Evaluation of a single serological screening of dairy herds for *Neospora caninum* antibodies

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Abstract

Twenty-one dairy herds with a history of *Neospora caninum*-associated abortions were used for a longitudinal serological study. A total of 1676 animals were blood sampled 3 times and used to evaluate a single serological screening for *N. caninum* antibodies. The results of the first serological screening were compared with the results based on three consecutive samples, whereby two or more positive or negative test results per animal were considered to determine its serological status as positive or negative, respectively. In both test regimes 95.3% of the animals had the same interpretation, of which 33.9% were seropositive, and 61.3% seronegative. Relative sensitivity of one-time sampling compared to three consecutive samplings was 94.7%, while relative specificity was 95.6%. Relative specificity differed between herds. Predictive values positive and negative of one-time sampling were 92.4 and 97%, respectively. The agreement between one-time sampling and three consecutive samplings, kappa, was 0.90. For evaluation of discrepant results age distribution and pedigree data were used to provide clues regarding likelihood of transmission. Age clustering of seropositive animals was interpreted to indicate a point source infection. Daughter–mother relationships were used for the interpretation of congenital infections. The proportion of congenital infection decreased with increasing parity of the mother. Seropositive heifers had 80% congenitally infected offspring, while in older cows 66% of the offspring was congenitally infected, possibly due an increased immunity to transplacental infection with age. It is concluded that a single serological screening of a whole herd in connection with an analysis of age distribution and pedigree data is a rapid and valid method to

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interpret the serologic status of individual animals and to study the mode of transmission of
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1. Introduction

Neospora caninum has been recognised as the most important cause of abortion in cattle in many countries (Anderson et al., 2000). Individual serological testing is used to diagnose N. caninum as a possible cause of abortion and to screen cattle for N. caninum antibodies at occasions of purchase or sale (Williams and Trees, 2000).

There are several serological tests for N. caninum antibodies, including the indirect fluorescent antibody test, the direct agglutination test, and various enzyme-linked immunosorbent assays (Björkman and Uggla, 1999; Atkinson et al., 2000). Usually serological tests are validated against the indirect immunofluorescent antibody test, or by using post-abortion sera of cattle with confirmed identification of N. caninum in their aborted foetuses (Anderson et al., 2000). However, due to fluctuation of titres, the sensitivity of serological tests for random sera is lower than for post-abortion sera. This antibody titre fluctuation is a major problem in chronically infected animals (Dannatt, 1997; Schares et al., 1999; Stenlund et al., 1999; Maley et al., 2001). Furthermore, the sensitivity depends on the serological test used, the cut-off value, and the test sample dilution (Björkman and Uggla, 1999; Wouda et al., 1998a).

If a herd is suspected of infection with N. caninum, blood samples of all animals can be collected for determination of the antibody status. However, the predictive value of a one-time sampling on herd and individual animal level is not known. Because 81 and 95% of N. caninum-infected cows transmit the infection to their offspring (Paré et al., 1996, 1997; Schares et al., 1998; Wouda et al., 1998b; Davison et al., 1999), it appears to be useful to include pedigree information in the evaluation of test results to increase the predictive value of the outcome of the test. Furthermore, the age distribution of positive cases can be helpful for the interpretation of test results. In particular, the presence of age clusters with a high seroprevalence has been shown to indicate post-natal infection of these age groups by a point source exposure (Dijkstra et al., 2001, 2002).

The main objective of the present study was to compare the relative sensitivity and specificity of one-time testing versus three-time testing of cattle for N. caninum antibodies. The second objective was to evaluate the use of age distribution of seropositive animals and pedigree data in the interpretation of conflicting results between the two test regimes.

2. Materials and methods

2.1. Herds and animals

The study was done between November 1998 and December 2001. All animals of 21 Dutch dairy herds with a history of sporadic, endemic or epidemic N. caninum-associated
Abortions were blood sampled. Interval between samplings was on average 11 months, ranging from 6 to 20 months. At the first, second and third sampling, 2642, 2668 and 2677 animals were blood sampled, respectively, and data from these animals were used for the pedigree and age distribution analysis. A total of 1676 animals, which were consistently present in the 21 herds at all 3 samplings, were used for the evaluation of serostatuses. All animals were of the Holstein Friesian breed. In all herds the calves were only fed colostrum of their own mother. Thus, false-positive results due to the feeding of pooled colostrum could be excluded.

2.2. Blood sample collection

Blood samples were taken using disposable needles and 8.5 ml SST™ Gel and Clot Activator Vacutainer® Plus serum-tubes (Becton Dickinson Vacutainer Systems Europe). All samples were immediately transported to the laboratory of the Animal Health Service (Deventer, The Netherlands). Serum was removed after centrifugation of clotted samples at 2000 \( \times g \) for 10 min.

2.3. Serology

All sera were tested for antibodies to *N. caninum* using the ELISA of the Animal Health Service (Deventer, The Netherlands). This ELISA is based on a detergent lysate of whole sonicated tachyzoite antigens and detects all Ig classes. This test has a sensitivity of 98% (95% CI: 93–100%) using post-abortion sera and a specificity of 92% (95% CI: 85–98%) using non-suspect sera. No cross-reactivity of the ELISA to closely related protozoa such as *Toxoplasma gondii*, *Sarcocystis cruzi*, *Cryptosporidium parvum*, *Babesia bovis*, *Babesia bigemina*, *Babesia divergens*, and *Eimeria alabamenis* was found, except for a transient reaction in one *B. bovis*-infected calf (Wouda et al., 1998a). The results of the ELISA kit were calculated as S/P ratio = \((\text{OD test sample} - \text{OD negative control}) / (\text{OD positive control} - \text{OD negative control})\). A cut-off S/P ratio of <0.5 was defined as negative, a S/P ratio of 0.5 to 1.5 as low positive, and a S/P ratio ≥1.5 as high positive.

2.4. Analyses

The results of the first serological screening of the 1676 animals that were serially blood sampled were compared with the results based on all three consecutive samples of the same 1676 animals. The serological status of an animal based on three consecutive samplings was done as follows. An animal was considered to be seronegative if two or more of the test results were negative. An animal was considered to be seropositive if two or more of the test results were positive.

Relative sensitivity of one-time sampling was defined as the percentage of the animals that was defined as seropositive using the three consecutive samplings, that also was seropositive at the first sampling. Relative specificity of one-time sampling was defined as the percentage of the animals that was defined as seronegative using the three consecutive samplings, that also was seronegative at the first sampling. The predictive value positive of one-time sampling was defined as the percentage of the animals that was seropositive at the first
sampling, that also was defined as seropositive using the three consecutive samplings. The predictive value negative of one-time sampling was defined as the percentage of the animals that was seronegative at the first sampling, that was also defined as seronegative using the three consecutive samplings.

Relative sensitivity, relative specificity, predictive value positive and negative were calculated using Statistix 2.0 for Windows. The serological result of the first sampling and the serologic status of the longitudinal sampling were compared using McNemar’s test for paired samples and using kappa (Martin et al., 1987). Distribution in congenital infection across parities, and difference in relative sensitivity and specificity between herds was compared using $\chi^2$ analysis on contingency tables (Rothman and Greenland, 1998). Statistical significance was defined at $P \leq 0.05$.

To facilitate the interpretation of serological test results of a single herd screening, software was designed linking all serologic test results to the data of the Dutch Identification and Registration system (Nielen et al., 1996). Date of birth, date of purchase and culling and pedigree were available for all herds (Royal Dutch Dairy Syndicate, Arnhem, The Netherlands). The software enhanced the interpretation of the serological test results by listing all seronegative and seropositive test results of all animals in a herd sorted by date of birth indicating clusters of infections, and listing all seronegative and seropositive test results within daughter–mother relationships, e.g. $D-M-, D+M-, D-M+ \text{ and } D+M+$. In case of embryo transplantation the mother who gave birth to the daughter was taken in account instead of the biological mother because congenital infections of $N. \text{caninum}$ will occur during gestation (Baillargeon et al., 2001; Bielanski et al., 2002). A seropositive test result at the first sampling was interpreted as: (1) post-natally infected (part of a seropositive age-cluster); (2) congenitally infected; (3) maternal immunity; or (4) false-positive. A seronegative test result at the first sampling was interpreted: as (1) negative; or (2) false-negative.

Subsequently the software assigned a parity number of birth to all daughters based upon the calving number, e.g. parity, of the mother. In this way missing data of bull calves would not affect assigning the parity number of birth. With the evaluation of congenitally infected offspring, a starting point, based upon the moment on which known $M+D+$ relations appear, was estimated and this was used together with the parity number of birth.

3. Results

At the first sampling, 616 (36.8%) animals were seropositive, whereas 601 (35.9%) animals were defined as seropositive based on three consecutive samplings ($P = 0.09$; Table 1). Of the seropositive animals at the first sampling 24% (148/616) had S/P ratios of 0.5 to 1.5 (low positive). Mean herd-level prevalence based on three consecutive samplings was 36.0%, ranging between 4.8 and 80.3%. Relative sensitivity of one-time sampling was 94.7% (95% CI: 92.9–96.5%), while relative specificity was 95.6% (95% CI: 94.4–96.8%). Predictive values positive and negative of one-time sampling were 92.4% (95% CI: 90.3–94.5%) and 97.0% (95% CI: 95.9–98.0%), respectively. The observed proportion of agreement between one-time sampling and three consecutive samplings was 95.3%. The agreement between one-time sampling and three consecutive samplings, kappa, was 0.90 (95% CI: 0.88–0.92).
Table 1
Comparison of serological status for *Neospora caninum* of cattle after a single serological sampling and based on three consecutive samplings

<table>
<thead>
<tr>
<th>Serostatus based on three consecutive samplings</th>
<th>Negative</th>
<th>Positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serostatus based on first sampling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1028</td>
<td>32</td>
<td>1060</td>
</tr>
<tr>
<td>Positive</td>
<td>47</td>
<td>569</td>
<td>616</td>
</tr>
<tr>
<td>Total</td>
<td>1075</td>
<td>601</td>
<td>1676</td>
</tr>
</tbody>
</table>

In 32 animals, the serostatus changed from negative at first sampling to positive at the second and third samplings (Tables 1 and 2). Nine seronegative animals at first sampling and seropositive at later samplings, were part of a seropositive age-cluster at the second and third sampling and therefore probably infected after the first sampling. Eleven animals with seropositive mothers or seropositive offspring were interpreted as false-negatives. Six animals with seronegative mothers or offspring were interpreted as negatives.

In 47 animals the serostatus changed from positive at first sampling to negative at the second and third samplings (Tables 1 and 2). Of these 47 animals, 5 had a seropositive mother and were younger than 6 months old at the first sampling. These positive test results can thus be explained by the presence of maternal antibodies. Seropositive animals were interpreted as positives if they were part of a seropositive age cluster indicating a point source post-natal infection or if they had seropositive mothers or offspring. All other seropositive animals with seronegative mothers or offspring were interpreted as false-positives. Of the 19 false-positive animals 13 had low positive and 6 had high positive test results at the first sampling.

Although 19 of the 32 seronegatives were found in four herds, relative sensitivity did not differ significantly between herds \(\chi^2 = 24.8, 20\) d.f., \(P = 0.21\). Relative specificity differed between herds \(\chi^2 = 61.3, 20\) d.f., \(P < 0.0001\); 24 of the 47 seropositives were found in 3 herds.

Seropositive mothers had on average 73% (363/500) seropositive offspring suggesting congenital infection. The percentage of congenitally infected offspring was 80% (144/180) at first parity, 71% (95/133) at second parity, 67% (63/94) at third parity and 66% (61/93) at parity >3. This decreasing tendency with increasing parity of the mothers was statistically significant \(\chi^2 = 8.8, 1\) d.f., \(P = 0.03\).

4. Discussion

In this study, a one-time testing for *N. caninum* antibodies using an ELISA was compared with three sequential testings. The absence of gold standard determination of the animals’ true infection status can result in a misclassification also when using the results of the three testings depending on the chosen cut-off value. However, this test had a high sensitivity, a high specificity in a previous validation (Wouda et al., 1998a), and was successfully used
Table 2
Interpretation of discrepant serostatuses for *Neospora caninum* in cattle obtained by single sampling versus serial sampling, using age distribution and pedigree data

<table>
<thead>
<tr>
<th>Age distribution and pedigree data</th>
<th>Seronegative at first sampling and seropositive at later samplings</th>
<th>Interpretation of test results at first sampling</th>
<th>Seropositive at first sampling and seronegative at later samplings</th>
<th>Interpretation of test results at first sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother seropositive, ≤6 months of age</td>
<td>0</td>
<td>Negative (post-natal infection after first sampling)</td>
<td>5</td>
<td>Maternal antibodies</td>
</tr>
<tr>
<td>Part of seropositive age-cluster</td>
<td>9</td>
<td>False-negative</td>
<td>3</td>
<td>Positive (post-natally infected)</td>
</tr>
<tr>
<td>Mother seropositive, &gt;6 months of age</td>
<td>9</td>
<td>Negative (post-natal infection after first sampling)</td>
<td>5</td>
<td>Positive</td>
</tr>
<tr>
<td>Mother seronegative</td>
<td>5</td>
<td>False-negative</td>
<td>11</td>
<td>False-positive</td>
</tr>
<tr>
<td>Serostatus mother unknown, offspring seropositive</td>
<td>2</td>
<td>Negative</td>
<td>4</td>
<td>Positive</td>
</tr>
<tr>
<td>Serostatus mother unknown, offspring seronegative</td>
<td>1</td>
<td>Negative</td>
<td>8</td>
<td>False-positive</td>
</tr>
<tr>
<td>Serostatus mother and offspring unknown</td>
<td>6</td>
<td>No interpretation</td>
<td>11</td>
<td>No interpretation</td>
</tr>
<tr>
<td>Total</td>
<td>32</td>
<td></td>
<td>47</td>
<td></td>
</tr>
</tbody>
</table>
for detection of post-natally infected age groups and congenitally infected animals (Dijkstra et al., 2001).

The present study shows that a single whole herd screening for *N. caninum* antibodies will result in misinterpretation of the serostatus of some individuals, but in only a minor proportion of the total herd. Relative sensitivity and relative specificity of one-time sampling was 94.7 and 95.6%, respectively. This means that 5.3% of the animals changed from seronegative at first sampling to seropositive at the later samplings, and 4.4% of the animals changed from seropositive at first sampling to seronegative at the later samplings. Analysis of age distribution data showed that 28% (9/32) of the fluctuations from seronegative to seropositive were probably caused by post-natal infections. Eleven percent (5/47) of the fluctuations from seropositive to seronegative were obviously caused by maternal antibodies which disappear within 6 months post-partum. By analysing daughter–mother relationships most of the other discrepant test results between one- and three-times testing could be interpreted, whereby one-time testing apparently produced false-negative or -positive results more frequently than three-times testing. However, in a minority of cases the pedigree data suggested that the results of the first testing were more reliable than the results of the later testings.

Relative specificity differed between herds. This may be due to differences in abortion pattern between the various herds. This is in accordance with a German study, which showed that the abortion pattern influenced the test results. ELISA indices of IFAT and IB positive dams were significantly lower in herds with epidemic abortions than in herds with endemic abortions (Schares et al., 1999).

It is useful to differentiate between low and high positive test results. High antibody titres are found in post-abortion sera and during the second part of pregnancy (Schares et al., 1999; Stenlund et al., 1999). In our study most fluctuations were observed in the low positive test results, which comprised 24% of the positive test results. Of 19 positive test results, which were interpreted as false-positives (Table 2), 13 were low positive. Considering the high level of congenital infections, interpretation of such low positive test results will be supported when they are compared with the test results of the mother and offspring. However, congenital infection occurs in less than 100% of the cases. This can explain the occurrence of seronegative daughters with seropositive mothers. Post-natal infection can explain the occurrence of seropositive daughters with seronegative mothers. However, post-natal infections are rare if these seropositives were not part of a seropositive age-cluster. Both false-positives and -negatives were especially seen in seropositive daughter/seronegative mother relations. It may be worth trying to solve discrepancies in serostatus between mother and offspring, especially in case of low positive test results, by retesting mother and offspring after a period of 3–4 weeks. However, deviations in serostatus of daughters and mothers may still occur because in a small minority of cases seronegative mothers have been shown to give birth to congenitally infected calves (Sager et al., 2001).

The mean calculated proportion of congenital infection of 73% in this study is lower than 81–95% congenital infection reported in other studies (Paré et al., 1996, 1997; Schares et al., 1998; Wouda et al., 1998b; Davison et al., 1999). The proportion of congenital infection decreased with increasing parity of the mother. In other studies no distinction between parities was made. The decreased percentage of congenitally infected calves may be explained by an increase of protective immunity against transplacental transmission.
Other investigators have found indications for development of protective immunity against abortion in cows that had a prior infection with *N. caninum* (McAllister et al., 2000).

It is our conclusion that a single whole herd screening for *N. caninum* antibodies in connection with an analysis of pedigree and age distribution data yields valuable information on the prevalence of infection in the herd and the routes of transmission. The software linking the serologic test results to the Identification and Registration data including pedigree information has been proven to be very helpful for a rapid analysis.

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**References**


