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Epidemiology of neosporosis in dairy cattle in Galicia (NW Spain)

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Abstract This comprehensive study of neosporosis in dairy cattle in Galicia (NW Spain) included: (1) a comparative study of three serological techniques for detection of *Neospora caninum* antibodies (direct agglutination, enzyme-linked immunosorbent assay and indirect immunofluorescence); (2) a cross-sectional serological survey in which 276 herds and 5,196 animals were tested; (3) a study of *N. caninum* antibody dynamics; (4) the isolation of viable tachyzoites of *N. caninum*. Data were analysed to determine the risk factors associated with the infection. A total of 219 herds (79.3%) and 816 heads of cattle (15.7%) were found to be seropositive. Seropositivity was higher on farms with dogs than on farms without dogs, and there was a negative correlation between the size of the herds and seroprevalence. Co-infection with *Toxoplasma gondii* increased the risk of seropositivity. Cows infected with *N. caninum* were 5.3 times more likely to abort than non-infected cows. The dynamics study showed an increase in anti-*N. caninum* antibody titres during the third trimester of pregnancy. Viable tachyzoites were isolated from brain samples. These results indicate that the economic impact of *N. caninum* is high in Galicia, and therefore, the inclusion of control measures for neosporosis in the official control health programmes is strongly recommended.

Introduction

Neospora caninum is amongst the most important protozoan parasites of cattle in terms of pathology, geographical distribution and economic losses incurred. Cattle and a wide range of other warm-blooded animals act as intermediate hosts, and dogs, coyotes and foxes are the only recognized definitive hosts (McAllister et al. 1998; Gondim 2006; Wapenaar et al. 2006). Infection of cattle has been associated with abortion, neonatal mortality and a 3–4% decrease in milk production (Hernández et al. 2001; Dubey and Schares 2006), but there are still several unknown factors regarding the epidemiology of neosporosis in cows.

Diagnosis of the infection in live animals can be achieved by detection of anti-*N. caninum* specific antibodies by different serological tests [e.g. indirect fluorescence antibody test (IFAT), *Neospora* agglutination test (NAT), enzyme-linked immunosorbent assay (ELISA), Western blotting], which must be validated in individual laboratories. Serodiagnosis has also been used to identify the risk factors associated with neosporosis (Ould-Amrouche et al. 1999), so that seroprevalence studies are necessary both to evaluate existing control programmes and to design new ones.

The seroprevalence of neosporosis in bovine livestock varies depending on the country and region under study. Thus, in a comparative study carried out by Bartels et al. (2006), seroprevalences on European dairy farms ranged between 0.5% in Sweden and 16.2% in Spain. Prevalences have been estimated at between 14.1 and 40.4% in America (Paré et al. 1996; Chi et al. 2002; Moore 2005), 5.7–35.6% in Asia (Hur et al. 1998; Koiwai et al. 2005) and 6.0–21.1% in Oceania (Reichel 1998; Hall et al. 2006).

At present, there are no treatments for neosporosis, and the development of vaccines is essential to have biological material to carry out studies of genetic variability, infectivity, virulence and immune responses. However, isolation of viable forms is difficult, and few isolates of *N. caninum* have been obtained from cattle worldwide (Dubey and Schares 2006).

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The aim of the present study was to carry out a comprehensive investigation of neosporosis in dairy cattle in Galicia, the largest dairy-producing region in Spain. Information about the applicability of serological techniques, distribution of *N. caninum* infection in the region, as well as risk factors and dynamics of antibodies is presented. Field isolates of *N. caninum* were obtained from bovine brains.

Materials and methods

Experimental design

The study was carried out in Galicia, in northwest Spain, where there is a high density of domestic ruminants. To determine the prevalence of infection, a transverse study was carried out, with the herd as the initial sampling unit. Epidemiological software (Win Episcope 2.0; Thrusfield et al. 2001) was used to calculate that 385 herds would need to be sampled to estimate the prevalence with a margin of error of 5% and a confidence interval of 95%, and assuming maximum standard deviation. However, access was available to only 276 farms distributed in the four provinces of Galicia: A Coruña (133), Lugo (98), Ourense (15) and Pontevedra (30), which enabled estimation of the prevalence of infection with an acceptable margin of error (5.9%). Between 20 and 100% of the animals on each farm were sampled, depending on the size of the farm; in each herd, a minimum of ten adult cows and three heifers (females between 1 and 2 years old) were sampled. A total of 5,196 blood samples were collected from 3,983 cows and 1,213 heifers. At the start of the study, a questionnaire was completed about each farm to obtain the following information: size of farm, presence/absence of dogs, and occurrence/non-occurrence of abortions in the cows.

At the same time, a longitudinal study was carried out on farms on which cows were chronically infected with *N. caninum*. For this, four farms with a history of abortion were selected, and blood samples were taken from the cows every 3 months during 1 year. The number of animals sampled varied depending on the size of the farm; on three of the farms (<40 cows) all of the animals were sampled, whereas on the fourth (120 cows), an initial analysis was carried out of all the animals, and 20 seropositive and 20 seronegative cows were selected for posterior analysis. Cows in the herd maintained at the *Centro de Investigacións Agrarias de Mabegondo* were used as controls. Twenty-five cows were selected from the herd and were also sampled every 3 months. In all cases, blood and faecal samples were also collected from any dogs on the farms. On all five farms, individual record sheets were completed to provide the following data about the sampled cows: date of birth, genealogy, date of last insemination and date of latest birth or abortion. From this data, the time of gestation at the moment of sampling and the interval between births were calculated.

Diagnostic techniques

Blood samples were taken from the caudal vein. They were left to clot, centrifuged and the serum separated and frozen at −20°C until used for antibody determination.

Three serological techniques were used for the detection of specific anti-*N. caninum* antibodies: direct agglutination (NAT), an immunoenzymatic assay (ELISA) and indirect immunofluorescence (IFAT).

For the NAT, the technique of Romand et al. (1998) was used with tachyzoites of *N. caninum* obtained from Swiss Webster mice previously immunosuppressed by administration of dexamethasone in the drinking water (10 μg/ml), and experimentally infected with tachyzoites of strain NC1. Four dilutions were made of each serum (1:20–1:160, dilution factor 2) in a reducing solution [0.2 M 2-mercaptoethanol in 0.01 M saline phosphate buffer (PBS), pH 7.4] previously deposited in 96-well, round-bottom plates. Whole tachyzoites of *N. caninum* were diluted in bovine albumin borate saline buffer [0.12 M NaCl; 0.05 M H_3BO_3; 0.024 M NaOH; 0.4% bovine serum albumin; 0.1% sodium azide, ph 8.95] to a final concentration of 0.5×10^4 tachyzoites/μl. Serum samples with antibody titres >1:40 were considered positive (Canada et al. 2004). All analyses included positive and negative sera (for both *N. caninum* and *Toxoplasma gondii*) as controls.

The ELISA was carried out with a CIVTEST® commercial kit (HIPRA S.A., Spain), with a soluble extract of *N. caninum* tachyzoites as the antigen. The serum samples were diluted 1:100, according to the manufacturer's instructions. Readings were taken at 405 nm in a microplate reader (Tecan Spectra). A value of more than 10 U was taken as the threshold for seropositivity.

IFAT was carried out by the method described by Schares et al. (1998). For this, tachyzoites (10^5/well) of strain NC-1 were placed on a glass slide and incubated in a moist chamber with the different dilutions of the sera (1:25–1:400; dilution factor 2) for 1 h at 37°C. The slides were then washed with saline buffer (FA) [0.025 M Na_2CO_3; 0.1 M $NaHCO_3$; 0.035 M NaCl, pH 9.0], and the conjugate was added (rabbit anti-bovine IgG serum labelled with fluoroscein isothiocyanate; Sigma, Spain) diluted 1:200 in PBS with 0.05% Evans blue and incubated for 1 h at 37°C. The samples were examined in a fluorescence microscope with B-2A filters (450–520 nm). Sera with antibody titres >1:50 were considered positive.

Sera were analysed for *T. gondii* antibodies using the direct agglutination test according to Desmonts and Remington (1980) and Canada et al. (2002). Serum samples with antibody titres >1:40 were considered positive.

Faecal samples were collected from the dogs by rectal sampling and were processed in the laboratory within 24 h of collection. A diphasic concentration method was used with the dog faeces to enable detection of *N. caninum* oocysts. The faecal samples were first concentrated in distilled water diethyl ether (2:1; by centrifugation at $1,250 \times g$ for 5 min). Aliquots of the sediment were examined by light microscopy ($\times 40$).

All analyses were carried out in duplicate.

Isolation of *N. caninum* tachyzoites from brain tissue

A total of 48 aborted bovine foetuses and 5 dead calves (<1 month) were obtained from 50 farms. At the moment of collection, blood samples were also obtained from the mothers, and the date of the last insemination was noted to enable calculation of the age of each foetus. The brains were extracted under sterile conditions and used for isolation of *N. caninum* according to the procedure of Canada et al. (2002). Tachyzoites were identified as *N. caninum* when reacted with anti-*N. caninum* antibodies but not with anti-*T. gondii* antibodies using an IFAT.

Statistical analysis

The degree of concordance among the three analytical techniques applied was evaluated by calculation of the Kappa value (Thrusfield 1995).

To establish the risk factors associated with infection, a univariate logistic regression was carried out, in which the dependent variable was seropositivity to *N. caninum* and the independent variables were the age of the animals and the presence of anti-*T. gondii* antibodies. The same analysis was used to determine the risk of abortion associated with the presence anti-*N. caninum* antibodies. To establish the association between the size of the farm or the presence of dogs and the level of seroprevalence, a Spearman's correlation test and a Wilcoxon's test, respectively, were used.

All analyses were carried out with SAS for Windows (version 5.1. 2006, 2007). Results were considered statistically significant at $P<0.05$.

Results

Comparison between NAT and indirect ELISA

The first 660 serum samples collected for the transverse study were analysed in parallel by NAT and ELISA. The degree of concordance between the results of the two techniques was high (kappa=0.91); however, analysis of the discordant samples by IFAT revealed a low concordance between this

Table 1 Percentage of seropositive herds in the four provinces in Galicia

Province	Number of farms investigated	Prevalence of infection (%)
A Coruña	133	79
Lugo	98	80.6
Ourense	15	80.0
Pontevedra	30	76.7
Galicia	276	79.3

technique and the other two techniques (kappa=0.476 and 0.25, respectively).

Risk factors associated with seroprevalence of *N. caninum*

Analysis by NAT showed that 15.8% (822/5,196) of the animals and 81.5% (225/276) of the herds sampled were positive. In the case of farms with only one seropositive animal (13.8%), a second analysis was carried out by ELISA, and animals were only considered infected if seropositivity was confirmed by both techniques. After the second analysis, the prevalence per animal and per herd was 15.7 and 79.3%, respectively. There were no differences among the four provinces in terms of the percentage of infected herds (Table 1). On seropositive farms, the within-herd prevalence ranged between 1.2 and 69.2%, with a predominance of herds with prevalence of infection of less than 40% (Fig. 1).

Seroprevalence of neosporosis was significantly higher ($P<0.001$) in cows (16.7%) than in heifers (12.3%), and the probability of seropositivity was 1.5 times higher (CI 95%, 1.2–1.8, $P<0.001$) in older animals.

Other factors related to seroprevalence of *N. caninum* in the herds were the size of the farm and the presence of dogs. There was, therefore, a negative and statistically significant correlation ($P<0.05$) between the number of cattle and seroprevalence. Mean seroprevalence was also
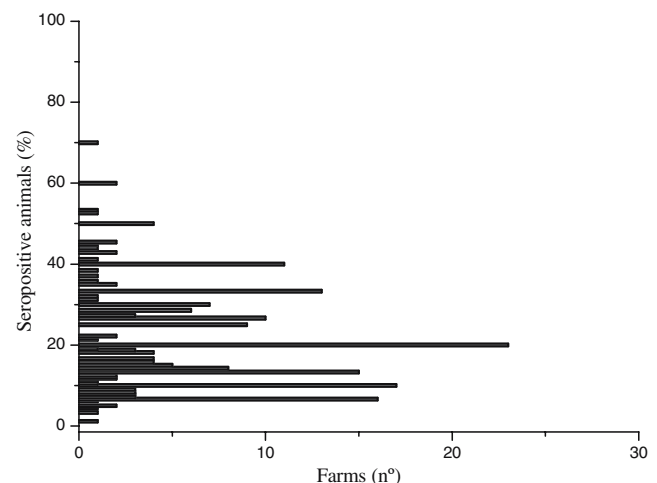

Fig. 1 Within-herd prevalence of infection in dairy herds in Galicia

higher ($P<0.05$) on farms that had dogs. However, the presence of the definitive host did not significantly increase the risk of seropositivity in the herd.

When the presence of other protozoans was taken into consideration, it was found that the probability of infection by *N. caninum* was 6.1 times higher (CI 95%, 4.2–8.9; $P<0.0001$) in animals infected with *T. gondii*. However, the seroprevalence of this parasite on Galician farms was very low (1.6%).

The reproductive history of 283 cows was recorded during the study (85 seropositive and 198 seronegative). When the presence/absence of anti-*N. caninum* antibodies was related to the appearance of abortions, it was found that 70% of abortions occurred in seropositive cows and that the risk of abortion in these cows was 5.3 times higher (CI 95%, 3.1–9.1; $P<0.0001$) than in seronegative cows. Tachyzoites of *N. caninum* were isolated from four foetuses (4–6 months) and one stillborn calf (1 month). Surprisingly, one of these isolates was obtained from the brain tissue of a foetus from a cow identified as seronegative by NAT, ELISA and IFAT.

Dynamics of antibodies in chronically infected cows

To study the dynamics of antibodies, four farms classified as seropositive (Farms 1–4) and one as seronegative (Farm 5) were selected, and serial samples were collected from cows (as indicated in "Experimental design") and analysed by ELISA. There were no significant variations in seroprevalence throughout the year on any of the farms (Fig. 2). The study of individual data revealed that, in 97.3% of the cows (108/111), the antibody titres generally remained positive or negative throughout the sampling, except in samples from three cows (2.7%).

As regards the change in the levels of antibodies, in the 27 seropositive cows in which the period of collection of samples and gestation coincided, there was only a signif-
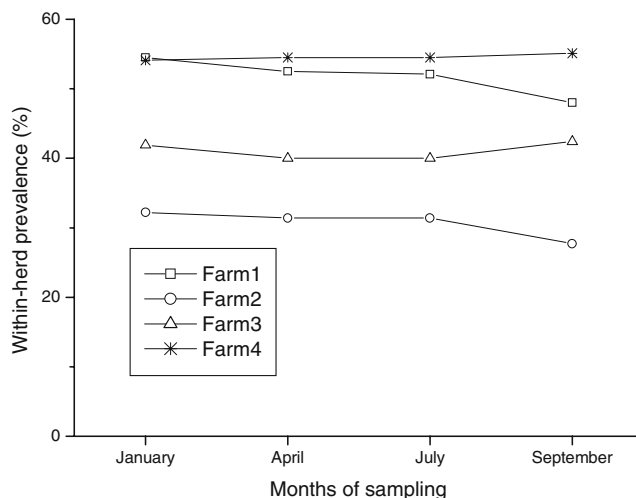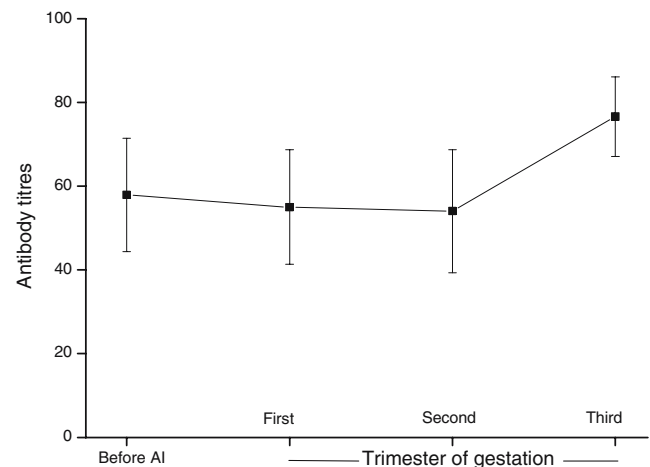

Fig. 3 Dynamics of anti-*N. caninum* antibodies in dairy cows

icant increase in antibody levels ($P<0.05$) in the third trimester of pregnancy (Fig. 3).

For 47 cows (24 seronegative and 23 seropositive), information was available about the dates that they gave birth in the year before sampling, which enabled calculation of the interval between births. The mean interval was significantly higher in seropositive cows (478 days compared with 396 days; $P<0.05$).

Furthermore, the genealogical study allowed identification of 67 cows of more than 8 months old, with progenitors on the same farm, and of known serology (35 seropositive and 32 seronegative). Some 97.1% of the positive cows had seropositive mothers, and 90.6% of the seronegative cows had seronegative mothers. Three cows with neosporosis transmitted the disease to three to four descendents in two to three successive gestations.

Finally, on the four seropositive farms, serological analyses revealed the presence of anti-*N. caninum* antibodies in two dogs on the same farm; all other dogs tested were seronegative. *N. caninum* oocysts were not detected in the faeces of any of the dogs.

Discussion

At present, there are several serological techniques that allow determination of the status of infection by *N. caninum* in live animals (Von Blumröder et al. 2004; Wapenaar et al. 2007), although there is no reference technique available. In the present study, comparison was made of two techniques, one based on the use of whole tachyzoites (NAT) and the other on the use of a soluble extract of tachyzoites (ELISA-CIVTEST®), and a high concordance between the two techniques was found, which indicates that they could be used interchangeably for the analysis of bovine livestock. However, there are differences in the techniques that would determine the choice of which

Fig. 2 Within-herd prevalence on the four seropositive farms

to use: The direct agglutination test allows analysis of samples from different species, with the same reagents, but does not allow quantification of the levels of antibodies; the ELISA is a quantitative technique that requires different reagents for each animal species. Therefore, the type of study (detection of infection or dynamics of antibodies), the species of animal analysed, the costs involved and the availability of the tests all determine the method chosen. Several authors have observed a high degree of concordance between the IFAT and NAT (Packham et al. 1998) and between IFAT and ELISA (Von Blumröder et al. 2004). However, in the present study, analysis by IFAT of the samples that had shown different results by NAT and ELISA ($n=27$) showed very low levels of concordance, and it was not therefore possible to confirm the positivity or negativity of the discordant samples (4%).

In the present study, to estimate the seroprevalence of bovine neosporosis, the direct agglutination technique was chosen. It was observed that the percentage of infected herds was high (79.3%), although there was a predominance of farms with moderate within-herd prevalences (<40%), and low individual seroprevalence was estimated (15.7%). Other studies of dairy cattle in NW Spain estimated higher individual seroprevalences (30.6 and 35.9%), possibly due to the fact that selection of the herds was based on the history of abortions (Quintanilla-Gozalo et al. 1999; Mainar-Jaime et al. 1999).

In a study carried out by Bartels et al. (2006) in four European countries, the estimates of individual and intra-herd prevalences were similar to those obtained in the present study. However, these authors detected a lower percentage (63.0%) of infected herds, which may be due to differences in sampling, as only small herds and animals more than 2 years old were analysed. However, the size of Galician dairy herds is currently increasing, and as there are no official programmes for prevention and control of bovine neosporosis in replacement livestock, it is also possible that the present results reflect the increase in number of infected herds in the period between the two studies (1999–2005).

Several features of the biological cycle of N. caninum still remain unknown (Pérez-Zaballos et al. 2005; Dubey et al. 2006), and to enable reasonable control measures to be established, it is essential to identify the risk factors associated with the epidemiology of this parasitic infection in different areas. The present study revealed a higher percentage of seropositive animals in older animals and that the mean prevalence of infection in herds was significantly higher in farms that had dogs. However, the presence of the definitive host was not a determining factor in the appearance of infection. Although coprological and serological studies were carried out on the dogs on the four seropositive farms, the small number of samples analysed and the difficulty in detecting the infection in these animals

(Schares et al. 2005) meant that we were unable to reach any conclusions about the importance of the definitive host in the transmission of infection. Furthermore, seroconversion was not observed in the cattle on these farms.

As regards the size of the farm, it was found that seroprevalence increased as the number of animals in the herd decreased. This finding is consistent with the results of the study carried out in Spain by Bartels et al. (2006), who reported that, on the small farms (<20 animals) that predominate in the province of Pontevedra, the risk of seropositivity was higher than on farms in the other provinces studied. The lower prevalence of the parasitic infection on larger, more specialised farms can probably be explained by better hygiene-sanitary conditions and handling practices.

Higher susceptibility to infection by N. caninum in cattle infected by T. gondii has not been demonstrated. The low seroprevalence (1.6%) of T. gondii observed in this work was similar to that obtained by Yu et al. (2007), also in dairy cattle. However, in contrast to the latter in which co-infection with the two protozoans was not reported, in the present study, it was found that animals infected with T. gondii showed higher seroprevalence of N. caninum. Although N. caninum is closely related to T. gondii and other Apicomplexan, cross-reactivity has not been a major issue (Dubey 2003), and the specificity of surface antigens has been previously confirmed (Dubey et al. 1996). The association observed in the present study, described for the first time in Spain, may be due to an increase in susceptibility to infection with N. caninum, or to deficient handling conditions that favour transmission of both of these parasitic infections (contact with definitive hosts, contamination of water and food, inadequate storage of fodder).

Furthermore, reproductive disorders cause important economic losses on dairy farms. Previous studies have shown an association between infection by N. caninum and the occurrence of abortions (Dubey et al. 1997; Paré et al. 1997; Mainar-Jaime et al. 1999; Anderson et al. 2000; López-Gatius et al. 2004a, b). In the present study, comparison between cows that were seropositive and seronegative for N. caninum demonstrated that the risk of abortion was 5.3 times higher in the former than in the latter. Moreover, tachyzoites of N. caninum were isolated in 5/53 (9.4%) samples of brain tissue from four foetuses and one stillborn calf, and surprisingly one of these samples was from a foetus from a seronegative cow. This questions the validity of the protocols used by several veterinary diagnostic laboratories, in which only those foetuses from cows that have been shown to have anti-N. caninum antibodies are analysed (by histochemistry or polymerase chain reaction) to detect the presence of the parasite.

The antibody response induced by N. caninum may persist throughout the entire life of an animal. However, there are fluctuations in the levels of antibodies that

occasionally fall below the limits of detection of serological techniques (Dubey and Schares 2006). The study of the dynamics of antibodies allows better interpretation of the serological analyses. In the present study, there were no significant variations in the seroprevalence of neosporosis on the five farms during the period of the study. The levels of antibodies in seropositive cows remained constant until the third trimester of pregnancy, when there was a significant increase in the levels. Several authors have related this increase to the birth of congenitally infected calves (Paré et al. 1997; Quintanilla-Gozalo et al. 2000). The close association between the serological status of mothers and daughters observed in the present study also demonstrates the importance of the congenital route of transmission of neosporosis in cattle herds. The increase in the levels of specific antibodies may be the result of the recrudescence of latent infection during gestation.

It was also found that there was a significant increase in the interval between births in seropositive cows. Infection by *N. caninum* has not been shown to produce a decrease in fertility (López-Gatius et al. 2005), and to date, abortion is the only clinical symptom described in adult cows, which suggests that absorptions of the embryo probably occur (Williams et al. 2000).

In conclusion, in light of the wide distribution of bovine neosporosis in Galicia, official control programmes should be established to include: (1) serological analysis of new cows used to restock a farm, and annually of the whole herd; (2) sacrifice of seropositive animals on farms with low seroprevalence; (3) avoidance of restocking with daughters of seropositive cows; (4) establishment of safety measures to improve farm installations and the storage conditions for water and foodstuff. López-Gatius et al. (2005) recommended that, on farms with high prevalence of infection, seropositive cows should be inseminated with semen from beef cattle.

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