

## ***Toxoplasma gondii* infection in slaughtered ewes in Khorramabad, western Iran: A preliminary molecular study**

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### **Key words:**

frequency, PCR, protozoa, sheep, *Toxoplasma gondii*

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### **Abstract:**

**BACKGROUND:** The parasitic protozoa *Toxoplasma gondii* is widely prevalent in humans and warm-blooded animals. Humans are usually infected with *T. gondii* by ingesting oocysts shed by cats or by ingesting viable tissue cysts present in raw or undercooked meat. **OBJECTIVES:** This preliminary study was conducted to assess the frequency of *Toxoplasma gondii* infection in tissue samples of ewes slaughtered in Khorramabad, in the west of Iran. **METHODS:** We examined the brain tissue, diaphragm, tongue and masseter muscles of 30 ewes. A nested-PCR which targets the 25-50 copies of B1 sequence has been used for tissue samples. **RESULTS:** The parasite was identified in 21 brain samples (70%) and 8 muscle samples (26.6%). Twenty-three sheep (76.6%) were infected with *T. gondii*. **CONCLUSIONS:** *T. gondii* might be considered as one of the major causes of ovine abortion in this region. According to the result, edible parts of sheep may play a greater role as a source of infection for individuals living in this area.

### **Introduction**

*Toxoplasma gondii* is a cosmopolitan zoonotic parasite, which is widely prevalent in humans and other warm-blooded animals. This parasite is an important cause of abortion in sheep and is a significant cause of economic loss to sheep industry. Furthermore, *T. gondii* infection is listed as the third-biggest cause of life threatening food-borne diseases (Kravetz and Federman, 2005). Up to one-third of the human world population is chronically infected with this obligate intracellular protozoan parasite (Tenter et al., 2000). The life cycle of *T. gondii* is facultatively heteroxenous and divides into an asexual cycle with little host specificity and a sexual cycle resulting

in the production of oocysts by cats (Tenter et al., 2000). Humans are usually infected with the parasite as a result of consuming raw or undercooked meat containing viable tissue cysts (Dubey, 2009).

Sheep meat is an important source of food for humans living in developing countries such as Iran. Numerous studies on prevalence of *T. gondii* in sheep have been carried out in different countries. Most of these studies have revealed a wide variation in the prevalence rates ranging between 3% and 95.7% within and across different countries (Dubey, 2009). In general, the variation in the prevalence rates among different regions is related to several factors such as the presence of oocysts (density of stray cats or wild felines), climatic condi-

tions, farm management, age distribution as well as the different techniques employed for the sampling and diagnosis (Bayarri et al., 2012; Silva et al., 2013; Tenter et al., 2000).

To our knowledge, little is known about the prevalence and molecular detection of *T. gondii* in sheep in Khorramabad, in the west of Iran. Therefore, the present preliminary study was conducted to assess the frequency of infection in several tissue samples of ewes slaughtered for food in Khorramabad.

**Materials and Methods**

**Sample collection:** Samples were collected randomly from Khorramabad slaughterhouse, during a three month period, between April and June 2015. Totally 60 tissue samples were obtained from 30 slaughtered ewes, directly from skulls and diaphragms. Brain samples and meat samples (containing diaphragm, tongue and masseter muscles) were removed from each examined sheep. The tissue samples were immediately transported to the laboratory and kept at 4 °C until further processing. Firstly, 50 g each of meat and of brain were separately minced into small pieces. For each sample, freshly cleaned instruments were used to prevent crossover contamination of DNA. Subsequently, 1 g of each minced sample was thoroughly powdered under liquid nitrogen, transferred to a 1.5 ml sterile tube and stored at -20 °C until DNA extraction.

**DNA extraction:** DNA extraction was performed using a commercial DNA isolation kit (MBST, Iran) following the supplier’s instructions. Briefly, 180 µl lysis buffer and 20 µl proteinase K were added per 50 mg powdered tissue sample, followed by incubation at 55 °C as long as no vis-

Table 1. Results of B1 gene detection in different tissue samples. \*Mix of diaphragm, tongue and masseter muscles.

Tested samples	Nested-PCR positive samples		Nested-PCR negative samples		Total
	No.	(%)	No.	(%)	
Brain	21	(70)	9	(30)	30
Meat*	8	(26.7)	22	(73.3)	30
Total	29	(48.3)	31	(51.7)	60

Table 2. Percentage and number of infected tissues in examined ewes. \*Mix of diaphragm, tongue and masseter muscles.

Infected tissues	No.	Percentage
Brain	15	50%
Meat*	2	6.6%
Mixed infection (brain & meat)	6	20%
Total	23	76.6%

ible indigested fibers were observed. After adding 360 µl binding buffer and incubation for 10 min at 70 °C, 270 µl ethanol was added to the solution and mixed by gentle vortexing. Subsequently, the complete volume was transferred to the MBST column. The MBST column was first centrifuged and then washed twice with 500 µl washing buffer. Finally, DNA was eluted from the carrier with 60 µl elution buffer and stored at -20 °C.

**Nested PCR:** A nested-PCR which targets the 25-50 copies of B1 sequence has been used for all DNA samples. PCR reaction was performed as previously described using specific primers P1 (5’ GGAAGTGCATCCGTTTCATGAG 3’) and P4 (5’ TCTTTAAAGCGTTCGTGGTC 3’) to amplify a 193 bp fragment of the B1 gene. The internal primer pairs used for secondary round were P2 (5’ TGCATAGGTTGCAGTCACTG 3’) and P3 (5’ GGCGACCAATCTGCGAATACACC 3’) to amplify a 94 bp DNA fragment (Wastling et al., 1993).

Nested-PCR was carried out on 2-5  $\mu$ l of DNA solution as template for the initial PCR reaction and 1  $\mu$ l of each PCR product as template for the second round in a total volume of 50  $\mu$ l, including 5  $\mu$ l PCR buffer (10X PCR buffer), 1.5 U Taq polymerase, 2 mM MgCl<sub>2</sub>, 0.2  $\mu$ M each primer (SinaClon, Iran) and 100  $\mu$ M of each dNTP (Fermentas, Germany), using a Primus thermal cycler (MWG-Biotech, Germany). The following program was performed for each PCR round: initial denaturation at 94 °C for 5 min; 35 cycles of 15 s at 94 °C, 30 s at 56 °C and 45 s at 72 °C; final extension at 72 °C for 5 min (Mason et al., 2010).

In each PCR experiment DNA isolated from *T. gondii* (RH strain, obtained from Pasteur Institute of Iran) was used as positive control template and also sterile distilled water was used as negative control template. PCR runs in which a negative control indicated contamination were discarded.

## Results

From 60 tissue samples that were examined by two pairs of primers in two rounds of amplification, 29 samples (48.3%) were positive. *Toxoplasma* DNA was detected in 21 (70%) brain samples and 8 (26.7%) meat samples (Table 1). In the first round, the amplified DNA fragment (193 bp) was not always visible. Tissue samples were considered positive if the secondary round band (94bp) appeared (Fig. 1).

Results of B1-PCR revealed the presence of *T. gondii* in 76.6% (23 out of 30) of the examined ewes. In 15 ewes (50%), *Toxoplasma* DNA was detected only in brain. Mixed infection of meat and brain tissues was found in 6 animals (20%). In 2 ewes

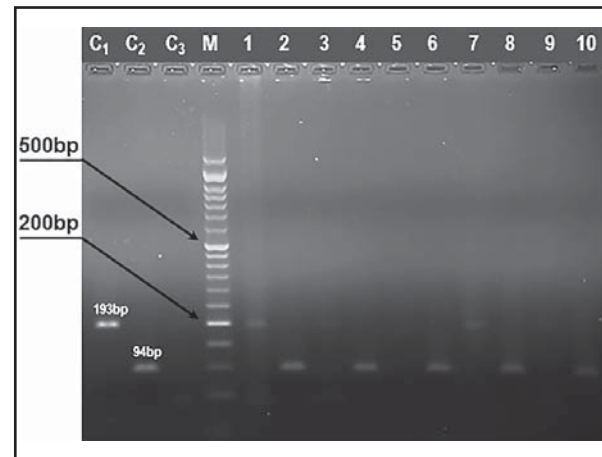


Figure 1. Electrophoretic separation of nested B1-PCR products. C1: positive control (RH strain, first round); C2: positive control (second round); C3: negative control (distilled water, second round); M: DNA ladder (50 bp); lanes 1, 3, 5, 7 and 9: tissue samples (first round, 193 bp which was not always visible); lanes 2, 4, 6, 8 and 10: positive tissue samples (second round, 94 bp).

(6.6%), *Toxoplasma* DNA was found only in muscles (Table 2). The results of present study showed that the nested-PCR is very sensitive to detection of parasite DNA, especially in samples harboring low amount of parasite.

## Discussion

Ovine meat is the most common red meat type consumed in Iran as well as many other Muslim countries. Therefore, edible parts of sheep probably should be considered as the main sources of *Toxoplasma* infection in humans living in these countries. Results from the present study indicate that 76.6% of the examined sheep are infected with *T. gondii* which currently appears to be the highest infection rate reported in Iran.

These results may reflect some epidemiologic factors such as traditional sheep farming, wide distribution of stray cats and high relative humidity which can affect on the viability and sporulation of oocysts. In contrast, prior studies have shown that these

factors are common in some other regions of the country with lower prevalence rates. In this regard, Asgari et al. (2011) analyzed several tissue samples taken from 56 sheep in Fars province (South Iran) and found that only 21 sheep (37.5%) were *Toxoplasma* DNA positive.

The majority of reports relating to the prevalence of the parasite are achieved from serological methods. High seroprevalence levels in mature sheep were found in the USA (65.5%) (Dubey and Kirkbride, 1989), France (65.6%) (Dumètre et al., 2006), Scotland (73.8%) (Katzner et al., 2011), Serbia (84.5%) (Klun et al., 2006) and Turkey (88%) (Yildiz et al., 2014). Similar seroprevalences were obtained in previous studies performed in Ahvaz, southwestern Iran (72.6%) (Hamidinejat et al., 2008) and Roudsar, northern Iran (62.2%) (Havakhah et al., 2014); while lower values (from 21.1% to 35%) were observed in some other regions of the country (Raeghi et al., 2011; Sharif et al., 2007). These differences can be attributed mainly to the age of the animals (Asgari et al., 2011; Dubey and Kirkbride, 1989; Dumètre et al., 2006; Halos et al., 2010; Katzner et al., 2011). Berger-Schoch et al. (2011) reported the seropositivity of animals slaughtered for meat production in Switzerland and found that increasing age of the sheep is a more important risk factor than housing conditions. In this study, all tissue samples were obtained from mature ewes that were older than two years. These results are expected because aged animals are exposed to oocysts for longer periods.

The difference in frequency among the present and previous studies may be partly due to the different sample preparation methods used for the PCR. In this study, each tissue sample (up to 50 mg) was taken

from the homogenate of a large sample (50 g) containing brain or a pool of diaphragm, tongue and masseter muscles to increase the chance of detecting the parasite in each animal. In this respect, our findings seem more comprehensive than those of some previous studies in which samples were obtained by direct sampling and/or from only one sheep's tissue.

Another explanation for these differences is the employment of different techniques for *T. gondii* DNA detection. It is well known that DNA extracts from tissues contain a high ratio of host:parasite genome, which might lead to inhibition of the PCR. In the present study, a second PCR cycle using nested-PCR primers was performed to increase the probability of isolating parasite DNA. A number of PCR assays based on different target genes, including B1, ITS1, SAG1, 3'SAG2, 5'SAG2, SAG3 and 529 bp repeat element, have been used for the detection of *T. gondii* in different tissue samples (Aspinall et al., 2002; Burrells et al., 2013; Homan et al., 2000; Jalal et al., 2004). Some previous studies have shown that the B1-PCR is more sensitive than other PCR assays (Mason et al., 2010; Wastling et al., 1993).

The present study showed that the *Toxoplasma* infection was significantly high in the brain samples (70%) compared to the meat samples (26.6%). Sheep brain is occasionally consumed by Iranian consumers and it is often well cooked. Therefore, consumption of undercooked meat is a more potent risk factor than consumption of brain. It is now widely accepted that ingestion of inadequately cooked meat is a major source of infection for humans in the United States and Europe (Cook et al., 2000; Dubey et al., 2011). It has been estimated that up to 63%



of *Toxoplasma* infections in pregnant women in Europe could be attributed to meat consumption (Cook et al., 2000). However, the importance of ingestion of infected meat in the epidemiology of human toxoplasmosis in Iran is unknown.

In the period of 2006 to 2007, a serological study of 390 pregnant women was performed in Khorramabad. Anti-*T. gondii* IgG antibodies were identified in 31%, which was higher than the country's average level (Cheraghipour et al., 2010). These results are somewhat in line with our findings. However, the study does not contain any information to show that the infection was acquired by ingestion of undercooked meat.

Taken together, our findings suggest that the range of problems associated with toxoplasmosis in humans and sheep in Khorramabad is higher than what has been thought so far. The high frequency of infection among examined ewes indicates that edible parts of sheep may play a greater role as a source of infection for individuals living in this area. Furthermore, *T. gondii* is might be considered as one of the major causes of ovine abortion in this region.

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## آلودگی به توکسوپلازما گوندی در میش‌های کشتار شده در خرم‌آباد، غرب ایران: مطالعه مولکولی مقدماتی

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### چکیده

زمینه مطالعه: تک‌یاخته انگلی توکسوپلازما گوندی به طور گسترده‌ای در بین انسان و حیوانات خونگرم شایع است. انسان معمولاً بدنبال بلع اووسیست‌های دفع شده توسط گریه و یا بلعیدن کیست‌های نسجی زنده موجود در گوشت خام یا نیم‌پز به توکسوپلازما گوندی آلوده می‌شود. هدف: این مطالعه مقدماتی با هدف بررسی فراوانی آلودگی به توکسوپلازما گوندی در نمونه‌های بافتی میش‌های کشتار شده در خرم‌آباد واقع در غرب ایران صورت گرفت. روش کار: در این تحقیق بافت مغز، دیافراگم، زبان و عضلات جوشی ۳۰ رأس میش مورد بررسی قرار گرفت. از nested-PCR با هدف قرار دادن توالی B۱ دارای ۵۰-۲۵ تکرار برای نمونه‌های بافتی استفاده شد. نتایج: انگل در ۲۱ نمونه مغز (۷۰٪) و ۸ نمونه عضله (۲۶/۶٪) شناسایی گردید. بیست و سه گوسفند (۷۶/۶٪) آلوده به توکسوپلازما بودند. نتیجه‌گیری نهایی: توکسوپلازما گوندی را می‌توان به عنوان یکی از عوامل مهم سقط جنین گوسفند در این ناحیه در نظر گرفت. براساس نتایج، ضمایم خوراکی گوسفند می‌توانند به عنوان یک منبع آلودگی نقش مهمتری برای افراد ساکن در این ناحیه ایفا کنند.

واژه‌های کلیدی: فراوانی، PCR، تک‌یاخته، گوسفند، توکسوپلازما گوندی