Serodiagnosis of Neosporosis in Individual Cows and Dairy Herds: A Comparative Study of Three Enzyme-Linked Immunosorbent Assays

W. WOUDA,1* J. BRINKHOF,2 C. VAN MAANEN,2 A. L. W. DE GEE,1 AND A. R. MOEN1

Animal Health Service, 9200 AJ Drachten,1 and 7400 AA Deventer,2 The Netherlands

Received 11 March 1998/Returned for modification 12 May 1998/Accepted 23 June 1998

The performance of three enzyme-linked immunosorbent assays (ELISA) for detection of antibodies to Neospora caninum in bovine sera was evaluated by using various categories of sera. Two commercial ELISA methods, one based on chemically fixed intact tachyzoites and one based on a sonicate lysate of whole tachyzoites, were compared with an in-house ELISA based on a detergent lysate of whole tachyzoites. A brief description of the development of the latter ELISA is also given. There was good agreement among all three tests with regard to postabortion sera. By using acute-phase abortion sera from cows with confirmed N. caninum-induced and non-N. caninum-induced abortions, satisfactory levels of sensitivity and specificity were calculated for all tests. In addition, similar test results were obtained with postpartum samples from dams and calves. However, considerable differences were found between test results of sequential samples and cross-sectional and total-herd samples. Apparently, these discrepancies were due to different sensitivities of the tests for detection of low antibody levels in chronically infected animals. It is suggested that these differences were primarily due to the use of different antigens and different test sample dilutions. It is concluded that all tests are applicable as an additional diagnostic tool in cases of abortion in cattle and for monitoring of congenitally infected calves. For herd screening, the lysate-based ELISAs appear to be more adequate because of their higher sensitivities.

Neosporosis is a newly recognized protozoan disease. The etiological agent was first isolated from a paralyzed dog and was named Neospora caninum (11). Later it was discovered that Neospora-like protozoa may cause abortion in cattle (27). The bovine and the canine parasite were shown to be identical (16, 20). At present neosporosis is recognized as a major cause of bovine abortion throughout the world (for a review, see reference 13).

The diagnosis of the infection in aborted fetuses is primarily based on the characteristic histological lesions and the immunohistochemical identification of the parasites in fetal tissues (1, 2, 32).

The complete life cycle of N. caninum is not known. At present the only described mode of infection is transplacental, from cow to calf (3, 17, 25, 31). This vertical transmission may contribute significantly to the persistence of the infection in the herd (25, 31). Congenitally infected calves sporadically show neurological signs (3, 12) but usually are in good health (25). However, several studies indicate that chronically infected cows have an increased risk of abortion (21, 26, 29, 31). The only way to identify chronically infected animals is by detection of antibodies in the blood.

The immunofluorescent antibody test (IFAT) has been widely used to detect antibodies against N. caninum (9, 23) but has subsequently been superseded by enzyme-linked immunosorbent assays (ELISAs) (5, 7, 10, 14, 18, 19, 24, 30). Different antigens have been used in the various ELISAs: extracted tachyzoite membrane proteins incorporated into immunostimulating complexes (7), whole-tachyzoite lysate antigens (10, 14, 24), recombinant antigens (18, 19), and tachyzoite surface antigens made available by chemical fixation of whole tachyzoites onto the plates (30). A monoclonal antibody-based competitive inhibition ELISA was developed by Baszler et al. (5). Recently, Dubey et al. (12) evaluated IFAT and ELISA performance in various laboratories, using sera from a herd which had experienced an outbreak of N. caninum-induced abortions. They found considerable differences between different tests and concluded that no test could be used to establish definitively that N. caninum caused the abortion in an individual cow.

In this study, we compared three ELISA methods for detection of bovine antibodies to N. caninum, using various categories of sera. Two ELISAs, referred to as test A (MAST Diagnostics, Bootel, United Kingdom) and test B (IDEXX Laboratories, Westbrook, Maine), were obtained commercially, and each was performed according to the manufacturer’s instructions. The third ELISA (test C) was developed at the laboratory of the Animal Health Service (AHS). The aims of the study were (i) to determine the diagnostic relevance of the three ELISA methods in abortion cases in cattle and (ii) to evaluate their applicability for herd prevalence studies and monitoring of congenital infections.

MATERIALS AND METHODS

Commercial ELISA methods (tests A and B). Test A was described by Williams et al. (30). Test B was derived from the ELISA originally described by Paré et al. (24). Both methods were performed entirely according to the manufacturer’s instructions supplied with the kits. The recommended test sample dilutions (1:400 for test A and 1:100 for test B) were used. Positive and negative controls were provided with the kits (a high and a low positive for test A and a single positive for test B). Results were expressed as sample/positive control (S/P) ratios. For interpretation of the results, the cutoff values recommended by the manufacturers were used. The main characteristics of each ELISA are summarized in Table 1.

AHS ELISA (test C). (i) Antigen preparation. N. caninum (isolate NC1) tachyzoites were cultured on Vero cells. Monolayers were maintained in 75-cm² or 162-cm² tissue culture flasks at 37°C by using Hank’s minimal essential medium with l-glutamine and 5% inactivated horse serum and were passaged at 7-day intervals. The flasks were seeded with 10⁶ to 10⁷ tachyzoites. Infected cultures were inspected daily with an inverted microscope. When numerous free tachyzoites were seen in the medium and >80% of the monolayer was destroyed,
the remaining cells adhering to the plastic were scraped into the culture medium by means of a rubber policeman. The suspension was then homogenized by means of a Potter-Elvehjem device, followed by filtration through a 5-µm-pore-size filter. Tachyzoites were washed twice in sterile phosphate-buffered saline (PBS) by centrifugation (for 15 min at 1,000 × g). After removal of the supernatant, the resulting pellet was resuspended in PBS. Tachyzoite concentration (10^7 to 10^8 parasites per ml) was estimated by using a hemocytometer. Tachyzoites were then pelleted again through a 20% sucrose cushion in PBS for 1 h at 13,000 × g at 4°C. Tachyzoite pellets were suspended in PBS containing 1% (vol/vol) Triton X-100. After overnight incubation at 4°C, sodium azide was added to a final concentration of 0.025%, and the antigen preparation was aliquoted and stored at −20°C.

(ii) ELISA technique. Optimal ELISA concentrations for coating and conjugate concentration were determined by means of checkerboard titration. For coating, Nunc Polysorp microtiter plates were used. Determination of the cutoff value was based on the mean extinction plus 3 times the standard deviation for 50 bovine sera from herds with no history of *N. caninum* abortions and was further refined by using a frequency distribution of extinction values obtained by assaying *N. caninum*-infected herds and herds not suspected of having *N. caninum* infection. After assays at dilutions of 1:50, 1:100, 1:200 and 1:400, a dilution of 1:50 was chosen for test sera because optimal sensitivity was achieved at this dilution.

Possible cross-reactivity of the ELISA with related parasites was determined by using sera from calves experimentally infected with Toxoplasma gondii, *Sarcocystis cruzi*, Cryptosporidium parvum, *Babesia bovis*, *Babesia bigemina*, *Babesia divergens*, and *Eimeria alabamensis*. A transient response was found for a *B. bovis*-infected calf, which was sampled serially during 65 days postinfection. In one experiment, samples had been taken from the herd (herd 1; see below) immediately after the onset of the abortion storm. Cows 32 and 38 aborted 3 and 10 days, respectively, after the herd sampling and were monitored for 6 months. Four cows (cows 21, 37, 45, 93) which had abortions caused by *N. caninum* were identified in different herds in which abortions were sporadic. These four cows were monitored until calving. Cow 93, which was not reinseminated, was monitored for another 12 months postpartum.

(iii) Cross-sectional and total-herd samples. In two dairy herds (herds 1 and 2) with acute *N. caninum* abortion outbreaks, 20% of the animals (51 and 31 animals, respectively) were bled immediately after the onset of the outbreak. In three herds (herds 3 through 5) with histories of ongoing *N. caninum* abortions, suggesting endemic neoparasporosis, sera were collected from all animals (190, 115, and 72 animals, respectively). In one herd (herd 6) with no history of *N. caninum* abortions, sera were obtained from all 80 cows. The sera from this herd were used for the estimation of test specificity. The distribution of the S/P ratios of the postpartum sample set 7 were below the cutoff in test A but above the cutoff level of the test, but at 4 weeks postinfection it had decreased below this level. None of the other sera from these experimentally infected cats showed reactivity in the ELISA.

Categories of test sera. (i) Postabortion samples. Postabortion sera were collected from 59 dairy cows which aborted fetuses with histological evidence of *N. caninum* infection (immuno histochemically confirmed) and from 16 cows which aborted fetuses with no histological evidence of *N. caninum* infection and for which other agents were identified: *Actinomyces pyogenes* (*n* = 9), *Listeria monocytogenes* (*n* = 2), *Salmonella dublin* (*n* = 2), *Escherichia coli* (*n* = 1), *Chlamydia sp.* (*n* = 1), and bovine herpesvirus 1 (*n* = 1). All sera were obtained from dairy herds in the northern part of the Netherlands. Sera were collected within 14 days after the abortion. All fetuses had been submitted to the laboratory of the AHS and had been examined by using a standard protocol as described previously (32).

(ii) Postpartum samples. Precolostral blood samples were collected from 20 calves born to dams which had previously aborted *N. caninum*-infected fetuses. Samples were taken immediately after parturition and before nursing. In addition, blood and colostrum samples were taken from the dams.

(iii) Sequential calf samples. Sequential sera were taken from two calves (calves 1 and 2) which had high precostral antibody levels, indicating congenital infection, and from two calves (calves 3 and 4) that were seronegative at birth but received colostrum from seropositive dams. Sampling was started immediately after birth and was continued at 3-week intervals for 8 to 10 months.

(iv) Sequential samples from aborting cows. Sequential sera were taken at 3- to 4-week intervals from six cows which had aborted *N. caninum*-infected fetuses.

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**TABLE 1. Characteristics of three ELISA methods for detection of antibodies to *N. caninum* in bovine sera**

| Test | Producer | Antigen prep | Bovine antibody class detected | Sample dilution | S/P cutoff |
|------|----------|--------------|-------------------------------|----------------|-----------|
| A    | MAST     | Whole fixed tachyzoites | IgG                          | 1:400          | 25%*      |
| B    | IDEXX    | Sonicate tachyzoite lysate | IgG                          | 1:100          | 0.5       |
| C    | AHS      | Detergent tachyzoite lysate | All Ig                       | 1:50           | 0.7       |

* Samples with S/P ratios of 20 to 25% were considered suspect.

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**TABLE 2. Proportions of positive postabortion sera as determined with three different ELISAs**

| Etiology                 | No. of positive samples/no. tested by: |
|--------------------------|----------------------------------------|
|                          | Test A | Test B | Test C |
| *N. caninum*             | 52/59  | 58/59  | 58/59  |
| Other identified cause   | 0/16   | 2/16   | 0/16   |

* Sera were from 59 cows which aborted *N. caninum*-infected fetuses and from 16 cows aborting due to other identified causes.

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**TABLE 3. Proportions of positive postpartum samples taken postpartum from calves, cows, and colostrum, as determined with three different ELISAs for detection of antibodies to *N. caninum***

| Source                  | No. of positive samples/no. tested by: |
|-------------------------|----------------------------------------|
|                         | Test A | Test B | Test C |
| Calves                  | 13/20  | 14/20  | 14/20  |
| Cows                    | 13/20  | 14/20  | 14/20  |
| Colostrum               | 12/18  | 12/18  | 12/18  |

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**RESULTS**

**Postabortion samples.** The proportions of positive postabortion samples for each ELISA are presented in Table 2. One of the 59 cows in the *N. caninum* abortion group was negative in all three tests.

**Postpartum samples.** The proportions of positive postpartum samples for each ELISA are presented in Table 3. The distribution of the S/P ratios of the postpartum sample sets, as assessed with test A, is presented in Fig. 1. Similar distributions were obtained with tests B and C. The values for cow-and-calf sample set 7 were below the cutoff in test A but above the cutoff in tests B and C (data not shown). With all tests it was found that positive calves had positive dams and negative calves had negative dams.

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**FIG. 1. Precolostral *N. caninum* antibodies (ELISA test A) in 20 calves compared with serum and colostrum antibodies of their dams, which had histories of *N. caninum* abortions (colostrum samples 4 and 14 are missing).**
Sequential calf samples. Results obtained in each ELISA for sequential samples from two congenitally infected calves and two calves with colostrum-derived antibodies are presented in Fig. 2.

Sequential samples from cows which aborted N. caninum-infected fetuses. Results obtained in each ELISA for sequential samples from aborting cows are presented in Fig. 3 (data from two cows monitored for 6 months) and Fig. 4 (data from four cows monitored until calving or longer). Cows 21, 45, and 93 gave birth to calves which had high levels of precolostral antibodies to N. caninum, indicating congenital infection (data not shown), whereas cow 37 gave birth to a seronegative calf.

Cross-sectional and total-herd samples. Considerable differences were found between the various ELISAs when the cross-sectional and total-herd samples were tested (Table 4). In particular, the assessed seroprevalences of the three herds (herds 3 through 5) with endemic neosporosis showed great variations depending on which ELISA was used (Table 4). For all six herds, there was a high level of agreement between tests B and C ($\kappa = 0.83$). Agreement of test A with tests B and C was low ($\kappa = 0.39$ for test B and $\kappa = 0.34$ for test C; these values increased to 0.47 and 0.42, respectively, if the samples that were suspect with test A were included in the calculation).

**DISCUSSION**

Good agreement between the three ELISAs was found with postabortion sera, as shown in Table 2. Antibodies were detected in a high proportion of cows which had recently aborted N. caninum-infected fetuses. These results were similar to those obtained by IFAT by other researchers testing acute-phase abortion sera (22). Sensitivity and specificity estimates with the postabortion sera were satisfactory for all tests. Sensitivity was slightly lower in test A, but this was not statistically significant. Likewise, precolostral calf sera gave similar results in all three ELISAs (Table 3).

**Sensitivity and specificity.** Sensitivity and specificity estimates for the three ELISAs, obtained by using 75 postabortion sera and 80 sera from a herd not suspected of having N. caninum infection, are presented in Table 5.

**Labor intensiveness.** There were no significant differences between the three ELISAs in labor intensiveness with respect to personnel required, the length of the assay, and numbers of samples run at a given time.

**FIG. 2.** Profiles of antibodies to N. caninum, as determined with three different ELISAs, in two congenitally infected calves (calves 1 and 2) and two calves (calves 3 and 4) which received colostrum from N. caninum-seropositive dams.

**FIG. 3.** Profiles of immune response to N. caninum, as determined with three different ELISAs, in two cows which aborted during an abortion storm.
These cows were from herds in which abortions were sporadic. as determined with three different ELISAs during 1 to 2 years postabortion. In the course of infection, whereas membrane antigens are recognized antigens of \textit{N. caninum} compared to a membrane antigen-based ELISA. In addition, the recognized antigens of \textit{N. caninum} may vary among cows (4, 5). This could explain the fact that one of the four aborting cows from herds with sporadic abortions was mainly negative in test A, which is in strong contrast with the data from tests B and C (Fig. 4). In the third place, the use of different conjugates may have contributed to the variation in test results. In the two commercial tests an anti-immunoglobulin G (IgG) conjugate was used, whereas in test C an anti-Ig conjugate was used. However, differences were particularly evident during the chronic stage of infection, when probably only IgG was detectable. Other researchers have also found discrepant results with different ELISAs, particularly for low antibody titers, but they did not determine the sensitivities and specificities of the different tests (12).

In some cows a sharp rise in antibodies was evident during gestation, most clearly with test A. Such a rise was seen in cows 38 (Fig. 3) and in cows 21, 45, and 93 (Fig. 4). This increasing immune response suggests a recrudescence of the infection (26). This may have caused the intrauterine infection of these cows’ calves, as evidenced by the presence of precolostral antibodies (3, 17, 25, 26, 31). The birth of a seronegative calf to cow 37, which showed no increase in antibodies during gestation, most clearly with test A. Such a rise was seen in cows

![Graph](image)

**FIG. 4.** Profiles of immune response to \textit{N. caninum} in four cows that aborted, as determined with three different ELISAs during 1 to 2 years postabortion. These cows were from herds in which abortions were sporadic.

However, considerable differences among the tests were found in the antibody profiles of serial samples (Fig. 2 through 4) and in the seroprevalences based on cross-sectional and total-herd sampling (Table 4). Apparently, the sensitivity of test A was inadequate to detect low antibody levels in some animals during chronic stages of the infection.

Several factors may have contributed to these discrepant results. In the first place, the sample dilution may have been important. In test A a dilution of 1:400 was used, whereas in tests B and C dilutions of 1:100 and 1:50, respectively, were used. Secondly, differences in antigen preparation may have played a role. In test A intact tachyzoites were used, and therefore mainly external-membrane antigen determinants and possibly secretory antigens adhering to the surface were exposed. In the other two tests whole-tachyzoite lysates were prepared either by sonication (test B) or by detergent solubilization (test C). Lysates contain soluble cytoplasmatic antigens as well as membrane antigens. It has been suggested that antibodies to soluble cytoplasmic antigens are formed later in the course of infection, whereas membrane antigens are likely to be recognized first (15). This could account for the longer duration of reactivity with a lysate-based ELISA, compared to a membrane antigen-based ELISA. In addition, the recognized antigens of \textit{N. caninum} may vary among cows (4, 5).

![Table](image)

**TABLE 4.** Proportions of positive samples with three different ELISAs in five dairy herds with \textit{N. caninum} abortions and one herd with no history of \textit{N. caninum} abortions

| Herd | Epidemic abortions | Endemic abortions | No abortions |
|------|-------------------|-------------------|-------------|
| 1    | 10 (19.6) 11 (35.5) 48 (25.3) 28 (24.3) 13 (18.1) 2 (1.6) |
| 2    | 14 (27.5) 13 (41.9) 61 (32.1) 32 (27.8) 16 (22.2) 3 (2.4) |
| 3    | 27 (52.9) 13 (41.9) 136 (71.6) 62 (53.9) 33 (45.8) 6 (4.8) |
| 4    | 26 (50.1) 18 (58.1) 144 (75.8) 57 (49.6) 35 (48.6) 6 (4.8) |
| 5    | 14 (27.5) 13 (41.9) 61 (32.1) 32 (27.8) 16 (22.2) 3 (2.4) |
| 6    | 10 (19.6) 11 (35.5) 48 (25.3) 28 (24.3) 13 (18.1) 2 (1.6) |

* Test A* includes suspect samples.

In some cows a sharp rise in antibodies was evident during gestation, most clearly with test A. Such a rise was seen in cows 38 (Fig. 3) and in cows 21, 45, and 93 (Fig. 4). This increasing immune response suggests a recrudescence of the infection (26). This may have caused the intrauterine infection of these cows’ calves, as evidenced by the presence of precolostral antibodies (3, 17, 25, 26, 31). The birth of a seronegative calf to cow 37, which showed no increase in antibodies during gestation, most clearly with test A. Such a rise was seen in cows

![Table](image)

**TABLE 5.** Sensitivities and specificities of three ELISAs for detection of antibodies to \textit{N. caninum} based on postabortion sera and sera from a herd in which \textit{N. caninum} is not suspected

| Test | Sensitivity (95% CI) | Specificity (95% CI) |
|------|---------------------|----------------------|
| A    | 0.88 (0.78–0.95)    | 1.00 (0.93–1.00)     |
| B    | 0.98 (0.93–1.00)    | 0.87 (0.63–1.00)     |
| C    | 0.98 (0.93–1.00)    | 1.00 (0.85–0.98)     |
The use of the detergent in the antigen preparation did not result in detectable cross-reactivity in the ELISA developed. Only Björkman et al. (7) mentioned poor sensitivity with ELISA utilizing extract-based antigen for Neospora caninum in their experimental transmission. Anderson, M. L., P. C. Blanchard, B. C. Barr, J. P. Dubey, R. L. Hoffman, B. A. Mathison, and P. A. Conrad. 1997. Serological response over time to recombinant Neospora caninum antigens in cattle after a neosporosis-induced abortion. Clin. Diagn. Lab. Immunol. 4:270–274.

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