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Evaluation by different diagnostic techniques of bovine abortion associated with *Neospora caninum* in Spain

J. Pereira-Bueno [a], A. Quintanilla-Gozalo [a], V. Pérez-Pérez [b], A. Espi-Felgueroso [c], G. Álvarez-García [d], E. Collantes-Fernández [d], L.M. Ortega-Mora [d,*]

[a] *Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de León, 24007 León, Spain*
[b] *Departamento de Medicina Animal, Facultad de Veterinaria, Universidad de León, 24007 León, Spain*
[c] *Laboratorio de Sanidad Animal, Consejería de Agricultura, 33299 Gijón, Spain*
[d] *Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Ciudad Universitaria s/n, E-28040 Madrid, Spain*

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Abstract

Eighty foetuses from some of the main cattle-producing regions in Spain were analysed to investigate the participation of *Neospora caninum* in cases of bovine abortion. Diagnosis of the infection was determined by histopathological analysis complemented with immunohistochemistry, serology (IFAT and ELISA) and PCR tests. A total of 38.8% of the bovine foetuses analysed were considered to be infected by at least one of the diagnostic techniques used. Microscopic lesions consistent with *Neospora* infection in brain were identified in 31.3% of the samples, whereas only 10.7 and 15.3% were positive using serological and PCR analysis, respectively. Perfect agreement was shown between IFAT and ELISA, although there was little agreement among results of the other diagnostic techniques. Gestational age of aborted foetuses checked ranged from <3 to 9 months, with a mean of 5.9 months, and no difference in age was found between infected and non-infected foetuses ($P > 0.05$). This study confirms the importance of *N. caninum* as a cause of abortion in Spain and underlines the need to use different diagnostic techniques to increase the chance to detect the infection in aborted foetuses.

Keywords: Neospora caninum; Cattle–protozoa; Abortion; Histopathology; Immunohistochemistry; IFAT; ELISA; PCR

* Corresponding author. Tel.: +34-91-394-4069; fax: +34-91-394-3908.
E-mail address: luis.ortega@vet.ucm.es (L.M. Ortega-Mora).

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1. Introduction

Neospora caninum is a protozoan parasite which was first associated with bovine abortion in the USA in 1989 (Thilsted and Dubey, 1989). Since then, this parasite has been recognised as a major cause of infectious bovine abortion world-wide (Dubey, 1999; Trees et al., 1999). In Europe, a theoretical estimate of abortions attributable to neosporosis in the UK gives a percentage of 12.5% of the total number of abortions (Davison et al., 1999a). Moreover, the percentages of *Neospora* foetal infection are also high in several other countries such as Switzerland (21–29%) (Gottstein et al., 1998; Sager et al., 2001) and France (21%) (Pitel et al., 2001). In Spain, in a recent study on 81 aborted bovine foetuses the prevalence of neosporosis oscillated between 32 and 57% depending on the diagnostic technique used (González et al., 1999).

A range of diagnostic tools has been proved to be useful to detect infection in aborted foetuses. One of the most widely used techniques is foetal histopathology of brain and some other organs such as heart and liver in an attempt to detect lesions characteristic or consistent of protozoal infection, combined or not with immunohistochemical examination of positive tissues (Anderson et al., 1991; Barr et al., 1991; González et al., 1999). Foetal serology has also been demonstrated to be a good diagnostic tool to indirectly detect the presence of parasite infection (Barr et al., 1995; Buxton et al., 1997; Osawa et al., 1998; Söndgen et al., 2001). Moreover, different PCR techniques have been used to detect parasite-DNA in target tissues from cases of bovine abortions (Gottstein et al., 1998; Baszler et al., 1999; Ellis et al., 1999; Schock et al., 2000).

The aim of this study was to investigate the participation of *N. caninum* in cases of bovine abortion in some cattle-producing areas in Spain by means of some of the most widely used techniques for diagnosis of the infection in foetuses (histopathology, immunohistochemistry, serology—IFAT and ELISA—and a nested-PCR) and comparing the value in the diagnosis of *N. caninum* infection in the bovine foetus.

2. Materials and methods

2.1. Samples and data recovered

Samples from bovine aborted foetuses were collected at the Regional Diagnostic Laboratories of Asturias, Galicia and León during 1996 and 1997 and submitted to our laboratory for analysis. All foetal samples arriving during this period were included in the study. This sampling area was selected because includes some of the main cattle-producing regions in Spain. In total, specimens from 80 foetuses were investigated, 35 from Asturias, 22 from Galicia, 20 from Castilla-León, and 3 from Madrid. Most of the foetuses submitted for diagnosis belonged to the Holstein–Friesian breed, although a small number of foetuses from bovine beef breeds were also studied. Before necropsy, general status of the foetus (autolysed, mummified, etc.), presence of macroscopic lesions and age of the foetuses (estimated by the measurement of crown–rump length) were recorded. Foetal sera or thoracic fluids were recovered from the thoracic cavity using sterile syringes. Samples were centrifuged at $500 \times g$ for 10 min, to eliminate cellular debris, and the serum was aliquoted and stored at −20 °C until analysed. Brain and heart—the latter was not always available—were recovered and samples from both organs were fixed for histopathology in 10% neutral buffered formalin. Also, an aliquot of brain (approximately 1 g of tissue taking randomly from different parts of this organ) was collected in aseptic conditions for PCR.

2.2. Parasite production and antigen preparation

N. caninum tachyzoites from the Nc-1 isolate (Dubey et al., 1988) were cultured in Vero cells in Minimum Essential Medium (MEM; Sebak, Aidenbach, Germany) supplemented with 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2.2 g l^{-1} sodium bicarbonate and 2% (v/v) heat-inactivated equine serum (Sebak), at 37 °C in a 5% CO_2 humidified incubator. Tachyzoites were harvested from tissue culture, washed three times in sterile PBS (pH 7.4) and separated from host cells by passing the mixture through a 27-gauge needle, followed by passage through a 5 μm polycarbonate filter. Tachyzoite soluble proteins to be used in the ELISA were prepared as follows. Approximately 2×10^9 tachyzoites were pelleted and re-suspended in 4 ml of 10 mM Tris hydrochloride containing 2 mM of the enzyme inhibitor phenylmethylsulfonyl fluoride. The tachyzoites were disrupted by two cycles of freezing and thawing followed by five to six cycles of ultrasound treatment in an ice-bath; the material was centrifuged at $10{,}000 \times g$ for 30 min at 4 °C and the supernatant recovered, aliquoted and cryopreserved at −80 °C. The protein content was determined using the bicinchoninic acid method (Smith et al., 1985).

2.3. Histopathological and immunohistochemical studies

From the 80 foetuses, 7 and 3 different sections from brain and heart, respectively, were processed by routine histological methods. Tissues were fixed in 10% neutral buffered formalin and dehydrated through graded alcohols before being embedded in paraffin wax. At least three sections, 4 μm thick, were cut from each sample and stained with haematoxylin and eosin. Observed brain lesions were classified according to previous descriptions (Barr et al., 1990; González et al., 1999), and the results obtained in this work were classified as none detected/unrelated (−), consistent with (+) or characteristic (++) of bovine neosporosis. Immunohistochemistry was carried out on brain sections from 13 foetuses. Only brain tissues with characteristic or consistent with lesions were selected. The number of samples included in this part of the study was determined by their availability, since in some cases only the haematoxylin and eosin stained section instead of the paraffin embedded block were submitted to our laboratory. From each block, at least two sections (separated by 50 μm) were mounted on poly(L-lysine) coated glass slides and processed. The primary antibody to *N. caninum* was a caprine polyclonal antiserum (VMRD Inc., Pullman, WA, USA) used at 1:3000 dilution. The secondary antibody was a biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA) and the detection system was an avidin–biotin complex immunoperoxidase system (Vectastain ABC Elite, Vector Laboratories, Burlingame, CA) used according to the manufacturer's recommendations. The stain was developed using diaminobenzidine as chromogen.

2.4. Foetal serology

Serum samples from 56 aborted foetuses were analysed by IFAT and ELISA. In this study, a slightly modified version of the ELISA described by Osawa et al. (1998) was used to detect the existence of specific anti-*N. caninum* IgG. Foetal serum samples with O.D. equal to or higher than 0.17 were considered as *Neospora* antibody-positive (Osawa et al., 1998). For the IFAT, tachyzoites were obtained from cell cultures as described above and subsequently coated onto 10-spot IFAT glass slides (bioMérieux España, Madrid, Spain) and air dried. Basically, the procedure was carried out as described by Trees et al. (1994) and a diagnostic cut-off point of 1:64 (Buxton et al., 1997) was used.

2.5. PCR technique

A total of 59 foetal brains samples taken at autopsy from aborted foetuses were also analysed using a nested-PCR technique similar to that used by Buxton et al. (1998). Briefly, DNA from the foetal brain samples (1 g of brain sample) was prepared by extraction with phenol and ethanol precipitation. Amplification of the internal transcribed spacer 1 (ITS1) region of *N. caninum* was carried out with four oligonucleotides. Oligonucleotide primers NN1 (5′-TCAACCTTTGAATCCCAA) and NN2 (5′-CGAGCCAAGACATCCATT) (Pharmacia Biotech, Uppsala, Sweden) were used for the primary amplification, followed by primers NP1 (5′-TACTACTCCCTGTGAGTTG) and NP2 (5′-TCTCTTCCCTCAAACGCT) (Pharmacia Biotech) to amplify a 213 bp fragment. PCR reactions contained 10 mM Tris, pH 8.3, 2 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 200 μM dNTPs (Pharmacia Biotech), 0.15 μM each primer and 1.15 units of Taq DNA polymerase (Boehringer Mannheim, Barcelona, Spain). Thermocycling consisted of 25 cycles of the following: denaturation, 1 min at 94 °C; annealing, 1 min at 48 °C; extension, 72 °C for 1.5 min. After 25 amplification cycles, the final extension step was continued for an additional 5 min at 72 °C. After the primary amplification, PCR reactions were diluted 1:15 in sterile water and 5 μl added to the secondary amplification reaction mix. In each amplification, DNA equivalent to 10^3 tachyzoites was employed as positive control. Aliquots of amplification product obtained in the PCR assay were analysed by agarose gel (1.8%) electrophoresis and the products were stained with ethidium bromide and visualised under UV light.

2.6. Analysis of data

The degree of agreement between the different techniques used was estimated by calculating the kappa value (Thrusfield, 1995). Comparison of gestational ages of the positive and negative bovine foetuses and comparison of ages of infected foetuses diagnosed by the different techniques were performed by non-parametric Mann–Whitney *U*-test and Kruskal–Wallis *H*-test, respectively, using Statistica v.5 (StatSoft Inc., OK, USA).

3. Results

A total of 38.8% of the bovine foetuses studied were determined as infected by *N. caninum* by at least one of the diagnostic techniques used (Table 1). A higher percentage of foetuses

Table 1
Percentages of foetuses infected by *N. caninum* according to the different diagnostic techniques

Region	Number of foetuses	Percentage (positives/analysed)					
		Histology	IH[a]	Serology		PCR	Total
				IFAT	ELISA		
Asturias	35	37.1 (13/35)	100 (1/1)	12.9 (4/31)	12.9 (4/31)	11.8 (4/34)	37.1 (13/35)
Galicia	22	40.9 (9/22)	55.6 (5/9)	22.2 (2/9)	22.2 (2/9)	(0/3)	50.0 (11/22)
Castilla-León	20	15.0 (3/20)	33.3 (1/3)	(0/16)	(0/16)	20.0 (4/20)	30.0 (6/20)
Madrid	3	(0/3)	–	–	–	50.0 (1/2)	33.3 (1/3)
Total	80	31.3 (25/80)	53.8 (7/13)	10.7 (6/56)	10.7 (6/56)	15.3 (9/59)	38.8 (31/80)

[a] Immunohistochemistry on 13 foetuses showing histological lesions.

were diagnosed as infected by detecting lesions in brain (31.3%) than by using serological (IFAT and ELISA) or PCR techniques (10.7 and 15.3%, respectively).

In 21 out of 25 foetuses with foetal pathology associated with *N. caninum* infection, lesions were defined as "characteristic" and formed by a non-suppurative multifocal encephalitis. In the myocardium, a non-suppurative myocarditis was also observed, with groups of lymphocytes and macrophages infiltrating among the cardiac muscle fibres. In the remaining four foetuses, lesions were described as "consistent with" and were also formed by non-suppurative multifocal inflammatory foci, but necrosis was not a prominent feature and appeared to a lesser extent throughout the brain parenchyma. When present, few inflammatory cells were observed in the myocardium. In infected foetuses, lesions were observed in the brain—mainly in the midbrain area, less frequently in the cortex and rarely in the medulla oblongata and cerebellum—and with the exception of two foetuses (from the "consistent with" group of lesions), in the myocardium. No parasitic forms were detected by routine histological methods in any tissue sample with lesions. In 7 out of 13 foetuses evidence of parasite was observed in the brain lesions by immunohistochemistry. Six out of seven positive foetuses were from the group showing "characteristic" lesions. In all cases, foetal fluids positive by IFAT were also positive by ELISA (Table 1). Antibody titres were low (between 1:64 and 1:128) in the majority of cases (four out of six cases) and were higher than this in only two cases (1:512 and 1:4096).

Detection of *N. caninum* infection by histology, serology and nested-PCR was carried out in 49 of the 80 bovine foetuses (Table 2). A perfect agreement was observed between IFAT and ELISA but, on the contrary, when histology was compared with serology and PCR techniques, there was little agreement in either case ($\kappa = 0.23$). Similarly, when PCR was compared with serology there was also little agreement ($\kappa = 0.24$).

Gestational age of aborted foetuses analysed by all *Neospora* diagnostic tests could be determined in 43 cases and ranged from <3 to 9 months and averaged 5.9 months (Fig. 1). The ages of the positive and negative foetuses ranged from <3 months to 9 months, with a mean of 5.7 months ($n = 19$) and 6 months ($n = 24$), respectively. No significant differences were observed when ages of the infected and non-infected foetuses were compared ($P > 0.05$, Mann–Whitney *U*-test) or when the ages of the foetuses considered as positive by the different diagnostic techniques were compared ($P > 0.05$, Kruskal–Wallis *H*-test).

Table 2
Detection of *N. caninum* from 49 foetuses analysed by histology (H), serology (S) and PCR

	Percentage (positives/analysed)
H	20.4 (10/49)
S	4.1 (2/49)
PCR	4.1 (2/49)
H & S	4.1 (2/49)
H & PCR	4.1 (2/49)
H & S & PCR	4.1 (2/49)
Total	40.8 (20/49)

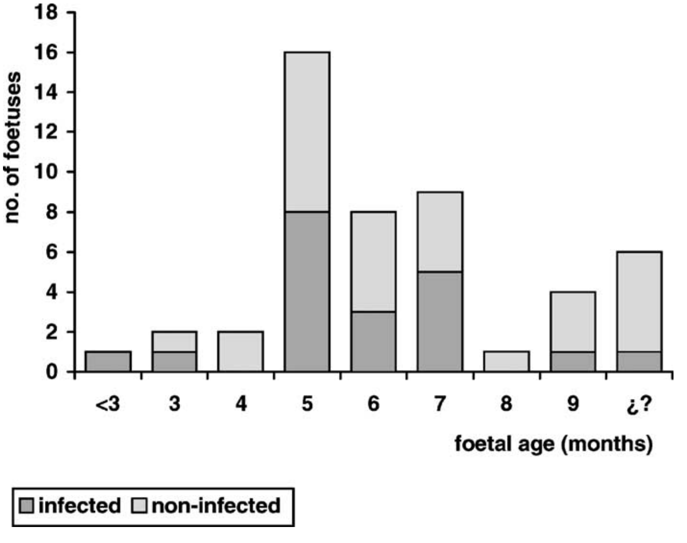

Fig. 1. Age distribution of the 49 bovine foetuses analysed by histology, serology and PCR.

4. Discussion

In this study, a total of 38.8% of bovine foetuses studied were diagnosed as infected by *N. caninum* by at least one of the diagnostic techniques used. Percentage of foetal infection found in this study is high but similar to those recorded in previous studies in Northern Spain (32–57%) (González et al., 1999) and in several other countries such as Switzerland (around 40%) (Gottstein et al., 1998; Sager et al., 2001), Mexico (34.6%) (Morales et al., 2001) and Brazil (39.1%) (Corbellini et al., 2002). On the contrary, lower percentages of foetal infection have been found in other areas of Europe such as France (21.2%) (Pitel et al., 2001), The Netherlands (17%) (Wouda et al., 1997b) and the UK (10.5–13%) (Otter et al., 1995; Schock et al., 2000).

The percentage of foetuses with lesions suggestive of neosporosis obtained in the present study (31.3%) was similar to those reported by Morales et al. (2001) (34.6%) and Sager et al.

(2001) (34.1%). Multifocal non-suppurative encephalitis and myocarditis in the aborted bovine foetus, although not pathogenomonic, are considered as indicative of infection by *N. caninum* (Barr et al., 1991; Wouda et al., 1997b). Moreover, histopathology has been used as a reference technique for the comparison of others (Baszler et al., 1999), although corroborative evidence for the presence of the parasite is necessary, since other protozoa may cause similar lesions (Jenkins et al., 2002). Because of this, confirmation of the infection by immunohistochemistry, serology or PCR is necessary.

Although an immunohistochemical technique was not done on all foetuses showing histological lesions suggestive of neosporosis, the percentage of positives (53.8%) was similar to that reported previously by González et al. (1999) (50%) and Otter et al. (1995) (40%) in Spain and UK, respectively, but was considerably lower than in other previously published works in which it ranged from 66.7 to 87% (Anderson et al., 1995; Corbellini et al., 2002; McNamee et al., 1996; Morales et al., 2001). Although the presence of parasites can be related to the lesions, our result was consistent with evidence for a low number of organisms in the affected foetal tissues (Nietfeld et al., 1992) and the demonstration of parasite antigens by immunohistochemistry depends to a large extent on the number of sections made and the time spent on microscopic examination (Wouda et al., 1997b). This lack of sensitivity is one of the reasons why immunohistochemistry has not been considered a useful tool in the diagnosis of foetal neosporosis (Gottstein et al., 1998).

In the 49 aborted foetuses analysed by all *Neospora* diagnostic tests used in this study, perfect agreement was observed between IFAT and ELISA. On the contrary, little agreement and low sensitivity (25%) and high specificity (94%) were observed when histology was compared with serology and PCR techniques used in this study.

The lack of sensitivity of serology and PCR to detect *Neospora* infection in the foetuses with lesions suggestive of neosporosis may be due to different factors. In previous reports (Barr et al., 1995; Otter et al., 1997; Wouda et al., 1997a), a high percentage of foetuses (>50%) with confirmed or presumptive (Barr et al., 1990, 1991) diagnoses of neosporosis also turned out to be positive by the IFAT technique. In contrast, in the present study only 25% of the 16 foetuses with lesions presumptive of neosporosis were seropositive. The foetal immaturity in the two younger seronegative foetuses (2–4 months gestational age) and other factors in the remaining ten such as death of the foetus during the acute phase of the infection (Gottstein et al., 1998), the cut-off (1:64) selected to detect *Neospora* antibodies in foetal fluids, the source of the examined fluid (Söndgen et al., 2001) and the detection of only specific IgG instead of IgG and IgM (González et al., 1999) are some of the possible reasons for the discrepancy between the serological and histological findings. Several suggestions have been made to improve foetal serology. In our laboratory, IFAT with a cut-off titre of 1:16 gave 100% diagnostic sensitivity and specificity in the case of the foetuses (unpublished observations), and testing for IgM might help to detect more recent infections as bovine foetuses develop the ability to mount an IgM response before an IgG response (Buxton et al., 1997; Slotved et al., 1999). On the other hand, *Neospora* antibodies were also found in 2 out of 33 negative foetuses by histology and PCR. In these two cases, the foetal death could not be attributed to neosporosis, since one of the criteria to define an abortion cause by *N. caninum* is the presence and extent of specific pathologic findings in the foetal brain (Gottstein et al., 1998; Jenkins et al., 2002) and the occurrence of transplacental transmission of the infection in asymptomatic and apparently healthy calves has proven to be high (Davison et al., 1999b). The discordance with the PCR results could be due, as has been pointed out previously (Gottstein et al., 1998; Baszler et al., 1999), to the focal parasite distribution, low parasite numbers and sampling methodology.

PCR was considered a highly specific and sensitive technique for the detection of foetal infection (Sager et al., 2001), but a poor agreement between PCR and histopathology was observed in the present study. In only 4 out of 16 foetuses with compatible histopathological lesions, positive reactions by PCR were observed, which were associated with the presence of *Neospora* antibodies in 2 cases. Parasite-DNA was also detected in brain samples from two foetuses without neosporosis being demonstrated by histological and serological techniques. Probably differences in sampling (i.e. source and size sample) for PCR and for histological examinations could explain the lack of sensitivity observed based on the focal distribution of parasites and lesions in the affected tissues.

Little agreement was also observed between PCR and serology. *Neospora*-DNA could be detected by PCR in the brain of only two of the seropositive foetuses. In four of the six PCR positive foetuses no specific antibodies were detected at the time of abortion probably due to foetal death during the acute phase of infection (Gottstein et al., 1998).

With regard to the gestational age, 95% (19/20) of infected foetuses ranged from 10 to 30 weeks, coinciding with the most likely period for *N. caninum*-induced abortion according to Williams et al. (2000). What is more, although a small number of the foetuses had detectable *Neospora* antibodies, there is a tendency for seroprevalence and titres to increase with gestational age (Barr et al., 1995; Wouda et al., 1997a; Álvarez-García et al., 2002).

In conclusion, our findings confirm the presence of *N. caninum* in aborted bovine foetuses in the study area (northern Spain). Moreover, the percentages of positive foetuses could be considered to be similar or slightly higher than in other European countries. In relation to diagnoses of *Neospora* infection, the poor agreement observed when histology, PCR and serology were compared underlines the need to use different and complementary techniques if we want to increase the probability of detecting infection in aborted foetuses. Moreover, more work needs to be done to quantify the operating characteristics of the tests and to define the sampling methodology before definitive recommendations can be drawn for *N. caninum* detection in the aborted foetuses, although a common feature of all diagnostic techniques is their lack of sensitivity, though all of them have proved to be highly specific. Diagnostic criteria to distinguish foetal infection and *Neospora*-associated abortion need to be defined and based not only on the demonstration of foetal infection, but also, as has been suggested previously by Jenkins et al. (2002), on the extent and severity of the lesions in the foetus, the foetal age and the assessment of neosporosis on a herd level.

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