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Serum antibody profile and reproductive performance during two consecutive pregnancies of cows naturally infected with *Neospora caninum*

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Abstract

The objective of this study was to record how the antibody levels change over time during pregnancy in dairy cows naturally infected with the protozoan parasite *Neospora caninum*, and relate this to the reproductive performance. Eighteen cows with antibodies to *N. caninum* were serum sampled monthly during their first pregnancy and 13 of them were also followed for a second pregnancy. In all, five pregnancies ended in abortion and two in stillbirth. Antibodies to *N. caninum* in serum were demonstrated by immune stimulating complex enzyme-linked immunosorbent assay (iscom ELISA). The *N. caninum* antibody titres remained well above the 1 : 100 cut-off limit for the test used during 2 years in all cows. In the non-aborting cows, mean values of antibody titres to *N. caninum* rose 1.5–2.5 dilution steps to reach a plateau 4–5 months before parturition, and thereafter decreased from 2 months before parturition. These changes were statistically significant ($p < 0.001$). The same pattern was seen in the aborting cows. The consistent pattern of rise in antibody titres observed during both pregnancies in all cows indicated a reactivation rather than a reinfection of the parasite at mid-gestation. ©1999 Elsevier Science B.V. All rights reserved.

Keywords: *Neospora caninum*; Cattle — Protozoa; Antibodies; Pregnancy; Time; Reproductive performance

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1. Introduction

The cyst-forming coccidian parasite *Neospora caninum* is associated with bovine congenital infection and abortion in many parts of the world (Anderson et al., 1991; Thornton et al., 1991; Holmdahl et al., 1995; Dubey and Lindsay, 1996). *N. caninum* related abortions can occur throughout pregnancy, but a majority of them occur in mid to late gestation (Anderson et al., 1995; Otter, 1997). The pathogenesis of *N. caninum* induced abortion is not fully understood. The dog was recently recognised as the definitive host of *N. caninum* (McAllister et al., 1998). Yet, all aspects regarding transmission of the parasite to the foetus are not known (Dubey and Lindsay, 1996). Unlike the closely related parasite *Toxoplasma gondii*, *N. caninum* can be transmitted from the dam to its progeny at several consecutive pregnancies (Björkman et al., 1996).

Presence of antibodies to *N. caninum* in serum is indicative of infection. *N. caninum* antibodies in bovine blood can be demonstrated by serological assays such as the indirect fluorescent antibody test (IFAT) (Dubey et al., 1988), direct agglutination test (Romand et al., 1998), or enzyme linked immunosorbent assay (ELISA)(Björkman et al., 1997; Dubey et al., 1997). Some authors have found fluctuating *N. caninum* antibody levels during pregnancy in naturally infected cows, occasionally even with values below the cut-off for the tests used (Conrad et al., 1993; Dannatt, 1997). Variations in *Neospora* IFAT titres during pregnancy were observed in naturally infected cows, whose titres rose to peak levels around 6–8 months of gestation (Paré et al., 1997). This increase in antibody titres may reflect either a reinfection or a reactivation of *Neospora* parasites encysted in the dam's tissues after initial infection.

Our aim was to characterise how the antibody levels change over time during pregnancy in cattle naturally infected with *N. caninum* and relate this to the reproductive performance. Therefore, in a long-term study, antibody activity was measured in serum samples collected monthly during the first two pregnancies of a group of *N. caninum* seropositive cows.

2. Material and methods

2.1. Animals

Eighteen cows from a Swedish Red and White breed dairy herd in which *N. caninum* had been isolated from a stillborn calf (Stenlund et al., 1997) were included in this study. The animals were as heifers reared for dairy replacement, and were artificially inseminated for each pregnancy. When examined at 13–15 months of age they all had antibodies to *N. caninum* as determined by ELISA (Björkman et al., 1997). The herd was declared free from bovine virus diarrhoea virus (BVDV) infection, the main recognised cause of bovine abortion in Sweden.

2.2. Blood sampling

Blood samples were collected from the 18 animals every month from 1 month before the first insemination until 1 month after the second parturition or until culling. Fourteen

heifer calves were blood sampled twice, at 6 and 18 months of age. Coccygeal or jugular vein blood was collected in plain vacutainer tubes and was sent by mail to our laboratory. After centrifugation at 1000 × *g* for 15 min, serum was removed and stored at −20°C until analysis.

2.3. Serological analyses

All serum samples were diluted 1 : 100 in phosphate buffered saline, pH 7.4 with 0.05% Tween-20 (PBST) and analysed for presence of IgG_1 antibodies to *N. caninum* by the iscom ELISA described by Björkman et al. (1997). Sera with absorbance values above the cut off level 0.20 absorbance units were subsequently diluted in PBST in two-fold serial dilutions, from 1 : 100 to 1 : 25,600 and re-analysed. The highest dilution in which the absorbance was above the cut-off level was given as titre for the sample.

In order to find out if the rise in *N. caninum* antibody titres reflected a rise in total IgG_1 antibody levels, all samples from the first pregnancy of six cows were analysed for presence of IgG_1 antibodies using a radial immunodiffusion test kit (Binding Site, Birmingham, UK) (Mancini and Vaerman, 1964).

2.4. Data collection

Data regarding animal identities and dates for insemination, and calving or abortion were obtained from the Milk Recording Service of the Swedish Association for Livestock Breeding and Production, and from the farm records. Abortion was defined as premature parturition when occurring between days 42 and 260 of gestation (Anonymous, 1972).

2.5. Data processing and statistics

For the statistical calculation the pregnancies were divided into 28-day months, i.e. 10 months ±10 days for a full term pregnancy. The sampling dates were related to calving dates, where samples from 14 days before to 14 days after calving were regarded as collected at calving (month zero), samples from between 15 and 42 days before calving regarded as collected 1 month before calving, etc. Consequently, in some cases there were no results for a particular month.

The means of *N. caninum* reciprocal titres were calculated for each month for all full term pregnancies. Effects of time (month before or after parturition) and pregnancy (first or second) on titre level were analysed by analysis of covariance (ANCOVA), using a general linear model (because the model was unbalanced). Time (13 months) and cow identity (18 cows) were the independent variables in the model, pregnancy was the co-variable, and titre (7 classes) was the dependent variable. Homogeneity of the variances and outlying values were tested for by plotting the residuals, and no violations of the ANCOVA assumptions were detected. The means of total IgG_1 levels were calculated for each month for the pregnancies of six cows.

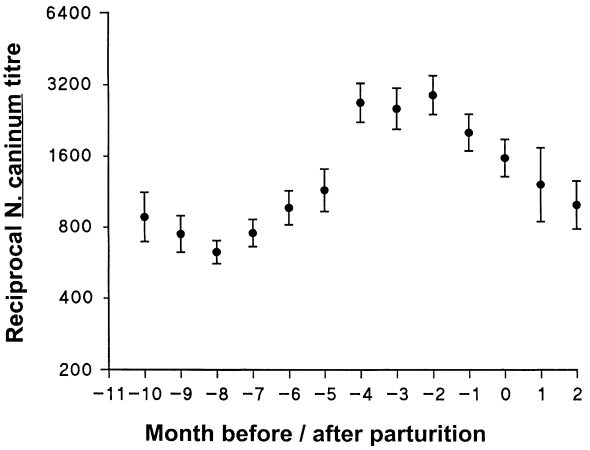

Fig. 1. Observed mean and 95% confidence interval of *N. caninum* antibody titre classes (averaged across both first and second pregnancy) for the 26 completed pregnancies of 18 cows.

3. Results

3.1. Serum antibodies

Screening of the samples for *N. caninum* antibodies by the iscom ELISA at 1 : 100 dilution revealed that all the cows had absorbance values above 0.4 at each sampling. The mean absorbance values of the non-aborting cows followed a uniform pattern during both pregnancies, with a rise 4–5 months before parturition followed by a decrease before parturition (results not shown). The mean antibody titres also showed the same pattern during both pregnancies. An increase of 1.5–2.5 dilution steps was seen 4–5 months before parturition, followed by a decrease from 2 months before parturition (Fig. 1). These changes in titre levels over time were significant ($p < 0.001$) during both pregnancies, and there was no significant difference in titres between the two pregnancies.

A similar pattern of *N. caninum* antibody titres with a peak 4–5 months before parturition was also seen in the pregnancies that ended in abortion. However, high titres (1 : 12,800) were observed more frequently in the pregnancies ending in abortion, 3 out of 5, than in the normal pregnancies, 2 out of 22 (Table 1).

In the six cows for which total IgG_1 was analysed, the mean total IgG_1 concentration varied between 8 and 17 g/l and tended to decrease during the course of pregnancy. The 14 heifer calves born to the cows studied were found seropositive to *N. caninum* at both 6 and 18 months of age.

3.2. Reproductive performance

The mean number of inseminations was 1.7 and 2.2 for the first and second pregnancy, respectively. Eighteen first, and 13 second, pregnancies were diagnosed during the observa-

Table 1
Number of inseminations, pregnancy outcome and maximal *N. caninum* titre for 18 cows studied during one and two pregnancies

Cow No	No. of inseminations		Outcome[a]		Max. reciprocal titre	
	Pregnancy 1	Pregnancy 2	Pregnancy 1	Pregnancy 2	Pregnancy 1	Pregnancy 2
555	1	1	N	N	6400	3200
556	1	2	A (8)	N	12800	6400
557	3	2	A (7)	N	12800	6400
558	3	2	N	N	6400	12800
559	1	3	N	–	3200	–
560	1	–	S	–	6400	–
561	1	3	N	A (5)	3200	3200
562	3	3	N	A (7)	6400	12800
563	1	1	N	N	1600	1600
564	3	3	N	N	6400	6400
565	3	4	N	N	3200	3200
566	1	–	N	–	6400	–
569	4	4	N	S	3200	3200
570	1	–	N	–	6400	–
572	1	1	N	N	3200	6400
573	1	1	A (5)	N	6400	12800
574	1	1	N	N	6400	6400
575	1	–	N	–	800	–

[a] N: Normal; A: Abortion (month); S: Stillbirth.

tion period. Seven (23%) of the pregnancies ended in abortion or stillbirth (Table 1). Three of the cows aborted their foetuses during their first pregnancy and 2 during their second pregnancy. Three abortions occurred earlier than 2.5 months before expected parturition, and 2 between 1.5 and 2.5 months before expected parturition. One full term calf was stillborn in a first pregnancy, and one in a second pregnancy.

4. Discussion

In this investigation, a group of cows naturally infected with *N. caninum* were monitored during their first two pregnancies to study the kinetics of serum antibodies to the parasite. The antibody levels remained high in all cows throughout both pregnancies. The iscom ELISA absorbance values were never below 0.4 and were thus at all times well above the cut off absorbance value 0.2. This is consistent with a previous report of high titres maintained for 8–15 months in 10 naturally infected cows (Jenkins et al., 1997). In a British study, however, the antibody levels of 25% of 40 *N. caninum* seropositive animals in a herd of dairy cattle were at least once during a 12-month observation period below the detection limit for their test (Dannatt, 1997). It has been shown, though, that the ELISA system used by Dannatt (1997) has a lower sensitivity than many other ELISAs (Wouda et al., 1998).

Even though all animals remained seropositive throughout the two years, there was a statistically significant variation in antibody levels during pregnancy. The rise in mean titres 4–5 months before parturition (i.e. 5–6 months of gestation), and the later decrease, was

consistent during both pregnancies. Similar observations were done in two naturally infected cows monitored during one pregnancy (Conrad et al., 1993). Their *N. caninum* antibody titres increased to peak levels where they, however, remained until parturition. In another study, the antibody levels were found to fluctuate in 11 out of 24 *N. caninum* seropositive animals showing a peak at 6–7.5 months of gestation and then declining although still remaining positive (Dannatt, 1997). In our study, the *N. caninum* antibodies did not follow the same pattern as did the total IgG_1 antibodies in the six specifically studied cows. We therefore assume that the rise in *N. caninum* antibody titres was related to the parasite and not to a general increase of antibodies in serum.

The pregnancies in the current study were not coordinated in time, but rather spread over the year. Consequently the titre-increases occurred at different times of the year in different animals. This, and the fact that the same pattern was seen during two consecutive pregnancies, suggests that the rise was not an effect of reinfection. If that would be the case, the cows would need to be particularly susceptible to infection at mid-pregnancy, and, also, an external source of infection would have to be present in the herd throughout the year causing each animal to be constantly at risk. The consistent pattern in the antibody kinetics observed in all animals during both pregnancies in the present study thus suggests a mid-pregnancy reactivation of the parasite in chronically infected cows, rather than a reinfection.

Oestrogen concentrations in plasma, urine and faeces of healthy cows have been found to increase at approximately 4 months of gestation, with a second increase at 8 months (Hoffman et al., 1997). Several studies suggest that a rise in oestradiol concentration suppresses cell-mediated immunity and enhances the formation of systemic antibodies to infectious agents (Styrt and Sugarman, 1991). Hence, the increased antibody titres in our study could reflect either an increased release of parasites from the cells of the cows due to suppressed immunity, a hormone triggered antibody production, or both. Alternatively, the rise in antibody titres might reflect an antigenic stimulation created by the *N. caninum* infected foetus. It was not established whether all cows in this study carried infected foetuses, but this was likely since all the 14 calves that were tested were seropositive to *N. caninum*, and previous studies have shown that a majority of calves born to *N. caninum* seropositive cows are seropositive (Paré et al., 1997; Anderson et al., 1997). Further, high antibody levels at 8 months of gestation combined with an increase from 3 months of gestation has been suggested to predict a large risk of transmission of the parasite to the foetus in naturally infected cows (Paré et al., 1997). A parallel can be drawn to BVDV for which it has been suggested that the infected foetus stimulates the dam to produce antibodies. In one study, BVDV antibody titres in dams of congenitally BVDV-infected calves rose continuously until parturition (Lindberg et al., 1997), after which the antibody levels decreased. In the last trimester the antibody levels were significantly higher than those of the dams carrying non-infected foetuses (Lindberg, personal communication).

In our study, 7 (23%) out of 31 pregnancies ended in abortion or stillbirth. This is considerably higher than the 5% in Swedish dairy herds reported by the Swedish Association for Livestock Breeding and Production for 1996 (SHS Årsstatistik, 1995–1996) and the 10.5% abortion rate reported for normal Holstein dairy cows in the USA (Forar et al., 1996). Cows with high *N. caninum* titres have previously been shown to run a significantly higher risk of aborting than cows with low titres (Paré et al., 1997). Also, congenitally *N. caninum*

infected cows have been suggested to have a 7.4 times higher risk of abortion during their initial pregnancy than non-infected cows (Thurmond and Hietala, 1997). The mean number of inseminations for the second pregnancy of the current group of cows was 2.2 whereas the mean number of inseminations per pregnancy for dairy cows in Sweden for 1996 was 1.7 (SHS Årsstatistik, 1995–1996). These results indicate that also other effects of *N. caninum* infection than abortion and stillbirth may affect the reproduction in cattle latently infected with *N. caninum*.

To conclude, this study has shown that *N. caninum* antibody titres can remain at high levels for at least two years in naturally infected cattle. Further, the consistent pattern of elevated antibody titres observed in late pregnancy indicates a reactivation rather than a reinfection of the parasite at mid-pregnancy.

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