Short communication

Occurrence of Neospora caninum and Toxoplasma gondii infections in ovine and caprine abortions

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ARTICLE INFO

Article history:
Received 10 November 2011
Received in revised form
15 December 2011
Accepted 29 December 2011

Keywords:
Neospora caninum
Toxoplasma gondii
Abortion
Ovine
Caprine

ABSTRACT

Neospora caninum and Toxoplasma gondii are closely related cyst-forming apicomplexan parasites identified as important causes of reproductive failure in cattle and small ruminants, respectively. Protozoan abortion in small ruminants is traditionally associated with T. gondii, but the importance of N. caninum remains uncertain. The aim of this study was to investigate the presence of N. caninum and T. gondii infections in abortion cases in small ruminants submitted for diagnosis. For this purpose, 74 ovine and 26 caprine aborted foetuses were recovered from different areas in Spain. Foetal histopathology was used to detect the presence of protozoal-associated lesions in brain. The presence of N. caninum and T. gondii was confirmed by PCR. Protozoal infection was detected in 17 out of 100 (17%) foetuses examined by at least one of the diagnostic techniques used. Lesions suggestive of protozoal infection were observed in 10.8% (8/74) and 15.4% (4/26) of the ovine and caprine abortions respectively. N. caninum and T. gondii infection was detected by PCR in 6.8% (5/74) and 5.4% (4/74) of sheep foetuses, respectively, of which five showed protozoal-associated lesions. N. caninum DNA was detected in 1.5% (3/26) of goat foetuses, of which two showed protozoal-associated lesions, whereas T. gondii DNA was detected in one goat foetus with no lesions. The simultaneous presence of N. caninum and T. gondii DNA was detected in one sheep foetus with severe lesions. This study demonstrates that N. caninum plays a significant role in abortion in small ruminants in the studied population. In addition, our results highlight the importance of differentiating between protozoa whenever characteristic lesions are observed.

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1. Introduction

The productivity of small ruminant flocks depends greatly on their reproductive efficiency. Abortion is a major cause of economic losses in the livestock industry due to its high prevalence. In a large number of abortions, the precise cause cannot be determined because a broad range of factors must be considered. Although other causes may exist, infectious aetiologies seem to predominate in sheep and goats. The diagnosis of an infection cause of abortion is often difficult and should be made in a specialised veterinary laboratory; thus, a high percentage of cases remain undiagnosed (Kirkbride, 1993; Moeller, 2001). Definitive diagnosis rate of between 40% and 62% are achieved in good diagnostic laboratories (Oporto et al., 2006; Edmondson et al., 2012). A variety of pathogens have been classically reported as being abortive in these species, some of which also have zoonotic potential.
including *Brucella melitensis*, *Chlamydiaphila abortus*, *Toxoplasma gondii*, *Coxiella burnetii* and the emerging pathogen *Campylobacter jejuni*. In addition, due to the high number of cases that remain undiagnosed (Edmondson et al., 2012), it is important to search for new pathogens or agents not typically associated with abortions in these species.

*Neospora caninum* and *T. gondii* are closely related cyst-forming apicomplexan parasites identified as important causes of reproductive failure in cattle (Dubey et al., 2007; Dubey and Scharer, 2011) and small ruminants, respectively (Buxton, 1998; Hurtado et al., 2001; Pereira-Bueno et al., 2004; Masala et al., 2007; Dubey and Scharer, 2011). Protozoan abortion in sheep and goats is traditionally associated with *T. gondii*, where characteristic necrotic lesions in the central nervous system are usually observed (Buxton, 1998; Hurtado et al., 2001; Dubey, 2003). However, similar lesions can also be observed in *Neospora*-associated abortion; therefore, discrimination between these infections is difficult from a pathological point of view (McAllister et al., 1996; Moreno, personal communication; Conraths and Gottstein, 2007). Nevertheless, in small ruminants, *N. caninum* seems to be much less relevant and its importance remains unclear (Otter et al., 1997; Buxton, 1998; Moeller, 2001; Dubey, 2003; Moore, 2005; Givens and Marley, 2008). Serological surveys in both sheep (Helmick et al., 2002; Reichel et al., 2008; Bártová et al., 2009; Panadero et al., 2010) and goats (Abo-Shehada and Abu-Halaweh, 2010; Dubey and Scharer, 2011) have shown a variable prevalence ranging from 0.6% to 30.8%. Moreover, abortion cases attributed to *N. caninum* infection have been occasionally reported in small ruminants (Barr et al., 1992; Dubey et al., 1996; Corbellini et al., 2001; Kobayashi et al., 2001; Dubey, 2003; Eleni et al., 2004). Much less frequently, *N. caninum* has been associated with epizootic abortion (Moreno, personal communication; Moeller, 2001; Hässig et al., 2003). In addition, *Neospora* infection has occasionally been detected in neonates, adult animals with nervous signs (Dubey et al., 1992; Corbellini et al., 2001; Bishop et al., 2010) and asymptomatic animals (Koyama et al., 2007). These studies indicate the presence of *N. caninum* infection and the potential for transplacental transmission in small ruminants; however, the frequency and aetiology of abortion due to this parasite remain unclear.

The aim of this article is to provide data about the occurrence of *T. gondii* and *N. caninum* in cases of small ruminant abortion in Spain by means of some of the most widely used techniques for diagnosis of infections in foetuses.

# 2. Materials and methods

## 2.1. Samples

Seventy-four sheep foetuses and twenty-six goat foetuses were sent to the Diagnostic Service of Exopol in 2008 and 2009 for general aetiological diagnosis. In addition to *T. gondii* and *N. caninum*, typical pathogens associated with abortion in sheep and goats, including *C. abortus*, *Coxiella burnetii*, *Leptospira* spp., *B. melitensis*, Border disease virus, *Campylobacter* spp., *Salmonella abortus ovis* and *Listeria* spp., were also analysed. All foetuses were obtained from abortion cases that took place on farms in different areas of Spain. A complete necropsy was performed on all foetuses submitted, and the presence of macroscopic lesions was also evaluated. Samples of brain, lung, heart, liver, spleen and kidney were obtained and fixed in 10% neutral buffered formalin at room temperature for histopathology. In addition, samples of anterior brain, mesencephalon, cerebellum and pons were subjected to PCR for the detection of *N. caninum* and *T. gondii*.

## 2.2. Histopathological analysis

For the histopathological study, fixed tissues were dehydrated through graded alcohols before being embedded in paraffin wax using routine procedures. Blocks were cut in 4 μm sections, deparaffinised, rehydrated, stained with haematoxylin and eosin (H/E) and examined by light microscopy. Organs were examined for protozoal-associated lesions. For brain tissues, microscopic examination was done on the mesencephalon, cerebellum and pons. Identified lesions were classified as previously reported (Pereira-Bueno et al., 2004). Briefly, in brain tissues, “characteristic” lesions contained multifocal necrotic foci surrounded by inflammatory cells and/or multiple foci of non-suppurative infiltrates, while “compatible” lesions were less severe, with scarce glial foci, diffuse gliosis and no necrotic areas. Additionally, based on the number of necrotic foci, “characteristic” lesions were classified as mild (<2 necrotic foci), moderate (2–5 necrotic foci) or severe (>5 necrotic lesions). “Compatible” lesions were classified as mild. Heart, lung, liver, kidney and spleen tissues were also examined for the presence of mononuclear infiltration and/or necrotic foci. These lesions were classified as mild (+), moderate (++ or severe (+++) as described above.

## 2.3. DNA extraction and nested PCR for the detection of *N. caninum* and *T. gondii*

Genomic DNA extraction was performed on three different samples of all brain sections recovered at necropsy. DNA was extracted from 20 mg of brain tissue using a commercial kit (Real Pure, Durviz, Spain) according to the manufacturer’s protocol. DNA concentrations were determined by spectrophotometric analysis at A260/280, and all samples were diluted to a final concentration of 60 ng/μl. DNA samples were stored at −20°C prior to PCR analysis.

Nested PCR was performed on all samples to detect *N. caninum* and *T. gondii* DNA. For *N. caninum*, four oligonucleotide primers were used to amplify the internal transcribed spacer (ITS1) region, as described by Buxton et al. (1998). For *T. gondii*, a single-tube nested-PCR assay was performed as previously described (Hurtado et al., 2001). Positive controls (purified *N. caninum* or *T. gondii* tachyzoite DNA) and negative controls (double-distilled water) were included in each set of PCR reactions. To avoid false-positive reactions, DNA extraction, PCR sample preparation and electrophoresis were performed in separate rooms with different sets of instruments, and aerosol barrier tips and disposable gloves were employed. Amplification products were separated by 1.8% agarose gel.
2.4. \textit{N. caninum} microsatellite analysis

Microsatellite analysis was performed on \textit{N. caninum} PCR-positive DNA samples extracted from aborted foetuses as previously reported (Pedraza-Diaz et al., 2009). Briefly, MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12 and MS21 markers were amplified using specific primers and nested-PCR conditions as described by Pedraza-Diaz et al. (2009). The sizes of the PCR products for all microsatellites were also determined using a 48-capillary 3730 DNA Analyser (Applied Biosystems, Foster City, CA) with GeneScan-500 (LIZ) Size Standards (Applied Biosystems) at the Unidad Genómica del Parque Científico de Madrid, and the results were analysed with GeneMapper1 v3.5 software. Additionally, the markers MS7 and MS10 were sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a 3730 DNA Analyser (Applied Biosystems) at the Unidad Genómica del Parque Científico de Madrid. Sequences were analysed using BioEdit Sequence Alignment Editor v.7.0.1 software (Copyright 1997-2004 Tom Hall, Ibis Therapeutics, Carlsbad, CA 92008, USA).

3. Results

Protozoal infections were detected by at least one of the diagnostic techniques in 17 out of 100 (17%) foetuses examined. Of the infected foetuses, 14.9% (11/74) and 23% (6/26) were ovine and caprine foetuses, respectively (Table 1). In ovine foetuses, “characteristic” lesions of protozoal abortion were observed in the brain tissues of seven foetuses (9.5%; 7/74) and “compatible” lesions were observed in one brain (1.4%; 1/74). \textit{N. caninum} was detected by specific PCR in 5 out of 74 foetuses (6.8%), three of which showed “characteristic” lesions of protozoal infection in the brain (Fig. 1). \textit{T. gondii} DNA was detected in four out of 74 foetuses (5.4%), three of which showed protozoal-associated lesions. Of the goat foetuses, three (11.5%; 3/26) showed characteristic lesions and one (3.8%; 1/26) showed compatible lesions.

\textit{N. caninum} DNA was detected in 11.5% (3/26) of goat foetuses, of which two also showed protozoal-associated lesions (Fig. 2a). \textit{T. gondii} DNA was detected in one foetus with no lesions. The simultaneous presence of \textit{N. caninum} and \textit{T. gondii} DNA was detected in only one sheep foetus, which also showed the most severe lesions. In two sheep foetuses with severe lesions, cyst-like structures were observed close to inflammatory foci in the nervous tissue of one foetus and in the myocardium of the other.

According to our classification of severity based on the presence and number of necrotic foci in the brain, the most severe lesions were found in sheep. Characteristic necrotic lesions were observed in six sheep foetuses and in zero goat foetuses (Table 1). In goats, lesions mainly corresponded with glial proliferation in the brain. Histological lesions associated with protozoal infection were also observed in other organs in most of the foetuses, and they were mainly characterised by several degrees of mononuclear inflammatory infiltrates (Table 1). However, in one goat foetus infected by \textit{N. caninum}, an atypical pattern of moderate diffuse gliosis in the brain, with no necrotic lesions and numerous necrotic foci in the lung, liver and spleen, was observed (Fig. 2b).
Amplification of *N. caninum* microsatellite markers was only achieved using DNA from brain samples of an ovine foetus that also tested positive by PCR (Table 2). New alleles were not identified after satellite marker analysis. However, comparison of this multilocus profile with others previously described for *N. caninum* isolates and infected clinical samples showed a unique multilocus pattern for this sample.

### 4. Discussion

*T. gondii* is considered to be the primary parasite responsible for both sheep and goat abortion. However, the economic, clinical and epidemiological importance of *N. caninum* infection in these species remains uncertain (Dubey and Scharcs, 2011). To date, this work is the largest study that has been performed to investigate the involvement of *N. caninum* and *T. gondii* in cases of small ruminant abortion, both in terms of the number of analysed foetuses and the range of diagnostic techniques applied. In this study, we used foetal histopathology combined with PCR to detect the presence of protozoal infection in aborted foetuses from sheep and goats. Confirmation of the presence of the etiologic agent using specific techniques such as PCR is needed because *N. caninum* and *T. gondii* may cause similar lesions.

Protozoal infection was present based on at least one of the diagnostic techniques in 17% (11 sheep and 6 goats) of the 100 foetuses analysed. In 12 foetuses (8 sheep and 4 goat foetuses), lesions suggestive of protozoal infection were observed, and in the remaining five, only protozoal DNA was detected. Our results indicate that protozoal infection is an important cause of abortion in sheep and goats, as is often reported in the literature (Dubey, 2003; Masala et al., 2007; Pereira-Bueno et al., 2004; Dubey, 2009). We also provide presumptive evidence that *N. caninum* could be an important abortifacient in small ruminants, as *N. caninum* DNA associated with characteristic lesions was found as frequently as *T. gondii* in the studied animals. *N. caninum* has been sporadically associated with abortions in small ruminants (Conraths and Gottstein, 2007; Dubey and Scharcs, 2011). *N. caninum* was detected in the brains of 3 out of 18 aborted ovine foetuses in New Zealand (Howe et al., 2008), in 18.5% of 74 aborted ovine foetuses in England (Hughes et al., 2006) and in 2% of 292 aborted sheep foetuses in Italy (Masala et al., 2007). In goat abortions, *N. caninum* was found in 8.6% of 23 aborted foetuses in Italy (Masala et al., 2007) and in 8.3% of 12 aborted foetuses in Spain (Moreno, personal communication). On the other hand, *T. gondii* DNA was only detected in four (5.4%) ovine foetuses and one (3.8%) caprine foetus in the present study. These figures are lower than those previously reported in Spain, in which *T. gondii* was detected in 15–23% of aborted sheep foetuses (Hurtado et al., 2001; Pereira-Bueno et al., 2004; Oporto et al., 2006) and in 8.3% of goat abortions (Moreno, personal communication). These differences may be influenced by geographical distribution of infection because those studies were mainly focused on abortions recovered from Northern Spain, whereas in this study *T. gondii* was detected in abortions from all Spanish regions. Furthermore, they may be also influenced by the different methodologies employed, as Pereira-Bueno et al. (2004) used serological techniques and Moreno (personal communication) histological and immunohistochemical techniques.

In this study, we observed characteristic lesions of protozoal infection in 9.5% and 11.5% of ovine and caprine foetuses, respectively. These data are similar to those reported in previous studies in Spain (Moreno, personal communication; Hurtado et al., 2001; Pereira-Bueno et al., 2004) and elsewhere (Moeller, 2001; Dubey, 2003). In these studies, protozoal-associated lesions associated with
T. gondii infection were found in 9.4% and 11.1% of aborted ovine foetuses (Hurtado et al., 2001; Pereira-Bueno et al., 2004) and in 16.7% of caprine abortion cases (Moreno, personal communication). However, N. caninum detection was not performed in these studies. Interestingly, Hurtado et al. (2001) found a 100% correlation between lesions and T. gondii DNA detection; however, this is not the rule, as very often PCR and lesions present a fair correspondence (Pereira-Bueno et al., 2004). This poor agreement is thought to be related to the uneven localisation and low levels of the parasite (Pereira-Bueno et al., 2004). In our study, abortion cases with characteristic lesions were mainly associated with the presence of N. caninum (62.5%) and less often with T. gondii (37.5%). It has been suggested that N. caninum may have been misidentified as T. gondii in previous studies (Dubey, 2003). Lesions induced by N. caninum and T. gondii in abortion cases are similar, so specific techniques must be used to detect the pathogen involved. Consequently, N. caninum infection in small ruminant abortions may be underdiagnosed, and its importance remains largely unknown (Dubey, 2003; Conraths and Gottstein, 2007).

Another aim of this study was to compare lesions between N. caninum and T. gondii. However, in both sheep and goats, the small number of foetuses with lesions precluded a comparison between ruminant species and between parasites. In sheep, characteristic necrotic foci were typically seen, while in goats only glial reactions were observed. Interestingly, in goats, similar to previous observations (Moreno, personal communication), N. caninum-associated lesions mainly corresponded with glial proliferation and very rare necrotic foci. While our study failed to detect necrotic lesions, in a previous investigation only a small necrotic focus in the mesencephalon was found (Moreno, personal communication). Some of the few pathologic descriptions of N. caninum-associated abortions in goats have also described only glial reactions (Corbellini et al., 2001) and less often the presence of necrotic foci (Eleni et al., 2004). More studies with an increased number of foetuses will be needed to determine whether pathologic differences exist between N. caninum and T. gondii infections in sheep and goats.

In addition, it is not clear whether an epidemiological association exists between small ruminant and cattle Neospora infections. Taking into account that N. caninum is one of the most important causes of abortion in cattle in Spain, and that some farms have sheep, goats and cattle in the same fields, an epidemiological relationship may be involved. Indeed, a previous study investigated this possibility, although no relationship was found (Spilovská et al., 2009). A molecular characterisation and comparison of small ruminant and bovine isolates of N. caninum would improve our knowledge of the epidemiology of this pathogen. Microsatellite markers have been demonstrated to be a suitable tool for genotyping N. caninum from clinical samples (Pedraza-Díaz et al., 2009). Thus, with the aim of investigating N. caninum isolates disseminated in ovine and caprine abortions, microsatellite genotyping was performed in the present study. However, we were able to determine only a single microsatellite profile, from an ovine sample, and were therefore unable to perform any
comparisons, although this genetic profile was different from those previously found in bovine samples (Regidor-Cerrillo et al., 2006, 2008; Rojo-Montejo et al., 2009; Basso et al., 2009a,b; Pedraza-Díaz et al., 2009).

Our results confirm that transplacental transmission of N. caninum occurs in sheep and goats and indicate that N. caninum could be a causative agent of abortion in domestic small ruminants. Consequently, neosporosis should be included in a differential diagnosis of causes of abortion in these species; however, further studies are necessary to investigate the role of N. caninum as a cause of abortion in sheep and goats and to evaluate the resulting economic losses to the industry.

Acknowledgements

We thank Carmen Cuevas (Animal Health Department, Complutense University of Madrid) for her excellent technical assistance. This work was funded by Programa de Creación y Consolidación de Grupos de Investigación Universitaria Complutense–Comunidad de Madrid (GR35-10).

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