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## **A Serological and Molecular study on *Toxoplasma gondii* infection in sheep and goat in Tabriz**

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### **ABSTRACT**

Toxoplasmosis is an important zoonotic diseases in human and animals. The disease caused by the protozoan *Toxoplasma gondii*. To determine the infection rate of toxoplasmosis in sheep and goats in east Azarbaijan province of Iran a total of 186 sera, 13 fetal brains, 13 cotyledons and 34 whole blood samples were collected from sheep and goats in Tabriz abattoir during the year 2010. Serum samples were tested for IgG antibodies against *Toxoplasma gondii* by Enzyme Linked Immunosorbent Assay (ELISA). This test is used in conjunction with direct and nested PCR techniques for detection of *T. gondii* DNA in blood, cotyledon and brain of fetuses, using 4 pairs of universal and specific primers, 18SrRNA, ITS-1, (Tg1,Tg2) and (Tg3,Tg4). Antibodies against *T. gondii* were found in 18.3% serum samples. DNA of *T. gondii* was detected in 69% of fetal brains, 23% of cotyledons and 14.7% of blood samples. The prevalence rate of *T. gondii* in sheep was 24.8% and in goats was 10.6% and the significant difference was observed between sheep and goats groups ( $p \leq 0.05$ ). The prevalence of toxoplasmosis antibodies was significantly higher in adult sheep and goats than younger animals. There was significant difference between male (10.5%) and female (19.2%) sheep and goat was observed ( $p \leq 0.05$ ). Detection of *T. gondii* DNA using PCR minimizes the problems which the researcher may face when using serological methods and facilitates diagnosis in complex cases. The presence of *T. gondii* in blood, fetal tissues and neonatal specimens strongly suggests active and or congenital infection.

**Keywords:** *Toxoplasma gondii*, ELISA, PCR, sheep, goat, Iran.

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### **INTRODUCTION**

Toxoplasmosis is a parasitic disease of mammals, birds and reptiles affecting primarily the central nervous system and also reproductive system and

sometimes visceral organs (Dubey 2004). Toxoplasmosis is one of the most important zoonotic diseases in human and animals (Lappin 2006). The disease caused by the protozoan *Toxoplasma gondii*. All felids family, mostly the common domestic cats, are the definitive host of the parasite. A broad spectrum of animals can be infected by ingestion of raw or

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undercooked meat containing viable tissue cysts or by ingesting food or water contaminated with oocysts from the feces of infected cats (Dubey 2004, Lake *et al* 2002). Cats complete the coccidian life cycle and pass environmentally resistance oocysts in their feces. (Frenkel & smith 1982, Dubey & Beatie 1988, Malmasi *et al* 2009). Another known mode of transmission of *Toxoplasma* in sheep and goat is vertical transmission from pregnant animal to the fetus (Dubey 2004). The majority of infections are asymptomatic and inapparent or latent, but in sheep and goat clinical toxoplasmosis most often reported. Infections during pregnancy can cause abortions, stillbirths, mummification or resorption of the fetus (Nurse 1986). Congenital infected lambs have shown clinical signs of incoordination, weakness and a high mortality rate. Human toxoplasmosis can be acquired both through ingestion of sporulated oocysts and via ingestion of bradyzoites in the tissues of numerous food animals. The infection is also transmitted transplacentally (Dubey 2004, OIE 2008).

The diagnosis of *Toxoplasma* infection is conventionally made by the direct demonstration or isolation of the parasite from biopsy or autopsy material, but such techniques are unsuitable for use in large-scale surveys. Therefore, recourse has been made to immunoserological tests for specific host antibody, and a variety of tests such as PCR has been described (Anderson & Remington 1975, Jauregui *et al* 2001). Numerous reports from various countries exist on the prevalence of toxoplasmosis. It is estimated that 35-40% of the world's adult population have been infected with *T. gondii* (smith, 1991). The prevalence rates in sheep and goats have been varied among countries, as have the diagnostic method used (Hashemi-Fesharaki 1996, Pita Gondim 1999, Tenter *et al* 2000, Hove 2005). In Iran, these rates were reported in variable number on regional basis (Hashemi-Fesharaki 1996). The prevalence of *T. gondii* in sheep and goats in North-west of Iran has been unknown, thereby making it impossible to assess their potential significance to public health. The aim of the present study was to

investigate the occurrence of antibodies to *T. gondii* among sheep and goats in this region and diagnose the presence of parasite by PCR.

## MATERIALS AND METHODS

In this study ELISA was used to detect the anti-*T. gondii* antibodies in sheep and goats sera, and Nested-PCR technique was carried out to show the presence of *T. gondii* parasite in different tissues. The samples were collected from sheep and goats in Tabriz abattoir during the year 2010. A total of 186 serum samples were collected randomly from sheep and goats in abattoir during 6 months (winter and spring seasons of 2010) for ELISA test. The notifications that recorded during sampling from animals were consist of: sheep or goat, male or female and age of animals. The age range was from 6 months to 4 years. Sheep and goats from different regions of East Azarbaijan were sampled by a simple random sampling method. The sera were separated from blood samples by centrifugation and stored at -20°C until used. A total of 60 tissue samples were collected including: 34 whole blood samples, 13 cotyledon samples and 13 fetal brain samples from uterine and fetus of slaughtered pregnant sheep and goats for PCR test. All samples stored at -18°C until used. East Azarbaijan province is located in North-west of Iran. Tabriz (center of east Azarbaijan province) has a mild climate. The average annual temperature and precipitation are 13°C and 380mm, respectively.

**Serological test.** All serum samples were tested for anti-*T. gondii* IgG antibodies with indirect ELISA test using a commercial ELISA kit (*Institute Pourquier, France*). Sera with S/P% (corrected OD 450<sup>nm</sup> of the samples/the mean corrected OD 450<sup>nm</sup> of the 2 positive controls×100) equal or greater than 50% are considered to be from animals that have been in contact with *T. gondii*. Positive and negative control sera included on each plate. Results were analyzed statistically using the Chi-square test to determine significance of the value in the present study.

**Molecular test.** DNA extraction: DNA extraction was performed in two different ways: 1. DNA extraction kit

(DNP™ CinnaGen, Iran) 2. Phenol/chloroform extraction method. This method consists of extracting DNA by using the conventional phenol/chloroform method: First, the 200  $\mu$ l of each homogenized tissue sample were placed in a 1.5 ml microcentrifuge tube and mixed with 700  $\mu$ l of lysis buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, 20 mM EDTA pH 8.0 and 1% SDS). The next step was to add 20  $\mu$ l of proteinase K (20 mg/ml; QIAGEN) and the mixture was then incubated at 56°C for an hour. The solution was added to 600  $\mu$ l of phenol/chloroform/isoamyl alcohol (25:24:1) solution and was mixed and then was placed at -20 °C for 10 minutes. The solution was centrifuged for 10 minutes at 12,000 rpm. The aqueous phase was transferred to a new 1.5ml tube and mixed with another 600  $\mu$ l of phenol/chloroform/isoamyl alcohol (25:24:1) solution, then kept at -20 °C for 10 minutes and centrifuged again at 12,000 rpm for 10 minutes. The aqueous phase was then transferred into a new 1.5ml tube and 30  $\mu$ l of 3 M sodium acetate (pH 5.2) were added followed by 600  $\mu$ l of absolute ethanol. The mixture was placed at -20 °C for 30 minutes and then was centrifuged at 12,000 rpm for 30 minutes. The supernatant was discarded. The precipitated DNA was washed in 500  $\mu$ l of 70% ethanol and then sedimented at 12,000 rpm for 5 minutes. The ethanol was removed and DNA was dried and dissolved in 100  $\mu$ l of TE buffer pH 7.6 (10 mM Tris, 1 mM EDTA) (Sambrook et al 2001). The presence of DNA was evaluated by 0.8% agarose gel electrophoresis, and nanodrop spectrophotometry method used for determining of DNA purity. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as pure for DNA (Glaser 1995).

**Oligonucleotides.** The primer concentration that used was 10 pmol/ $\mu$ l. Universal 18S rRNA primer for detection of protozoa (Jauregui et al 2001) (GenBank accession no.X75429.1, RH strain), Toxo ITS-1 primer for detection of *Toxoplasma gondii* or *Neospora caninum* or *Hammondia hammondi* (Jauregui et al 2001), Tg1/Tg2 and Tg3/Tg4 primers used in nested-

PCR for detection of *T. gondii* (Grigg and Boothroyd 2001). The primers sequences have shown in table 1. Preparation of DNA sample: 5 $\mu$ l from 100ng/ $\mu$ l DNA sample was used for each PCR reaction.

**PCR.** PCR was performed on a final volume of 50  $\mu$ l containing 1x PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 2.5U Taq DNA polymerase, 0.2 mM of each primer and 5 $\mu$ l from 100 ng/ $\mu$ l of DNA sample for each PCR reaction. PCR reaction was conducted in a Gradient Mastercycler (Eppendorf, Germany). The PCR program was run with an initial denaturation at 95 °C for 5 minutes as hot start PCR, followed by 30 cycles of amplification. Each amplification cycle was consisted of denaturation at 94 °C for 30 seconds, primer annealing at 50 °C for 1 minute, and primer extension at 72 °C for 2 minutes. Finally, after 10 minutes incubation at 72 °C the PCR products were examined by electrophoresis in 1.5% agarose gel, stained with ethidium bromide solution, visualized under UV transilluminator and photographed.

## RESULTS

In total 34 (18.3%) samples by ELISA were found to be seropositive out of 186 blood samples. The seroprevalence of *T. gondii* in sheep (24.8%) was higher than in goats (10.6%) (table 2). The association of age with presence of infection is shown in table 3. In this region of Iran, seroprevalence was high in all ages, but the highest was in the range 1.5-2.5 years old. Thirty-two (19.2%) of 167 female animals and 2 (10.5%) of 19 male animals tested were seropositive for toxoplasmosis. There was significant difference between these two male and female groups ( $p \leq 0.05$ ). Twenty-four (25.8%) of 93 female sheep, only 1 (12.5%) of 8 male sheep, 8 (10.8%) of 74 female goat and only 1 (9.1%) of 11 male goat tested were seropositive for toxoplasmosis. There was significant difference between male and female sheep, but no significant difference between male and female goats.

**PCR results.** Test 1: DNA amplification of protozoa using Universal 18S rRNA primer produced a 205bp fragment and showed the parasites in 10 fetal brain, 10

cotyledon and 9 blood samples (Figure 1). Test 2: DNA amplification of *Toxoplasma gondii* or *Neospora caninum* or *Hammondia hammondi* using Toxo ITS-1 primer produced a 333bp fragment and showed them in 8 fetal brain, 3 cotyledon and 5 blood samples (Figure 2). Test 3: Results of Nested-PCR for DNA amplification of *T. gondii* using Tg1/Tg2 primer in step1 produced a 580bp fragment. This product (after dilution) used as a DNA sample for step2. In step2 of Nested-PCR using Tg3/Tg4 primer, produced a 531bp

fragment that detected *T. gondii* in 9 fetal brain (69%), 3 cotyledon (23%) and 5 whole blood samples (14.7%) (Figure 3).

## DISCUSSION

Toxoplasmosis is a zoonotic disease with global distribution that is caused by *T. gondii* (Dubey and Beatie 1988). A number of different, mainly serological, diagnostic tests have been used by different workers to detect infection of farm animals with this

**Table 1.** Characterization of primers used in PCR method.

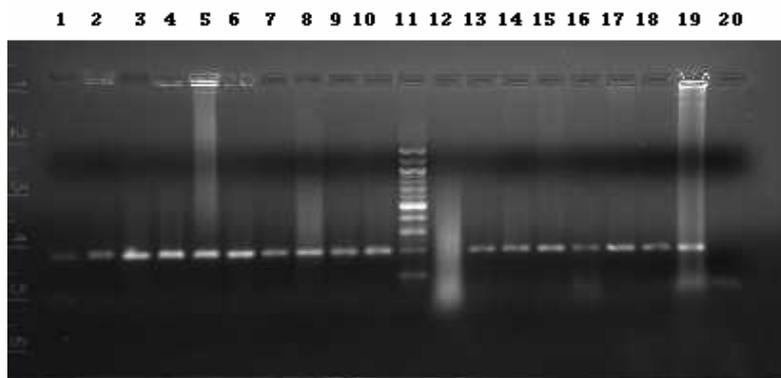
	Primer name		sequence	size
1	Universal 18S rRNA	forward	5'-CGGCTACCACATCTAAGG-3'	205 bp
		reverse	5'-TATACGCTATTGGAGCTGG-3'	
2	Toxo ITS-1	forward	5'-GATTTGCATTCAAGAAGCGTGATAGTA-t-3'	333 bp
		reverse	5'-AGTTTAGGAAGCAATCTGAAAGCACATC-3'	
3	Tg1	forward	5'-TGTTCTGTCTATCGCAACG-3'	580 bp
	Tg2	reverse	5'-ACGATGCAGTTCCTTCTG-3'	
	Tg3	forward	5'-TCTTCCCAGACGTGGATTTTC-3'	531 bp
	Tg4	reverse	5'-CTCGACAATACGCTGCTTGA-3'	

**Table 2.** Seroprevalence rate of anti *T. gondii* antibody in sheep and goat.

	samples	negative	positive	positive%
sheep	101	76	25	24.8
goat	85	76	9	10.6

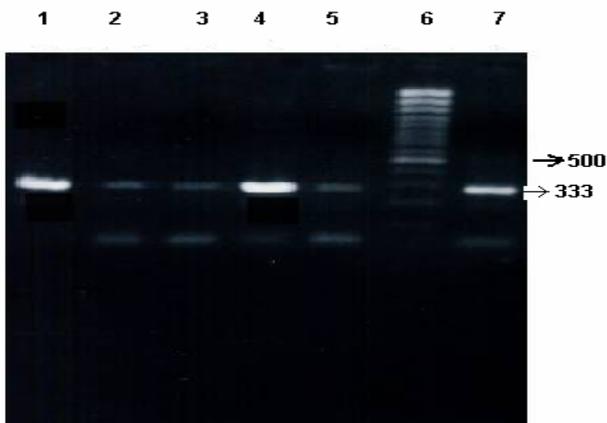
**Table 3.** Age distribution of anti-*T. gondii* antibody.

age	1	1.5	2	2.5	3	3.5	4
All animals	10	16	35	40	52	22	11
negative	9	13	27	28	47	18	10
positive	1	3	8	12	5	4	1
Seropositive rate%	10	18.75	22.85	30	9.6	18.2	9.1

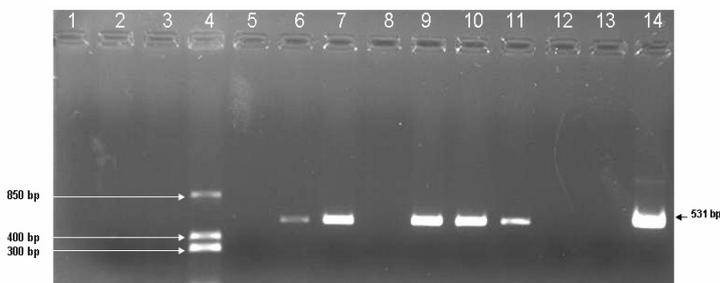


**Figure 1.** PCR amplification by Universal 18S rRNA primer for detection of protozoa. Lanes 1,2,3,6,7,9,12,13,14,16 cotyledon samples, lanes 4,5,8,10,15 fetal brain samples, lanes 17,18 whole blood samples, lane 11 100bp DNA ladder, lane 19 positive control and lane 20 negative control.

pathogen, therefore comparative studies on global epidemiology of toxoplasmosis is not easily possible. While the 24.8% frequency of toxoplasmosis in Tabriz sheep herds we are reporting here is lower than similar reports from Canada (57.6%), Ghana (33.2%), Turkey (31%), Brazil (29.4%) and Morocco (27.6%), a higher frequency is observed in Greece (23%).



**Figure 2.** PCR amplification by Toxo ITS-1 primer for detection of *Toxoplasma gondii* or *Neospora caninum* or *Hammondia hammondi*. Lane 1 *T. gondii* DNA sample, lanes 2,3 cotyledon samples, lanes 4,7 fetal brain samples, lanes 5 whole blood sample and lane 6 100bp DNA ladder.



**Figure 3.** PCR amplification by Tg3/Tg4 primer for detection of *T. gondii*. Lanes 1,2,3,7cotyledon samples, lane 4 a simple size marker, lanes 5,8,9,10,11,12 fetal brain samples, lane 6 whole blood sample, lane 13 negative control and lane 14 positive control.

This finding which is in accord with previous works of other Iranian researchers (Hashemi-Fesharaki 1996) indicates toxoplasmosis is widely distributed in the study region. Host age (Oncell and Vural 2006, Opsteegh *et al* 2010) and sex (Alexander and Stinson (1988), Oncel and Vural (2006)) are the two physiological elements that their impact on frequency of toxoplasmosis has been previously acknowledged. Accordingly, in the present study we observed the

highest prevalence in female animals over one year of age. A significant difference was observed between male and female groups ( $p \leq 0.05$ ). Prevalence rate in females was 19.2% (25.8% for ewes and 10.8% for does) and this for males was 10.5% (12.5% for rams and 9.1% for bucks). Besides, observations of the present work are in support of a significantly ( $p \leq 0.05$ ) higher frequency of *T. gondii* infection in sheep (24.8%) compared to goat (10.6%). We assume the high prevalence of *T. gondii* antibodies in sheep and goat herds in Tabriz might be attributed to the frequent visit of domestic cats to their farms as infected young cats are very likely to shed oocysts while roaming between farms (OIE 2008). Humans are typically acquired the infection through consumption of undercooked meat or meat products (Dubey 2004, Lake *et al* 2002, OIE 2008, Opsteegh *et al* 2010). Lamb, mutton and chevon are the favorite meats for majority of Iranians raising risk of infection with *T. gondii* in Iran for the consumers. Therefore, the prevalence of *T. gondii* infection in sheep and goats can help the health authorities to better epidemiological understand of epidemiology of this infection in human in any given region. One intriguing observation of the present work is the high level of similarity between PCR findings from toxo ITS-1 system (Figure 2) and Nested-PCR approach (Figure 3), an indication of accuracy of toxo ITS-1-PCR in detection of *T. gondii* infection. Further, our results confirm that PCR and serological tests (ELISA) are accurate and perfectly suitable for control and prevention measures and also treatment of the infection. The fact that ELISA-detected infection rate based on serum specimen analysis in the present work was much lower (18.3%) than that of PCR results (69%) from analysis of fetal brain specimens, one explanation is that there are cases of infection with *T. gondii* where serum levels of antibody are still not enough to trigger ELISA-sensitive reactions. If this is case, then one could assume that serology tests are not necessarily suitable to trace all infection cases. What might be the reason for absence of antibody in *T. gondii* infection? It has been understood that some of the

diseases such as theileriosis are able to suppress immune system. Accidental intake of particular food toxins such as aflatoxins and treatment of animals with immunosuppressive drugs are well-known to down regulate the immune system responses. Nevertheless, such reactions are also observed at the onset of the infection. Although the indirect ELISA detects IgG antibody in the serum, but at the beginning of infection the IgM is typically produce and its serum level is raised therefore, ELISA is unable to detect the infection at early stages of infection. Again, in acute phase of infection *T. gondii* when death can happen in only few days, the disease progression is too fast to let immune system of animal to produce ELISA-detectable levels of antibody (Nguyen *et al* 1996).

Finally, while PCR findings of the present work show toxoplasmosis infects many of sheep and goats in Tabriz, the antibody level in serum of a large portion these animals is detectable by ELISA, so these results strongly suggests active and or congenital infection. Measures are required to be taken by health professionals protecting local citizens against the disease transmission risk.

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