular weight 730bp of C. parvum, showed two positive samples in N. meleagris

DISCUSSION

To our knowledge, this study is the first in Al-Najaf province to detect C. parvum 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 PCR.

Numida meleagris The results of the PCR test showed that the infection rate of C. parvum in N. meleagris was 8% (2 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, which are considered a source of infection with the C. parvum, as well as close to other animals in a closed environment (Badparva et al. 2015).

Gallinula chloropus

The results of the PCR test showed that the infection rate of C. parvum in G. chloropus was 4% (1 positive sample), 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).

Alectoris chukar

The results of the PCR test showed that the infection rate of C. parvum in G. chloropus was 4% (1 positive samples), the cause of infection in this bird is probably because it prefers to live in high places where many parasites can survive at higher altitudes where environmental conditions become more suitable for the parasite (Rodriguez et al. 2021).

Pterocles alchata

The results of the PCR test showed that the infection rate of C. parvum in P. alchata was 7.5% (3 positive samples). The reason is that these birds live in the desert environment (Hinsley et al., 1993), therefore, the lack of plants and arthropods leads to a lack of food availability for these birds, and consequently, the difficult access to food, in turn, leads to immunosuppression and increased infection with parasites (Cabodevilla et al., 2020).

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