Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

The prevalence of *Toxoplasma gondii* in sheep carcasses with special reference to its sustainable control using mice bioassay

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ARTICLE INFO

Keywords

Toxoplasma gondii

Mice bioassay

Sheep carcass

Slaughterhouse

Zoonotic parasites

Received 10/01/2025
Accepted 21/02/2025
Available On-Line 01/04/2025

ABSTRACT

The study was conducted to estimate *Toxoplasma gondii* cyst prevalence in three hundred slaughtered sheep carcasses slaughtered in Benha city, Qalyubia governorate abattoirs. Sample types were divided depending on the rearing system of sheep to random grazing and closed rearing system. Tissue samples were collected from the muscles of the heart, diaphragm, and tongue of the examined sheep carcass. Results revealed that *T. gondii* was detected in higher prevalence in the samples collected from sheep of random grazing rearing system (39/150: 26.0%), than those of closed system rearing (23/150: 15.3%). Heart muscle revealed the highest prevalence of *T. gondii* (13.7%), followed by the diaphragm (5.0%) and tongue (2.0%) samples, respectively. Moreover, the effect of different thermal treatments on *T. gondii* cysts was assessed by feeding experimental-free mice on Toxoplasma-infected tissues that were previously exposed to chilling, boiling, or microwave cooking. It was proved that boiling and freezing had the potential to inactivate the infectivity of Toxoplasma cyst depending on the temperature degree and time of treatment; while, chilling, and microwave cooking treatments could not affect the infectivity of the cyst. Referring to the recorded results, and the independence of the expected hypothesis of statistical analyses, *T. gondii* was detected in a relatively higher prevalence than the expected prevalence for random grazing examined carcasses, while in lower prevalence in closed system reared carcasses. On the other hand, thermal treatments appeared to be effective in inactivating the infective cyst making the possible infected meat safe for human consumption.

1. INTRODUCTION

Toxoplasma gondii is a protozoan parasite of warm-blooded animals, including humans (Dubey, 1996). Humans become infected by ingesting food contaminated with feces from cats, which are the definitive host, or by ingesting undercooked meat from infected food animals such as sheep (Stelzer et al., 2019).

Toxoplasma gondii is a parasite with veterinary and medical importance that can be transmitted through food and water, making epidemiological surveys crucial for understanding and managing its spread (Almeria et al., 2021). These surveys provide data on the prevalence of T. gondii in both humans and animals, helping to identify potential sources and range of infection localization (Tenter et al., 2000). The information gleaned from these studies is essential for public health workers, informing suggestive planning for control strategies to reduce the risk of infection, especially through eating infected meat and meat products (Holec-Gąsior et al., 2013).

Toxoplasma gondii infection poses significant health hazards to the consumers causing toxoplasmosis, which is often asymptomatic but can cause severe complications in immunocompromised individuals (Mose et al., 2020). These individuals are at risk for life-threatening conditions like encephalitis (Wang et al., 2017); while, pregnant women are particularly vulnerable as the parasite can cross the placenta potentially resulting in pregnancy disturbances even miscarriage, stillbirth, or severe congenital disabilities in the newborn (Arranz-Solís et al., 2021). Some people may experience flu-like symptoms, such as fever, headache, sore throat, aching body, swollen glands, feeling tired, and feeling sick. More severe symptoms can include confusion, blurred vision, slurred speech, and unsteady walking (CDC, 2024).

Previous studies were conducted seeking inactivation of T. gondii tissue cysts, infective stage, and infectivity using high temperature, and concluded that cooking of contaminated meat to an internal core temperature up to 67ºC for 10 minutes was sufficient to kill tissue cysts in contaminated meat (El-Nawawi et al., 2008; Yang et al., 2020; Marín-García et al., 2022). Cysts of T. gondii can also be inactivated by low temperatures; when stored at -18°C, experimentally infected meat loses its infectious properties (Rani and Pradhan, 2021). Moreover, cats given -12°C stored contaminated minced beef for up to four days did not shed T. gondii oocysts, according to Mirza Alizadeh et al. (2018). On the other hand, using microwaves for rapid cooking as a common household practice has demonstrated that encysted *T. gondii* from infected sheep meat was still infective even after processing in a microwave oven (El-Nawawi *et al.*, 2008 and Zhang *et al.*, 2022).

The mouse bioassay is a critical method for identifying the activity of T. gondii cysts in various tissues (Opsteegh et al., 2020); that technique involves infecting laboratory mice with tissue samples suspected to contain T. gondii cysts, allowing researchers to observe the development of infection in the host. This approach not only confirms the presence

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of T. gondii but also provides insights into the infectivity and potential public health risks associated with consuming contaminated meat products. Therefore, the current study aimed to determine the prevalence of *T. gondii* in slaughtered sheep carcasses, followed by the thermal inactivating experiment of the tissue cyst incorporating mice bioassay for evaluating the virulence of Toxoplasma.

2. MATERIAL AND METHODS

The protocols of this study were approved by the Experimental Animal Welfare Ethics Committee of Animal Health Research Institute, Agriculture Research Center, Egypt (No: ARC-AHRI-93-24). Euthanasia was a part of the study design for the experimentally infected mice using CO_2.

1.1. Samples collection

The current study covered a period of four months starting from August to November 2024 in Qalyubia governorate, Egypt. Post-slaughtering, three samples from the heart, diaphragm, and tongue muscles of each of the 300 sheep carcasses that were slaughtered at Benha City Central slaughterhouse were collected, pooled, and prepared for examination. Sheep carcasses were categorized according to their rearing system to random grazing and closed system rearing systems (150 of each).

It is worth noting that sample size calculation was determined using the Biomath website (http://www.biomath.info/power/prt.htm) following the resource equation method.

2.2. Preparation and examination of muscle samples

All of the collected samples were crushed and homogenized using a pestle and mortar. With certain modifications (Dubey's, 1998), 10 grams were extracted after mixing, and a concentration process using an acid pepsin digestion procedure was employed (Bayarri et al., 2010).

In a mortar, ten grams of each muscle sample was ground up and mixed with twenty milliliters of 0.9% NaCl. Then, 20 milliliters of pepsin solution (pH 1.1–1.2) were added to the mixture. The samples were stirred at 37°C for 30 minutes. To neutralize the pepsin and stop the parasite from being destroyed, the contents were filtered, centrifuged, and then mixed with 25 mL of 1.2% sodium bicarbonate (NaHCO3) solution (pH = 8.3). Ultimately, the materials were reconstituted in 2 milliliters of PBS and two PBS washes were done. After processing the samples were kept in clean disinfected tubes. The extracts were examined under the light microscope (×1000) aiming for detection of *T. gondii* tissue cyst.

2.3. Thermal processing of positive muscle samples

2.3.1. Experimental design

Positive *T. gondii* infected meat samples, from different carcasses, were grouped into ten groups based on the type of thermal treatment as follows: G1: Control positive without thermal treatment, G2: infected muscle samples chilled at 4ºc for one week, G3: infected muscle samples chilled at 4ºc for two weeks, G4: infected muscle samples were frozen at -10ºc for three days., G5: infected muscle samples were frozen at -20ºc for three days, G6: infected muscle samples were boiled at 100ºc for five minutes, G7: infected muscle samples were boiled at 100ºc for ten minutes, G8: infected muscle samples were thermally treated with moderate-power microwave treatment for five minutes, G9: infected muscle samples were thermally treated with high-power microwave treatment for five minutes, and G10: Control negative (collected from *T. gondii*-negative carcass).

2.3.2. Experimental infection of laboratory mice

For this experiment, thirty Swiss mice, 40 days of age, weighing between 100 to 150 g, were obtained from a local supplier in Egypt and divided into ten groups (3 mice per group). Four Kg and 50 g of infected meat were collected from different infected carcasses and grouped according to the thermal treatment. A 50 g portion of the grouped muscle samples was given orally to the experimental mice, after thermal treatment, in three meals to infect them (Kotula et al., 1983). For 120 days after infection, the experimental mice were given regular *ad libitum* food and drink.

2.3.3. Detection of T. gondii in the experimental mice

After 120 days post-infection (El-Nawawi et al., 2008), experimental mice were euthanized using carbon dioxide following the American Veterinary Medical Association (AVMA) guidelines. Cardiac muscle was collected and treated as previously described according to Dubey (1998), with some modifications (Bayarri et al., 2010), followed by light-microscopic examination (400 µm scale bar) for detection of *T. gondii* tissue cyst.

2.4. Statistical analysis

The significance between the examined samples in relation to the rearing system was assessed using descriptive statistics (represented by the calculation of positive and negative ratios), the chi-square test for the calculation of the expected prevalence hypothesis, and a comparison of nominal data with respect to the rearing system of the examined carcasses using SPSS software 20.0th version.

3. RESULTS

Referring to the recorded data in Table (1), the statistical hypothesis of positive prevalence in comparison with the current obtained results of the present study revealed relatively higher positive detection of *T. gondii* cyst in the random-grazing examined sheep carcasses; while it came in lower correlation in the closed system reared examined carcasses.

Referring to the recorded results in Table 2, *T. gondii* was detected in higher prevalence in the samples collected from sheep of random grazing rearing system (39/150; 26.0%) than those of closed system rearing (23/150: 15. 3%). Heart muscle was found to be more infected by *T. gondii* followed by diaphragm (5%) and tongue samples (2%).

Table (1). Statistical hypothesis and current prevalence in different examined samples of different carcass rearing system

Groups	prevalence	Heart	Diaphragm	Tongue
Random-grazing reared group	Study prevalence	26	9	4
	Expected prevalence	20.5	7.5	3.0
Closed-system rearing group	Study prevalence	15	6	2
	Expected prevalence	20.5	7.5	3.0

Table (2). Prevalence of *T. gondii* in the examined sheep meat samples (n=150)

Type of rearing	No. of examined carcasses	Heart		Diaphragm		Tongue		Total	
		No.	%	No.	%	No.	%	No.	%
Random grazing system	150	26	17.3	9	6.0	4	2.7	39	26.0
Closed rearing system	150	15	10.0	6	4.0	2	1.3	23	15.3
Total	300	41	13.7	15	5.0	6	2.0	62	20.7

Regarding the statistical analyses for obtaining the correlation between the rearing system of the examined carcasses, Table (3) showed that there was no significant difference between the examined samples in relation to the rearing system.

Table (3). Statistical analyses of the obtained results in relation to the rearing system

	Heart	Diaphragm	Tongue
X^2 value	3.418	0.632	0.680
df	1	1	1
P value	0.064	0.427	0.409

X^2: chi-square value, df: Degree of freedom, *P* value: value lower than 0.05 was considered significantly different

Under light microscope, *T. gondii* tissue cyst appeared thin-wall, oval in shape; where, many crescent-shaped organisms appeared within the cyst represented the bradyzoites with granular matrix filled the space between them (Fig., 1).

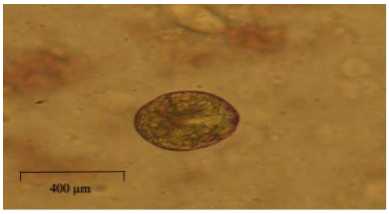

Fig. (1). Toxoplasma gondii tissue cyst in infected sheep muscles (x1000)

The assessment of the infectivity of *T. gondii* cysts to experimental mice after the cysts' exposure to chilling, freezing, or boiling, verified that boiling of meat samples, at 100 ^{O}C for 10 minutes and freezing at -10 and -20^{O}C for 3 days had the potential to inactivate the tissue cyst; while, chilling for 4^{O}C for 1-2 weeks, and different powers of microwave cooking treatments for 5 minutes didn't affect the infectivity of Toxoplasma cyst (Table 4).

Table (4). Effect of meat thermal processing on the infectivity of *Toxoplasma gondii* tissue cysts in muscles of infected sheep indicated by feeding of experimental mice on the treated infected tissues

Method	Degree	Time	Infectivity of *T. gondii* tissue cyst
Chilling	4^{O}C	One week	Not affected
		Two weeks	Not affected
Freezing	-10 ^{O}C	3 days	Affected
	-20^{O}C	3 days	Affected
Boiling	100 ^{O}C	5 minutes	Not affected
		10 minutes	Affected
Microwave cooking	Medium power	5 minutes	Not affected
	High power	5 minutes	Not affected

4. DISCUSSION

Toxoplasmosis is a disease caused by a known protozoan called *Toxoplasma gondii*, which is able to infect a wide range of warm-blooded animals, including humans (Brito et al., 2023). The three infectious phases of *T. gondii* in humans and other warm-blooded animals are tachyzoites, bradyzoites, and sporozoites (FAO, 2004). Bradyzoites or oocysts are the primary means of infection for both humans and animals (Al-Malki, 2021).

Food handlers who work directly or indirectly with the production, preparation, processing, and distribution of foods across communities such as farmers, vendors, butchers, or housewives are typically shown to be the most susceptible hosts to human toxoplasmosis infection (Slany et al., 2019). In sheep, infection often occurs through the eating of oocysts present in contaminated feed or water. The parasite can cause significant reproductive issues, including abortion and stillbirths, posing economic challenges for livestock producers (Holec-Gąsior and Sołowińska, 2023).

Referring to the recorded results in this current study, Al-Kappany et al. (2018) recorded that the sheep carcasses that were slaughtered at Cairo abattoirs revealed a lower prevalence (4.1%), than those of Dakahlia, Giza, and Sharkia governorates (26%, 23% and 12%, respectively). On the other hand, in El Fayoum province, Nageib and Mohamed (2021) recorded a higher prevalence of *T. gondii* cysts (67.7%) in sheep meat samples. Furthermore, *T. gondii* cyst was previously encountered in the tissue of slaughtered sheep (14.4%, 24.5%) in Iran and Spain; respectively (Bahreh et al., 2021; Peris et al., 2024).

The collected samples in this study showed a higher prevalence of *T. gondii* cyst in cardiac muscles compared to diaphragm and tongue samples. This came in agreement with the recorded findings of Bahreh et al. (2021) who found *T. gondii* in 11.1 and 12.2% of the examined diaphragmatic and cardiac muscle samples. The variation in the recorded results may be attributed to the variation in the geographical location of sheep, the system of sheep rearing, the type of examined tissues, the season of the study, and the cyst detection technique (Al-Kappany et al., 2018). The current results proved that the collected samples from randomly grazed sheep carcasses revealed a higher prevalence of *T. gondii* tissue cysts than those reared in the closed-rearing system. This may be ascribed to the higher chance of contracting *T. gondii* oocysts from contaminated grass with feline feces as it was proven that *T. gondii* oocysts can survive in soil, contaminated vegetables, water, and soil for up to two years (Hussain et al., 2017). On the contrary, the higher sanitation and clean feeding applied in the closed rearing system (Paștiu et al., 2023) inhibits *T. gondii* infection in sheep.

Regarding the assessment of the infectivity of T. *gondii* cysts to experimental mice after the cysts' exposure to chilling, freezing, or boiling, it was noted that freezing for up to 3 days and boiling for up to 5 and 10 minutes had the potential to inactivate the infectivity of *T. gondii* tissue cyst and the mice appeared cyst free upon examination for 120 days. In this respect, these results agreed with those of El-Nawawi et al. (2008) who demonstrated that heating of meat to 100°C for 10 minutes effectively killed *T. gondii* cysts, with no viable infective stages isolated from treated samples. Generally, it was proven that meat and meat products' shelf life can be significantly increased by heat processing which reduces or eliminates infective stages of the zoonotic parasites (Mirza Alizadeh et al., 2018; Lorenzo et al., 2018; Munekata et al., 2021). Also, extreme heat can kill or inactivate T. gondii oocysts of both sporulated and unsporulated strains (El-Nawawi et al., 2008; Mirza Alizadeh et al., 2018; Pinto-Ferreira et al., 2021; Arranz-Solís et al., 2023). On the other side, Mirza Alizadeh et al. (2018) reported that freezing at -20°C for 2 days was sufficient to inactivate *T. gondii* tissue cysts. The longer freezing applied in our study highlighted that longer freezing durations for 3 days, further ensured the inactivation of the parasite. This agreed with previous researchers (Opsteegh et al., 2020, El-Nawawi et al., 2008; Hill et al., 2018; and Mirza Alizadeh et al., 2018) who ensured that freezing meat at -20°C for 3 days effectively rendered *T. gondii* cysts non-infectious.

The cause of the inactivation of *T. gondii* cysts in meat through boiling and freezing involves specific mechanisms that disrupt the viability of the parasite. Boiling meat at high temperatures (around 100°C) leads to the denaturation of proteins within the *T. gondii* cysts. This process disrupts the structural integrity of the cyst wall and the proteins necessary for the parasite's survival, effectively killing the cysts (Pinto-Ferreira et al., 2021); besides that, even heat

penetration may agglutinate the inter-cystic fluids lead to its death (Mirza Alizadeh et al., 2018). Moreover, freezing induces cryoinjury to *T. gondii* cysts. When meat is frozen at temperatures around -20°C, ice crystals form within and outside the cysts, causing physical damage to their cellular structure. This mechanical disruption can lead to leakage of cellular contents and eventual death of the parasite (Hill et al., 2018).

In the current study, chilling of *T. gondii* cysts for up to 2 weeks and microwave cooking for up to 5 minutes did not affect the infectivity of *T. gondii* cysts. These results came in agreement with Tenter (2009) who demonstrated that *T. gondii* cysts could survive in refrigerated conditions (1-4°C) for up to three weeks, indicating a significant resilience to low-temperature storage. Additionally, Lundén and Uggla (1992) and Mirza Alizadeh *et al.* (2018) found that microwave cooking might not uniformly heat meat, leading to the survival of *T. gondii* cysts if the meat is not heated evenly throughout. This uneven cooking can result in some areas of the meat remaining below effective killing temperatures, thereby allowing for the potential survival of the parasite.

5. CONCLUSIONS

In the current study, *T. gondii* was detected in relatively higher than the expected prevalence for random grazing examined carcasses, while in lower prevalence in closed system reared carcasses. The prevalence of *T. gondii* was significantly higher in the random grazing reared sheep than those who were reared in a closed rearing system. Cardiac muscle samples revealed a higher prevalence of *T. gondii* cyst than diaphragm and tongue samples. Thermal treatments revealed the potential ability to inactivate the infectivity potency of *T. gondii* tissue cyst especially when boiling and freezing were applied. On the other hand, microwave cooking and refrigeration could not affect the cyst infectivity. So, thorough cooking and even heat treatment are recommended for safe meat meal processing.

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