Turkish Journal of Veterinary & Animal Sciences

Volume 39 | Number 3 Article 12

1-1-2015

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KADER YILDIZ

FATMA ÇİĞDEM PİŞKİN

ARMAĞAN ERDEM ÜTÜK

SAMİ GÖKPINAR

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YILDIZ, KADER; PİŞKİN, FATMA ÇİĞDEM; ÜTÜK, ARMAĞAN ERDEM; and GÖKPINAR, SAMİ (2015) "Prevalence of Toxoplasma gondii in sheep meats purchased from retailstores in Central Anatolia, Turkey," *Turkish Journal of Veterinary & Animal Sciences*: Vol. 39: No. 3, Article 12. https://doi.org/10.3906/vet-1412-63

Available at: https://journals.tubitak.gov.tr/veterinary/vol39/iss3/12

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TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

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Research Article

Turk J Vet Anim Sci
(2015) 39: 328-332
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doi:10.3906/vet-1412-63

Prevalence of Toxoplasma gondii in sheep meats purchased from retail stores in Central Anatolia, Turkey

Kader YILDIZ[1,*], Fatma Çiğdem PİŞKİN[2], Armağan Erdem ÜTÜK[3], Sami GÖKPINAR[1]

[1]Department of Parasitology, Faculty of Veterinary Medicine, Kırıkkale University, Kırıkkale, Turkey
[2]Parasitology and Bee Diseases Laboratory, Central Veterinary Control and Research Institute, Ankara, Turkey
[3]Department of Parasitology, Faculty of Ceyhan Veterinary Medicine, Çukurova University, Adana, Turkey

Received: 24.12.2014 • **Accepted/Published Online:** 13.03.2015 • **Printed:** 10.06.2015

Abstract: Toxoplasmosis is one of the most important foodborne parasitic diseases of humans. In particular, sheep muscles are significant sources of infection in the transmission of toxoplasmosis. Carnivorism is the most important transmission route for human populations. The aim of this study was to detect the prevalence of *Toxoplasma gondii* tissue cysts in sheep meats in retail stores of Turkey. A total of 250 boneless sheep meat samples were purchased from randomly selected retail stores in different locations of Ankara and Kırıkkale provinces of Turkey. The homogenized meat samples were centrifuged with Percoll dilutions. The tissue cysts were removed by pipette and analyzed under light microscope. Additionally, nested PCR was used to detect *T. gondii* DNA in the meat samples. Tissue cysts were observed in 21.2% of the meat samples with Percoll gradient centrifugation. The prevalences of the tissue cysts were detected as 20.8% in the meat samples obtained from Ankara and 22.4% from Kırıkkale ($P > 0.05$). *T. gondii* DNA was detected in 40.8% of the meat samples with nested PCR.

Key words: *Toxoplasma gondii*, Apicomplexa, sheep, meat, tissue cyst, Turkey

1. Introduction

Toxoplasma gondii is the only species in the family Toxoplasmatidae (1). Felids are both final and intermediate hosts, while humans and different animal species are intermediate hosts of the parasite (2). Humans are infected either through eating raw or undercooked meat infected with tissue cysts or by ingestion of food or water contaminated with oocysts; congenital transmission is another significant transmission route (1).

Tissue cysts develop in different organs such as the liver, lungs, brain, myocardium, and skeletal muscles during the chronic period of the illness (1,3). The cysts filled with bradyzoites are responsible for foodborne toxoplasmosis in humans, since bradyzoites are resistant to the hosts' digestive enzymes (4).

Tissue cysts of *T. gondii* are generally resistant to temperature changes (5). Cysts may die at –9.4 °C or colder (6). However, some tissue cysts may remain viable even under storage at –12 °C (7) and in microwave-cooked meat due to uneven heating (8).

In Turkey, the major source of toxoplasmosis in consumers is thought to be fresh sheep meat purchased from retail stores. There are many seroprevalence studies concerning sheep toxoplasmosis in different provinces of Turkey (9–14); nevertheless, there is no comprehensive survey examining the presence of *T. gondii* tissue cysts in sheep meat sold at retail stores. The aim of the present study was to detect the prevalence of *T. gondii* in sheep meat samples collected from retail stores in Central Anatolia, Turkey. The retail stores in metropolitan cities and small cities have different meat supply chains and storage times. Therefore, study samples were obtained from Ankara, a metropolitan city, and Kırıkkale, a small city, with populations of 5,045,083 and 274,658, respectively, according to 2013 data (http://www.nufusu.com).

2. Materials and methods

2.1. Tissue samples

During the period of August 2011 to April 2012, a total of 250 boneless sheep meat samples were purchased weekly from retail meat stores selected randomly at different locations in Ankara and Kırıkkale provinces of Central Anatolia, Turkey. Samples were aseptically collected in a sterile plastic bag by sterile scalpel. Connective tissues and fat were removed from meat samples. The minimum sample unit was 100 g for each sample; 50 g of each sample

* Correspondence: kaderyildiz@hotmail.com

was used for Percoll gradient centrifugation and the remaining was stored at –20 °C for DNA extraction.

2.2. Percoll gradient centrifugation

Briefly, 5 g of meat sample was cut with sterile scissors and added to 20 mL of PBS. The samples were then homogenized using a high-speed tissue homogenizer (OMNI Tip, USA). The homogenizer was cleaned in boiled water before every consecutive homogenization. The homogenates were sieved in a centrifuge tube using separate cheesecloths. From Percoll stock solution (Sigma-Aldrich, USA), 90% and 30% diluted solutions in distilled water and NaCl were prepared. Percoll dilutions and the homogenates were centrifuged (Nuve, Turkey) at 4,000 × *g* for 20 min. Following centrifugation, the cysts found in between the Percoll layers were removed using a Pasteur pipette and analyzed for presence of tissue cysts by light microscope (Olympus BX 50).

2.3. *Toxoplasma gondii* genomic DNA isolation

One gram of each meat sample was cut into small pieces before being thoroughly ground into a fine powder under liquid nitrogen. Genomic DNA was extracted by the QIAamp DNA Mini Kit (QIAGEN, Germany) with some modifications, and then 500 mg of the powder was transferred to an Eppendorf tube and digested in 30 µL of proteinase K (25 mg/mL, MBI Fermentas), 180 µL of ATL buffer from the extraction kit, and 500 µL of DNase-free, RNase-free sterile distilled water (BioBasic, Inc.) at 56 °C overnight in a dry block (Labnet, D-1200). The DNA extraction was then performed with 200 µL of supernatant following centrifugation at 12,000 × *g* for 3 min, according to the manufacturer.

2.4. Nested PCR amplification

Nested PCR was performed to confirm the presence of the *T. gondii B1* gene in the sheep meat homogenates. The *B1* gene is a 35-fold repetitive gene and is highly conserved in *T. gondii* (15). While in the first round of nested PCR, a 194-bp DNA fragment of the *B1* gene of *T. gondii* was amplified using Toxo 1 for: 5'-GGAACTGCATCCGTTCATGAG-3' and Toxo 2 rev: 5'-TCTTTAAAGCGTTCGTGGTC-3' primers, in the second round, a specific 97-bp part of the *T. gondii B1* gene was amplified from previously amplified template DNA using Toxo 3 for: 5'-TGCATAGGTTGCCAGTCACTG-3' and Toxo 4 rev: 5'-GGCGACCAATCTGCGAATACACC-3' primers.

PCR was carried out in a final volume of 50 µL. Master mixes of both PCR reactions consisted of 5 µL of 10X PCR buffer, 5 µL of 25 mM $MgCl_2$, 4 µL of 1 mM dNTP mix, 1.5 µL of each primer (30 pmol), 0.25 µL of Taq DNA polymerase (1.25 IU; MBI Fermentas), and 5 µL and 2 µL of template for each round, respectively.

Amplifications were carried out in a thermal cycler (SensoQuest, Labcycler) and the PCR conditions consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min, with a final elongation at 72 °C for 5 min. In every reaction, DNA from tachyzoites of the *T. gondii* RH strain was included as a positive control and ultrapure water (BioBasic, Inc.) as a negative control.

The PCR products were separated on agarose gels (1.5%; Sigma-Aldrich) stained with ethidium bromide (Sigma-Aldrich) and visualized on a UV transilluminator (UVP, M-20V).

2.5. Statistical analysis

Statistical analysis of the data was performed by using SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Chi-square test was used to compare the data. $P < 0.05$ was considered significant.

3. Results

In this study, 250 sheep meat samples from Ankara (n: 192) and Kırıkkale (n: 58) were analyzed for *T. gondii* (Table). Tissue cysts were observed in 21.2% of the meat samples with Percoll gradient centrifugation. The prevalences of the tissue cysts in the meat samples obtained from Ankara and Kırıkkale were 20.8% and 22.4%, respectively ($P > 0.05$). The spherical tissue cysts were measured as 30–36.5 × 25.5–32 µm in diameter (mean: 32.5 × 27.5 µm). The wall thickness of cysts was measured as <1 µm. The tissue cysts were confirmed as *T. gondii* by nested PCR. Genomic DNA of *T. gondii* was detected in 40.8% of the meat samples with nested PCR (Figure). The prevalences

Table. Distribution of *T. gondii* in meat samples of sheep from Ankara and Kırıkkale.

Province	Number of samples	*T. gondii* tissue cysts positivity		*T. gondii* PCR positivity	
	(n)	(n)	%	(n)	%
Ankara	192	40	20.8	89	46.3
Kırıkkale	58	13	22.4	13	22.4
Total	250	53	21.2	102	40.8

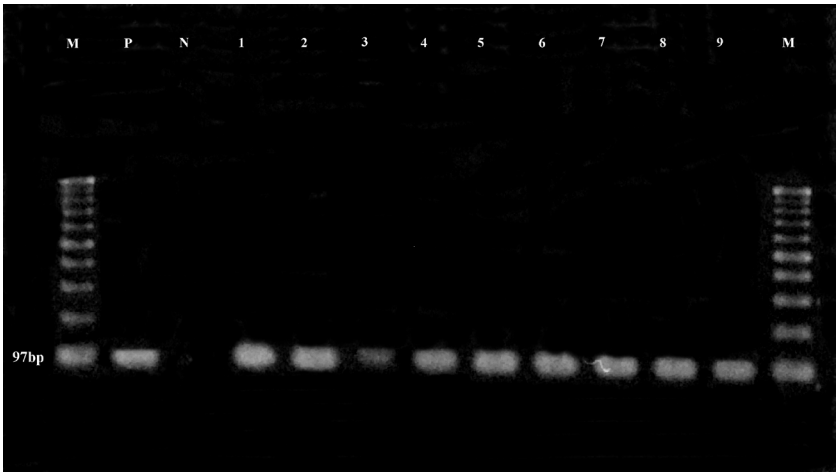

Figure. *T. gondii B1* gene amplification results of the tissue cyst samples selected randomly. Lane M: marker (100 bp), N: negative control, P: positive control. Lanes 1–9: tissue cyst samples (97-bp bands).

of *T. gondii* DNA in the samples were 46.3% and 22.4% in Ankara and Kırıkkale, respectively ($P < 0.001$).

4. Discussion

Toxoplasma gondii causes tissue cyst formation in different organs of intermediate hosts (3). These cysts are isolated from the tongue, intercostal, and leg muscles in sheep (5,16) and are important for human infections (1). Real-time PCR-positive reactions were reported in pig meat samples (18.03%) and only one viable isolate was obtained from PCR-positive pork meat sampled in retail stores in China (17). The prevalence of viable *T. gondii* in retail meat is very low and viable parasites were only detected in pig meat samples in the United States (18). *T. gondii* was reported in diaphragm samples of cattle at the highest rate using real-time PCR (19). In the present study, intact *T. gondii* tissue cysts were observed at 21.2% in the sheep meat samples. Viability of these tissue cysts was unknown since bioassay trials could not performed in this study.

Oral transmission is the major route for human toxoplasmosis (2). Nested PCR is a useful technique for the detection of *T. gondii* DNA in tissue samples (20). However, the presence of the parasite DNA in the tissues does not reveal the infection through carnivorism. It is well known that PCR cannot distinguish between living and dead organisms (1). In the present study, the molecular prevalence in meat samples collected from Ankara was higher when compared with Kırıkkale (46.3% vs. 22.4%). The viability of tissue cysts in meat is affected from storage temperature and duration in the retail stores (21). In Turkey, metropolitan retail stores supply meat generally from big suburban farms. After the slaughtering process, meats are transported in bulk to metropolitan cities with cold chain and stored in suitable refrigerators in markets. However, small city markets supply limited amounts of meat from the closest small farms and sell it in a short time without freezing. Freezing of meat for 2 days at –12 °C is sufficient for the death of *T. gondii* tissue cysts (22), even if some of them are highly resistant to temperature changes (7). Furthermore, tissue cysts may be infectious in meat after storage at temperatures of 1–4 °C during 3-week periods (7). *T. gondii* tissue cyst and parasites DNA were detected in frozen buffalo meat samples with use of microscopic and molecular techniques, respectively (23).

In general, raw or undercooked meat is considered as the most important source for human foodborne toxoplasmosis (22). This has been confirmed by epidemiological studies based on the monitoring of toxoplasmosis (24,25). In the present study, *T. gondii* tissue cysts of sheep meat samples were observed at 20.8% in Ankara and 22.4% in Kırıkkale. The seroprevalence rates of human toxoplasmosis in these provinces were reported as 28% and 22.1%, respectively (26,27). The seropositivity rate of *T. gondii* in humans may be related to traditional eating habits. In Turkey, the traditional raw meatball called "çiğ köfte" prepared with raw sheep meat can be an important mode of transmission. In addition to cultural eating habits, cross-contamination of cutting boards and knives after preparing meat in the kitchen may be significant in the transmission of toxoplasmosis to humans.

According to epidemiological surveys on toxoplasmosis in Turkey, the seropositivity rates have been gradually increasing in farm animals (9,11,13,14,28). In previous reports, sheep toxoplasmosis was reported between 14.7% and 45.4% (9,11), whereas the seropositivity rates have dramatically increased to 97.4%–98.92% in sheep based on recent published reports (13,14,28). Increasing

seropositivity rates in farm animals suggest that *T. gondii* may be an important health problem for humans in the future. Although *T. gondii* is one of the most studied parasites, there are still many questions that have to be addressed. Today, in respect to public health, one of the most important issues is to prevent transmission of toxoplasmosis among hosts including humans. In addition to the basic control measurements, public awareness is necessary to prevent the transmission of toxoplasmosis.

Acknowledgments

This work was supported by a grant from the Kırıkkale University Scientific Research Unit (Project number: 2011–32). This research was presented at the 12th International Symposium Prospects for the 3rd Millennium Agriculture University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania, 26–28 September 2013. The authors would like to thank Dr Cahit Babür of the Public Health Institution of Turkey.

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