INVESTIGATION OF TOXOPLASMA GONDII AND NEOSPORA CANINUM AS CAUSE OF OVINE ABORTION IN AFFECTED FLOCKS OF URMIA, NORTHWEST OF IRAN

A. KHODADADI¹, F. MALEKIFARD¹ & R. A. BATAVANI²

¹Department of Pathobiology, ²Department of Theriogenology; Faculty of Veterinary Medicine, Urmia University, Urmia, West Azarbaijan, Iran

Summary

Toxoplasma gondii and Neospora caninum, two obligatory intracellular protozoan parasites, are important causes of neonatal mortality and abortion in farmed ruminants worldwide. Previously, protozoan abortions in small ruminants were attributed to T. gondii, but the role of N. caninum in these abortions was uncertain. The aim of this study was to investigate the role of T. gondii and N. caninum in ovine abortion in Urmia, northwest of Iran using a molecular method. Overall, 130 placenta and brain samples of aborted ovine foetuses were collected. Extracted DNA from placenta and CNS tissues of the aborted foetuses were analysed using PCR with primers specific for T. gondii and N. caninum. The association of the frequency of T. gondii and N. caninum infection of aborted foetuses with age and breed in flocks was also studied. The results showed that out of the 130 examined ovine foetuses, 5.3 and 2.3 % were PCR-positive for T. gondii and N. caninum DNA, respectively. In this study, no significant differences were recorded relating to Toxoplasma and Neospora infection in different age groups in flocks and among sheep breeds included in the present study (P>0.05). The results of this study proved the importance of T. gondii and N. caninum as reasons of abortion in the studied area.

Key words: abortion, foetuses, Neospora caninum, sheep, Toxoplasma gondii, Urmia

INTRODUCTION
Failures in reproduction as a result of infectious factors is one of the main causes of poor performance in ruminants. Neospora caninum and Toxoplasma gondii are respectively the main causes of failures in reproduction of cattle (Dubey & Schares, 2011) and small ruminants (Buxton, 1998; Dubey & Schares, 2011; Hurtado et al., 2001; Masala et al., 2007; Pereira-Bueno et al., 2004).

Hartley et al. were the first to report T. gondii as the cause of abortion in sheep in New Zealand which was then confirmed in different countries (Buxton et al.,
This intracellular protozoan parasite does not lead to any clinical disease in sheep, however congenital infections can engender a series of disorders like early death of the embryo, mummification, stillbirth, foetal resorption, stillbirth, foetal death in sheep (Blewett & Watson, 1983; Johnston, 1988; Givens & Marley, 2008). The existing evidence show that this method of transmission may have more significance than it was previously assumed (Duncanson et al., 2001; Williams et al., 2005; Hide et al., 2009).

Neospora caninum which causes neosporosis is one of the most considerable reasons of bovine abortion all over the world (Anderson et al., 1991). Despite causing congenital infections in sheep and mortality in newborn lambs, N. caninum is not considered as a significant cause of abortion in sheep (Buxton, 1998; Dubey & Lindsay, 1990; Dubey et al., 1990; Georgieva et al., 2006). The results obtained from recent studies show that neosporosis may lead to a higher abortion risk in sheep than previously thought (Hässig et al., 2003; Dubey & Schares, 2011; Howe et al., 2012; Moreno et al., 2012; González-Warleta et al., 2014).

For a long time, histopathological evaluations of foetal tissues were used for the diagnosis of ovine abortion caused by protozoa. It should be noted that histopathological methods are not able to accurately differentiate T. gondii from N. caninum, since both of them share the same morphological features and induce similar lesions which leads to errors in diagnosis (Buxton et al., 1997; Buxton, 1998; Dubey, 2009; Dubey & Schares, 2011; Moreno et al., 2012; Edwards & Dubey, 2013). Hence, it is reasonable to use PCR in order to confirm the diagnosis of infectious agents causing abortion (González-Warleta et al., 2014).

In Iran, PCR and bioassay methods are generally used to confirm the etiology of abortions caused by toxoplasmosis and neosporosis (Habibi et al., 2005; Zia-Ali et al., 2007; Razmi et al., 2010; Rassouli et al., 2011). This study aims to use PCR in order to find out the presence of the genome of the parasite in various foetal tissues and to this end, provides data on the occurrence of T. gondii and N. caninum in aborted foetuses in Urmia, Iran as well as determines various risk factors related to neosporosis and toxoplasmosis.

**MATERIALS AND METHODS**

**Study area**

The study was conducted on 130 aborted ovine foetuses during the breeding seasons of 2016–2017 in Urmia, West Azerbajan province in northwest of Iran. The region of the study is an agriculturally fertile area located between 37º 32´ N and 45º 04´ E and the area is estimated to be around 8000 km². The temperature of the study area varies from −3.8 ºC to +23.4 ºC during different seasons. The area is bordered in with Iraq and Turkey (Yakhchali & Hosseine, 2006).

**Collection of samples and tissues**

Placenta tissue and brain samples were collected from 130 aborted ovine foetuses for analysis. Data regarding the breed and the foetal age were recorded (Table 1). Table 1 shows the age and breed of the examined ewes. There were three different breeds (Makuii, Ghezel and Haraki) in this study. The foetuses were examined for autolysis, freshness, mummification and other lesions. Crown-rump length was used for evaluation of conception age (Evans & Sack, 1973). The collected samples from the brain and placenta were
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DNA extraction of tissues

Genomic DNA of the samples taken from brain and placenta were extracted from the aborted foetuses (Rassouli et al., 2011; Asadpour et al., 2013). About 5 to 10 g of the samples taken from different tissues were homogenised and powdered using liquid nitrogen and then transferred to microtubes. DNA was extracted from 1 g homogenate sample using the commercial kit (Cinnagen Inc., Iran) according to the manufacturer’s instructions. The resulting DNA was stored at −20°C to conduct PCR analysis.

PCR amplification

Nc5 region was chosen as the main sequence for the amplification of the DNA in order to diagnose N. caninum using PCR (Kaufmann et al., 1996). The PCR method explained by Müller et al. (1996) was used for N. caninum diagnosis. The amplification was done by using N. caninum primers Np21plus (5’- CCCAGT GCGTCAAATCCTGTAAAC-3’) and Np6plus (5’-CTCGCCAGTCAACCTAC GTCT TCT-3’) and 337 bp DNA fragment was amplified (Müller et al., 1996).

The PCR method used by Bretagne et al. (1993) was applied for the detection of T. gondii. It should be noted that 35-fold-repetitive B1 gene was chosen as the target for DNA amplification (Burg et al., 1989). T. gondii specific primers TOXQB22 5’- AACGGGCGAGTAGCCTGAGGAGA and TOXOB23 5’- TGGGTCTACGTCGATGGCATGACAC were used to amplify the 115 bp DNA fragment (Bretagne et al., 1993). The positive controls of N. caninum and T. gondii were obtained from Tehran University; distilled water was the negative control. Two percent agarose gel electrophoresis was used to separate the products of amplification and then they were stained with ethidium bromide and UV light was used to visualise them.

Statistical analysis

The analysis of the results was conducted with Chi-square in SPSS software (v. 21.0) and the differences were considered as significant at P<0.05.

RESULTS

PCR test showed that 3 out of 130 aborted samples (2.3%) were positive for N. caninum. Np21plus and Np6plus primers were utilised for the amplification of a 337 bp section of the repetitive area in the genome of the parasite (Fig. 1). The mean gestational time of the abortions varied from 3 to 5 months.

PCR diagnosed Toxoplasma in 7 out of 130 foetal samples (5.3%) and detected 115 bp bands in all of the positive samples (Fig. 2). Table 2 shows the age and breed of the dam and the age of the aborted foetuses with T. gondii and N. caninum. In this study, no significant differences were found between Toxoplasma and Neospora infection in different age groups in flocks.

<table>
<thead>
<tr>
<th>Aborted ewes</th>
<th>Age</th>
<th>Breed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 3</td>
<td>≥ 3</td>
</tr>
<tr>
<td>130</td>
<td>51</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>61</td>
</tr>
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<td></td>
<td>47</td>
<td></td>
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</tbody>
</table>

Table 1. Number of examined aborted ewes based on age and breed

CCTGAGGAGA-3’ and TOXOB23 5’-TGGGTCTACGTCGATGGCATGACAC AC-3’ was used to amplify the 115 bp DNA fragment (Bretagne et al., 1993).
and among sheep breeds included in the present study (P>0.05, Table 2).

Table 3 shows the results of detecting *N. caninum* and *T. gondii* in different samples obtained from aborted foetuses by PCR. It is notable that brain was mostly affected by *N. caninum* and *T. gondii*, hence it is advisable to examine the brain of aborted foetuses to diagnose protozoan ovine abortions.

**DISCUSSION**

*Toxoplasma gondii* is one of the main parasites causing abortions in both sheep and goat populations. The economic, epidemiological, and clinical influences of *Neospora caninum* is unknown in these species (Dubey & Schares, 2011). To date, this study has been performed to investigate the role of *N. caninum* and
Investigation of Toxoplasma gondii and Neospora caninum as cause of ovine abortion in affected cases of ewe’s abortion. To this end, we used PCR for detecting these protozoan infections in the aborted samples. Confirmation of the presence of the etiologic agent using specific techniques such as PCR is needed because N. caninum and T. gondii may cause similar lesions (Moreno et al., 2012).

In the current study, 7.69% (10 out of 130 foetuses) had protozoal DNA. The results showed that these infections are the main reason for abortions in sheep herds which has been reported in previous studies (Dubey, 2003; Pereira-Bueno et al., 2004; Masala et al., 2007; Hide et al., 2009; Moreno et al., 2012).

In the studied flock consisting of various breeds, the frequency of T. gondii was 5.3% (7/130). The prevalence of T. gondii in previous studies conducted in Iran was higher and in two studies it was reported as 16.07% and 18.2% (Rassouli et al., 2011; Danechchin et al., 2017). The differences can be justified by the geographical distribution of the infection, since the aforementioned studies were conducted on recovered foetuses from Northeast of Iran. Comparison of our results with those obtained in other countries shows that the rate of infections caused by T. gondii in Urmia, Iran was lower compared to other countries which were 17.5% in north central United States (Dubey & Kirkbride, 1990), 18.1% in Italy (Masala et al., 2003) and 48.2% in Bulgaria (Prelezov et al., 2008). Different results of the PCR are due to various factors including different PCR methods and climatic conditions of different countries (Danechchin et al., 2017).

In this study, the DNA of T. gondii was seen in 3.84% (5 out of 130) samples taken from brain and its presence in placenta samples was 0.76%. It should be noted that 0.76% of the parasitic DNA was detected in both placenta and brain samples obtained from aborted foetuses. Skeletal muscles, brain, liver, cardiac muscle, lung, spleen and placenta are very useful in detecting T. gondii but due to higher frequency of brain infections, it is the most common tissue used for diagnosis of Toxoplasma-induced ovine abortions (Esteban-Redondo & Innes, 1998; Hurtado et al., 2001; Pereira-Bueno et al., 2004; Dubey, 2009). A study conducted by Gutierrez et al. (2010) showed that placenta and lung tissues can be helpful in identifying the infection, yet Duncanson et al. (2001) and Sreekumar et al. (2004) did not detect any parasites in the lung.

The majority of foetuses infected with Toxoplasma in this study were older than 3 months. The clinical signs of ovine toxoplasmosis in pregnant animals is relative to the age and immunity status of the foetus. The foetal immune system is relatively immature during the first trimester of pregnancy and this leads to higher foetal death rate during this period. In cases

### Table 3. The PCR amplification results for different foetal tissues samples corresponding to protozoa-infected foetuses

<table>
<thead>
<tr>
<th>Samples</th>
<th>N. caninum-infected samples</th>
<th>T. gondii-infected samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain (%)</td>
<td>2 (1.53)</td>
<td>5 (3.84)</td>
</tr>
<tr>
<td>Placenta (%)</td>
<td>1 (0.76)</td>
<td>1 (0.76)</td>
</tr>
<tr>
<td>Placenta and brain (%)</td>
<td>–</td>
<td>1 (0.76)</td>
</tr>
<tr>
<td>Total positive samples in PCR (%)</td>
<td>3 (2.30)</td>
<td>7 (5.30)</td>
</tr>
</tbody>
</table>
in which the infection occurs in the middle of the gestation period, the result would be the birth of stillborn or weak lambs. It is also notable that infections in later stages lead to birth of physically normal but infected lamb (Scott et al., 2007; Innes et al., 2009). Some other studies point out that most of the abortions caused by toxoplasmosis occur during mid-pregnancy (Pereira-Bueno et al., 2004) and the foetuses are mostly aborted at days 110–130 (Danehchin et al., 2017). There was no evidence for any case happening before 60 days since foetal resorption is the commonest event during this period (Blewett & Watson, 1983; Johnston, 1988; Givens & Marley, 2008).

The reports regarding abortions in small ruminants caused by N. caninum are sporadic (Dubey & Schares, 2011). N. caninum was detected in the brains of 3 out of 18 aborted ovine foetuses in New Zealand (Howe et al., 2008), in 18.9% of 74 aborted ovine foetuses in England (Hughes et al., 2006), in 2% of 292 aborted sheep foetuses in Italy (Masala et al., 2007) and 5.4% (4/74) of sheep foetuses in Spain (Moreno et al., 2012). The results of our study indicate the existence of N. caninum DNA in 2.3% of ovine foetuses. Another study conducted by Asadpour et al. (2013) in Iran reported 8.5% which is higher than the percentage obtained by our study. This difference can be attributed to keeping sheep and beef cattle in the same place, age, environment, and use of farm working dogs in flocks (Asadpour et al., 2013). Furthermore, our results showed that the samples were not co-infected with both T. gondii and N. caninum, but other studies with larger sample sizes are necessary to confirm this issue.

The results of PCR showed that 2 out of 3 positive samples for N. caninum were identified in the brain and the third was found in the placenta. This is in line with previous studies showing tissue parasites detected most frequently in ovine brain by PCR (Masala et al., 2007; Silva et al., 2009; Bishop et al., 2010; Asadpour et al., 2013; Sasani et al., 2013).

The current study showed no significant correlation between infection rates of T. gondii and N. caninum in different age groups in flocks. Similar conclusion has also been reported by other studies (Rassouli et al., 2011; Asadpour et al., 2013). It is interesting to note that there was no association between breed and infection rate in our study. There are some reports on higher seroprevalence of N. caninum in pure breeds compared to crossbred sheep, showing that imported animals were more endangered compared to the local breeds (Guimarães Jr et al., 2004; Akca et al., 2005).

In conclusion, the results of this study proved the significance of T. gondii and N. caninum as causes of abortion in the studied area and enhanced our understanding of the role of T. gondii and N. caninum in abortions of flocks affected by these pathogens. Due to the shared source of water and pasture in sheep and cattle, it is possible to observe a correlation between both species in the epidemiology of these parasites (González-Warleta et al., 2014), therefore additional research is needed to determine the expansion of abortions caused by Neospora and Toxoplasma in dairy cows of the studied area.

ACKNOWLEDGEMENTS

We would like to sincerely thank the members of the Faculty of Veterinary Medicine and Urmia University Research Council for the approval and support of this research.
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strains from different hosts in Iran. 

Paper received 08.05.2020; accepted for publication 20.06.2020

Correspondence:
Farnaz Malekifard,
Department of Pathobiology,
Faculty of Veterinary Medicine,
Urmia University, P.O. Box 1177, Urmia, Iran.
tel: +98-44-32972654,
fax: +98-44-32770508,
e-mail: f.malekifard@urmia.ac.ir