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CASE REPORT

Ovine Abortion by *Neospora caninum*: First Case Reported in Argentina

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Abstract

Purpose The aim of this study was to describe for the first time a natural case of ovine abortion associated with *Neospora caninum* in a flock with reproductive losses in Argentina.

Materials and Methods The analyzed flock consisted of 256 Texel sheep, of which 134 had been mated. A single blood sample was obtained by jugular vein puncture from 220 ewes (116 adult ewes, 104 yearling ewes) and 93 lambs. Serum samples and fetal fluid were tested using the indirect fluorescence antibody test to detect antibodies against *N. caninum* and *T. gondii*. Fetal and placental tissues from aborted fetus were examined by standard gross pathology procedures and were tested using histopathology and immunohistochemistry. Moreover, DNA from fetal and placental tissues was isolated and a PCR assay to detect *N. caninum*, *T. gondii* and *Chlamydia* spp. was used.

Results and Discussion The pregnancy rate was 89% (119/134), the abortion rate was 8.4% (10/119) whereas the perinatal mortality rate was 15% (16/109). Out of 116 adult ewes sampled, 34.48% presented anti-*N. caninum* antibodies. Ten ewes had aborted, and one fetus was directly submitted to the diagnostic laboratory for further processing. Antibodies against *N. caninum* were detected in fetal fluid and in the aborted dam. Histopathological analysis in fetal tissues showed multifocal lymphohistiocytic glossitis, diffuse mild lymphohistiocytic endocarditis, pericarditis and focally extensive myocarditis. Severe multifocal necrotizing placentitis and diffuse mild lymphohistiocytic placentitis with the presence of lymphohistiocytic vasculitis were observed in placenta. *N. caninum* was immunolabeled in the placenta and fetal tongue. In addition, *N. caninum* DNA was detected in placenta, central neural system, lung and heart of the aborted fetus. There was no evidence of other infectious abortifacients in the aborted fetus.

Conclusion The present study described for the first time an ovine abortion caused by *N. caninum* in Argentina. Further investigations at a larger scale are required to establish the role of *N. caninum* as an important cause of reproductive losses in sheep flocks from the region.

Keywords *Neospora caninum* · Reproductive losses · Abortion · Ovine

Introduction

Sheep industry has an important socio-economic impact in Argentina. There are 14,746,566 sheep heads in Argentina and 16% of them are located in the Humid Pampa region [27]. The productivity of the sheep flocks depends on their reproductive efficiency. Ovine abortion is a major cause of economic losses; nevertheless, research on the etiology of ovine abortion is scarce in Argentina. Only few works have reported other abortifacient agents such as *Brucella ovis*, *Leptospira* spp., *Campylobacter fetus* and *Toxoplasma gondii* in Argentinean flocks [12, 15, 25].

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Neospora caninum is an obligate intracellular protozoan parasite similar, as regards structure and development, to *T. gondii* [10]. *N. caninum* and *T. gondii* cause abortions and due to their similarity in the morphology and in the lesions induced, histopathological techniques do not enable accurate differentiation between both parasites [10]. *T. gondii* is one of the most common causes of reproductive failure in sheep worldwide and ovine abortions are often associated with this parasite [8]. Clinical manifestation of ovine neosporosis is like ovine toxoplasmosis although *N. caninum* has shown to be a more common cause of abortion in cattle [8]. For this reason, the economic, clinical, and epidemiologic importance of *N. caninum* infection in sheep remains under study [9, 10]. Bishop et al. [3] found *N. caninum* DNA in the brain of a ewe with encephalitis suggesting that the parasite might cause clinical neosporosis in adult sheep. However, few studies have reported the potential involvement of *N. caninum* as cause of reproductive failure in sheep flocks [1, 13, 14, 16, 19–21, 24]. At the present, there are no reports in Latin America that confirm *N. caninum* as causal of ovine abortions. This work describes for the first time a natural case of ovine abortion associated with *N. caninum* in a flock with reproductive losses in Argentina.

Materials and Methods

Description of the Flock

The analyzed flock consisted of 256 Texel sheep, of which 134 had been mated. The farm was located in Ranchos, Buenos Aires province, Argentina (35°31′24.96″S 58°44.16″W). The farmer had observed a reduction in the reproductive performance during the previous lambing season. Animals were grazing native grasslands, supplemented with conserved forage (hay and silage) and concentrate during the breeding season. The affected flock was brucellosis- and tuberculosis-free. The mating period extended from March to May 2017. Ewes were synchronized for estrus and artificially inseminated. Non-pregnant ewes were resynchronized followed by natural breeding using one ram.

Sampling and Data Collection from the Animals

On March 2018, a single blood sample was obtained by jugular vein puncture from 220 ewes (116 adult ewes, 104 yearling ewes) and 93 lambs. Furthermore, there were three adult dogs and 11 cats in the farm co-habiting with the ewes. Serum samples were also obtained from the three dogs (identified as dog A, B and C), but unfortunately, it was not possible to obtain serum samples from the cats. The sera were separated by centrifugation and stored frozen (− 20 °C) until analysis for the detection of antibodies to *N. caninum* and *T. gondii*.

During the study period, there were recorded data about reproductive history of the flock. One aborted fetus was recovered and submitted to the diagnostic laboratory for diagnosis purposes. Animal procedures were performed according to standard protocols and guidelines from the Animal Ethics Committee (CICUAE#008/2015) at INTA, Argentina.

Serology

All serum samples and fetal fluid were evaluated for anti-*N. caninum* and anti-*T. gondii* specific IgG by IFAT using slides prepared with whole *N. caninum* and *T. gondii* tachyzoites, respectively. Cut-off titers of ≥ 1:50 and 1 ≥ 10 were defined for serum and fetal fluid, respectively [17]. The highest serological dilution with complete peripheral positive reactions was considered the end-point titer. Positive control sera were obtained from *N. caninum* and *T. gondii* experimentally infected ewes. Negative control sera were obtained from *T. gondii* and *N. caninum* free ewes [28]. Dogs sera were tested by IFAT as previously mentioned Basso et al. [2] using a cut-off titer of ≥ 1:50.

Necropsy and Sampling from the Aborted Fetus and Placenta

Fetal and placental tissues were examined by standard gross pathology procedures [5]. Fetal tissue samples including lung, spleen and abomasal content were extracted to determine the presence of aerobic and microaerophilic bacteria according to the methods described by Campero et al. [5]. Fetal cavity fluids were collected and stored at − 20 °C until assessment of antibodies to *N. caninum* and *T. gondii* by IFAT. Placental and fetal tissue samples (central neural system, heart, lung, liver, tongue, forelimb and hind limbs muscles) were collected for DNA extraction and histopathological analysis according to Hecker et al. [18].

Histopathology and Immunohistochemistry

Five µm-thick sections of each fetal tissue were cut, mounted on glass microscope slides and stained with hematoxylin and eosin (H&E). Tissues that had compatible lesions with *N. caninum* and *T. gondii* infection were selected and analyzed by immunohistochemistry (IHC) using polyclonal antibodies against *N. caninum* (kindly provided by Dr. M. Anderson, UCDavis, USA) and *T. gondii* (kindly provided by Dr. M.C. Venturini, inmunoparasitology laboratory (LAINPA), FCV, UNLP, Argentina) and the Dako Envision kit (Carpinteria, CA, USA), employed according to the manufacturer's instructions.

DNA Extraction and PCR

DNA was isolated using a commercially available kit according to the manufacturer's recommendations (DNeasy Tissue Kit, QIAGEN Group, Germany). DNA concentration was measured using an Epoch micro-volume spectrophotometer system (Epoc, Bioteck® Instruments, Inc., Vermont, USA). The concentration of DNA for all samples was adjusted to 50–100 ng/µL.

For the detection of *Neospora* DNA, an adapted single-tube nested-PCR on the internal transcribed spacer (ITS1) region of *N. caninum* was carried out with four oligonucleotides as described by Buxton et al. [4]. In addition, a PCR assay on the B1 gene [23] and on *omp 1* gene [26] was used to detect *T. gondii* and *Chlamydia* spp., respectively. Purified *N. caninum, T. gondii* and *Chlamydia* spp. DNA were used as positive controls. Secondary amplification products were visualized in a 1.8% agarose gel electrophoresis and SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) under UV light.

Statistical Analysis

The association between serological status and reproductive performance was evaluated by applying Chi-square test or Fisher's exact test. P values < 0.05 were required to demonstrate statistical significance. All statistical analyses were performed using Graph-Pad Prism 5 v.5.01 (GraphPad Software, San Diego, CA, USA).

Results

Description of the Flock

The pregnancy rate was 89% (119/134), the abortion rate was 8.4% (10/119) whereas the perinatal mortality rate was 15% (16/109). At the end of the lambing season (September 2017 to November 2017), 93 ewes delivered 143 lambs: 50 ewes delivered twins and 43 delivered single lambs. During the study period, 10 ewes aborted but only one fetus was recovered and submitted to the diagnostic laboratory for diagnosis purposes. The aborted dam was 1.5 years old and it was its first gestation.

Serological Study

Neospora caninum and *T. gondii* global seroprevalence was 26.8% and 59.1%, respectively. Detailed information on prevalence for each category is provided in Table 1. Ewes (adult and yearling) were more likely to be *N. caninum* and *T. gondii* seropositive in comparison with lambs ($P < 0.05$). Furthermore, adult ewes had more likely higher antibody´s levels against *T. gondii* than yearling ewes ($P < 0.05$).

The serum of the aborted dam had high antibody titers against *N. caninum* (1:12,800) and was *T. gondii*-seronegative. Antibody titers against *N. caninum* were higher in seropositive aborted ewes when compared with those seropositive non-aborted ewes ($P < 0.05$), although there was no association between reproductive losses and seropositivity to *N. caninum* or *T. gondii* ($P > 0.05$). There was a positive correlation between the levels of antibodies against both protozoa in lambs, indicating that as levels of antibodies to *N. caninum* increased, also antibodies levels to *T. gondii* increased ($P < 0.001$). Moreover, there was a significant association between dams and lambs *T. gondii* serostatus, but this effect was not observed with *N. caninum* serostatus.

Finally, dog A was *N. caninum*-seropositive (1:800) and *T. gondii*-seronegative; dog B was *T. gondii*–seropositive (1:200) and *N. caninum*-seronegative; and dog C was *N. caninum* (1:400) and *T. gondii* (1:200) seropositive.

Fetal Study

The aborted fetus recovered was a twin and had 112 days of gestation. Fetal central neural system (CNS) was liquid, therefore, it could not be processed for histopathological and immunohistochemical studies. Other fetal tissues and the placenta had a moderate state of mummification (Fig. 1a).

Histopathological analysis in fetal tissues showed multifocal lymphohistiocytic glossitis (Fig. 1b), diffuse mild lymphohistiocytic endocarditis, pericarditis and focally extensive myocarditis. Severe multifocal necrotizing placentitis and diffuse mild lymphohistiocytic placentitis with

Table 1 Seroprevalence and range of antibody titers to *Neospora caninum* and *Toxoplasma gondii* in ewes and lambs

Categories	*N*	*N. caninum* seroprevalence (number of positive animals)	Positive range to *N. caninum*	*T. gondii* seroprevalence (number of positive animals)	Positive range to *T. gondii*	Positive to both protozoa
Adult ewes	116	34.48% (40)[a,*]	50–12,800	77.59% (90)[a]	1600–12,800	24.13% (28)[a]
Yearling ewes	104	27.88% (29)[a]	200–12,800	65.38% (68)[b]	1600–12,800	22.11% (23)[a]
Lambs	93	16.12% (15)[b]	50–6400	29.09% (27)[c]	800–12,800	10.75% (10)[b]

*Different letters indicate significant statistical differences

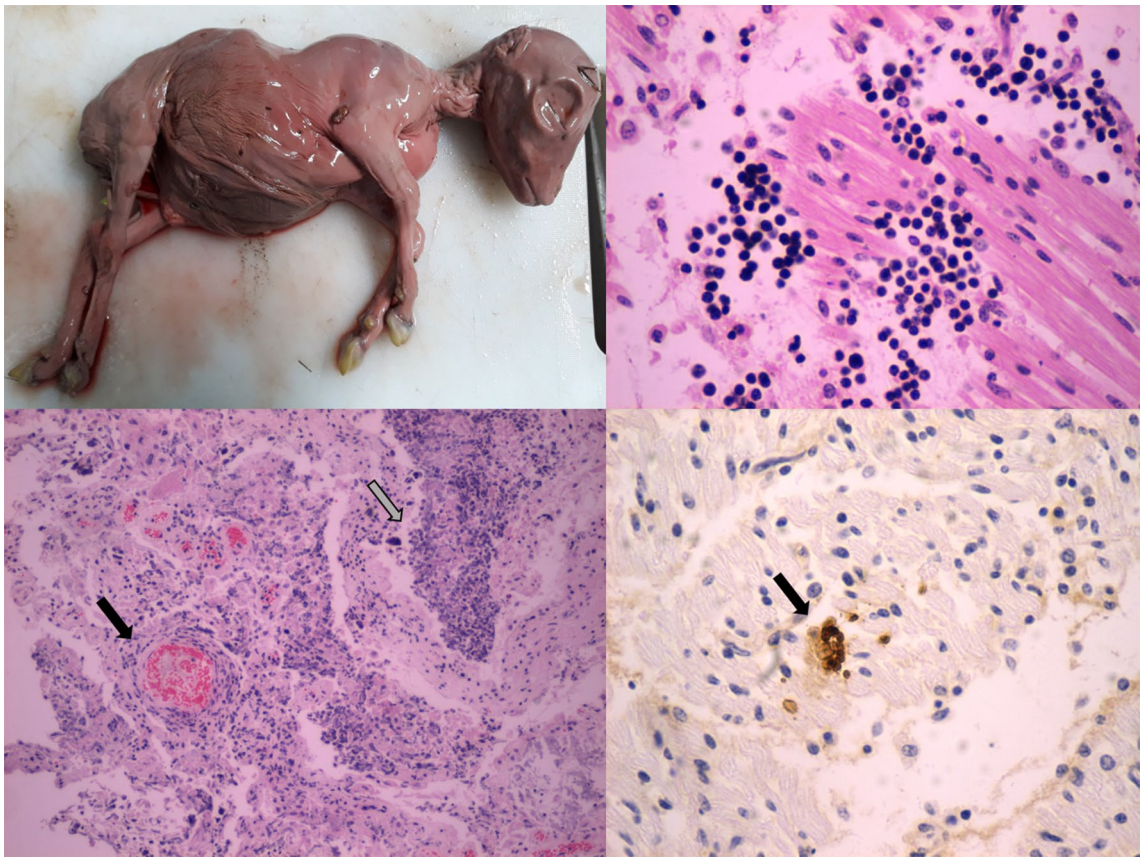

Fig. 1 Macroscopic and microscopic findings in the aborted ovine fetus. **a** Moderate state of mummification and autolysis. **b** Multifocal lymphohistiocytic glossitis (HE; 400×). **c** Severe multifocal necrotizing placentitis (grey arrow) and diffuse mild lymphohistiocytic placentitis with the presence of lymphohistiocytic vasculitis (black arrow) (HE; 100×). **d** Immunohistochemical labelling of several *N. caninum* protozoa in the fetal tongue (black arrow) (IHC, 400×)

the presence of lymphohistiocytic vasculitis were observed in placenta (Fig. 1c). Positive *N. caninum* immunolabelling was detected in tongue (Fig. 1d) and placenta. In addition, antibodies against *N. caninum* were detected in fetal fluid (1:800) and *Neospora* DNA was detected in CNS, heart, lung and placenta.

Immunohistochemistry for *T. gondii* was negative in placenta and all fetal tissues analyzed and no serological evidence of *T. gondii* was detected in the fetal fluid. No evidence of other abortigenic pathogens (*Brucella* spp., *Campylobacter* spp, *Salmonella* spp., *Chlamydia* spp.) was found in the tested tissues.

Discussion

Ovine neosporosis was first described in sheep in 1990 associated with congenital infection in a lamb [7]. Afterwards, *N. caninum* has been sporadically reported as cause of reproductive disorders in sheep [1, 13, 14, 16, 20, 21, 29]. The present study is the first description of an ovine abortion caused by *N. caninum* in Argentina. The characteristic fetal lesions, intralesional identification of the parasite by IHC, detection of *N. caninum* DNA and serology confirmed the diagnosis in the analyzed fetus. Moreover, there was no evidence of other infectious abortifacients in the aborted fetus.

Several authors have described foci of leukomalacia, isolated granulomas or necrogranulomas (some with mineralized centers) and occasional tissue cysts in CNS of ewes aborted fetuses due to *N. caninum* [16, 22, 30]. Unfortunately, neural tissues were not available for histopathological examination. However, the presence of *N. caninum* DNA in fetal CNS was confirmed by PCR. In addition, characteristic lesions of neosporosis were found in placenta, fetal heart and tongue; and the IHC confirmed the presence of the parasite in placenta and tongue.

West et al. [29] related *N. caninum* as a cause of abortion in maiden ewes of New Zealand. These authors found positive *N. caninum* titers in fetal fluids and compatible histopathological lesions in aborted fetuses, but they described that the aborted ewe showed low antibody titers compared with those found in aborted cows. In the present

work, high *N. caninum* antibody titers were found in the aborted ewe and fetus, in agreement with previous reports in cattle and goats [6, 9].

In the present study a higher flock *N. caninum* seroprevalence was found compared with previous reports in other countries [8, 11, 28]. Previous works of our group reported low *N. caninum* seroprevalence in a dairy (3%) and Texel flock (1.54%) from Humid Pampa, Argentina [17, 19]. In agreement with our results, Gonzalez-Warleta et al. [13] reported a high seroprevalence in a Spanish flock where *N. caninum* infection was the cause of the low reproductive performance. Based on our serological findings and the presence of *N. caninum* in the aborted fetuses, we can hypothesize that *N. caninum* is an important cause of reproductive losses in this flock. However, we cannot assume that all reproductive losses in the flock were caused by *N. caninum* because other aborted fetuses were not available for analysis and no significant association was detected between seropositive animals to *N. caninum* and reproductive losses. Although the flock was brucellosis and tuberculosis free, the authors cannot rule out the presence of other infectious abortifacients.

Toxoplasma gondii seroprevalence in the studied flock was high (59.10%) compared with seroprevalences previously reported in Argentina [15, 17, 19] and in other countries [8]. In the analyzed farm there were nine cats cohabiting with the sheep. Unfortunately, these cats were not sampled, in order to confirm them as the possible source of infection. The high *T. gondii* seroprevalence is probably due to the intensive husbandry conditions of this flock and the more probable exposition to contaminated feed or water with oocysts of *T. gondii*. Although there was not association between *T. gondii* seropositivity and reproductive losses, and *T. gondii* was not detected in the aborted fetus, it is difficult to discard the possibility role of this protozoon as a cause of reproductive losses in this flock.

The results of this study showed that *N. caninum* and *T. gondii* seroprevalence increased with the age of animals, indicating that the horizontal transmission of both infections was occurring in this flock. This hypothesis could be strengthened for the fact that the analyzed dogs were seropositive to *N. caninum* and *T. gondii* and that these animals and cats were co-habiting with the sheep and their lambs.

The present study described for the first time an ovine abortion caused by *N. caninum* in Argentina. In addition, high seroprevalence were detected against *N. caninum* and *T. gondii* in the flock, indicating that the reproductive losses could be caused by these parasites. Further investigations at a larger scale are required to continue providing detailed information and establish the impact of neosporosis and toxoplasmosis on sheep reproductive losses of farms from Argentina.

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Compliance with ethical standards

Conflict of interest There are no financial or personal relationships with other people or organizations that could inappropriately influence this work.

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