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Toxoplasma gondii in sheep and goat livers: Risks for human consumption

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ABSTRACT. Toxoplasmosis is one of the most important zoonotic diseases worldwide and is caused by the protozoan *Toxoplasma gondii*. Besides vertical transmission during pregnancy, humans can become infected post-natally either by oral uptake of sporulated *Toxoplasma* oocysts or through ingestion of tissue cysts upon consumption of raw or undercooked meat. The aim of this study was to approximate the risk of human infection via liver consumption by estimating the seroprevalence and molecular prevalence of *T. gondii* in slaughtered sheep and goats in Iran. In the present study, livers from 150 sheep and 150 goats were collected at slaughter. In- house enzyme-linked immunosorbent assay was performed in *T. gondii* liver juice. Parasite-specific polymerase chain reaction was carried out on all samples obtained from liver tissues. Antibodies against *T. gondii* were detected by in-house ELISA in 32.6% sheep and 48% goat livers and 8% and 11.3% of sheep and goat livers were positive for the presence of *T. gondii* DNA, respectively. The results of this study provide baseline information on the presence of *T. gondii* in sheep and goats livers and imply an important human health and hygienic risk associated with the consumption of raw or undercooked liver from these animal species.

Keywords: *Toxoplasma gondii*, Liver, In house ELISA, PCR, Sheep, Goat

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INTRODUCTION

The apicomplexan parasite *Toxoplasma gondii* is a prevalent pathogen in wild and domestic animals worldwide, and is transmitted through the food chain by carnivorous feeding and scavenging (Dubey & Jones, 2008). It can be transmitted not only between intermediate and definitive hosts (sexual cycle) but also between intermediate hosts via carnivores (asexual cycle) or even between definitive hosts (Barros et al., 2018). Up to one- third of the human population in the world is chronically infected and toxoplasmosis has been considered one among the five parasitic diseases of priority for public health action (Montoya & Liesenfeld, 2004). Humans can become infected through three routes: (i) uptake of sporulated oocysts from the environment, (ii) consumption of raw or undercooked meat containing tissue cysts and (iii) pre-natal infection (Dubey, 2010). Infections in humans are primarily asymptomatic, but lymphadenopathy or ocular toxoplasmosis may occur in some patients. *T. gondii* infection in pregnant women may lead to abortion, stillbirth, or other serious consequences in newborns (Weiss & Dubey, 2009). In immunocompromised patients, toxoplasmosis can be fatal if left untreated, and the reactivation of a latent infection can cause life- threatening encephalitis (Machala et al., 2015). Among food animals, sheep and goats are well-known sources of human infection. The European Food Safety Authority (EFSA) has recognized toxoplasmosis as the parasitic zoonoses with the highest human incidence and has recently published a scientific opinion that clearly states the need of representative data on toxoplasmosis in Europe (EFSA, 2007). Furthermore, different options of obtaining *Toxoplasma*-free meat are being discussed since 2008 (Kijlstra & Jongert, 2008). *Toxoplasma* infection in sheep and goats at slaughter cannot be diagnosed because infected animals are asymptomatic and the cysts are too small to be visually detectable during meat inspection. Other rapid, reliable and cost-effective control methods for large-scale monitoring and surveys are therefore required. Serological testing of toxoplasmosis has been shown to be the most practical method for monitoring the exposure status of farms and the efficacy of the implemented control measures. Various serological methods, such as the modified agglutination test (MAT), immunofluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), have been used to detect *Toxoplasma* IgG antibodies in blood and tissue samples (Basso et al., 2013). ELISA tests, which can be semi-automated, are cost-effective and convenient diagnostic tools for large-scale screening. Serological studies confirmed that meat juice serology has proven to be an excellent method of detection for *T. gondii* infection at slaughter in different species, including sheep, pigs, poultry, and has been proved to correlate well with serum serology (Basso et al., 2013; Meemken et al., 2014; Bacci et al., 2015). Several molecular tests to detect parasites have been developed in the last decade. Their specificity and sensitivity have gradually increased, and parasitic diseases that were previously difficult to diagnose using conventional techniques have begun to be identified by molecular techniques (Bastein, 2002). In some countries, people based on ancient beliefs consume liver for iron supplementation and elimination of anemia. Accordingly, pregnant women in Iran consume liver (especially undercooked) during their pregnancy. Due to the risk of *Toxoplasma* infection from undercooked liver consumption, the aim of the present study was therefore to evaluate Toxoplasma infection of sheep and goats liver using juice serological and molecular methods in livers, which can be conveniently collected at slaughter.

MATERIALS AND METHODS

Collection of samples

During a six month period (December 2016-May 2017), a slaughterhouse in the South-western part of Iran (Ahvaz, Khuzestan province) was visited once a week and liver samples were collected randomly from a total of 150 sheep and 150 goats. Immediately after opening the carcass, liver samples were obtained with sterile single use surgical blades and transferred into sterile plastic bags. A two-gram piece of each liver sample was aseptically transferred to a sterile 50 ml falcon tube and gently crushed using a sterile glass rod. Then 18 ml of sterile physiological saline solution was added to the falcon and vortexed for 20 min. The homogenates were filtered through two layers of sterile gauze and centrifuged at 4000 rpm for 30 min. The

supernatants were discarded, and the resulting pellets were suspended in 1 ml of sterile physiological saline solution and divided into two microcentrifuge tubes for serological and molecular study.

Liver juice serology

The assay was optimized using formalin-fixed *T. gondii* tachyzoites. The optimum dilutions were determined by checkerboard titration of antigen, serum and conjugate. Microplates (Greiner, Germany) were coated with 50 µL of tachyzoites of *T. gondii* RH strain (approximately 105 tachyzoites), diluted 1:50 in carbonate buffer (pH = 9.6), and then incubated overnight at 4°C. The plates were washed three times with 300 µL PBS containing 0.1% Tween 20 and blocked with 5% nonfat dry milk for 60 min at 37°C. After washing, 100 µL of liver juice sample, diluted 1:10 in PBS, was added and the microplate was incubated for 120 min at 37°C. After rinsing as above, 50 µL of alkaline phosphatase-labeled anti-goat/sheep conjugate (Sigma-Aldrich), diluted 1:500 in PBS was added to the wells, and then the microplates were incubated at 37°C for 60 minutes. Following washing, 50 µL of substrate solution (10 mg/mL 4-nitrophenylphosphate in 10 ml diethanolamine buffer, pH = 9.6) was added and the microplate was left for 30 min at room temperature. The reaction was stopped with 50 µL of 20% hydrochloric acid, and the optical density at 450 nm was read in an ELISA reader (ELX800-Biotec). The cutoff value of optical densities (OD) was determined by the method of Hillyer et al. (1992): the mean OD of negative control sera plus two standard deviations.

MOLECULAR STUDY

For DNA extraction, an aliquot of the resuspended pellet (500 µl) was transferred to a microcentrifuge tube and centrifuged at 12000 rpm for 5 min. The pellet was suspended again in 200 µl lysis buffer, stored at room temperature for 30 min and then heated at 100 °C for 10 min. Subsequently DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitatation with ethanol and resuspension in sterile distilled water. The extracted DNA was frozen until PCR analysis. For detection of T. gondii, primers targeting B1 gene were selected from the literature (Jalal et al., 2014). Primers used in the reaction were the forward primer with the sequence 5'-GAGACCGCGGAGCCGAAGTGC-3' and the reverse primer with the sequence 5'-CCTCCTCCTCCCTTCGTCCAAG-3', yielding a 469 bp product. All PCR were performed in 25 µl reactions, containing 12.5 µl Taq DNA polymerase master mix Red (Amplicon, Denmark), 1 µM primers and 50 ng DNA templates. PCR cycling included an initial denaturation at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 45s, annealing at 52ºC for 45s, extension at 72ºC for 60s. This was followed by a final extension at 72ºC for 5 min. PCR reactions included a negative control, consisting of the reaction mix and 2 µl of DNase/RNase-free water instead of DNA and a positive control consisting of DNA sample from the *T. gondii* tachyzoites (RH strain). PCR products were electrophoresed in 1.5% agarose (SinaClon Bioscience, Iran) in Tris–acetate–EDTA (TAE) buffer, stained with Green Safe stain (SinaClon Bioscience, Iran) and visualized under ultraviolet light. Positive samples showed a band of approximately 469 bp.

RESULTS

Based on the results of liver juice serology 49 sheep (32.6%) and 72 goats (48%) were positive for *T. gondii,* respectively. In 12 (8%) samples of 150 examined sheep livers, the PCR was positive and a band of approximately 469 bp was observed on the agarose gel which was considered as infection with *T. gondii* (Fig 1). Furthermore, parasite DNA amplification was obtained in 17 out of 150 (11.3%) goat livers. Table 1 represents frequency of *T. gondii* in sheep and goats livers as found by liver juice serology and molecular method.

After comparing the results of sheep liver juice serology and PCR, it was found that ten livers were positive in both methods while two livers were diagnosed PCR positive and serologically negative. Also, 39 livers were serologically positive but their PCR results were negative.

Comparing the results of goats' liver juice serology and PCR, it was demonstrated that 13 livers were serologically and molecularly positive for *T. gondii.* While four livers were only PCR positive, 59 livers were found positive only by juice serology method.

Table 2 represents results of liver juice serology and PCR in sheep and goats.

DISCUSSION

Foodborne diseases are caused by a number of agents, varying in severity from weak to chronic or acute disturbances that can affect or compromise the life of the consumer, and the agents of biological origin (bacteria, viruses, parasites) are the major cause of these diseases. Parasites, including *T. gondii*, are reported less frequently in humans, and have caused fewer outbreaks than bacteria and viruses. However, in many instances, their impact (severe illness, disability, death, and costs related to diagnostic procedures, hospitalization and treatment) on vulnerable groups of the population, and often in immunocompetent people, has been considerable (EFSA, 2007). Small outbreaks of toxoplasmosis have been associated with the consumption of raw meat in Korea, USA, France, French Guiana and New Zealand (Kijlstra & Jongert, 2008). Most farm animals that are naturally infected with *T. gondii* have been shown to carry infectious parasites in their meat. There are several new ready-to eat smallgoods which are meat products that may represent a source of *T. gondii* infection (Mie et al., 2008). In Iran, consumption of undercooked liver is common in pregnant women and since congenital toxoplasmosis can cause serious health problems in the fetus, the aim of this study was therefore to evaluate *Toxoplasma* infection in sheep and goats liver. Bioassay and molecular biology tests used to search for cysts in meat are complex and time consuming, due to the uneven distribution of cysts in the carcass, which often results in false negative results (Lundén et al., 2002). Antibodies usually reflect the exposure of the hosts to the parasite and could also reflect the infective status of meat (Dubey et al., 2008). ELISA is a large scale, simple and sensitive serological assay method that is useful for the surveillance and control of toxoplasmosis (Ferguson et al., 1989). This diagnostic test has been conducted for sanitary control using serum samples taken from slaughtered animals, despite presenting a sampling associated problems. This preventive approach is safe and useful, but frequently the only available sample for testing is already meat, which contains exudates, formed after the retail processing, and consisting mainly of blood and interstitial fluid. As reported elsewhere, there is a good correlation between ELISA results obtained for anti-*T. gondii* antibodies detected in meat juices and in serum samples (Lundén et al., 2002; Wingstrand et al., 1997). In the present study, an ELISA test was designed for detection of anti-*T. gondii* specific IgG in sheep and goat livers, using liver juices in order to determine the presumptive risk for consumers, acknowledging at the same time that seropositivity does not directly imply infectivity. Based on the liver juice ELISA results, 49 sheep (32.6%) and 72 goats (48%) were positive for *T. gondii*, respectively. Liver juice is a matrix easily available from sheep and goats at slaughter, and can also be used for the detection of other public health hazards such as Salmonellae, pathogenic Yersiniae and etc. Since it seems that the levels of *T. gondii* specific IgG are lower in liver juice compared to serum and to compensate this, a lower dilution factor was used for the liver juice samples. In accordance with the approach described by Wingstrand et al. (1997), who found excellent correlations for *Toxoplasma* antibodies between meat juice and serum, liver juice samples were 10 times less diluted than serum samples. Briefly, liver juice samples were diluted 1: 10. The overall estimate of seroprevalence of *T. gondii* in sheep and goats was generally high. Nevertheless, this count does not necessarily represent a hazard for the population as a seropositive animal does not compulsively harbour active tissue cysts with infective parasites (de A Dos Santos et al., 2005; Halos et al., 2010). For the parasite detection we used primers targeting B1 gene (a marker of *T. gondii*) as they were described as the sensitive ones even in tissues (Martínez-Flores et al., 2017). PCR can be considered as a useful method to assess *T. gondii* prevalence in tissues. Based on PCR results, parasite DNA was detected in 8% and 11.3% of sheep and goat livers in the present study. The presence of DNA shows that the meat originates from a *Toxoplasma*-infected animal but this does not necessarily mean that the product contains infectious organisms. In China, liver, lung and lymph nodes from 403 Yunnan black goats were

collected randomly from different administrative regions in Yunnan province, and B1 gene was identified using PCR in 20 (5%) animals (Miao et al., 2015).

After comparing the results of sheep liver juice serology and PCR, it was found that ten livers were positive in both methods while two livers were diagnosed PCR positive and serologically negative. Furthermore, 39 livers were serologically positive but their PCR results were negative. Comparing the results of goats' liver juice serology and PCR demonstrated that 13 livers were serologically and molecularly positive for T. gondii. While four livers were only PCR positive, 59 livers were found positive only by juice serology method. Table 2 represents results of liver juice serology and PCR in sheep and goats. Recent infection with no pervious exposure may explain negative ELISA samples with positive PCR results.

The distribution of *T. gondii* parasites within the same tissue is random, and parasite density may be low. Therefore, a negative result has to be interpreted carefully due to the possibility that the parasite could be present in unexamined parts of the target tissue. The mentioned explanation can be considered for positive ELISA samples with negative PCR results.

Finally, since there is a globalization in the trade of animals and food worldwide, rules of trading meat and meat products ought to guarantee that all imports and exports fulfill high standards that ensure food safety. These rules should also be extended to animal health status and high standards of meat and meat products in order to avoid human toxoplasmosis. Besides measures focusing on pre-harvest food safety (e.g. surveillance and monitoring in animals), post-harvest strategies at slaughter and during food processing have become increasingly important in recent years. With regard to meat processing, demands of consumers for pathogen free meat products have focused the attention of meat industry on food safety and the necessity to produce meat that is wholesome, safe, and of high quality, using the appropriate technological treatments. Furthermore, liver should be considered as a potent source for *Toxoplasma* infection.

CONCLUSIONS

Overall, the results of our study show that Toxoplasma infections are prevalent in sheep and goats livers. Pregnant women who are found seronegative for *T. gondii* and other susceptible categories of people in Iran should therefore be very careful when preparing and consuming liver and they should make sure that they strictly apply to the recommendations for food hygiene and safety, such as washing hands after the preparation of liver and consuming only well-cooked liver.

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CONFLICT OF INTEREST

There is no conflict of interest.

Figure 1. Amplication of *T. gondii* DNA. Lane M is a 100-bp ladder. Lane P is a positive control DNA lane. Lane N is a negative control DNA lane. Lanes 1,3 represent negative samples. Lanes 2 represents a positive sample.

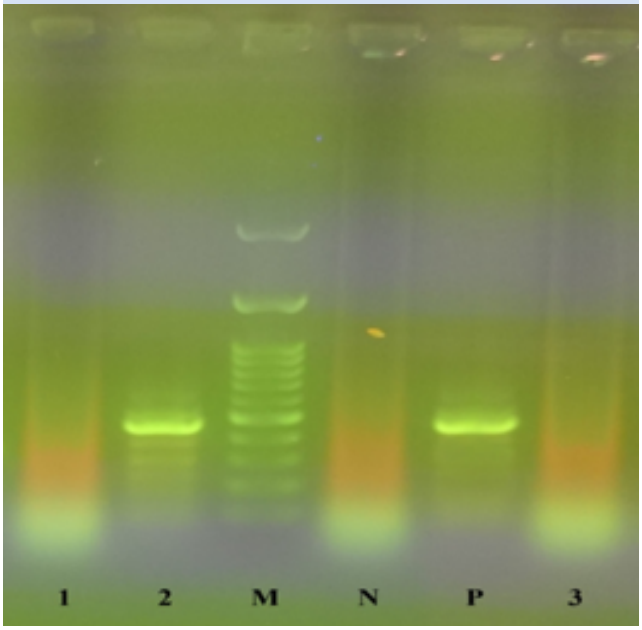

Table 1. Liver juice in- house ELISA and PCR results for *T. gondii* infection.

		Positive (%)	Negative (%)	No. examined
ELISA	Sheep	49 (32.6)	101 (67.4)	150
	Goat	72 (48)	78 (52)	150
PCR	Sheep	12 (8)	138 (92)	150
	Goat	17 (11.3)	133 (88.7)	150

Table 2. Comparison of results of liver juice serology and PCR in sheep and goats.

			Liver juice ELISA	
Test	Result	No/Total	+	-
Sheep- PCR	+	12/150	10	2
	-	138/150	39	99
Goat- PCR	+	17/150	13	4
	-	133/150	59	74

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