Molecular evidence of *Toxoplasma gondii* from the tissue and blood of naturally infected sheep

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Abstract

Toxoplasmosis is a cosmopolitan zoonotic infection that has significant effects on public health and causes economic losses in the livestock industry. The current study was designed to detect the *Toxoplasma* parasite in sheep blood samples and tissue samples of slaughtered sheep at the Sulaimani abattoir using molecular technique. A total of 300 peripheral sheep blood samples were randomly collected from 20 small ruminant flocks at 4 locations in the Sulaymaniyah province, northern Iraq. Also, 150 meat samples from thigh muscle, heart, and diaphragm were collected from slaughtered sheep. All collected blood samples were subjected to polymerase chain reaction (PCR) amplification to confirm *Toxoplasma* infection; in addition, meat samples were also analyzed for *Toxoplasma* by PCR following the digestion process. Of the 300 amplified blood samples, 94 were considered positive for *Toxoplasma gondii*, with a prevalence rate of 31.3%. The overall prevalence of *Toxoplasma* among meat samples was 34%. The diaphragm reported a higher infection rate (46%) than the heart (32%), while the femoral muscle reported an infection rate of 24%. Aged animals (older than 24 months) presented a higher infection rate (32.8%) than younger animals (28.9%). Contact with or consumption of uncooked meat from infected sheep increases the chance of parasite transmission to humans.

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Introduction

Toxoplasmosis is caused by *Toxoplasma gondii* (a coccidian parasite belonging to the phylum Apicomplexa). *T. gondii* can infect almost all warm-blood animals as well as humans (Amdouni *et al*. 2017). Toxoplasmosis was stated as the most prevalent parasite-origin zoonotic disease worldwide (Tenter *et al.,* 2000). It was reported from all continents, with various infection rates. Cats in particular are a definitive host for the parasite, and other infected animals and humans serve as intermediate hosts (Dubey, 2010). Several millions of resistant oocysts are expelled by the definitive host during the early stages of infection (Opsteegh *et al.,* 2010). The sporulation of oocysts occurs over 1-5 days under natural environmental conditions (Motarjemi *et al*., 2014).

T. gondii may transmit either horizontally or vertically. During vertical transmission, tachyzoites infect the fetus from the mother through the placenta and cause congenital toxoplasmosis. Horizontal infection occurs after eating meat (or organs) from an infected intermediate host that harbors the tissue cysts (Guo *et al*., 2015) or through the ingestion of sporulated oocyst shed in cats' feces from soil, water, and plants (Motarjemi *et al*., 2014). Toxoplasmosis results in significant financial losses in the sheep industry on a global scale due to abortions and other reproductive failures (Ferreira Da Silva *et al*., 2013).

Risk factors for infection by *T. gondii* include outdoor admittance following exposure to soil and water and animal feed kept in a location where cat excrement might be present (Klun *et al.,* 2006).

Livestock animals, as sources of meat for human food, serve as a reservoir for *T. gondii* (Boyer *et al*., 2005). The main source of parasite infection in humans is raw or undercooked meat from slaughtered animals (Sroka *et al*., 2020).

Infection in humans is mostly asymptomatic; however, congenital toxoplasmosis might be associated with serious complications such as abortion, hydrocephalus, chronic ocular disease, lymphadenopathy, and death in newborns. Moreover, retinitis and

encephalitis might occur in immunosuppressed patients (Hill and Dubey, 2002). In ruminants, abortion and stillbirth could occur as a consequence of toxoplasmosis, which accompanies significant economic losses in the animal industry (Cenci-Goga *et al*., 2011).

Toxoplasma-infected sheep are thought to be a primary parasite infection source for both humans and predatory animals (Dubey, 2009). To diagnose toxoplasmosis, various serological and molecular methods have been employed in both humans and animals worldwide (Armand *et al*., 2016).

Raw or undercooked meat from warm-blooded animals, including sheep and goats, allows the infection to enter the human food chain. Because the parasite stages are visible only under a microscope, they are not detected during meat inspection (Shapiro *et al*., 2019). The impossibility of detecting *Toxoplasma* infection from contaminated carcasses by visual inspection in the slaughterhouse necessitates the application of various laboratory methods. The molecular methods offer the advantages of prominent sensitivity and high specificity with high speed in diagnosis (Anvari *et al.,* 2018).

Polymerase chain reaction (PCR)-based techniques have been applied for the identification of *Toxoplasma* DNA in meat and meat-producing animals (Guo *et al*., 2015). The parasite appears to be easier to recognize with the *Toxoplasma* 529-bp fragment (Yang *et al*. 2009). Various studies determined *Toxoplasma* seropositivity in small ruminants, while its molecular estimation from the blood and tissue of food animals was not well defined. So, the current study aimed to find out the infection rate of toxoplasmosis in sheep, and besides that, it tried to clarify the *Toxoplasma*'s existence in meat samples of slaughtered sheep, which are used as a main source of food in the study area.

Materials and Methods

Animals and sample collection

A total of 300 sheep were selected randomly from 20 small ruminant flocks from the Sulaymaniyah province for the collection of blood samples between June and December 2021. The selected farms belonged to four regions, and animals were reared under a semi-intensive rearing system, with traditional management.

Approximately 5 mL of blood was drawn from the jugular vein of each animal and collected in a tube with an anticoagulant. Samples were transported in cold condition to the laboratory at the College of Veterinary Medicine of Sulaimani University and stored at -80°C until DNA extraction.

Tissue samples from the heart, diaphragm, and femoral muscle were collected from slaughtered sheep in the Sulaimani abattoir. Samples were randomly selected among the slaughtered animals; approximately 10 g of tissue samples were collected from 150 sheep carcasses using sterile disposable blades and transferred to plastic bags. Then, they were transferred under cold condition to the laboratory and stored at 4°C until performing tissue digestion procedures over 2-4 days.

DNA extraction

DNA extraction was performed for all blood samples collected using Ge Net Bio Kit (Daejeon, South Korea). According to the manufacturer's instruction protocol, extracted DNA aliquots were stored at -80°C until PCR assay. All meat tissue samples were digested with pepsin solution according to the method described by Dubey and Beattie (1988). Approximately 5 g of each tissue sample was cut with a sterile scalpel and mixed with 50 mL of acid pepsin solution composed of 2.6 gm of pepsin, 7 mL of HCl, 0.9% of NaCl and filled up with distilled water to 500 mL with a pH 1.1-1.2. Samples were left for digestion at room temperature for 90 minutes. Digested materials were filtered through gauze and centrifuged at 1200×g for 10 minutes. Pellets were collected and resuspended in 10 mL of phosphate buffer saline (pH 7.4), again centrifuged at 1200×g for 10 minutes. The supernatant was removed, and the sediment was transferred to a sterile Eppendorf, resuspended in 1 mL of normal saline, and stored at -80°C for DNA extraction in the next step. Genomic DNA was extracted from tissue suspension samples using the extraction kit from Trans Gen Biotech (Beijing, China). A volume of 0.5 mL from the homogenate was used for DNA extraction and suspended in 200 µL lysis buffer in a 1.5 microcentrifuge tube; 20 µL of proteinase K was added and followed the extraction procedures. All DNA aliquots were stored at -80 ºC for performing PCR reaction.

Polymerase chain reaction assay

All extracted DNA samples were subjected to PCR assay by applying previously used primer sets TOX4 (5'CGCT-GCAGGGAGGAAGACGAAAGTTG3') and TOX5 (5'CGCT-GCAGACACAGTGCATCTGG ATT3'), which amplified a nearly 530 bp fragment of the *T. gondii* genome (Homan *et al*., 2000).

PCR was carried out in a programmable Thermal Cycler (Prime, UK) and ran out in a 20 µL reaction volume consisting of 10 µL of master mix from (Ge Net Bio, Daejeon, South Korea), 5 µL of sample genomic DNA with 1 µL from each Sens and anti-Sens primers, and the volume was completed with double-deionized water. The amplification conditions were the following: initial denaturation at 93°C for 5 minutes, followed by 30 cycles of denaturation at 93°C for 30 seconds, annealing at 55ºC for 30 seconds, and extension at 72ºC for 30 seconds, the reaction then completed by a final extension at 72°C for 5 minutes. After amplification, DNA fragments were analyzed by electrophoresis and identified on a 1.5% agarose gel, stained with ethidium bromide under ultraviolet illumination.

Data analysis

A Chi-square test ($\chi 2$) of significance was used for data comparison using the SPSS package (V22) (IBM, Armonk, NY, USA).

Results

The overall infection rate of *T. gondii* among the examined sheep was 31.3%. Although toxoplasmosis was estimated in slaughtered sheep meat samples at 34%. According to the selected regions, a higher prevalence of toxoplasmosis was reported in Sharazoor (34.8%) and a lower prevalence in Said Sadiq (27.4%). Tissue-wise prevalence represents a higher prevalence rate in the diaphragm (46%) and a lower one (24%) in the thigh muscle. Regarding the frequency of toxoplasmosis in selected age groups, a higher infection rate of 32.8% was found among animals older than 24 months rather than younger animals, with no statistically significant association ($p>0.05$), as shown in Table 1.

Discussion

Small ruminants' meat, especially lamb and mutton, was considered the major meat source consumed in the study area. The current study data revealed the moderate existence of toxoplasmosis

among sheep. Consuming raw meat or animal products that contain tachyzoites or bradyzoites continues to be the principal way that *Toxoplasma* is transmitted to humans (Dubey, 2009).

By molecular findings using PCR, the prevalence of toxoplasmosis among sheep was revealed to be 31.3% in Sulaymaniyah province (northern region of Iraq); similarly, toxoplasmosis has been reported from sheep with various prevalence rates: 21.7% (Mikaeel and Al-Saeed, 2020), a low prevalence of 8.75%, and 9% was reported from other regions of Iraq by Al-Shaibani *et al.* (2019) and Al-Abodi (2021), respectively.

The current study data also highlight the existence of the *Toxoplasma* parasite in meat samples of slaughtered sheep at the Sulaimani abattoir. The molecular findings by PCR revealed infection in 34% of tissue samples collected from the heart, diaphragm, and thigh muscle. In agreement with current data, higher prevalence rates of 52.5% and 34.32% were reported by Kareshk *et al.* (2017) and Armand *et al.* (2016), respectively. Contrary to the present data, a low prevalence of 14.4% and 14.6% was found by Bahreh *et al.* (2021) and Amouei *et al*. (2022) respectively.

Regarding toxoplasmosis prevalence rates in different regions, a higher infection rate was reported in Sharazoor at 34.8%, followed by 32.4% in Sitak and 31.2% in Bazian, with a lower reported rate of 27.4% in Said Sadiq, although no significant association was detected statistically. Such variation might relate to the management systems applied on different farms regarding hygienic conditions and the availability of cats. The degree of pasture environment contaminated by oocysts, the physiology, and the health status of the animals might affect the frequency of *Toxoplasma* positivity.

Among the tissue samples, a higher prevalence was demonstrated from the diaphragm (44%), followed by the heart (32%), and a lower level (24%) was stated from the femoral muscle. Similar to current findings, in the study by Firouzeh *et al*. (2021), a higher detected infection rate was found in the diaphragm (47.8%) than in the heart tissues (26.1%) of slaughtered sheep. Other related studies reported various prevalence rates of *Toxoplasma* existence from different tissue samples. In a study performed by Yousefvand *et al.* (2021), a higher infection rate of 32% was demonstrated in the heart, followed by 22% in muscle tissue and 17.3% in the liver of slaughtered sheep, which is in harmony with the present findings. Furthermore, different from the current data, lower infection rates of 17.8% from the heart (Rasti *et al*., 2018) and 11.1% from the diaphragm (Bahreh *et al*., 2021), with a higher infection rate of 28% from the femoral muscle (Azizi *et al*., 2014), were reported previously. The higher incidence of toxoplasmosis could be due to animals' lifetime exposure to the parasite on a regular basis (Verma *et al.,* 2017).

Various factors can influence the parasitic invasion of different tissues of infected hosts. It was found that parasite distribution in the various tissues of infected hosts may change depending on the length of the infection. At the beginning of infection, parasites load at a higher rate in the brain, liver, and blood. Thereafter, it gradually grows in the heart and skeletal muscles (Azizi *et al*., 2014; Verma *et al.*, 2017). The strain of the parasite or the infection stage (oocysts or bradyzoites) may have an impact on the variation in the location of parasitic cysts in the various tissues of the host carcass (Swierzy *et al*., 2014).

The study data presented a variation in *Toxoplasma* infection rate according to the age groups of selected animals. 32.8% were reported from the >24 months age group, and 28.9% were reported from ≤24 months animals; however, the difference was not significant. In accordance with the study findings, a higher prevalence of toxoplasmosis in adult sheep than in younger animals (less than 2 years) was found by Abdallah *et al.* (2019) and Gebremedhin *et al*. (2014). Previous data presented a significant relation between toxoplasmosis and the age of infected hosts; the higher incidence rate among adults than young animals could be due to the further exposure of animals to contaminated environments during their lives. Animals mostly acquire *Toxoplasma* infection *via* ingestion of oocysts from soil and water that they shed by domestic or feral cats (Tonouhewa *et al*., 2017).

Livestock animal exposure to *Toxoplasma* has been linked to several risk factors, including type of farm, food supply, existence of cats, quality of water, and carcass handling techniques (Guo *et al.,* 2015). Moreover, traditional husbandry and pasture feeding significantly increase the risk of toxoplasmosis occurrence (Anvari *et al.*, 2018). Livestock production is widely dispersed in the study regions, and semi-extensive production is the main farming system for small ruminants.

Cattle and sheep are more susceptible to contracting feline oocyst infections due to feeding practices and environmental hygiene. In outdoor management systems, livestock animals have frequently become infected due to excessive levels of parasitic oocysts in the environment brought on by infected stray cats that were released on poorly maintained farms (Abd El-Razik *et al.,* 2018).

Due to the ability of an infected cat to shed a large number of oocysts, it is probable that a few cats are sufficient for contaminating a wide field area in a short period of time, since millions of oocysts can be released by a single infected cat (Tilahun *et al*.,

Table 1. Frequency of *Toxoplasma* infection in blood and tissue samples of sheep.

Parameters	Determinants	Total examined No.	No. of positive	Prevalence %	Chi-square value [p]
Sample	Blood	300	94	31.3	0.325
	Tissue	150	51	34	[0.5]
Regions	Bazian	77	24	31.2	0.860
	Said Sadiq	73	20	27.4	[0.8]
	Sharazoor	79	27	34.8	
	Sitak	71	23	32.4	
Tissues	Heart	50	16	32	5.525
	Diaphragm	50	23	46	[0.6]
	Muscle	50	12	24	
Age	≤24 months	114	33	28.9	0.486
	>24 months	186	61	32.8	[0.4]

2018). *Toxoplasma*-infected animals' placentas and aborted fetuses should be disposed of properly to prevent the parasite's life cycle from continuing and resulting in the shedding of additional oocysts. This increases the risk that cats will become infected by eating these items (Hamilton *et al*., 2014).

The possible threat to human health becomes evident in areas where small ruminants have a high infection rate (Gebremedhin *et al*., 2014). Consumption of undercooked meat from infected sheep plays a significant role in human infection (Kijlstra and Jongert, 2008). It was verified that *Toxoplasma* tissue cyst is extremely strong, maintains its viability at temperatures of 1-4°C for weeks, and requires a temperature over 67°C or below 12°C for significant loss of viability (Kotula *et al*., 1991).

Typically, undercooked lamb meat is considered a significant source of infection. However, the meat of adult sheep is frequently well-cooked, which likely reduces the risk of infection for customers (Kijlstra and Jongert, 2008). In addition, the recent rise in consumption of barbecued meat (kebab), which is made from the meat of adult animals, represented a significant risk of a higher infection rate (Armand *et al*., 2016). *Toxoplasma* infection rates in animals can help estimate the frequency of toxoplasmosis in humans (Tonouhewa *et al*., 2017).

The free-ranging production practices used for small ruminant rearing make it difficult to control the disease, but education and training can readily reduce infection among humans, which is crucial for pregnant women and those with immune system disorders (Gorji *et al*., 2018).

Conclusions

According to data from the current investigation, toxoplasmosis affects sheep broadly in different regions of the study area, which is considered a cause of economic impairment in the livestock industry. In addition, the existence of *Toxoplasma* has been confirmed from meat and tissue samples of slaughtered sheep from the Sulaimani abattoir at a moderate rate. Due to the involvement of sheep in the transmission of the parasite to humans through the consumption of meat and meat products, it might be likely that infected sheep become a significant source of *T. gondii* infection among individuals who belong to the studied area. The zoonotic risk aspect of *Toxoplasma* infection for human health is clear, especially for pregnant and immunodeficient people. However, frozen meat might reduce the hazard of parasite transmission. Moreover, control of toxoplasmosis in farm animals may result in a decline in the human infection rate.

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