Research Article

The relationship between seropositivity and tissue cysts in sheep naturally infected with Toxoplasma gondii*

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Abstract: Skeletal muscles (tongue, masseter, leg, intercostal, and diaphragmatic muscles) and brain samples of 100 sheep at slaughter were analysed for the presence of *T. gondii* tissue cysts along with serum IgG titres. Two methods of isolation by percoll gradient centrifugation and tissue microarray (TMA) technique with immunoperoxidase staining were used. Seropositivity was detected in 88% (88/100) of sheep sera analysed by indirect fluorescent antibody test. Tissue cysts were observed in 46 (52.3%, 46/88) and 15 (17%, 15/88) of the seropositive sheep with the isolation technique and TMA and immunoperoxidase staining, respectively. The diameters of the tissue cysts were 25–58 × 25–62 (mean 34 × 36) μm. The relationship between the presence of tissue cysts and seropositivity in sheep was statistically significant at 1/16 (P < 0.01) and at 1/64 and 1/128 (P < 0.001) serum dilutions.

Key words: *Toxoplasma gondii*, tissue cyst, percoll gradient centrifugation, tissue microarray, serology, sheep, Turkey

1. Introduction

The only species in the family Toxoplasmatidae, *Toxoplasma gondii*, is one of the most prevalent apicomplexan parasites found in various animal species and humans (1). Members of Felidae, especially cats, serve as the final hosts of the parasite, while humans and warm-blooded animals, including cats, play a role as intermediate hosts (1,2). It was estimated that about one third of the people worldwide are exposed to *T. gondii* (2).

T. gondii has 3 consecutive infectious stages: tachyzoite, bradyzoite, and sporozoite (1,2). After infection, during the chronic phase of toxoplasmosis, tissue cysts can develop in different tissues of the hosts (1–3). Tissue cysts of *T. gondii* are about 5–70 μm in diameter, and harbour a number of bradyzoites that are resistant to gastric digestion and responsible for foodborne transmission to the intermediate hosts (1,2,4). Tissue cysts usually remain intact throughout the hosts' life (3,5). However, the exact factors affecting the form of tissue cysts are unknown and in some instances, as the tissue cyst ages, degeneration of bradyzoites may

occur (3). People are infected by consumption of raw or undercooked various animal tissues harbouring the tissue cysts (6). Nearly 50% of all human toxoplasmosis cases are related to foodborne transmission (7) and the meat of sheep is highly implicated in southern European countries (8).

Diagnosis of toxoplasmosis is generally based on serological and histological analysis (9) and serology is preferred in live hosts (1,9). Most farm animals are seropositive for *T. gondii* and serological studies of widespread *T. gondii* infection in sheep have been reported (10–13). Seropositivity shows whether a host is infected with *T. gondii*, but it fails to determine the presence of infectious tissue cysts. Thus, understanding the relation of seropositivity with presence of tissue cysts in meat originating from seropositive sheep offered for human consumption has extreme importance for the epidemiology of toxoplasmosis in humans. With the present study, we aimed to determine the relationship between seropositivity and presence of tissue cysts in the skeletal muscles and brains of sheep.

2. Materials and methods

2.1. Collection of tissue samples

Sampling was carried out in slaughterhouses in Kırıkkale Province, located in the Central Anatolia region of Turkey, between March and September 2011, with consecutive visits. With each visit in consecutive weeks, up to 5 sheep that showed no signs of clinical disease in the antemortem inspection were picked for randomisation of sampling. Blood samples prior to slaughter were collected from 100 sheep (77 female and 23 male), most of them of Akkaraman breed (n: 97), into centrifuge tubes without anticoagulant. After slaughter, the brain and skeletal muscles (tongue, diaphragm, masseter, leg, and intercostal muscles) of sheep were sampled into separate sterile bags. The present study was approved by the Ethical Committee of Animal Research of Kırıkkale University (No: 10/02-10).

2.2. Percoll gradient centrifugation

Percoll gradient centrifugation is a method capable of purifying *T. gondii* tissue cysts from tissues (14,15). Tissue cysts were isolated according to Eggleston et al. (16). Briefly, aliquots of 5 g of tissue samples from each tissue (muscles and brains) were cut with sterile scissors and added to 20 mL of PBS. Then the samples were homogenised using a high speed tissue homogeniser (OMNI Tip, USA) that was washed in boiled water before every consecutive homogenisation. The homogenates were sieved in a centrifuge tube using separate cheesecloths. Percoll stock solution was diluted to 90% and 30% with distilled water and NaCl. Percoll dilutions and the homogenates were centrifuged at $4000 \times g$ for 20 min. Following centrifugation, percoll layers were taken on slides using a Pasteur pipette and analysed for presence of tissue cysts by a binocular light microscope (Olympus BX 50).

2.3. Tissue microarray (TMA) technique and immunoperoxidase staining

Tissue samples fixed in 4% buffered paraformaldehyde were dehydrated through degraded alcohol and xylene series, and were embedded in paraffin wax. Circular spots 2 mm in diameter from each tissue sample in paraffin wax were taken by the Tissue-Tek Quick-Ray System (Sakura Inc, USA) and re-embedded in arrayed master blocks with an automatic microarrayer (Integrated Biosystem, Galileo CK3500, Italy). Arrayed master blocks were sectioned at a thickness of 4–5 μm and stained with hematoxylin–eosin. An immunoperoxidase test (LSAB 2 System, HRP, Daco Cytomation, Denmark) was used to demonstrate the presence of *T. gondii* antigen in paraffin-embedded sections. Following the deparaffinisation in xylene and rehydration in alcohol series, the tissue sections were treated with proteinase K for 7 min. Endogenous peroxidase activity was quenched in 0.3% hydrogen peroxide in methanol and nonspecific staining was reduced by application of normal goat serum. Then tissues were incubated with goat anti-*T. gondii* specific polyclonal antibody (VMRD Inc., Pullman, USA) for 60 min in a humidity chamber. Finally, a biotinylated antigoat secondary antibody (Invitrogen, USA) and AEC chromogen (Labvision Corp., Fremont, CA, USA) were applied. Immunoperoxidase staining results were evaluated using a binocular light microscope (Olympus BX 50, Japan).

2.4. *Toxoplasma gondii* genomic DNA isolation and nested PCR protocol

For genomic DNA isolation a Chelex resin based technique was used. Tissue cysts in percoll dilutions were centrifuged at $3000 \times g$ for 10 min (Hettich, Universal 32R, Tuttlingen, Germany). Sediments were taken into microcentrifuge tubes and washed 3 times with ultrapure water for removal of the percoll dilutions. The sediments were digested in 200 μL of Chelex-100 (Bio-Rad, Hercules, CA, USA) and 2 μL of proteinase K (70 mg/mL, AppliChem GmbH, Darmstadt, Germany) at 56 °C overnight and held in a Thermo-shaker (1500 rpm, ALLS, Msc-100, China) at 95 °C for 10 min for inactivation of proteinase K. Following centrifugation (Beckman Coulter 22R Centrifuge, Fullerton, CA, USA) at $12,000 \times g$ for 10 min, supernatants were used as template DNA.

Presence of *T. gondii* tissue cysts was confirmed by nested PCR. While the first round targeted the 197 bp part of the B1 gene of *T. gondii* using Toxo 1 for: 5'-GGAACTGCATCCGTTCATGAG-3' and Toxo 2 rev: 5'-TCTTTAAAGAGTTCGTGGTC-3' primers, the second round targeted a 97 bp part of previously amplified template DNA with Toxo 3 for: 3'-TGCATAGGTTGCCAGTCACTG-3' and Toxo 4 rev: 5'-GGCGACCAATCTGCGAATACACC-3' primers. Master mixes of both PCR reactions consisted of 1X PCR Buffer (100 mM Tris-HCl [pH 8.8 at 25 °C], 500 mM KCl, 0.8% Nonidet P40), 2 mM $MgCl_2$, 10 pmol of each primer, 200 μM of each dNTP, 2 U of *Taq* DNA polymerase (Fermentas, EP0402), and 10 μL and 2 μL of template for each round, respectively. Amplifications were carried out in a thermal cycler (Eppendorf Mastercycler Gradient, Hamburg, Germany) and the PCR conditions consisted of initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C (56 °C for the second round) for 30 s, and elongation at 72 °C for 60 s, with a final elongation at 72 °C for 7 min. In every reaction, DNA from tachyzoites of *T. gondii* RH Ankara strain was included as a positive control and ultrapure water as a negative control.

A 10-μL aliquot of each resultant PCR product with 2 μL of 6X loading dye (Promega, Madison, USA) was further analysed by agarose gel (1.5% Agarose-Basica LE, Prona, Spain) electrophoresis (CSL MSMixi-Duo, Corston, UK), stained with 0.1 μg/mL ethidium bromide (BioChemica GmbH, Darmstadt, Germany) at 120 V for 30 min, and visualised by a gel documentation and analysis system (Syngene InGenius, Cambridge, UK).

2.5. Examination of blood sera by indirect fluorescent antibody test (IFAT) and Sabin–Feldman dye test (SFDT)

Serum samples were examined with IFAT and SFDT to determine the presence of *T. gondii* specific IgG. For preparation of IFA test slides, whole *T. gondii* tachyzoite preparations were placed into each well of slides and allowed to air dry. Serum PBS dilutions starting from 1/16 were added to wells along with a positive and negative control serum and incubated at room temperature for 30 min. Slides washed twice in PBS and stained with antisheep IgG conjugated to FITC (Sigma) that was diluted 1/500 in 0.2% filtered Evans blue dye in PBS were incubated at room temperature for 30 min. Stained slides were washed 3 times with PBS and examined using a fluorescence microscope (Olympus BX50-FLA reflected light fluorescence attachment) fitted with ×40 objective lenses. An unbroken green band of fluorescence within at least 80% of a given well that surrounds a fluorescent red parasite was determined as positive, while a green fluorescent cap at the parasite pole was evaluated as nonspecific polar fluorescence.

The SFDT was performed at the Laboratory of Parasitology in Ankara Refik Saydam National Institute of Hygiene. Serum samples were incubated with live *T. gondii* tachyzoites and complement at 37 °C for 1 h, stained with methylene blue, and examined under a light microscope. The SFDT results were accepted as positive if more than 50% of tachyzoites remained unstained. To eliminate the cross reactivity and false positive results, only 1/16 and above titres were accepted as positive.

2.6. Statistical analysis

The chi-square test was used to compare the antibody titres and the presence of tissue cysts in sheep. Statistical analysis was done with SPSS version 15.0. P values less than 0.05 were considered significant.

3. Results

Using the percoll gradient centrifugation, *T. gondii* tissue cysts were detected in 46% (46/100) of sheep examined. Table 1 shows the distribution rates of detected tissue cysts in various tissue samples of sheep. Tissue cysts were found in 36% and 32% of the brain and skeletal muscles of all sampled animals, while of the 46 tissue cyst positive sheep, 78.2% and 69.5% of the brain and skeletal muscle samples were harbouring tissue cysts, respectively. The cysts were spherical and 25–58 × 25–62 (mean 34 × 36) μm in diameter. The wall thickness of the tissue cysts was 1 μm. All *T. gondii* tissue cyst samples were confirmed by detection of 97 bp bands of *T. gondii* B1 gene by nested PCR (Figures 1A and 1B).

Tissue cysts were detected in 45.4% (35/77) of ewes and 47.8% (11/23) of rams. Statistically, no relationship between the prevalence of tissue cysts and sex was observed

Table 1. Distribution of *T. gondii* tissue cysts in brain and muscle samples of sheep using percoll gradient centrifugation.

Tissue	Tissue cyst positive sheep (n: 46)	
	n	%
Brain	36	78.2
Muscles	32	69.5
(masseter)	(9)	(28.1)
(tongue)	(9)	(28.1)
(diaphragm)	(14)	(43.7)
(intercostal)	(15)	(46.8)
(leg)	(14)	(43.7)

(P > 0.05). Furthermore, tissue cysts were observed in 55.5% (35/63) of 1-year-old, 30% (7/21) of 2-year-old, 25% (3/12) of 3-year-old, and 25% (1/4) of 4-year-old sheep with a significant reduction in sheep older than 2 years of age (P < 0.05) (Table 2).

Seropositivity was detected in 88% (88/100) of sheep examined by IFAT, all 46 sheep with cysts were in this group (52.3%). The relationship difference between the presence of tissue cysts and seropositivity in sheep was statistically significant at 1/16 (P < 0.01) and at 1/64 and 1/128 (P < 0.001) serum dilutions. However, tissue cysts were significantly more prevalent in sheep with a high titre of IgG in serum samples (P < 0.001).

Using the TMA and immunoperoxidase staining, tissue cysts were detected in 17% of seropositive sheep (15/88). The most prevalent pathological sign in histological examinations was nonpurulent interstitial myositis consisting of a variety of cell infiltrations, the majority being macrophages and lymphocytes (Figure 2A). Immunopositive reactions in the serial tissue sections after immunoperoxidase staining were observed in the brain and skeletal muscles in both bradyzoites and the cyst walls of *T. gondii* tissue cysts (Figures 2B and 2C), which were present in sarcoplasms as oval, elliptical, and fusiform parasites.

4. Discussion

Different techniques based on serological examinations have been performed to detect toxoplasmosis in live farm animals (9). However, serological findings are only indicators of the *T. gondii* infection of the animal and do not give any clear information about the presence of tissue cysts. Since one of the major transmission routes of toxoplasmosis, especially in human populations, is consumption of various tissues of intermediate hosts harbouring tissue
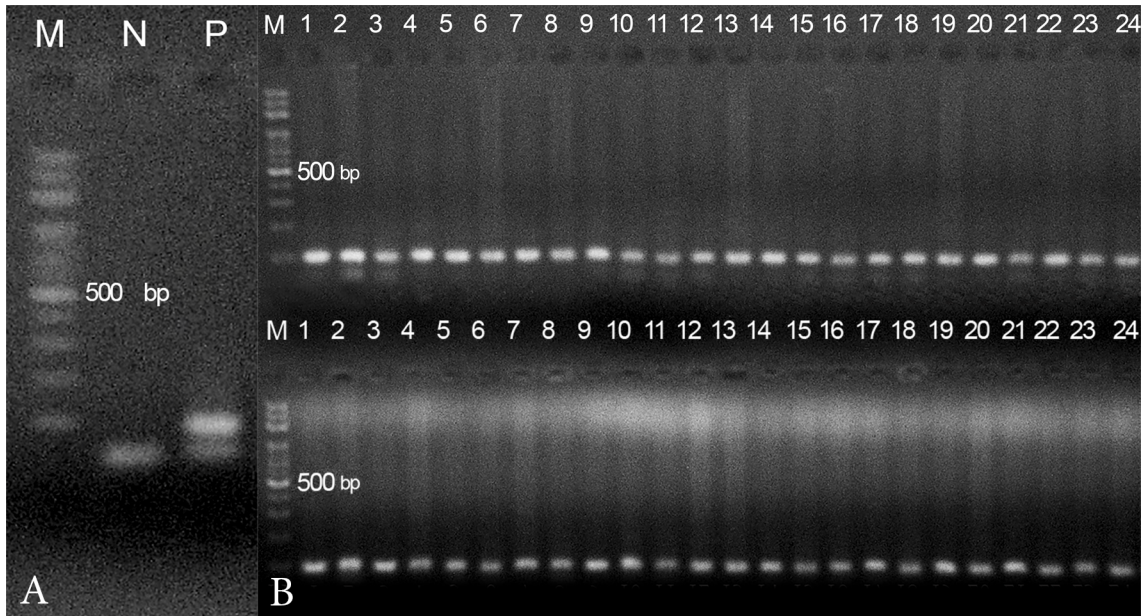
Figure 1. (**A**). *T. gondii* B1 gene amplification result. Lane M: Marker (100 bp), N: Negative control, P: Positive control. *T. gondii* B1 gene amplification results of the tissue cysts samples selected randomly (**B**). Lane M: Marker (100 bp), Lanes 1–24: Tissue cyst samples (97 bp bands).

Table 2. The relationship differences among age, seropositivity, and tissue cysts positivity in sheep examined.

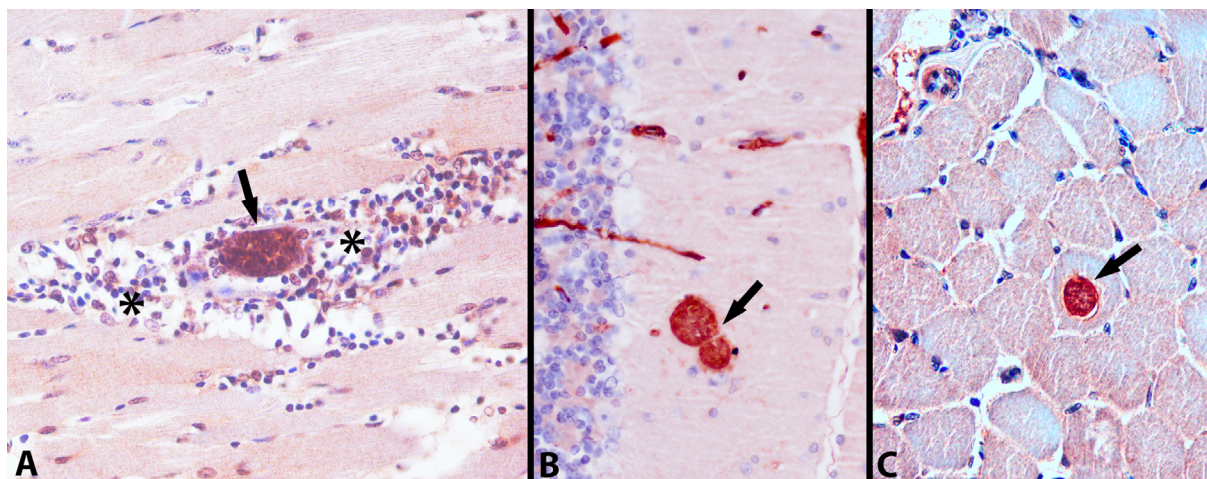| Age	Serum dilution steps	Seropositive	Tissue cysts positive	P value
		n	n	
1 (n: 63)	1/16	32	16	
	1/64	18	14	
	1/128	6	5	P < 0.05
	1/256	1	-	
2 (n: 21)	1/16	7	2	
	1/64	7	2	NA
	1/128	3	3	
	1/256	2	-	
3 (n: 12)	1/16	5	3	
	1/64	4	-	
	1/128	-	-	NA
	1/256	-	-	
4 (n: 4)	1/16	3	1	
	1/64	-	-	
	1/128	-	-	NA
	1/256	-	-	
Total: 100		88	46	

NA: Not applicable

Figure 2. (**A**) *T. gondii* tissue cyst (arrow) and nonpurulent interstitial myositis consisting of a variety of cell infiltrations (asterisks), the majority being macrophages and lymphocytes. Immunopositive reactions in brain (**B**) and skeletal muscle (**C**), both in bradyzoites and the cyst walls of *T. gondii* tissue cysts (arrows). Immunohistochemistry, anti-*T. gondii* antibody, AEC chromogen, and hematoxylin counterstain. Magnification 400×.

cysts with live bradyzoites (1,17), understanding the relationship between seropositivity and the presence of *T. gondii* tissue cysts is important.

Tissue cysts may not always be found in tissues of seropositive hosts (18). In accordance, tissue cysts in our study were only observed in 52.3% and 17% of seropositive sheep by the percoll gradient centrifugation and TMA, respectively. The difference between the techniques in terms of detection of tissue cysts was thought to be related to the number of samples analysed since histopathology was not also capable of detecting tissue cysts of *T. gondii* in tissue samples (19). Though TMA is a fast and cost-effective method to evaluate large numbers of tissue samples simultaneously by histochemical staining (20), the sensitivity of TMA in our study was proved to be lower than that of percoll gradient centrifugation for detection of *T. gondii* tissue cysts in host tissues (52.3% versus 17%). There remains the possibility that some of the tissues contain a few cysts, which might have been lower than the percoll gradient centrifugation's capability of detection as well, possibly explaining the failure of tissue cyst isolation in all of the seropositive sheep.

Raw or undercooked lamb meat and meat products are considered a taste of preference in some countries and therefore implicated as an important source of infection (21). In the present study, tissue cysts were mostly detected in the tissues of animals 1-year old (55.5%), while the detection rate was significantly reduced in sheep older than 2 years of age (P < 0.05). However, this could be attributed to the relatively low number of 2-year-old sheep sampled. Either way, tissue cysts can survive in different tissues of intermediate hosts throughout their life and the factors affecting

the viability and the time span of survival of tissue cysts are not well known (1–3). However, even though the life span of tissue cysts is not fully understood, toxoplasmosis seropositivity does continue throughout the host's lifetime (1–3) and the intensity of antibody response may be independent of the parasite burden of the host (22). In accordance, the isolation rate of parasites from the tissues did increase with increasing antibody titre in the host (23) as tissue cysts were significantly more prevalent in sheep with a high titre of IgG in serum samples (P < 0.001) in our study.

Today, bioassay either in cats or mice is preferred for detection of the viability of *T. gondii* tissue cysts in the intermediate host tissues. Isolation rates of *T. gondii* by bioassay from seropositivity hosts show differences (23–26) as *T. gondii* was isolated from 53 of 68 seropositive lamb tissues (24) and only from 29 of 112 seropositive goat tissues by bioassay (26). The relationship between antibody titres and *T. gondii* isolation from the same animal by bioassay was recently reported (23), but a bioassay was not used in this study because of the ethical restriction that arose due to the high numbers of sampled animals.

It is well known that the tissue cysts of *T. gondii* may develop in the brain, lung, liver, kidney, heart, and skeletal muscles of intermediate hosts during the chronic phase of toxoplasmosis (1). Tissue cysts were also isolated from the leg, tongue, and intercostal muscles of lambs (27). The highest prevalence of *T. gondii* was reported in the diaphragm of cattle, while the lowest prevalence was observed in sheep and bear diaphragms (28). The prevalence of *T. gondii* tissue cysts in the brain was lower than in the muscle of sheep (29). However, in our study, tissue cysts were observed in 78.2% of the brains and 69.5% of

the skeletal muscles of 46 sheep that were determined to be positive for *T. gondii*. Sheep brain may play a limited role in human toxoplasmosis as it is rarely consumed by humans; however, skeletal muscles have utmost importance in the foodborne transmission of toxoplasmosis. The present study highlighted the potential public health risk since tissue cysts were mostly detected in the intercostal, leg, and diaphragmatic muscles of sheep, which are frequently consumed by humans.

The size of *T. gondii* tissue cysts may vary depending on the suspension medium used and the pressure the tissue faces during the preparation and processing of the samples (1,2). In addition, they are affected by tissue fixation procedures. Depending on the cell morphology in histological sections, tissue cysts of *T. gondii* were reported to be spherical and 60 μm in diameter from brain tissues but much longer in shape with a size up to 100 μm in muscle cells (1). The tissue cysts observed by percoll

gradient centrifugation were spherical and 25–58 × 25–62 (mean 34 × 36) μm in diameter. In contrast to histological features reported in muscle cells, no differences were observed between the tissue cysts from muscle and brain samples with regards to morphology and size, as the tissue cysts obtained from the muscles with the percoll gradient centrifugation were also spherical.

Our study showed that almost half of the sheep brought to slaughterhouses were harbouring tissue cysts, presenting a high risk for consumers. The significant relationship between serum dilutions and the presence of tissue cysts observed in this study may be of help in the determination of sheep harbouring tissue cysts, but further studies regarding this finding are needed.

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