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Molecular Detection of *Toxoplasma Gondii* in Naturally Infected Cats in Mosul City

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TOXOPLASMA gondii, an obligatory intracellular protozoan parasite, that has the ability to causes toxoplasmosis in wide range of host, including humans and various warm-blooded animals as intermediate hosts. The parasite has a complex life cycle include sexual and asexual replication in members of the cat family (Felidae).

In this study, 45 blood samples were collected from stray cats that caught with special traps from Mosul's residential neighbour hoods, during the period extended from September 2022 to February 2023, the results of the current study showed that all cat serums gave positive to Direct Latex agglutination test, While, the percentage of infection with the parasite by ELISA was (26.6) .According to gender the percentage of infection in females was (15.5%) more than males (11.1%). In order to confirm the infection, the blood samples collected during the study were subjected to polymerase chain reaction (PCR) detection using primers targeting B1 gene. Molecular results showed that the infection rate was 22% (10 out of 45). Bioinformatics and phylogenetic analysis revealed the presence of SNPs in the sequence of the nitrogenous bases of the two isolates that were recorded during this study for the first time in the city of Mosul. It was registered in the name of the two researchers in the International Gene Bank with serial number LC749846.1 RK1 and the second isolate LC749847.1 RK2.By comparing the sequences of the two isolates, it was noted that there is a single nucleotide polymorphism (SNP), as well as some mutations (inversion and insertion mutations).

Keywords: *Toxoplasma gondii*, B1 Gene, Elisa, Mosul city, Stray cats.

Introduction

Toxoplasmosis is considered one of the global zoonotic diseases, it caused by a "successful" parasite called *Toxoplasma gondii* [1]. This coccidian parasite has a wide variety of intermediate hosts such as humans and a great majority of homoeothermic animals [2-4], and the family felidae is considered a definitive host [5]. The disease is transmitted mainly by the consumption of food and water contaminated by sporulated oocysts or tissue cysts, mainly from undercooked meat [6-8]. In addition to more than 2 billion people infected by this parasite [9,10]. The infection causes huge economic losses. Cats can spill millions of oocysts and infection rates in cats reflect to the rate in local areas and populations [11,12]. *Toxoplasma gondii* is usually more prevalent, especially in moist, warm, and low-altitude regions, which is associated with longer viability of sporulated oocysts [13-15] The prevalence of toxoplasmosis in male and female domestic cats was higher in Asia, Europe, Africa, and Australia [16, 17]. According to the CDC, *T. gondii* accounts for approximately 24% of all estimated deaths due to food-borne pathogens in the USA [18-20]. Although, many studies have been conducted that clinical presentations are not specific for diagnosis of toxoplasmosis, many laboratory methods were developed to detect infection. The most commonly applied diagnostic methods may be established by serologic tests, PCR, and isolation of the

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parasite from tissues by histological examination. Enzyme-linked immunosorbent assay (ELISA) is accurate to identify *T. gondii* infection due to a rapid and cost-effective with considerable number of samples in a short time[21]. while conventional PCR (polymerase chain reaction), nested PCR (n-PCR), and real-time PCR, considered an early assay approaches for the diagnosis toxoplasmosis ,that can examine a wide variety of samples [7] In Iraq, toxoplasmosis is well studied in many intermediate hosts, information about the prevalence of *T. gondii* or infection and epidemiology in cats is little, and not exhaustively detailed [22]. So far, molecular techniques readily used to detect the parasite in blood and faeces cat by conventional PCR, thus very important to find the suitable genomic sequences for sensitive and specific detection, common genomic targets used is B1 gene[23].

Therefore, the aim of the present work was carried out to estimate the prevalence followed by genotypes of *T. gondii* and DNA sequencing of *T. gondii* to determine the genetic diversity of the -positive samples in blood cats, with phylogenetic analysis in stray cats for the first time in Mosul city, Iraq.

Material and Methods

Study area and collection of Samples

Forty five stray cats were randomly obtained from different areas in Mosul city (Northern of Iraq). This city is away about 400km from Baghdad, located between longitudes 42°56` - 43°22` and 36°16` - 36°30` latitudes (Figure 1), during the period from September 2022 to February 2023, using special traps prepared for this purpose. The information of each cat, which includes the approximate age, weight, and gender, was recorded with the help of a specialized veterinarian. As well as the type of living (stray) and geographical location.

Blood samples collection

After anesthetizing the cats by ketamine 0.1mg/Kg of the cat's body weight, 4 ml of blood specimens was collected from the heart as shown in figure 2. All animals were manipulated within a suitable standards and ethical issues considered to cats by veterinary and animal health recommendations. The blood Specimens was divided into two equal parts, the first part containing EDTA material, which was kept at 4°C, until nucleic acid extraction within 24 hours, and the second part, free of EDTA, was centrifuged at 3000g for 15 minutes, and then frozen at (-20°C)[24].

Ethical approve; University of Mosul, College of Education for Girl's animal care committee approved each step of the above protocol.

Serological analyses

A. Direct Latex agglutination test

Fig.1. Satellite map shows the locations for collecting cat in Mosul city

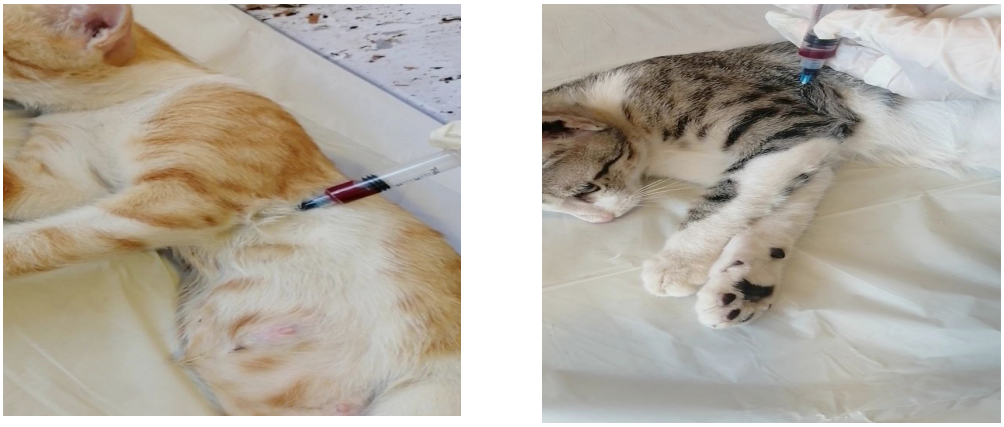

Fig. 2. Collection of blood samples from heart of cats

A latex examination was conducted for all cat serums to confirm the presence of the *T.gondii* parasite. Latex particles made of polystyrene covered with the antigen of the *T. gondii* parasite were used. Agglutination occurs as a result of the presence of antibodies in the cat serum [25].

B. ELISA test

All serum blood samples previously isolated were utilized to test the infection of *T.gondii* in the serum examined with a commercial Elisa kit by ID.Vet *Toxoplasma* Indirect Multi-species French company [26].and the optical densities (OP) were recorded at 450 nm.

Molecular analyses

A. DNA extraction from the blood of cats

Whole blood samples were collected in EDTA tubes, were used to extracted DNA from blood cats, depending on method manufacturer's instructions kit of the Korean company: Add Bio (add prepGenomic DNA) Extraction kit [27].

B. Amplification B1gene region by conventional PCR

The polymerase chain reaction technique were used to detect B1gene region in order to diagnosis *T.gondii* in blood cat samples , by dilution with Borat, EDTA buffer Tris solution to obtain the required concentration for conducting the PCR reactions at (50) ng/microliter for each sample.

The primers targeting *B1* gene were used according to Mohammed et al.[27] The master mixture reaction volume were done in a final volume PCR reaction fixed to 20 µl, The reaction mixture contained 4µl DNA template , 4µl DW and 1µl of 10pmol of each of the forward and reverse primers. The PCR cycler condition was installed according to the guide as shown in (Table 2)

C. Agarose gel electrophoresis of DNA products

Separation of the PCR products was done in 2% agarose gel electrophoresis, The electrophoresis samples were prepared by mixing 5 µl of DNA sample with 3 µl of loading solution then 4 µl of DNA ladder (100 bp) were add. Finaly, PCR product were electrophoresed with 80 volts for 60 minutes and the results were visualized under UV light [27].

D. Sequence and phologintic analysis

The sequencing was done depending on national instrumentation centre for environmental management which online at (http://nicem.snu.ac.kr/main/?en_skin=index.html). PCR product were used to the sequencing of 10 samples of *T. gondii* isolated from blood cats.

Results

Serological analyses

A. Direct Latex agglutination test

The results of the current study, 45 isolated blood samples from stray cats which hunted from random areas of the Mosul city were examined by Direct Latex agglutination test, all samples of cat serums gave positive to infection of *T. gondii.*

B. ELISA test

By following up the results of the ELISA assay to detect IGg antibodies to the *Toxoplasma gondii* parasite in the cat's sera, the results showed that there were 12 positive samples out of 45 with a percentage of 26.6% and 33 negative with a percentage of 73%, as in Figure (3) Table (3).

According to the outcome of ELISA assay, the positive infection in samples of cats serum were distributed by gender between males with a percentage of 11% and females with a percentage 15%. As shown in Table (4).

TABLE 1. Shows the primers targeting of B1 gene were used to detected *Toxoplasma gondii*

No.	Sequence of nitrogenous bases	Temperature	Length	Primer
1	TTTTGACTCGGGCCCAGC	60	18	Forward
2	GTCCAAGCCTCCGACTCT	58	18	Revers

TABLE 2. The cycler conditions for *B1 gene of Toxoplasma gondii*

No.	Stage	Temperature	Time	Cycle number
1	Initial denaturation	95	6 min.	1
2	Denaturation	95	45 sec.	35
3	Annealing	56	1 min.	
4	Extension	72	1 min.	
5	Final extension	72	5 min.	1

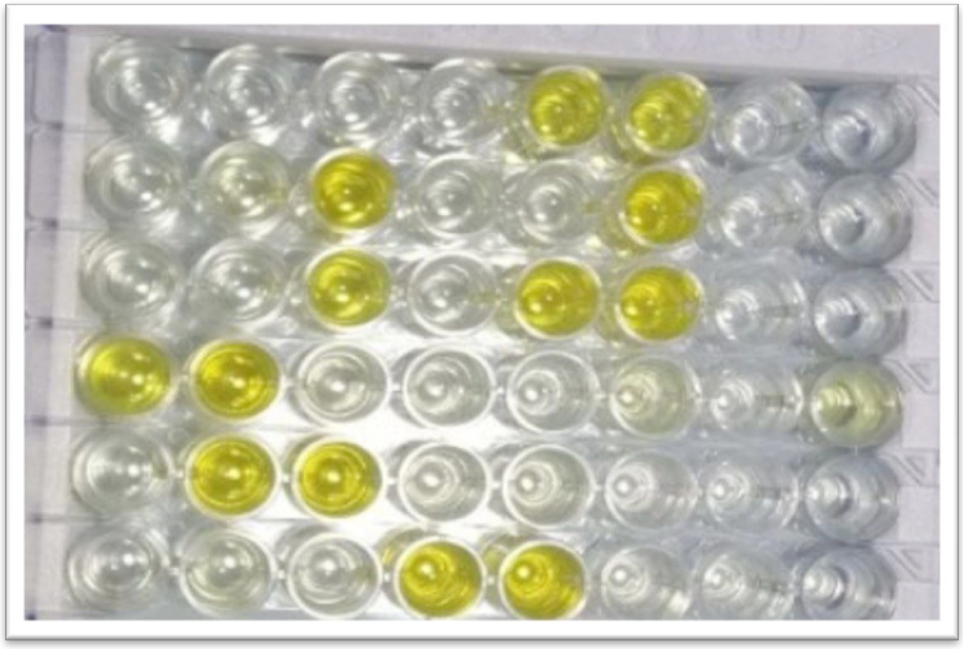

Fig. 3. ELISA assay of infected cat sera containing antibodies of *Toxoplasma gondii*

TABLE 3. Shows the number of positive and negative cat sera samples using the ELISA assay.

The total number of cat samples	Positive sample using ELISA	Percentage%	Negative sample using ELISA	Percentage%
45	12	26.6%	33	73.3%

Molecular analyses

The results of the present study, purity of DNA was confirmed DNA extraction of parasite isolates from blood cats a high purity at a rate from 1.8 to 2 by A_{260}/A_{280} as shown in Figure (4).

In the present study, molecular detection of *T. gondii* by using *B1* gene by conventional polymerase chain reaction (PCR) ,were conducted, 45 DNA samples of *T. gondii* which were extracted from the blood of cats using specific primers B1 gene of 420 bp fragment, the current study recorded an infection rate of 22% in (10) positive samples, while 35 negative samples, with a percentage of 78%, as shown in Table (5).

In order to identify the molecular biological aspects molecular sequencing of the DNA product was carried out in our study, analysis of alignment via online examined sequence fragment when compared with the database of Genbank of *Toxoplasma gondii* accessible data (Table 6).also, it was evident and interesting that there are presence of SNPs in the sequence of the nitrogenous bases of the two isolates that were recorded during the study.

One of the fascinating novel outcome in this results that appeared during the current study as well, the sequence of B1 of *Toxoplasma gondii* was genotype and verified as accession No. LC749846.1 RK1 and the second isolate LC749847.1 RK2 is recording in GenBank (https://www.ncbi.nlm.nih.gov/nuccore/MG792129).

In order to identify the origin of the gene of the study samples RK1 and RK2; the phylogenetic tree was designed as shown in Figure (7) based on the study isolate. designed according to our Iraqi isolate of *Toxoplasma gondii* and compared with global isolates .The results showed that most likeness to the Australian isolate and closest to the current isolate that was recorded during this study, followed by the Brazilian isolate, then the Iranian isolate, with some closeness to other global isolates as shown in Figure (7).

In order to investigate the possibility of differences between the two isolates RK1 and RK2 diagnosed during the current study, and by comparing the sequences of the two isolates,

TABLE 4. Distribution of the infection rates accoding to gender by using ELISA assay.

Total Number of Cats	Gender	Number of positive samples	Percentage%	Number of negative samples	Percentage%
45	Males	5	11.1%	10	22%
	Females	7	15.5%	23	51.1%
Total No.	45	12	26.6	33	73.3

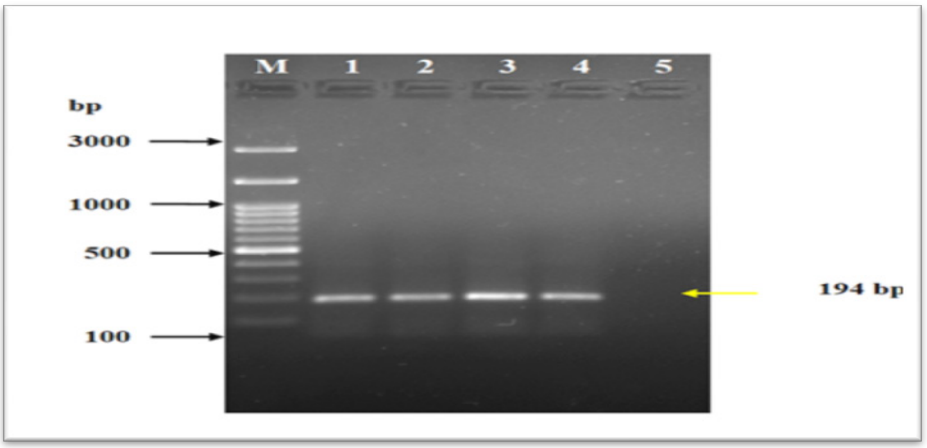

Fig. 4. The electrophoresis of agarose gel and bands produced. The path M (Marker) shows the size of 100 bp. The path 1-4 represent positive blood samples from infected cats with a size of 194 bp. The path 5 represents the negative control

TABLE 5. Positive and negative samples for infection with *Toxoplasma gondii* isolated from the blood of cats using molecular examination.

Total Number	Positive samples	Percentage%	Negative samples	Percentage%
45	10	22%	35	78%

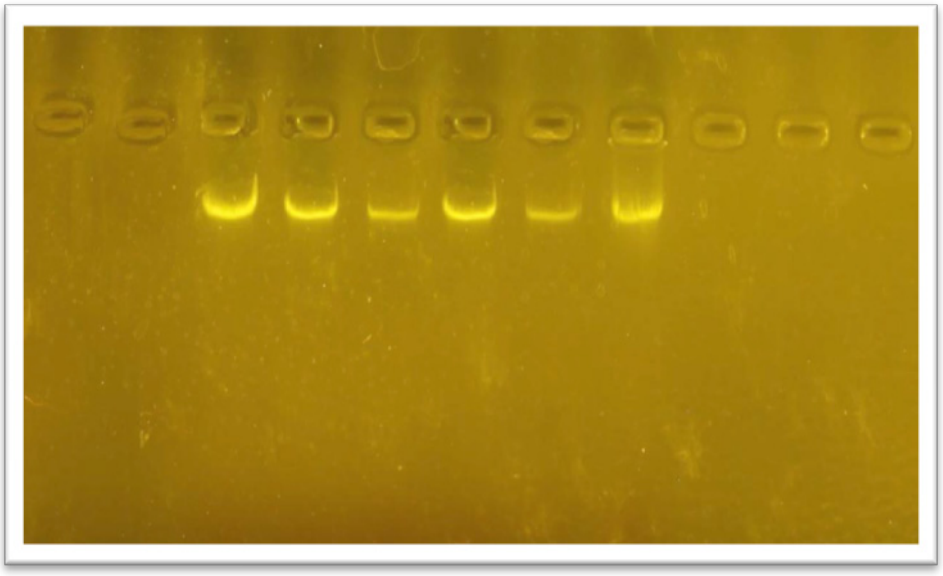

Fig. 5. Agarose gel electrophoresis of reaction bands by PCR technique using the B1gene of ***Toxoplasma gondii*** **isolated from the blood of cats**

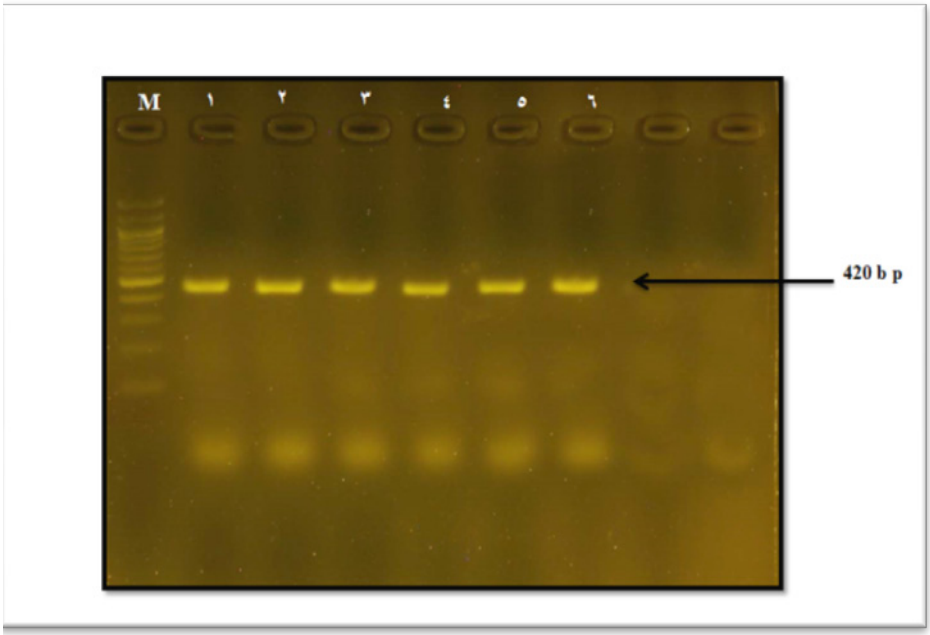

Fig. 6. The product of the PCR reaction for the B1 gene of the Toxoplasma gondii parasite in cat blood shows a reaction result of 420 bp, as M represents the DNA ladder and the samples (1,2,3,4,5,6) represent the positive samples in which the bands of parasite's DNA that were electrophoresed with agarose gel appeared

TABLE 6. Sequenced and submitted samples of *Toxoplasma gondii* by B1 gene to GenBank

Description	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession
***T.gondii* (Sailie) 17S, 5.8S, 26S and 5S rRNA genes**	1301	1301	100%	0	99.17	8352	X75430.1
***T.gondii* 16S-like Rrna**	1230	1230	100%	0	97.4	1788	X65508.1
***Toxoplasma gondii 18S ribosomal (18S rRNA*) gene**	1334	1334	99%	0	100	1723	L24381.1
***Toxoplasma gondii* 18S ribosomal RNA gene, complete sequence**	1338	1338	100%	0	100	1738	L37415.1
***Toxoplasma gondii* 5S ribosomal RNA (5S rRNA), 3' end, and ribosomal RNA small subunit, 5' end, genes**	1201	1201	91%	0	99.25	2398	M97809.1
***Toxoplasma gondii* isolate Tg10 18S ribosomal RNA gene, partial sequence**	1338	1338	100%	0	100	1738	KX008033.1
***Toxoplasma gondii* ME49 18S ribosomal RNA Rrna**	1338	1338	100%	0	100	1079	XR_001974310.1
***Toxoplasma gondii* ME49 18S ribosomal RNA Rrna**	1332	1332	100%	0	99.86	1312	XR_001974253.1
***Toxoplasma gondii* ME49 18S ribosomal RNA rRNA**	1332	1332	100%	0	99.86	866	XR_001974188.1
***Toxoplasma gondii* RK1 gene for 18S rRNA, partial sequence**	1338	1338	100%	0	100	724	LC749846.1
***Toxoplasma gondii* S48 18S rRNA gene, partial sequence**	1338	1338	100%	0	100	1732	U12138.1
***Toxoplasma gondii* strain RH ribosomal RNA small subunit gene, complete sequence**	1334	1334	100%	0	99.86	1795	M97703.1

TABLE 7. Genetic and SNP analysis of B1 gene of *Toxoplasma gondii* from blood cats in Iraq

Variant P-Value (approxim...	Variant Frequency	Polymorphism Type	Coverage	Change	Length	Maximum	Minimum
0.030	33.3%	SNP (transition)	3	G -> A	1	477	477
0.020	50.0%	Insertion (tandem repeat)	2	(G)3 -> (G)4	0	1,092	1,092
0.020	50.0%	Insertion (tandem repeat)	1	(G)2 -> (G)3	0	1,097	1,098

it was noted that there was a single nucleotide polymorphism (SNP), as well as a codons substitution mutations (inversion and insertion mutations) as shown in Table (7).

Discussion

Toxoplasmosis is still the most zoonotic disease significant and critical in worldwide. The documented infection in cats in Iraq are very

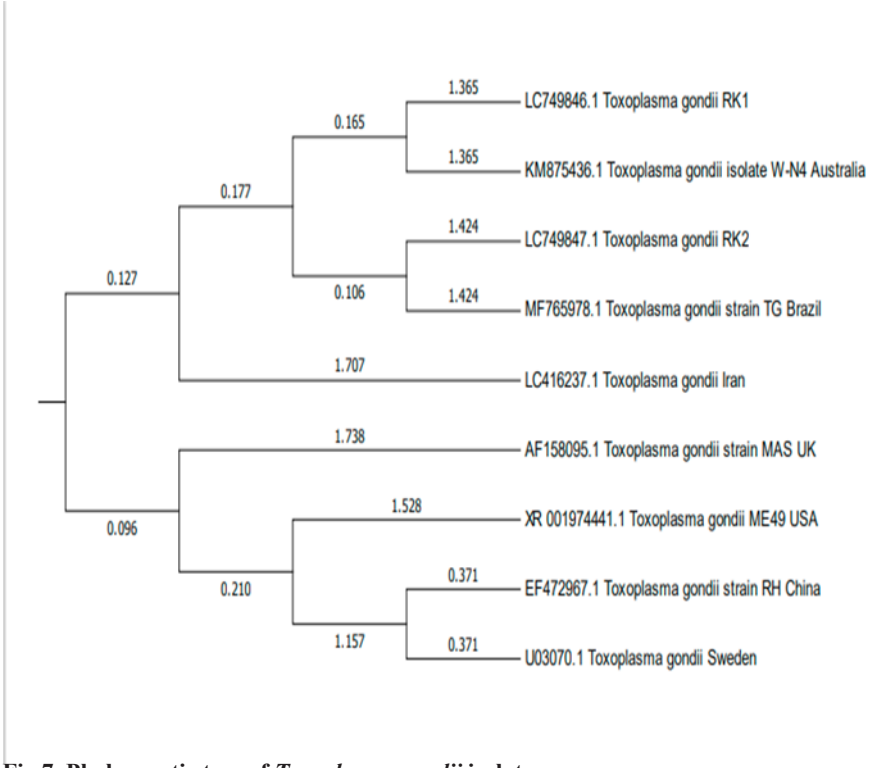

Fig.7. Phylogenetic tree of *Toxoplasma gondii* isolates

rare, despite it being the main source of human infection, and given the importance of this parasite due to the economic and health losses [28-30]. This disease may cause abortion of fetuses in pregnant women [31,32]. This study focused on detecting the presence of the *Toxoplasma gondii* parasite in stray cats in the city of Mosul. The current results of the ELISA test for cat sera showed seropositivity for toxoplasma antibodies for 12 samples, at a rate of 48% out of a total of 45, this study agreed [33]. As the high prevalence of Toxoplasmosis in stray cats can be a cause of environmental pollution by shedding the egg cysts that leads to infection of humans through soil and water [34], and agreed with the study of some authors [35]. Our results shown that the rate of infection in female cats was higher than that of males. The relationship was found between infection and the hunting habit of stray cats, whose diet includes such as rodents, and indoor and wet weather in Iraq. From the observation in this results, the highest and variations were observed in the rates of infection using the latex agglutination test compared to the molecular examination and the ELISA examination, and this gives an indication of the inaccuracy of the results using this method, perhaps due to its interference with other parasitic diseases.

Also, the results were close to Fábrega et al.[36], with a rate of 50% for the appearance of seropositivity with ELISA, while Hadi and Faraj [37] found that 3.75% of cats were infected while they were using a stool test and agreed with Hotez [13]. The results of the current study showed through the ELISA test that the oldest cats were more infected than the younger ones, and this may be due to the chances of their exposure to infection through the diversity of the expanding hunting areas and their movement in more places, the chance of infection for young cats may increase over time during acquired and perfect hunting operations [38,18,39] as the greater the age stage of cats, the more they are exposed to injury because experienced cats will be able to hunt a larger number of animals such as mice and birds that may be carriers of the infection, and the environmental and social factors specific to each region may affect the increase in the rates of infection with *Toxoplasma gondii* . The serological negative results by ELISA assay may be due to one of the possible explanations that kittens aged one month or more, are less susceptible to infection, due to the transmission of antibodies derived from the mother cat that is previously exposed to infection [40,41]. The results of the molecular examination study using

polymerase chain reaction by targeting the *B1* gene and amplifying it in this article agree with most previous studies with Hadi and Faraj [37]. The infection was detected in the blood of cats and the presence of a positive result for 10 blood samples of cats out of 45, with a percentage of 22%, and it agreed with Mawloodet al.[42]. The higher prevalence infection with *T. gondii* may be due to climate in Iraq , which plays an important role in the conservation of oocysts, the occurrence of *the* infection are related to higher temperature, lower precipitation .

The evolutionary history of *T. gondii* investigated by phylogenetic tree analysis agree with Azimpour-Ardakan et al. [43]. that *B1* gene was good an attractive target for rapid detection of *T. gondii* parasites.

Conclusion

This study indicated the high infection of *T. gondii* in stray cat in Mosul city, this reveal high prevalence of toxoplasmosis in this region may affect the increase in the rates of infection with *Toxoplasma gondi*. Furthermore, more work are needed to identify the origin and phylogenetic tree and their virulent of *Toxoplasma gondii* in cats .

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Conflict of interest

There is no conflict of interest in the current study.

Funding statement

The authors approved that there was no funding support this work.

Contribution of Authors

S.K.AL-Zori and R.N. Hamoo contributed equally to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

High lights

1- Toxoplasmosis, is a diseases caused by parasites called *Toxoplasma gondii* .

2. The importance use molecular methods and serological methods in diagnosis of parasite infection.

3- Cats, being the main source of human infection.

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الكشف الجزيئي عن طفيلي المقوسة الكوندية في القطط المصابة طبيعيا في مدينة الموصل

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طفيلي المقوسة الكوندية طفيلي أولي إجباري داخل الخلايا ، لديه القدرة على احداث داء المقوسات في مجموعة واسعة من المضائف بضمنهم الانسان وعدد كبير من الحيوانات ذوات الدم الحار كمضيف وسطي للطفيلي دورة حياة معقدة أذ يحدث التكاثر الجنسي واللاجنسي في أفراد عائلة القط (سنوريات).

تم جمع 45 عينة دم من القطط الضالة التي تم اصطيادها بفخاخ خاصة من أحياء مدينة الموصل ، خلال الفترة الممتدة من سبتمبر 2022 إلى فبراير 2023 ، وأظهرت نتائج الدراسة أن جميع العينات المفحوصة أعطت نتيجة موجبة بأستخدام أختبارتلازن اللاتكس المباشرفي حين كانت نسبة الإصابة بالطفيلي بأستخدام تقنية الاليزا ٪11 (5 من 45) في القطط الذكور، في حين كانت النسبة في الإناث (٪15) ، كما تم إخضاع عينات الدم التي جمعت أثناء الدراسة للكشف عن الاصابة من خلال تفاعل البلمرة المتسلسل (PCR) باستخدام بادئات تستهدف جينB1 ، أظهرت النتائج الجزيئية أن معدل الإصابة كان ٪22 (10 من 45). وكشف تحليل المعلوماتية الحيوية عن وجود تعدد الأشكال في تسلسل القواعد النيتروجينية للعزلتين اللتين تم تسجيلهما أثناء الدراسة لأول مرة في مدينة الموصل ، وتم تسجيلها باسم الباحثتين في بنك الجينات بالرقم التسلسلي LC749846.1 RK1 والعزلة الثانيةبالرقم LC749847.1 RK2. ، وبمقارنة تسلسل العزلين ، لوحظ أن هناك تعدد أشكال للنيوكليوتيدات، مع بعض الطفرات (طفرات الانقلاب والغرز).

الكلمات المفتاحية: المقوسة الكوندية ، جين B1 ، القطط االضالة ، الاليزا ، مدينة الموصل .