Molecular detection of *Toxoplasma gondii* infection in aborted fetuses in sheep in Khorasan Razavi province, Iran

Danehchin, L.¹, Razmi, Gh.²*, Naghibi, A.³

¹Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
²Center of Excellence in Ruminant Abortion and Neonatal Mortality, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
³Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

**Key words:** abortion, brain, nested-PCR, sheep, *Toxoplasma gondii*

**Correspondence**
Razmi, Gh.
Center of Excellence in Ruminant Abortion and Neonatal Mortality, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
Tel: +98(51) 38788944
Fax: +98(51) 38763853
Email: razmi@um.ac.ir

Received: 26 October 2016
Accepted: 14 January 2017

**Introduction**

*Toxoplasma gondii* was recognized for the first time as a significant cause of abortion in sheep in New Zealand and later in many countries (Buxton et al. 2007; Dubey, 2009). *T. gondii* infection during pregnancy can result in embryo/early fetal loss, fetal death and abortion/mummification and birth of weak lambs. (Buxton 1991; Scott 2007). This parasite is transmitted to sheep via ingesting food and water contaminated with oocysts which are excreted by cats or by other transmission rout from mother to fetus (Buxton et al. 2007). Furthermore, recent molecular studies showed that repeated transmission of *T. gondii* from dam to fetuses may be more common than previously observed in sheep (Duncanson et al. 2001; Morley et al. 2007; Hide et al. 2009).

The diagnosis of *T. gondii* infection is usually based on serological assay, histopathological examination and mouse bioassay. Many epidemiological studies have been carried out on ovine toxoplasmosis in different regions of Iran. The se-
The prevalence of ovine toxoplasmosis was reported between 13.8% -35% in different areas of Iran (Ghorbani et al. 1983; Rahbari&Razmi1992; Hashemi-Fesharki et al. 1996; Sharif et al. 2007). *T.gondii* infection was also detected in ovine fetuses using PCR and bioassay methods from Iran (Rasouli et al. 2011; Razmi et al. 2010; Zia-ali et al. 2007; Habibi et al. 2012). The aim of this study was to determine the frequency of Toxoplasma infection in aborted ovine fetuses in the province using molecular method.

**Materials and Methods**

The field of study: Khorasan Razavi province is located in northeastern Iran between 33°30’-37°41’ N latitude and 56°19’-61°18’ E longitude, with an area of more than 127,000 km2. The northern part of the province has a mountainous climate and suitable conditions for agricultural activity and animal husbandry. The southern part is mostly semi-desert and desert, with poor vegetation cover. **Sampling:** From June 2009 to July 2013, one hundred-twelve aborted ovine fetuses were examined. The fetuses and fetal membranes were grossly examined for any macroscopic lesions and the age of the aborted fetuses was also determined by crown-rump length (Evans & Sack 1973).

The fetuses were necropsied and the brain samples were collected and stored at −20 °C for PCR analysis. The frozen fetal brain tissues were homogenized by mortar and pestle in Tris-HCl (pH 8•0) followed by treatment with proteinase K (Fermentas®, Lithuania) at a final concentration of 200 μg/ml. The phenol-chloroform and ethanol precipitation method was used for DNA extraction procedures. The purified DNA samples were resuspended in 20 μl of TE (10mM Tris and 1mM EDTA, pH8•0) and stored at −20 °C. DNA extracted from 10⁶ particles of the RH strain of *T. gondii* was used as a source of positive control sample. DNA samples were tested by nested-PCR based on the amplification of B1 gene (35 copies per parasite) using 2 sets of oligonucleotide primers as was described by Burg et al. (1989). Amplification was performed in 20 μl reaction volumes (Accupower PCR premix kit,Bioneer®, South Korea) with a final concentration of each dNTP of 250 μM in 10mM Tris-HCl pH 9•0, 30mM KCl and 2mM MgCl2, 1U Taq DNA polymerase and 10 pmol of each PCR primer(Denazist. Iran). Then 1 μl of DNA template (250-500 ng) was added to each reaction and the remaining 20 μl reaction volume was filled with sterile distilled water. The PCR conditions were slightly modified. The following cycling conditions using a Bio-Rad thermocycler were applied: The first step of amplification was 3min of denaturation at 94 °C, this step was followed by 38 cycles, with 1 cycle consisting of 1min at 94 °C, 1min of annealing at 50°Cfor each pair of primers, and1min at 72 °C, an extension step of 7min at 72 °C was planned after the eventual cycle. The PCR primer pair was derived from the B1 gene sequence (Burgetal.1989) , B1F0:5’GGAACTGCATCCGTTCATGAG3′ and BIR0:5’TCTTTAACGCGTTGCTGGGTC-3′ (position 694-714 and 887-868 nucleotide respectively).The PCR products were electrophoresed through a 1.8% agarose gel to assess the presence of a 193 bp band (if any) in the first round. All PCR products were included in the second round of PCR. The nested-PCR reaction condition was similar to the first round, except for the annealing temperature which was 52 °C,
the number of cycles was 30. The resulted amplification products were diluted to 1:10 in water and then a second amplification was done with the internal primers (Burg et al 1989), B1F1:5’-TGCATAGGTTG-CAGTCACTG-3andB1R1:5’-GGCGAC-CAATCTGCGAATACACC-3’ (position 757-776 and 853-831 nucleotide, respectively), using 1µl of the diluted product as the template nested PCR. The amplified products were detected in 1.8% gel stained by electrophoresis and viewed under ultraviolet light.

The presence of specific bands of 193 bp in primary PCR and 96 bp in nested-PCR on agarose gel was considered as positive sample.

**Statistical analysis:** The Chi-square test was used to analyze the association between the *T.gondii* infection with fetal age and geographic region.

All risk factors were analyzed in a multivariable logistic regression model and Odds-ratios (OR) with 95% confidence intervals are calculated. Statistical analyses were performed with the statistics package SPSS, version 16.0 for Windows (IBM, New York, USA).

**Results**

*T.gondii* DNA was detected in 18 out of 112 (16.07%) brain samples of ovine fetuses by nested-PCR (Table.1) Three positive samples demonstrated a detectable reaction in both rounds of PCR (Fig.2) and 15 positive samples were detected in second round (Fig. 3). Based on the geographical location, all Toxoplasma infection was detected in the aborted fetuses of sheep in the north areas (p<0.01, Table 2). In this study, significant differences were found between Toxoplasma infection in different age groups of fetus (p<0.05, Table 2). No significant difference was shown between the frequency of Toxoplasma infection in different time periods (p>0.05, Table2).

**Discussion**

Several PCR methods have been devel-
Molecular detection of *T. gondii* infection

Danehchin, L.

Figure 1. Electrophoretic analysis of PCR-amplified products stained with ethidium bromide in 1.8 % agarose gel. (M) Lane M, 50 bp DNA ladder marker; lane 1, negative control ; Lane2, positive control DNA sample of *T. gondii* (193 bp) ; lane 1-3 samples : DNA samples of blood.

Figure 2. Electrophoretic analysis of PCR-amplified products stained with ethidium bromide in 1.8 % agarose gel. (M) Lane M, 50 bp DNA ladder marker; (P) lane P, positive control ; LaneN, negative control DNA sample of *T. gondii* (96 bp) ; lane 1-5 samples : DNA samples of blood.

Among the regions studied in Rio Grande do Norte, seroprevalence varied from 17.8% in the Central Potiguar region to 26.3% in the Leste Potiguar region (24).

In Iran, *T. gondii* DNA has been detected in aborted fetuses to range between 13.5% to 69% in Khorasan, Qazvin and East Azarbeijan provinces by different PCR assays (Rassouli et al. 2009; Habibi et al. 2011; Mooazeni Jula et al. 2013). These results show that the moutainous climate of Qazvin and East Azarbeijan provinces are more favorable conditions to the development and maintenance of *T. gondii* oocysts in the environment. Oocysts can survive in the environment for months, depending on moisture and temperature. Thus, low humidity and high temperatures of the Khorasan Razavi province is deleterious to the viability of Toxoplasma oocysts in the pasture. In other countries, *T. gondii* PCR positivity was detected to range between 3.5 and 11.1% in Italy (Masala et al. 2003; Pereira-Bueno et al. 2004; Chessa et al. 2014), 5.4 to 16.9% in Spain (Hurtado et al. 2001, Moreno et al. 2012), 14% in Ireland (Gutierrez et al. 2012), 14.3% in Brazil (de Moraes, et al. 2011) and 29.8% in Jordan (Abu-Dalbouh et al. 2012). The differences of PCR results would be related to many factors such as different PCR methods, climatic condition in each country.

All infected fetuses in this study belonged to the areas that are located in the northern part of the province. The northern part of the province has mountainous climate and mean annual rainfall is more than the southern areas with semi desert climate. The mountainous climate confers favorable conditions for Toxoplasma oocysts survival, because the viability of *T. gondii* oocysts is dependent on the moisture and temperature of the soil (Lélu et al. 2012; Du et al. 2012 ;). The higher prevalence of ovine toxoplasm-
Toxoplasmosis has been reported in the humid and temperate areas compared to dry areas (Kamani et al. 2010; Alvarado-Esquivel et al. 2013; Gebremedhin et al. 2013; Andrade et al. 2013; Hamidinejat et al. 2008).

In the present study, most of the infected fetuses had more than 120 days of age. The clinical signs of ovine toxoplasmosis in pregnant ewes depends on the age and immune status of fetus. Fetal death is more likely to occur from infection during the first trimester of pregnancy when the fetal immune system is relatively immature. Infection at mid-gestation can result in birth of a stillborn or weak lamb which may have an accompanying small mummified fetus, whereas infection in later gestation may result in birth of a live, clinically normal, but infected lamb (Buxton 1991; Scott 2007; Innes et al. 2009). Other studies have shown that the abortion associated ovine toxoplasmosis generally occurs during the mid pregnancy (Pereira-Bueno et al. 2004) when the _T. gondii_ aborted fetuses have 110-130 days of age (Giadinis et al. 2011).

Based on our results, the moderate frequency of _T.gondii_ infection was detected among ovine aborted fetuses in this area. However, a definite diagnosis of Toxoplasma infection in causing abortion in sheep needs to combine pathological and molecular examination

**Conflict of interest:** The authors declare that they have no conflict of interest

**Acknowledgments**

This study was supported by a grant VPRTFM-3.27748 from the Vice President of Research and Technology of Ferdowsi University of Mashhad, Iran. We would like to thank Dr Zahra Naseri for help sampling and Dr Fazaeli kindly provided the control positive PCR from the Institute of Public Health Research, Tehran University of Medical Sciences, Tehran, Iran.

**References**


Molecular detection of *T. gondii* infection

Danehchin, L.

gondii diagnosis in ovine aborted fetuses and stillborns in the State of Pernambuco, Brazil. Vet Parasitol. 183: 152-5.


Danehchin, L.

1887-1894.


تعیین ملکولی آلودگی توکسوپلاسما گوندی در جنین‌های سقطی گوسفندان استان خراسان رضوی

لیلا دانی، چین، غلامرضا رزمی، ابوascalام نقیبی

زمینه مطالعه: توکسوپلاسما گوندی به علت مهم سقط جنین در گوسفندان و بزان در دنیا می‌باشد. روشهای تشخیص آلودگی توکسوپلاسما گوندی بسیار کم و کاربرد ندارد و مطالعات در این زمینه کمتری است.

هدف مطالعه: تحقیق و تعیین افراد آلوده به آلودگی توکسوپلاسما گوندی در جنین‌های سقطی گوسفندان استان خراسان رضوی.

روش کار: جنین‌های سقوط شده گوسفندان در استان خراسان رضوی جمع‌آوری شدند و تعداد 121 نمونه مغز جنین که از سایر مناطق استان خراسان رضوی برگرفته شدند به روش PCR تحلیل شد. این آزمایش برای تعیین آلودگی توکسوپلاسما گوندی که شامل DNA و RNA می‌باشد انجام شد و با استفاده از نمونه مغز جنین شناسایی و تعیین آلودگی به توکسوپلاسما گوندی شد. این روش شامل دو مرحله گروه نسبی یا Multi PCR و نسبی یا nested-PCR می‌باشد.

نتایج: در نمونه مغز جنین‌های سقوط شده گوسفندان استان خراسان رضوی، نتایج آزمایش PCR نشان داد که 12 نمونه از آنها آلوده به آلودگی توکسوپلاسما گوندی بودند. این نتایج نشان می‌دهد که آلودگی توکسوپلاسما گوندی در جنین‌های سقطی گوسفندان استان خراسان رضوی بیشتر می‌باشد.

واژه‌های کلیدی: سقط جنین، مغز، توکسوپلاسما، PCR، Nested-PCR، گوسفندان

Email: razmi@um.ac.ir 9898(51) 2387685633 98(51) 3878849444 98(51)