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Neospora caninum in Aborted Bovine Fetuses in Trakya Region, Turkey - Histopathological, Immunohistochemical and Molecular Detection

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ABSTRACT

***Background*:** Being the major cause of bovine abortion in the world, Neosporosis is considered to be a very important protozoal infection in dairy cattle. Vertical transplacental transmission is the major route of the infection causing either abortion or birth of calves with persistent infection. As the seropositivity in individual cows and in fetal serology only indicate exposure to the protozoa, the diagnosis of the infection has to be based on histopathology of aborted fetuses. Additional techniques such as immunohistochemistry (IHC) and PCR are required for the detection of the etiological agent. The purpose of the current study was to diagnose *Neospora caninum* infection in aborted bovine fetuses in Trakya Region of Turkey. For this purpose, serological, histopathological, IHC, and PCR methods were used.

***Materials, Methods & Results*:** The blood samples and the fetuses of 55 aborted dairy cattle from various farms located in 3 provinces of Trakya, Turkey constituted the material of the present study. The sera obtained from the blood samples were tested using a *Neospora caninum* Antibody Test Kit cELISA and anti-*N. caninum* antibodies were detected in the sera of the dams of the 8 aborted fetuses (8/55; 14.54%). Following the necropsy, samples from the brain, heart, liver, lung, kidney, spleen, and placenta of 55 fetuses were routinely processed for histopathological examination and evaluated under a light microscope. Nonsuppurative encephalitis (15/55; 27.27%), necrosis (5/55; 9%) and gliosis (1/55; 1.8%) in the brain, mild to severe nonsuppurative myocarditis and epicarditis (14/55; 25.45%), and portal to mid-zonal nonsuppurative hepatitis (13/55; 23.63%) were the relevant findings. PCR analysis was performed on fresh frozen fetal tissues. Nested PCR detected *N. caninum* DNA in the brain, heart, liver, lung, and kidney tissues of 6 fetuses (6/55; 10.9%). IHC was performed on the brain, heart, and liver tissues of all the fetuses using avidin-biotin-complex peroxidase method. Immunoreactivity was observed in the brain of 1 fetus (1/55; 1.8%).

***Discussion*:** In the present study, histopathological, immunohistochemical and PCR analyses were performed to detect *N. caninum* in 55 spontenously aborted bovine fetuses in Trakya Region, Turkey. Histopathologic hallmark of the study was nonsuppurative inflammation found mostly in the brain, heart and liver followed by kidneys and lungs. No protozoa was observed in the microscopic examination supporting the fact that definitive diagnosis of *N. caninum* infection requires ancillary techniques such as IHC and PCR. Nested PCR detected *N. caninum* DNA in the tissues of 6 fetuses (6/55; 10.9%). Brain was the most reliable organ for detection by PCR (6/6; 100%), compatible with the previous reports. IHC diagnosis revealed only 1.8% positivity in the present study which was remarkably lower than found in the previous studies. Even though histopathology in conjunction with IHC are accepted as the "gold standard" methods to detect *N. caninum* infection in aborted bovine fetuses, there are studies claiming that IHC is relatively insensitive in the diagnosis of neosporosis as parasite numbers can be low and thus, false negative results can be obtained. Other factors affecting the sensitivity of the technique are thoroughly discussed by many authors. Supportively, the findings of the current study showed that using both IHC and PCR as complementary techniques, increases the success of detection of *N. caninum* as recommended in previous studies. In conclusion, the present study demonstrated the first molecular diagnosis of *Neospora caninum* infection in bovine aborted fetuses in Trakya Region of Turkey which has a critical geographical location bordering Europe.

Keywords: bovine abortion, protozoal infection, histopathology, immunohistochemistry, PCR, *Neospora caninum*.

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INTRODUCTION

Neospora caninum is a protozoan parasite which has a wide range of intermediate hosts including many species of ruminants and equids [4,16,22,32]. Canids, mostly domestic dogs, are the only known definitive host for *N. caninum* [3,12]. It causes neuromuscular paralysis in dogs and abortion in cattle, sheep, horses and goats [8,17,22,23]. The life cycle stages of *N. caninum* in the intermediate hosts consist of tachyzoites and tissue cysts. Tachyzoites are found in various cell types such as neural cells, vascular endothelial cells, hepatocytes, myocytes and macrophages whereas, tissue cysts are found mostly in neural tissues [10,11].

Neospora caninum infection has been identified as the major cause of bovine abortion all around the world and thus plays an important role in the economy of many countries [13,20,28,29]. Vertical transplacental transmission is the main route causing either abortion or birth of calves with persistent infection with or without clinical symptoms. Although abortions mostly take place in the fifth and sixth months of gestation, they can occur at any time following the third month [4,12].

There are many studies that report the seropositivity of *N. caninum* in dairy cattle ranging between 6% to 33% in Turkey [2,14,15,19,21]. However, the number of the studies including histopathological, immunohistochemical and molecular detection of the protozoan in aborted fetuses is limited [1,20,25,26].

The aim of the present study is to investigate *Neospora caninum* in dairy cattle and in their aborted fetuses in Trakya Region of Turkey by histopathology, immunohistochemistry (IHC), polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Material of the study

The blood samples and the fetuses of 55 aborted dairy cattles from the dairy farms in Tekirdağ, Edirne and Kırklareli Provinces in Trakya Region of Turkey constituted the material of the present study. The blood samples and fetuses were carried to the Pathology Laboratory of Veterinary Faculty of Istanbul Universtiy- Cerrahpasa with cold chain.

Serological examination

The sera obtained from the blood samples of the dams of the 55 aborted fetuses were tested using a *Neospora caninum* Antibody Test Kit cELISA[1] according to the manufacturer's recommendation. Percent inhibition values with ≥ 30 indicated a positive result, whereas < 30 indicated a negative result.

Necropsy and histopathological examination

At necropsy, carcasses were weighed, the gross findings including age were recorded. Gestational ages of the fetuses were determined by the crown-rump length [5] and in conjunction with the breeding dates, when available. Following necropsy, samples from the brain, heart, liver, lung, kidney, spleen and placenta were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin for histopathological examination.

PCR analysis

The genomic DNA was extracted from fresh and frozen tissues of brain, kidney, heart, liver and lung using a commercial kit[2]. Nested- PCR protocol was conducted using primers from the Nc5 region of the genomic DNA. The amplification was a modification of a previous study [18]. First and second round PCR was conducted using the primers Np21PLUS: 5'- GGG TGT GCG TCC AAT CCT GTA AC 3', and Np6PLUS: 5'- CTC GCC AGT CAA CCT ACG TCT TCT 3' and NP6: 5'- CAG TCA ACC TAC GTC TTC T 3' and NP7: 5' – GGG TGA ACC GAG GGA GTT G 3', respectively. The PCR conditions were programmed according to the literature. PCR products were electrophoresed through 2% agorase gels containing ethidium bromide (10 mg/mL) and the expected DNA fragments (337bp, 227 bp) were visualized with an ultraviolet transilluminator. All positive PCR products were purified using the High Pure PCR Product Purification kit[3] from the gel according to the instruction of manufacturer and sent to a private company[4] for sequencing in order to confirm the validity of PCR amplifications. Sequences were compared with GenBank by Blast analysis.

Immunohistochemical examination

IHC was performed on brain, heart, liver, kidney and lung tissues of all the fetuses using avidin-biotin-complex peroxidase method at the Pathology Laboratory of Veterinary Faculty of Istanbul-Cerrahpasa University. Anti-*N. caninum* antibody[1] diluted at 1:1000 was used as the primary antibody. The antibody was proved that it does not have cross-reactivity for Toxoplasma or Sarcocystis. The IHC protocol was performed as previously described [25] with the exception of using 0.06 % Pronase[5] for antigen retrieval. Additionally, paraffin blocks of brain, heart and liver tissues of 16 cases, including fetuses of the 8 dams that were seropositive for

cELISA (Nos. 16, 18, 25, 35, 40, 46, 49, 55) and 8 fetuses that showed histopathological findings suggestive of *N. caninum* infection (Nos. 7, 8, 22, 32, 41, 48, 50, 51) were submitted to the California Animal Health and Food Safety Laboratory Davis System (CAHFS), Davis, California, USA (UC Davis) for an extra immunohistochemical staining in which a rabbit polyclonal antibody[6] was used as a primary antibody, diluted at 1:200. The IHC protocol was performed as previously described [24].

RESULTS

Serology

Anti-*N.caninum* antibodies were detected in the blood sera of the dams of 8 aborted fetuses (8/55; 14.54%) as shown in Table 1.

Post mortem *findings, histopathology*

Post mortem examination was performed on all 55 aborted fetuses. The fetuses were autolyzed in varying degrees. Edema, congestion and reddish fluid in body cavities were the most consistent gross findings. There were pale areas in the liver of 2 fetuses (Nos. 10, 41). Moderately enlarged lymp nodes were noticed in 3 fetuses (Nos. 7, 32, 43). Gestational ages of the fetuses ranging from 3 to 8 months was as follows: 3-months (5/55; Nos. 8, 23, 28, 37, 51), 4-months (11/55; Nos. 3, 4, 7, 15, 29, 38-40, 44, 47, 48), 5-months (10/55; Nos. 5, 18, 21, 22, 27, 32, 35, 45, 49, 53), 6-months (14/55; Nos. 1, 6, 12, 16, 17, 19, 20, 26, 30, 31, 36, 42, 54, 55), 7-months (10/55; Nos. 9-11, 24, 25, 33, 34, 46, 50, 52), 8-months (5/55; Nos. 2, 13, 14, 41, 43). Histopathological lesions suggestive of a protozoal infection were found in the brain, heart, liver, lung and kidney tissues characterized by nonsuppurative inflammation with or without necrosis (Table 2). The lesions detected in the brain tissues (Figure 1) consisted of nonsuppurative encephalitis (15/55; 27.27%), necrosis (5/55; 9%) and gliosis (1/55; 1.8%). Mild to severe nonsuppurative myocarditis and epicarditis was present in 14 fetuses (14/55; 25.45%) and portal to mid-zonal nonsuppurative hepatitis was present in 13 fetuses (13/55; 23.63%). Mononuclear cell infiltrates were observed in the lungs of 11 fetuses (11/55; 20%) and in the kidneys of 10 fetuses (10/55; 18.18%). Protozoa were not observed in any of the tissues.

PCR

Neospora caninum was found positive by nested PCR in 6 fetuses (6/55; 10.9%; Nos.16, 18, 40, 46, 48, 49) [Table 2]. PCR detected *N. caninum* DNA in brain, heart, liver, lung and kidney tissues (Figure 2).

Immunohistochemistry

IHC was performed on the brain, heart, liver, kidney and lung tissues. Immunoreactivity was observed in the brain of 1 fetus (1/55; 1.8%; No.35), revealing immunopositive cysts (Figure 3). Immunopositive labeling by the monoclonal antibody was not observed in any of the tissues of 55 fetuses.

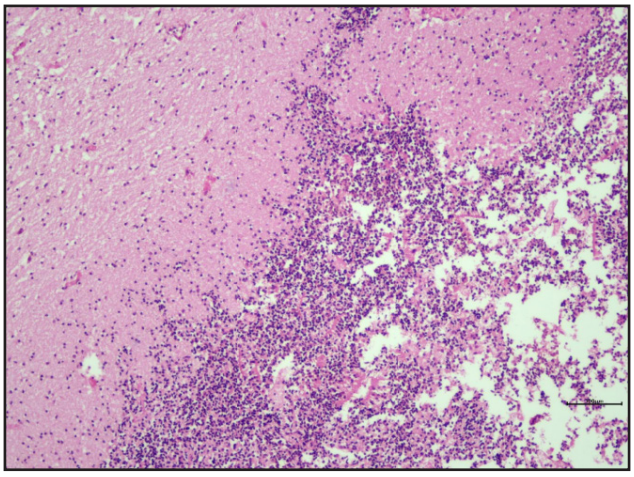

Figure 1. Lesions detected in the brain tissues. Fetus No. 48. Severe lymphoplasmacytic encephalitis [HE; Bar= 100 µm].

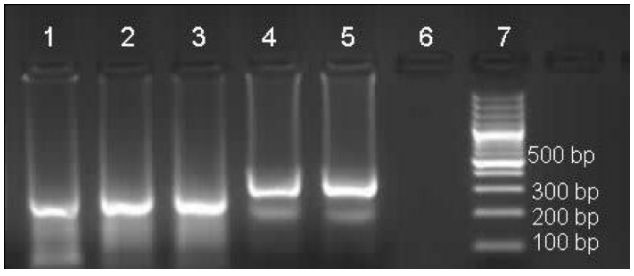

Figure 2. Results of nested PCR products belonging to positive *Neospora caninum* samples electrophoresed through 1.5 % agarose gel. 1,2. bands: positive samples amplified by second round nested PCR (227 bp), 3 band: *N. caninum* positive sample belongs to the present study (227 bp), 4. band: positive samples amplified by first round nested PCR (337bp), 5. band: *N. caninum* positive sample belongs to the present study (337 bp), 6. band: negative control, 7. band: DNA marker (100 bp).

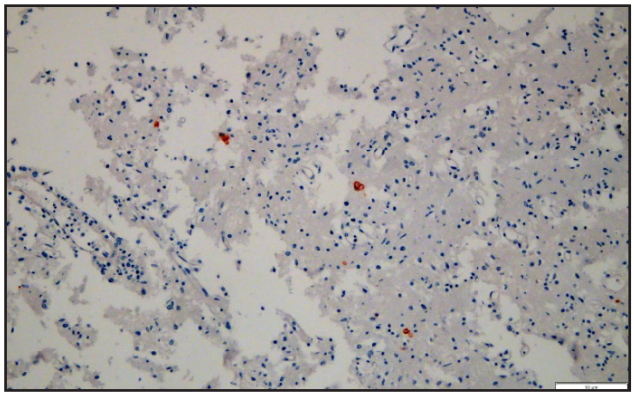

Figure 3. Brain. Fetus No. 35. Positive immunohistochemical labeling for *Neospora caninum*. Anti-*N. caninum* antibody [streptavidin peroxidase method / Mayer's counterstain; Bar= 100 µm].

Table 1. Seropositivity of *Neospora caninum* in the dams of the aborted fetuses by ELISA. Inhibition % was calculated according to the procedure of *N. caninum* Ab Test Kit, cELISA. Inhibition % ≥ 30 indicates a positive result.

Aborted Fetus N°	Inhibition
16	62.2 %
18	49.0 %
25	35.3 %
35	51.7 %
40	51.9 %
46	44.2 %
49	43.2 %
55	37.6%

Table 2. Gestational age, histopathological findings, tissue distribution of *Neospora caninum* DNA in the nested PCR positive bovine fetuses and IHC positivity.

Fetus No	Gestational Age	Histopathological Findings[a]	PCR positivity[b]	IHC positivity
16	6-months	NSE&N, NSM	B, H, Li, Lu	-
18	5-months	NSE, MCIK	B, Lu	-
35	5-months	NSE&N, NSM, NSH, MCIL	-	B
40	4-months	NSE, NSM, NSH, MCIK	B, K	-
46	7-months	NSM, MCIK	B	-
48	4-months	NSE, N, NSM, NSH	B, H, Li, Lu	-
49	5-months	NSE&N, NSM, NSH	B, H, Li, K	-

[a]Histopathological Findings = NSE: Nonsuppurative encephalitis; N: Necrosis; NSM: Nonsuppurative myocarditis; MCIK: Mononuclear cell infiltrates in kidneys; NSH: Nonsuppurative hepatitis; MCIL: Mononuclear cell infiltrates in lungs. [b]PCR positivity = B: Brain; H: Heart; Li: Liver; Lu: Lung; (-): negative; K: Kidney.

DISCUSSION

Neospora caninum is considered to be a very important infectious agent in cattle as being the main cause of abortion, worldwide [10]. Although, there have been many seroprevalence studies on detection of Neosporosis in cattle in Turkey [2,3,14,15,19,21], the number of the studies including histopathological and molecular methods are still limited.

In the present study, histopathological, IHC and PCR analyses were performed to detect *N. caninum* in 55 spontenously aborted bovine fetuses in Trakya Region, Turkey. Additionally, blood samples from the aborting cows were tested for seropositivity by ELISA.

Neospora caninum seropositivity was detected in the dams of 8 aborted fetuses (14.54%). Although, it is well known that maternal serology does not confirm the existence of neosporosis, it is accepted as an indication of a contact with the protozoan. *N. caninum* seropositive cows are reported to have a higher risk of abortion compared to seronegative cows [12]. Mean gestational age of the fetuses of the 8 seropositive dams in the present study was 5.6 months consistent with many authors suggesting that *Neospora* abortions mostly take place in the 4th-6th months of gestation [4,12]. Aborted fetuses are usually found to be autolyzed with serosanguinous pleural and abdominal fluid accumulation [10,11,13]. Pale white foci in the skeletal and heart muscles are counted for *N. caninum* infection related gross lesions. However, they are reported rarely [4]. Consistent with the literature, there were no specific macroscopic findings observed in the present study and the fetuses were autolyzed in varying degrees, as expected.

Histopathologic hallmark of the current study was nonsuppurative inflammation found mostly in the brain, heart and liver followed by kidneys and lungs. No protozoa was observed in the microscopic examination, in correspondence with the previous studies in which none or very low number of parasites were found in the histopathological evaluation of fetal tissues, and the definitive diagnosis of *N. caninum* infection was

made by the parasite detection techniques such as IHC and PCR [12,28,30,31].

In the current study, all of the PCR analysis were performed on fresh frozen fetal tissues. Nested PCR detected *N. caninum* DNA in the tissues of 6 fetuses (6/55; 10.9%). Brain, heart, liver, lungs and kidneys were the PCR positive organs. Brain was the most reliable organ for detection by PCR (6/6; 100%), compatible with previous studies [6,11]. Histopathological findings of the 6 PCR positive fetuses were; nonsuppurative encephalitis (6/6; 100%), nonsuppurative myocarditis (5/6; 83.3%), nonsuppurative hepatitis (3/6; 50%) and mononuclear cell infiltrates in the kidneys (3/6; 50%), which were consistent with findings of previously reported *N. caninum* infections in bovine fetuses [10,31,33].

IHC was performed on tissues of all fetuses. At the first step, anti-*N. caninum* monoclonal antibody was used as the primary antibody, in order to increase specificity in staining, in the brain, heart, liver, kidney and lung tissues of 55 fetuses. However, as there were no fetuses positive on IHC, polyclonal antibody was used at the second step, to have a higher ability for detecting any low quantity anti-*N. caninum* proteins. Strong and prominent staining with anti-*N. caninum* polyclonal antibody was obtained only in the brain of 1 fetus (No. 35), out of brain, heart and kidney tissues of 16 fetuses, that were either PCR positive (n:6) or just showed histopathological lesions suggestive of *N. caninum* infection (n:10). Although, histopathological lesions, including nonsuppurative inflammation in the brain, heart, liver and lung tissues, of fetus No.35 were compatible with *N. caninum* infection, all the tissues were PCR negative.

IHC diagnosis revealed only 1.8% positivity in the present study which was lower than found in the previous studies [7,9,26]. In spite of the fact that, histopathology and IHC are accepted as the "gold standard" methods to detect *N. caninum* infection in aborted bovine fetuses [11,27,30,33], there are studies suggesting that, confirmation of histopathological diagnosis by PCR gives more sensitive results compared to IHC [6,7,31]. Even though IHC plays an important role in the specific identification of protozoons of *N. caninum* infections, it is also claimed to be relatively insensitive in the diagnosis of neosporosis as parasite numbers can be low and they can be present in any region of the brain and thus, false negative results can be obtained inspite of multiple sectioning of the tissues [6]. Additionally, IHC is known to be a specific technique which is sensitive and effective in case that is applied to unautolyzed, well preserved tissues in formalin. Many additional variables are claimed to play an important role in the immunopositivity such as the experience of the operator applying the technique [31]. Thus, it is recommended to use both IHC and PCR as complementary techniques, in order to increase the success in detection of *N. caninum*, when available [6].

In a previous study, an interlaboratory comparision of IHC and PCR methods for detection of *N. caninum* was carried out in bovine fetal tissues. According to the results, PCR techniques were found to be more sensitive and specific. Also, the agreement between the results of IHC and PCR methods was remarkably low [31]. Similarly, in a study that was carried out in Brazil, IHC detected *Neospora* infections in 8.6% of 105 bovine fetuses, while nested-PCR Plus revealed 20.9% positive results [7].

In spite of the fact that serological methods have been frequently used in the detection of *N. caninum* infections in different parts of Turkey, there are still relatively few reports with the definite diagnosis provided by IHC and/or PCR [1,20,25,26]. A study that was carried out in the Black Sea Region and Sivas Province in Turkey revealed 48.9% (43/88) PCR positivity in 42 aborted bovine fetuses and 1 water buffalo fetus [1]. Meanwhile, detection of *N. caninum* was performed by IHC and PCR in 102 aborted bovine fetuses in Elazığ, a city located in the east of Turkey, and the positivity ratio was 17.64% and 25.49%, respectively [26]. In the current study, the very low positivity of IHC makes the comparision with PCR results unavailing. However, detection of *N. caninum* DNA in 6 fetuses (10.9%) and their compatability with the histopathological lesions indicate the presence of *N. caninum* infection in the Trakya Region of Turkey.

CONCLUSION

To the best of authors' knowledge, the present study demonstrates the first molecular diagnosis of *Neospora caninum* infection in bovine aborted fetuses in Trakya Region of Turkey. *N. caninum* infection was confirmed by PCR in 10.9% of 55 specimens indicating that neosporosis is an important cause of bovine abortion in the northwest of the country which has a critical geographical location as being a bridge between Europe and Asia.

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***Ethical approval*.** All animal procedures were carried out in accordance with the approval of the Unit Ethical Committee at Istanbul University-Cerrahpaşa Faculty of Veterinary Medicine (Approval number: 72796624-604.02.01-191607).

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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