Serological Response over Time to Recombinant *Neospora caninum* Antigens in Cattle after a Neosporosis-Induced Abortion

M. C. JENKINS, W. WOUĐA, AND J. P. DUBEY
Parasite Biology and Epidemiology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705, and Gezondheidsdienst Voor Dieren, Locatie Drachten, 9200 AJ Drachten, The Netherlands

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Recombinant *Neospora caninum* tachyzoite antigens were evaluated in an enzyme-linked immunosorbent assay (ELISA) for recognition by serum antibodies (Ab) from *Neospora*-infected cattle. Serum samples were obtained every 2 to 3 weeks for 8 to 15 months from 10 cows with histories of *Neospora*-associated abortion. Serum samples were also obtained from offspring of these animals and from a large number of cows that had aborted a fetus, due to infection by *Neospora* or other organisms, at various times during gestation. All 10 cows had positive ELISA Ab titers to both recombinant *N. caninum* tachyzoite antigens after abortion, during subsequent gestation, and after parturition. In three cows, there was a noticeable peak in Ab titers early in gestation. Calves born to *Neospora*-infected cows also had positive titers of Ab to the recombinant tachyzoite antigens, and these titers remained elevated for at least 4 months after birth. A portion of the serum immunoglobulin in calves may have been derived from colostrum of infected cows. A calf born from a seronegative mother had a positive ELISA titer only after being fed colostrum from a seropositive cow. However, precolostral titers in calves born from *Neospora*-infected cows were high at birth, suggesting that the parasite was transmitted to the fetus via the placenta and induced a humoral immune response therein. The recombinant tachyzoite antigens were also useful for corroborating clinical diagnoses of *Neospora*-induced abortion. A significant difference (*P < 0.05*) between anti-recombinant antigen Ab titers in cows that aborted due to *Neospora* and those in cows that aborted from other causes was found.

Neosporosis is a parasitic disease that is a major cause of abortion in cattle (1–3, 15, 17). The causative organism, *Neospora caninum*, is a protozoan similar in many respects to *Toxoplasma gondii* (9, 10, 13). Although a definitive host of the parasite has not yet been identified, congenital infection is an important route in *Neospora* transmission (1–5). Cows which aborted during a previous pregnancy due to neosporosis can abort again, or they can give birth to diseased calves or calves that either are not infected or have a subclinical *Neospora* infection (6).

Our laboratory has developed an enzyme-linked immunosorbent assay (ELISA) that uses recombinant *Neospora* tachyzoite antigens for identifying cows infected with *Neospora* and for corroborating clinical diagnoses of neosporosis (12). These recombinant antigens proved to be useful for detecting antibodies (Ab) to the parasite in both experimental and natural *Neospora* infections (12). Previous studies were limited by the testing of sera from only one time point in a group of cows that aborted because of a natural *Neospora* infection. The present study describes the use of the recombinant-antigen-based ELISA to monitor natural *Neospora* infections in 10 cows and their offspring for various lengths of time. Further, the diagnostic value of the test was determined by comparing postabortion sera from 70 cows which had aborted a *Neospora*-infected fetus with sera from 30 cows which had aborted due to infections with other organisms or unknown causes.

**MATERIALS AND METHODS**

**Sera.** Venous blood was obtained from 10 cows in nine dairy herds from The Netherlands every 2 to 3 weeks for 8 to 15 months, after confirmed *Neospora*-induced abortions. In each abortion case, the diagnosis of neosporosis was confirmed by immunohistochemical analysis (IHC) by identifying *N. caninum* in fetal tissues (18). Three cows (cows 1, 2, and 3) had aborted twice during successive gestations, before serial bleeding was started. A *Neospora* infection was diagnosed in both fetuses from cow 2. The first aborted fetuses from cows 1 and 3 were not examined. Eight cows were rebred successfully after abortion. Seven cows delivered full-term healthy calves after uncomplicated pregnancies. The calf from cow 1 was stillborn. Cow 2 was inseminated three times but remained empty; cow 7 was not rebred. A blood sample was obtained before colostrum feeding from each of the seven liveborn calves and from the stillborn calf. Sequential blood samples were taken from several calves after colostrum feeding as long as the calves were available. Sequential blood samples were also obtained from one calf born to a seronegative dam. This calf was fed colostrum from a seropositive cow (cow 3).

Venous blood samples were also obtained at a single time point from 100 cows from dairy herds in The Netherlands experiencing sporadic or multiple abortions. A blood sample was taken from each dam within 1 week after diagnostic evaluation of its aborted fetus; 70 cases in which a *Neospora* infection was diagnosed, 15 cases in which other pathogens were identified, and 15 cases in which no pathogenic agent was detected were selected. Other organisms detected were *Actinomyces pyogenes* (n = 9), *Listeria* (n = 2), *Proteus* (n = 1), *Chlamydia* (n = 1), *Escherichia coli* (n = 2), and bovine herpesvirus (n = 1). As in the procedure described above, the diagnosis of neosporosis-associated abortion was confirmed by IHC (18). Sera were collected from blood samples and stored at −70°C.

**ELISA.** Serum anti-*Neospora* Ab titers were measured by a recombinant-antigen-based ELISA method as described previously (12), with a few modifications. Recombinant Ne4.1 and Ne14.1 antigens were expressed as polyhistidine fusion proteins in *E. coli* and purified by passage through a Ni-nitrilotriacetic acid column as described previously (12). Both recombinant tachyzoite antigens were adsorbed to the surfaces of enzyme immunoassay-radioimmunoassay microtiter plates (Costar, Cambridge, Mass.) at 50 ng per well in 0.05 M sodium carbonate (pH 9.5) buffer for 1 h at 37°C followed by overnight incubation at 4°C. Recombinant antigens not adhering to the plate surface were removed by two washes with phosphate-buffered saline (PBS). Nonspecific Ab binding in subsequent steps was minimized by treating the wells with PBS containing 1% bovine serum albumin. In the primary ELISA, experimental and negative-control bovine sera were assayed at a 1:200 dilution. A *Neospora* positive-control sample was prepared by pooling sera from a similar herd of cows that had a high incidence of neosporosis-induced abortion. The positive-control sample was assayed at serial twofold dilutions from 1:200 to 1:2,560. All serum dilutions and ELISA plate washings were performed with PBS containing 0.05% Tween 20. Immunoglob-
ulinf binding was assessed with peroxidase-labeled anti-bovine immunoglobulin G (heavy- plus light-chain specific; Kirkegaard & Perry, Inc., Gaithersburg, Md.) followed by 0.1-mg/ml 0-phenylenediamine-0.01% H$_2$O$_2$ substrate. Color development was stopped after 10 min with 1% H$_2$SO$_4$. The readings were read with a microtiter plate reader at optical densities at 492 nm (OD$_{492}$), and blank readings (PBS controls) were subtracted from all OD$_{492}$ values. By using a previously described procedure (11), serum titers for all natural-infection sera were estimated from the linear regression of the positive-control OD$_{492}$ values versus serum dilutions. Serum samples from Neospora-infected cows that produced an OD$_{492}$ value greater than the positive control were applied at a higher dilution and, if necessary, at higher dilutions until the OD$_{492}$ value was within the linear range of the curve generated from serial dilutions of the positive control. This ELISA method was chosen because it allows estimation of serum titers relative to a positive-control sample obtained from a pool of sera from a similar population of Neospora-infected cows. The ELISA is designed so that the amount of antigen in each well is never limiting (i.e., a lower dilution of serum will produce a greater OD value). IFA. Indirect immunofluorescence assays (IFA) with whole N. caninum tachyzoites were performed on a number of sera for the purpose of validating the recombinant-antigen-based ELISA (7). Multwell microscope slides containing culture-derived air-dried Neospora tachyzoites were obtained from a commercial source (Veterinary Medical Research Diagnostics Inc., Pulman, Wash.). Initial, midpoint, and final sera from each of the 10 cows that were bled after abortion for 8 to 15 months were assayed by IFA at a dilution of 1:400 to 1:12,800 by using standard procedures (7). In addition, all 100 sera from the other cows that aborted a fetus were assayed at a 1:200 dilution. All dilutions of sera and washings were performed with PBS. Ab binding was assessed by secondary staining with fluorescein isothiocyanate-conjugated anti-bovine immunoglobulin G (heavy- plus light-chain specific; Kirkegaard & Perry, Inc.). IFA reactions were scored between 0 and +4 with an epiphrenoscope microscope.

**Statistical analyses.** Means and standard deviations of titers of Ab to recombinant Neospora antigen for groups of animals were determined by Kruskal-Wallis one-way analysis of variance on ranks by using the SigmaStat program package (Jandel Scientific Inc., San Rafael, Calif.). The ELISA data from the 100 individual sera did not follow a normal distribution, thus requiring the use of a nonparametric statistical comparison. Correlation coefficients between titers of anti-Neospora Ab to both recombinant antigens were calculated by Spearman rank order correlation by using the SigmaStat program package.

**RESULTS**

**Serological response to Neospora infection over time.** All cows with confirmed neosporosis-induced abortion had prolonged positive titers of Ab to both recombinant tachyzoite antigens (Fig. 1). The sera that were from cows which had had neosporosis-induced abortions and that had a positive ELISA titer (>1,000) were also positive by IFA at a serum dilution of 1:200 (data not shown). Anti-Neospora tachyzoite IFA titers ranged between 3,200 and 12,800 for these animals. For most cows, the titers of Abs to both recombinant antigens were similar at each time point (Fig. 1B to F and H to J). For three cows, there appeared to be an increase in anti-recombinant-tachyzoite Ab titer early in gestation followed by a second increase late in gestation (Fig. 1H to J). Two cows had a slowly increasing Ab titer during late gestation (cows 4 and 5). In one pregnant cow (cow 6) and in the two nonpregnant animals (cows 2 and 7), Ab titers were between 1,000 and 10,000 during the entire sampling period.

Six of seven live-born calves from seropositive dams had positive precolostral ELISA Ab titers that were higher than the Ab titers in the dam (Fig. 1C [calf-1], D, E, and H to J). Also, serum anti-Neospora Ab titers remained positive in these five calves at all time points after colostrum feeding. A calf born from a Neospora-negative cow exhibited a positive anti-Neospora Ab titer only after being fed colostrum from a Neospora-positive cow (Fig. 1C [calf 2]). It appears that high titers of Ab to recombinant Nc4.1 and Nc14.1 antigens may not always be observed in calves born to Neospora-infected cows (Fig. 1F). Ab titers increased in some calves after being fed colostrum. The stillborn calf from cow 1 had a high Ab titer as well (Fig. 1A). On histological examination of this calf, a mild multifocal encephalitis was found and an N. caninum tissue cyst was identified in the brain by IHC (18).

**Anti-Neospora Ab titers in cows at abortion.** A high correlation ($r = 0.88$) was observed between titers of Ab to both recombinant antigens when sera from 100 cows that experienced an abortion were evaluated by ELISA. When these data were divided into groups based on cause of abortion, sera from cows which aborted due to Neospora had two- to sevenfold-greater ($P < 0.05$) mean anti-recombinant-tachyzoite Ab titers than sera from cows that aborted a fetus due to other causes (Fig. 2). No significant difference ($P > 0.05$) was observed in the mean titers of Ab against either recombinant antigen among groups of cows that aborted due to causes other than Neospora infection.

For comparison, all 100 sera assayed by ELISA were also assayed by IFA at a 1:200 dilution. Except for two sera, there was complete agreement between ELISA and IFA results; sera positive by ELISA (cutoff value $= 1,000$) were positive by IFA (at least $+1$ at a 1:200 dilution). Two sera had high titers of Ab to both recombinant antigens but were negative by IFA. Three of the abortion cases for which no cause was found yielded positive Ab titers by IFA and by ELISA.

**DISCUSSION**

The results demonstrate that cows which had aborted a fetus with confirmed neosporosis had prolonged positive titers of Ab to recombinant Neospora tachyzoite antigens. Positive serum Ab titers were observed during the gestation and after the birth of a clinically normal calf. This observation is consistent with the observed serological responses of sheep and goats to the related protozoan T. gondii (8). The positive anti-Neospora Ab titers may be due to a number of infection parameters, including continual release of antigens from tissue cyst stages and/or recrudescence of a latent maternal infection (5). The prolonged responses to both recombinant tachyzoite antigens in natural Neospora infections differ from those observed in previous studies of experimental neosporosis (12). Titers in sera in experimental infections returned to background levels by about 3 months post-Neospora inoculation, which suggests that the N. caninum infection was short lived in that experiment. In the present study, three of the cows (cows 1, 2, and 3) aborted twice. It appears that for at least one cow (cow 2), Neospora was the causative agent in both abortions. Whether the subsequent unsuccessful breeding of cow 2 was related to Neospora infection is unknown. It is also unknown whether the first abortions for cows 1 and 3 were due to neosporosis since fetal tissue was examined only for the second abortions.

With one exception (cow 6), calves born to Neospora-infected cows also had positive titers of Ab to both recombinant antigens. Our data indicate that serum Ab to Neospora in calves may arise both from a congenital infection and from receiving high-titer anti-Neospora colostrum. The negligible titers of Ab to both recombinant antigens in the calf from Neospora-infected cow 6 may indicate that Neospora is not always transmitted to the fetus during pregnancy. Previous studies have shown that not all calves born to Neospora-positive cows have high precolostral titers of Ab to the parasite at birth (16). Although most cows (7 of 10) in this study and in other studies (6) gave birth to clinically normal calves, two cows (cows 1 and 2) continued to have reproductive deficiencies. One animal (cow 2) aborted a fetus during the second pregnancy. Histological examination of brain from this fetus revealed lesions suggestive of Neospora infection. A stillborn calf delivered by cow 1 during the second pregnancy had high titers of Ab to both recombinant antigens in serum. These results indicate that cows which have a high serum Ab titer are capable of transmitting Neospora to the fetus via the placenta.
It is unknown why chronic neosporosis leads to fetal death in some cases (14, 18) and to subclinical (16) or clinical (5) congenital infection in other cases.

The recombinant Nc4.1 and Nc14.1 antigens appear to be useful for detecting Neospora-specific Ab in sera from Neospora-infected cows. The high correlation ($r = 0.88$) between ELISA titers of Ab to these two antigens indicates that related native tachyzoite antigens induce similar responses during a natural Neospora infection. Our recent immunogold labeling of tachyzoites with antisera against recombinant Nc4.1 and Nc14.1 proteins showed that both antigens are components of dense granules in N. caninum tachyzoites (unpublished observations). Based on the present study, the two recombinant antigens are now being combined in a single ELISA for estimating anti-Neospora Ab titers. The agreement between recombinant-antigen ELISA and whole-tachyzoite IFA indicates that the former is useful for detecting anti-Neospora Ab in infected cows. The use of recombinant Neospora antigens produced in E. coli eliminates the need to culture N. caninum tachyzoites for IFA or native-antigen ELISA. Extensive studies in our laboratory have failed to show any cross-reactivity of either recombinant antigen with other parasites (e.g., Sarcozystis and T. gondii) or with noneukaryotic organisms (e.g., E. coli and Salmonella) (12).

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**REFERENCES**


