A Modified Agglutination Test for *Neospora caninum*: Development, Optimization, and Comparison to the Indirect Fluorescent-Antibody Test and Enzyme-Linked Immunosorbent Assay

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Current serologic tests used to detect antibodies to *Neospora caninum* require species-specific secondary antibodies, limiting the number of species that can be tested. In order to examine a wide variety of animal species that may be infected with *N. caninum*, a modified direct agglutination test (N-MAT) similar to the *Toxoplasma gondii* modified direct agglutination test (T-MAT) was developed. This test measures the direct agglutination of parasites by *N. caninum*-specific antibodies in serum, thus eliminating the need for secondary host-specific anti-isotype sera. The N-MAT was compared to the indirect fluorescent-antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) with a “gold standard” serum panel from species for which secondary antibodies were available (n = 547). All positive samples tested were from animals with histologically confirmed infections. Up to 16 different species were tested. The N-MAT gave a higher sensitivity (100%) and specificity (97%) than the ELISA (74 and 94%, respectively) and had a higher sensitivity but a lower specificity than the IFAT (98 and 99%, respectively). The reduced specificity of the N-MAT was due to false-positive reactions in testing fetal fluids with particulate matter or severely hemolyzed serum. Overall, the N-MAT proved to be highly sensitive and specific for both naturally and experimentally infected animals, highly reproducible between and within readers, easy to use on large sample sizes without requiring special equipment, and useful in testing serum from any species without modification.

*Neospora caninum* is a pathogenic protozoan that was first identified in 1988 as a new genus of *Toxoplasma*-like apicomplexan. Although first associated with congenital encephalomyelitis in puppies (20, 21), it has since been described for cattle, sheep, goats, and horses (18). It is very similar to *Toxoplasma gondii* in morphology and biological behavior, but its antigenic and ultrastructural characteristics differ (7, 20, 21). *N. caninum* has been found to be the major cause of bovine abortion in the United States (2), New Zealand (45), The Netherlands (53), and Great Britain (49). Abortions resulting from *N. caninum* infections are also recognized in several other countries (4). Approximately 42% of abortions in California dairy cattle alone have been attributed to *N. caninum* infection (3).

The host range and life cycle of *N. caninum* remain unknown (7). To date, the only known mode of transmission is vertical transmission, whereby the parasite passes transplacentally from an infected dam to her offspring (5). Because of its similarities to *T. gondii*, *N. caninum* is thought to possess a similar life cycle with horizontal transmission via the ingestion of oocysts shed in the feces of the unknown definitive host (8). Recent evidence of a point source exposure to *N. caninum* in dairies in South Dakota (54) and California (40) is consistent with the assumption that *N. caninum* is spread horizontally by a definitive host. In both instances, exposure to the organism was thought to be through contaminated feed which was incorporated into a total mixed ration. Another study gave evidence for congenital transmission in an *N. caninum*-endemic herd but found that most cows aborting during an epidemic were infected postnatally (48).

Currently, there is no effective means for treatment or control of this disease, particularly in dairies where bovine neosporosis is a major disease problem (8, 47). Culling of seropositive cows is the only current means of control (47), and this may or may not be beneficial depending on several factors, such as the risk of horizontal transmission, the reproductive history and costs and benefits of culling each animal, and herd seroprevalence. Implementation of more effective control procedures would be facilitated by the identification of the definitive host(s) for *N. caninum*. To date, the definitive host is completely unknown. One study suggested that, as for *T. gondii*, cats might also be the definitive host for *N. caninum* (1). However, this proposal has not been supported by other investigators (19, 39). Results of laboratory and field studies looking at dogs, cats, rats, and mice have all proved negative (11a), suggesting that the definitive host may be some other wildlife species. If so, finding this definitive host will be extremely difficult without some methodology to screen various wildlife species for evidence of exposure to the *N. caninum* protozoan.

The purpose of this study was to develop and evaluate a modified agglutination test (MAT) that could be used to
screen for antibodies to *N. caninum* (N-MAT) in a wide variety of wildlife species suspected of being possible definitive hosts for the parasite. Unlike most serologic tests, the agglutination test does not require species-specific conjugates, and so any animal species could conceivably be tested for the presence of *N. caninum* antibodies. Direct agglutination (DA) is based on the principle that formalin-treated organisms agglutinate at the presence of specific immunoglobulin G (IgG) antibodies (28). A *Toxoplasma* DA test was first described by Fulton et al. (27–29) and then modified and adapted by Desmonts and Remington (17) to improve the sensitivity and specificity. Today, the modified DA test for *T. gondii* (T-MAT) is widely used for research purposes in all species and as a screening test for humans in Europe (17, 33, 41, 52). The test is rapid and easy to perform, does not require sophisticated equipment, and can be performed on sera from any species with a modification (6, 17, 27, 29, 33, 44, 52). Such a test could be used in extensive seroprevalence studies to help pinpoint possible definitive hosts for *N. caninum*, particularly for those species found in dairies with endemic *N. caninum* infection. This would in turn allow for the development and implementation of proper preventive measures against *N. caninum*.

In this study, *N. caninum* antigen was prepared and testing conditions were optimized for the development of the N-MAT. In order to determine the diagnostic capabilities of the test, an extensive panel of “gold standard” sera from histologically confirmed infected and uninfected animals was used to compare results to those obtained from the more commonly used indirect fluorescent-antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA).

**MATERIALS AND METHODS**

**MAT antigen preparation and testing methods.** The *N. caninum* isolate (BPA-1) was obtained from an aborted bovine fetus (15) and maintained in vitro on a stationary monolayer of Vero cells (green monkey kidney) cells. Tachyzoites were harvested when ≥80% of the Vero cells were infected. The monolayer was dislodged from the flask into the cell culture medium with a cell scraper (Costar Corp., Cambridge, Mass.). This suspension was then centrifuged at 1,500 × *g* for 10 min, the supernatant was discarded, and the pellet was resuspended in 2 to 3 ml of 37% formaldehyde. After the last wash, the pellet was decanted, and the pellet was resuspended in 2 to 3 ml of 37% formaldehyde. The resulting suspension was added to each well. Two wells were reserved for positive and negative controls. The negative control well contained PBS with no serum added. The well was regarded as a positive agglutination reaction, whereas a negatively well-exhibited a central discrete opaque dot or button. Following this initial rapid screen, an endpoint titration was set up for any sample with a positive reaction at 1:40 with twofold serial dilutions from 1:40 to 1:20,480. Mercaptoethanol and antigen were then added to all wells as described above. The endpoint titers were determined by using the dilution of the last well that showed a positive reaction.

**IFAT antigen preparation and testing methods.** The IFAT was performed as previously described (14) with antigen slides (Cell Line Associates, Newfield, N.J.) that were prepared with tachyzoites of the BPA-1 *N. caninum* isolate. The fluorescein isothiocyanate-labeled, affinity-purified antibodies directed against species-specific IgG were diluted 1:300 for bovine antisera (Jackson Immunoresearch, Inc., West Grove, Pa.) and 1:100 for monkey, dog, (used for both domestic dog and coyote), rabbit, cat (used for both domestic cat and mountain lion), rat, mouse, horse, goat, and chicken antisera (all from Jackson ImmunoResearch, except for monkey antisera, which were from Sigma, St. Louis, Mo.) in PBS and added in 10-μl aliquots to each well. For testing positive sera, an unconjugated serum (Nordic Immunology, Tilburg, The Netherlands) was directed against *IgM* was used with a fluorescein-labeled rabbit antibody (Jackson Immunoresearch) as a tertiary antibody. A fluorescein isothiocyanate-conjugated polyclonal anti-psittacine Ig antibody produced by immunization of a rabbit with ammonium sulfate-precipitated psittacine serum from multiple psittacine species (kindly provided by C. Cray, University of Miami School of Medicine) was used as the conjugating antibody for all bird samples (blackbirds, starlings, and budgerigars) for which a secondary antibody was not commercially available. The bird conjugate was diluted 1:10 in PBS and added to the slides in 10-μl aliquots for each well. The endpoint titer was the last serum dilution showing distinct, whole-parasite fluorescence (14).

**ELISA antigen preparation and testing methods.** The whole-parasite lysate ELISA was performed as previously described (26) with a few modifications. Briefly, plates were coated with supernatant antigens (1 to 2 μg) by incubation, washed, and dried at 37°C. After overnight incubation, plates were covered and incubated at 37°C with 5% CO2. The next day, plates were washed three times, covered, and incubated overnight at 37°C with 5% CO2. The following day, the plates were washed and dried as described above. The wells were then mixed thoroughly by pipetting them up and down 10 times, covered, and incubated at 37°C for 1 h. The next day, the plates were washed and dried as described above. The wells were then mixed thoroughly by pipetting them up and down several times, covered, and incubated overnight at 37°C with 5% CO2. The results were read the next morning. Sera from confirmed positive animals had elevated serum antibody titers to *N. caninum* as measured initially by the IFAT. For all experimental inoculations, the tachyzoites were derived from naturally infected tissues confirmed by postmortem examination of tissues from either the animal or its fetus or newborn offspring. Infection was confirmed by immunofluorescence (IFAT) antigen preparation and testing methods.

For all experimental inoculations, the tachyzoites were derived from tissue culture as described above with either Vero or cardiopulmonary aortic endothelial cells as the feeder layer. Experimentally inoculated cattle were challenged with a range of tachyzoite doses (1.5 × 10⁶ to 8 × 10⁶ tachyzoites) mixed with 100 μl of 5% heat-inactivated HS in PBS-T, with no serum added. Since bovine samples were the only samples that could be run on this ELISA, the same bovine serum samples used by Louis et al. (34) were used to compare this ELISA with the IFAT and MAT (34).

**OD reading standard.** Confirmed *N. caninum*-positive animals were defined as animals experimentally infected with live parasites (tachyzoites or bradyzoites) and naturally infected animals confirmed as infected by postmortem examination of tissues. Tissues were placed in PBS with 0.05% Tween (PBS-T), and washed with 200 μl of 5% heat-inactivated HS in PBS-T for 1 h at 37°C. Plates were then rinsed and dried completely before being stored at 4°C. Prior to use, test samples were diluted 1:10 in PBS-T containing 1% heat-inactivated HS. The plates were incubated for 30 min at 37°C with 100 μl of sample per well and then washed four times with PBS-T. Peroxidase-labeled rabbit anti-bovine IgG (Jackson Immunoresearch) was diluted 1:5,000 in 0.1 M phosphate-buffered saline (PBS). The plates were washed a final four times with PBS-T and developed with 100 μl of ABTS [2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt]–1-Step (Pierce, Rockford, Ill.) for 30 min at room temperature. The reaction was stopped with 100 μl of 1% sodium dodecyl sulfate, and the optical density (OD) was determined at a wavelength of 405 nm with a reference wavelength of 490 nm. Final ODs were obtained by subtracting the OD values of the samples from the OD values of the blanks (1% heat-inactivated HS in PBS-T, with no serum added). Since bovine samples were the only samples that could be run on this ELISA, the same bovine serum samples used by Louis et al. (34) were used to compare this ELISA with the IFAT and MAT (34).
1-week intervals. Experimentally inoculated mice were challenged orally or by various parenteral routes with $8 \times 10^5$ to $5 \times 10^6$ tachyzoites (some of which were kindly provided by L. Choromanski).

Sera from naturally infected cows were taken at the time of abortion between 1991 and 1995 from animals in 11 different California dairies (3, 5). All cows had aborted *N. caninum*-infected fetuses as confirmed by the presence of characteristic lesions and tachyzoites in fetal tissues. Sera from naturally infected calves came from four different California dairies and were drawn within 5 days after birth. Bovine fetal fluid samples ($n = 17$) were collected from aborted fetuses submitted to the California Veterinary Diagnostic Laboratory System (CVDLS) at the University of California, Davis, for necropsy. All calves and fetuses were also confirmed positive for *N. caninum* at necropsy. Naturally infected horse samples came from two horses with equine protozoal myeloencephalitis-like symptoms that were later diagnosed with *N. caninum* infections based on IPX staining (16, 35). The naturally infected dogs were Rhodesian Ridgebacks that gave birth to puppies with clinical central nervous system symptoms that were confirmed as positive *N. caninum* infections at necropsy by IPX staining. The pygmy goat aborted an *N. caninum*-positive fetus as confirmed at necropsy by IPX staining.

N-MAT sera ($n = 245$) were collected from cattle ($n = 56$), rhesus macaque monkeys ($n = 54$), cats ($n = 7$), blackbirds ($n = 14$), starlings ($n = 6$), a budgerigar ($n = 1$), mice ($n = 75$), a rat ($n = 1$), pigeons ($n = 10$), coyotes ($n = 2$), and mountain lions ($n = 19$). The cattle were from herds with no history of *N. caninum*-like abortion, including beef heifers kept in a closed speleic-pathogen-free herd in Nebraska, beef heifers maintained on pasture in California, and dairy cows maintained at the University of California, Davis. Five of the cows were from California dairies with an *N. caninum* abortion problem but had remained seronegative over a 3-year period and had produced *N. caninum* negative calves or had unrelated abortions with fetuses showing no signs of *N. caninum* infection at necropsy (5). The rhesus macaque monkeys were housed at the California Regional Primate Center on the University of California campus in Davis and had no history of *N. caninum* infection. Serum samples from the cats, blackbirds, starlings, budgerigars, mice, and rats were either preinoculation samples from experimentally inoculated animals or samples from contact control animals. The pigeons, coyotes, and mountain lions were necropsied at the CVDLS with no infectious disease diagnosed.

Additional negative sera ($n = 58$) used to evaluate the specificity of the N-MAT came from sera of animals infected with other pathogens, including rabbits ($n = 4$) experimentally inoculated with *T. gondii* (ME-49 strain), *H. hexamita* (kindly provided by J. P. Dubey), and *Sarcocystis cruzi* (32) (kindly provided by J. P. Dubey) or *Sarcocystis neurona* (9); cats ($n = 3$) experimentally inoculated orally with bradyzoites of the T-265 or T-263 strain of *T. gondii* (kindly provided by L. Choromanski); a mouse experimentally inoculated with *S. neurona* ($n = 1$) (36); blackbirds ($n = 2$) experimentally inoculated with $6 \times 10^6$ *Sarcocystis falcatula* merozoites i.m. or 500 *S. falcata* sporozoites i.m.; budgerigars ($n = 3$) experimentally inoculated with *S. neurona* or *S. falcata* (37); chickens ($n = 4$) experimentally inoculated with four different strains of infectious bronchitis virus; a goat ($n = 1$) infected with *Toxoplasma* (ToxoPLassa DA kit [bioMerieux SA, Marcy l’Etoile, France]); horses diagnosed with *S. neurona* ($n = 7$) or an unrelated neurologic disease ($n = 3$); Mexican beef bulls ($n = 3$) with *Trichomonas foetus* infection; and bovine fetuses ($n = 27$) submitted to the CVDLS with no evidence of *N. caninum* infection but diagnosed with other diseases (bovine viral diarrhea, epizootic bovine abortion, and infectious bovine rhinotracheitis).

**DETERMINATION OF CUTOFF VALUES.** The positive cutoff value for the ELISA was calculated at 2 standard deviations above the negative sample average. Positive cutoff values for the N-MAT and the IFAT were determined by calculating the sensitivity, specificity, and predictive values of each test at three different titer cutoff values. The titer with the highest sensitivity and specificity results was used as the positive cutoff value. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated for all three tests by the following equations: sensitivity = (number of animals both test positive and disease positive)/(number of animals disease positive), specificity = (number of animals both test negative and disease negative)/(number of animals disease negative), positive predictive value = (number of animals both test positive and disease positive)/(number of animals test positive), and negative predictive value = (number of animals both test negative and disease negative)/(number of animals test negative) (50). *P*-values were determined by running McNemar’s chi-square ($\chi^2$) test at a achieving a standard statistical $\chi^2$ table with 1 df (38).

**MEASUREMENT OF REPRODUCIBILITY.** One hundred tests of serum from a naturally infected cow who had a history of repeat abortions were run by two different trained technicians on the IFAT and N-MAT. Results were compared between and within readers to determine reproducibility for both testing methods.

**RESULTS**

Cutoff values were determined with gold standard-negative ($n = 76$) and -positive ($n = 116$) naturally infected cattle samples only. A cutoff titer of 1:80 for the N-MAT gave the greatest sensitivity and specificity values. Selecting a cutoff titer lower than 1:80 resulted in decreased specificity due to increased false-positive reactions. Sensitivity significantly ($P < 0.05$) dropped while specificity remained the same if a cutoff value of 1:160 was used. A cutoff titer of 1:160 for the IFAT gave the greatest sensitivity and specificity values. The specificity was slightly lower at the cutoff value of 1:160 than at the 1:320 value but not enough to make up for the significant ($P < 0.05$) drop in sensitivity at 1:320. The mean OD of the negative samples ($n = 52$) used on the ELISA was 0.181 nm with a standard deviation of 0.106 nm, yielding a cutoff value at an OD of 0.393 nm.

The ELISA gave the lowest sensitivity and specificity results compared to the N-MAT and IFAT with a select group of positive ($n = 63$) and negative ($n = 52$) cattle samples (Table 1). Both the N-MAT and the IFAT gave significantly better results than the ELISA ($P < 0.05$), but there was no significant difference between the N-MAT and the IFAT, even though the N-MAT had a slightly higher specificity and positive predictive value.

Further comparison of the N-MAT and the IFAT with a larger and more diverse serum panel, including numerous animal species, revealed that, while the N-MAT gave a higher sensitivity and negative predictive value, the IFAT gave a higher specificity and positive predictive value (Table 2). These differences were not, however, statistically significant at the 5% level. When the serum panel was broken down into groups where the naturally infected-cattle, the naturally infected-other-species, and the experimentally infected-animal samples were analyzed separately (Table 3), both the N-MAT and the IFAT performed perfectly for the experimentally infected animals but gave either false-positive or false-negative results for

**TABLE 1. Comparison of N-MAT, IFAT, and ELISA with selected sera from confirmed *N. caninum*-positive ($n = 63$) and -negative ($n = 52$) cattle**

| Characteristic | N-MAT | IFAT | ELISA |
|---------------|-------|------|------|
| Sensitivity   | 100 (63/63) | 100 (63/63) | 74 (47/63) |
| Specificity   | 100 (52/52) | 98 (51/52) | 94 (49/52) |
| Positive predictive value | 100 (63/63) | 98 (63/64) | 94 (47/50) |
| Negative predictive value | 100 (52/52) | 100 (51/51) | 75 (49/65) |

a Values in parentheses are as follows: for sensitivity, number of samples both test and disease positive/number disease positive; for specificity, number both test and disease negative/number disease negative; for positive predictive value, number both test and disease positive/number test positive; for negative predictive value, number both test and disease negative/number test negative.

| Characteristic | Value (%) for testa |
|---------------|---------------------|
| Sensitivity   | 100 (244/244) |
| Specificity   | 98 (296/303) |
| Positive predictive value | 98 (244/251) |
| Negative predictive value | 100 (296/296) |

a Values in parentheses are as follows: for sensitivity, number of samples both test and disease positive/number disease positive; for specificity, number both test and disease negative/number disease negative; for positive predictive value, number both test and disease positive/number test positive; for negative predictive value, number both test and disease negative/number test negative.
TABLE 3. Comparison of N-MAT and IFAT with results for naturally and experimentally infected cattle and other species within a gold standard serum panel

| Group and characteristic                  | Value (%) for test<sup>a</sup> | N-MAT | IFAT |
|------------------------------------------|---------------------------------|-------|------|
| Naturally infected cattle                |                                 |       |      |
| Sensitivity                              | 100 (116/116)                   | 97 (113/116)<sup>b</sup> |      |
| Specificity                              | 96 (73/76)<sup>b</sup>          | 98 (75/76) |      |
| Positive predictive value                | 97 (116/119)                    | 99 (113/114) |      |
| Negative predictive value                | 100 (73/73)                     | 96 (75/78) |      |
| Naturally infected other species         |                                 |       |      |
| Sensitivity                              | 100 (8/8)                       | 100 (8/8) |      |
| Specificity                              | 95 (90/94)<sup>c</sup>         | 100 (94/94) |      |
| Positive predictive value                | 66 (8/12)                       | 100 (8/8) |      |
| Negative predictive value                | 100 (94/94)                     | 100 (94/94) |      |
| Experimentally infected animals          |                                 |       |      |
| Sensitivity                              | 100 (120/120)                   | 100 (120/120) |      |
| Specificity                              | 100 (133/133)                   | 100 (133/133) |      |
| Positive predictive value                | 100 (120/120)                   | 100 (120/120) |      |
| Negative predictive value                | 100 (133/133)                   | 100 (133/133) |      |

<sup>a</sup> Naturally infected cattle are the only group that includes fetal fluids in addition to postnatal sera. Experimentally infected animals include all species. Values in parentheses are as follows: for sensitivity, number of samples both test and disease positive/number disease positive; for specificity, number both test and disease negative/number disease negative; for positive predictive value, number both test and disease positive/number test positive; for negative predictive value, number both test and disease negative/number test negative.

<sup>b</sup> 3 of 17 fetal fluid samples yielded negative results from confirmed infected fetuses.

<sup>c</sup> 3 of 44 fetal fluid samples yielded false-positive MAT results.

<sup>d</sup> Four of eight extensively hemolyzed serum samples yielded false-positive MAT results.

The naturally infected-animal samples. The N-MAT gave three false-positive results in the naturally infected-cattle group and four false-positive results in the naturally infected-other-species group while the IFAT performed perfectly on the naturally infected-other-species group but gave one false-positive and three false-negative results for the naturally infected-cattle group (Table 3). The seven false-positive samples by the N-MAT were either fetal fluid samples that contained particulate matter or extensively hemolyzed serum samples from mountain lions that had been frozen and/or dead for several hours before the samples were taken. The three samples falsely negative by the IFAT were also fetal fluid samples, but the one false-positive sample was from a mature cow.

Had the IFAT been used as the standard test to determine the diagnostic capabilities of the N-MAT instead of histological results being used to confirm infection, both the sensitivity and the specificity of the N-MAT would have been lower (Table 4), although not significantly at the 5% level.

In comparing the N-MAT and IFAT endpoint titers, 26% of the positive titers for the gold standard panel were in agreement. The remaining 74% were 1 to 4 dilutions off, with the N-MAT having a tendency to produce lower titers than the IFAT. Two trained readers examining 100 tests of the same positive sample were in total agreement for 41% of the tests on the N-MAT and 57% of the tests on the IFAT. The readers were within 0 to 1 dilutions of each other for 88% of the time with the N-MAT and for 95% of the time with the IFAT. Within each reader, the titers seemed to vary slightly more with the IFAT than with the N-MAT.

### DISCUSSION

This is one of the first reports on the development of a serologic test for *N. caninum* that does not require species-specific secondary antibody conjugates. The test was adapted from the modified direct agglutination test for *T. gondii* (T-MAT) described by Desmonts and Remington (17), which is now marketed as the bioMerieux Toxo DA kit. While the antigen preparation techniques and testing methods are very similar, there were several adaptations, involving changes in antigen preparation, serum volume, medium, and indicator dyes.

The *T. gondii* antigen used in the T-MAT was prepared from parasites harvested from trypsinized sarcoma cells grown in mice, but for a simpler and more economical approach, the *N. caninum* antigen used in the N-MAT was prepared from parasites harvested from Vero cells grown in tissue culture. The alkaline buffer used to make the *T. gondii* antigen contained bovine serum albumin, but due to possible preexisting Igs from *N. caninum* exposure in bovine derived products, it was replaced with HS albumin for this study. False-positive results have been observed with an IFAT when antigen slides prepared with parasites maintained in medium containing fetal bovine serum were used, but switching to medium with HS eliminated the problem (6a). The *N. caninum* tachyzoite concentration was also higher than the *T. gondii* tachyzoite concentration. Desmonts and Remington (17) suggested using only 20,000 tachyzoites/μl as the antigen concentration for the T-MAT; however, the optimal antigen concentration for the N-MAT was found to be no less than 30,000 and no more than 40,000 tachyzoites/μl. If the concentration of the tachyzoites in the antigen was too low, reactions were not detectable. Conversely, if the antigen concentration was too high, the negative buttons began to appear fuzzy, looking more like weakly positive reactions than like true-negative reactions, and resulted in false-positive readings.

As stated by Desmonts and Remington (17) for the *T. gondii* antigen, preservation of the parasite integrity and reduction of cell culture debris in the antigen preparation were very important for the *N. caninum* antigen to work properly. The parasites had to retain a normal crescent shape to maintain optimum test sensitivity, and as a result, tachyzoite preparations could not be passed through a needle to break up intact cells, could not be frozen for later use, and could not be filtered through a 5-μm-pore-size filter. The parasites had to be drawn...
through a special column (Pharmacia Biotech) that reduced cell debris without decreasing parasite numbers or disturbing the tachyzoite morphology. This requirement for undisturbed *N. caninum* tachyzoites strongly suggests that an intact cell membrane is necessary for accurate N-MAT results. It also suggests that significant parasite-specific antigen epitopes are present on the outer parasitic membrane but that nonspecific antigens that cross-react with other related parasites are present within the parasitic cytoplasm and are exposed following the rupture of the tachyzoite cell membrane. Similar findings have been reported by Williams et al. (51) and Bjorkman et al. (13) in dealing with cross-reactivity problems of an *N. caninum* ELISA.

The bioMerieux Toxo DA kit uses an initial serum volume of 100 μl to prepare serial dilutions for running the test. With the N-MAT, we found that only 5 μl of each serum sample was sufficient to prepare dilutions without compromising the test results. The T-MAT kit also has a dye (name not given) added to the alkaline buffer that stains the negative pellet and makes the test easier to read and more reproducible. However, when the dye used in the bioMerieux Toxo DA kit was used in the N-MAT, the parasites did not stain adequately. Up to 15 different dyes were tested in an effort to find one that would work. Eosin Y gave the best results, although it did not stain the *N. caninum* pellets as distinctly as the *T. gondii* pellets were stained in the bioMerieux Toxo DA kit. Overall, when the N-MAT was compared to the T-MAT, the *T. gondii* agglutination reactions were more distinct and easier to read. This may be due to differences in the major surface proteins between the two parasites (12, 30). In addition, results from the T-MAT kit could be read after letting the test plate sit at room temperature undisturbed for 5 h, but the N-MAT results were readable only after an overnight incubation, which remains the only major drawback to this test.

The determined cutoff values established for the N-MAT, *Neospora* IFAT, and ELISA in this study were comparable to what others have suggested for T-MAT and *Neospora* IFAT and ELISA (24, 26, 43, 46). Dubey et al. (24) arbitrarily selected a cutoff of 1:25 for the T-MAT, although Thuilliez (46) found that the specificity of the agglutination reaction was optimal at 1:40 and that the test started to give false-positive results below this dilution. Our results indicate that the highest sensitivity and specificity were obtained for the N-MAT at a cutoff value of 1:80. These various chosen cutoff values may be one of the reasons that Dubey et al. obtained a lower specificity (90%) with the T-MAT (24) than we did with the N-MAT in this study (98%). Selection of differing cutoff values would also partially explain reported sensitivity differences for the *N. caninum* IFAT. Paré et al. (43) reported a sensitivity of 77% for their *N. caninum* IFAT with a cutoff value of 1:640, while our sensitivity was 98% with a chosen cutoff value of 1:160. Data from another study comparing the IFAT to an immunostimulating complex iscom (ELISA) approximates our reported 98% sensitivity for the IFAT (13). The cutoff value for the *N. caninum* ELISA developed by Dubey et al. (26) was at an OD value of 0.40 nm, which was very close to the cutoff OD value in this study at 0.39 nm.

Compared to the *N. caninum* ELISA and IFAT, the N-MAT produced the highest sensitivity (Tables 1 and 2), which is consistent with other reports comparing the T-MAT to the *T. gondii* ELISA (24, 44) and IFAT (24, 44). Comparisons of the T-MAT to other *T. gondii* serologic tests have shown it to be more sensitive than the dye test (17, 22–24), latex agglutination test (22–25), and indirect hemaggulination test (22–25) as well. However, the N-MAT did have a lower specificity compared to that of the *N. caninum* IFAT, which is also similar to reports on the T-MAT (24, 29). The *N. caninum* ELISA used in this study had a significantly lower sensitivity and specificity than both the N-MAT and the IFAT, which might be expected with a whole-parasite lysate, although higher values have been reported with a similar whole-parasite lysate ELISA (42). Low sensitivity and specificity values for the whole-parasite lysate ELISAs would not be unexpected since parasite lysis would lead to the release of numerous cytoplasmic antigens, including nonspecific group antigens for the Apicomplexa. Further, as stated above, our studies with the N-MAT do suggest that nonspecific reactions may be associated more with internal parasitic antigens than with surface antigens. More recently developed second-generation ELISAs give greatly improved sensitivities and specificities; however, they are still lower than the N-MAT and IFAT values for *N. caninum* in this study. These second-generation ELISAs rely on the selection of single *N. caninum*-specific antigens or groups of specific antigens. Such tests include the iscom ELISA (13), the intact formalin-fixed *N. caninum* ELISA (51), and the recombinant *N. caninum* ELISA (31, 34).

The sensitivities and specificities of the N-MAT among different groups of animals are summarized in Table 3. The test was highly sensitive for all groups of animals, but the specificity was reduced in naturally versus experimentally, infected animals. This might be due to cross-reacting antibodies from exposure to closely related but unknown members of the Apicomplexa that have shared antigen epitopes. However, when animals with known infections with closely related apicomplexan parasites were tested by the N-MAT in this study, no cross-reactivity was observed. Therefore, another possible explanation for this variability between experimental and natural infections is the difference in the time course of infection. The experimental animals invariably had acute or recent infections compared to those of the naturally infected animals, many of which were chronically infected. Further, our definition for infection may be too stringent and thus biased. In this study, the definition of infected required confirmation of infection by the presence of lesions or organisms but ignored the possibility of animals having latent, undetected natural infection or exposure. Finally, the serum collected from animals with experimental infections was in optimal condition compared to that from the naturally infected animals. False-positive results were increased as a result, especially with severely hemolyzed sera or fetal fluid samples (Table 3).

The naturally infected-calf and the naturally infected-other-species groups (Table 3) had similar sensitivities and specificities when tested by the N-MAT, but the positive predictive value was greatly reduced in the naturally infected-other-species group due to low numbers of confirmed infections in this group (Table 3). Results from a much larger sample size for each species would allow for increased positive predictive values and confidence intervals.

The IFAT actually performed better overall for the naturally infected-other group than for the naturally infected-cattle group. This disparity may be a reflection of small sample size in the naturally infected-other group (Table 3). Additionally, it may be an artifact resulting from our working definition of positive within the group of *N. caninum*-infected bovine fetuses tested. Within this bovine fetal group, three histologically confirmed infected fetuses tested negative for antibodies to *N. caninum* by the IFAT but positive by the N-MAT. We chose to include these as positives since they had confirmed infections by histology; however, this may be incorrect since the production of fetal *N. caninum* antibodies requires the infected fetus to be sufficiently developed enough to have a mature immune system (~130 to 145 days of gestation) and to remain viable.
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