How to Cite:
Hussein, H. I. J., & Jasim, G. A. (2022). Detection of toxoplasmosis in (Free living and Industrial) chicken of Qadisiyah province, Iraq. *International Journal of Health Sciences*, *6*(S5), 3162–3171. https://doi.org/10.53730/ijhs.v6nS5.9324

Detection of toxoplasmosis in (Free living and Industrial) chicken of Qadisiyah province, Iraq

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***Abstract*---**The study aimed to investigate the differences in the genetic tree of types infected (Free living and Industrial chickens) with the Toxoplasma parasite, (brain and liver) collecting samples in Al-Qadisiyah province during the period from October 2021 until the end of March 2022. A total of 90 samples of chicken were collected, 60 samples of brain and 30 samples of liver. The purpose of diagnosis, the impression method was used. It was found that 60 samples of free living chickens were all positive for examination. As for industrial chickens, 20 samples from 30 were positive at rate 88.8%, in rate of (83.3%) 50/60 from the brain and (66.6%) 20/30 from the liver. Nested PCR targeting aspecific region within SAG3 gene technique applied for detect the genotype of *T.gondii*, the results showed that 30/48 of the chickens (brain and liver) (62.5%) were positive. A genetic match was found between *Toxoplasma gondii* in chickens SAG3 gene and the gene isolates according to NCBI – BLAST.

Keywords*---Toxoplasma gondii,* PCR, parasite.

Introduction

Toxoplasma gondii is an obligatory intracellular protozoan parasite that causes serious disease in humans, small ruminants, other warm-blooded mammals, and birds (Yektaeian *et al.*,2021). It is distributed all around the world (Woyesa *et al.*,2021), *Toxoplasma gondii* has been isolated from a variety of geographical regions (Yang et al.,2021). According to estimates, this parasite threatens one-third of the world's population (Bandelj *et al.*,2021). The parasite which is common in both humans and animals, causes the disease depending on where they reside, 15 to 85 percent of the human population is asymptomatically infected (Mose *et al.*,2020). Feline species, which acts as a definitive and

intermediate host, all animal species act as intermediate hosts (Elsheikha *et al.*,2021). The apicomplexa zoonotic parasite has three infective stages: sporozoites in sporulated oocysts, which are shed in unsporulated form into the environment by infected felids; tissue cysts containing bradyzoites, and fast replicating tachyzoites that observed take place in acute toxoplasmosis (García *et al.*,2021). It has a complex life cycle and infected people with a range of diseases (Overstreet, 2021). Clinical toxoplasmosis in poultry is uncommon since the disease rarely manifests clinically, and there are few publications detailing the symptoms and morphological changes associated with toxoplasma in poultry caused by *Toxoplasma gondii* infection (El-Sayed *et al.*,2021). The parasite infection in free-range (FR) Poultry essential because FR hens are one of the strongest indications for *T.gondii* oocyst contamination in soil because they eat from the ground, and tissues from infected chickens are regarded a good source of infection for cats. *T.gondii* infection in humans and other animals can also be spread through the consumption of infected chicken flesh, Toxoplasmosis can induce clinical illness in chickens on rare occasions (Stelzer *et al.*,2019). Poultry that have access to the outdoors will take up a lot of soil and become infected with Toxoplasma. As a result, free-ranging hens are currently utilized as sentinels around the world to isolate and characterize Toxoplasma strains (Aguirre *et al.*,2019). Since the prevalence of parasite in chickens is a good indicator for soil contamination with *T. gondii* oocysts. In otherwise detect the parasite by molecular and serologic study for infections in chickens (Minutti *et al.*,2021). The birds infected by feed on the ground, human infection is spread through the consumption of raw contaminated poultry and bird meat, aside from the tissue of diseased chickens, cats can contract the disease from infected chickens (Shokri *et al.*,2017).

Aim of Study

The main purpose of the current study was Detection of toxoplasmosis in free living and industrial chicken in AL-Qadisiyah province and the genetic tree of types by Molecular method.

Materials and Methods

Sample:

The study was carried out in 90 samples of free living and industrial chickens were collected from different areas in AL-Qadisiyah province, during the period October 2021, to March 2022, and samples were taken from (brain, liver) and examined by impression method and keep the samples at (-20°C) until they are used for DNA extraction.

Diagnosis of parasite

1-Impression smear.
A primary diagnostic method by noting the parasite stages in prepared stamp the tissue on the glass slid and left in air to dray and methanol alcohol 90% was added for ten minute to fixating then was stained with Giemsa stain for 15 mints

and was washed by tab water then left to dray, after drying they were examined under the oil immersion lens of light microscope (Zhang *et al.*,2019).
2- Diagnosis of parasite by Nested PCR.

Genomic DNA Extraction: according to (Grigg *et al.*,2001)

The aim of using a primer is to amplify a specific gene segment SAG3, to detect *Toxoplasma gondii* in free living and industrial chicken samples. These primers were designed by (Grigg et al.,2001).), The primers were synthesized by (Macrogen company, Korea), The Nested PCR technique was performed for detection *T. gondii* targeting a specific region based Surface antigen 3 gene(SAG3) from free living and industrial chicken tissue samples to 48 samples were positive to impression method to toxoplasma which confirmed by electrophoresis analysis by strands of DNA which successful binding then gene sequence and analysis (sequencing phylogenetic tree analysis) to compare with NCBI-BLAST data base.

Primers

Table (1): primer of SAG3 gene of *T.gondii*

Name	Sequence 5-----3	Annealing	Amplicon size
SAG3ExF	CAACTCTCACCATTCCACCC	55 C	450 bp
SAG3ExR	GCGCGTTGTTAGACAAGACA		
SAG3InF	TCTTGTCGGGTGTTCACTCA	56 C	225 bp
SAG3InR	CACAAGGAGACCGAGAAGGA		

PCR amplification of DNA samples in the first round
PCR components in the first round:

PCR component	Volume/ µl
H2O	11.8
10x buffer	2
DNTPs	2
Forward primer (0.5 pmol/ 20 µl)	1
Reverse primer (0.5 pmol/ 20 µl)	1
Taq Polymerase	0.2
DNA template (About 100 ng)	2
Total	20

PCR amplification of DNA samples in the second round
PCR components in the second round:

PCR component	Volume/ µl
H2O	11.8
10x buffer	2
DNTPs	2
Forward primer (0.5 pmol/ 20 µl)	1
Reverse primer (0.5 pmol/ 20 µl)	1

Taq Polymerase	0.2
DNA template	1
Total	20

Thermo cycling conditions:

The first and second rounds of PCR were conducted using a PCR thermo cycler system, with the first and second rounds being similar to those listed in the table (2).

Table (2): PCR thermo cycler conditions

PCR step	Temperature	Time	Repeat
Initial denaturation	95°C	10 min	1
Denaturation	95°C	45 sec	39
Annealing	56 °C As mentioned in the primers table	40 sec	
Extension	72°C	50 sec	
Final extension	72°C	5min	1

Statistical Analysis

Use the percentage to analyze the results that were reached in the current study to prove the existence of a relationship between toxoplasmosis infection in two type of living chickens.

Results

Microscope examination by using impression smears

Out of 90 sample from FR and Industrial chickens 88.8% (60/60) by 100%, (20/30) by 66.6% were positive to infection by impression smears respectively table (1).

Table (1) show the percentage of infection by impression smears.

Type of sample	No.	(+ve)	%
FR chickens	60	60	100%
Industrial ch.	30	20	66.6%
Total	90	80	88.8%

A total of 90 samples of FR and industrial chickens. 50 were positive from 60 brains from FR and industrial chickens (83.3%) and 20 were positive from 30 liver in (66.66%) by organ impression smear method. In the table (2). figure (1,2).

Table (2) infection according to organ by *T. gondii* by impression smears.

Organ	No.	(+ve)	%
Brain	60	50	83.3%
Liver	30	20	66.66%
Total	90	70	77.7%

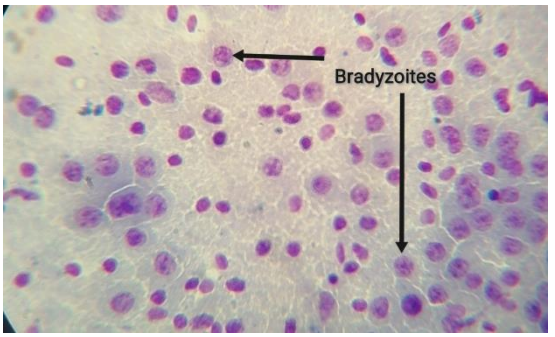

Figure (1): Microscopic image of tissue cysts of impression smears of liver chicken stained with Giemsa stain (100x).

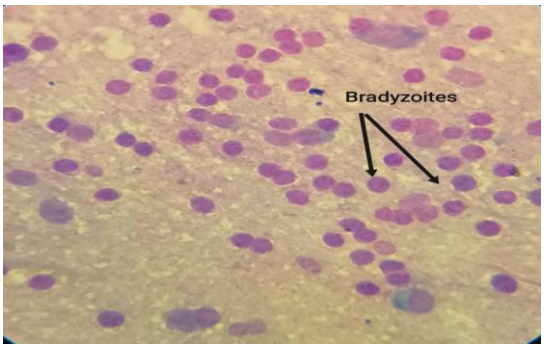

Figure (2): Microscopic image of tissue cysts of impression smears of brain chicken stained with Giemsa stain (100x).

Detection of *T. gondii* using Molecular method (nPCR)

The results of PCR amplification which was performed on the DNA extracted of SAG3 gene of *Toxopalasma gondii*, the samples were taken from 90 samples which were positive result with impression method as (Liver 30 and brain 60), respectively the studied isolates confirmed by electrophoresis analysis. The result of nPCR in present study of chickens as (Liver and brain 30/48) 62.5% from positive samples to impression method. Out of 48 sample from liver and brain were positive in impression smear performed on the DNA extracted and by Nested PCR to targeting SAG3 gene by primers the result show 30/48 62% positive.

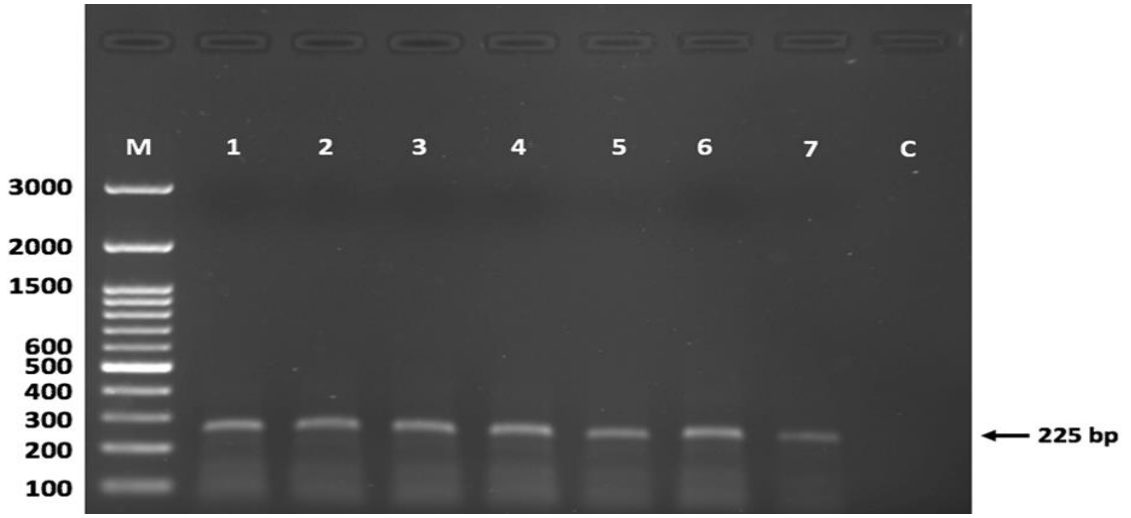

Figure (3): - Agarose gel electrophoresis image (2 %Agarose) shows the positive amplicons (1-7) of *Toxoplasma gondii* targeting SAG3 gene (size= 225 bp) originated from testing chicken samples. C is control negative in which similar PCR reaction mixture was used except H2O was added instead of DNA. M is molecular marker from Genedirex, Korea.

DNA sequencing, genetic tree analysis, genetic polymorphisms and genotyping (SAG3)

As for the results of isolates of toxoplasmosis from chickens, isolates No. (1, 2, 3, 5, 6, 7 and 8) showed, as for the results of isolates of toxoplasmosis from chickens, isolates No. (1, 2, 4, 5, 6, 7 and 8) obtained from chickens (brain and liver); Close kinship with NCBI-BLAST for the parasite *T. gondii* type (MT358429, MT358429, MK127861, MH744785, MH744782, MT358429, MH744785), match ratio (99.45-100%) for samples (1, 2, 4, 5, 6, 7 and 8), while isolates No. (3, 9 and 10) showed a genetic kinship with NCBI isolates of the Toxoplasma parasite, type (MT358429, MT358429, MT358429), match ratio (98.36-100%). The identification of genetic polymorphisms in chicken (41) C/A ON240973, (98) T/C ON240964, ON240967, ON240968, ON240969, ON240970 and ON240971, (107) G/A ON240965, (130) G/C ON240966. While Results multiple alignment analysis of the identified *Toxoplasma gondii* amino acids sequences(SAG3) in chicken, there was a single nucleotide alteration that occurred of T /C at position (98) (ON240964, ON240967, ON240968, ON240969, ON240970 and ON240971) of the SAG3 of *T. gondii*, where nucleotide replaced resulted in change of a single nucleotide residue in the deduced amino acid sequence at position 33 as Leucine (L) instead of Serine (S) obtained accession numbers (ON240967, ON240968, ON240969, ON240970, ON240971), at the position (107) substitute to amino acid sequence at position (36) as Glycine (G) instead of Glutamic acid (E) obtained accession numbers (ON240965, ON240966), and at position (130) substitute to amino acid sequence at position (44) as Glycine (G) instead of Glutamic acid (E) obtained accession numbers (ON240966, ON240972 and ON240973) table (3).

Table (3): the NCBI-BLAST Homology Sequence identity (%) between local *Toxoplasma gondii* isolated from Chicken cases that have been deposited in the NCBI under the following accession numbers (ON240964, ON240965, ON240966, ON240967, ON240968, ON240969, ON240970, ON240971, ON240972, and ON240973) and NCBI-BLAST deposited other global strains.

Toxoplasma gondii sequence	Obtained Accession Number	NCBI-BLAST Homology Sequence identity (%)			
		Identical to	Genbank Accession number	Country	Identity (%)
1	ON240964	*Toxoplasma gondii*	MT358429	Spain	99.45
2	ON240965	*Toxoplasma gondii*	MT358429	Spain	99.45
3	ON240966	*Toxoplasma gondii*	MT358429	Spain	98.91
4	ON240967	*Toxoplasma gondii*	MK127861	Mexico	99.45
5	ON240968	*Toxoplasma gondii*	MH744785	USA	99.45
6	ON240969	*Toxoplasma gondii*	MH744782	USA	99.45
7	ON240970	*Toxoplasma gondii*	MT358429	Spain	99.45
8	ON240971	*Toxoplasma gondii*	MH744785	USA	99.45
9	ON240972	*Toxoplasma gondii*	MT358429	Spain	98.91
10	ON240973	*Toxoplasma gondii*	MT358429	Spain	98.36

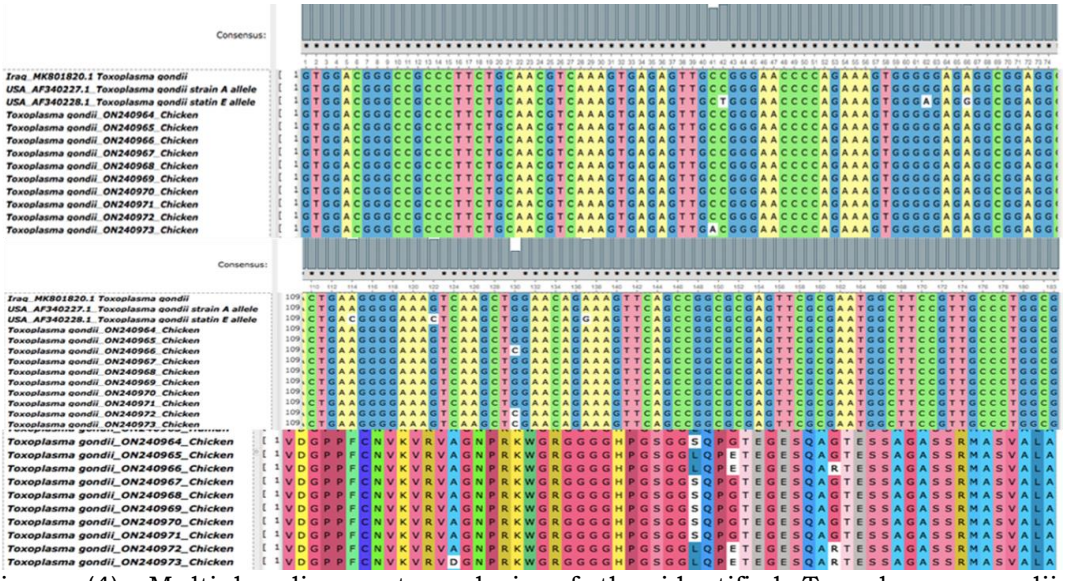

Figure (4): Multiple alignment analysis of the identified Toxoplasma gondii sequences and amino acids sequences (SAG3 gene) followed by the obtained accession numbers and the host from which originated (chicken and eggs) that shows the similarity and differences among these sequences which compared with global deposited sequences. This was carried out by MegaX software.

Discussion

Out of 90 samples of free living chickens, industrial chickens were examined by using the impression survey method 88.8% positive the percentage of total s.

While organ, (brain and liver) impression smear method 50 were positive from 60 brains from FR and industrial chickens (83.3%) and 20 were positive from 30 livers in (66.66%). Based on our results, *T. gondii* can persist in tissues of Chickens. Therefore, chicken's meat should be cooked thoroughly before human or animal consumption. Because *T. gondii* is disseminated in the environment through feces of infected cats, cats should not have access to food and water consumed by chickens. *T. gondii* infects and multiplies in a variety of organs in infected birds, resulting in a considerable increase in circulating antibodies (Almuhana and Al-Hamairy,2021). Despite the fact that the disease generally manifests without symptoms in hens, infected chickens can keep the parasite in their organs for months after infection, indicating their potential role as reservoirs and vectors of the parasite (SOGUT and TEKELIOGLU,2021). The findings of this investigation matched those of (Lindsay and Dubey,2020), who found tissue cysts in the brain, heart, lung, liver, and spleen of Gallus domestics. The findings of this study are consistent with those of (Busselman and Hamer,2021), who noted the possibility of diagnosing *T. gondii* in various organs in Zoo birds.

The percentage of chickens with Toxoplasmosis differs (Brain and Liver), that recorded in current study by conventional and nPCR, may be attributed to that nPCR was more sensitive and specific for detection *T. gondii* in tissues than from the rest of the tests, and distinguish several protozoan parasites closely related to *T. gondii* (Minutti *et al.*,2021). When clinical tissue samples have low copy counts of targets against a high background of host tissue DNA and inhibitors of DNA polymerases, nPCR can typically address detection problems (Martínez-García *et al.*,2021). The results of current study were high than that recorded (10.3% and 17.1%) in chickens of Brazil and United state respectively (Obonyo *et al.*,2022). The current study showed that the strands of DNA that arise from successful binding between specified primers and extracted DNA of isolates appear as a single band together with the 225 pb band product size in this analysis. As for the results of isolates of toxoplasmosis from chickens, as in the table (5) isolates No. (1, 2, 3, 5, 6, 7 and 8) , isolates No. (1, 2, 4, 5, 6, 7 and 8) obtained from chickens (brain and liver); Close kinship with NCBI-BLAST for the parasite *T. gondii* type (MT358429, MT358429, MK127861, MH744785, MH744782, MT358429, MH744785), match ratio (99.45-100%) for samples (1, 2, 4, 5, 6, 7 and 8), while isolates No. (3, 9 and 10) showed a genetic kinship with NCBI isolates of the Toxoplasma parasite, type (MT358429, MT358429, MT358429), match ratio (98.36-100%), after make the alignment samples chicken , it shows has three-position polymorphism at site with accession number in the strains (41) C/A ON240973, (98) T/C ON240964, ON240967, ON240968, ON240969, ON240970 and ON240971, (107) G/A ON240965, (130) G/C ON240966.

In the figure (4) chicken, there was a single nucleotide alteration that occurred of T /C at position (98) (ON240964, ON240967, ON240968, ON240969, ON240970 and ON240971) of the SAG3 of *T. gondii*, where nucleotide replaced resulted in change of a single nucleotide residue in the deduced amino acid sequence at position 33 as Leucine (L) instead of Serine (S) obtained accession numbers (ON240967, ON240968, ON240969, ON240970, ON240971), at the position (107) substitute to amino acid sequence at position (36) as Glycine (G) instead of Glutamic acid (E) obtained accession numbers (ON240965, ON240966), and at position (130) substitute to amino acid sequence at position (44) as Glycine (G)

instead of Glutamic acid (E) obtained accession numbers (ON240966, ON240972 and ON240973).

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