Original Article

Molecular Study of Toxoplasma Gondii Infection in Sheep at Basrah Province

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Abstract

Toxoplasma gondii is the causative agent of toxoplasmosis, one of the most common parasitic infections of humans and pets. In this study, the parasite was isolated from chopped diaphragm muscles from naturally infected sheep in Basrah Province. 196 meat samples were collected from local sheep during the period from February 2020 to March 2021. The samples were examined microscopically and (115) positive samples containing the parasite were recorded. The percentage of infection was (59%). The current study included a genetic detection of T. gondii in the sheep to find out the infection rate spread of the parasite in local sheep meat and to determine the main cause of transmission of infection to humans. Nested Polymerase chain reaction technique using specific nested primers was used for genetic detection of the parasite in sheep. The PCR-positive samples recorded an infection rate of (28.8%). The DNA sequences were studied to determine the sequence of the nitrogenous bases of the DNA of the parasite. The genetic tree of these samples comparing with isolated hosts was drawn.

Keywords: Basrah, Molecular Study, Nested PCR Sheep, Toxoplasma Gondii.

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INTRODUCTION

Livestock animals, when infected with *T. gondii* during pregnancy will suffer from parasitemia, infect the placenta and fetus and ultimately result in fetal resorption, miscarriage and death. Most *T. gondii* infections in sheep occur through the ingestion of oocysts, a stage of the parasite, which is very stable and can survive in favorable conditions in the environment for over 12 months, contaminating pasture, feeds and drinking water (Innes *et al*., 2009) (1). It is a public health problem owing its transmission to humans (Raeghi *et al*., 2011) (2). In sheep and goats infection not only it results in significant reproductive looses, but also it has an implication of public health since consumption of infected meat and milk can facilitate zoonotic transmission (Bisson *et al*.,2000) (3). Many diagnostic techniques used for identification of toxoplasmosis infection: isolation of the parasite, microscopic examination, serological and molecular technique (Mahajan *et al*., 1977) (4).

MATERIALS AND METHODS

Meat sample collection

Meat samples were collected after slaughtering from the sheep in butcher shops in Basrah province. Meat samples were collected in clean plastic sterile containers, kept in cool container, and transported immediately to the laboratory. Each sample was treated separately, and after removing adipose tissues from them they were cut in to small pieces (7-26) gm in weight.

Isolation of parasite from meat

Meat samples were minced thoroughly in sterilized mincer containers (20ml). Mixed with equal volume of normal saline, the solution was filtered by using gauze to avoid large particles. Then placed in clean test tube centrifuged at 3000 pm for 10 minutes, the supernatant discarded and the sediment was then resuspended by normal saline, these process were repeated 3 times. Pencillines 1000 l.U and 100 mg of streptomycin were added into the solution to prevent contamination. Many clean dry smear slides were prepared from the sediments of the above samples.

A drop of the same sediment was placed on a clean glass slide for preparing smear, left to dry and after fixation by methyl alcohol 90% for ten minutes, stained for 30 minutes with 10% Giemsa stain for further details and examined and photographed under light Leica microscope supplied with digital camera drawing scale.

Microscopic test of T.gondii

1. Examination and imaging of specimens under a

light microscope

T. gondii samples were photographed in sheep in the Parasitological Laboratory of Graduate Studies at the College of Education for Pure Sciences using a Leica optical microscope with a digital camera and scale.

2. Electron microscopy of T. gondii.

The scanning electron microscope (FESEM- EDS- Zeiss Supra 55(VP) Germany/ Pharmacy College, Basrah University was used to photograph the parasite specimens.

The samples were first loaded on specimen stub using double side carbon tape and stick down the mask layers on it then tight all stubs on specimen's holder after blowing to remove non- adherent pieces. Prepared specimen were loaded on SEM via air lock door depend on low voltage to exceed the coating technique and avoid charging. By using secondary electron detector (SE2) for high resolution and sharp image with fix magnification and well calibrated Quantax EDSX Flash 6/60 Burker (Germany) (Kazmiruk, 2012)(5).

Molecular study (PCR)

Nested Polymerase chain reaction technique using specific nested primers was used for genetic detection of the parasite.

Nested PCR

Nested PCR primers for *Toxoplasma gondii* were provided by Macrogen company south Korea.

First primer: (500 bp)

Forward (F1): 5'-TCAAGCAGCGTATTGTCGAG 663-682

Reverse (R1): 5'-CCGCAGCGACTTCTATCTCT 949-930

Second primer: (200 bp)

Forward (F2): 5'-GGAACTGCATCCGTTCATGAG 694-714

Reverse (R2): 5'-TCTTTAAAGCGTTCGTGGTC 887-868

DNA Extraction

DNA was extracted by using Geneid kit U.S.A and done according to company manual instruction as follows:

1. Dry clean smear were scraped from the slide and added to 200µl lysis buffer in 1.5 ml micro centrifuge tube.
2. 20 µl of Proteinase K added, then mix by pipetting and incubated at 60ºC for 5 minutes.
3. 200 µl of GSB Buffer added then mixed by shaking vigorously, incubated at 60ºC for 5minutes then inverted the tube every 2 minutes.
4. During incubation, required volume of Elution Buffer (200µl/sample) was transferred to a 1.5 ml micro centrifuge tube and heated to 60C.
5. 200 µl of absolute ethanol were added to the sample lysate and mixed immediately by shaking vigorously for 10 seconds. If precipitate appeared, it broke up as much as possible with a pipette.
6. A GS Column placed in a 2 ml Collection tube, all of the mixture (including any insoluble precipitate) transferred to the GS Column, centrifuged at 14-16,000 x g for 1 minute. Following centrifugation, if the mixture did not flow through the GS Column membrane, the centrifuge time increased until it passed completely. The 2 ml Collection tube discarded which containing the flow-through then the GS Column transferred to a new 2 ml Collection tube. It is important to noticed that the lysate and ethanol are mixed thoroughly to yielded a homogeneous solution.
7. 400 µl of W1 Buffer were added to the GS Column, centrifuged at 14-16,000 x g for 30 seconds then discarded the flow-through. The GS Column placed back in the 2 ml Collection tube. 600 µl of Wash Buffer (it important to make sure that absolute ethanol was added) added to the GS Column, centrifuged at 14-16,000 x g for 30 seconds then discarded the flow-through. The GS Column placed back in the 2 ml Collection tube, centrifuged again for 3 minutes at 14-16,000 x g to dry the column matrix.
8. The dried GS Column transferred to a clean 1.5 ml micro centrifuge tube. 100 µl of pre-heated Elution Buffer, TE Buffer or water added into the center of the column matrix. Let stand for at least 3 minutes to allow Elution Buffer, TE Buffer or water to be completely absorbed. Centrifuge at 14-16,000 x g for 30 seconds to elute purified DNA.

DNA Examination

The extracted DNA was checked by using Nanodrop spectrophotometer (U.S.A) which measured DNA concentration (µg/µl) and purity by reading the absorbance at 260/280nm.

Nested PCR reagents preparation

Master mix preparation

Nested PCR master mix was done by using PCR premix kit (Bioneer, USA) and according to company instructions.

Primary round PCR

The Material	Volume
PCR master mix	25 µl
Template DNA	10 µl
F1: (Forward primer 10 pmol/ µl)	2µl
R1: (Reverse primer 10 pmol/ µl)	2µ
D.D. water	11µl
Total volume	50 µl

Master Mix contents

Component	Reaction size 50µl reaction
Top DNA polymerase	2.5U
dNTP(dATP,dCTP,dGTP,dTTP)	Each 250µM
Reaction Buffer,with 1.5mM $MgCl_2$	1X

PCR Thermo cycler

First round of amplification was carried out with Nested PCR (10 µl template DNA) as follows:

No.	Steps	Time	Temperature	Cycle
1	Initial Denaturation	5 min	94ºC	30 cycle
2	Denaturation	20 sec.	94ºC	30 cycle
	Annealing	20 sec.	53ºC	
	Extension	20 sec.	72ºC	
3	Final Extension	5 min	72ºC	-----

Secondary round PCR

PCR master mix	**Volume**
PCR master mix	25 µl
Primary PCR product	10µl
F2: (Forward primer 10 pmol/ µl)	2µl
R2: (Reverse primer 10 pmol/ µl)	2µ
D.D. water	11µl
Total volume	50µl

The secondary round of amplification was started with 2 ml template from first reaction.

PCR Thermo cycler

Round of amplification was carried out as follows:

No.	Steps	Time	Temperature	Cycle
1	Initial Denaturation	2 min	94ºC	35 cycle
2	Denaturation	20 sec.	94ºC	30 cycle
	Annealing	20 sec.	45ºC	
	Extension	20 sec.	72ºC	
3	Final Extension	5 min	72ºC	1 cycle

Gel Electrophoresis

PCR products were analyzed by loading in 1.5% Agarose as following steps:

1. 1.5% Agarose gel was prepared in using 1X TBE and dissolving in water bath at 100ºC for 15 minutes, after that, left to cool 50ºC.
2. Then 2µl of ethidium bromide stain were added into agarose gel solution.
3. Agarose gel solution was poured in tray after fixed the comb in proper position, after that, left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray and 5µl of PCR product were added into each comb well and 5µl of (100pb Ladder) in one well.
4. The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volt and 80 AM for 50 minutes.
5. PCR products were visualized by using ultraviolet transiluminator and the bands were photographed.

Gel purification

Before PCR products were sent to sequencing, The gel bands were purified by using gel extraction kit as follows:

The DNA bands were cut from the gel using sharp blade. The gel transferred into 1.5 ml eppendrof tube. GSB buffer was added, incubated at 55^0C for few minutes till the gel was dissolved, Then other steps of procedure were followed.

DNA Sequencing

To identify the genetic variation (Genotype) between *T. gondii* isolate as well as standard NCBI isolates 25 µl volume of PCR product and 17 pica mole for each of the first and second Nested primers were send to Macrogen company, South Korea. The analysis of DNA sequence were done according to genetic analysis (Mega X) and multiple sequence alignment. Phylogenetic tree was drown according to VPGMA programme.

Statistical analysis

Chi- square were used to analyzed data obtained in the present study with probability $P \leq 0.05$.

RESULTS

The percentage of positive cases of Toxoplasma gondii infection in sheep meat in Basrah province.

A significant difference was found between percentage of *Toxoplasma gondii* in sheep according to months of years ($p \leq 0.000$). The higher percentage is (100%) and the lower percentage is (25%) table (1).

Table (1): The number of tested and infected samples and the percentage of T. gondii parasite in sheep according to the months of the year.

Month	Sheep		
	Examined No.	Positive No.	Percentage %
January 2020	5	5	100%
February	10	5	50%
March	10	10	100%
April	6	6	100%
May	4	1	25%
June	21	11	52%
July	24	9	38%
August	45	20	44%
September	49	34	63%
October	10	9	90%
November	7	7	100%
December	5	5	100%
$X^2 = 183.518$, DF = 11, $p \leq 0.000$			

The samples photographed under light Leica microscope and Scanning Electron Microscope as shown in Figures (1), (2.A), (2.B) and (2.C).

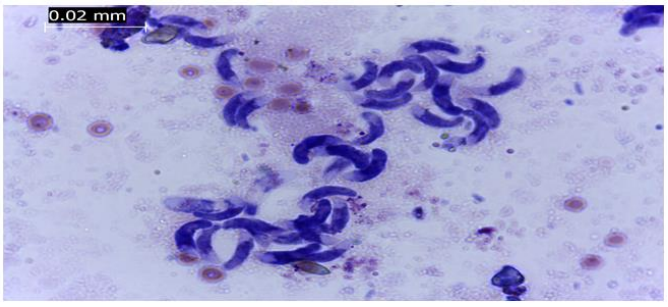

Figure (1): Tachyzoite parasite T. gondii in sheep diaphragm muscle smear (under Leica Microscope).

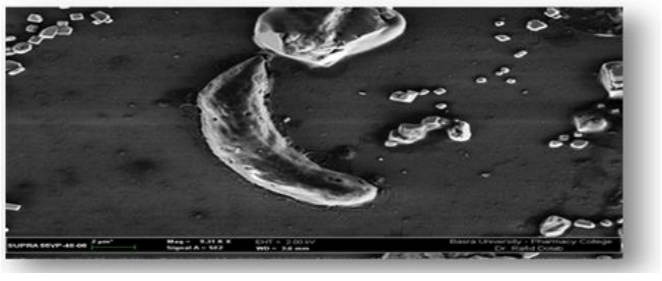

Figure (2.A): tachyzoite parasite T. gondii in sheep muscle smear under a Scanning Electron Microscope showing the external surface structure.

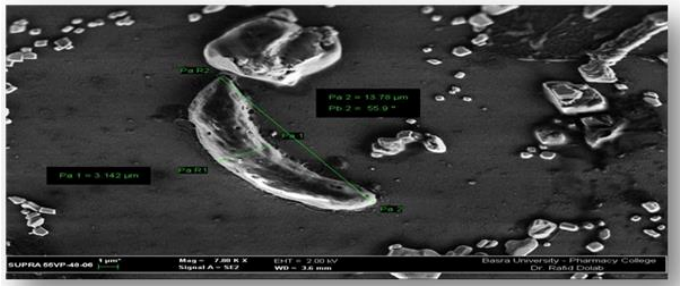

Figure (2.B): T. gondii parasite in sheep muscle smear under a scanning electron microscope showing the parasite dimensions

Figure (2.C): Magnified fragment of the introduction of T. gondii parasite in sheep muscle smear under a scanning electron microscope

Molecular study (PCR)

Nested PCR of T. gondii tachyzoite (dry smear) from sheep meat on 1.5% agarose gel.

Genomic DNA was isolate from sheep diaphragmatic muscle samples subjected to molecular analysis by using PCR for detection of B1 gene. Nested primers were used to identify the genotypes of *T. gondii*. Samples used in present study show a distinct single band of 194bp from the PCR product on agarose gel (Fig. 3).

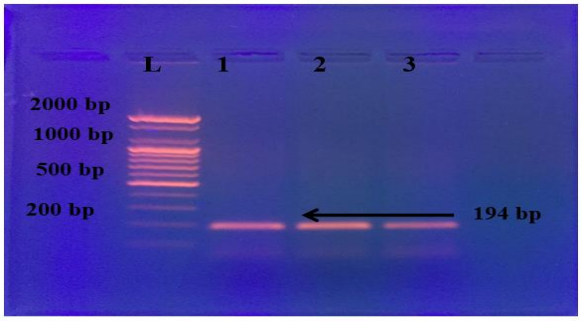

Fig (3): Nested PCR (Second round) amplification of T. gondii tachyzoite (dry smear) from sheep meat on 1.5% agarose gel.

L: Ladder (100 2000bp)

Lane 1-3: Positive samples (194bp)

Partial sequence of T. gondii

The sequence of the local sheep *T. gondii* isolate showed 100% identity. Four samples were taken. It was deposited at the gene Bank data base MW233648.1 (Table 2).

Table (2): Partial sequence of 194 bp T. gondii isolate UBE- A51 GAPDH (glycerol -3- phosphate dehydrogenase) (B1) gene.

No.	Sequence (5'-3')	bp	Identity	Note
1	**GGAACTGCATCCGTTCATGAGTATAAGAAAAAAATGTGGGAAT GAAAGAGACGCTAATGTATTTGCATAGGTTGCAGTCACTGACG AGCTCCCCTCTGCTGGCGAAAAGTGAAATTCATGAGTATCTGT GCAACTTTGGTGTATTCGCAGATTGGTCGCCTGCAATCGATAGT TGACCACGAACGCTTTAAAGA**	**194**	***Toxoplasma gondii* (KX270373.1 and MK507731.1)** **GAPDH (B1) gene: 100%**	**deposited at the Gene Bank database. MW233648.1**

Sequencing identity of local sheep T. gondii compared with NCBI- Blast homology.

From table (3). The identity was 100% identical to Mexico sheep skeletal muscle (KX270373.1) and to 100% of blood from Iran. 97.42-99.48% identity was found in other different countries (Iran-Mexico- India- USA). 98.97% identity was found in USA high virulence human culture strain = RH.

Table (3): Sequence identity % between current local sheep T. gondii isolate and NCBI- Blast homology from different countries.

No	Accession No.	Country	Host	Organ	Identity %
1	MK507731.1	Iran	-	Blood	100
2	KX270373.1	Mexico	Sheep	Skeletal muscle	100
3	MN542678.1	Iran	Human	Blood from bone marrow	99.48
4	MK507732.1	Iran	-	Blood	99.48
5	MK521885.1	Iran	-	Blood	99.48
6	MK521884.1	Iran	-	Blood	99.48
7	MK521883.1	Iran	-	Blood	99.48
8	MK521882.1	Iran	-	Blood	99.48
9	MK521881.1	Iran	-	Blood	99.48
10	MK031701.1	Iran	Human	Blood	99.48
11	MK031700.1	Iran	Human	Blood	99.48
12	MK031699.1	Iran	Human	Blood	99.48
13	MK031698.1	Iran	Human	Blood	99.48
14	KX270388.1	Mexico	Sheep	Skeletal muscle	99.48
15	KX270387.1	Mexico	Sheep	Skeletal muscle	99.48
16	KX270386.1	Mexico	Sheep	Skeletal muscle	99.48
17	KX270385.1	Mexico	Sheep	Skeletal muscle	99.48
18	KX270384.1	Mexico	Sheep	Skeletal muscle	99.48
19	KX270383.1	Mexico	Sheep	Skeletal muscle	99.48
20	KX270382.1	Mexico	Sheep	Skeletal muscle	99.48
21	KX270381.1	Mexico	Sheep	Skeletal muscle	99.48
22	KX270380.1	Mexico	Sheep	Skeletal muscle	99.48
23	KX270379.1	Mexico	Sheep	Skeletal muscle	99.48
24	KX270378.1	Mexico	Sheep	Skeletal muscle	99.48
25	KX270377.1	Mexico	Sheep	Skeletal muscle	99.48
26	KX270376	Mexico	Sheep	Skeletal muscle	99.48
27	KX270374.1	Mexico	Sheep	Skeletal muscle	99.48
28	KX270371.1	Mexico	Sheep	Skeletal muscle	99.48
29	KX270370.1	Mexico	Sheep	Skeletal muscle	99.48
30	KX270369.1	Mexico	Sheep	Skeletal muscle	99.48
31	KX270368.1	Mexico	Sheep	Skeletal muscle	99.48
32	KX270367.1	Mexico	Sheep	Skeletal muscle	99.48
33	KX270366.1	Mexico	Sheep	Skeletal muscle	99.48
34	KX270365.1	Mexico	Sheep	Skeletal muscle	99.48
35	KX270364.1	Mexico	Sheep	Skeletal muscle	99.48
36	KX270363.1	Mexico	Sheep	Skeletal muscle	99.48
37	KC607827.1	India	Mice	-	99.48
38	AF179871.1	USA	Human	Culture, Strain = RH	99.48
39	KX270372.1	Mexico	Sheep	Skeletal muscle	98.97
40	KX270375.1	Mexico	Sheep	Skeletal muscle	97.42

Phylogenetic tree

The phylogenetic tree was done to show the relationship between NCBI reference sequence of the glycerol 3-phosphate dehydrogenase (GADPH, B1) isolates study. Analysis of phylogenetic tree was done using UPGMA tree (MEGAG version) (Fig 4).

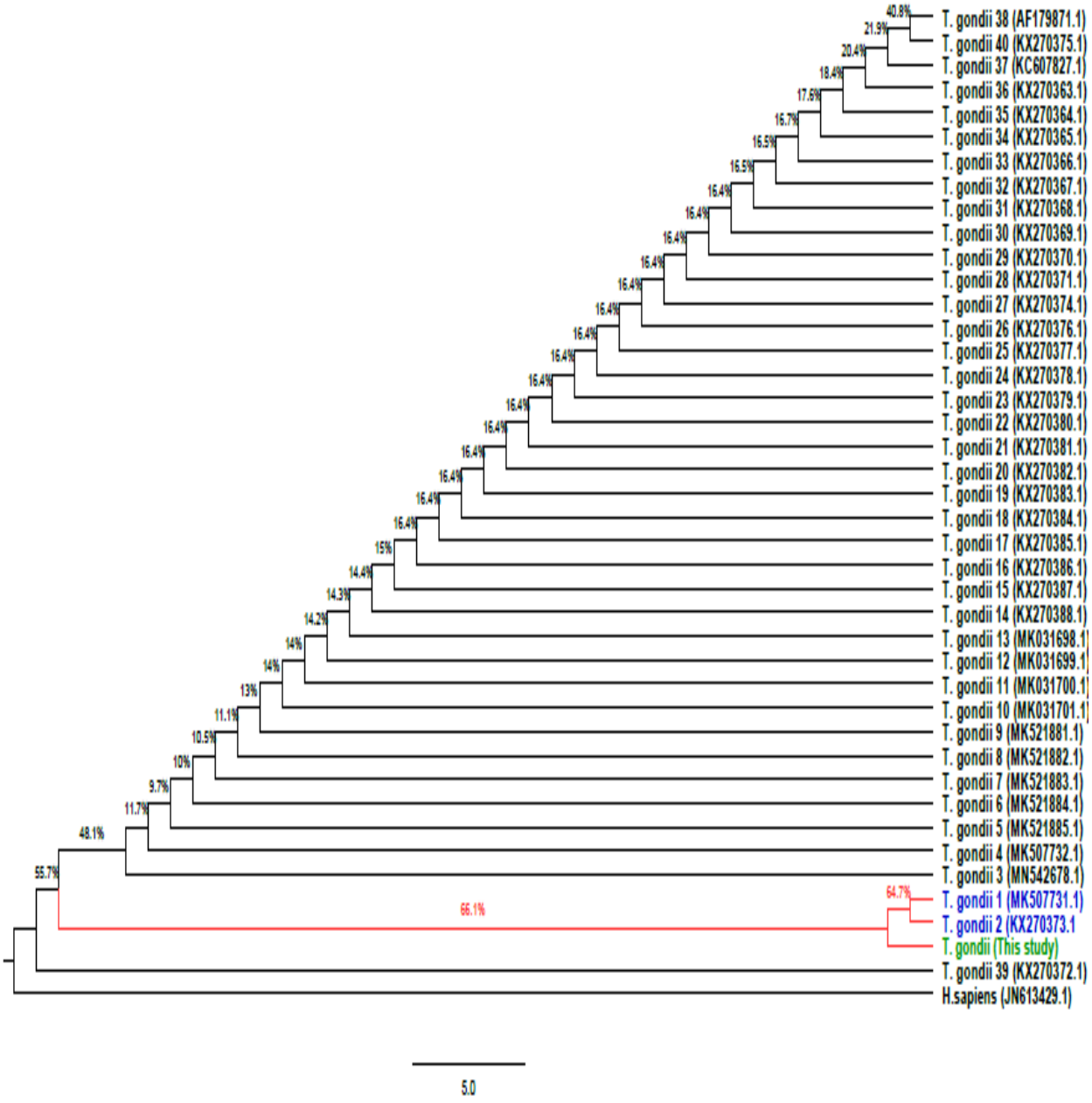

Figure (4): A phylogenetic tree showing the relationships between references of the glycerol -3- phosphate dehydrogenase (GADPH, B1) genes from Toxoplasma gondii isolates with the GADPH sequence of the present study isolate.

Nucleotide sequence alignment of GADPH of T. gondii

A nucleotide sequence was done to show the alignment GADPH genes from *T. gondii* reference isolates. The GADPH sequence from the present study isolates shown in Figure (5).

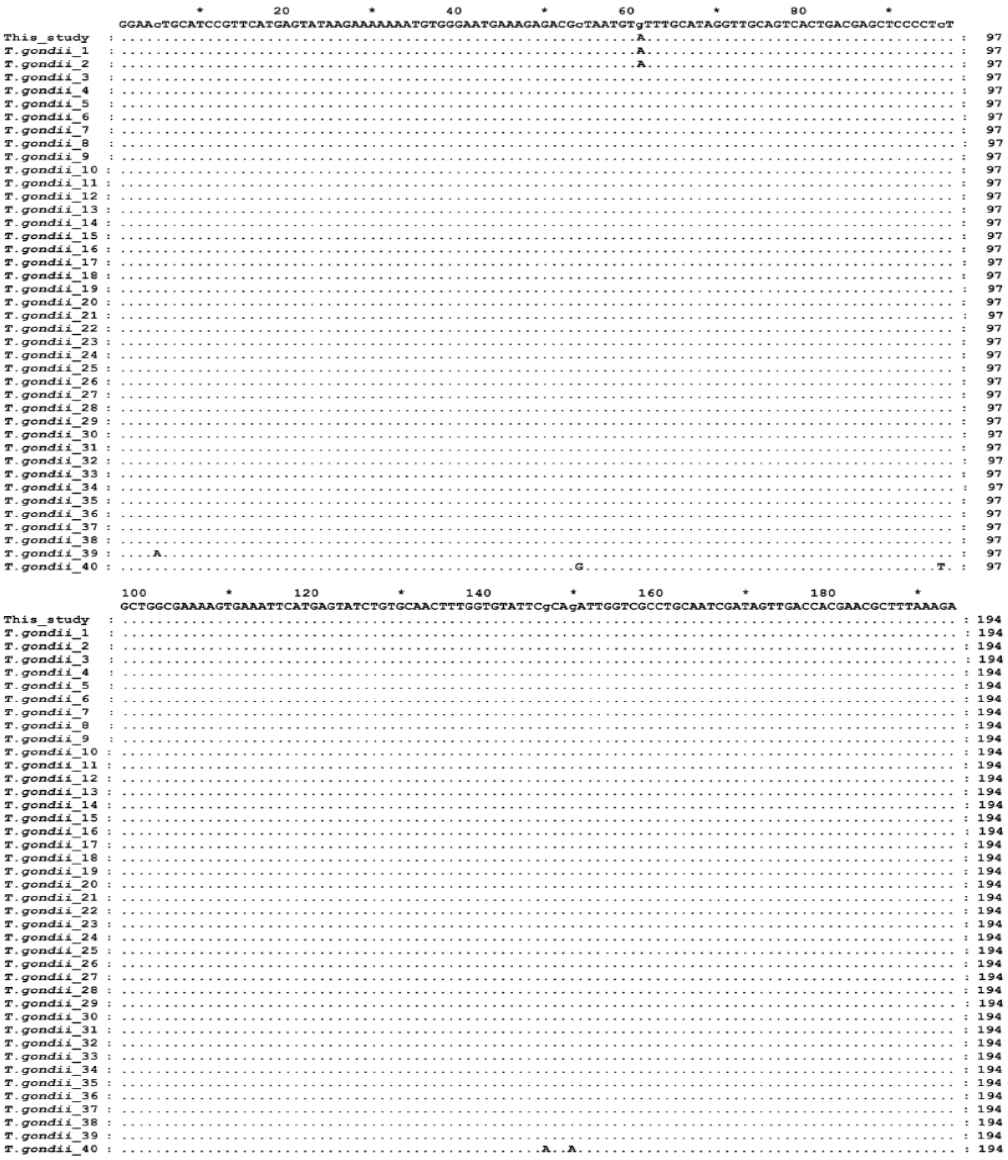

Figure (5): A nucleotide sequence alignment of GADPH genes from T. gondii reference isolates and the GADPH sequence from the present study isolates

The phylogenetic network of T. gondii

Figure (6) showed the phylogenetic network the relationship between *T. gondii* haplotypes from 1- 4.

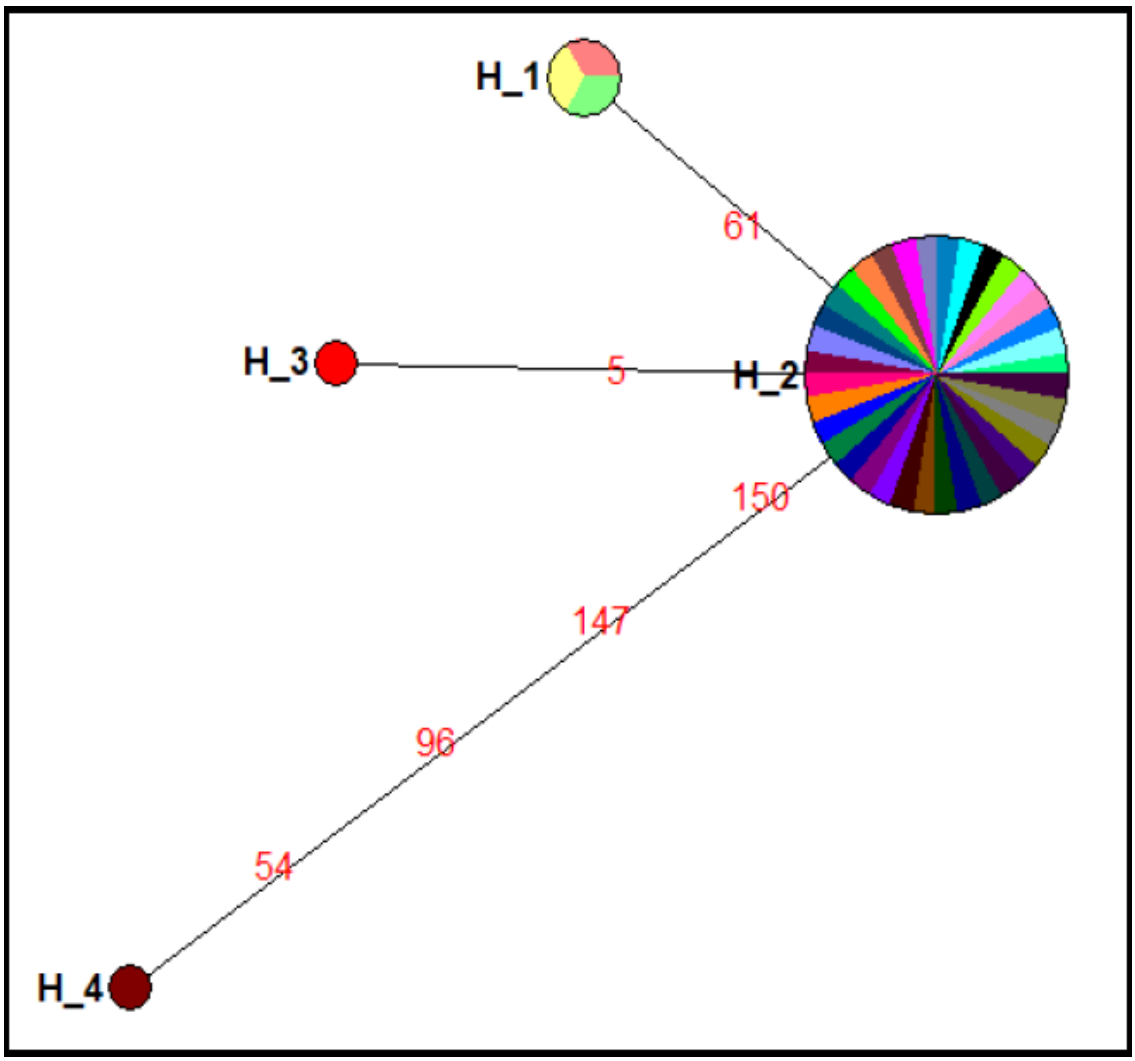

Figure (6): A phylogenetic network showing the relationships between T. gondii haplotypes. Each color represents an isolate.

Haplotype_1 (H_1) includes the study isolates, *T. gondii*_1 and *T. gondii*_2

Haplotype_2 (H_2) includes *T. gondii*_3 to 38.

Haplotype_3 (H_3) includes *T. gondii*_39.

Haplotype_4 (H_4) includes *T. gondii*_40.

Nucleotide sequence alignment of T. gondii haplotypes.

Figure (7) showed the nucleotide sequence alignment of *T. gondii* haplotypes and the differences in base pairs 5,54,61,96,147,150 as shown in Table (4).

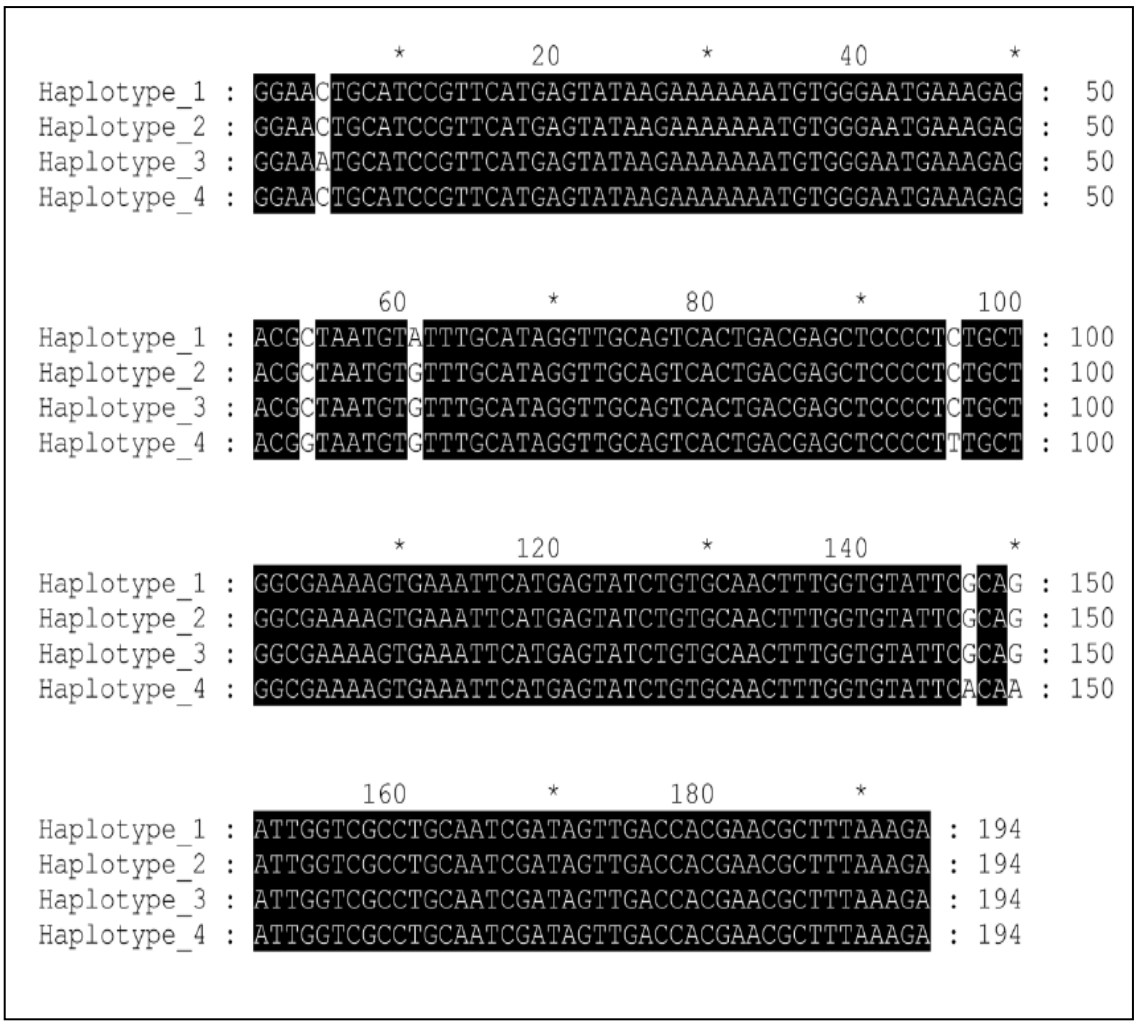

Figure (7): A nucleotide sequence alignment of T. gondii haplotypes.

Table (4): Nucleotide sequence differences of GADPH haplotypes.

	Nucleotide position					
Haplotype	**5**	**54**	**61**	**96**	**147**	**150**
H_1	C	C	A	C	G	G
H_2	C	C	G	C	G	G
H_3	A	C	G	C	G	G
H_4	C	G	G	T	A	A

The percentage of DNA identity among haplotypes.

The percentage of DNA identity between haplotype 1 is 100, between 1 and 2 was 99.48, between 1 and 3 was 99.48 between 1 and 4 was 97.42, between 2 and 4 was 97.94 and between 3 and 4 was 97.42 as shown in table (5).

Table (5): The percentage of DNA identity among haplotypes.

	Haplotype_1	Haplotype_2	Haplotype_3	Haplotype_4
Haplotype_1	100	99.48	98.97	97.42
Haplotype_2		100	99.48	97.94
Haplotype_3			100	97.42
Haplotype_4				100

The sequence in position 61 change from A to G in the present study Haplotype 3 and the American human high virulence culture isolate (Fig 8).

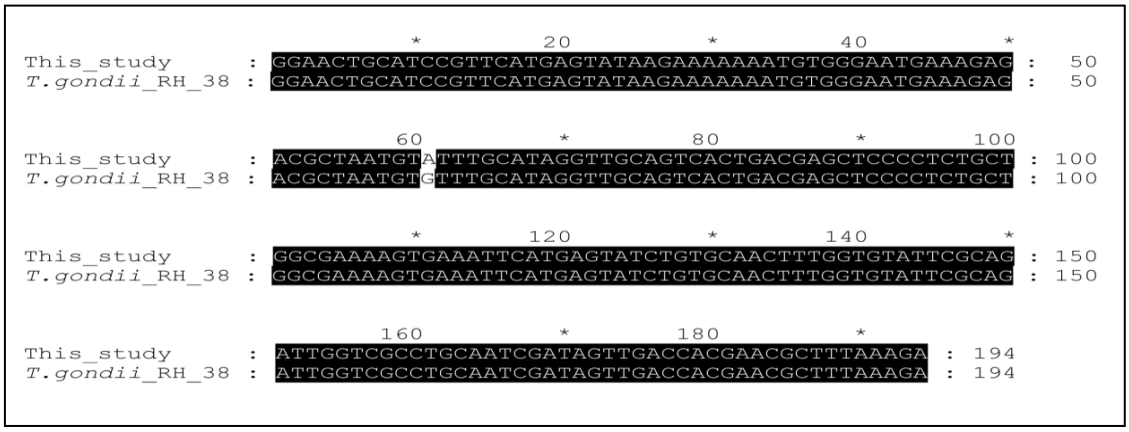

Figure (8): The relationship between present sequence Haplotype3 and the American human high virulence culture isolate

DISCUSSION

The meat of slaughtered animals is considered as one of the most important sources of transmission of the *T. gondii* parasite to human (Hill and Dubey, 2002) (6).

According to the months of the year, in the current study, the highest percentage (100%) of *T. gondii* was recorded in sheep in November, December, January (winter), then March and April, and the lowest percentage (25%) in May.

The sheep muscle samples that were examined by PCR reaction in this study recorded an infection rate (28.8%), (30/104) and this result was close to what was recorded by Yildiz *et al*. (2015)(7) Which recorded to (20.8%) and (22.4%) in (Ankara and Kirikal) in Turkey using PCR technique on tissue cysts. The study of Al-taie and Abdullah (2008)(8) in Sulaymaniyah had an infection rate of (58.33%) (7/13).

Sheep regarded as a food and industrial source for human, Sheep infected with *T. gondii* parasite have a role in the spread of infection with this parasite for humans and meat-eating animals. Several studies were conducted to find out the percentage of sheep infection with *T. gondii* parasite in different areas of world, which ranged from 3% to 95% (Dubey, 2009)(9).

The reason for the high rate of infection of animals with *T. gondii* parasite may be due to the grazing method and the method of storing food. The high rate of infection is also due to the type of animal and its sensitivity to infection Bisson *et al*. (2000) (3) as well as the age of the animal, its sex and breed, the nature of the surrounding environmental conditions, the type of technology used in the examination to determine the prevalence rates and health education standards (Kamani *et al*., 2010) (10).

The method of isolation of parasite DNA by PCR technique is a relatively simple method, sensitive, reproducible and low cost Su *et al*. (2010) (11)compared to the biological model with mice that lasts 3 to 6 weeks or cell culture that takes 4 to 10 days to show results (Liu *et al*., 2015)(12). The sensitivity of this method depends on the technique of DNA extraction, sample processing and the nature of the parameters (primers) used in the amplification reaction (Switaj *et al*., 2005)(13).

The meat samples analyzed in the current study were taken from adult animals and therefore were more susceptible to *T. gondii* parasite, as studies showed that age is a risk factor for toxoplasmosis in sheep (Hamilton *et al*., 2014; Amdouni *et al*., 2017)(14), (15).

The parasite was diagnosed more frequently in skeletal muscle than in any other tissue in experimentally infected sheep (Dubey and Sharma, 1980) (16). It was found that *T.*

gondii DNA is detected more frequently in the sheep brain and heart than in skeletal muscle (Esteban *et al*., 1999) (17).

The differences between the percentages infection recorded in the current study and other studies can be attributed to the different origin of the samples used. It was found that the incidence of toxoplasmosis in sheep is relatively high in Wasit Province. *T. gondii* infection is important among animals (sheep), as some of them play a distinct role as a source of human infection.

The variation in infection rates among animals in the different governorates of Iraq may be due to the way of grazing the animals, the extent of the presence of cats in the areas, their contact with animals, the number of oocysts presented, and how to dispose of dead and aborted animals (Tenter *et al*., 2000; Oncel and Vural 2006)(18),(19) or it may be due to the type and race of animals, some of them are more sensitive to infection and some of them are resistant. Bisson *et al*. (2000) (3) showed that the immune status, age and climate have a role in the occurrence of infection.

The molecular sequence of sheep muscle in the current study showed a close relationship to NCBI-BLAST (MW233648.1) with a matching percentage of 100%,(194bp), these results were identical to that obtained in blood sample / Iran (The source of the sample taken from it was not mentioned) also, the molecular sequence of sheep muscle in the current study agrees with what was recorded in Mexico 100% (803bp).

Conclusion

It was concluded from the current study that eating undercooked sheep meat is one of the important sources of toxoplasmosis infection in Basrah. The reading of the phylogenetic tree showed that the sheep sample was identical with the main branch of the phylogenetic tree carrying the sequence MW233648.1 in the global genebank.

Acknowledgments

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Conflict of interest

The authors declare that no conflict of interest exists.

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Article Highlight

1. Given the importance of toxoplasmosis, as a zoonotic disease and its close relationship to community health, the study was conducted to isolate and identify the local strain of *T. gondii* from the diaphragm muscles of naturally infected sheep in Basrah Province using PCR (Nested PCR).
2. The DNA sequencing of the B1 gene of *T. gondii* and the phylogenetic tree were studied in this study to determine the genetic relationship between local isolation and global spread.