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Short communication

Histologically, immunohistochemically, ultrastructurally, and molecularly confirmed neosporosis abortion in an aborted equine fetus

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ABSTRACT

Neosporosis is a common cause of abortion in cattle worldwide but is rare in horses. Here, the first case of histologically, ultrastructurally, immunohistochemically, and molecularly confirmed equine abortion caused by neosporosis is reported. Samples of lung, heart, liver, skeletal muscle, tongue, brain, and the placenta from a female fetus aborted at 280 days of gestation were fixed in formalin and submitted for diagnosis. Histologically, there was disseminated neosporosis with severe lesions in lungs, liver and the heart. Protozoal tachyzoites in all tissues reacted with polyclonal anti-*Neospora caninum* rabbit antibodies. Transmission electron microscopic observation on lung tissue revealed tachyzoites consistent with *Neospora*, including many rhoptries. Polymerase-chain reaction (PCR) using primers designed to amplify the rRNA gene internal transcribed spacer 1 (ITS1) of the Sarcocystidae was performed on DNA extracted from fetal tissues. Comparison of the ITS1 amplified from the foal tissue to sequences available in GenBank revealed 100% sequence identity to the ITS1 from three isolates of *Neospora hughesi*.

1. Introduction

Neosporosis is a common cause of abortion in cattle worldwide (reviewed in Dubey et al., 2017). The etiologic protozoan, *Neospora caninum*, is very efficiently transmitted transplacentally; up to 90% of calves from infected dams can be born infected. *N. caninum* also causes neonatal mortality in other species of livestock and wildlife. Canids (dog, coyote, red wolf) are its definitive hosts that can excrete environmentally resistant oocysts in their feces. Currently, there are two described species of *Neospora*, *N. caninum* and *N. hughesi* (Dubey et al., 1988; Marsh et al., 1998). The definitive host for *N. hughesi* is unknown and infection has been reported only in horses. Although epitope differences in individual antigens have been identified (Marsh et al., 1999), there is extensive serologic cross-reactivity between the two species of *Neospora*.

Numerous serological surveys, mostly using *N. caninum* as antigen, have revealed an asymptomatic *Neospora* infections in horses (Dubey et al., 2017). However, unlike neosporosis abortion in cattle to date *Neospora*-associated abortion in horses has not been readily recognized nor have extensive studies that evaluated this etiology of equine abortion been performed (Duarte et al., 2004; Locatelli-Dittrich et al., 2006; Antonello et al., 2012). In one study, congenital neosporosis was reported in seven foals but they were healthy (Pusterla et al., 2011, 2014). Additionally, *Neospora* DNA was detected by PCR in equine fetal tissues from France (Pronost et al., 1999; Veronesi et al., 2008; Leon et al., 2012). Suggestive evidence for neosporosis abortion was provided by comparing seropositivity in aborting and non-aborting mares (Pitel et al., 2003; Villalobos et al., 2006; Abreu et al., 2014).

There is only one previous report of finding *Neospora* tachyzoites in histological section of fetal equine lung by immunohistochemistry but no other tissue was examined (Dubey and Porterfield, 1990). Here, we present conclusive evidence for neosporosis associated abortion in an equine fetus.

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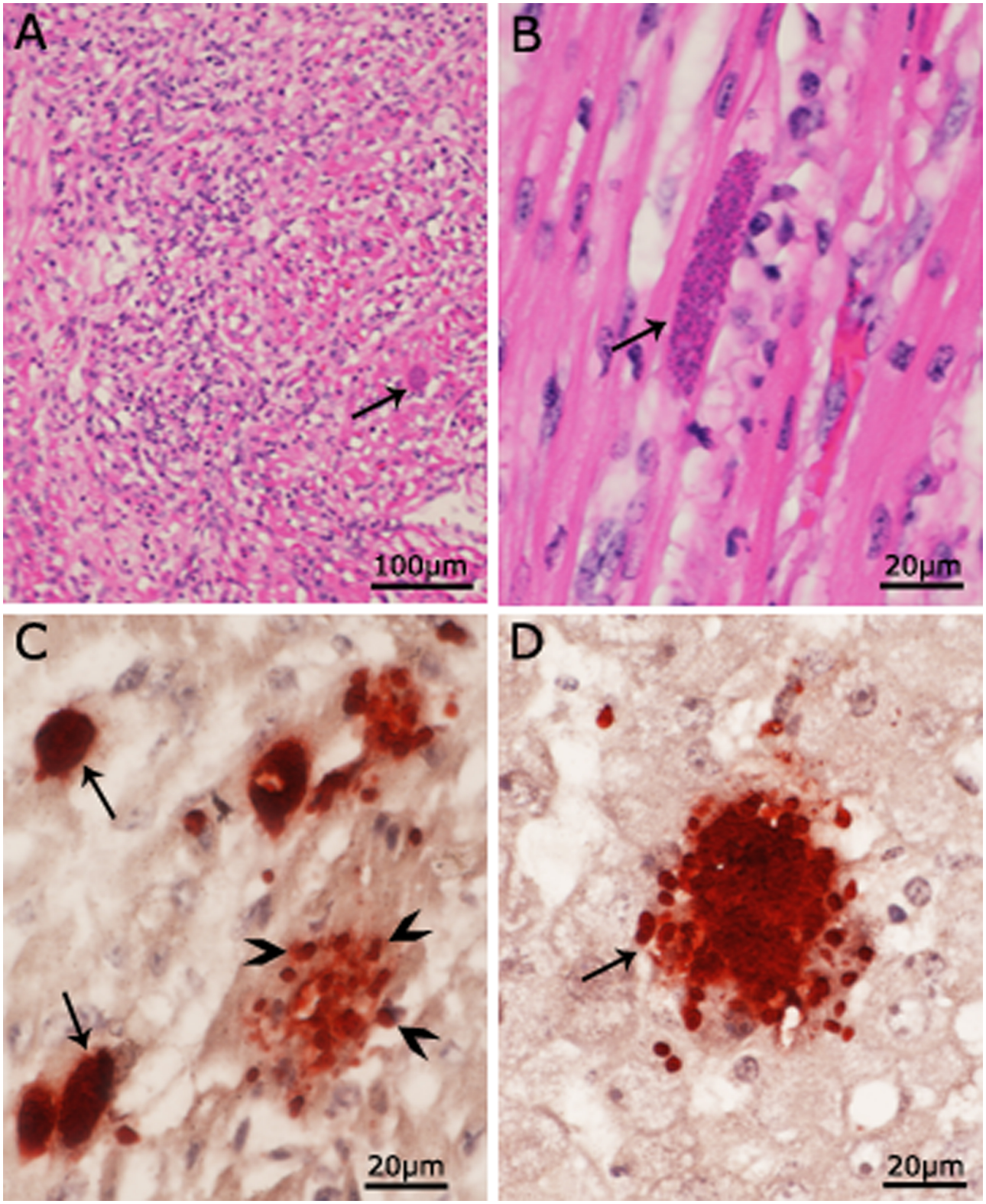

Fig. 1. Neosporosis myocarditis and hepatitis in the aborted equine fetus. (**A, B**) Myocarditis and large groups of tachyzoites (arrows). Hematoxylin and eosin stain. (**C**) Numerous intracellular and extracellular tachyzoites (arrows). Immunohistochemical staining with polyclonal *Neospora caninum* antibodies. (**D**) A large group of tachyzoites (arrow) in liver parenchyma. Immunohistochemical staining with polyclonal *N. caninum* antibodies.

2. Materials and methods

2.1. Horse

In early 2018, a seven-year-old Quarter Horse aborted a female fetus at 280 days of gestation. The mare was housed with 10 other horses on a small farm in Montana, USA. Other than a low progesterone level measured at days 14, 75, and 150, the pregnancy had been unremarkable. The mare was receiving progestin since the low progesterone measurement at day 14. Multiple previous pregnancies of this

mare were uneventful with one exception. Two to three years prior, a foal was born weak and died at three months old. A post-mortem examination was not performed nor was there a record of diagnostic testing. The most recent pregnancy, one year prior to the 2018 abortion, produced a viable foal that was clinically healthy. Since the abortion reported herein, the mare has again become pregnant and at the most recent recheck at 65 days gestation, the mare and fetus were clinically healthy.

The aborted foal was necropsied by the attending veterinarian on the day of abortion and samples of fetal lung, heart, liver, skeletal muscle, tongue, brain, and the placenta were collected and submitted to the Montana Veterinary Diagnostic Laboratory (MVDL), Bozeman, Montana for diagnosis. At MVDL, tissues were processed for histopathology using routine protocols. After a preliminary diagnosis of protozoal etiology, paraffin blocks and hematoxylin and eosin-stained slides were submitted to Joint Pathology Center, Silver Spring, Maryland (JPC) for consultation. Subsequently samples were sent to Animal Parasitic Diseases Laboratory, US Department of Agriculture, Beltsville, Maryland (APDL) for definitive diagnosis.

2.2. Histopathology and immunohistochemistry

Paraffin embedded sections were cut at 5 µm thick and examined after staining with hematoxylin and eosin (HE). Immunohistochemistry was performed at APDL using rabbit polyclonal rabbit *Toxoplasma gondii* and *N. caninum* antibodies as described previously (Dubey, 2010; Dubey et al., 2017).

2.3. Transmission electron microscopy (TEM)

Formalin-fixed paraffin embedded tissue blocks of fetal lung were submitted for TEM. Blocks were placed in 2% glutaraldehyde then tissues were embedded in epoxy resin. Thin sections of 900 ångströms were cut and stained with uranyl acetate and lead citrate and examined with the JEOL JEM-1400 Electron Microscope and Gatan Digital Micrograph.

2.4. Polymerase-chain reaction (PCR) and sequencing

DNA was extracted from lung tissue in paraffin at APDL using QiAamp® DNA FFPE Tissue (Qiagen Inc., Valencia, CA, USA), per manufacturer's instructions. PCR was performed using primers designed to amplify a 258 bp portion of the rRNA gene internal transcribed spacer 1 (ITS1) of the Sarcocystidae (Gjerde, 2014; Gjerde and Josefsen, 2015). To account for potential nucleotide changes introduced by PCR, the amplified ITS1 from three independent reactions were cloned and sequenced.

3. Results

3.1. Hematoxylin and eosin-stained sections

Based on histologic examination alone, this foal had disseminated protozoal infection most likely due to either neosporosis, toxoplasmosis, or sarcocystosis. The most prominent lesion was necrotizing interstitial pneumonia (Supplementary Fig. 1A). Both lungs were consolidated. Alveolar septa were fragmented and thickened up to five times normal by macrophages, neutrophils, fibrin, hemorrhage, edema, and necrotic cellular and karyorrhectic debris. Additionally, hyperplastic type II pneumocytes lined the septa. Alveolar spaces and terminal bronchioles were partially to completely filled with an exudate consisting of alveolar macrophages, fibrin, edema, and necrotic material. Lobular septa and the pleural surfaces were thickened up to ten times normal by additional edema, mild hemorrhage, fibrin, congestion, and scattered lymphocytes, plasma cells, and macrophages. Multifocally alveolar epithelial cells were enlarged and contained groups of protozoal tachyzoites (Supplementary Fig.1).

The myocarditis was characterized by marked atrophy and loss of cardiomyocytes with replacement by mature fibrous connective tissue and reactive fibroblasts, aggregates of lymphocytes, plasma cells, macrophages, and fewer neutrophils (Fig. 1A, B). Cardiomyocytes within and surrounding these regions of fibrosis were often degenerate with swollen vacuolated sarcoplasm, or necrotic with shrunken hypereosinophilic sarcoplasm and a pyknotic or karyorrhectic nucleus. Large groups of protozoal tachyzoites were seen in foci of myocarditis and normal cardiac tissue. Multiple foci of necrosis and mononuclear cell infiltration in the liver associated with large groups of tachyzoites (consistent with a necrotizing hepatitis) (Fig. 1D) were also observed.

Initially no protozoa were identified on routine HE sections of the placenta and skeletal muscle although each was affected by a severe necrotizing placentitis and lymphohistiocytic myositis, respectively. Lesions were not detected in the tongue and brain following initial examination. After evaluation of *N. caninum* immunostaining, minor focal inflammatory lesions were detected in the tongue, skeletal muscles and brain.

3.2. Immunohistochemistry

Protozoa in all tissues reacted positively to *N. caninum* antibodies but not to *T. gondii* antibodies. The most important observation was the extent of tissue parasitization that was missed by examination of HE-stained sections (Supplementary Fig.1). Large numbers of tachyzoites were present in virtually all elements of lungs, including epithelium of alveoli and bronchioles (Supplementary Fig. 1B). One prominent feature in the liver and the heart was the large groups of tachyzoites (Fig. 1).

Although the placenta was autolyzed, tachyzoites were evident in placental villi (Fig. 2A). An unexpected finding was the presence of tachyzoites in muscular tissue of placenta (Fig. 2B, C). The tachyzoites were concentrated around the periphery of blood vessels. An intensive search revealed these tachyzoites also in HE stained sections (Fig. 2C)

3.3. TEM

TEM of the lung identified multiple intracellular and extracellular protozoal tachyzoites with multiple rhoptries, and micronemes (Supplementary Fig.2). The rhoptries were numerous, their contents were electron dense.

3.4. PCR

Comparison of the ITS1 amplified from the foal tissue to sequences available in GenBank revealed 100% sequence identity to the ITS1 from two isolates of *N. hughesi* (accessions EU290461.1, DQ997621.1) while three single-nucleotide polymorphisms (SNPs) and a 2-nucleotide insertion/deletion (indel) were evident when aligned with the ITS1 from two *N. caninum* isolates (accessions AY259037.1 and AY665715.1) (see supplementary Fig. 3). The ITS1 sequence from a third isolate of *N. hughesi* (NE1; AF038859.1) shared the three SNPs with the foal sample but had the 2-nucleotide indel present in the two *N. caninum* sequences.

4. Discussion

The present case of equine abortion was diagnosed as neosporosis based on light microscopy and TEM findings, immunohistochemistry, and molecular characterization. By light microscopy, the case was initially confused with toxoplasmosis. However, TEM, immunohistochemistry, and PCR excluded toxoplasmosis. Ultrastructurally, *T. gondii* tachyzoite rhoptries are different than *Neospora* spp.; *T. gondii* has only few rhoptries, and their contents are electron lucent (Dubey, 2010; Dubey et al., 2017). The present case is also not due to another related protozoan, *Sarcocystis neurona,* because *S. neurona* merozoites lack rhoptries (Dubey et al., 2015).

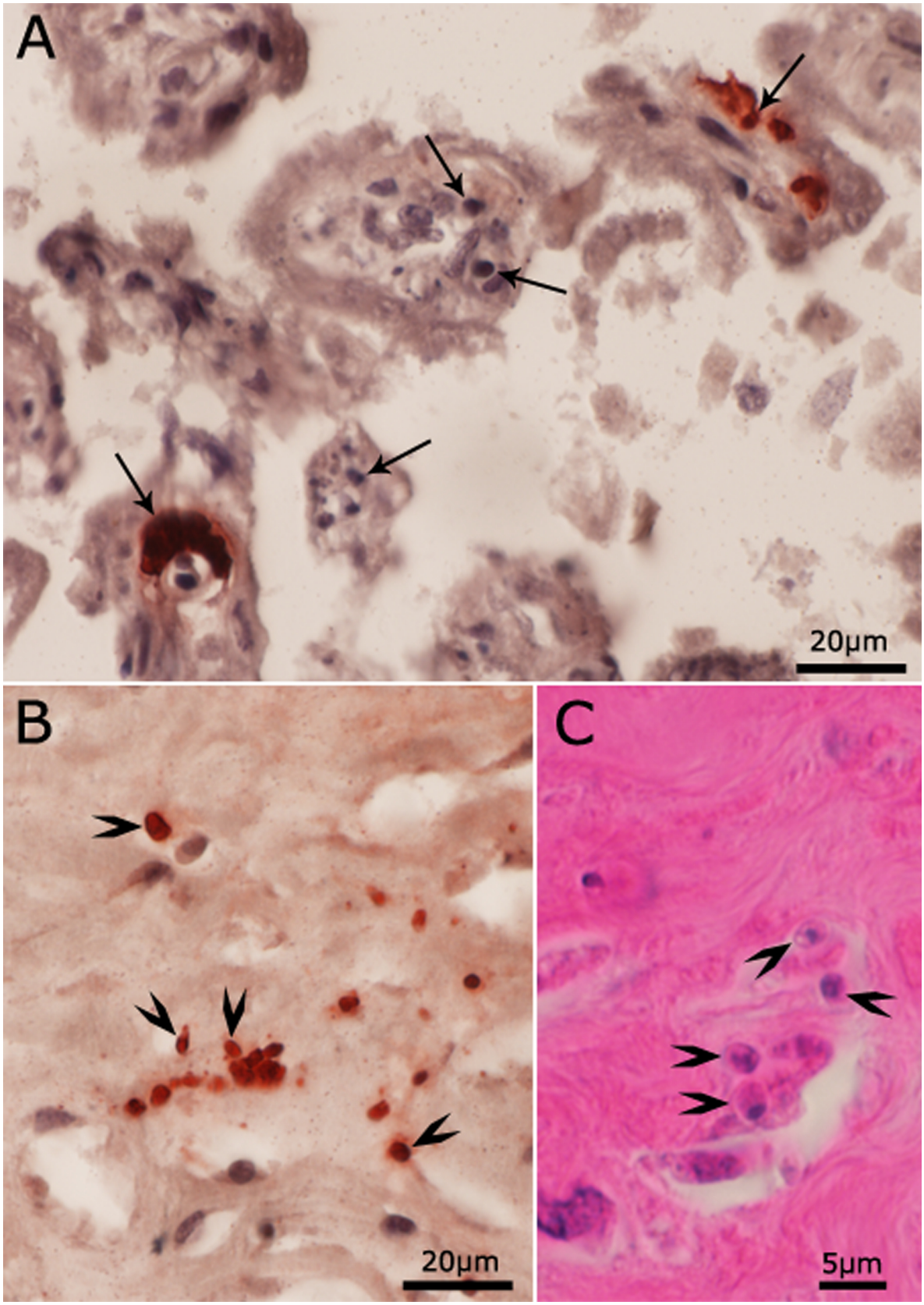

Fig. 2. Placenta of the aborted equine fetus. (**A**) Numerous tachyzoites (arrows) in villous. Immunohistochemical staining with polyclonal *Neospora caninum* antibodies. (B) Myositis with numerous tachyzoites (arrows). Immunohistochemical staining with polyclonal *N. caninum* antibodies. (**C**) Individual tachyzoites in muscular tissue. Hematoxylin and eosin stain.

In the present case, the species of *Neospora* was not determined definitively, although the ITS1 sequence was fully consistent with two *N. hughesi* isolate sequences from GenBank. Currently, there are two species of *Neospora*, *N. caninum* with a wide host range, and *N. hughesi* with host range limited to horses (Marsh et al., 1998; Dubey et al., 2017). The distinction between these organisms based on histology, serology, and immunohistochemistry is difficult bur antigenic and molecular differences have been reported (Marsh et al., 1998, 1999; Spencer et al., 2000; Dubey et al., 2001; McInnes et al., 2006; Dubey et al., 2017). Both organisms cross-react extensively by both serology and immunohistochemistry.

In the present case of equine abortion, pneumonia and myocarditis were the predominant lesions. In *N. caninum*-associated abortions in cattle, the predominant lesions are in the brain (see Dubey et al., 2017). Finding of no or minor lesions in the brain of the equine fetus suggests that the fetus became recently infected. Therefore, for the diagnosis of neosporosis abortion multiple tissues or the whole fetus should be submitted for diagnosis. The lesions described here should help in the diagnosis of equine abortion.

Conflict of interest

None.

Ethical statement

No experiment on animals was performed.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.04.009.

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