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Investigation of *Toxoplasma gondii* and *Neospora caninum* in different tissues of aborted foetuses of sheep in Van Province, Türkiye: Analysis by nested PCR, histopathological and immunohistochemical methods

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Abstract

Toxoplasma gondii and *Neospora caninum* are protozoon parasites from the intracellular apicomplexan family. *Toxoplasma gondii* is the cause of health and economic problems in the sheep industry worldwide. *Neospora caninum* is usually reported in cows and leads to infections causing abortions; however, its prevalence in sheep is not clear. The present study aimed to investigate the prevalence and pathology of *T. gondii* and *N. caninum* by PCR, histopathological and immune-histochemical methods in aborted sheep foetuses collected at different sheep flocks in the Van Province, Türkiye, in 2021. Firstly, the DNA of *T. gondii* and *N. caninum* were investigated by PCR in the brain, heart, and peritoneal fluid samples from 42 sheep foetuses. *Toxoplasma gondii* DNA was proved in 35.7% (15/42) of foetuses whereas *N. caninum* DNA was not determined in any of the samples. Histopathologically, all *T. gondii* positive brain tissue samples showed lymphohistiocytic multifocal encephalomyelitis and additional findings included necrotizing myocarditis in the positive heart samples. *Toxoplasma gondii* tachyzoites were identified in the lesions (diffuse or focal mononuclear cell infiltration in the meninges, and microglia proliferation, myocarditis with oedema) by anti-*T. gondii* antibodies by the immunohistochemical method. Based on our results, we can conclude that *T. gondii* is an important agent in sheep abortions and the PCR method is a suitable method for diagnosis which can also be used in heart tissue in pathological studies.

Ovine, abortion, protozoon parasites

Toxoplasma gondii and *Neospora caninum* are obligate intracellular protozoan parasites from the Apicomplexa phylum (Arraes-Santos et al. 2016). *Toxoplasma gondii* is a protozoan causing toxoplasmosis, which is among the most common parasitic diseases in humans and animals in the world (Tenter et al. 2000; Wang et al. 2011). Infected cats, the definitive host of *T. gondii*, may spread millions of oocysts in their faeces and thus contaminate the environment that can become the source of infection for herbivorous animals during grazing (Mor and Arslan 2007; Innes et al. 2009; Can 2010). *Toxoplasma gondii* infection can lead to abortions and reproductive disorders in sheep. When pregnant sheep develop acute toxoplasmosis, their placenta is invaded by tachyzoids followed by the infection of the foetus (Moraes et al. 2011; Ibrahim et al. 2017). Neosporosis is a parasitic disease of great importance in livestock caused by the obligate intracellular parasite *N. caninum* that was first isolated in puppies with congenital encephalomyelitis in Norway in 1984 (Dubey et al. 2007; Uzêda et al. 2007). Domestic and wild canids

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are the definitive host of *N. caninum*. Intermediate hosts, like herbivores (cattle, sheep, goat, horse, bison, and deer) become infected by ingesting infected oocysts spreading in the faeces of definitive hosts (Nath-Sharma et al. 2015; Gharekhani et al. 2016). In many hosts, contamination occurs by the transplacental route. This disease, which causes neuromuscular disorders, paralysis, and death in dogs, causes abortion and neonatal death in sheep and goats. Since *T. gondii* and *N. caninum* are very similar in structure, it has been suggested that misdiagnoses have been made for years, especially in terms of *N. caninum* (Dubey 2003; Figliuolo et al. 2004; Uzêda et al. 2007; Filho et al. 2017). Although *N. caninum* causes infections, especially in cattle and dogs, its presence has been reported in many warm-blooded animals including goats, sheep, buffalo, equids, and other domestic and wild carnivores. Bovine neosporosis is an important cause of abortion in cattle. It also causes reproductive problems in cattle. Abortions due to *N. caninum* in sheep have been rarely reported (Bártová et al. 2009; Ueno et al. 2009).

Abortions caused by *T. gondii* cause serious economic losses in sheep breeding (Ahmed et al. 2008; Moreno et al. 2012). Although *N. caninum*-related abortions have been reported in sheep (Pereira-Bueno et al. 2004; Ueno et al. 2009; Ezatpour et al. 2015), the number of studies on the subject is insufficient. Careful diagnosis of *T. gondii* and *N. caninum* in sheep abortions is of great importance in understanding reproductive disorders in flocks, and preventing abortions and economic losses. For the diagnosis of these two agents, serological tests and histopathological tests are frequently used. However, the morphological similarity of these two agents increases the possibility of error in histopathological tests. Molecular methods used for detection of the DNA of these two agents and its further genetic characterizations have been reported as the best method for the diagnosis of *T. gondii* and *N. caninum* from ovine foetuses and placenta (Moraes et al. 2011; Moreno et al. 2012; Ezatpour et al. 2015).

The aim of the study was to detect *T. gondii* and *N. caninum* by PCR in tissues of 42 aborted sheep foetuses in the Van Province in Türkiye, and to study the pathology of these two parasites using histopathological and immunohistochemical methods. In addition, this study aimed to determine the the usability of the peritoneal fluid and heart tissue as an alternative to brain tissue for diagnosis of *T. gondii* and *N. caninum* in pathology studies.

Materials and Methods

The study area and sample collection

The present study was conducted in the Van Province in Türkiye, located in the Eastern Anatolian Region and bordering with Iran (Fig. 1). The samples consisted of 42 sheep foetuses aborted during the first 1–3 months of pregnancy in different sheep flocks bred in different locations of the Van Province in 2021. All the sheep from which the samples were taken were of the Morkaraman breed. A total of 126 tissue samples (42 brain, 42 heart, and 42 peritoneal fluid samples) were obtained from 42 foetuses. The obtained samples were stored at −20 °C until the PCR analyses. Tissue fragments from the brain and heart tissue, except frozen samples, were fixed in 10% buffered formalin for histopathological and immunohistochemical analysis.

DNA extraction, PCR amplification, and sequence analysis

The DNA extraction was carried from the brain, heart, and peritoneal fluid of 42 ovine foetuses by the Pure Link™ Genomic DNA Mini Kit (Thermo Fisher, Carlsbad, CA, USA).

Amplification of the 529-bp repetitive sequence of *T. gondii* was carried out using TgTox4F (5-CGCTGCAGGGAGGAAGACGAAAGTTG-3′) and TgTox4R (5’-CGCTGCAGACACAGTGCATCTGGATT-3′) primers (Sah et al. 2019). In both reactions, 25 pmol forward and reverse primers, 200 µM dNTPs, 2 mM $MgCl_2$, 1U Hot Start TAQ DNA Polymerase, 10X PCR buffer (0.8 M Tris-HCl, 0.2 M $(NH_4)_2SO_4$), Nuclease Free Water and 2 µl DNA were used in 25 µl Mastermix. The PCR was as follows: 15 min at 95 °C, 35 cycles (60 s at 95 °C, 60 s at 60 °C, 60 s at 72 °C), and 10 min at 72 °C.

For amplification of the Nc5 gene region of *N. caninum*, nested PCR was performed using external (5’-CTGCTGACGTGTCGTTGTTG-3′) forward and (5’-CATCTACCAGGCCGCTCTTC-3′) reverse primers inner (5’-GCGTCAGGGTGAGGACAGTG-3′) forward and (5’-CTCTCCGTTCGCCAGCAGTG-3′) reverse primers (Fish et al. 2007). In both reactions, 10 pmol forward and reverse primers, 200 µM dNTPs, 2 mM $MgCl_2$, 1U Hot Start TAQ DNA Polymerase, 10X PCR buffer (0.8 M Tris-HCl, 0.2 M $(NH_4)_2SO_4$), Nuclease Free Water

Fig. 1. The area in Türkiye where aborted sheep foetuses were collected for this study.

and 2 µl DNA were used in 25 µl mastermix. For the first step of nested PCR, the reaction was the following: 15 min at 95 °C, 30 cycles (30 s at 95 °C, 30 s at 56 °C, 40 s at 72 °C), and 10 min at 72 °C. For the second step of nested PCR, the reaction was the following: 15 min of pre-denaturation at 95 °C, 30 cycles (30 s at 95 °C, 30 s at 57 °C, 3 min at 72 °C), and final elongation (5 min at 72 °C). The reaction was performed using gradient PCR, the SuperCycler (Kyratec, Australia) device. Subsequently, 1.5% agarose gel was prepared and stained with redSafeTM Nucleic Acid Staining Solution, PCR products were run at agarose gel, and images were obtained by gel imaging device (Syngene bioimaging system). Positive samples were sent in a double-way sequence (BM Labosis, Ankara, Türkiye). Blasting and alignment were carried out and compared with the related reference genes at GenBank.

Histopathology

PCR-positive tissue samples were washed under running water for removing formalin. Afterward, routine pathological tissue tracing was performed and passed from graded alcohol (50%, 75%, 96%, 100%) and xylol series, and embedded in paraffin blocks. Tissue sections of a size of 5 µm were placed onto slides with Leica RM 2125 RT (the first 3 sections and each 10th section). The preparations were treated with alcohol and xylol series and stained with haematoxylin-eosin (HE). All samples were examined under a high-resolution light microscope (Olympus DP-73 camera, Olympus BX53-DIC microscope, Tokyo, Japan).

Immunohistochemistry

For the immunohistochemical examination, 4 µm thick sections were obtained from the paraffin-embedded tissue blocks and placed on poly-L-lysine-coated glass slides that were stained with the streptavidin-biotin-

peroxidase complex (ABC) technique after routine deparaffinization and rehydration procedures. Antigen retrieval was performed in a microwave oven with citrate buffer (pH 6.0) (700 W, 20 min). Endogenous peroxidase activation in the tissues was blocked for 15 min with 0.3% hydrogen peroxide (H_2O_2) in 0.01 mol/l PBS in methanol at room temperature. Before applying the primary antibody, the tissues were incubated for 20 min with 5% goat blocking serum for protein blocking. Then, the sections were incubated with primary anti-*T. gondii* antibodies for 1 h at room temperature. Tissues were kept in rabbit anti-mouse biontinylated secondary antibody for 30 min after removing the unbound primary antibody. Then, the sections were made to react with horseradish peroxidase streptavidin for 30 min. After washing with PBS, the sections were treated and incubated with DAB (3,3'-Diaminobenzidine, Dako, Glostrup, Denmark) for 5 min. Finally, the background of the tissue sections was stained with haematoxylin. For negative controls, PBS was used instead of the primary antibody. All staining steps were carried out at 37 °C and in humidity cabinets. PBS solution was used as a wash-away solution during all the staining steps.

Results

In this study, heart, brain, and peritoneal fluids from 42 aborted foetuses (in total 126 samples) were examined. *Toxoplasma gondii* was detected in 15 (35.7%) foetuses (in the brain of 3 foetuses, in the heart of 3 foetuses, and in both the brain and the heart of 9 foetuses) while peritoneal fluid was negative. All tissue samples were negative for *N. caninum*. The 529-bp repeat element regions of sequenced *T. gondii* were compared with reference genes in the GenBank (Table 1). The results of the sequences performed with the F primers were not evaluated because they did not have the quality to be analysed. The 230 bp region in the results of the sequences performed with the R primers was checked with BLAST. Except for sample 11, all other samples showed 100% overlap with *T. gondii* sequences. G-C transversion was observed in sample 11 at position 138. The nucleotide sequence of *Toxoplasma* positive samples in this study is given in Table 2.

In the histopathological and immunohistochemical analyses performed with positive brain samples, the lesions were characterized by perivascular mononuclear cell infiltration, diffuse or focal mononuclear cell infiltration in the meninges, and macrophages/microglia proliferation (Plate VII, Fig. 2a). The inflammatory scores are presented in Fig. 2a. Tissue cysts and tachyzoites were observed in all *T. gondii* positive animals (Plate VII, Fig. 2b, 2c). Inflammatory lesions in the brain were more pronounced at the beginning of the

Table 1. Comparison of the sequence results of this study with the reference gene in the NCBI Nucleotide Database and similarity rates.

Samples in this study	Compared access code	Similarity rate
1	LC547467, LC547463, MW509775	100%
2	LC547467, LC547463, MW509775	100%
3	LC547467, LC547463, MW509775	100%
4	MW509775, MW509772, LC547467	100%
5	LC547467, LC547463, MW509775	100%
6	LC547467, LC547463, MW509775	100%
7	LC547467, LC547463, MH884740	100%
8	LC547467, LC547463, MW509775	100%
9	LC547467, LC547463, MH884740	100%
10	LC547467, LC547463, MH884740	100%
11	KX781159, MW509771, MW509769	100%
12	LC547467, LC547463, MH884740	100%
13	MW509775, MW509772, LC547467	100%
14	MW509775, MW509772, LC547467	100%
15	LC547467, LC547463, MH884740	100%

Table 2. The nucleotide sequences performed with the R primers of *Toxoplasma gondii* positive samples in this study.

Samples 1-10,12-15
TTTTTTTTGTTTTTTCACAGGCGAGCTCGCCTGTGCTTGGAGCCACAGAAGGGACAGAAGTCGAG GGGACTACAGACGCGATGCCGCTCCTCCAGCCGTCTTGGAGGAGAGATATCAGGACTGTAGATGA AGGCGAGGGTGAGGATGAGGGGGTGGCGTGGTTGGGAAGCGACGAGAGTCGGAGAGGGAGAA GATGTTTCCGGCTTGGCTGCTTTTCCTGGAGGGTGGA

Sample 11
TTTTTTTTGTTTTTTCACAGGCGAGCTCGCCTGTGCTTGGAGCCACAGAAGGGACAGAAGTCGAA GGGGACTACAGACGCGATGCCGCTCCTCCAGCCGTCTTGGAGGAGAGATATCAGGACTGTAGAT GAAGGCGACGGTGAGGATGAGGGGGTGGCGTGGTTGGGAAGCGACGAGAGTCGGAGAGGGAGA AGATGTTTCCGGCTTGGCTGCTTTTCCTGGAGGGTGGA

Unlike other examples, G-C transversion was observed in sample 11 at position 138.

infection and during the established chronic infection. Anti-*T. gondii* immunopositivity was mainly observed in cells located in areas characterized by glial proliferation (Fig. 2a) and perivascular mononuclear cell infiltration as demonstrated by histopathology. Lymphocytic myocarditis, myocardial inflammation with oedema and mononuclear cells, destruction of the cardiomyocyte, perivascular mononuclear cell infiltration, and diffuse or focal mononuclear cell infiltration, necrosis and tachyzoites were identified in heart tissue (Plate VII, Fig. 2d).

Discussion

Sheep breeding constitutes the largest part of animal husbandry in Türkiye. The Van Province and the neighboring regions are locations where sheep breeding is carried out most. Abortions are among the most important factors that negatively affect sheep breeding. *Toxoplasma gondii* was found to be one of the most often determined causes of abortion (Edmondson et al. 2012). Toxoplasmosis causes severe losses in the sheep industry. In previous studies investigating the prevalence of *T. gondi* in sheep, positivity was found at different rates. The prevalence rates determined world-wide are as follows: 3% in China (Wang et al. 2011), 51.8% in Egypt (Ibrahim et al. 2017), 36.2% in Nepal (Subedi et al. 2018), 38.2% in Brazil (Ueno et al. 2009), 1.6% in Iran (Raeghi et al. 2011), 3.8% in India (Sharma et al. 2008) and 42.1% in Lithuania (Stimbirys et al. 2007). In studies conducted in Türkiye, 45.4% were detected in Yozgat (Babur et al. 2001), 48.4% in Mersin (Öztürk et al. 2002), 54.7% in Afyonkarahisar (Çiçek et al. 2004) and 98.9% in Afyonkarahisar (Çiçek et al. 2011), 66.7% in Yalova (Öncel et al. 2005), 95.7% in Kars (Mor and Arslan 2007), 13% in Konya (Aköz et al. 2009), 53.8% in Hatay (Muz et al. 2013), 97% in Silopi (Leblebicier and Yıldız 2014), 10% in Nevşehir (Çakmak and Karatepe 2017) and 78.6% in Adana (Ekşi et al. 2018). In a study conducted by Irehan et al. (2022) on 30 cattle, 18 sheep and 7 goat foetuses, the total prevalence of *T. gondii* was 10.9%. In the present study, *T. gondii* DNA was detected by PCR in 15 (35.7%) foetuses. The findings of this study are similar to the results of studies on sheep in Brazil and Nepal (Ueno et al. 2009; Subedi et al. 2018).

Although *N. caninum* has been reported to cause abortions in sheep and congenital infections and deaths in newborn lambs, it is not considered among the main causes of abortion in sheep (Innes et al. 2001; Koyama et al. 2001; Hässig et al. 2003). The prevalence of *N. caninum* tested in sheep by PCR in the world is the following: 27.7% in Pakistan (Nasir et al. 2012), 8.8% (Ueno et al. 2009) and 62.2% in Brazil (Filho et al. 2017),

12% in the Czech Republic (Bártová et al. 2009), 10.1% in Spain (Panadero et al. 2010), 1.53% in Iran (Ezatpour et al. 2015) and 16.8% in Greece (Anastasia et al. 2013). In Türkiye, the prevalence of *N. caninum* in sheep was 0.8%, 2.7%, and 0% in Karaman, Konya, and Zonguldak, respectively (Zhou et al. 2016), 12.4% in Adana (Ekşi et al. 2018), 0% in Van (Har and Başbuğan 2019) and Elazig (Özkaraca et al. 2016) and 2.1% in Kars (Gökçe et al. 2015). In our study, *N. caninum* was not detected in any of the 42 foetal tissue samples, and this result is consistent with the results of the study by Özkaraca et al. (2016).

In sheep, protozoal abortions are often associated with *T. gondii* infection. The effect of *N. caninum* on sheep abortions is still obscure. The signs and lesions of toxoplasmosis and neosporosis are similar. Hence, serological tests performed in maternal blood or molecular tests performed in the tissues directly obtained from foetuses play an important role. The PCR test was reported to be the most specific test to reveal the aetiological agents. Histopathological tests performed after these tests increase the reliability (Hässig et al. 2003; Moreno et al. 2012; Irehan et al. 2022). Varying rates of *T. gondii* and *N. caninum* have been reported in previous studies conducted with PCR tests in tissue samples of aborted foetuses. In previous studies, the tissue used for PCR was the brain. Hässig et al. (2003), reported *N. caninum* in four of the brain tissues of 20 aborted foetuses, and *T. gondii* in three of them; Moreno et al. (2012) reported *N. caninum* in five (6.8%), and *T. gondii* in four (5.4%) of 74 sheep foetuses; Shahbazi et al. (2019) reported *T. gondii* in 48 (64%) out of 75 sheep foetuses; Partoandazanpoor et al. (2020) reported *T. gondii* in nine (8.10%) out of 111 sheep foetuses; Howe et al. (2008) reported *N. caninum* in three out of 18 foetuses; Hughes et al. (2006) reported *N. caninum* in 18.9% of 74 foetuses; Irehan et al. (2022) reported *T. gondii* in one of 18 and *N. caninum* in six of 18 aborted foetuses; Hurtado et al. (2001) reported *T. gondii* in nine out of 53 sheep foetuses. In this study, *T. gondii* DNA was determined in 15 (35.7%) out of 42 sheep foetuses; however, *N. caninum* was not determined. *Toxoplasma gondii* DNA was detected in a total of 15 foetuses (both heart and brain tissue of nine foetuses, in only the brain tissue of 3 foetuses, and only the heart tissue of 3 foetuses) and it was concluded that heart tissue was a suitable tissue for molecular studies. We suggest that the different results among the studies may have resulted from the environmental conditions, the distribution of the definitive hosts, the susceptibility of the sheep, and the suitability of the primers to be used in PCR studies. It has been reported that the earlier the pregnancy period of pregnant sheep with toxoplasmosis, the more severe the consequences, and the more waste and re-infections (Dubey 2009). The material of this study comprised early aborted (1–3 months) foetuses as reported by Dubey (2009) indicating that *T. gondii* should be considered first among the aetiological factors in aborted foetuses.

Protozoan abortion in sheep is traditionally associated with *T. gondii*. The characteristic necrotic lesions of toxoplasmosis are usually observed in the central nervous system (Dubey 2003; Partoandazanpoor et al. 2020). *Toxoplasma gondii* invades astrocytes, neurons, and other neuroglia, causing diffuse inflammatory foci, blood vessel clamping and inflammatory meningeal cell infiltrates. Previous studies have reported gliosis foci, necrosis foci, focal large non-suppurative encephalitis areas, mononuclear cell increase, encephalitis findings, tachyzoite, bradyzoite, and pseudocysts in the brain of foetuses aborted due to toxoplasmosis (Hurtado et al. 2001; Pereira-Bueno et al. 2004; Moreno et al. 2012; Castaño et al. 2016; Atmaca et al. 2019). The findings reported in previous studies were identified in 12 foetal brain tissues in the current study.

In some cases, the lesions in foetal tissues are not extensive but may cause abortion, whereas in other cases lambs with toxoplasmosis born with severely damaged placentas may appear healthy. Therefore, examination of various foetal tissues is important in understanding the mechanism of miscarriage (Dubey 2009; Castaño et al. 2016).

Toxoplasma gondii-related lesions are most intensive in the brain and the placenta, and mainly the brain, placenta, and liver are the most commonly used tissues for molecular and pathological examinations. However, the spread of lesions is time-related and autolysis in these tissues may make diagnosis impossible (Hurtado et al. 2001; Castaño et al. 2016). The number of studies investigating *T. gondii* in different tissues of the foetus is very insufficient. Hurtado et al. (2001) investigated *T. gondii* DNA by PCR in 145 tissue samples (lung, spleen, liver, placenta, foetal fluid, brain) obtained from 53 sheep foetuses and a stillborn lamb, and found positivity in nine foetuses and one stillborn lamb. These investigators detected agents in all nine histopathologically examined PCR-positive brain samples, seven of ten spleen samples, eight of nine lung samples, all three of three placental samples, five of seven liver samples, one of two kidney samples, and three of eight foetal fluid samples. The same investigators reported that lung, spleen, and liver tissues could be used in molecular and pathological examinations, following the brain and the placenta. Moreno et al. (2012) detected *T. gondii* and *N. caninum* DNA in aborted sheep foetus samples by PCR and histopathologically determined *T. gondii*-related lesions mainly in brain tissue, and also in the heart, lung, liver, and kidney samples. In this study, the lesions were detected in both the heart and brain tissue of nine PCR positive foetuses, only in the brain tissue of three foetuses, and only in the heart tissue of three foetuses. In the heart tissue, histopathologically and immunohistochemically, lymphocytic myocarditis, myocardial inflammation with oedema and mononuclear cells, destruction of the cardiomyocyte, perivascular mononuclear cell infiltration, necrosis and diffuse or focal mononuclear cell infiltration were determined (Fig. 2D). These results indicate that the heart tissue is suitable for use in investigations for *T. gondii*. Similar results may also be observed in *Neospora caninum*-associated abortion; therefore, it is difficult to discriminate from a pathological perspective and the PCR method is the gold standard for discrimination (McAllister et al. 1996; Hurtado et al. 2001; Moreno et al. 2012).

In conclusion, based on the results of this study, it can be stated that *T. gondii* is an important cause of abortion in sheep in this region. It was also concluded that the PCR method is an important tool to diagnose protozoal abortion agents and that the heart tissue may also be used for histopathological and immunohistochemical analysis when brain tissue is not available. The use of peritoneal fluid was not found to be suitable for the diagnosis of *T. gondii*. Although *N. caninum* was not detected in aborted sheep foetuses in this study, further studies are needed to determine the role of *N. caninum* in abortions in sheep.

Conflict of Interest

There is no conflict of interest.

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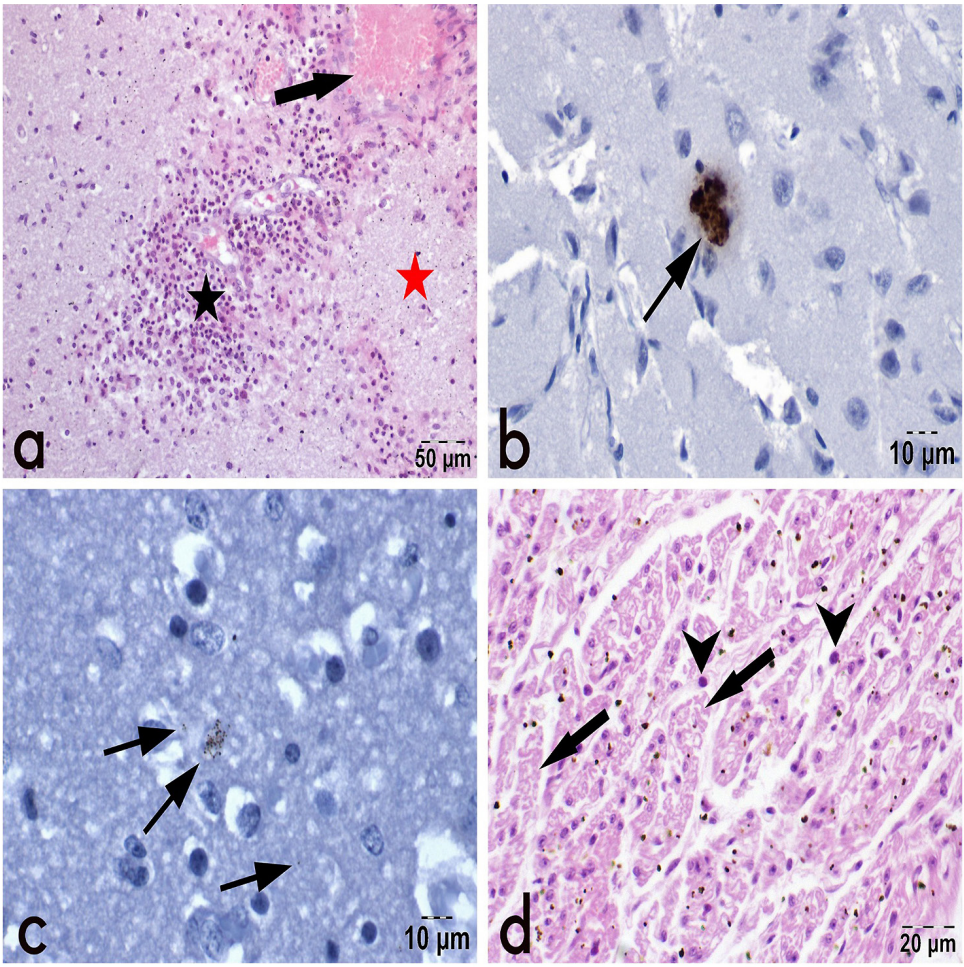

Fig. 2. A - Brain of the sheep infected with *Toxoplasma gondii* showing severe lymphohistiocytic encephalitis (black star), necrosis (red star), and severe hemorragia (arrow). Haematoxylin and eosin staining. B - Immunohistochemical labelling of *Toxoplasma gondii* tissue cysts (arrow) in the brain of the infected sheep. Immunohistochemical staining. C - *Toxoplasma gondii* tachyzoites (arrow) in brain. Immunohistochemical staining. D - Heart tissue of the sheep infected with *Toxoplasma gondii* showing cellular degeneration (arrows) with intralesional *Toxoplasma gondii* tachyzoites (arrow heads). Haematoxylin and eosin staining.