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First case report of *Toxoplasma gondii*-induced abortions and stillbirths in sheep in Argentina

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ABSTRACT

The aim of this study is to report an episode of reproductive losses due to toxoplasmosis in a sheep flock in Argentina. A total of 15 abortions and 9 stillbirths were recorded in a flock of 190 Texel ewes. The affected ewes were more likely to be seropositive for *Toxoplasma gondii* (15/24) than ewes that delivered normal lambs (5/34, OR = 9.6, 95%CI = 2.7–34.0, p = 0.0004). A pair of aborted twins was recovered for diagnostic investigation. One of these fetuses and its dam were seropositive for *T. gondii*. Histological examination of the two fetuses revealed non-suppurative myocarditis and epicarditis, portal hepatitis and multifocal necrotizing encephalitis with protozoal cysts in the brain. *T. gondii* was detected intralesionally by immunohistochemistry in one fetus and by PCR in both. Further investigations are necessary to evaluate the economic losses due to *T. gondii* in the Argentinean ovine industry.

1. Introduction

Toxoplasmosis causes considerable economic losses to the sheep industry worldwide (Dubey, 2010). *Toxoplasma gondii*-induced abortion can occur in ewes of all ages infected for the first time during gestation. Fetuses from infected ewes may be mummified, macerated, aborted or resorbed; lambs may be born dead or weak; these usually die within a week of birth. Those that survive the first week generally grow normally to adulthood and produce *T. gondii*-free lambs. Infected ewes themselves do not have clinical signs but *T. gondii* remains encysted in their tissues, mainly in muscles. Toxoplasmosis is a neglected parasitic zoonosis. Ingestion of uncooked or undercooked meat is a risk factor for human toxoplasmosis, especially in pregnant women (Dubey, 2010).

Sheep are the second most frequent domestic mammalian species in Argentina, with a national stock of 14.746.566 sheep in March 2017 (SENASA, 2017). A recent seroepidemiological survey conducted on a dairy sheep flock in the Argentinean Pampas revealed that 17.3% of 704 animals had circulating anti-*T. gondii* antibodies (Hecker et al., 2013), however there are no reports on reproductive losses associated with natural *T. gondii* infection in sheep in this country. Here we report an episode of reproductive losses due to toxoplasmosis in a commercial sheep flock raised for meat production in Argentina.

2. Materials and methods

2.1. Antecedents, animals and sampling

Animal procedures were performed according to standard protocols and guidelines from the Animal Ethics Committee at INTA, Argentina. Fifteen abortions and 9 stillbirths were recorded in a commercial sheep flock of 190 Texel ewes and 6 rams. The flock was in the Southeast of Buenos Aires province, Argentina (region known as the humid pampas), and was raised for meat production under an extensive, pasture-based system. The mating period extended from April 15 to June 15. The pregnancy rate was 96.84% (184/190), the abortion rate was 8.15% (15/184) whereas the perinatal mortality rate was 4.9% (9/184). At the end of the lambing season (September 15 to November 15), 166 ewes delivered 217 lambs. One ewe delivered triplets, 49 delivered twins,

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and 116 delivered single lambs. Only two (twin) fetuses of an estimated gestational age of 17–19 weeks were recovered on September 1, for postmortem examination and diagnostic investigation. The dam was two years old and was in its second gestational period. On December 2, serum samples were obtained from the 24 affected ewes and 34 ewes that delivered normal lambs. Tissue samples (cerebral hemispheres, midbrain, medulla oblongata, cerebellum, heart, lung and liver) from the two aborted fetuses were recovered for routine histological examination. Samples were fixed in 10% neutral buffered formalin, embedded in paraffin, microtome-sectioned at 4 µm, stained with hematoxylin and examined by light microscopy (Olympus Optical, Japan). Fresh fetal tissue (brain, heart, lung, liver, kidney, mesenteric lymph node and placenta) and thoracic fluid samples were collected and stored at − 80 °C until DNA extraction.

2.2. Immunohistochemistry and PCR

Selected formalin-fixed paraffin-embedded sections of brain and striated muscle were assessed by immunohistochemistry (IHC). Briefly, antigen unmasking was accomplished by heat-induced epitope retrieval using a decloaking chamber (Biocare Medical). A polyclonal rabbit antibody against *T. gondii* (NeoMarkers RB-282-A) was used as a primary antibody; EnVision + /HRP goat anti-rabbit IgG conjugate (Dako, HRP-labeled polymer conjugate) was used as a detection system; and 3-amino-9-ethylcarbazole + (AEC +, Dako K3469) was used as the chromogen. Sera from the sheep and thoracic fluids from the twin fetuses were tested for the presence of anti-*T. gondii* specific IgG by indirect immunofluorescence antibody test (IFAT). The cutoff titer used was 1:50 (Hecker et al., 2013). Positive reactions that had the highest serological dilution were considered the end-point titer. A polyclonal rabbit anti-sheep IgG labeled with fluorescein isothiocyanate (SIGMA, St. Louis, USA) was used as conjugate.

The DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) was used following the manufacturer's instructions, with the samples being finally eluted in 100 µl of DNase/RNase free water. A nested-PCR assay was performed to detect *T. gondii* DNA, as described by Herrmann et al. (2010). Positive (purified *T. gondii* DNA) and negative controls, and secondary amplification products were visualized by 1.8% agarose gel electrophoresis and SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) under UV light.

2.3. Other ancillary laboratory procedures for differential diagnosis

For bacterial isolation, abomasal content, lung, spleen and liver from the aborted twin fetuses were incubated at 37 °C for 24–48 h in MacConkey agar and Columbia agar supplemented with 7% of bovine blood, in aerobic and microaerobic (10% CO_2) atmosphere, respectively. Additionally, an aliquot of these samples was incubated in tetrathionate enrichment broth for *Salmonella* spp. isolation, with subcultures to XLD agar plates, every other day, for one week. All serum samples were assessed for *Brucella ovis* antibodies by an indirect ELISA (Estein et al., 2009).

For the identification of *Leptospira* spp., direct fluorescent antibody test (DFAT) was performed in fetal liver, lung, and kidney imprints and aqueous humor smears of both fetuses. Fluorescein-labeled rabbit polyclonal antiserum (NVLS, USA), was used at a dilution of 1:5, and slides were examined in a fluorescence microscope (Nikon Fluophot, Japan). *Campylobacter* spp. DFAT was performed on smears of abomasal content, as described by Campero et al. (2003), and examined under the same fluorescence microscope.

A *Chlamydiaceae*-specific real-time PCR targeting the 23S rRNA gene was performed with ABI 7500 (Applied Biosystems) using a Universal Master Mix (Applied Biosystems), primers Ch23S-F and Ch23S-R (300 nM) and the probe Ch23S-p (200 nM) (Ehricht et al., 2006).

For viral isolation, spleen homogenates were inoculated onto cultures of Madin-Darby bovine kidney (MDBK) cell cultures. After four blind passages, the cultures where tested for bovine viral diarrhea virus (BVDV) and border disease virus (BDV) by an indirect fluorescent antibody procedure with a polyclonal antibody (VMRD, Pullman, TN, USA).

All serum samples were evaluated for anti-*N. caninum* specific IgG by IFAT using slides prepared with whole *N. caninum* tachyzoites. A cutoff titer of 1:50 was used (Hecker et al., 2013). Additionally, *N. caninum* DNA was assessed in fetal brain by a nested-PCR targeting the internal transcribed spacer (ITS) 1 region, as described by Buxton et al. (1998). Positive (purified *N. caninum*) and negative controls, and secondary amplification products were visualized as described for *T. gondii* PCR above.

2.4. Statistical analysis

The association between serological status and reproductive performance was determined using odds ratio (OR). P-values < 0.05 were required to demonstrate statistical significance. Data were processed by the use of Med Calc programme (Med Calc, 2017).

3. Results

Both fetuses were autolyzed and partially mummified, weighed 0.75 (fetus 1) and 1 (fetus 2) kg and had a crown-to-rump length of 15 cm. Fetus 1 was wrapped in the placenta and had a more advanced degree of mummification and autolysis (Fig. 1A). Microscopically, both fetuses had multifocal non-suppurative myocarditis and epicarditis and portal hepatitis. Additionally, fetus 1 had multifocal necrotizing encephalitis with mononuclear cell infiltration and glial aggregates with occasional foci of mineralization in the neuroparenchyma (Fig. 1B) and multifocal moderate non-suppurative myositis. *Toxoplasma gondii*-like tissue cysts, ranging from 13 to 17 µm in diameter, were seen in the brain of this fetus (Fig. 1C). In addition, *T. gondii* zoites were detected intralesionally by IHC in the brain, and *T. gondii* cysts were detected by the same technique within the sarcoplasm of cardiomyocytes (Fig. 1D).

Fetus 1 and the dam had IFAT titers of 1:50 and 1:1600, respectively, confirming *in utero* exposure to *T. gondii* in the fetuses. The PCR assay was positive for *T. gondii* in the thoracic fluid and heart of both fetuses and mesenteric lymph node of fetus 2, whereas the brain, liver, lung and kidney of both fetuses and the mesenteric lymph node and placenta of fetus 1 were negative. *T. gondii* genotyping by nested PCR-RFLP was attempted with inconclusive results due to the low amount of DNA in the samples.

Antibodies to *T. gondii* were detected in 24 of 58 (41.28%) ewes tested, with higher proportion of seropositive animals in the group of ewes with a recent history of reproductive loss (Table 1). Ewes that aborted or delivered stillborn lambs were more likely to be seropositive to *T. gondii* than ewes that delivered normal lambs, and there was statistical association between reproductive loss and seropositive status (OR = 9.6, 95%CI = 2.7–34.0, p = 0.0004). *Neospora caninum* PCR and IFAT were negative in all samples, as well as tests for bacterial and viral detection. None of the ewes tested positive for *Brucella ovis* antibodies by ELISA.

4. Discussion

Toxoplasmosis was diagnosed in aborted twins based on characteristic lesions, intralesional identification of the parasite by IHC, detection of *T. gondii* DNA and serology. Noteworthy, there was a statistical association between the *T. gondii* seropositivity and the reproductive losses suggesting a flock problem rather than an individual abortion. Additionally, there was no evidence of other infectious abortifacients. Although data regarding to this case report was described preliminary (Gual et al., 2015), here we provided the complete study about that first report of *T. gondii* abortion in sheep in Argentina.

Although the source of *T. gondii* infection remained unclear in this