

Toxoplasma gondii Strains: Bioinformatic Analysis of Heat Shock Protein 60 (Hsp60) Gene in Experimentally Infected Mice

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Background and study aim: There is a need for more detailed characterisation of *T. gondii* strains to determine the level of genetic variability which is useful in effective diagnosis, treatment and control of toxoplasmosis. This work aimed to assess the difference between the two sequences of Hsp60 gene of both RH and ME 49 strains of *T. gondii* and to investigate its role in the virulence of the parasite.

Materials and Methods: Two strains of *T. gondii* parasite RH and ME-49 were used for experimental infection of mice groups which were divided into group I (infected with RH strain), group II (infected with ME-49), and group III (non-infected non treated group).

Results: Histopathological examination showed that there was a statistically significant increase in the intensity of infection in liver and spleen of group I and in kidney and brain of group II, with no difference between both groups organs. Molecular and Bioinformatics studies included Primers design using primer 3 program, primer 2 (P2) was selected and

used in PCR amplification according to its highest product size 247 bp. The results of Insilico PCR amplification program that was carried out to amplify Hsp60 gene theoretically using primer 2 (P2) showed higher number of positive samples that detected in group II than group I, there was significant statistical increase in frequency of brain infection in group II.

Conclusion: Our results showed that PCR was a highly sensitive, specific and rapid technique for detecting *T. gondii*. It revealed higher positive results in chronic strain (ME49) especially in brain tissue than acute strain (RH) but it may show false negative results in some samples. Both PCR technique and histopathological examination showed that the preferred tissue for detection of *T. gondii* in experimentally infected mice was liver & spleen in acute strain (RH) and brain & kidney in chronic strain (ME49). Bioinformatic analysis of Hsp60 gene sequences of both (RH & ME 49) strains of *T. gondii* showed that there was a polymorphism in some nucleotides between the 2 sequences.

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is an intracellular protozoan that can infect cells of all warm-blooded animals including birds, livestock, marine mammals and humans [1]. Although toxoplasmosis is generally asymptomatic, primary infection during pregnancy in women and animals may cause abortion, congenital anomalies, or perinatal death [2]. Infection in humans generally occurs either by accidental ingestion of food or water contaminated with sporulated oocysts

from faeces of infected cats which is considered as important reservoir or undercooked meats bearing bradyzoite tissue cysts [3]. In addition, *T. gondii* can be also transmitted vertically from an infected mother to her baby during her first gestation [4]. In experimental infection, the severity of toxoplasmosis depends on host and parasite factors as well as the dose and route of inoculation. Oral infection with tissue cysts in mice has been widely used for

pathological studies, and the small intestine, liver, lungs, spleen and brain are often found to be affected [5]. There are various techniques for diagnosis of toxoplasmosis, but the molecular methods are more sensitive and specific to determine the presence of parasites in clinical sample specially in prenatal diagnosis of congenital toxoplasmosis and in immunocompromised patients, as they do not depend on the immunological status of the host and they are less time consuming [6]. Molecular diagnosis of toxoplasmosis is usually based on the detection of a specific DNA sequence principally from highly conserved regions. It was described that *T. gondii* has a clonal population structure consisting of three genetic lineages i.e., Type I, Type II and Type III [7]. Studies on mice have shown that infections with the different clonal lineages of *T. gondii* result in very different outcomes. Type I strains are highly virulent, whereas type II and type III are relatively nonvirulent. The enhanced virulence of type I strains is in part due to overstimulation of a Th1 immune response that leads to pathology [8]. However, it remains uncertain to what extent the genotype of the parasite directly contributes to the clinical severity of human toxoplasmosis [9]. The variability in the strains has led to speculation that some strains of *T. gondii* might be closely related species with differing pathogenesis in host tissue, therefore, there is a need for more detailed characterization of *T. gondii* strains to determine the level of genetic variation within strains. Knowledge about genetic variability will be useful in the effective diagnosis, treatment and control of toxoplasmosis in humans and animals [10]. Hsp60 plays an important role in intracellular survival, in the differentiation of *T. gondii* and also mediates its virulence [11]. As Hsp60 acts as a mitochondrial chaperone so it is involved in mitochondrial electron transport chain that promote replication of intracellular tachyzoites [12]. Therefore, specific inhibition of the mitochondrial electron transport chain by such drugs as atovaquone and oligomycin, are capable of blocking the replication of tachyzoites, also promote their conversion into encysted bradyzoites so become less virulent [13]. Comparison of genes within a species or between different species can show similarities among protein functions, or relations between species. Sequence alignment is the main process in bioinformatics which is used for measuring the similarity between biological sequences (DNA/ protein), it compares sequences

by matching their nucleotides bases to give maximum possible alignment [14].

The aim of the present work is to assess the difference between the two sequences of Hsp60 gene of both RH and ME 49 strains of *T. gondii* and to investigate its role in the virulence of the parasite parasitologically, histopathologically, molecular studies and by using bioinformatic analysis in experimentally infected mice.

MATERIALS AND METHODS

In this experimental study, two strains RH, ME-49 strains were obtained from "Zoonotic Diseases Department, National Research center (NRC)", El Buhouth St., Dokki, Cairo, Egypt. This study was completed in Parasitology Department, Faculty of medicine, Zagazig University, also the molecular study was performed in lab of molecular medical parasitology (LMMP), Medical Parasitology Department, Kasr Al-Ainy Faculty of Medicine, Cairo University. during the period from September 2016 to October 2017.

Ethical consideration:

Mice were maintained in accordance with the research protocols following the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals, Faculty of Medicine, Zagazig University.

Study design:

Toxoplasma-free Swiss albino male mice, one-month-old weighing 25-30 gm were used. All mice were kept in clean cages, fed on *Toxoplasma* free food and clean water, subsequently infected intraperitoneally with *T. gondii* strains and divided to the following groups: group I: (10 mice) was infected by human RH strain of *T. gondii*, group II: (10 mice) was infected with ME49 strain of *T. gondii* and group III: negative control group (10 mice). All groups were subjected to The following:

1- Clinical examination: Was performed by Dr. Ashraf Barakat and Dr. Abdelnasser Madboli, group I (RH strain), mice were followed up daily for any apparent clinical signs of acute toxoplasmosis. The predicted signs may be varied between raised and rough hair coat, pendulous abdomen, severe ascites, dullness, tachypnea, nervous manifestations, evidence of early emaciation and dehydration or death

usually within 72-84 hours according to **Mercier [15]**.

2- Parasitological examination: Was performed to detect presence or absence of *T. gondii* tachyzoite, bradyzoites or tissue cysts. Group I (RH strain) peritoneal exudate was collected according to **Rougier and Ambroise - Thomas [16]** and Giemsa staining of peritoneal fluid for tachyzoites according to **Brown [17]**. Maintenance of infection for RH strain was carried out through serial intraperitoneal passage in mice according to **Moreland [18]**. For group II (ME 49 strain) collection, homogenization and examination of brain samples after 45th.60th day post inoculation were performed according to **Dubey and Beattie [19]**. Tissue cysts were counted microscopically in 50 µl of the homogenates, and the count was multiplied by 20 to obtain the number of tissue cysts per one gram.

3- Histopathological study, Suvarna [20]: In group I (RH strain) & group II (ME 49 strain), tissue samples from liver, spleen, kidney, lung, heart and brain were collected and divided into two compartments; the first compartment fixed in 10% formalin and kept as a paraffin blocks. Microtome sections were cut at a thickness of 4 µm. Sections were stained with haematoxylin and eosin (H&E), then all slides were examined microscopically. For detection of free tachyzoites, tissue cysts or pseudocyst, while the second one was kept frozen at (-20°C) in aluminium foil (for all samples except brain samples) for further *T. gondii* DNA extraction [21]. The 2nd compartment of brain samples were placed in 1ml of sterile saline and grounded using glass manual homogenizer to release tissue cysts then placed in Eppendorf tubes and kept frozen at -20 °C for further *T. gondii* DNA extraction. Regarding group III (control group), mice were sacrificed and tissue samples from liver, spleen, kidney, lung, heart and brain were collected and stored in 10 % formal saline solution for comparative histopathological study only.

4- Molecular and Bioinformatic studies:

a- Primer design using Primer 3 program according to **Rozen and Skaletsky [22]** that helped us to decide which portion of the large sequence makes the best primers. The browser was pointed to biotools.

umassmed.edu/. This page offering access to several sequence-analysis programs. The Hsp60 gene sequence of *T. gondii* at <https://www.ncbi.nlm.nih.gov/gene/7901362> was copied in FASTA format and pasted in the sequence window.

b- In silico PCR amplification according to San Millán [23]: Calculation of theoretical PCR results by using up-to-date sequenced parasites genomes <http://insilico.ehu.es/pcr/> was done . The selected Hsp60 gene was uploaded after saving it in a text/plain file (".txt" extension) with Fasta format then PCR was clicked. After that ATCATCGACCCACCAAAGT was pasted in the Forward Primer text box and also CAAGCGCTGAGATCCCTTTC pasted in the Reverse Primer text box. Finally, all the default settings were kept and amplify was selected.

c- Polymerase chain reaction (PCR) technique according to Jones [24] was used for detection of *T.gondii* DNA from frozen tissue samples of both strains. Processing of DNA by PCR involved first DNA extraction from tissues by Quick-gDNA™ Miniprep Kit, Cat. Nos. D3025, Zymo research corporation, followed by *T. gondii* DNA amplification using one of the primers designed based on Hsp60 gene <https://www.ncbi.nlm.nih.gov/gene/7901362>. Primers was ordered from, (Invitrogen, Thermo Fisher Scientific, USA), lastly detection of the amplified products by agarose gel electrophoresis (1.5 %).

Identification and sequencing of the gene was carried out for only 2 samples, liver sample of RH strain (group I) and brain sample of ME49 strain (group II). Sequencing was performed in both directions (forward and reverse direction by direct, automated sequencing (BigDye® Terminator v.3.1 chemistry, Applied Biosystems, USA), using the same primers employed in PCR.

Alignment analysis of the gene sequences was done by two programs; LALIGN and BLAST programs. In LALIGN program available at: https://embnet.vital-it.ch/software/LALIGN_form.html, the two sequences were pasted in one of the supported formats in the sequence fields. In BLAST program, available at: <https://blast.ncbi.nlm.nih.gov/Blast.cgi> the two sequences were pasted in one of the supported formats into the sequence fields.

Statistical analysis:

Collected data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 25.0. Chi

square test was used to calculate difference between qualitative variables in two different groups and fisher exact correction was used when one of the cells had value less than 5.

RESULTS

I- Clinical examination results were shown in table (1) and (2):

Table (1): Frequency of different clinical signs among group I:

Signs	n=10		+ve		-ve	
	N	(%)	N	(%)	N	(%)
Raised & rough hair coat	2	(20)	8	(80)		
Tachypnea	3	(30)	7	(70)		
Ascites	6	(60)	4	(40)		
Fatigue	5	(50)	5	(50)		
Loss of appetite & Emaciation	5	(50)	5	(50)		
Death	0	(0)	10	(100)		

Table (2): Frequency of different clinical signs among group II

Signs	n=10		+ve		-ve	
	N	(%)	N	(%)	N	(%)
Asymptomatic:	6	(60)	4	(40)		
Nervous manifestations: climbing on wall and rearing (raise itself upright on its hind legs).	4	(40)	6	(60)		
Partial off food:	2	(20)	8	(80)		

II- Parasitological results: Shown in Table (3) and Fig. (1, 2).

Table (3): Parasitological Examination of Group I & Group II.

Sample	Group I (RH strain)			Group II (ME 49 strain)			P- value
	+ve	-ve	% +ve	+ve	-ve	% +ve	
Parasitological examination	9	1	90	8	2	80	0.53 NS

Fisher exact test

NS: Non-significant ($p > 0.05$)

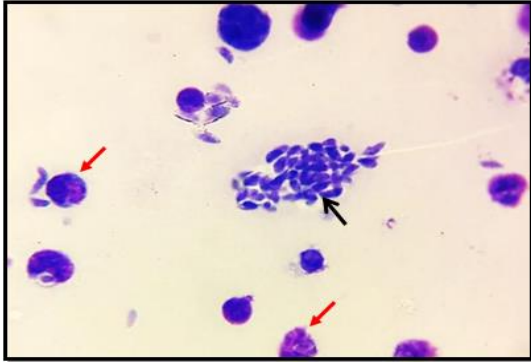


Fig. (1): Peritoneal fluid stained smear of group I (RH strain) exhibited banana shaped *T. gondii* tachyzoite clusters (black arrow) and intact macrophages filled with dividing tachyzoites (pseudocysts) (red arrow). Giemsa stain X 1000

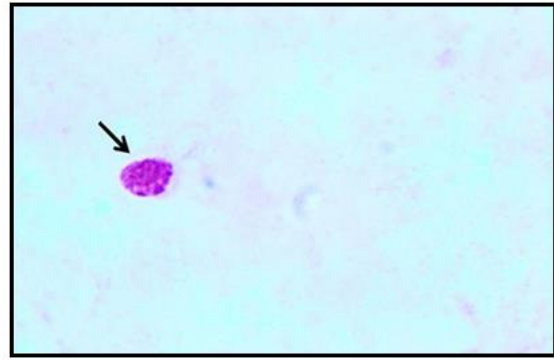


Fig. (2): Stained smear from the brain tissue homogenate of group II (ME 49 strain) showed stained *T. gondii* cyst which is rounded in shape containing many bradyzoites (arrow). Giemsa stain X 1000.

III- Histopathological results: Were presented in Fig. (3,4,5,6,7,8,9 and 10).

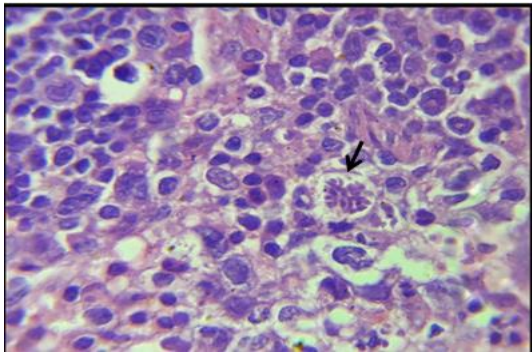


Fig. (3): Spleen section of group I (RH strain) revealed the presence of multifocal clusters of tachyzoites as a pseudocyst of *T. gondii* (black arrow) in the white pulp of spleen. H&E X 400.

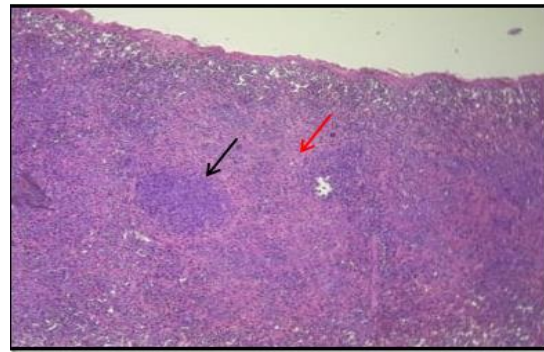


Fig. (4): Spleen section of group III (control group) showed normal architecture of white pulp formed of lymphoid follicle containing many inflammatory cells mainly lymphocytes (black arrow) and red pulp formed of blood sinusoids (red arrow) H&E X 200.

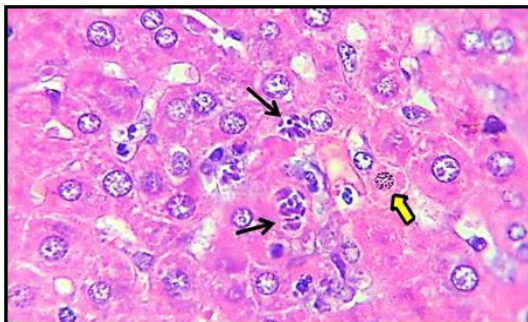


Fig. (5): Liver section of group I (RH strain) showed multiple pseudocyst of *T. gondii* among the hepatic cords (black arrows). Karyorrhexis in nuclei of some hepatocytes were observed (yellow arrow) with degeneration of hepatocytes H&E X 400.

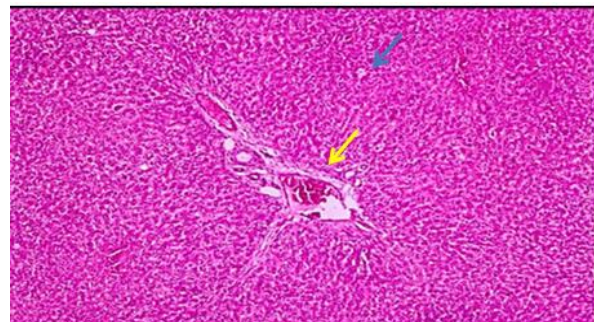


Fig. (6): Liver section of group III (control group) showed normal hepato-portal area (yellow arrow) and thin walled central vein (blue arrow) with normal sized hepatocytes. H&E X 200.

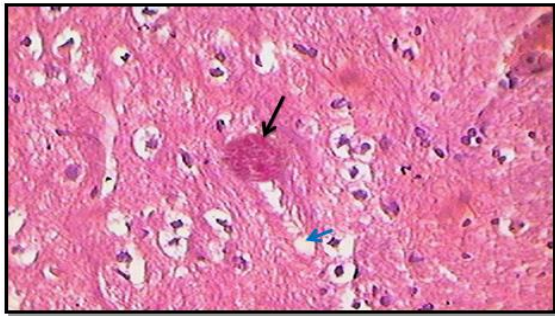


Fig. (7): Brain section of group II (ME 49 strain) showed *T.gondii* cyst containing many bradyzoites (black arrow) with mild neuronal loss and vacuolar degeneration (blue arrow). H&E X 400.

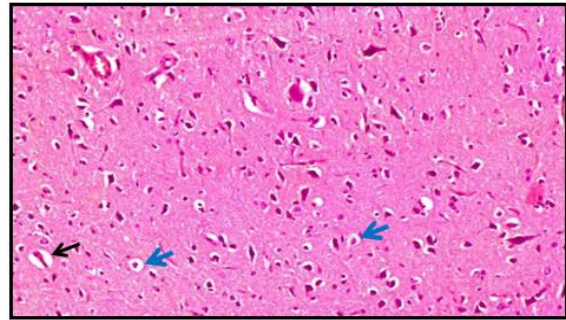


Fig. (8): Brain section of group III (control group) showed completely normal brain tissue as normal glial cells (blue arrows) and capillaries (black arrow) without clear histopathological changes. H&E X 200.

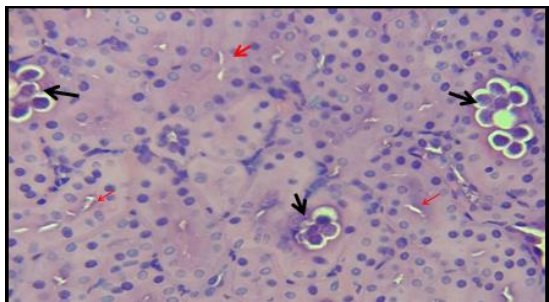


Fig. (9): Kidney section of group II (ME 49 strain) exhibited multiple cysts of *T. gondii* containing bradyzoites, nearly occluded the lumen of the degenerated proximal convoluted tubules (black arrows) and obliterated tubules with no lumen (Red arrows). H&E X 400.

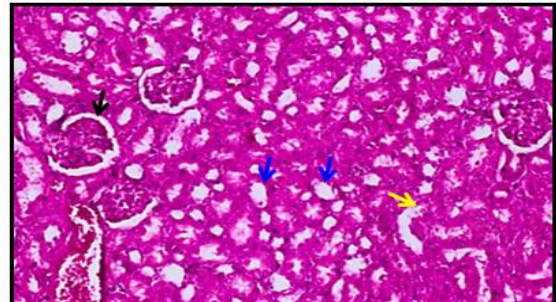


Fig. (10): Kidney section of group III (control group) showed the normal histological structure of the renal glomeruli (black arrow). The proximal convoluted tubules (blue arrow) and distal convoluted tubules (yellow arrow) appear completely normal. H&E X 200.

Table (4): Comparison between Histopathological Examination (HPE) findings in studied groups

Infected group Tissue Sample	Group I (10 mice) (RH strain)			Group II (10 mice) (ME 49 strain)			P value
	+ve	-ve	% +ve	+ve	-ve	% +ve	
Liver	5	5	50	1	9	10	0.04*
Spleen	5	5	50	1	9	10	0.04*
Kidney	2	8	20	6	4	60	0.04*
Lung	2	8	20	1	9	10	0.99 NS
Heart	1	9	10	3	7	30	0.58 NS
Brain	1	9	10	5	5	50	0.04*

Fisher exact test NS: Non-significant ($p > 0.05$) *: Significant ($P < 0.05$)

There was a statistically significant increase in the intensity of infection in liver and spleen of group I and in kidney and brain of group II, with no significant difference between both groups in lung and heart.

IV: Molecular and Bioinformatic Results:

- 1- Primer design results: table (5) showed a map of the best oligonucleotide pair forward (F) and reverse (R) primers also called left and right primers respectively. The position, length, predicted melting temperature and GC percentage were given for each primer. In addition, four additional primer pairs were proposed. After the primers were designed, primer 2 (P2) was selected and used in PCR amplification according to its highest product size 247 base pair (bp).

Table (5): The primer designed with primer 3 program and its additional four primer pairs with the specific characters for each

	Sequence	Direction	Product size	Corresponding cDNA nucleotides (bp)	GC %	T _M °C
P1	TGCACATCTACAAGGGCTGA	F	227	239-466	50	59.02
	CAACAACCACAAAACGCCAC	R			50	59.00
P2	ATCATCGACCCCACCAAAGT	F	247	2872-3119	50	59.01
	CAAGCGCTGAGATCCCTTTC	R			55	58.99
P3	CGTAGGAGCTCGCTTAGTCA	F	200	700-900	55	58.98
	GATCTTGGACAATTCGCGCT	R			50	58.99
P4	TTGTGAGAGTCTTCGCTGGT	F	225	495-720	50	58.96
	TGACTAAGCGAGCTCCTACG	R			55	58.98
P5	GAGTTCGTCGCTGTGAATCC	F	204	205-409	55	59.01
	GCAACTGACGGCAACAATA	R			50	59.06

- 2- In silico PCR Amplification results: In silico PCR amplification program that used to amplify Hsp60 gene theoretically by primer 2 (P2). The length of amplicon was 247 bp and the starting position for amplification was 2872.
- 3- PCR technique (extraction and amplification of Hsp60 gene): Figures (11, 12) showed gel electrophoresis of PCR reaction that targeted Hsp60 of both infected groups (I & II). The best results were observed in one mouse as a sample compared to other infected mice of the same group. The positive bands appeared in the gel between 200bp and 300bp as the amplified fragment was 247 bp in length according to the best primer selected. Regarding group I Fig. (11) showed also the positive tissue samples :liver, spleen, kidney, lung and brain, while the heart tissue samples were negative in all infected mice of group I. Regarding group II, Fig. (12), the positive tissue samples were liver, spleen, kidney, heart and brain, while the lung tissue samples were negative in all infected mice of group II.

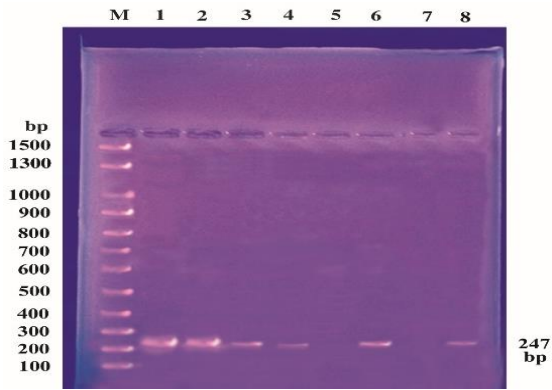


Fig. (11): Agarose gel electrophoresis for products of PCR targeting Hsp60 gene of *T. gondii* (RH strain) in tissue samples of one mouse at 247bp. Lane 1: liver positive sample, Lane 2: spleen positive sample. Lane 3: kidney positive sample, Lane 4: lung positive sample. Lane 5: heart negative sample, Lane 6: brain positive sample. Lane 7: negative control, Lane 8: positive control. M (marker): DNA molecular size marker

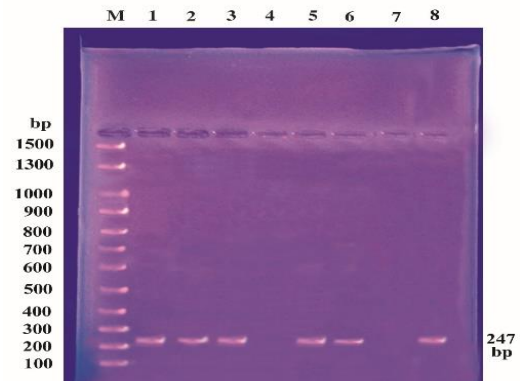


Fig. (12): Agarose gel electrophoresis for products of PCR targeting Hsp60 gene of *T. gondii* (ME49 strain) in tissue samples of one mouse at 247 bp. Lane 1: liver positive samples, Lane 2: spleen positive samples. Lane 3: kidney positive samples, Lane 4: lung negative samples. Lane 5: heart positive samples, Lane 6: brain positive samples. Lane 7: negative control, Lane 8: positive control. M (marker): DNA molecular size marker

Table (6): Comparison between PCR findings in studied groups:

PCR Tissue Sample	Group I (10 mice) (RH strain)			Group II (10 mice) (ME 49 strain)			P- value
	+ve	-ve	% +ve	+ve	-ve	% +ve	
Liver	6	4	60	7	3	70	0.64 (NS)
Spleen	5	5	50	5	5	50	1 (NS)
Kidney	2	8	20	6	4	60	0.17 (NS)
Lung	3	7	30	0	10	0	0.21 (NS)
Heart	0	10	0	3	7	30	0.21 (NS)
Brain	1	9	10	6	4	60	0.03*

Fisher exact test NS: Nonsignificant ($p > 0.05$) *: Significant ($P < 0.05$)

Table (6) shows PCR findings in GI and GII, higher positive results were found in most organ samples of group II than group I as the percentage of liver, spleen, kidney, lung, heart and brain positive samples in group I were 60%, 50%, 20%, 30%, 0% and 10% respectively, while in group II the percentage were 70%, 50%, 60%, 0%, 30% and 60% respectively with significant increase in brain infection (60%) in group II.

Table (7): Validity of PCR in diagnosis of infection in liver compared to parasitological study as Gold standard in Group I.

PCR	Parasitological Study		Total
	+ve	-ve	
+ve	6	0	6
-ve	3	1	4
Total	9	1	10
Validity	Sensitivity: 66.7% PPV: 100 %		Specificity: 100% NPV: 25 %
Accuracy	70 %		

Table (7) showed that all parasitological study positive samples were also positive by PCR except 3 samples were PCR negative. There was only one negative sample by both parasitology and PCR. The sensitivity of PCR was (66.7%), specificity was (100%), Positive predictive value (P.P.V) was (100%), Negative predictive value (N.P.V) was (25%) and the accuracy of PCR in relation to parasitological study was 70%.

Table (8): Validity of PCR in diagnosis of infection in liver compared to parasitological study as gold standard in Group II

PCR	Parasitological Study		Total
	+ve	-ve	
+ve	7	0	7
-ve	1	2	3
Total	8	2	10
Validity	Sensitivity: 87.5% Specificity: 100%		
	PPV: 100 % NPV: 66.7 %		
Accuracy	90%		

NB: **Sensitivity**= true positives / (true positive + false negative). **Specificity**=true negatives/ (true negative + false positives). **PPV**= true positive/ (true positive + false positive). **NPV**= true negatives/ (true negatives +false negatives). **Accuracy**= (true positive + true negative)/total no.

Table (8) showed that all parasitological study positive samples were also positive by PCR except 1 were negative by PCR. There were 2 negative samples by both parasitology and PCR. The sensitivity of PCR was (87.5%), specificity was (100%), Positive predictive value (P.P.V) was (100%), Negative predictive value (N.P.V) was (66.7 %) and the accuracy of PCR in relation to parasitological study was 90%.

- 4- Sequencing and Alignment analysis of Hsp60 gene. Sequencing was carried out for only one positive sample of each mouse in group I (RH strain) and one positive sample of each mouse in group II (ME49 strain). Sequencing was performed in both directions (forward and reverse direction by direct, automated sequencing using the same primers employed in PCR. Then Alignment analysis of Hsp60 gene sequences By LALIGN program as shown Fig (13) showed a total of 5 nucleotide position differences between both RH and ME49 strains of *T. gondii*. There were 2 nucleotide substitutions detected between the 2 strains at position 95-96 bp, it was (GG) in RH strain sequence and (AT) in ME49 strain. In addition, 3 nucleotide substitutions detected between the 2 strains at position 192-194 bp, it was (TTT) in RH strain sequence and (ACC) in ME49 strain. Similar results were obtained using BLAST program.

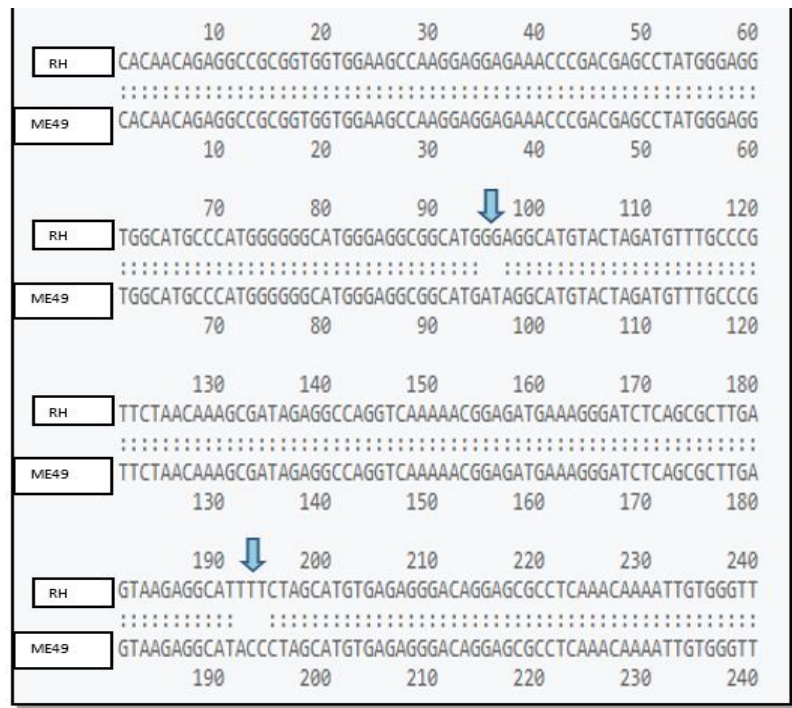


Fig. (13): Analysis of **LALIGN** program showing polymorphism between RH strain sequence and ME 49 strain sequence in two sites (blue arrow).

DISCUSSION

Genetic variation among different strains of *T. gondii* has been illustrated through many genetic studies including DNA sequence comparisons [25]. In this study, firstly the bioinformatic studies were used to design the primers specific to *T. gondii* Hsp60 gene. After that, detection of the parasite using parasitological, histopathological and PCR technique was performed, then sequencing the targeted gene from the PCR amplified fragments of both RH (group I) and ME49 (group II) strains. Alignment analysis of the gene sequences was carried out using alignment programs to determine the difference of HSP60 gene sequence between both strains.

In the current study, clinically we observed some signs on both infected groups, the most observed sign in group I infected with *T. gondii* (RH strain) is ascites (60%) followed by fatigue (50%), loss of appetite and emaciation (50%), tachypnea (30%), raised and rough hair coat (20%). Finally, no death was found among the studied mice. These results are in agreement with findings of Waree [26] and Carneiro [27]. The occurrence of these signs can be explained by peritonitis and internal organ inflammation El

Fadaly [28]. In group II infected with *T. gondii* (ME49 strain) most of the mice were asymptomatic (60%) and this comes in agreement with Waree [26], followed by nervous manifestation (40%) that include climbing on wall and rearing (raise itself upright on its hind legs), these signs were in agreement with Carneiro [27] and El Fadaly [28]. The nervous manifestations were explained by encephalitis and brain parasite migration. The difference of clinical manifestation of both groups in this study may be due to the quantity and the parasite strain that was used for the infections El Fadaly [28] and also the habitat of each strain inside the infected mice.

Regarding the parasitological examination in this study; in group I, 90% of mice were positive for tachyzoites in the peritoneal fluid. While in group II, 80% of mice were positive for *T. gondii* cysts in brain homogenate with no statistically significant difference between the two groups of infected mice. The only negative sample detected in group I may be due to missed injection in other sites rather than the peritoneal cavity. Also the two negative samples by parasitological examination in group II may be explained that mice being sacrificed early at day 45 after oral inoculation before the infection settled in the

brain, Yai [29], the infection may also fail due to low or non-viability of parasite in tissue, Basso [30].

Our histopathological results showed high percentage of positive samples containing tachyzoites were detected in liver and spleen tissue samples in group I. The percentage was (50%) for both organs, while in group II were 10% for both organs too, with statistically significant difference between both infected groups (I & II). These results were explained that as these organs being adjacent to the peritoneal cavity where tachyzoites were inoculated by intraperitoneal route, it is well known that tachyzoites freely multiply in the lymph nodes and lymphoid tissue drained to liver and spleen, Dubey and Beattie [1]. Concerning the pathological changes in the liver of group I, it appeared as multiple pseudocyst of *T. gondii* among the hepatic cords and karyorrhexis in nuclei of some hepatocytes. Also, the pathological changes in the spleen of the same group appeared as multifocal clusters of tachyzoites as a pseudocyst of *T. gondii* in the white pulp of spleen, these findings are in agreement with Egan [31], Glatman-Zaretsky [32] and Etewa [33]. Pathology that occurred in the liver can be a result of tachyzoite multiplication in hepatocytes and occasionally in kupffer cells, tachyzoites attract inflammatory cells and, when activated, exacerbate the inflammatory response causing cell lysis in addition to the mechanical damage produced by the tachyzoites, Fuentes-Castro [34].

Regarding group II, we detected high percentage of positive samples containing *T. gondii* cysts in kidney (60%) & brain (50%) tissue samples, while in group I were (20%) in kidney & (10%) in brain tissues with statistically significant difference between both infected groups. The high positivity in brain tissue samples in group II point towards a preferential location of the parasite strain in mouse brains, Dubey [35] and these alterations are caused by parasite molecules that increase the permeability of the blood-barrier and facilitate the establishment of the parasite. When the parasite infects a large number of astrocytes and glial cells, their protective effect is diminished or lost, which aggravates tissue damage and the inflammatory process, favoring the formation of tissue cysts Strunk [36]. However, absence of brain cysts in group I was explained as brain is an immunologically privileged site, and it is believed that the brain is

the last organ to be reached by tachyzoites, in addition the blood-brain barrier slows the influx of mediators of inflammation and antibodies to the brain, which has greater pathological importance in the chronic phase of infection, Dubey [37]. Similar results were obtained by Smiley [38] and Djurkovic-Djakovic [39]. The pathological changes in brain of group II observed as *T. gondii* cyst containing many bradyzoites with mild neuronal loss and vacuolar degeneration, these findings are in agreement with Gulinello [40], Parlog [41] who observed evidence of inflammatory infiltration and presence of *T. gondii* cysts. These findings in brain were disagreed by McGavin and Zachary [42], Silva [43] and Nunes [44] who found pathological changes in brain without *T. gondii* cysts.

The high positive samples in renal tissues may be explained by the high vascularity of the kidney and so can be easily contaminated through the blood stream. Similar pathological changes were observed by El Fadaly [28] who observed that there were dilatation of renal blood vessels and degeneration of glomerular tufts and Miranda [45] who detected nephron loss with consequent kidney volume decrease in *T. gondii* infected mice (ME49 strain).

Molecular diagnosis of toxoplasmosis has been mainly performed using Hsp60 gene. The primers were designed to amplify portion of Hsp60 gene. Designing primers based on *T. gondii* ME49 heat shock protein Hsp60 sequence (Gene bank XM_002367081) to detect both RH strain (group I) & ME49 strain (group II) of *T. gondii*. Previous studies used the same primer to detect most of *T. gondii* strains, Anizah [46] and Rahumatullah [47].

Regarding PCR findings in GI and GII (Table 6), higher positive results were found in most organ samples of group II than group I with significant increase in brain infection (60%) in group II, this was attributed to the number of bradyzoites found inside a cyst has a magnitude order higher than the tachyzoites, favoring the DNA extraction and amplification of the mice organ samples. This finding was in agreement with Terra [48] who observed higher positive tissue samples in chronic infection group than acute infection group. Other study by Rahumatullah [47] disagreed with this finding, they observed that RH-infected mice generally showed higher parasite loads in all tissue organs as compared to ME49 strain-infected mice except with brain

sample. Our results showed that heart tissue samples did not show any amplification in group I (0%) and it could be attributed to absence of tachyzoites in these tissues owing to acute phase of infection and tachyzoites did not get enough time to reach to this organ. This finding is in agreement with Sudan [49]. On the other hand, Dadimoghaddam [50] found that the high parasite load in all tissues and the peak of DNA was observed in heart, kidney and liver in experimentally infected mice with *T. gondii* RH strain.

The ultimate significance of PCR based detection of toxoplasmosis is that PCR can detect the DNA of parasites even when the tissues available for testing are in state of decomposition; in contrast in histopathology it is quite difficult to identify the necrosed tachyzoites in the regions of marked generalized necrosis of the tissues induced by tachyzoites themselves, Hurtado [51] and Yai [29]. Absence of *T. gondii* parasite in histological sections however it was detected by PCR may be explained by the difficulty in detecting the parasite due to the uneven and clustered distribution of the parasite in tissue, Bezerra [52], or may be explained as only a small amount of tissue can be fixed on one slide and as few as one tissue cyst can be present in an entire organ, so the parasite may be present in unexamined tissues, Bouchard [53]. Selection of liver tissues to determine the PCR sensitivity depending on the highest intensity of infection in both infected groups as liver of mice infected with group I & II showed the highest parasite load, followed by other organs, this finding was in agreement with Rahumatullah [47] who showed highest parasite load in liver tissue of RH & ME49 strains.

The sensitivity of PCR was (66.7%) in group I, out of 9 positive samples by parasitological examination, 3 samples were not detected by PCR technique. This finding resulted from limited sample size, random distribution of tachyzoites in tissue or may be due to low number of parasites in tissues, Hill [54]. Other studies reported high PCR sensitivity, Hafid [55] (2 tachyzoites) and El sayed Khalifa [56] (1 tachyzoite).

The sensitivity of PCR was (87.5%) in group II, out of 8 positive samples by parasitological examination, only one sample was not detected by PCR technique. These results are in agreement with findings obtained from Hamidinejat [57] who detected 46.23% of chicken infected with *T.*

gondii by PCR compared to 27.36% by mouse bioassay, Also, Saki [58] reported 6% positive samples in rodents by PCR but 2% positive samples by mouse bioassay. On the contrary, the results obtained from Tsutsui [59] identified *T. gondii* in pork by mouse bioassay and PCR in 67.5% and 22.5% respectively.

Alignment analysis of Hsp60 gene sequences revealed a total of 5 nucleotide position differences between both RH and ME49 strains of *T. gondii*. There were 2 nucleotide substitutions detected between the 2 strains at position 95-96 bp, it was (GG) in RH strain sequence and (AT) in ME49 strain. In addition, 3 nucleotide substitutions detected between the 2 strains at position 192-194 bp, it was (TTT) in RH strain sequence and (ACC) in ME49 strain. Similar results were obtained by Lilue [60] which involved the comparison of 315 bp sequence from the 3' regions of the SAG1 gene from nine strains of *T. gondii* detected polymorphisms correlated with virulence.

These differences between the two sequences can provide valuable information in detecting mutational changes between closely related strains which might otherwise be concealed when looking at protein coding sequences or might change in protein function, Binias [61]. Also definition of a *T. gondii* strain as murine virulent or nonvirulent without needing to test it in vivo, Cheng [62]. Lyons and Johnson [63] reported that Hsp70 demonstrated after transcription in virulent strains of *T. gondii* and compared with avirulent strains suggesting that Hsp70 play an important role in the virulence of *T. gondii*. So, in this study, the sequence of *T. gondii* Hsp60 (RH strain) may be after gene expression, provide protection for this strain against cell damage associated with host cell invasion, allowing the virulent strain to persist as tachyzoites without the requirement for the encystation observed in avirulent strains.

As Hsp60 is thought to be located to the mitochondria of eukaryotes, where it helps in protein homeostasis, these chaperon systems involve multiple types of PPIs (protein interactions) that ensures proper protein folding and degradation, Koldewey [64]. Recent efforts have yielded chemical probes or inhibitors which are small molecules that inhibit (or promote) PPIs and so inhibit or promote molecular chaperone, Gestwicki and Shao [65]. Therefore, gene expression and protein translation of *T.*

gondii Hsp60 (ME 49 strain) could be smoothly happened but later on it may be inhibited by these chemical inhibitors.

So, the difference between the virulence of the two strains may be due to the difference in their sequence as approved by our results and consequently the difference in the sequence of amino acids and the produced protein, it may be also due to production of the same protein and then its inhibition by these chemical mediators.

Another explanation for avirulence of ME49 strains, as DNA molecule is a double helix, therefore either side can serve as a template to be copied by RNA, the genetic information is stored in a sequence of nitrogenous bases that get transcribed by mRNA. Every three bases is called a codon, the mRNA codon is actually the information that gets translated into protein, Sadowski and Jones [66]. Altered codon leads to insertion of an incorrect amino acid into a protein molecule during translation, so the protein function may be altered or the codon may be unaltered and codes for the same amino acid, Sauna and Kimchi-Sarfaty [67].

CONCLUSION

Our results showed that PCR is a highly sensitive, specific and rapid technique for detecting *T. gondii*. It revealed higher positive results in chronic strain (ME49) especially in brain tissue than acute strain (RH) but it may show false negative results in some samples. Both PCR technique and histopathological examination showed that the preferred tissue for detection of *T. gondii* in experimentally infected mice was liver & spleen in acute strain (RH) and brain & kidney in chronic strain (ME49). Bioinformatic analysis of Hsp60 gene sequences of both (RH & ME 49) strains of *T. gondii* showed that there was a polymorphism in some nucleotides between the 2 sequences. This polymorphism might change in protein function or may be concealed after protein translation. The results of the present study suggested that the coding region of the Hsp60 gene may represent a genetic marker for further intraspecies phylogenetic analyses of *T. gondii*. Further studies are needed to investigate whether Hsp 60 gene polymorphism affects *T. gondii* strains virulence or not.

Ethical considerations:

Mice were maintained in accordance with the research protocols following the recommendations of the National Institutes of Health Guide for

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Conflicts of interest:

There are no conflicts of interest.

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