The First Molecular Evidence of Toxoplasma gondii in the brain tissue of rats, Gaza, Palestine

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Research Article

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Abstract

**Background** *Toxoplasma gondii* is the causative agent of toxoplasmosis, its associated disease, which has significant economic, veterinary and medical importance. To determine the prevalence of *T. gondii* infection in the brain tissues rats trapped from local markets in Gaza, Palestine using nested-PCR assay.

**Methods** A total of 132 black rats (*Rattus rattus*) were analyzed. We used Polymerase Chain Reaction (nested-PCR). The present study showed that 86 out of 132 (65.2%) and 36 (27.3%) of the trapped rats were infected with *T. gondii* using B1 and SAG1 genes respectively.

**Results** The results showed that males have higher infection with *T. gondii* than females with a significant difference (P=0.02) when using B1 gene. In both B1 gene and SAG1 gene there was shared positive for 30 samples (22.7%).

**Conclusions** This finding indicated that *Rattus rattus* which is dominant in Gaza Strip could be a potential source of *T. gondii* for stray cats in the local markets and disseminating infection to the other parts of Gaza Strip. We recommend that Gaza municipality should take serious measures towards minimizing the number of roaming cats in the local markets of Gaza and other governorates, and changing policy towards the current site of Feras market.

**Highlights**
- This paper is the first of its kind that thoroughly investigates the molecular epidemiology of *Toxoplasma gondii* in rats in Gaza Strip, Palestine
- In particular, it investigates the status of such zoonotic diseases in rodents.
- Social and environmental risk factors related to this infectious disease are discussed.
- It sheds light on the presence of *T. gondii* in environments heavily populated with rodents and with poor sanitary conditions such as local markets in Gaza.
- This paper contributes to filling in a major gap in the literature
- Important recommendations are presented to those concerned, including policy- and decision makers, at governmental, nongovernmental, and academic institutions, especially for the Gaza municipality as well as in health and environmental sectors.

**Introduction**

Health and environmental challenges have shaped Gaza over the past decades. The Gaza Strip population is estimated to reach 2,106,745 individuals in 2021 overpopulating the small 365km² area [1]. Gaza Strip has maintained its traditional local markets selling vegetables, fruits and birds. Market floors
are covered with Zinco/Aspast or sometimes concrete which easily allow rodents, mainly rats, to enter the shops or burrow underground. These rats are the potential prey for cats which compete for food in the same area. The environment is thus suitable for completion of *T. gondii* life cycle. According to [1] poverty levels in Gaza strip reached 71.2%. It is estimated that only 70% of Gaza’s households are connected to the sewerage network, with the remaining sewage disposed of in cesspits. About 40,000 cesspits are in use in Gaza, and most of them are manually emptied due to the lack of connection to the sewerage network[2]. Intestinal parasites and other parasitic diseases are still endemic in the Gaza Strip, due to risk factors such as poor housing conditions, poor hygienic practices, especially among children, challenged sewer system infrastructure, and environmental contamination[3]. Common parasites identified in school children were *A. lumbricoides, E. vermicularis, T. tricbiura, S. stercoralis, E. histolytica, G. lamblia, Cryptosporidium spp, H. nana, and E. granulosus*[4].

*T. gondii* is a zoonotic parasite belonging to Phylum Apicomplexa with a world-wide distribution [5]. Most of the outbreaks of acute toxoplasmosis in humans are caused by oocysts [6]. *T. gondii* has a complex life cycle does not encompass all transmission routes and modes that can be used by the parasite to pass from definitive hosts, where sexual reproduction occurs, to intermediate hosts. The “classical” complex life cycle uses felids (domestic and wild-living cats) as definitive hosts and their prey as intermediate hosts [7]. Rodents, and in particular mice, are considered to be an important intermediate host as they are a potential prey species for cats [8]. They may play an important role in the completion of the life cycle of *T. gondii* by providing a source of infected meat for the cat which leads to subsequent infection of more secondary hosts. Despite this apparently important role, a number of serological studies have shown that *T. gondii* has a relatively low prevalence in mice [9] although some PCR-based studies of *T. gondii* in *Mus domesticus*, and other species of mice, have shown higher prevalences [10].

It was therefore hypothesized that rats in Gaza strip harbored different types of intestinal parasites [11].

In Palestine, the published reports on toxoplasmosis are entirely serologically-based studies that indicate varied seroprevalence across the country especially in Gaza Strip which is an overpopulated area, with limited resources and other potential risk factors [12].

The current study aimed to assess the molecular epidemiology of *T. gondii* among rats trapped from Gaza using two genetic markers that code for surface antigens SAG1 and B1 [13]. The study also looks to determine if rats in Gaza play a role as intermediate/reservoir host for *T. gondii*.

### Materials And Methods

#### Ethical considerations

Ethical approval was obtained from the Helsinki committee of the Palestinian Health Research Council, Gaza, Palestine.
Study Design

This is a prospective cross-sectional study where 132 brain tissue specimens were collected from *Rattus rattus* in local markets in Gaza Strip.

**Sampling**

Live trappings were performed at different local markets in Gaza. In each market traps were baited at night with food such as chocolate and butter nut. Traps were collected in the early morning from each market and were transported to the Medical laboratory sciences department. Sex determination was carried out as described by Roberts [14], following which, rats were anaesthetized and dissected as described by [15] brain tissue was removed from the skull and was stored at −20°C until use.

The frozen rat brain tissues were transported to Glasgow University under licence from the Scottish Government dated and Animal and Plant Agency.

**Molecular Examination At Glasgow University**

DNA was extracted from digested tissue samples of brain, using a spin column kit (QIAamp DNA tissue Mini Kit Qiagen) in accordance with manufacturer instructions. ~50 mg of each brain tissue was cut into small pieces, homogenized in 200 µl of DNA extraction buffer and proteinase K, and ingested at 55 °C for 2 hours. After the spin column is washed, DNA was eluted with 200 µl distilled water and used for PCR template. Detection of *T. gondii* DNA was carried out using nested-PCR assay through two genetic markers. Oligonucleotide sequences are shown in Table 1. Each marker has specific PCR conditions and PCR reaction.
Table 1
The genetic markers and their Oligonucleotide sequence

<table>
<thead>
<tr>
<th>Genetic Marker</th>
<th>Nested-PCR for \textit{T. gondii}</th>
<th>Oligonucleotide sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1gene</td>
<td>(1st Round PCR)</td>
<td>F/P 5/GGAAC\quad \quad GATCCGTT CATGAG-3</td>
<td>Nucleotide position</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R/P 5/ TCTTTAAAGCGTT\quad CGTGGTC-3</td>
<td>663–682, 949–930</td>
</tr>
<tr>
<td></td>
<td>(2nd Round PCR)</td>
<td>F/P 5/ TGC\quad \quad CATAGGTTCAGTCATG-3</td>
<td>694–714</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R/P 5/ GGCGAC\quad \quad CAATCTGCGAATACACC-3</td>
<td>887–868</td>
</tr>
<tr>
<td>SAG1</td>
<td>(1st Round PCR)</td>
<td>DS29 (5/TTGCCGCGCCCACACTGAG-3/)</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DS30 (5/CGCGACACAAGCTGCGATAG-3/)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2nd Round PCR)</td>
<td>DS38 (5/GACAGCCGCGGTCATTCTC-3/)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DS39 (5/GCAACCAGTCAGCGTCGCC-3/)</td>
<td></td>
</tr>
</tbody>
</table>

**Nested-PCR Assay**

Nested-PCR was performed in a final volume of 10 µl using Go Taq master mix (Promega, USA) with 100 ng/µL of DNA. The nucleotide sequences of the primers for the nested-PCR test targeting the B1 gene and SAG1 from are shown in Table 1. The nested-PCR assays were performed based on two genetic markers, B1 and SAG1 to detect \textit{T. gondii} DNA. The first round of PCR amplification contained 5 µl Gotaq buffer, 5 µM of each primer, and 100 ng/µL (2 µl) of extracted DNA. Reactions were started with an initial denaturation at 94°C for 5 min and then cycled 30 times with denaturation at 94°C for 20 s, followed by annealing at 53°C for 20 s for B1 gene and 55°C for 20 s and finally an extension step at 72°C for 20 s followed by a 5 min final extension at 72°C. DNA with \textit{T. gondii} was used as a positive control. A negative control containing no DNA was also included in the 1st and 2nd reaction reactions.

In the second round of PCR reactions the amplification mixtures consisted of 2 µl of first-round 1/25 diluted product, contained 5 µl Gotaq buffer, 5 µM of each primer. Nested PCR was started with an initial denaturation at 94°C for 5 min and cycled 35 times using a denaturation step of 94°C for 20s, followed by annealing at 45°C for 20s and a final 5 minute extension step at 72°C.
For SAG1 in the 1st and 2nd rounds of PCR reactions the amplification mixtures consisted of 2 µl of DNA, 5 µl Gotaq buffer and 10 pM/µl of each primer. Amplification was carried out using PCR Max thermocycler as follows: an initial denaturation step of 5 minutes at 95°C followed by 40 cycles of PCR performed for 40 sec at 95°C, 40 sec at 63°C, and 1 minute 10 seconds at 72°C, with a final extension step of 10 min at 72°C.

**Visualization And Confirmation Of PCR Amplification Product**

The PCR products were separated on a 1.5% Tris–acetate–EDTA (TAE) agarose gel and stained with Gel Red. Two molecular markers were included in each run, 100 bp and 1kb Plus marker (Invitrogen, USA). 7µl of B1 and SAG1 PCR-products were visualized using the gel documentation system from Synoptics Group, UK. PCR reactions were expected to yield 194bp and 522bp bands for B1 and SAG1 respectively.

**DNA Sequencing Of PCR Products For Both B1 And SAQ1 Genes**

PCR products were excised from agarose/TAE gels, and were then purified using QIAquick Gel Extraction kit (Qiagen, Germany), all sequences were carried out by Source bioscience company, UK.

**Statistical analysis**

Statistical analysis of the data was carried out using SPSS software, where a p-value < 0.05 was considered significant.

**Results**

The present study showed that 86 out of 132 (65.2%) and 36 (27.3%) of the trapped rats were infected with *T. gondii* using B1 and SAG1 genes respectively. Table 2. shows that males have higher infection with *T. gondii* than females with a significant difference (P = 0.02) when using B1 gene.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Brain tissues with <em>T. gondii</em> No. %</th>
<th>Brain tissues without <em>T. gondii</em> No. %</th>
<th>χ², P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B1 gene</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>53 (40.2)</td>
<td>37 (28.0)</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>33 (25.0)</td>
<td>9 (6.8)</td>
<td>4.886, 0.02*</td>
</tr>
<tr>
<td><strong>SAG1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>25 (18.9)</td>
<td>65 (49.2)</td>
<td>0.036, 0.51</td>
</tr>
<tr>
<td>Females</td>
<td>11 (30.6)</td>
<td>31 (23.5)</td>
<td></td>
</tr>
</tbody>
</table>
*P<0.05 is significant

**Amplification of B1 gene from T. gondii from rat brain tissue**

As shown in Fig. 1., the 2nd round of PCR amplification for DAN samples and positive control yielded the expected band 194 bp for B1.

As shown in Fig. 1., the samples were positive in 194bp location for B1 gene and 522bp for SAG1 gene.

As indicated in Fig. 3. In both B1 gene and SAG1 gene there was shared positive for 30 samples (22.7%).

**Sequencing results:**

We sent 6 samples for sequencing the results are below: This sequencing was used for three samples for Gene (SAG1) When using BLASTn Alignment results was 98% *Toxoplasma gondii*.

*Toxoplasma gondii* isolate M7 glycerol-3-phosphate dehydrogenase (B1) gene, partial cds

Sequence ID: MK521885.1Length: 287Number of Matches: 1

Range 1: 77 to 225

<table>
<thead>
<tr>
<th>Alignment statistics for match #1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score</td>
</tr>
<tr>
<td>276 bits(149)</td>
</tr>
</tbody>
</table>

Query 1  AGAGACGCTAATGTGTTTGCATAGGTTGCAGTCACTGAGCTCCCCTCTGCTGGCGAA 60

Sbjct 77  AGAGACGCTAATGTGTTTGCATAGGTTGCAGTCACTGAGCTCCCCTCTGCTGGCGAA 136

Query 61  AAGTGAAATTCACTGATATCTGTGCTGGTATTCGCAGATTGTCGCCTGCAAT 120

Sbjct 137  AAGTGAAATTCACTGATATCTGTGCTGGTATTCGCAGATTGTCGCCTGCAAT 19

Query 121  CGATAGTTGACCACGAACGCTTTAAGAA 149

Sbjct 197  CGATAGTTGACCACGAACGCTTTAAGAA 225

**Discussion**
Toxoplasma gondii is an intracellular parasite with a variable prevalence rate worldwide. It is estimated that T. gondii infects one third of the world human population [17].

This is the first molecular evidence showing that T. gondii is readily present in rats from local markets in Gaza, making them potential intermediate hosts for infecting the definitive host, the cat, which also populates local markets in Gaza. Previous reports suggest that it is possible for mice to act as a mutation hub for the evolution of new, virulent T. gondii strains [18]. In the present study the prevalence of T. gondii in rats was found to be 65.2% and 27.3% as measured by presence of B1 and SAG1 genes respectively. This was considered to be high given that the sample was taken from areas of high stray cat density such as Feras market, the local oldest market in Gaza. Rodents studied here were trapped from the local markets of Gaza, Feras, Als-Shejayaa, Jabalia, which are local, not modernized and old, with poor hygiene and sanitary measures, where sellers put their produce on wooden desks and spray unsterile water. In addition, large numbers of cats and rats are simultaneously populating the local markets, especially at night, hunting for prey. Thus, two parasitic hosts are present in the environment. Cat defecation may infect rats, rats are prey for cats, and cats disseminate oocysts in the Gaza local markets. This cycle is more important in species that live close to human habitats, because of the importance of its environment and human health. The role of rodents in the transmission cycle causes infected felids to release oocysts and thus the spread of contamination in the environment, increasing the infection risk of each of the parasitic hosts in the environment, including, importantly, of humans [19]. Wild rats tend to live in association with human environments. They live in social hierarchies consisting of a dominant male with several females and younger or submissive males [20]. Zoonotic parasitic diseases especially toxoplasmosis present among stray cats (5.4%) in Khan-Younis, Gaza Strip, where this study employed the conventional methods of [21]. Different reports on the Prevalence of T. gondii in rats from different countries either by serology or PCR were obtained. 8.8% in Brazil [22], 23.6% in Eastern China [23], 5.9% in Malaysia [24], 81.5 in Saudi Arabia [25], and 6% in Iran [26].

Numerous studies use PCR detection of T. gondii DNA from biological samples showing good specificity and sensitivity in the diagnosis of toxoplasmosis [27].

In the present study, 18 PCR positive rat brain tissues for T. gondii were blasted against the Toxoplasma database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), where all sequences were shown to be of T. gondii B1 gene and found to be identical and similarity of 100% in all blasted samples. 18 Toxoplasma positive by B1 gene results were matched to 18 samples from SAG1 gene. Rodents play an important role in the maintenance of the T. gondii life cycle and epidemiology of toxoplasmosis because they are considered as reservoirs and carriers of the disease and the main source of infection for cats and their relatives [28, 29].

We are still lacking molecular evidence for the existence of Toxoplasma gondii among pregnant women, women have had abortions in Gaza strip, where using molecular techniques will give more accurate results. Most diagnosis of Toxoplasmosis in laboratories and hospitals in Gaza was carried out through
serological methods, with an overall seroprevalence rate amongst the general population of 27.8% [30, 31].

The presence of roaming stray cats in local markets will facilitate the transmission cycle. Likewise, the dominant rats populating the same area also facilitate transmission despite efforts by Gaza municipality to control such rodents, with the structure of these markets adding logistical difficulty. The current study indicated that males (40.2%) were more infected with *Toxoplasma gondii* than females (25%) with statistically a significant level \( P = 0.02 \). A study in the USA showed that female rats showed behavioral modifications as a result of *T. gondii* infection similar to those previously reported in male rats [32].

A low seroprevalence was found over the whole town with only 1.96% of the rodents from Niger markets found seropositive [19].

By nested PCR, the brain and muscle tissues received from 3 black rats and a clouded leopard (1.37%) were found positive for *T. gondii* [33].

Out of the 100 rats captured, three rats were found to possess *T. gondii* cysts in their brains (3%) at the local Chow Kit market, the largest wet market in the city of Kuala Lumpur [34].

This sequencing was used for 14 samples for Gene B1. When using BLASTn Alignment results was 100%.

Evidence yielded adds value in filling the literature void and prompts further studies in this zoonotic disease in Palestine.

**Conclusions**

*T. gondii* in rat brain tissue was confirmed by PCR as an evidence in the reservoir host (rats) in Gaza Strip. This finding indicated that *Rattus rattus* which is dominant in Gaza Strip could be a potential source of *T. gondii* for stray cats in the local markets and disseminating infection to the other parts of Gaza Strip. There is discrepancy between the selected primers for identification of positive samples due to the sensitivity of B1 gene. The alignment showed 100% of *T. gondii* similarity using B1 gene.

**Recommendations**

Gaza municipality should take serious measures towards minimizing the number of roaming cats in the local markets of Gaza and other governorates. Changing policy towards the current site of Feras market. Finding effective control measures towards rats in local markets especially Feras market environmentally friendly. Finding strategies to minimize/eradicate the rats populations in the local markets of Gaza.

**Declarations**
Beneciaries
- Gaza Municipality, Health authorities, physicians, veterinaries, researchers and the local Palestinian community of Gaza strip.

Acknowledgement
The authors gratefully acknowledge the help and assistance provided by the Institute of Infection, Immunity and Inflammation, University of Glasgow (flow/imaging/bioinformatics) Facility in the generation of some of the data reported in this manuscript. We would like to gratefully acknowledge the team of students who contributed to the dissection of rats.

Ethical statement
Ethical approval was obtained from the Helsinki committee of the Palestinian Health Research Council, Gaza, Palestine.

Data availability
The datasets generated and/or analysed during the current study are presented along the manuscript any query should sent to the author.

Author contribution
As an author for this research work, I confirm that I collected the samples with my self and contributed in preparation literature review, designing, conceptualization, investigation, data curation, and critically reviewing the manuscript. data curation, editing, and critically reviewing the manuscript and approve it.

Conflicts of Interest
The authors declare that they have no conflict of interest.

Funding
This study was funded from Microbiology Society, London, UK and hosted by the University of Glasgow.

References


**Figures**

**Figure 1**

PCR amplification products of *T. gondii* B1 gene in rat brain tissue samples; 1 ladder 100 bp (2-6, 8 positive samples, 7 empty), 10 Positive control, 11 (empty), 12 negative control.
Figure 2

PCR amplification products of *T. gondii* SAG1 gene in rat brain tissue samples (1-marker 1Kp+, 2-3 positive samples, 4-5 negative samples, 6 positive control, 7 negative control)
Figure 3

Double positive due to sex

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MicrobiologySocietygrant442023.pdf
- WaivingAPCfromParasitesandVectors542023.pdf
- WaivingofAPC442023.docx