Pakistan J. Zool., vol. XX(0), pp 000-000, 2022. **DOI**: https://dx.doi.org/10.17582.....

Detection and Genotyping of *Toxoplasma gondii* in Beef and Mutton Consumed in Gaza, Palestine

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Article Information
Received 24 July 2022
Revised 16 August 2022
Accepted 21 September 2022
Available online
(early access)
Published

Authors' Contribution
.....?

Key words
***Toxoplasma gondii*, Mutton, Beef, PCR, ELISA, Gaza-Palestine**

ABSTRACT

Toxoplasmosis is a parasitic disease that is transmitted by a variety of routes, including the ingestion of raw or undercooked meat. It infects roughly one-third of the world's population and is caused by *Toxoplasma gondii*, an obligate intracellular parasite. The goal of this research is to detect the existence and genotypes of *T. gondii* in beef and mutton, two of the most widely consumed red meats in Gaza, Palestine, using both ELISA and PCR techniques. For this purpose, 60 red meat samples were collected from butcheries in Gaza city, during the period from January to March 2021. These samples were divided evenly between beef and mutton. This study found that beef is devoid of *T. gondii*, whether tested using ELISA or PCR. On the contrary, both approaches detected *T. gondii* in mutton; however, the percentage of positive samples reported differed. For example, whereas *T. gondii* was detected in 14 (46.66 %) of 30 samples using ELISA, only 5 (16.66 %) of positive samples were detected using PCR. The genotyping results of the current investigation showed that the three DNA isolates were *T. gondii* type II. A Chi-square test was also implemented to evaluate the prevalence of *T. gondii* and the type of red meat samples (mutton and beef) examined using PCR and ELISA. Similarly, in the detection of *T. gondii*, a comparison of the PCR approach and ELISA was conducted, and all of these relationships were shown to be statistically significant, with p values < 0.05. Meanwhile, this investigation found that beef samples were devoid of *T. gondii* infection. Regardless of whether it was examined with an ELISA or a PCR, this study revealed the occurrence of *T. gondii* in mutton. The current study also concluded that eating raw or undercooked mutton is a potential risk factor for the transmission of *T. gondii* infection to humans. Besides, the occurrence of *T. gondii* type II in the three genotyped ADNA isolates.

INTRODUCTION

A particular parasite's occurrence in food varies from country to another, and parasitic zoonotic diseases mainly affect the health of people and animals as well as total productivity (Slifko*et al*., 2000). It should be noted that parasitic zoonotic diseases are transmitted by the consumption of infected or contaminated meat, fish, vegetables, and contaminated water (Zhou*et al*., 2008; Bhatia *et al*., 2010). Likewise, meat-borne diseases are transmitted by the consumption of infected raw or undercooked meat, such as the meat of pigs, buffaloes, goats, and sheep, where the most frequent parasitic meat-borne diseases include sarcocytosis, trichinellosis, toxoplasmosis, hydatidosis, and cysticercosis (Sharma *et al*., 2019). Toxoplasmosis is a parasitic disease caused by *T. gondii*, an intracellular parasite that may infect all warm-blooded animals and has a complicated life cycle that includes two modes of reproduction: sexual and asexual. Numerous investigations have shown that many members of the feline family are

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0030-9923/2022/0001-0001 $ 9.00/0

capable of serving as the definitive hosts for *T. gondii*. On the other hand, all mammals, birds, and reptiles can serve as intermediate hosts, where asexual reproduction occurs (Dubey *et al*., 2009; Dubey, 2009b; Skariah *et al*., 2010). Additionally, tissue cysts found in meat or meat-derived products are important sources of *T. gondii* transmission to humans. However, it is crucial to highlight for public health concerns that the location of *T. gondii* tissue cysts and the amount of these tissue cysts generated in a specific organ vary depending on the intermediate host species. Livestock cysts, for example, are frequently seen in the tissues of infected pigs, sheep, and goats, and much more frequently in infected poultry, rabbits, dogs, and horses. On the other hand, tissue cysts in the skeletal muscles of buffaloes and cattle are extremely rare (Tenter *et al*., 2000). It is worth noting that eating undercooked, raw, or processed meat is one of the primary routes of toxoplasmosis transmission to humans, particularly in congenital cases, which account for 30-60% of infections during pregnancy. Several public health agencies across the world have implemented *T. gondii* surveillance prog in animals intended for human consumption, notably meat samples (Tenter *et al*., 2000; Juránková *et al*., 2013). Even though it has been determined that the consumption of raw or undercooked meat is a risk factor for *T. gondii* transmission in several studies, the relative significance of the risk factor and the type of meat associated with the most infectious varies from one country to another. For example, in France and Norway, many studies have reported that consuming undercooked lamb poses a greater risk factor than consuming undercooked pork (Kapperud*et al*., 1996; Baril *et al*., 1999; Cook *et al*., 2000). While in various locations, the most important risk factor varies. In Poland, for example, undercooked pork has been ascertained as the main risk factor for *T. gondii* transmission to humans in several investigations (Paul, 1998). *T. gondii* is not in the vanguard of foodborne pathogens, yet it is an important foodborne pathogen. It is also worth noting that numerous recent studies published in the United States have confirmed the existence of six pathogens that play a significant role in the transmission of almost 90% of foodborne diseases. *T. gondii* came in fifth in these investigations, following *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes,* and *norovirus* (Batz *et al*., 2011). Otherwise, the CDC estimates that toxoplasmosis is the second most common cause of mortality in the United States, after only salmonellosis, and that foodborne toxoplasmosis accounts for half of all cases in the United States (Scallan *et al*., 2011). Numerous studies and researches in the United States and the Netherlands have lately highlighted *T. gondii's* significance as a cause of foodborne diseases, ranking it second and third among the most significant pathogens in the United States and the Netherlands, respectively (Scallan *et al*., 2011; EFSA, 2018). Despite the fact that several techniques have been employed to identify *T. gondii*, the most relevant ones are serological tests such as ELISA and molecular, such as PCR. While serological tests of meat juice are regarded as a great and efficient approach for the detection of *T. gondii* in various types of slaughtered animals' meat, their combination with PCR is an effective and helpful method for the accurate diagnosis of *T. gondii* in meat (Dubey, 2009; Fallahi *et al*., 2014; Bacci *et al*., 2015). Numerous investigations have been performed in the study area to investigate the seroprevalence of *T. gondii* infection in pregnant and abortion-experiencing women. *T. gondii* specific antibodies were detected in 12.8 % of 312 women who had abortions in Gaza-Palestine, according to (Al-Hindi and Lubbad, 2009; Al-Hindi and Lubbad, 2009). Furthermore, Al-Jarousha (2012) found 30.9 % seropositivity among 255 pregnant women in Gaza (Al-Jarousha, 2012). Meanwhile, *T. gondii* oocytes were detected in 6.33% of 300 fresh samples of vegetables consumed raw in Gaza city, according to one research, while anti-*T. gondii* antibodies were detected in 64.00% of sheep and 26.0% of backyard chickens in the study region, according to another (Dardona *et al*., 2021, 2022). Moreover, the main purpose of this study is to detect the occurrence of *T. gondii* in beef and mutton using both serological technique (ELISA) and molecular technique (PCR) in Gaza-Palestine as well as the genotyping of some *T. gondii* DNA isolates.

MATERIALS AND METHODS

Ethical approval

On July 5, 2019, the ethical research committee of the Islamic University of Gaza approved an ethical clearance for this study, which planned to gather fresh red meat samples from the study area.

Sample collection

A total of 60 red meat samples were purchased from butcheries in Gaza city, Palestine, during the period from January to March 2021. These fresh samples were evenly distributed between beef and mutton, which are the most frequently, consumed red meats in the study area. Furthermore, these samples were taken from the animal's muscle and stored at a temperature of -20°C until they were utilized. Meanwhile, each sample weighed between 200 and 250 g of red meat. It should be noted that these samples contained neither lipids nor bones.

Meat juice

Subsequently, for collecting meat juice, the samples were thawed at room temperature after being deep frozen

for about a month, and the meat juice resulting from the thawing process was collected in Eppendorf tubes 2-3 ml to be tested for the occurrence of anti-*T. gondii* antibodies using an ELISA technique on the same day of thawing (Wallander *et al*., 2015).

Enzyme linked immunosorbent assay (ELISA)

Meat juice samples were examined using the Indirect Multi-Species ELISA for the detection of anti-*T. gondii* antibodies, (ID Screen® Toxoplasmosis Indirect Multi-species, France). Based on the instructions indicated by the manufacturer, the results were pronounced as optical density (OD). For more details, the absorbance was measured at 450 nm. The wells of each microplate were coated with *T. gondii* antigens, and subsequently, the peroxidase (HRP) conjugate was added. This HRP substance addition plays a crucial role in the formation of the antigen–antibody complex. In order to draw the validation purpose from each test, positive and negative controls supplied by the kit manufacturer were utilized. Samples with values of greater than 35 IU/ml were considered positive and vice versa for negative samples. It should be noted that the results obtained using this kit do not give the specific type of antibody, either IgM or IgG, but instead give the sum of these antibodies. In other words, these values are the sum of (IgM + IgG) antibodies without knowing the values of either of them.

Molecular identification of T. gondii

DNA extraction

To extract *T. gondii* DNA from meat samples, the procedure described by Rehedar *et al*. (2012), was followed, in which 50 g of meat were chopped from each sample, and the equipment used in the cutting process was sterilized before being used to prevent cross contamination. Following that, the meat was minced using an electric meat grinder, and 25–30 mg of each minced tissue were extracted according to the manufacturer's instructions for the commercial kit used for DNA extraction (Qiagen, Valencia, Ca, USA). The samples were then resuspended in 180µL of ATL buffer and 20µL of proteinase K (provided in the QIAamp DNA Mini Kit) to identify the relevant gene from pure DNA, the methodology indicated by the manufacturer for tissue samples was entirely followed to extract DNA from tissue samples (Rehedar *et al*., 2012).

PCR amplification

Targeting the 194 bp fragment of the 35 fold repetitive B1 gene of *T. gondii*, PCR reaction was performed using a pair of primers TOXO1 (5′ GGA ACT GCA TCC GTT CAT GAG 3′) and TOXO2 (5′ TCT TTA AAG CGT TCG TGG TC 3′). For that, reason amplification was performed according to the conditions that described by Schwab and McDevitt (2003), with the exception that the reaction mixture volume was 25 µl (Burg *et al*., 1989; Schwab and McDevitt, 2003). For positive results confirmation, all positive samples were also examined by PCR reaction with primers TOXO-F (5′ AGG CGA GGG TGA GGA TGA 3′) and TOXO-R (5′ TCG TCT CGT CTG GAT CGC AT 3′), which preplanned for targeting a fragment of the 200 to 300-fold-repetitive element (REP) sequence (AF146527) (Lass *et al*., 2009; Cassaing *et al*., 2006). Consequently, all components were added to AccuPower® PCR PreMix tube (Bioneer Corporation- Hylabs). For each isolate, PCR amplification of TOXO genes was performed in the thermal cycler (Biometra, Germany). The following conditions were considered: An initial denaturing step at 95◦C for 5 min, thermocycling for 30 cycles, where each cycle consisted of 30 s at 94◦C followed by 90 s at 55◦C for annealing, and 1 min at 72◦C for extension, and a final extension cycle of 10 min at 72◦C (Hassanain *et al*., 2013).

Genotyping

Three of the PCR-positive samples were genotyped using the PCR-RFLP approach described by Norouzi *et al*. (2016). The GRA6 primers (5'-GTAGCGTGCTTGTTGGCGAC-3) and (5'TACAAGACATAGAGTGCCCC-3) were used to amplify a 791 bp DNA fragment. The restriction enzyme *MseI* then digested the fragment. The digestive products distinguish *T. gondii* genotypes. Type I, type II, and type III are identified by products of 168 and 544 bp, 75 and 623 bp, and 97 and 544 bp fragments, respectively (Norouzi *et al*., 2016).

Statistical analysis

A statistical analysis was implemented using the SPSS (USA, II, Chicago, SPSS Inc) software package version 15.0 and the data gathered by the current investigation. Furthermore, a Chi-square test was used to compare the incidence of *T. gondii* and the type of red meat samples (mutton and beef) tested using PCR and ELISA. In addition, a comparison of the PCR method and the ELISA for the detection of *T. gondii* was conducted. A $P < 0.05$ was deemed significant.

RESULTS

Overall, the current study's findings clearly demonstrated that anti-*T. gondii* antibodies were detected in only 14 out of 60 samples (23.33%) that were tested for the existence of these antibodies using the ELISA technique. Despite the fact that all beef samples were determined to be devoid of anti-*T. gondii* antibodies, the results also revealed that 14 out of 30 mutton samples

were positive (46.66%). The current study's findings also revealed a statistically significant relationship between the occurrence of anti-*T. gondii* antibodies and the type of red meat samples (mutton and beef) examined using ELISA, with a *p* value of < 0.05 (Table I).

Table I. Occurrence of anti- *T. gondii* antibodies in red meat samples (beef and mutton), using ELISA technique.

Type of meat	No of samples	Antibodies (+) IgG/IgM	Antibodies (-) IgG/IgM	% Positive
Beef	30	0	30	0.00
Mutton	30	14	16	46.66
Total samples	60	14	46	23.33
$\chi 2 = 18.26$	P = 0.000< 0.05		Statistically significant	

The results of the PCR technique were much different, as PCR for detection of the B1 gene revealed that just five (8.33 %) of the sixty samples were found to be positive. These results also showed that all the beef samples were negative; however, five of the thirty mutton samples (16.66 %) were positive. The current study's findings also revealed a statistically significant relationship between the occurrence of *T. gondii* cyst and the type of red meat samples investigated (mutton and beef) using PCR, as indicated in Table II, while the PCR findings are also shown in Figure 1.

Table II. Occurrence of *T. gondii* in red meat samples (beef and mutton), using PCR technique.

Type of meat	No of samples	Positive (%)
Beef	30	0
Mutton	30	5(16.66)
Total samples	60	5 (8.33)
$\chi 2 = 5.445$	P =0.020 < 0.05	Statistically significant

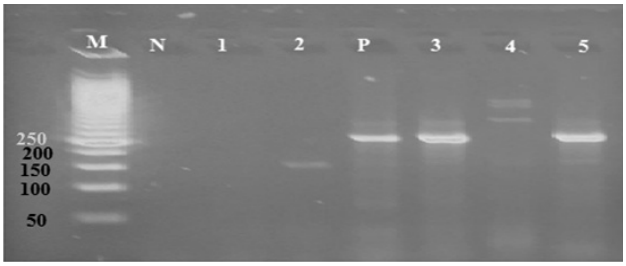

Fig. 1. Results of *T. gondii* recovery test of red meat samples (Beef and Mutton). B1 amplification products loaded on to 2% gel agarose. Lane M, molecular weight marker (Marker 50 bp, Bioline, Italy). Lane N, Negative control. Lane P positive control. Lanes 3 and 5 positive samples, and Lanes 1, 2, and 4, negative samples.

Regarding the PCR-RFLP genotyping, Figure 2 shows that the *MseI* digestion products are 75 and 623 bps thus indicating that the three samples tested are Toxo-type II.

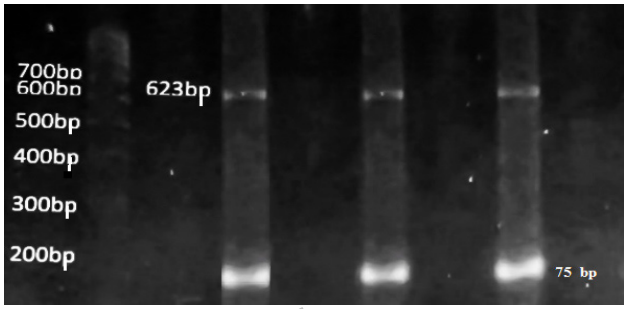

Fig. 2. PCR-RFLP pattern of the GRA6 gene cut by *MseI* endonuclease (75 and 623bp bands), corresponding to the pattern of type II *T. gondii* on a 2% agarose gel.

DISCUSSION

Consumption of any meat from warm-blooded animals or birds, which are intermediate hosts of *T. gondii*, is a substantial risk factor for *T. gondii* transmission. However, the risk ratio varies based on the species of animal (Asgari *et al*., 2006). Numerous studies performed across the world supported this perspective and confirmed the occurrence of several outbreaks, despite their small size, but they were attributed to consuming raw or undercooked meat as the main cause of these outbreaks (Choi, 1997; Cook *et al*., 2000; Tanter *et al*., 2000). The current study's findings clearly revealed a significant variation in the prevalence of *T. gondii* in the most frequently consumed red meat in the study region, as well as significant variances in the results obtained by the two methods utilized, ELISA and PCR. In more details, the current study's findings proved beyond any doubt that beef samples were devoid of *T. gondii*, both by ELISA and PCR. On the contrary, the results confirmed the existence of *T. gondii* in sheep meat (mutton), and while both techniques confirmed the occurrence of *T. gondii* in sheep meat, there was a significant difference in the results, as 46.66% of samples were found to be positive using ELISA, while only 16.66% were found to be positive using PCR. Furthermore, when comparing the results of the present study with those obtained by other similar studies, we found some similarities and some differences. For instance, in a study conducted in Egypt, a neighboring country to the study area, Abdel-Aziz *et al*. (2020) recorded that 45.8% of the sheep meat samples were found to be positive. This is nearly equal to the present research's results, but there is a notable and distinct variation in the results related to beef samples, where the Egyptian study documented the presence of *T.*

gondii in more than 31% of samples, the current study, on the other hand, confirmed that beef is completely free of *T. gondii*, and it is noteworthy that the Egyptian study used latex agglutination as well as microscopic examination, and the researchers justified the high results of *T. gondii* infection in the meat of those sheep by the way of grazing outdoors and to unsanitary conditions, in addition to their permanent exposure to the external environment that is potentially contaminated with *T. gondii* (Abdel-Aziz *et al*., 2020). It is interesting that the Egyptian research's arguments are so comparable to the characteristics and situations associated with sheep farming in the study area. Besides this, another study conducted in Scotland is consistent with the current study regarding the occurrence of *T. gondii* in mutton, but there was a difference in the recorded percentage of toxoplasmosis, as the Scottish study revealed *T. gondii* infection in 16.5% of mutton. Additionally, the Scottish study detected *T. gondii* in 5.30% of beef, in contrast to the current study, which did not find any beef positive samples (Plaza *et al*., 2020). One more study on this subject was conducted in Tunisia, and documented the incidence of *T. gondii* in adult sheep at a rate of more than 73.00% when examined serologically by the MAT technique. On the other hand, the occurrence of *T. gondii* was confirmed in 50.00% of ewes' tissue samples, and these results are consistent with the current study in the diagnosis of toxoplasmosis in mutton, with a difference in the percentage of the prevalence of *T. gondii* (Boughattas *et al*., 2014). Likewise, in an Italian investigation to investigate the prevalence of *T. gondii* in meat, the results agreed with the current study's results in terms of the occurrence of *T. gondii* in mutton using the ELISA technique. The current study showed anti-*T. gondii* antibodies in 46.66 % of the sheep meat samples using the ELISA technique, whereas the Italian investigation reported *T. gondii* in 28.6 % of the samples using the same approach (Gazzonis *et al*., 2020). In general, variances in *T. gondii* occurrence rates in meat in various countries around the world are related to changes in weather, climate, geographical locations, animal types, animal age, animal raising method, and management, as well as the quality of health prog (Kijlstra and Jongert, 2008; Dubey, 2016).

While the rates of toxoplasmosis in livestock fluctuate from one country to another, there is no doubt that the prevalence of *T. gondii* in meat is mainly related to the prevalence of *T. gondii* in meat-producing animals, conspicuously sheep. This is determined by a number of factors, including the age of the animal, the method of animal husbandry, animal care, the animal health system, and the management of animal farms. This difference also depends on the geographical area, so the incidence rate varies from one country to another (Dubey, 2016). From this current perspective, it is evident that there is a tight relationship between the incidence of toxoplasmosis in sheep and the meat they produce. For example, globally, the prevalence of *T. gondii* in sheep is very high for instance, in America, France, Turkey, and the United Kingdom, the rate ranges between 27 and 95%, which is a very high percentage (Kijlstra and Jongert, 2008; Dubey, 2010). It is surprising that the justifications for the occurrence of *T. gondii* infection in meat are very similar not only in the study area but also in other regions, as many studies mentioned some reasons that lead to the spread of *T. gondii* in meat specifically, as well as through foodborne diseases in general, such as livestock production management, hygienic practice in slaughterhouses, food processes and technology, and the density of cats or other felines, as well as climatic differences that aid in the occurrence of oocyst sporulation, such as heat, humidity, wind, and rain, which are environmental factors that also assist in the transmission of *T. gondii* oocysts to animals and human food, thus playing a crucial role in the transmission of the infection to animals, subsequently to their meat (Tenter, 2009). In addition to the abovementioned, the results pertaining to mutton can be supported by the high prevalence of infection in sheep in the research region. Najm (2019) and Dardona *et al*. (2022), conducted two of the few investigations in the research region that demonstrated the existence of anti- *T. gondii* antibodies in 46.70% and 64.00 % of sheep, respectively (Nigem, 2019; Dardona *et al*., 2022). The prevalence of *T. gondii* in sheep in the study area is attributed to several reasons, including what was mentioned previously. The most important of them is the way of grazing sheep outdoors, as well as their dependence heavily on grazing in those areas near garbage containers or close to sewage pumps, water swamps, and places where cats in general abound. It is also worth mentioning that this is the first study to assess the prevalence of *T. gondii* in meat in the study area. Using two different techniques, it was also able to document the occurrence of this parasite in sheep meat. Cattle, in contrast to sheep, are regarded as poor and undesirable hosts for *T. gondii*. Although *T. gondii* may infect cattle, as evidenced by numerous studies, two of which was conducted in the research area by Nigem (2019) as well as by Dardona *et al*. (2022), however, after a few weeks of infection, cattle can expel the parasite and eliminate it. This was explained by the presence of innate immunity capable of either expelling the parasite or lowering its amount in the host to undetectable and hence ineffectual levels (Munday and Corbould, 1979; Dubey, 1983, 1986; Nigem, 2019; Dardona *et al*., 2022). It is worthwhile noting that finding tissue cysts in big infected animals might be difficult. Bias can occur during the cutting of the

sample for investigation or while collecting meat samples from unfavorable regions for the parasite. As a result, the PCR test may provide a false negative; bigger samples in terms of quantity and weight are required to reliably detect the presence of *T. gondii* cysts in meat samples (Esteban-Redondo *et al*., 1999; Edvinsson *et al*., 2004). This illustrates why ELISA detects more positive samples than PCR in general, and specifically in this study. Because the tissue cyst does not form until the chronic stage of infection, rather than the acute stage, this cyst forms for the lifespan, and so the IgG antibody is present in the meat juice at this period (Ridley, 2012).To the best of the authors' knowledge, this is not only the first study of its kind in the study area that investigated the occurrence of *T. gondii* in meat, but it is also the first that genotyped some of *T. gondii* isolates, as the results of this study revealed that the three samples for which genotyping was performed were *T. gondii* type II, which is consistent with the findings of research conducted in Iran by Zia-Ali *et al*. (2007), to assess the genotyping of *T. gondii* in a variety of hosts, with the data revealing the existence of *T. gondii* types II and III (Zia-Ali *et al*., 2007). Even though much of the researches implies that *T. gondii* type I is the main cause of morbidity, other studies suggested that *T. gondii* type II is responsible for 70% of cases of toxoplasmosis in humans (Howe and Siby, 1995). Because of the economic relevance of *T. gondii*, particularly in meat production and animal husbandry processes, and in order to reduce the process of economic loss, numerous studies have been conducted to detect the prevalence of *T. gondii* in meat as well as its genotypes. For instance, in research conducted in the United Kingdom, meat samples included 57 pork, 9 muttons, 4 beef samples, and a blend of pork and beef samples were obtained from retail sellers. The study's findings revealed that 27 out of 71 samples were infected with toxoplasmosis using PCR. A genotyping investigation based on targeting the SAG2 gene revealed that 21 of the infected meats harbored Type I parasites. Six samples, on the other hand, had parasites of both Type I and Type III genotypes (Aspinall *et al*., 2002). Many European countries have a high prevalence of genotype II. For example, in one of the studies conducted in France, it was discovered that 85% of the 86 isolates of congenital toxoplasmosis were genotype II, besides the results in Poland were identical to those previously stated studies conducted in France. Furthermore, the first strain isolated from congenital toxoplasmosis in Serbia was classified as type II (Ajzenberg *et al*., 2002; Nowakowska *et al*., 2006; Djurkovi-Djakovi *et al*., 2006).

CONCLUSION

The present study revealed that beef, whether tested by ELISA or PCR, is devoid of *T. gondii*. On the other hand, both of the approaches utilized in this investigation successfully documented the occurrence of *T. gondii* in mutton. Although *T. gondii* was found in mutton using both techniques, the percentage of positive samples varied. ELISA technique confirmed that 46.6% of mutton samples were positive, whilst PCR revealed that only 16.66% were positive. This study also concluded that sheep meat (mutton) represents a potential risk factor for the transmission of *T. gondii* to humans in the study area, particularly those who consume raw or undercooked meat, as in some traditional meals in the study area, such as kebabs, shawarma, and grills, furthermore the occurrence of *T. gondii* type II in meat in the study area was revealed.

RECOMMENDATIONS

The current study strongly recommends avoiding consuming raw or undercooked meat, as well as encouraging appropriate cooking of meat used in various traditional cuisines in the research region, such as kebabs and shawarma. Furthermore, imported animals used for meat production from other countries should be assessed on a regular basis, and infected animals should be quarantined for further monitoring. Additionally, the present study recommends grazing local meat-producing animals away from garbage cans, landfills, wastewater and rainfall gathering ponds, and areas where cats and decaying animal corpses thrive, such as Wadi (valley) Gaza. Lastly, it is crucial to emphasize that restaurants that provide ready-made meals must maintain the greatest levels of hygiene and sanitization, as well as cooking meat at a high enough temperature to destroy *T. gondii* cysts.

ACKNOWLEDGEMENT

The authors of this article would like to express their appreciation and gratitude to their colleagues in the Genetics Laboratory at the Islamic University of Gaza, particularly Prof. Fadel A. Sharif and Mr. Muhammad Ashour, for their tremendous efforts in completing the part related to the genotyping process in this research; without their efforts, the genotyping part of the research would not have been completed.

Funding

The study received no external funding.

Ethical statement

Ethical Research Committee of the Islamic University of Gaza, Gaza, Palestine approved all the procedures of this study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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