Bulgarian Journal of Veterinary Medicine, 2022, **25**, No 2, 308–317
ISSN 1311-1477; DOI: 10.15547/bjvm.2020-0104

Original article

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

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Summary

Khodadadi, A., F. Malekifard & R. A. Batavani, 2022. Investigation of *Toxoplasma gondii* and *Neospora caninum* as cause of ovine abortion in affected flocks of Urmia, northwest of Iran. *Bulg. J. Vet. Med.*, **25**, No 2, 308–317.

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*.,

2007). 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, neonatal and 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 *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 Azerbaijan 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 km^2. 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

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

Aborted ewes	Age		Breed		
	< 3	≥ 3	Ghezel	Makuii	Haraki
130	51	79	22	61	47

stored at −20° C in order to conduct PCR analysis.

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 GCGTCCAATCCTGTAAC-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'- AACGGGCGAGTAGCA CCTGAGGAGA-3' and TOXOB23 5'-TGGGTCTACGTCGATGGCATGACA AC- 3' was 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

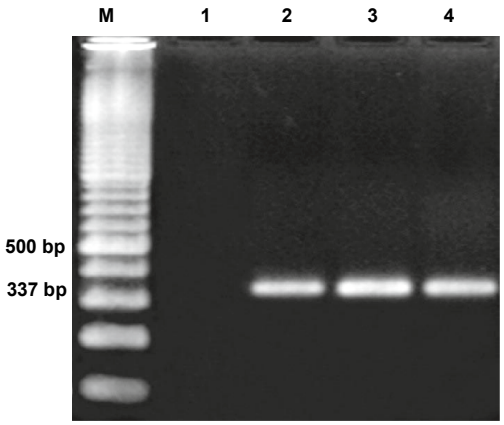

Fig. 1. PCR detection of *N. caninum* with specific primer (Np21plus and Np6plus) in aborted ewes of Urmia, Northwest of Iran. M: 100 bp DNA marker (Fermentas); Lane 1, Negative control; Lane 2, Positive control for *N.caninum*; Lane 3, positive placenta samples; Lane 4 positive brain samples.

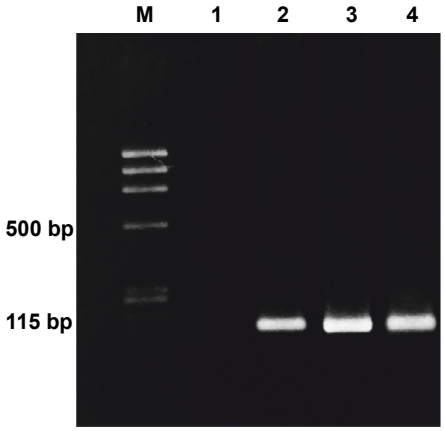

Fig. 2. PCR detection of *Toxoplasma gondii* in aborted ewes of Urmia, Northwest of Iran. M: 100 bp DNA marker (Fermentas); lane1, Negative control; Lane 2, Positive control for *T. gondii*; Lane 3, positive placenta samples; Lane 4 positive brain samples.

Table 2. PCR results obtained in samples corresponding to protozoa-infected foetuses

Infection	Sample no.	Age of ewe	Breed	Foetal age(months)
T. gondii	1	≥ 3	Ghezel	3
	2	< 3	Makuii	4
	3	≥ 3	Ghezel	4
	4	< 3	Makuii	4
	5	≥ 3	Haraki	3
	6	< 3	Haraki	Dead after birth
	7	< 3	Makuii	Dead after birth
N. caninum	8	< 3	Makuii	4
	9	< 3	Haraki	3
	10	≥ 3	Makuii	Dead after birth

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

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

Samples	*N. caninum*-infected samples	*T. gondii*-infected samples
Brain (%)	2 (1.53)	5 (3.84)
Placenta (%)	1 (0.76)	1 (0.76)
Placenta and brain (%)	–	1 (0.76)
Total positive samples in PCR (%)	3 (2.30)	7 (5.30)

T. gondii in cases of ewe's abortion. To this end, we used of 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; Danehchin *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 (Danehchin *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

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 leads 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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Paper received 08.05.2020; accepted for publication 20.06.2020

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