A potential infection source for humans: Frozen buffalo meat can harbour tissue cysts of *Toxoplasma gondii*

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**ABSTRACT**

Buffaloes are considered resistant to toxoplasmosis. Tissue cysts of *Toxoplasma gondii* found rarely in skeletal muscles of buffaloes. However, in the present study, we found tissue cyst of *T. gondii* in frozen boneless buffalo meat illegally imported to Turkey. Spherical tissue cysts of *T. gondii*, 27–34 to 30–32 (mean 31 × 30) μm in diameter, were found in 3 out of 20 (15%) spontaneously thawed meat samples analyzed by light microscopy of percoll dilutions. All positive samples were also confirmed by observation of 97 bp gene products of *T. gondii* repetitive B1 gene by nested PCR. The tissue cysts however, were not found following a preservation of two days at −18 °C. Present study shows that frozen buffalo meat can be a potential new infection source for human toxoplasmosis.

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1. Introduction

Toxoplasma gondii, a zoonotic Apicomplexan parasite, needs two hosts in its life cycle. While Felidae species are final hosts of *T. gondii*, an array of warm blooded animals, including humans and cats serve as intermediate hosts. During chronic phase of toxoplasmosis, intermediate hosts may harbour tissue cysts in their various tissues such as, skeletal and heart muscles (Dubey, 2010, chap. 9). *T. gondii* is different from other members of Apicomplexa due to the significant role of carnivorism in transmission of the parasite between the intermediate hosts. Thus, consumption of *T. gondii* tissue cysts harbouring undercooked or raw meat, meat-derived products or offal by humans as well as contact with unprocessed meat is an important route of infection for human toxoplasmosis (El-Tras, Tayel, & El-Kady, 2011; Kijlstra & Jongert, 2008; Tenter, Heckerth, & Weiss, 2000). Toxoplasmosis is generally detected by serological tests in animals and humans (Altintas, 2008; Dubey, 2009; Hutchinson, Wear, Lambton, Smith, & Pritchard, 2011; Ramos et al., 2011; Yildiz, Babur, Kihc, Aydenizoz, & Dalkilic, 2000; Yildiz et al., 2009). However, *T. gondii* seropositivity of animals is not generally sufficient to reveal the risk that those animals do constitute for public health. Sheep and goats may harbour large numbers of tissue cysts in their meats but even though *T. gondii* seropositivity is evident in cattle and buffalo, cysts are rarely found in their meats (Dubey, 2010, chap. 9; Tenter, 2009).

Meat prices are very high in Turkey with regards to USA and EU countries, mainly, due to the ascending animal feed prices. This increase also causes a gradual decrease in animal husbandry and breeding, resulting from low profit margin. This trend has brought forth meat import and thus triggered illegal meat import. Though Turkish government forces destruction of illegally imported meat, not every batch can be caught and continue to present public health concern. In the present study, we aimed to evaluate the presence of *T. gondii* in frozen buffalo meat cuts that were caught after crossing through the Iraqi border.

2. Materials and methods

Hundred gram aliquots from randomly selected spontaneously thawed 20 meat samples, which were imported to Turkey through Iraq border between March and April 2011, were used as materials. All of the samples were taken from batches of “fresh frozen boneless buffalo meat, originated from India” as indicated on their labels on vacuum-packaged plastic bags. Samples were aseptically collected in a sterile plastic bag throughout a vertical cross section...
of each randomly selected spontaneously thawed meat by sterile scalpel and were transported to laboratory in an icebox under cold chain, in less than 12 h. Meat samples were analyzed for presence of T. gondii tissue cysts before and after preservation at –18 °C for two days.

2.1. Isolation of T. gondii tissue cyst

Aliquots of 5 g meat samples from each package (n: 20) were analyzed for the presence of T. gondii tissue cysts as modified from Eggleston, Fitzpatrick, and Hager (2008). Briefly, random pieces of meat samples were cut with a sterile scissor, added to 20 ml of PBS (Amresco) and homogenised with a high speed tissue homogenizer (OMNI Tip, USA). Homogenizer was scrubbed in boiled water with detergent and dipped in absolute ethanol before every consecutive homogenization. The homogenates sieved in a centrifuge tube by a sterile Pasteur pipette were inspected with light microscope (Olympus BX50) for presence of T. gondii tissue cysts.

2.2. Physical characteristics of the buffalo meats

Whole meat cuts were physically inspected for colour, meat consistency, marbling, fat colour, fat consistency, location and carotene content of fat according to Singh and Neelam (2011).

2.3. Agar gel immunodiffusion (AGID) assay

To eliminate the possibility of meats being cattle or horse origin, agar gel immunodiffusion assay with anti-cattle and anti-horse specific serums purchased from Etlık Veterinary Control Central Research Institute, Ankara, Turkey was conducted.

Preparation of tissue antigens and AGID were done as adopted from FSIS (Anonymous, 2005). Briefly, small pieces of meats were mixed with saline (0.85%, NaCl) at a volume of 1:3 in a mortar and thoroughly pestled. Following an incubation of 2 h at 37 °C, mixtures were filtered through Whatman no.4 paper (Whatman Int., Maidstone, UK) into a clean flask. Extracts of buffalo meats and specific anti-serums were charged in appropriately prepared wells with autoclaved cheesecloths. Stock percoll solution (Sigma) was diluted with distilled water and 1.5 M NaCl to 30% and 90%. Mixture of percoll dilutions and the homogenates were centrifuged at 4000 × g for 20 min, and the dilutions taken from the percoll layers by a sterile Pasteur pipette were inspected with light microscope (Olympus BX50) for presence of T. gondii tissue cysts.

2.4. Confirmation of T. gondii by nested PCR analysis

2.4.1. DNA extraction

For genomic DNA isolation, DNeasy Blood and Tissue Kit (Qiagen, Cat No. 69504) was used with a modification, in order to increase the amount of meat sample taken into analyzes. Thus, rather than 25 mg of meat sample, pelleted 1 ml aliquots of PBS-homogenate mixtures that were taken into Eppendorf tubes following a centrifugation (Hettich, Universal 32R, Tuttingen, Germany) at 3000 × g for 10 min were used. One milliliter of each meat homogenates were washed three times with a ml of ultrapure water by centrifugation at 8000 × g for 5 min (Beckman Coulter 22R Centrifuge, Fullerton, CA, USA) for removal of PBS. The washed homogenates were digested in 10 ml of proteinase K (70 mg/ml, AppliChem GmbH, Darmstadt, Germany), 180 μl ATL buffer from extraction kit and 360 μl of ultrapure water at 56 °C overnight in a Thermo-shaker (1500 rpm/min, ALLS, Msc-100, China). Then, DNA extraction was performed with 200 μl of the supernatant, following centrifugation at 12,000 × g for 3 min, according to the manufacturer.

2.4.2. Nested PCR amplification

Nested PCR was performed according to Burg, Grover, Pouletty, and Boothroyd (1989) to confirm the presence of T. gondii B1 gene in the buffalo meat homogenates. While in the first round of nested PCR, 197 bp part of B1 gene of T. gondii was amplified using Toxo 1 for: 5′-GGAACTGACATCCGTATGAG-3′ and Toxo 2 rev: 5′-TTTAAAAAGCCGTGTGTC-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: 3′-TCAGTGGCCAGTCACGTAAG-3′ and Toxo 4 rev: 5′-GCGCAGCACTCCTGGAATAACC-3′ primers. Master mixes of both PCR reactions were consisted of: 1 X PCR Buffer (100 mM Tris–HCl [pH 8.8 at 25 °C], 500 mM KCl, 0.8% Nonidet P40) and 10 μl of template for each round, respectively. Amplifications were carried out in a thermal cycler (Eppendorf Mastercycler Gradient, Hamburg, Germany) and the PCR conditions were consisted of an 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, 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 positive control and ultrapure water as negative control.

2.4.3. Gel electrophoresis

A 10 μl aliquot of each resultant PCR products with 2 μl of loading dye (Promega, Madison, USA) were further analyzed by agarose gel (1.5% Agarose–Basica LE, Prona, Spain) electrophoresis (CST MSMiX-Duo, Corston, UK), stained with 0.1 μg ml−1 ethidium bromide (BioChemica GmbH, Darmstadt, Germany), at 120 V for 30 min and visualized by a gel documentation and analysis system (Sygene Ingenium, Cambridge, UK).

3. Results

3.1. Presence of T. gondii tissue cysts

Tissue cysts of T. gondii were found in 3 of 20 (15%) spontaneously thawed meat samples analyzed (Fig. 1). Observed tissue cysts were spherical and had diameters of 27 μm. The tissue cysts were not observed in the meat samples after preservation at –18 °C for two days. All positive samples were confirmed with detection of 97 bp bands of T. gondii B1 gene by nested PCR (Fig. 2).

3.2. Characteristics of buffalo meat

All of the spontaneously thawed meat samples included in the present study showed physical attributes characteristic for buffalo meat. They were dark red in colour, firm in consistency and showed no marbling. Fat was intermuscularly distributed, slightly firm and pure white in colour with absence of carotene. None of the samples analyzed showed immunoprecipitation lines with anti-cattle or anti-horse serums in AGID assay.

4. Discussion

Buffalo is a valuable animal and is raised mainly in Asia. It has a high dressing percentage and its meat has an increasing demand due to its low fat and cholesterol content (Kandeepan, Biswas, &
With around 1.5 million tonnes of estimated production and exportation of 32% of this production, India takes the first place in the ranking of the world’s buffalo meat production and exportation (Food and Agricultural Organization, 2009). Furthermore, exported buffalo meat has had a calculated annual growth rate around 10% between 1980 and 2007 showing the increasing demand especially in developing countries facing increasing meat prices along with increasing per capita consumption (El-Tras et al., 2011; Soliman & Bassiony, 2010). Moreover, some meat products (i.e. bresaola, salami, cacciatorini etc.) are being produced from buffalo meat in certain countries (Borghese, 2005, chap. 11) and cured meat products are implicated in acute toxoplasmosis, especially in pregnant women (Warnekulasuriya, Johnson, & Holliman, 1998). Since buffalo meat is not regularly consumed in Turkey, possibility of fraudulency and cheaper price with regards to beef could have played a role in illegal import mentioned in the present study.

Fig. 1. Isolated T. gondii tissue cyst. Bar: 30 μm.

Rajkumar, 2009). It was reported that, buffaloes are considered resistant to toxoplasmosis like cattle and tissue cysts are reported to be found rarely in skeletal muscles of buffaloes (Dubey, 2010, chap. 9; Tenter, 2009). Furthermore, even though experimental infections have been shown (Gautam, Chhabra, Gupta, & Mahajan, 1982; Oliviera, Costa, Bechara, & Sabatini, 2001) and seroprevalence of T. gondii in buffaloes is reported between 4.7 and 100% (Chaudhary, Ahmed, Hussain, & Shakoori, 2006; Navipour & Hghooghie-Rad, 1998; Selvaraj, Murali, Sarman, & Balachandran, 2007), to our knowledge, there has been one report on prevalence of T. gondii tissue cyst in both fresh buffalo meat and imported frozen buffalo meat (El-Tras et al., 2011). Thus, encountering tissue cysts of T. gondii in 3 out of 20 (15%) imported frozen buffalo meat samples analyzed was surprising.

Buffalo meat is exported either fresh or frozen (Biswas et al., 2008; Kandeepan et al., 2009) and freezing can either be quick/fast; within 30 min using frigid blasts of air or immersion in refrigerants, or slow, within 3–72 h aiming a decrease of foods' internal temperature to about –20 °C (Jay, Loessner, & Golden, 2005, chap. 16). It was reported that, buffalo meat produced for export are mainly blast frozen in India (Biswas et al., 2008). T. gondii tissue cysts are highly susceptible to low temperatures and lose their viability at temperatures below –12 °C (Djurkovic-Djakovic & Milenkovic, 2000; El-Nawawi, Tawfik, & Shaapan, 2008; Kotula et al., 1991; Lunden & Uggla, 1992). In our study, all of the examined meats had been frozen upon being caught and left for thawing in order to collect the samples. Having analyzed before a preservation of 2 days at –18 °C, we have found intact tissue cysts. Following the preservation however, no intact tissue cysts were found in accordance with previous reports. Thus, former observation can either be attributed to an insufficiency in freezing technology used by the producer, since marginally frozen meats did not guarantee the destruction of the tissue cysts, or strain specific resistance (Kotula et al., 1991; Tenter, 2009). El-Tras et al. (2011) evaluated both fresh and imported frozen buffalo meat for presence of T. gondii with bioassay and found 15.4% and 0% of prevalence, respectively. They concluded that the imported frozen buffalo meat is safe compared to fresh buffalo meat. Nevertheless, it is alarming for public health that imported frozen buffalo meat actually may be a source of T. gondii infection due to the intact tissue cysts that tolerate freezing.

Surveys relying on detection of T. gondii DNA from meat and meat products of various animal origins by PCR methods have extensively been reported (Ergin, Ciftcioglu, Midilli, Issa, & Gargili, 2009; Jauregui, Higgins, Zarlenoga, Dubey, & Lunney, 2001; Warnekulasuriya et al., 1998). Acknowledging the false-negative results that may rise due to non-homogenous distribution and low numbers of T. gondii tissue cysts per gram (Dubey et al., 1996), we modified the amount of meat used for DNA extraction in order to confirm our results with nested PCR of the repetitive B1 gene which was proved to be more sensitive than other targeted genes (Mason, Quinnell, & Smith, 2010). When relatively insufficient amount of samples (25 mg) taken into analyzes, we failed to confirm (data not shown) the presence of T. gondii DNA, while increasing the amount (250 mg/ml of PBS-meat homogenate) achieved the PCR products of 97 bp.

5. Conclusions

Our present study shows that buffalo meat may harbour viable T. gondii and serve as a potential infection source. To our knowledge, this is the first study that reports presence of T. gondii tissue cysts in imported frozen buffalo meats. While the world is facing ascending volumes of trade in buffalo meat, presence of T. gondii tissue cysts in frozen buffalo meat, either due to insufficiency of
freezing technology used or strain tolerance, is alarming. Thus, further studies on tolerance of T. gondii tissue cyst in frozen buffalo meat as well as meat products or mixtures derived from frozen buffalo meats should be conducted in order to regard frozen buffalo meat as safe.

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