How to Cite:
Lamy, S. A. Q., & Kawan, M. H. (2022). Molecular study of Toxoplasma gondii B1 Gene in Quails Birds (Coturnix Japonica) in Baghdad City, Iraq. *International Journal of Health Sciences*, *6*(S3), 11250–11262. https://doi.org/10.53730/ijhs.v6nS3.8635

Molecular study of Toxoplasma gondii B1 Gene in Quails Birds (Coturnix Japonica) in Baghdad City, Iraq

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***Abstract*---**Toxoplasmosis is a common zoonotic parasitic disease of medical and veterinary importance worldwide, caused by an obligate intracellular protozoan parasite Toxoplasma gondii. The study was conducted to investigate the infection rate of Toxoplasma gondii in 100 tissues samples of (25/150) seropositive quail bird organs (25 brain, 25 liver, 25 heart and 25 pectoral muscles) of both sex by Nested Polymerase Chain Reaction (N-PCR) based on B1 gene in some areas of Baghdad city during the period from the 1st January 2021, up to 30th September 2021. The total infection rate was 24% (24/100) and the highest percentage was recorded in pectoral muscles 28% (7/25) and the lowest percentage was recorded in the heart 20% (5/25) without significant difference. Regarding the sex the results revealed that the total infection rate of T. gondii in hens was 30.76% (16/52) more than cocks tissues samples 16.66% (8/48) with highly significant (P≤0.01) differences between organs type. In cock quail, the highest percentage was recorded in brain 33.33% (4/12) and the lowest percentage was recorded in the pectoral muscles and heart 8.33% (1/12). While in hen quail, the highest rate was recorded in the pectoral muscle 46.15% (6/13) and the lowest recorded in the brain 15.38% (2/13). The phylogenetic analysis of 10 local isolates of T. gondii in both sex quail birds were recorded in the National Center for Biotechnology Information (NCBI) with accession numbers OM160926, OM160927, OM160928, OM160929, OM160930, OM160931, OM160932, OM160933, OM160934 and OM160935 and their compatibility with other global isolates were 99.32-99.67%. Investigation of quail bird toxoplasmosis by N-PCR that included in this study was done for the first time in Iraq.

Manuscript submitted: 18 Feb 2022, Manuscript revised: 27 April 2022, Accepted for publication: 9 June 2022

***Keywords*---**toxoplasma, B1gene, Nested-PCR, tissue, quail Bird.

Introduction

Toxoplasma gondii is an obligate apicomplexan intracellular protozoan parasite that causes a significant zoonotic disease called toxoplasmosis. The *T. gondii* definitive host in which sexual reproduction occurs are strictly members of the felidae family only, but it is able to infect almost all warm blooded vertebrates, including humans and birds that harbor infective tissue cysts (Dubey, 2010 and Lorencova *et al.*, 2015). *Toxoplasma* is a cystforming coccidian parasite and have three infectious stages which are linked together in an intriguing lifecycle, the environmentally resistant oocysts containing two sporocysts and eight sporozoites, the tachyzoites (rapidly multiplying and circulating) and tissue cyst containing bradyzoites (Tenter *et al.*, 2000 and Jones *et al.*, 2001). Detection of *T. gondii* prevalence in ground feeding birds is considered as a sensitive indicator for environmental contamination with *T. gondii* oocysts excreted in cat feces. Different bird species, such as chickens, ducks, turkey, pigeons have been reported to be infected with *T. gondii* and are regarded as potential sources for human infection if not handled and cooked properly, so it regarded as a significant public health problem worldwide (Al Khalid, 2012; Dubey *et al.*, 2020; Mikaeel and Al-Saeed, 2020).

Molecular detection of toxoplasmosis usually depends on detection of parasite-specific DNA in biological samples using PCR-based molecular methods has gained popularity (Mikaeel and Al-Saeed, 2020). These methods have proved to be are simple, sensitive, reproducible and can be applied to all clinical samples (Contini *et al.*, 2005). The molecularbased assays like Nested-PCR can be further used to confirm the presence of the parasite in tissues. Targeting the *B1* gene for the detection of *T. gondii* was developed. The function of the *B1 gene* is unknown but it is used in diagnosis and epidemiological studies because of its specificity and sensitivity (Burg *et al.*, 1989; Ivović *et al.*, 2012). Al-khanak and Salman, (2021) mentioned that the N-PCR approach has been considered as the optimal approach compared to the other approaches that are utilized to diagnose the *T. gondii* with the use of the tissue sample and certain primer of the parasite.

Considering the importance of chickens in transmission of *T. gondii* to human and felids, since the prevalence of the parasite in poultry is a bio-indicator for soil contamination with *T. gondii* oocysts (Al Khalid, 2012; Dubey *et al.*, 2020) as well as study in Egypt highlights on the role of quail as a source of *Toxoplasma* infection for human (Hussein *et al.*, 2018), and according to previous knowledge, there is no information on *T.gondii* infection in quail birds intended for human consumption in Iraq is available. Therefore, we conducted the present molecular study for *T. gondii* infections in quail birds in Baghdad.

Materials and Methods

Samples collection
Quail birds

One hundred and fifty quail birds (75) cocks and (75) hens with age ranged from (less than sexual maturity to more than one year) purchased**,** from different areas in Baghdad city**,** during the period from the 1st January 2021**,** up to 30th September 2021**.**

Tissue samples

Three gram of each 100 tissue samples (25 brains, 25 hearts, 25 Livers and 25 pectoral muscles) from seropositive of (25/150) quail birds after slaughtered in a humanely way and dissected according to normal anatomical procedures and one hundred organs separated, kept with normal saline in a separated sterile plastic container labeled with date, tissue and quail bird identification and the sterile instruments (scalpel, thumb forceps and scissor) used for tissue specimens collection. The containers were immediately transported under cool conditions (ice bags) as soon as possible to laboratory of Kamal Al-Samarraee Hospital for DNA extraction. The extracted DNA was kept in a deep freezer at -20 C°, until used for nested polymerase chain reaction (Al-Khaled, 2012).

Nested-PCR (N-PCR) based on *B1* gene

Genomic DNA was extracted from the (liver, brain, heart and pectoral muscle) tissue samples of seropositive quail birds, according to the Tissue protocol of G-spin™ Genomic DNA Extraction Kit (Intron Biotechnology, korea). The extracted DNA quality was assessed by running on 1.5% agarose gel stained with ethidium bromide. The electrophoresis gel was examined in the UV transilluminator, and bands were visualized and photographed using a gel documentation system (Gel Doc. ATTO-Japan). The N-PCR primers that used for detection *T. gondii* based on *B1 gene* were designed using NCBI Genbank Sequence (KX270388.1) and Primer3 plus with at 586 bp product size. These primers were provided by (Scientific Reseracher. Co. Ltd /Iraq). The N-PCR technique was performed for detection *Toxoplasma gondii B1* gene from quail tissue samples, which included DNA extraction from tissue samples by using G-spin™ Genomic DNA Extraction Kit (Intron Biotechnology, korea) as described above. Primary PCR master mix preparation by using first primer pair: forward (5- GCCTTCTGTTCTGTTCGCTG -3) and reverse (5- TATCGATTGCAGGCGACCAA -3) (GoTaq ™ Green PCR Master Mix) (Promega, USA), then placed in PCR Thermocycler (Promega, USA). Secondary PCR master mix was prepared by using second primer pair: forward (5- GTCCCATGAAGTCGACCACC -3) and reverse (5- GATTGCAGGCGACCAATCTG -3) (GoTaq ™ Green PCR Master Mix). (Promega, USA), then placed in PCR Thermocycler (Bio-Rad/ USA).

Statistical Analysis

Statistical Analysis System-SAS (2012) used for detection the effect of infection on organs type and Chi-square test (x2) used to compare the significance ($P \leq 0.05$ or 0.01) difference in this study.

Results

DNA Extraction

The DNA extracted was very efficient and showed sharp bands on the agarose gel as clarified in Figure (1).

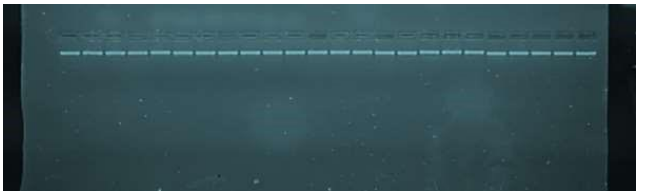

Figure (1). Genomic DNA in 1.5% agarose gel electrophoresis

Nested Polymerase Chain Reaction

Nested PCR products of 100 quail tissue samples were exhibited in distinct bands of 586bp on 1.5% agarose gel electrophoresis and the total infection rate of *T. gondii* from quail tissue samples was 24% (24/100), as clarified in Figure (2, 3, and 4).

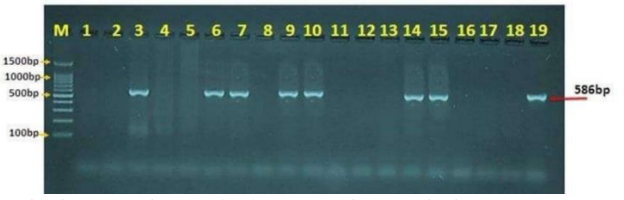

Figure 2. Agarose gel electrophoresis image showed the N-PCR product analysis of glycerol-3-phosphate dehydrogenase *(B1)* gene in *T. gondii* from quail tissue samples. Where M: marker (1500-100bp), Lanes (1-19) were showed some positive N-PCR amplification at (586bp) PCR product

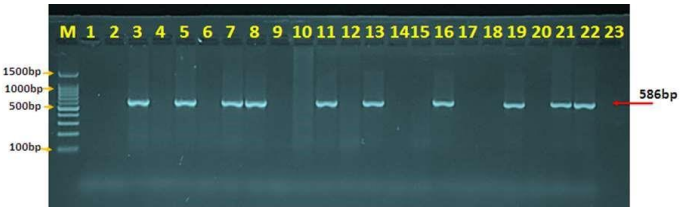

Figure 3. Agarose gel electrophoresis image showed the N-PCR product analysis of glycerol-3-phosphate dehydrogenase *(B1)* gene in *T. gondii* from quail tissue samples were M: marker (1500-100bp). Lanes (1-23) were showed some positive N-PCR amplification at (586bp) PCR product

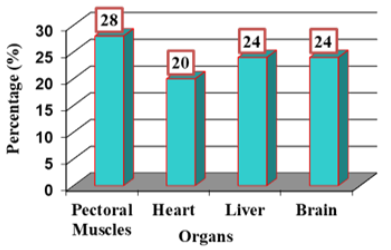

Figure 4: Infection rates of *T. gondii* according to organs type of quails by N-PCR

Figure 4. Agarose gel electrophoresis image showed the N-PCR product analysis of glycerol-3-phosphate dehydrogenase *(B1)* gene in *T. gondii* from quail tissue samples. Where M: marker (1500-100bp), Lanes (1-23) were showed some positive N-PCR amplification at (586bp) PCR product

according to organs type of quails by N-PCR: Out of one hundred tissues samples from four (25 brain, 25 heart, 25 liver & 25 pectoral muscle) organs of seropositive quail birds, only 24% (24/100) were positive by N-PCR. No significant differences was recorded between the organs type, the highest percentage was registered in pectoral muscles 28% (7/25) and the lowest percentage in the heart 20% (5/25), as clarified in Figure (5) and Table (1).

Organs

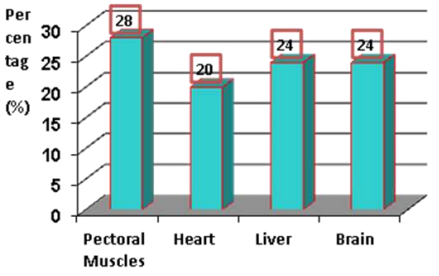

Figure 5. Infection rates of *T. gondii* according to organs type of quails by N-PCR

Table 1
Infection rates of *T. gondii* according to organs type of quails by N-PCR

Organs	Examined No.	Positive No.	Percentage (%)
Pectoral Muscles	25	7	28
Heart	25	5	20

Liver	25	6	24
Brain	25	6	24
Chi-Square (x^2)	0.333 NS		
P value	0.953		
	NS: Non-Significant		

according to organs type from cock quails by N- PCR: A highly significant** (P≤0.01) differences was recorded between the organs type in cock quail, the highest percentage was recorded in Brain 33.33% (4/12) and the lowest was recorded in the Pectoral Muscles and Heart 8.33% (1/12), as clarified in Figure (6) and Table (2).

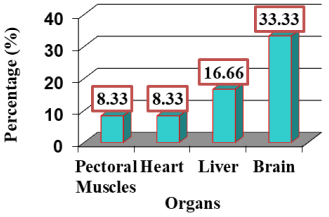

Figure 6: Infection rates of *T. gondii* according to organs type from cocks quails by N-PCR

Table 2
Infection rates of *T. gondii* according to organs type from cock quails by N-PCR

Organs	Examined No.	Positive No.	Percentage (%)
Pectoral Muscles	12	1	8.33
Heart	12	1	8.33
Liver	12	2	16.66
Brain	12	4	33.33
Chi-Square (x^2)	8.347 **		
P value	0.0074		
	** (P≤0.01).		

According to organs type from hen quails by N- PCR test: The results showed highly significant** (P≤0.01) differences recorded between types of organs in hen quails, the highest rate was recorded in the Pectoral Muscle 46.15% (6/13) while the lowest recorded in the Brain 15.38% (2/13), as clarified in Figure (7) & table (3).

Figure 7. Infection rates of T. gondii according to organs type from hens quails by N-PCR

Organs

Table 3
Infection rates of *T. gondii* according to organs type from hen quails by N-PCR.

Organs	Examined No.	Positive No.	Percentage (%)
Pectoral Muscles	13	6	46.15
Heart	13	4	30.76
Liver	13	4	30.76
Brain	13	2	15.38
Chi-Square (x^2)	8.503 **		
P value	0.0081		
** (P≤0.01).			

DNA Sequence & phylogenetic tree analysis

Ten local isolates of *T. gondii* from both sexes seropositive (24/100) quail birds tissue samples were sequences and analyzed by BLAST-NCBI program. The sequences were submitted to the NCBI Gen bank database under accession numbers: OM160926, OM160927, OM160928, OM160929, OM160930, OM160931, OM160932, OM160933, OM160934 and OM160935 and their compatibility with other global isolates were 99.3299.67%. The phylogenetic tree genetic relationship analysis showed that the local *T. gondii* quails isolates (IQ-No.1-IQ-No.10) were showed closed genetic related into NCBI BLAST *T. gondii* Iraq and Iran isolates (MK704513.1) and (MN275917.1), respectively at total genetic change (0.0060-0.0010%), as clarified in Fig (8) and Table (4) and (5).

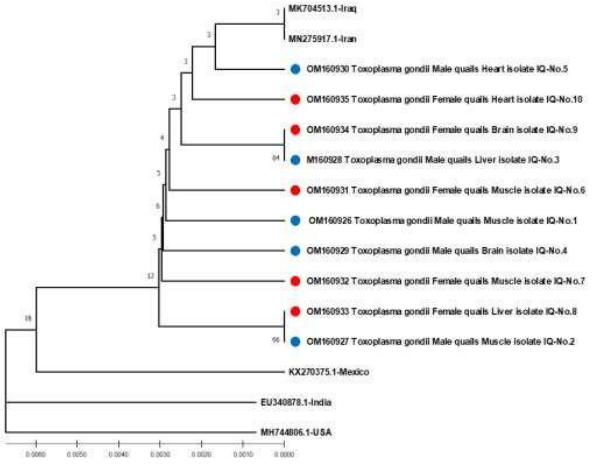

Fig 8. Phylogenetic analysis and identified *T. gondii* of the local isolates with the global NCBI isolates

Table 4
The NCBI-BLAST Homology Sequence Identity between local *T.gondii* Quails (IQ-No.1-IQ-No.10) isolates and NCBI-BLAST related Iraq isolate

local isolate	Genbank Accession number	Homology sequence identity (%)		
		Related country isolate	Accession number	Identity (%)
Male quails pectoral muscle isolate IQ-No.1	OM160926	Iraq	MK704513.1	99.67%
Male quails pectoral muscle isolate IQ-No.2	OM160927	Iraq	MK704513.1	99.67%
Male quails Liver isolate IQ-No.3	OM160928	Iraq	MK704513.1	99.67%
Male quails Brain isolate IQ-No.4	OM160929	Iraq	MK704513.1	99.32%
Male quails Heart isolate IQ-No.5	OM160930	Iraq	MK704513.1	99.34%
Female quails pectoral muscle isolate IQ-No.6	OM160931	Iraq	MK704513.1	99.67%
Female quails pectoral muscle isolate IQ-No.7	OM160932	Iraq	MK704513.1	99.34%
Female quails Liver isolate IQ-No.8	OM160933	Iraq	MK704513.1	99.32%
Female quails Brain isolate IQ-No.9	OM160934	Iraq	MK704513.1	99.67%
Female quails Heart isolate IQ-No.10	OM160935	Iraq	MK704513.1	99.67%

Table 5
The NCBI-BLAST Homology Sequence dentity between local *T.gondii* Quails (IQ-No.1-IQ-No.10) isolates and NCBI-BLAST related Iran isolate

local isolate	Genbank Accession number	Homology sequence identity (%)		
		Related country isolate	Accession number	Identity (%)
Male quails pectoral muscle isolate IQ-No.1	OM160926	Iran	MN275917.1	99.34%
Male quails pectoral muscle isolate IQ-No.2	OM160927	Iran	MN275917.1	99.64%
Male quails Liver isolate IQ-No.3	OM160928	Iran	MN275917.1	99.67%
Male quails Brain isolate IQ-No.4	OM160929	Iran	MN275917.1	99.32%
Male quails Heart isolate IQ-No.5	OM160930	Iran	MN275917.1	99.67%
Female quails pectoral muscle isolate IQ-No.6	OM160931	Iran	MN275917.1	99.67%
Female quails pectoral muscle isolate IQ-No.7	OM160932	Iran	MN275917.1	99.34%
Female quails Liver isolate IQ-No.8	OM160933	Iran	MN275917.1	99.32%
Female quails Brain isolate IQ No.9	OM160934	Iran	MN275917.1	99.67%
Female quails Heart isolate IQ-No.10	OM160935	Iran	MN275917.1	99.67%

Discussion

Molecular detection of *T. gondii* by N-PCR was appeared specific and highly sensitive assay (Mose *et al.*, 2016). Also, Al-khanak and Salman, (2021) mentioned that the N-PCR approach has been considered as the optimal approach compared to the other approaches that are utilized to diagnose the *T. gondii* with the use of the tissue sample and certain primer for the parasite. Nested PCR was chosen as a method in the present study, due to nested PCR had a respectable sensitivity, and distinguish several protozoan parasites closely related to *T. gondii*. The N-PCR can often solve the problems of detection that associated with clinical tissue samples containing a low copy number of targets against a high background of host tissue DNA and inhibitors of DNA polymerase (Albert and Fenyö, 1990). The *B1* gene was only an attractive target for rapid detection of *T. gondii* parasites (Azimpour-Ardakan *et al.*, 2021), so choosing the *B1* gene as a target was due to its amplification has some advantages, such as higher sensitivity than another targeting gene, did not amplify DNA from any other bacterial and fungal, and its sensitivity was not changed. This gene also had good gene conservation. Moreover, the *B1* gene was the most often gene used in toxoplasmosis molecular study (Vitale, 2013 and Halleyantoro *et al.*, 2019). So the N-PCR technique was done using glycerol-3-phosphate dehydrogenase *B1* gene that were amplified to confirm the diagnosis of *T. gondii* from quail tissue samples.

The results of present study 24% (24/100) agree with a number of studies has already shown that a positive PCR result is not always accompanied by positive serology indicating local synthesis of antibodies (Al Sanjary and Hussein, 2012). Nearly close to results (26.9%) have been reported in chickens in Iraq by Al-Sanjary and Hussein, (2012), due to the uniformity of climate and management practice in these areas. In other countries using the PCR technique, the current

study is higher than reported in Pakistan by Khan *et al.*, (2020) who detected that *Toxoplasma* DNA was 10.84% in chicken tissues samples by NPCR, in India 6.06% (Rajendran *et al.*, 2018) in Iran 8% (Mahami-Oskouei *et al.*, 2017), but lower than reported from Kenya 79%, Barzil 42% and Egypt 47% (Mose *et al.*, 2016), and Iraq 5(38.46%) and 56.9% by (Al-Khalid, 2012) and (Al-khanak and Salman, 2021), respectively. The variation in the infection rates between the results of the present study and the previous studies attributed to several risk factors associated with infection, including the number and age of chickens examined, sex, density of the final host, coinfections, and the environmental conditions that effect on the infection, sanitation conditions and management practice. These variable results may be due to the differences in the specimens used and their variable condition and data of studies. Also the laboratory conditions of uncontrolled is affecting on the polymerization chain reaction (Ayinmode and Dubey, 2012; Mose *et al.*, 2016).

The total 24% (100), *Toxo*-DNA was detected by N-PCR in the tissues of quails, there were no significant differences recorded between the types of organs, the highest percentage was recorded in pectoral muscles 28% (7/25) and the lowest was in the heart 20% (5/25). These results were incompatible with Cong *et al.,* (2017) in China who recorded an infection rate of 6.41% (25/390) in common quails muscle tissues by N-PCR and with Al-khanak and Salman, (2021) who recorded the highest percentage in pectoral muscles 58.3% & the lowest percentage was recorded in the brain 62.5% while liver (70.8%) by N-PCR, and with Khan *et al.*, (2020) in Pakistan was observed in chickens higher prevalence in liver 10.50% as compared to heart 9.5% and the lowest percentage was recorded in the muscles 7.11% by NPCR, and disagreed with Mose *et al.*, (2016) in Kenya who recorded 79.0% in the brain of chickens by N-PCR, and with Hassan *et al.*, (2020) in Egypt who revealed that the total infection rate was 7% in quail brain by histopathological examination. Finally disagreed with Al-Khalid, (2012) that recorded the prevalence rate in liver and heart was 57.14% and 25% respectively, while no amplification occurred in samples of brain by N-PCR that attributed to the capacity for tissue cyst formation varies among strains. The differences in the total rate of infection with *Toxoplasma* in quail organ type by N-PCR attributed to several factors such as the number of organ type samples collected, using different diagnostic methods, or probably the tissue cysts not been present in the part of the sample examined, host immunity and parasite strains.

Regarding the sex, the results showed highly significant (P≤0.01) differences recorded between types of organs in quails from both sexes. The highest rate in hens was recorded in the pectoral muscle 46.15% (6/13) while the lowest recorded in the brain 15.38% (2/13). In cocks the highest percentage was recorded in brain 33.33% (4/12) and the lowest percentage was recorded in the pectoral muscles and heart 8.33% (1/12). Also the highest percentage was recorded in hen tissue samples as compared with cock tissue samples. These results relatively agreement with Nazir *et al.*, (2018) who recorded 50% in female pectoral muscle of pigeons in Pakistan by PCR, while disagreement with percentage 23.3% in male pectoral muscle. The percentage was 36.4% recorded in female heart and 21.7% in male heart. The low or high rate of *toxoplasma* infection of the heart, brain, liver and pectoral muscles using the PCR method is

probably related to the presence of tissue cysts in the samples, and it has been reported that the female hormones play an important role in the susceptibility of the animals to *T. gondii* through reducing their immune responses to the infection, or could be attributed to genetic or endocrine reasons of animal. Dubey *et al.*, (1993) and (1994) reported the isolation of *T. gondii* from brain, heart and skeletal muscles of (bobwhite quail) *Golinus virginianus* and (Pheasants) *Phisanas colchircu*, respectively. The phylogenetic tree genetic relationship analysis showed that the local *T. gondii* quail isolates (IQ-No.1-IQ-No.10) was showed closed genetic related into NCBI BLAST *T.gondii* Iraq and Iran isolates (MK704513.1) and (MN275917.1), respectively at total genetic change (0.0060-0.0010%). and identity of local isolates between 99.32%-99.67% with the global NCBI isolates.

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