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Short communication

Microsatellite pattern analysis of *Neospora caninum* from a naturally infected goat fetus

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ABSTRACT

Neospora caninum is an apicomplexan protozoan responsible for abortion in ruminants. The present study aimed to diagnose an abortion from an Anglo Nubian goat from a dairy herd located in Buenos Aires province, Argentina. The goat delivered a fetus of approximately 3 months gestation that was studied by indirect fluorescent antibody test (IFAT), Immunoblot (IB), histopathology (HP), immunohistochemistry (IHC), and molecular assays (PCR, sequencing and microsatellite genotyping). Interferon gamma knock-out mice were inoculated with a pool of tissues for isolation attempts. The mother had IFAT titers of 1:3200 and 1:400 for *N. caninum* and *Toxoplasma gondii*, respectively, as well as positive IB reactions, whereas the fetus was seronegative to both parasites by IFAT and IB. The fetus had severe multifocal necrotizing myocarditis and hepatitis, moderate interstitial pneumonia, and nephritis. Myocardium sample resulted positive by IHC, evidencing clusters of *N. caninum* tachyzoites within myocardiocytes associated with histopathological lesions. *Neospora caninum*-DNA was detected by PCR in heart, liver, lungs, kidney, and muscle from the fetus, and was negative for *T. gondii* by PCR. NC-5 and 18 S rRNA gene fragment sequences showed 100% identity with *N. caninum*. Inoculated mice bled 30 days post-inoculation resulted seronegative to *N. caninum* and *T. gondii* by IFAT, and showed no clinical signs. Multilocus-microsatellite genotyping revealed a genetic profile that differed from previously reported *N. caninum* genotypes, with unique MS21 and MS10 alleles. These findings indicate that *N. caninum* was efficiently transmitted from the mother to the fetus. We report the first case of direct detection of *N. caninum* in a goat fetus in Argentina and *N. caninum* microsatellite genotyping in naturally infected goat.

1. Introduction

Neosporosis is a worldwide distributed disease caused by the intracellular protozoan parasite *Neospora caninum* and is considered one of the main causes of abortion in cattle (Dubey and Schares, 2011). However, little is known about *N. caninum* in goats and most studies consist on serological surveys with variable prevalence ranging from 0% to 26.6% (Dubey et al., 2017). Toxoplasmosis is a worldwide distributed zoonosis which can produce reproductive losses in small ruminants (Dubey, 2010; Unzaga et al., 2014). Morphological similarities between *Toxoplasma gondii* and *N. caninum* justify the need of specific differential diagnosis by immunological and molecular methods. *Neospora caninum* is considered a potential cause of abortion in goats (Eleni et al., 2004; Masala et al., 2007; Moreno et al., 2012), and several studies have demonstrated natural congenital transmission (Dubey, 2010; Unzaga et al., 2014; Mesquita et al., 2013). However, the parasite has not yet been isolated from goats (Dubey et al., 2017). Recently, an experimental study analyzed the outcome of *N. caninum* infection at different gestational stages in goats (Porto et al., 2016), and a *N. caninum* genotype from goat placentas was characterized in Brazil (Costa et al., 2017). The role of *N. caninum* as a cause of natural abortion in small ruminants from Argentina needs to be further investigated.

We report the detection of *N. caninum* in a naturally infected Anglo Nubian goat fetus and the characterization of a unique *N. caninum* genotype by multilocus-microsatellite analysis.

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2. Materials, methods and results

The goat belonged to a dairy herd ($n = 46$ adult goats, Anglo Nubian) located in Buenos Aires province, Argentina. The herd was free of brucellosis and tuberculosis with a seroprevalence of 9% for *N. caninum* and 76% for *T. gondii* determined by IFAT. The pregnant goat had purulent vulvar secretion, and a combination of antibiotics and oxytocin was applied by a private practitioner. Two days after treatment, a 13 cm length macerated fetus, with no body hair, was delivered with an estimated gestational age of approximately 3 months. The head of the fetus was partially eaten by scavenger animals; therefore, no brain sample was available. The fetus was sent to Immunoparasitology Laboratory, School of Veterinary Sciences in La Plata for necropsy where heart, liver, lungs, kidneys, and muscle samples were aseptically collected and processed for routine histological procedures and polymerase chain reaction (PCR) studies.

Serum sample from the mother and thoracic liquid from the fetus were used for serological screening. Indirect fluorescent antibody test (IFAT) was performed as previously described for *N. caninum* and *T. gondii* specific antibodies detection, using cut-off titers of 1:25 and 1:10 for the mother and fetus, respectively (Unzaga et al., 2014). The mother had IFAT titers of 1:3200 for *N. caninum* and 1:400 for *T. gondii*, while the fetus was seronegative to both parasites by IFAT. Immunoblot (IB) assay was performed as a confirmatory test (Campero et al., 2015a). Positive IB reactions for *N. caninum* and *T. gondii* were detected for the mother, whereas no IB reactions were detected for the fetus.

A pool of tissues collected during necropsy was processed for bioassays in gamma interferon knock out mice, and 30 days post-inoculation mice were bled and sera analyzed for antibody detection against *N. caninum* and *T. gondii* by IFAT (Campero et al., 2015b). Mice were seronegative to both parasites and showed no clinical signs of protozoan infection.

Histopathological examination of fetal tissues stained with hematoxylin and eosin (H/E) revealed severe multifocal necrotizing myocarditis and hepatitis. Moderate interstitial pneumonia and nephritis were also observed (Fig. 1). Tissues were processed by specific immunohistochemestry (IHC) for *N. caninum* by Avidin Biotin Complex using a commercial kit (ABC Elite ABC Peroxidase Complex Vector PK-601, Vector Laboratories, Burlingame CA, USA) as previously described (Campero et al., 2003). Myocardium sample resulted positive by IHC, evidencing clusters of *N. caninum* tachyzoites within myocardiocytes associated with histopathological lesions.

Fetal tissues DNA was extracted using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega) according to the manufacturer's protocol. Each routine of extraction was conducted with a control extraction sample (only kit solutions). Conventional PCR using the specific primers Np6 + /Np21 + and TOX5-TOX8 was performed for *N. caninum* and *T. gondii* DNA detection, respectively, using protocols previously described (Unzaga et al., 2014). Each amplification was conducted along with a positive control (NC-1 and RH strain DNA, respectively), an extraction control, and a NTC (No template control) sample. A fragment of the 18 S ribosomal RNA (rRNA) gene was amplified by PCR using the primers SarcoFext and SarcoRext as previously described (Moré et al., 2013). *Neospora caninum*-DNA was detected in fetus's heart, lungs, liver, kidney, and muscle, whereas *T. gondii* PCR was negative in all samples analyzed. Amplification products from NC-5 gene (Np6 + /Np21 + primers) and a fragment of the 18 S rRNA gene were submitted for sequencing to the Genomic Unit, Biotechnology Institute CICVyA – CNIA –INTA, Argentina. Sequences were analyzed using the Geneious software (R9 version). Consensus sequences obtained were compared with others reported in GenBank by BLASTn analysis (megablast). NC-5 gene fragment consensus sequences of 250 bp and 254 bp were obtained from fetal heart and liver samples, respectively. BLASTn analysis demonstrated 100% identity to *N. caninum* NC-5 gene sequences (LN714488, KU253799, and KF649847, among others). The sequences obtained from the goat fetal tissues were registered on the GenBank under accession numbers MG973171 and MG973172. Additionally, a 18 S rRNA gene fragment consensus sequence of 808 bp was obtained from the heart sample, and it showed 100% identity with others *N. caninum* 18 S rRNA gene sequences (GQ899206, U17345, L24380, and U03069). This sequence was registered under the accession number MG973173. The *N. caninum* positive PCR samples were used for genotyping by multilocus-microsatellite typing (MLST) for 9 microsatellites: MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, and MS21 of *N. caninum* (Regidor-Cerrillo et al., 2013). Complete genotyping was achieved from the heart sample. Microsatellites MS7 and MS10 were amplified and sequenced using primers and protocols described previously (Regidor-Cerrillo et al., 2013). Microsatellite genotyping revealed a unique genetic profile that differed from previously reported *N. caninum* genotypes (Table 1). MS21 and MS10 alleles are unique and the nucleotide sequence of MS10 has been deposited in the GenBank database under accession number MG383404.

3. Discussion

Results from this work indicate that *N. caninum* was efficiently transmitted to the goat fetus. The fetus had negative serological results which indicate lack of humoral response, probably associated with the immature immune system (Unzaga et al., 2014). However, specific antibodies to *N. caninum* and *T. gondii* were detected by IFAT and IB in the mother´s serum sample. In fact, the high IFAT titer for *N. caninum* (1:3200) detected in the mother sample is in agreement with previous studies where a peak of humoral response was detected at the time of abortion (Mesquita et al., 2013; Costa et al., 2017). Even though no gross lesions associated with infection were observed in the goat fetus, severe histopathological lesions were found in multiple organs. This is in agreement with other reported cases, where gross lesions associated

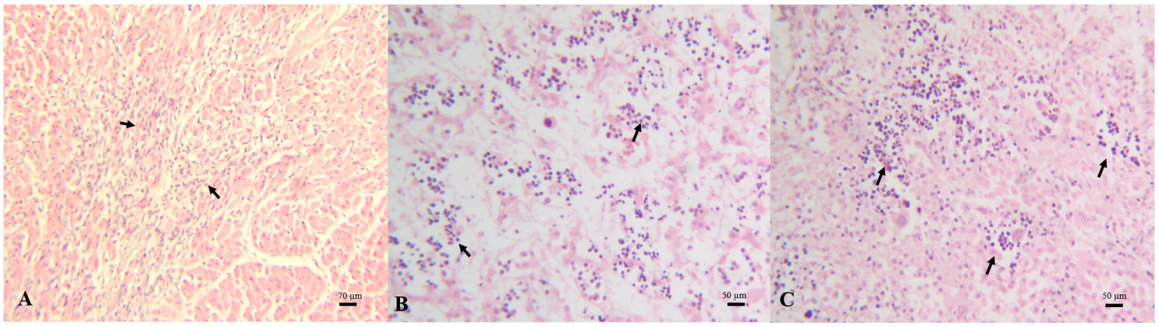

Fig. 1. Photomicrographs of tissues from aborted caprine fetus showing different lesions: (A) heart sample, foci of necrotizing myocarditis (arrows); (B) liver with multiple periportal mononuclear cells infiltrates (arrows) in autolyzed liver stroma; (C) kidney section showing multifocal interstitial nephritis with inflammatory mononuclear cells infiltrates (arrows)(H&E, 20X).

Table 1
Microsatellite (MS) genotyping of the *Neospora caninum* isolate from an Argentinean aborted goat fetus.

Organs	[#]MS4 GC-(AT)n-ACATTT-$(AT)_2$-AC	MS5 CG-(TA)n-TGTA-GG	MS6A GC-(TA)n-AC	MS6B CC-(AT)n-GT	MS7[*] ATAA-(TA)n	MS8 AC-(AT)n-GG	MS10[*] (ACT)x-(AGA)y-(TGA)z	MS12 GC-(GT)n-GC	MS21 TG-$(TACA)_3$-TACC-(TACA)n-TT
Liver/heart	14	15	16	12	9.1	13	5.14.8	16	7
SP-05-GAL-W-35	14	15	16	12	9.1	13	7.11.9	16	6

[#] Allele assignation for each of the microsatellite sequences are shown by the number or repeats (n and x-y-z for MS10) according to the size determined by fragment analysis.

[*] MS7 and MS10 microsatellites were also identified by sequencing.

with *N. caninum* infection were rarely detected (Corbellini et al., 2001; Eleni et al., 2004). However, hydrocephalus was described in goat abortions caused by *N. caninum* (Dubey et al., 1996; Varaschin et al., 2012). The presence of *N. caninum* cysts in central nervous system of the fetus has been reported (Corbellini et al., 2001; Eleni et al., 2004). Unfortunately, no brain sample was available in the present study. Despite the fact that the mother was seropositive to *T. gondii*, the parasite was not detected in fetal tissues. These facts suggest that *T. gondii* was probably not transmitted from the mother to the fetus and therefore, ruled out as the cause of abortion. On the other hand, the presence of *N. caninum* tachyzoites in myocardium (confirmed by IHC), associated with histopathological lesions, and the detection of *N. caninum* DNA in multiple organs suggests congenital infection. In addition, the species of the parasite present in tissues was confirmed by sequencing PCR products of NC-5 and 18 S rRNA gene fragments, which showed 100% identity with other *N. caninum* sequences. No differences were found with other sequences reported from several *N. caninum* strains/types isolated from different hosts and geographical regions, although NC-5 *locus* has been reported as polymorphic for *N. caninum* (Beck et al., 2009). Furthermore, *N. caninum* microsatellite genotyping was possible, probably due to high parasite DNA concentration in tissues, especially in heart, resulting in a unique profile different from those isolates reported on other animal species. Microsetellite pattern comparison showed the closest genetic relationship with the Spanish bovine genotype (SP-05-GAL-W-35) (Table 1). The *N. caninum* genotype reported in our work also differs from a recent *N. caninum* genotyping report on goat placentas where only MS10 was sequenced (Costa et al., 2017). In addition, we present a complete *N. caninum* genotype for nine MS, sequences of MS7 and MS10, and unique alleles for MS21 and MS10. Moreno et al. (2012) performed MLST on goat fetal tissues, but no successful results were achieved. The genetic pattern herein reported is relevant for future epidemiological studies, specially to study association between small ruminants and cattle infections as previously proposed (Moreno et al., 2012). Molecular characterization studies will be conducted (i.e. sequencing ITS-1 or SAG genes) to identify potential differences with other *N. caninum* isolates. We can presume the existence of potential goat adapted-*N. caninum* genotypes although higher number of samples are needed to confirm this hypothesis. The failure to isolate viable *N. caninum* parasites in mice could be related with the high degree of autolysis of the fetus affecting parasite viability. Therefore, biological studies and characterization of the reported strain were not plausible.

The present study reports the detection of *N. caninum* in a goat fetus associated with histopathological lesions in several tissues and the first *N. caninum* genotyping by multilocus MS from an aborted goat fetus.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this article.

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