DETECTION OF TOXOPLASMA GONDII
FROM MEAT AND MEAT PRODUCTS
BY THE NESTED-PCR METHOD
AND ITS RELATIONSHIP WITH SEROPREVALENCE IN SLAUGHTERED ANIMALS

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Abstract

Toxoplasma gondii DNA was detected at rates of 2%, 6%, 4.17%, 20%, and 19% in 50 bovine brains, 50 bovine muscles, 120 ovine brains, 20 ovine muscles, and in 100 fermented sausage samples, respectively. Sequenced positive samples showed 100% identity with T. gondii RH strain. Seropositivity rates were detected as 24% in cattle and 25% in sheep slaughtered in local slaughterhouses. The detection of the parasite in uncooked meat and commercial meat products, and the high ratio of seropositive slaughtered animals, emphasise that the risk still exists for food-transmitted toxoplasmosis. This is the first report from Turkey describing the detection of Toxoplasma gondii in food using the molecular method.

Key words: cattle, sheep, brain, muscles, fermented sausage, Toxoplasma gondii, PCR.

Toxoplasma gondii is an obligate, intracellular parasite, which is widely distributed in the world. The parasite is able to infect all warm-blooded hosts - including human - and all host cells except erythrocytes. Its infection has various manifestations in immunocompetent or immunocompromised patients and pregnant women. Immunocompetent individuals generally do not show clinical symptoms related to the infection. In immunocompromised patients such as HIV/AIDS patients, organ transplant recipients, and patients with malignancy, the infection may manifest itself as severe clinical forms and life-threatening disease. T. gondii has three types of transmission: by consuming food or water containing oocysts, by eating undercooked meats containing tissue cysts, and transplacentally (9).

Out of 225,000 toxoplasmosis cases in the United States, 750 were reported to have terminated with death. As a result of these cases, toxoplasmosis has been regarded as the most significant risk factor of primary infection during pregnancy (5, 7, 8, 19) It has been reported that up to 63% of seroconversions during pregnancy were subsequent to undercooked or cured meat consumption (8). In Norway, consumption of raw or undercooked meat has been reported as the major risk factor for toxoplasmosis in pregnant women (19). Raw and undercooked meats were reported as responsible for 50% of congenital toxoplasmosis (23). Serological surveys emphasised that toxoplasmosis has high prevalence among animals which are raised for meat production, such as pigs, sheep, and goats (31). The prevalence of toxoplasmosis has been detected as higher in old animals, which are the source of meat products such as fermented sausage, salami, and others (11). Some meat products may be composed of various animal meats and thus raw and undercooked meats have become an important risk factor in the transmission of toxoplasmosis (2).
High seropositivity scores (26.6%-88.7%) for *T. gondii* have been reported in slaughtered animals (3, 4) and the prevalence of infection varies between 1.2% and 81.6% in people in Turkey (26, 34).

Regarding the lack of the information on the *T. gondii* presence in meat and meat products in Turkey, an attempt has been made to detect the parasite in meat and meat products - which could increase the potential hazard to human health - to evaluate seroprevalence among slaughtered animals, and to compare the results of serological tests and PCR.

**Material and Methods**

**Specimen collections.** A hundred sheep brains, 33 traditional raw meat balls, and 100 fermented sausages, were collected from local grocery stores in Istanbul city. Brain, muscle, lymph node, and blood samples were also collected from 50 cattle and 20 sheep slaughtered in three different cities. The tissue samples were stored at -80ºC until work up. Serum samples were analysed to detect antibodies for *T. gondii* and to correlate the serological results with the presence of the parasite in tissues.

**DNA extraction.** Collected tissue samples and fermented sausages were crushed with a homogeniser. DNA was extracted from 25-30 mg of each crushed tissue using a commercial DNA extraction kit (Nucleospin tissue kit, Macherey-Nagel, Germany) following the manufacturer’s instructions. All DNA extracts were stored at -80ºC until use.

**PCR amplification and sequence analysis:** *T. gondii* DNA was determined via nested-PCR protocol. ATP Binding Cassette B1 gene region was targeted for the generation of specific primer sets for *T. gondii*. Nucleotides and positions of the primers are given in Table 1.

The PCR mixture contained 0.5 μM of each primer, 10 mM of Tris-HCl, 1.5 mM of MgCl₂, 50 mM of KCl (pH 8.3), 0.2 mM of each deoxynucleoside triphosphate (Fermentas®, Lithuania), and 1.25 U of *Tag* DNA polymerase (Fermentas®, Lithuania). The reaction volume was 50 μl containing 10 μl of DNA extracts. Amplification was carried out within a PTC-200 MJ Research thermal cycler. The first PCR (an initial step at 94°C for 3 min, 45 cycles of PCR amplification 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, final extension at 72°C for 8 min) was performed with Toxo-1 and Toxo-2 primers.

Three microlitres of the reaction product were used for nested-PCR (under the same conditions), which was performed with the primer pairs, Toxo-3 and Toxo-4, respectively, resulting in amplification of a 96 bp fragment. DNA samples of *Toxoplasma* cell culture extractions were used as positive control. Distilled water and fish DNA extractions (the only animal known not to be infected with *Toxoplasma*) were used as negative controls (2). Amplification products were run in 1.5 % agarose gel stained with ethidium bromide solution (2 μg/mL) and were viewed in a transilluminator under UV light.

One of the second-round PCR products was purified with a commercial PCR product purification kit (Roche®, Germany) according to the manufacturer’s instructions. Then it was subjected to cycle sequencing using the big-dye terminator kit (ABI®, USA). Following the clean-up procedure through sephadex-G50 fine columns, the cycle-sequencing product was run on an automated sequencer (ABI®, 310). The obtained sequence was edited and aligned using lasergene (DNA Star®) and Bioedit software packages (16), and then compared with data available in GenBank.

**Serological examination.** All the serum samples were tested for the presence of IgG antibodies for *T. gondii* by microtitration methods using a commercial latex test kit (Toxo Reagent RST 701, Mast Diagnostic, U.K.) (24, 32). Sera were screened at dilutions of 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1,024, and 1:2,048.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxo-1</td>
<td>ggaacgtcattgcatcag</td>
<td>(694-714)</td>
</tr>
<tr>
<td>Toxo-2</td>
<td>tct tta aag cgt tcg tcg</td>
<td>(868-887)</td>
</tr>
<tr>
<td>Toxo-3</td>
<td>tgcagagttgcagctagc</td>
<td>(757-776)</td>
</tr>
<tr>
<td>Toxo-4</td>
<td>ggcgaccaatgcgaatcacc</td>
<td>(831-853)</td>
</tr>
</tbody>
</table>
### Table 2

PCR results of meat products and samples collected from slaughterhouses

<table>
<thead>
<tr>
<th>Source</th>
<th>Sample type</th>
<th>Total</th>
<th>Positive sample (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slaughterhouse</td>
<td>Cattle brain</td>
<td>50</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Slaughterhouse</td>
<td>Cattle muscle</td>
<td>50</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>Slaughterhouse</td>
<td>Cattle lymph node</td>
<td>50</td>
<td>2 (4%)</td>
</tr>
<tr>
<td>Slaughterhouse</td>
<td>Positive cattle in total</td>
<td>50</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>Slaughterhouse</td>
<td>Sheep brain</td>
<td>20</td>
<td>3 (15%)</td>
</tr>
<tr>
<td>Slaughterhouse</td>
<td>Sheep muscle</td>
<td>20</td>
<td>4 (20%)</td>
</tr>
<tr>
<td>Slaughterhouse</td>
<td>Sheep lymph node</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Slaughterhouse</td>
<td>Positive sheep in total</td>
<td>20</td>
<td>5 (25%)</td>
</tr>
<tr>
<td>Commercial</td>
<td>Fermented sausage</td>
<td>100</td>
<td>19 (19%)</td>
</tr>
<tr>
<td>Commercial</td>
<td>Traditional raw meat balls</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td>Commercial</td>
<td>Sheep brain</td>
<td>100</td>
<td>2 (2%)</td>
</tr>
</tbody>
</table>

### Table 3

Number of animals with positive PCR and serology results

<table>
<thead>
<tr>
<th>Animals</th>
<th>Total number</th>
<th>Lymph node (PCR)</th>
<th>Muscles (PCR)</th>
<th>Brain (PCR)</th>
<th>Serology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cattle</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cattle</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Cattle</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cattle</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Cattle</td>
<td>36</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sheep</td>
<td>2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sheep</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Sheep</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sheep</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sheep</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Results

The PCR results obtained from the tissue samples of slaughtered animals and commercial meat products are given in Table 2. From the slaughterhouse samples, a total of five cattle out of 50 (10%) were found as positive for *T. gondii*. In one of them, multiple organ positivity was detected (brain and muscle). Brain and muscle samples of two sheep were found as positive simultaneously, and only muscle samples were found as positive in other two sheep, and a brain sample was found as positive in only one sheep. Total positivity rate was 25% (5/20) in sheep. Among the commercial samples, 19% of the fermented sausages and 2% of the sheep brains were positive. Total positivity rate for commercial and slaughterhouse brains from sheep was 4.17%. *T. gondii* DNA was not found in traditional raw meat balls.

One of the amplified PCR products was sequenced and the analysis of the sequence showed a 100% identity with *T. gondii* (AF179871) RH strain.

Out of 50 cattle, 12 (24%) were found as positive for IgG with the titres of 64 (seven animals), 128 (two animals), 256 (two animals), and 512 (one animal). IgG antibodies were detected in five out of 20 sheep (25%) with the titres of 64, 128, 256, 512, and 512 in particular sheep, respectively. The number of animals positive for PCR and/or serology are given in Table 3.

### Discussion

This is the first report on the detection of *T. gondii* by the PCR technique in meat and meat products in Turkey. Although the results obtained do not indicate the presence of a living parasite, it is known that cyst forms of the parasite may survive several weeks between 1°C and 4°C and can be inactivated only in processes over 67°C or below -12°C (2). In previous studies, seropositivity rates between 1.2% and 81.6% were reported in humans from Turkey (26, 34). Serosurveys in Istanbul City revealed that IgM seropositivity in pregnant women was 0.9% and IgG seropositivity was between 34% and 43% (20, 27). Toxoplasmosis resulted from cat-sourced fecal-oral transmission and the risk of meat and meat products were only mentioned according to the literature up to the present. The results of this study indicate the potential risk of uncured meat and meat products to human health.
Warnekulasuriya et al. (35) detected one PCR positive sample out of 67 cured meat samples, including dried and semi-dried sausages and hams in UK. The authors suggested that the detected level of parasite contamination would be sufficient to establish human infection following the consumption of a typical meal portion of cured meat. Aspinall et al. (2) detected with PCR 19 positive out of 57 samples of pork, six out of nine lamb, and one out of four beef, and one sample containing pork and beef in UK four years later. The authors claimed that there may have been a significant increase (38%) in the degree of contamination of commercially-available meat products over a short period.

*T. gondii* was detected in experimentally-infected sheep, particularly within brain and heart tissues, whereas parasites were not detected in experimentally-infected cattle (13). Although pork was considered as the major source of toxoplasmosis in humans in Europe and the USA (10, 31), Aspinall et al. (2) detected a higher contamination rate (six of nine, 66.7%) in sheep samples compared with pork samples (34.5%). Antibodies to *T. gondii* were found in 104 (27.2%) of 383 lambs in Maryland, Virginia, and West Virginia, USA.

It has been found that among all types of samples examined in this study, the highest proportion (4 of 20, 25%) of the *T. gondii*-positive are among sheep muscle samples. The second highest contamination rate was detected in fermented sausages (19%). The majority of these samples contained a mixture of meat from several animals and several species. More likely, beef and lamb, and tissues other than meat, of these animals, were used for fermented sausages, so the high contamination rate of *T. gondii* may be explained by the presence of lamb in these products. It has also been born in mind that contamination could play an important role during the whole manufacturing process of this product.

Twenty-four percent of the cattle and 25% of the sheep were found to be IgG seropositive in our study. Among these seropositive cattle and sheep, 25% and 40%, respectively were found to be also positive with PCR. Recent studies reported various seropositivity rates for sheep, such as 12.50%-26.92% in Brazil (15), 24.50% (17) and 35% in Iran (30), 33.2% in Ghana (28), and 78% in Italy (14). In Turkey, rates of toxoplasmosis seropositivity in sheep were reported as 42.74% in Sanlurfa (1), 63.9% in Kirikkale (33), and 65.08% in Yalova (25). Seroprevalence of toxoplasmosis amongst sheep have been reported to be increasing, at 20-30%, while among pigs it is reported to be falling (31). These findings, with the results of those reported from meat and meat-products concerning lambs (2, 12, 13), are of importance for public health, inferring that lamb may be a significant source of human toxoplasmosis. Although seropositivity of *T. gondii* in cattle was reported to be low, between 1.03% and 16.10% (6, 15, 18, 29) and 0% in some studies (17, 30), it was reported as 49.13% in Turkey (1).

In conclusion, the results of this study confirm the presence of *T. gondii* in slaughtered animals and commercially-available uncooked meat-products and also define the prevalence of seropositivity in slaughtered sheep and cattle. Although the risk in sheep meat is of greater concern, cattle meat also has remarkable importance regarding the transmission of *T. gondii* to humans. Therefore, the potential risk of the transmission of the disease through *T. gondii* containing meat should still be considered a public health threat.

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