A survey of *Neospora caninum*-associated abortion in dairy cattle of Romania

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Abstract. The first detection by immunohistochemistry of *Neospora caninum* parasitic forms in the tissues of aborted bovine fetuses in Romania is reported. Nine dairy cattle and their aborted fetuses and feces samples from 12 dogs were tested. Five dairy cattle were serologically positive for *N. caninum* by ELISA. The Nc-5 gene of *N. caninum* was amplified from samples of four aborted fetuses. In two fetuses immunohistochemistry showed parasitic forms stained positively with the mouse anti-*N. caninum* antibodies. Histopathology revealed multifocal non-suppurative encephalitis and myocarditis. *Neospora caninum* DNA was amplified in one feces dog sample.

Keywords: *Neospora caninum*; PCR; IHC; Cattle abortions; Dogs; Romania.

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Introduction

*Neospora caninum* is an apicomplexan protozoan parasite with a worldwide distribution. It was first reported in dogs (Bjerkas et al., 1984; Dubey et al., 1988) and it has been found to have a wide intermediate host range including cattle. Tachyzoites, tissue cysts and oocysts are considered to be the three infectious stages in its life-cycle (Dubey, 2003). Dogs are both intermediate and definitive hosts for *N. caninum*. Oocysts of the parasite are shed in the feces of dogs (McAllister et al., 1998), wolves (Dubey et al., 2011), coyotes (Gondim et al., 2004) and dingoes (King et al., 2010). Transplacental infection is thought to be the major route of its transmission in cattle (Scharles et al., 1998).

*Neospora caninum* is considered to be the major cause of abortion in cattle worldwide. Investigations in Romania for detecting *N. caninum* DNA by PCR revealed Nc-5 fragments of the expected size (about 327 bp) from the brain tissue samples (Şuteu et al., 2010). Serological investigation in dairy cattle from Romania, for detecting antibodies against *N. caninum* showed a prevalence between 27.7% and 41.7% (Gavrea et al., 2011; Mitrea et al., 2012; Imre et al., 2012). Serological studies in
goats from Romania revealed *N. caninum* prevalence in 2.3% (Iovu et al., 2012). However, there is no information on *N. caninum* infection associated abortion in Romania. The aim of this study was to prove in the aborted cattle the direct correlation between the positive serology and the presence in the abortions of *N. caninum* like parasitic forms by IHC.

### Materials and methods

#### Biological samples

During three months of 2011 the aborted fetuses from a dairy farm from Central region of Romania, were collected. From a total of 310 dairy cattle, 22 cattle aborted in this period. From the total abortions only nine aborted bovine fetuses, between 4 and 6 months, and blood serum samples from the dams (Holstein breed) were provided to the laboratory and examined. Blood samples were collected from the jugular vein at abortion day. Fetuses were collected in the first 12 hours after abortion and submitted to the laboratory for necropsy and histopathology. Samples of brain and heart were collected from eight fetuses while, from one fetus only placenta was available because the body was eaten by dogs. Immunohistochemistry and polymerase chain reaction was performed in brain, heart and placenta samples. The dairy cattle farm is exposed to stray dogs contact, 12 individual dog feces samples being collected from different places inside the farm.

#### Serology of the dairy cattle

The presence of *Neospora caninum* (BIO-X *Neospora caninum* ELISA KIT, Bio-X Diagnostics, Belgium) and *T. gondii* (Chekit Toxotest Antibody ELISA, Idexx-Bommeli, Switzerland) antibodies (IgG) was checked by indirect enzyme-linked immunosorbent assay (ELISA) using two commercial kits and following the manufacturer’s instructions.

Serum samples were diluted 1:100 for detecting *N. caninum* antibodies and the results were measured and quantified according with the producer’s instructions (Val=Delta OD spl*100/Delta OD pos). The sensitivity and specificity of *N. caninum* ELISA were 89% and 96% respectively.

In the case of *T. gondii* ELISA serum samples were diluted 1:400 and the results were measured as optical density percent-ages (OD% = OD sample – OD negative control/OD positive control – OD negative control × 100). Sensitivity and specificity of *T. gondii* ELISA were 90.5% and 97.8%, respectively (Mainar-Jaime and Barberan, 2007).

#### Histopathology and immunohistochemistry of fetal tissues

Samples of brain and heart tissues, collected from the eight aborted fetuses, were fixed in 10% neutral buffered formaldehyde and stained with hematoxylin and eosin (HE) for routine histologic examination. In one case only placenta was available. All tissues were processed by IHC.

Briefly, we used the following IHC protocol: after paraformaldehyde fixation, the samples were embedded in paraffin, then sectioned at 5 μm using a Leica RM 2125 RT microtome and mounted on slides embedded with APES (aminopropyl-tri-ethoxy-silane). For a better adherence, samples were incubated at 37°C for 24 hours. Dewaxing and rehydration were followed by antigen retrieval in a sodium citrate solution (10 mM pH 6). Samples were brought to boiling temperature, and then kept 10 minutes at sub-boiling temperature and 30 minutes cooled on benchtop. Peroxidase blocking was done with 3% hydrogen peroxide (x). A monoclonal anti-*N. caninum* primary antibody (BIO 319, BioX Diagnostics, Belgium) and, for the visualization, a secondary antibody included in the kit LSAB+System HRP was used. Nuclei were counterstained with Gill’s hematoxylin. Tissue sections stained exclusively with the secondary antibody were used as negative control.

#### Polymerase chain reaction (PCR)

Genomic DNA extraction of tissues from aborted fetuses was performed on brain, heart and placenta samples. DNA was extracted from 40 mg tissue using a commercial kit (Isolate Genomic DNA Kit, Bioline, UK), according to the
manufactures protocol. For DNA extraction from dog feces we used a commercial kit (Isolate Fecal DNA Kit, Bioline, UK). Extraction was performed on 180 mg dog feces following the manufacture’s protocol.

PCR was performed on all abortions samples to detect *N. caninum* and *T. gondii* DNA, and on all dog feces samples to detect *N. caninum* DNA. PCR protocol for detecting *N. caninum* was conducted using primers from the Nc-5 region of the genomic DNA. A pair of Np6/Np21 primers (5’ GGGTGTGCGTCCAATCCTGTAAC 3’–5’ CTGCCAGTCACCTAGTCTTTCT 3’) (Generi-Biotech, Czech Republic) was used to amplify the 327 bp DNA fragment (Yamage et al., 1996). PCR was carried out in a 25 μl reaction mixture consisting of 12.5 μl of MyTaq Red HS Mix (Bioline, UK) and 25 pM of each primer. The volumes of DNA template were 4 μl. The amplification was performed in BIO RAD C1000™ Thermal Cycler. Cycling conditions were: 1 min at 95°C; 15 s at 95°C, 1 min at 63°C, and 10 s at 72°C (40 cycles); and 2 min at 72°C. For detection of *T. gondii* we used a pair of Tox4/Tox5 specific primers (5’ CGC TGC AGG GAG GAA GAC GAA AGT TG 3’ – 5’ CGC TGC AGA CAC AGT GCA TCT GGA TT 3’) (Generi-Biotech, Czech Republic) from the ITS1 region of the ribosomal DNA (Homan et al., 2000). PCR was carried out in a 25 μl reaction mixture consisting of 12.5 μl of MyTaq Red HS Mix (Bioline, UK) and 25 pM of each primer. The volumes of DNA template were 4 μl. Cycling conditions were: 1 min at 95°C; 15 s at 95°C, 15 s at 60°C, and 10 s at 72°C (35 cycles); and 5 min at 72°C.

Aliquots of each PCR product were electrophoresed on 1.5% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen) and observed for the presence of the specific fragment under UV light (BIO DOC-ItTM Imagine System). DNA fragment size was compared with a standard molecular weight (100 bp DNA ladder – Fermentas). Three controls were performed: a positive control of *N. caninum* – Nc 1 strain (Şuteu et al., 2004), a positive control of *T. gondii* – RH strain and a negative control – distilled water.

### Results

**Serology of the dairy cattle**

Five of the nine dairy cattle (55%) tested were positive for *N. caninum*. Antibodies anti-*T. gondii* were detected in seven of the nine samples (77.7%) tested (table 1), three of them being positive for both *N. caninum* and *T. gondii*.

<table>
<thead>
<tr>
<th>Fetus no.</th>
<th>Serology of the cattle</th>
<th>Age of fetus (months)</th>
<th>PCR – fetuses</th>
<th>IHC – fetuses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>N. caninum</em></td>
<td><em>T. gondii</em></td>
<td><em>N. caninum</em></td>
<td><em>T. gondii</em></td>
</tr>
<tr>
<td>1</td>
<td>Negative</td>
<td>Positive</td>
<td>5</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>Negative</td>
<td>Positive</td>
<td>6</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>Positive</td>
<td>8</td>
<td>Negative</td>
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<tr>
<td>4</td>
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<td>7</td>
<td>Negative</td>
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<td>8</td>
<td>Positive</td>
<td>Negative</td>
<td>5</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>Positive</td>
<td>5</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*o.p.* – only placenta.

**Histopathology and immunohistochemistry of fetal tissues**

Of the nine bovine fetuses, only eight were fit for histological and immunohistochemistry examination. One of them was eaten by dogs and only the placenta was tested (table 1). Careful examination revealed histologic lesions in both organs examined, respectively the heart and the brain. Lesions were often...
obscured by autolysis, especially in the brain. Most significant lesion in the brain was multifocal non suppurative encephalitis. Also focal gliosis was seen in all affected brains.

Additionally, there were random foci of necrosis surrounded by rims of glial cells, varying in size, often in white matter. Sometimes scattered tachyzoites were observed in these foci.

Focal or diffuse non suppurative myocarditis was the main lesion in the heart. There were varying numbers of mononuclear cell infiltrate in the myocardium. Degeneration and necrosis of cardiomyocytes was observed but it could have been obscured by autolysis, as all of the tissues collected suffered different degrees of autolysis.

Neospora-like tissue cysts, tachyzoites in parasitophorous vacuoles and free tachyzoites were found in two of the eight fetuses processed by immunohistochemistry. In one of the fetuses immunohistochemistry revealed positive stained *N. caninum* parasitic forms in both tissues tested. Neospora-like tissue cysts, round in shape and about 21 µm in diameter (figure 1) were found in the brain tissue and the exam of the heart muscle revealed presence of specific Neospora-like parasitophorous vacuoles, oval in shape and about 14/8 µm in diameter (figure 2). In the other fetus Neospora-like parasitic forms (figure 3) and free tachyzoites were revealed in the brain tissue (figure 4). Parasitic forms were stained positively with the mouse anti-*N. caninum* antibodies (figure 2), and negatively with the mouse anti-*T. gondii* antibodies.

*Molecular analysis*

Nc-5 fragments of the expected size (about 327 bp) were amplified from the brain or heart tissues of four fetuses (44%) (table 1). Only in one case both brain and heart were positive. No specific *T. gondii* DNA fragments were amplified. *N. caninum* DNA was obtained in one of twelve dog feces samples (8.33%).

![Figure 1. N. caninum broken tissue cyst in the brain (IHC x 400)](image-url)
Discussion

Positive serological testing of individual dams only allows one to suspect *N. caninum* infection, but is no proof that *N. caninum* was involved in the reproductive failure. In our study, the diagnosis of abortion due to *N. caninum* was oriented by serology of the dams and based on the presence of histological lesions, positive reaction by IHC and PCR in abortions.

Because less than 10% of the cows aborted, we defined the pattern of abortions as sporadic (Wouda et al., 1999).
The most characteristic lesion in neosporosis is focal encephalitis characterized by necrosis and non suppurative inflammation (Wouda et al., 1997). Although a presumptive diagnosis may be done by examination of hematoxylin and eosin (HE) stained sections, IHC is necessary because there are often only a few *N. caninum* present in autolyzed tissues and these are often not visible in HE stained sections (Lindsay and Dubey, 1989). The IHC we performed revealed the presence of *N. caninum* forms in the brain and heart muscle in two from the eight of the tested aborted fetuses (25%).

Using the PCR method, Gottstein et al. (1998) examined 83 bovine fetuses from Switzerland for protozoal abortion. *Neospora*-specific DNA was found in 24 (29%), and *T. gondii*-specific DNA was found in 4 (5%) fetuses. Aborted fetuses investigated in Romania for detecting *N. caninum* DNA by PCR revealed *Nc*-5 fragments of the expected size (about 327 bp) from the brain tissue samples of three from nine aborted fetuses checked (Șuteu et al., 2010). PCR performed in our study revealed *N. caninum* DNA in four from nine (44%) abortions tested and no DNA fragment of *T. gondii*, excluding a possible toxoplasmosis abortion etiology.

**Figure 4.** Focal area of necrosis in brain, with scattered tachyzoites (IHC x 400)

We confirm the theory shown in several studies which indicate that brain tissue is the most suitable for the detection of *N. caninum* DNA by PCR (Gottstein et al., 1998; Baszler et al., 1999; Buxton et al., 1998). In our test, *N. caninum* DNA was mainly found in brain samples which proved to be positive in four cases instead of only one heart positive sample. Only in one case both brain and heart were positive.

Bovine neosporosis can persist for generations (Pare et al., 1996) and this can be a source of infection with *N. caninum* infection for dogs (Wouda et al., 1999). There are studies that showed a positive correlation between presence of dogs in farm’s area and cattle abortion induced by *N. caninum* (Pare et al., 1998). We obtained *N. caninum* DNA in one of twelve dog feces samples. The DNA found in the dog feces might originate in oocysts, meaning that in this case the dog was definitive host. Because these dogs have a permanent access inside the farm we can suppose that the dog represented a reservoir of *N. caninum* for cattle. However, the DNA found in the dog feces can originate also from the ingestion of a *N.
*caninum* infected tissue, in this case the dog playing just a vector role.

In conclusion, the diagnosis of abortion due to *N. caninum* was oriented by serology of the dams, and in the aborted fetuses tested by histopathology, immunohistochemistry and PCR, *N. caninum* infection was confirmed, by all three techniques, in two of nine specimens (22%), suggesting that *N. caninum* is one of the pathogen involved in abortion in dairy cattle farm from Romania.

Infection of *N. caninum* in cattle in Romania has been confirmed. Further work is needed to isolate the tachyzoites of *N. caninum* from bovine tissues obtained from dairy farms in Romania.

Acknowledgments

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References


