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Study on outbreak of *Neospora caninum*-associated abortion in dairy cows in Tabriz (Northwest Iran) by serological, molecular and histopathologic methods

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ABSTRACT

Objective: To determine *Neospora caninum* (*N. caninum*) as a cause of bovine abortion in dairy cows by ELISA, PCR and Pathological methods in Tabriz, Northwest of Iran. **Methods:** For study of outbreak of neosporosis, blood samples were collected from 76 Holstein aborted dairy cows in Tabriz (Northwest Iran). Antibodies to *N. caninum* were assayed by using a commercially ELISA kit (IDEXX, USA). IgG against *N. caninum* were found in 14 (18.4%) cases. Aborted fetuses of these seropositive dams were proposed for histopathological and molecular investigations. Brains, spinal cords and placentas of the fetuses were fixed in 10% buffered formalin for histopathology. Also 5-10 g of brain tissue was sampled for DNA extraction. In 6 out of 14 (42.8%) fetuses, brain tissue was positive in PCR. All dams of these fetuses were serologically seropositive in ELISA test. **Results:** Histopathologically, the lesions consistent with *N. caninum* were observed in brains, spinal cords and placenta of all fetuses. Lesions in CNS included severe congestion, perivascular and perineuronal edema, status spongiosis, perivascular cuffing, focal gliosis, nourophagy and focal necrosis. There were some Neospora-like cysts in brain. In placentas, severe congestion, perivascular infiltration of mononuclear cells, vascular thrombosis, focal placentitis and necrotic foci in cotyledons were noticed. **Conclusions:** The results of present study agreed the results of similar studies about serological, histopathological and molecular results of other studies about neosporosis and it seems to support the outbreak of *N. caninum*-associated abortion in dairy cows in Tabriz (Northwest Iran).

1. Introduction

Neospora caninum (*N. caninum*) is an apicomplexan parasite considered as a significant cause of bovine abortion worldwide[1]. Abortion can occurred at any stage of pregnancy, but usually occur at 5-6 months of gestation, and may occur more than once in the reproductive seasons[2]. In some geographic regions, up to 42.5% of abortions are attributable to neosporosis. The economic impact will depend on the indirect costs, as well as on the value of fetuses lost. Indirect costs include professional help and costs associated with establishing diagnosis, re-breeding, increase lactation time, possible loss of milk yield, and replacement costs if aborted cows are culled[3].

Several methods can be used to detect *N. caninum* including indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) and in aborted fetuses such as histopathology (HP), immunohistochemistry (IHC) and Polymerase chain

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reaction (PCR). In order to reliably diagnosis of *N. caninum*-associated abortion problems, serology alone is inadequate. There is need to demonstrate the presence of the parasite in the fetus, and, if technically feasible, to include (histo) pathological aspects[4]. Concerning fetal diagnosis, detection of compatible lesions by histology and parasites by PCR in brain (as well as heart and liver) are the best choices for fetal diagnosis[5]. Examination of the fetus is necessary for a definitive diagnosis of neosporosis. Ideally, the entire fetus should be submitted but if this is not possible, then samples from brain, heart, and liver should be examined for histopathological changes and body fluids or blood serum for serological evaluation. Although, *N. caninum* infection can cause lesions in several organs, fetal brain is the most consistently affected tissue. The most characteristic lesion is focal encephalitis characterized by necrosis and nonsuppurative inflammation particularly in the brain and to lesser extent in the cord[6]. In advanced lesions, the necrotic areas may be completely replaced by macrophages and a few glial cells, which make the lesions, appear as discrete granulosoma[7]. Furthermore, several PCR methods have been reported to detect *N. caninum* DNA. These methods generally have a higher sensitivity and specificity than other methods and the ability to amplify small amounts of Neospora DNA in a larger quantity of tissue[8].

In Iran, results based on seroprevalence of *N. caninum* in aborted dairy cattle showed that neosporosis should be regarded as a cause of economic loss in dairy cattle[9-11]. The objective of this study was to determine *N. caninum* as a cause of bovine abortion in dairy cows by ELISA, PCR and Pathological methods in Tabriz, Northwest of Iran.

2. Materials and methods

2.1. Study site

This study was carried out in Tabriz (northwest Iran) during 2008-2010. Tabriz is located in the East-Azerbaijan province ($36^0 43' - 39^0 25'$ N and $45^0 3' - 48^0 19'$ E). The region is mountainous, with altitudes 1351.4 m. The climate is temperate. Summers are relatively hot and dry while winters are cold. Samples were collected from cows on dairy farms in around of Tabriz.

2.2. Sampling and ELISA

Blood samples were collected from 76 Holstein aborted cows in plain vacationer tubes and were transported to the laboratory. After centrifugation at 1 000×*g* for 15 min, serum was removed and were analyzed for antibody activity to *N. caninum* using a commercially ELISA kit (IDEXX, USA). The tests were performed according to manufacturer's instruction. OD of 0.15 was considered as cut-off based on the instruction of manufacture and the ratio of sample for positive control was ⩾0.2 OD. IgG to *N. caninum* were found in 14 out of 76 (18.4%) sera. Aborted fetuses of these seropositive cows were objected for histopathological and molecular studies. For this purpose, the fetuses were immediately transported on cold conditions. The fetuses were necropsied and samples from brain, spinal cord, liver, heart and placenta were obtained and fixed in 10% buffered formalin (pH=7.2). Also, 5-10 g of brain tissue were randomly taken from different regions and homogenized in 20 mL of PBS containing 5% antibiotic and were stored at -20 ℃ in microtubes for DNA extraction.

2.3. DNA extraction

DNA extraction from frozen tissue samples was performed using a commercial kit (Accuprep Genomic DNA Extraction Kit, Bioneer, South Korea) following the manufacture's instructions. Briefly, 100 μL of thawed brain tissue were mixed with 600 μL of Tissue Lysis Solution and homogenized for 10 s. Samples were incubated at 65 ℃ for 30 min, followed by addition of 17.5 μL proteinase K (20 mg/mL) and incubation at 60 ℃ for 3 h, overtaxing every 30 min. Three microliters of RNase A (4 mg/mL) were added, the samples were mixed and incubated at 37 ℃ for 30 min. After cooling, 200 μL of Protein Precipitation Solution were added, followed by overtaxing and centrifugation at 13 000×*g* for 4 min. The supernatant was transferred to a new microtube with 600 μL of isopropanol, mixed and centrifuged at 13 000×*g* for 3 min. The supernatant was discarded and the pellet was washed with 600 μL of 70% ethanol, followed by a final centrifugation at 13 000×*g* for 3 min. Each pellet was dissolved in 100 μL of DNA Rehydration Solution by incubating at 65 ℃ for 1 h.

DNA quality was assessed by a spectrophotometery and PCR amplification of an internal control (Prolactin gene). Samples that did not yield a Prolactin amplicon or had DNA concentration lower than 100 ng/μL as assessed by spectrophotometery were excluded from further analysis.

2.4. PCR assay

2.4.1. Primers and thermal cycling conditions

The primers were designed based on the report of Kaufmann *et al*(1991). The primers Np6+ (5-gggtgaggacagtgtgtcaa-3) and Np21+ (5-tcccatacctggatgctttc-3) were predicted to produce

a 213 bp region of *N. caninum* genome. Furthermore, two primers HL033 (5-cgagtccttatgagcttgattctt-3) and HL035 (5-gccttccagaagtcgtttgttttc-3) that target part of the bovine Prolactin gene were considered as an internal control[12].

2.4.2. Amplification

PCR was performed using DNA with brain, spinal cord and placenta of samples collected pathology division of the University of Tabriz.

The PCR assay was conducted in a Perimus 96 thermal cycler (Peqlab, Germany). The reaction mixture (25 μL) contained $MgCl_2$ (4 mM), 1 mM dNTP mix (Bioline), 2 mM each primer, 0.2 U *Taq* (Bioline), and 2 μL of extracted DNA. The thermal protocol was as 5 min at 95 ℃ for initial DNA denaturation, followed by 30 cycles programmed as follows: 95 ℃ for 30 s (denaturation step), 60 ℃ for one minute (annealing step), and 72 ℃ for 30 s (extension step) and 10 min for final extension at 72 ℃. The products of PCR were separated on a 2 percent (w/w) agarose gel, in TAE buffer and stained with ethidium bromide. A 50 bp DNA ladder (Fermentase, Ukrain) was used as a size marker. The gel photos were recorded by a Syngene gel documentation system.

2.5. Histopathology

The formaldehyde-fixed tissue specimens from brains, spinal cords, placenta, liver and heart were processed in a tissue processor (Jung histokinette 2000, Leica, Germany), paraffin blocks were made, and 5-6 microns thick sections were serially cut with a microtome (Jung histocuts, Leica, Germany) and stained with hematoxylin and eosin (H&E) (21). Histopathologically, the sections were examined by light microscopy (Olympus, CH36RF200, Japan) and digitally photographed with a photomicrograph (Olympus DP12, U-TVO.5XC-2, Japan).

3. Results

Seventy six sera of aborted cows were tested. IgG to *N. caninum* were found in 14 out of 76 (18.4%). Aborted fetuses of these seropositive dams were proposed for histopathological and molecular investigations. In 6 out of 14 (42.8%) fetuses, brain tissue was positive in PCR.

Products of ~370 bp were amplified by using primer pair Np6 and Np21 (Figure 1). The size of the bands matched the positive control. Amplification of the prolactin gene with primer pair (HL033 and HL035) gave product of ~156 bp in the all DNA samples.

Histopathologically, the lesions consistent with *N. caninum* were observed in brains, spinal cords and placenta and heart of all fetuses. Lesions in CNS included severe congestion, perivascular and perineuronal edema, status spongiosis, perivascular cuffing, focal gliosis, nourophagy and focal necrosis. There were some Neospora-like cysts in brain (Figure 2 & 3). In placentas, vasculitis, severe congestion, perivascular infiltration of mononuclear cells, vascular thrombosis, focal placentitis and necrotic foci in cotyledons were noticed. In heart edema, focal epicarditis and focal hemorrhages were observed (Figure 4).

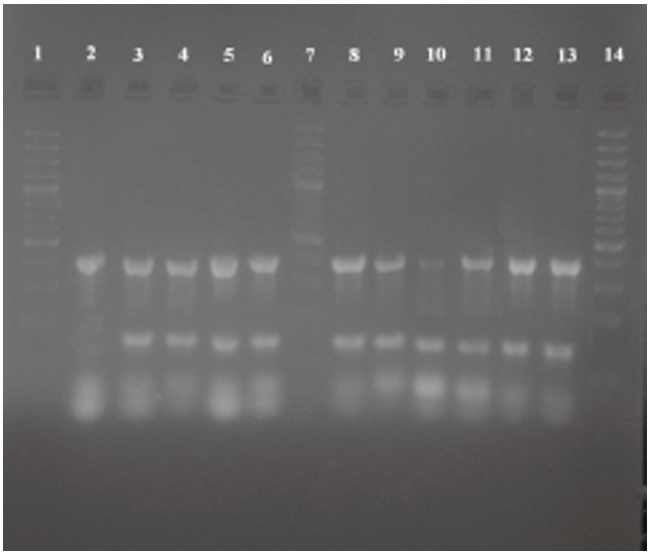

Figure 1. Agarose gel electrophoresis of PCR products (Np6 plus and Np21 plus primers) from brain tissues of aboted fetuses (Lanes 3-6 & 8-13).
Positive control DNA prepared from cultured *N. caninum* NC5 (lane 2). Lanes 1, 7 & 14 represent PCR 100 bp Low Ladder marker (Bioneer Co Ltd. S. Korea). In each lane PCR product of prolactin gene is also included.

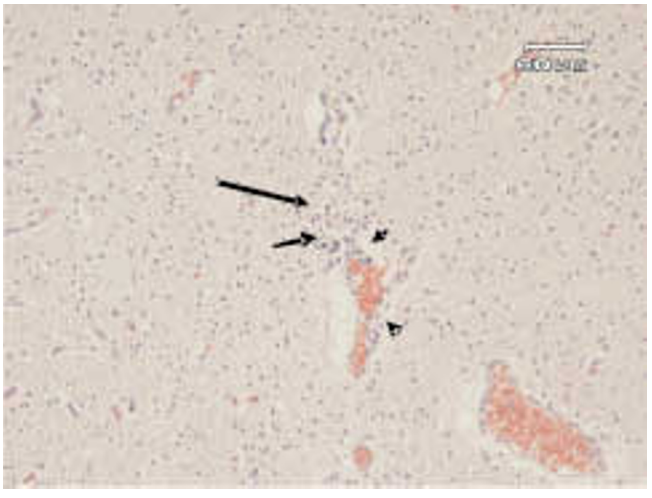

Figure 2. A microscopic section of brain of aborted calf due to neosporosis, hyperemia, focal gliosis (long arrow), a small neospora-like cyst (short arrow) and mild nonsuppurative encephalitis with lymphohistiocytic perivascular cuffing (arrowheads) are present (H&E, 200×).

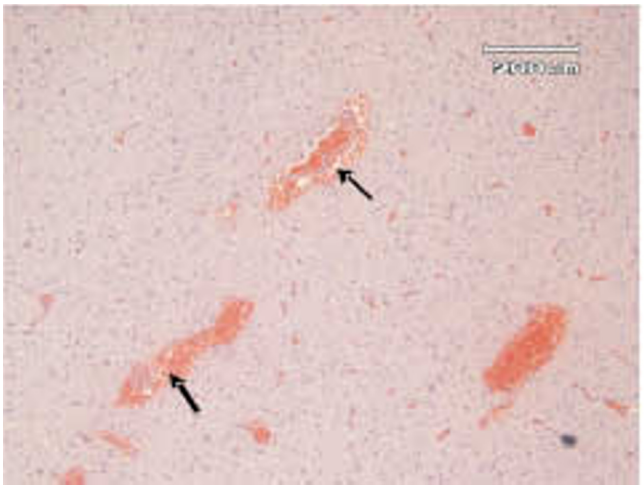

Figure 3. Microscopic section of CNS, severe hyperemia and perivascular hemorrhage (arrows) are observed in brain of aborted calf due to neosporosis (H&E, 100×).

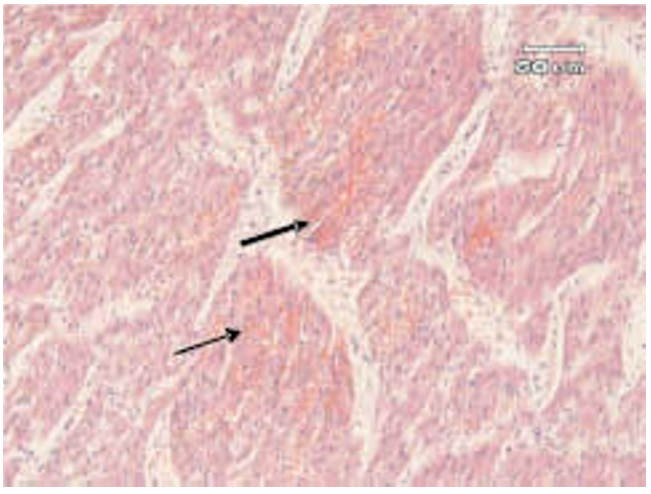

Figure 4. Histopathologic section of heart of aborted calf due to neosporosis, hyperemia, edema and focal hemorrhages (arrow) are seen (H&E, 200×).

4. Discussion

Bovine neosporosis can be diagnosed in adult cattle using indirect methods such as indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), while in fetuses, direct methods such as histopathology, immunohistochemistry (IHC) and, recently, PCR probes are more often used. The fetal brain is the most consistently affected organ, but heart and liver are also commonly affected[6]. PCR is considered a highly specific and sensitive technique for detection of fetal infection[4]. In this study ELISA method, PCR technique and histopathology were used for diagnosis of neosporosis. From 76 sera of aborted cows, IgG to *N. caninum* were found in 14 (18.4%) cases and 6 out of 14 (42%) of aborted fetuses of these cows were positive in PCR and the lesions consistent with *N. caninum* were observed in all these fetuses.

This study shows that the seroprevalence of antibody to *N. caninum* is 18.4% in dairy cattle in Tabriz. Also, percentage of *N. caninum*-related abortions in seropositive cattle is 42.8% and all dams of these fetuses were serologically seropositive in ELISA test. Although, the presence of antibodies against *N. caninum* in cattle only indicate expose to the parasite, probability of abortion in seropositive cattle was twice higher than in seronegative cattle[3]. In other investigation in Iran with *N. caninum*, serological results showed a considerable variance. The prevalence of infection in the present study seemed comparable with Sadrbazzaz *et al* (2004) and Hajikolaei *et al* (2008), in which they presented the seroprevalence of *N. caninum* in dairy cattle was 18% and 21% in Mashhad and Ahvaz[9-11]. This difference may be due to type of test used, their cut-off points and other characteristics. In other studies in Iran about *N. caninum* the seroprevalence of the parasite was denoted in healthy and aborted cattle[9,13].

Recently DNA of *N. caninum* was demonstrated in tissues of seropositive cattle by PCR[14]. Thus providing another potential tool for the diagnosis of *N. caninum* infections in cattle. In the present study, *N. caninum* DNA was found in brain tissue of 6 out of 14 (42.8%) of *N. caninum*-related abortions. This differences between results of ELISA and PCR methods suggested that all detected abortions in this study were not due to neosporosis. It may be seropositivity in ELISA test was due to presence of other coccidian organisms (Neospora-like organism such as Toxoplasma) in farm of cows.

In the present study, 6 out of 14 (42.8%) of *N. caninum* fetal infection-cases were diagnosed by PCR. This value is higher than the 33% previously reported by Sadrebazzaz *et al* in 2007 from Iran[13]. The results of this study also showed lower prevalence than those reported by Dubey (2003) and other studies using PCR diagnosis[6,13-15].

In the present study, fair agreement was observed between PCR and histopathology in all 6 fetuses which were positive by PCR method. This high correlation between PCR and diagnosis of necrotic lesions in histopathology provided a solid evidence for *N. caninum* being the cause of abortion. This result was agreement by similar studies in Iran and world[12,13,15] but Salehi *et al* (2009) reported histopathologic changes in 3 out of 12 aborted fetuses[10].

In this study, pathological changes were observed in brain, spinal cord, placenta and heart of 6 PCR-positive fetuses. These changes were agreement by suggestive lesions of neosporosis by other studies[4,16]. Also, focal microgliosis, focal non-suppurative, necrotizing encephalitis and myocarditis are considered as indicative of infection by *N. caninum*, although corroborative evidence for the presence of the parasite is necessary, since other protozoa may cause similar lesions[17].

In conclusion, the results of present study agreed the results of similar studies about serological, histopathological and molecular results of other studies about neosporosis and it seems to support the outbreak of *N. caninum*-associated abortion in dairy cows in Tabriz (Northwest Iran).

Conflict of interest statement

We declare that we have no conflict of interest.

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