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# Detection of serum antibody responses in cattle with natural or experimental Neospora infections

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**Abstract.** Parasite-specific antibody responses were detected using an indirect fluorescent antibody (IFA) test in cattle that were naturally or experimentally infected with *Neospora* parasites. The test was developed using *Neospora* tachyzoites isolated from an aborted bovine fetus and grown in bovine cell cultures (isolate BPA1). In all cases, infections were confirmed by the identification of *Neospora* tachyzoites and/or bradyzoite cysts in fetal or calf tissues using an immunoperoxidase test procedure. Fifty-five naturally infected cows that aborted Neospora-infected fetuses had titers of 320-5,120 at the time of abortion. The titer of 6 cows that were serologically monitored over a prolonged period decreased to 160-640 within 150 days after they aborted infected fetuses. Two of the cows showed an increase in their *Neospora* titers during their subsequent pregnancy, and they gave birth to congenitally infected calves that had precolostral titers of 10,240-20,480. Postcolostral titers of these calves and of 4 other calves with congenital *Neospora* infections were all 25,120, whereas calves with no detectable parasites had titers ≤ 160. Two pregnant heifers that were experimentally infected with the BPA1 isolate at approximately 120 days gestation seroconverted to *Neospora* antigens within 9 days and developed peak titers of 5,120 and 20,480 within 32 days of infection. The fetus taken by caesarian section 32 days postinfection from 1 heifer and the full-term calf born to the other had *Neospora* titers of 640 and 10,240, respectively. Nine cows that aborted uninfected fetuses and 61 adult cattle maintained under pasture or feedlot conditions, where risk of exposure to *Neospora* was considered to be low, had titers ≤ 320. Some of the feedlot cattle tested had serologic reactivity that was restricted to antigens at the apical end of both *Neospora* and *Toxoplasma gondii* tachyzoites. This type of reactivity, which may result from serologic cross-reactivity between conserved apical complex antigens of closely related sporozoan parasites, differed from the whole parasite fluorescence that was observed with sera from Neospora-infected animals. The significance of these results and the potential application of the IFA test for the diagnosis of *Neospora* infections in cattle are discussed.

*Neospora* is a newly recognized tissue-cyst forming parasite that was first reported in cattle as an unknown sporozoan in calves [16,20,21] and subsequently recognized as a cause of abortion in dairy cattle. [1,2,23] The parasite was identified as a *Neospora* species based on the morphologic similarity of the thick-walled tissue cysts to those of *Neospora caninum* in dogs[6-8,11] and reactivity of the tachyzoite and/or cyst stages in cattle with antisera to *N. caninum*[3,5,10,12,14]

At present, the diagnosis of *Neospora* infections is based on the identification of characteristic lesions and/or tissue cysts in hematoxylin and eosin (HE)-stained histopathologic sections, combined with immunohistochemical evaluation to detect tachyzoites and confirm the identity of the parasites by their reactivity with *N. caninum* antisera. [1,3] Using this approach, Neospora infections have been confirmed in aborted bovine fetuses and/or congenitally infected calves in New Zealand 24 Australia, [12,16,18] Britain, [10,20] The Netherlands,[25] Japan,[19] and 17 states in the United States[15,17,22] (M. Anderson, unpublished data), and in Canada and Mexice (M. Anderson, unpublished data).

An indirect fluorescent antibody (IFA) test, developed using culture-derived tachyzoites, has been successfully employed to identify N. *caninum* infections in dogs.[13] However, the development of a similar test using the bovine *Neospora* parasite has not been reported. In the absence of a serologic test, there is no method for antemortem identification of *Neospora*-infected cattle.

The purpose of this study was to determine whether cattle that were naturally or experimentally infected with *Neospora* developed detectable antibody titers to a bovine *Neospora* isolate. The parasite isolate (BPA1) used as antigen in this test was recently obtained from

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an aborted bovine fetus and established in continuously growing in vitro tachyzoite cultures.[9]

## Materials and methods

*Parasites and antigen slide preparation.* Antigen slides were prepared using tachyzoites of the BPA1 *Neospora* isolate, which was obtained from an aborted bovine fetus and grown continuously in stationary monolayer cultures of bovine cardiopulmonary aortic endothelial (CPAE) cells (ATCC #CCL209)[a] as described previously.[9] Culture medium consisted of Dulbecco's Minimum Essential Medium (DMEM)[b] supplemented with 10% (v/v) heat-inactivated adult equine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin (DMEM-HS). Tachyzoites of *Toxoplasma gondii* (RH isolate; provided by J. Boothroyd) were obtained from CPAE cell monolayer cultures grown in the same medium except that 10% (v/v) heat-inactivated fetal bovine serum was used instead of equine serum. Parasite-infected cultures were maintained in 25 or 75cm$^2$ flasks incubated at 37 C in an atmosphere of 5% $CO_2$.

Parasites were harvested for antigen preparation when 280% of the CPAE cells in the culture flask were infected with clusters of tachyzoites. The infected monolayer was removed from the flask by scraping into the medium and then passed 3 times through a 25-ga needle to disrupt the cells. The suspension was passed through a 5-µm filter to remove cellular debris, and tachyzoites were pelleted by centrifugation at 1,300 x g for 10 min. After removing the supernatant, the pellet was washed twice in sterile phosphate-buffered saline (PBS) (pH 7.2) and then resuspended in a modified PBS saline (137 mM NaCl, 3 mM KCl, 3 mM $Na_3C_6H_5O_7$-$2H_20$, 0.4 mM NaH2PO4·$H_2O$, 12 mM $NaHCO_3$, 6 mM glucose) to a final concentration of approximately 2,000 tachyzoites/µl. Aliquots of 10 µ1 of tachyzoite suspension were dispensed into each 4-mm well on 12-well heavy-teflon-coated (HTC) antigen slides.[c] Slides were air dried at room temperature and stored at -70 C.

*Cattle.* Test sera were obtained from naturally infected cows that aborted Neospora-infected fetuses and from congenitally infected calves. In addition, sera was obtained from 2 pregnant heifers that were experimentally infected at approximately 120 days gestation with tachyzoites of the BPA1 isolate derived from CPAE cell cultures. Tachyzoites were obtained from cultures using the procedure described for harvesting tachyzoites for antigen preparation, except that the parasites were not washed in PBS and only the inoculum given to each heifer intravenously was filtered to remove cellular debris. After centrifugation, tachyzoites were resuspended in DMEM and administered by inoculation to each heifer so that 3 x $10^6$ tachyzoites were given intravenously and 5 x $10^6$ were given intramuscularly. A control heifer from the same herd and at the same stage of gestation was inoculated with an equivalent amount of uninfected CPAE cell culture material that was prepared and administered using the same procedures as for the infected heifers. Natural or experimental infections were confirmed by identification of *Neospora* tachyzoites and/or tissue cysts in fetal or calf tissues using an immunoperoxidase test procedure. [1,3]

For serologic comparison with samples from *Neospora*-infected cattle, sera were obtained from the following additional sources: 1) cows that aborted fetuses that did not have lesions or parasites typical of *Neospora* infections; 2) weak calves that were suspected of having *Neospora* infections but showed no lesions or parasites on postmortem histopathologic examination; 3) 20 heifers that were purchased as weanlings from a closed beef herd in Todd County, Nebraska, and maintained under strict isolation on range conditions at the Agricultural Research Development Center, University of Nebraska-Lincoln, in Mead, Nebraska; 4) 20 pregnant heifers that were maintained on pasture in California; and 5) 21 adult beef bulls or cows that were originally on pasture and then maintained in the same feedlot as the experimentally infected heifers.

*Serum collection and testing.* Test and control sera were obtained from blood samples that were collected by venipuncture into vacutainer tubes without anticoagulant. After storage at 4 C for 2-12 hr, the blood was centrifuged at 500 x g for 10 min and the serum was removed. Sera were stored either at 4 C for <48 hr or frozen at - 70 C until tested.

Antigen slides were thawed at room temperature immediately prior to use. Sera were initially titrated in 2-fold dilutions from 1:40 to 1:40,960 to determine the end-point titer. Ten microliters of diluted test or control sera were placed in separate wells on the antigen slides. Slides were incubated at 37 C for 1 hr in a moist chamber, washed 3 times for 5 min each in PBS, and then tapped gently to remove excess PBS. Fluorescein-labeled affinity-purified rabbit anti-bovine IgG[d] diluted 1:500 in PBS was added in 10-µ1 aliquots to each well. Slides were incubated at 37 C for 30 min, washed 3 times with PBS for 5 min each, tapped to remove excess PBS, cover-slipped with buffered glycerol (25% [w/v] glycerine in Tris-HC1 [pH 9.0]), and examined at 200 x using a fluorescence microscope. The end-point titer was the last serum dilution showing distinct, whole parasite fluorescence.

## Results

*Natural infections.* Sera collected at the time of abortion from 64 cows were tested for serologic reactivity to *Neospora* antigen (isolate BPA1) using the IFA test procedure. Aborted fetuses from 55 of these cows had nonsuppurative encephalitis and/or myocarditis that was consistent with a protozoa1 infection. In addition tachyzoite and/or cyst stages of *Neospora* were identified by immunohistochemical evaluation in the tissues of these 55 fetuses (Table 1). In the remaining 9 fetuses, there was no indication of encephalitis and/or myocarditis and no detectable protozoal parasites. All of the cows that aborted Neospora-infected fetuses had titers of 320-5,120 to *Neospora* antigens (Table 1). Eight of the cows that aborted fetuses with no detectable *Neospora* parasites had titers ≤ 160, and 1 had a titer of 320.

Six of the cows that aborted Neospora-infected fetuses were maintained on their 4 dairies of origin so that these cows could be tested repeatedly over a 6-12-month period to determine changes in the *Neospora*

**Table 1.** Serologic titers to bovine *Neospora* (BPA1 isolate) antigens after abortion of Neospora-infected fetuses.

| | | *Neospora* tissue stages in fetus | | |
|---|---|---|---|---|
| *Neospora* titer | No. cows | Cysts | Tachyzoites | Cysts + Tachyzoites |
| 320 | 1 | 0 | 1 | 0 |
| 640 | 12 | 2 | 8 | 2 |
| 1,280 | 12 | 2 | 10 | 0 |
| 2,560 | 15 | 2 | 11 | 2 |
| 5,120 | 15 | 5 | 7 | 3 |

titer. Peak titers of 640-2,560 were apparent within the first 20 days after abortion in all of the cows (Figs. 1, 2). Subsequently, the titers of 4 of the cows (Fig. 1, cows 9,970, and 522; Fig. 2, cow 578) decreased to 640, whereas the titers of cow 3 (Fig. 1) and cow 1328 (Fig. 2) dropped to 160 within 150 days postabortion. Cows 578 and 1328 were rebred and became pregnant again within approximately 50-70 days of aborting Neospora-infected fetuses. When these cows were approximately 4-5 months pregnant, their *Neospora* titers increased to their original peak levels of 1,280 and remained at this level until the cows gave birth to full-term calves (Fig. 2). The calf born to cow 1328 had a precolostral titer of 20,480, and twin calves born to cow 578 both had precolostral titers of 10,240 to the bovine *Neospora* isolate. Upon necropsy at 2-6 days of age, these calves showed mild nonsuppurative encephalomyelitis or focal mononuclear cell infiltrates in the brain parenchyma.[4] *Neospora* tissue cysts were seen in association with inflammatory lesions in all three calves. The postcolostral titers of sera taken from each calf prior to euthanasia were the same as their precolostral titers.

Serologic titers were determined for 4 additional calves that were diagnosed as having congenital Neospora infections based on the presence of characteristic cyst stages in the brain and/or spinal cord, which reacted immunohistochemically with antisera to the

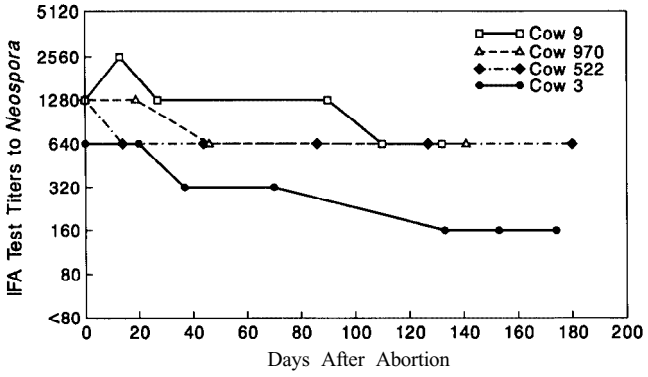


**Figure 1.** IFA test titers of serial samples from four cows that aborted Neospora-infected fetuses.

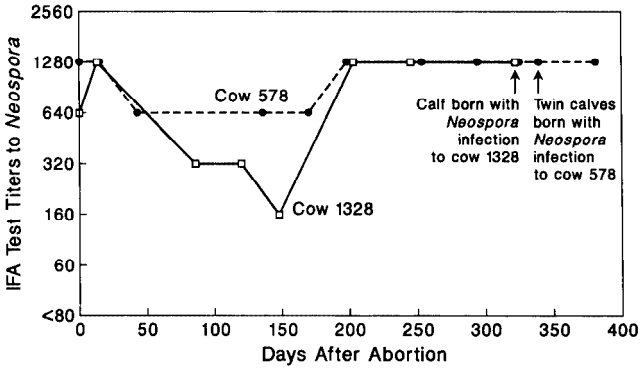


**Figure 2.** IFA test titers of serial samples from two cows that aborted Neospora-infected fetuses and subsequently delivered congenitally infected calves.

BPAl bovine *Neospora* isolate. *Neospora* was isolated from the brains and/or spinal cords of calves 1-3, and the parasites were grown continuously in vitro, using a previously described method for isolation of *Neospora* from aborted bovine fetuses.[9] At necropsy, calves 1 and 2 had *Neospora* titers of 20,480, calf 3 had a titer of 10,240, and calf 4 had a titer of 5,120. Serum collected from the dam of calf 4 at calving had a *Neospora* titer of 2,560. Precolostral calf sera and sera from the dams of the calves 1-3 were not available for testing.

The titers observed in the 7 congenitally infected calves with confirmed *Neospora* infections were markedly greater than those obtained from sera of 4 weak 1-5-day-old calves that were suspected of having Neospora infections but showed no histopathologic evidence of characteristic lesions or parasites on postmortem examination. One of these uninfected calves had a titer of 160, whereas the others had titers <80 to bovine *Neospora* antigens. Whether or not these calves had received colostrum was not known.

*Experimental infections.* Repeated serum samples taken from the 3 pregnant heifers prior to experimental inoculation on day 43 had titers of <80 to *Neospora* BPAl antigens. The 2 heifers that were infected with culture-derived tachyzoites of the BPAl bovine isolate developed *Neospora* titers of 640 by day 9 and 1,280 by day 18 after parasite inoculation (Fig. 3). The heifer that received uninfected cell culture material had titers <80 to *Neospora* antigens throughout the experiment. She was euthanized 32 days after inoculation to remove her fetus, which was viable, histologically normal, and uninfected, with no detectable titer to *Neospora*. Peak titers for both infected heifers were detected 32 days after parasite inoculation, at which time the fetus of heifer 413 was removed by caesarian section. Histologically, the fetus had inflammatory lesions and numerous *Neospora* tachyzoites in its central nervous system. In addition, *Neospora* tachyzoites were isolat-

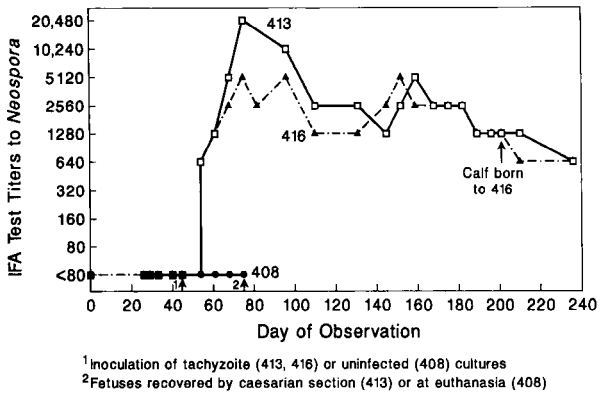


**Figure 3.** Seroconversion by heifers experimentally infected with *Neospora* (BPA1 isolate).

**Table 2.** Serologic titers to bovine *Neospora* and *Toxoplasma gondii* in cattle that have no evidence of *Neospora* infection.

| | | | *Neospora* | | *Toxoplasma* | |
|---|---|---|---|---|---|---|
| Cattle | No. | Location | Titer | No. | Titer | No. |
| Heifers | 20 | Nebraska pasture | ≤80 | 16 | ≤80 | 14 |
| | | | 160 | 3 | 160 | 6 |
| | | | 320 | 1 | ... | ... |
| Heifers | 20 | California pasture | ≤80 | 17 | ≤80 | 13 |
| | | | 160 | 3 | 160 | 3 |
| | | | | | not done | 4 |
| Cows | 9 | UCD* feedlot | <80 | 9 | ≤80 | 7 |
| | | | | | 160 | 2 |
| Bulls | 12 | UCD feedlot | ≤80 | 11 | ≤80 | 10 |
| | | | 160 | 1 | 160 | 2 |

* UCD = University of California, Davis.

ed from fetal tissues and grown continuously in cell culture. Serum collected from the fetus had a titer of 640 to *Neospora* antigens. After her fetus was removed, the *Neospora* titer of heifer 413 fluctuated between 1,280 and 5,120 until day 193 postinfection, when it dropped to 640 (Fig. 3). Heifer 416 calved 158 days after parasite inoculation, at which time she had a *Neospora* titer of 1,280 (Fig. 3). The calf had a precolostral *Neospora* titer of 10,240, which was the same as the sample collected 2 days later, after ingestion of the dam's colostrum. Clinically, the calf appeared normal except that it had decreased conscious proprioception in all 4 limbs when examined prior to euthanasia at 2 days of age. There were minimal histologic lesions, consisting of focal gliosis in the central nervous system, but no parasites were detected in fetal tissues.

*Uninfected cattle.* Fifty-three of the 61 (87%) adult cattle tested that had no history of *Neospora* infection had titers of ≤ 80, and all but 1 animal had titers ≤ 160 to both *Neospora* and *Toxoplasma* antigens (Table 2). The pastured cattle that were moved and subsequently maintained under feedlot conditions did not have higher serologic titers to tachyzoites of bovine *Neospora* or *Toxoplasma gondii* than did those kept on pasture. End-point titer determinations of all samples from infected or uninfected cattle were always based on the whole tachyzoite fluorescence. However, in testing the apparently uninfected animals, serum samples from 3 of the cows and 7 of the bulls that were housed in the feedlot had parasite fluorescence that was restricted to the apical end of the parasite. This reaction was particularly marked with the 7 sera from bulls that had apical fluorescence titers of 160-320 to both *Neospora* and *Toxoplasma,* whereas the whole parasite fluorescence titer was ≤ 80.

Figure 4 shows the serologic titers of the 61 uninfected adult cattle plus the 9 cows that aborted fetuses without evidence of *Neospora* infections as compared with the titers of Neospora-infected cows at the time of abortion or calving. Although a majority of the infected cattle had titers ≥ 1,280 to *Neospora* and most of the cattle that had no evidence of infection had titers 180, there was some overlap between these groups in the 160-640 titer range (Fig. 4).

## Discussion

This in-depth study is the first to show that cattle naturally or experimentally infected with *Neospora* develop a detectable serologic antibody response to the parasite. In addition, this is the first report of the use of the newly isolated bovine *Neospora* parasite (BPA1)[9] as antigen in an indirect fluorescent antibody test, which is similar to the test that was developed for the serodiagnosis of *Neospora caninum* infections in dogs.[13] The present study includes a large group of cattle with natural *Neospora* infections that were confirmed by the positive identification of *Neospora* cysts and/or tachyzoites in the tissues of their aborted fetuses. Sera taken at the time of abortion from these cows showed titers of 320-5,120 to *Neospora* antigens.

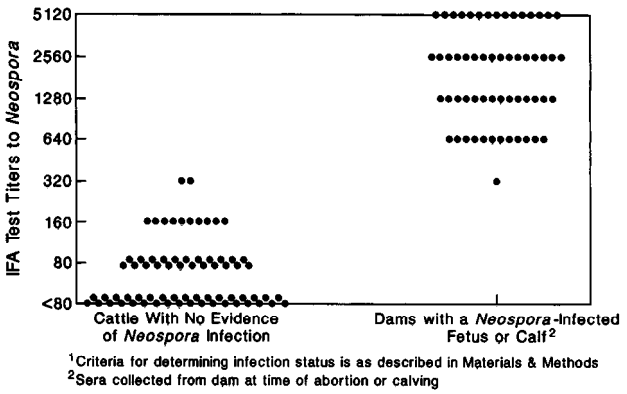


**Figure 4.** IFA test titers to *Neospora:* Comparing cattle with no evidence of infection to dams with Neospora-infected fetuses or calves.[1]

Since most large drylot dairies in California cull cows that abort, we were only able to repeatedly sample 6 of the 55 cows after their fetuses were diagnosed with *Neospora* infections. The *Neospora* titers of all of the cows declined over a 1-5-month period after abortion. However, the titers of the 2 cows that were successfully rebred returned to their original peak levels of 1,280 when the cows were approximately 4-5 months pregnant. This change could be due to reinfection of the pregnant cows, because both cows were maintained at the same time on the same dairy. However, the elevation in titer may have been in response to a reactivation of *Neospora* parasites that encysted in the dams' tissues after initial infection. Evidence from a prospective study involving 4 cows that were rebred following abortion of Neospora-infected fetuses suggests that reactivation of a latent infection during pregnancy is likely.[4] The release of the active, rapidly dividing tachyzoites might provide sufficient antigenic stimulation to trigger an increased antibody response in the dam as the organisms invade her tissues and infect the fetus.

Cows that have previously aborted Neospora-infected fetuses can subsequently abort another infected fetus (M. Anderson and B. Barr, unpublished data) or give birth to a congenitally infected calf.[4] This possibility is of considerable importance to veterinarians and dairy managers working with infected herds. Further experiments are needed to determine whether cows that have been infected with *Neospora* are susceptible to reinfection or whether they undergo parasite reactivation, perhaps induced by hormonal and/or immunologic alterations, during subsequent pregnancies. Results of the present study indicate that the IFA test could be employed as a means of monitoring reexposure of infected cows to tachyzoite antigens during pregnancy.

The *Neospora* IFA test was particularly useful for the antemortem diagnosis of congenital infections in calves. Precolostral serum samples obtained from 3 of the naturally infected calves had titers of 10,240-20,480, and all 7 calves that had *Neospora* tissue cysts had postcolostral titers $\geq$ 5,120. By contrast, the calves with titers of $\leq$ 160 showed no evidence of *Neospora* parasites or characteristic lesions in their tissues. Serodiagnosis by this method was used to rapidly identify infected calves so that tissues could be obtained for in vitro cultivation. Thus, time, effort, and money was not spent on trying to isolate organisms from uninfected calves, and the number of successful parasite culture isolations was increased.

Experimental infection of pregnant heifers with the BPAl bovine isolate of *Neospora* were undertaken to study the maternal and fetal immune response to the parasite. Tachyzoites inoculated into the dam were transmitted transplacentally, resulting in pathologic changes in the fetus that was taken by caesarian section and the live calf from the infected heifers. Both infected heifers seroconverted to *Neospora,* reaching peak titers of 5,120 and 20,480 by day 32 postinfection, after which their titers decreased to levels that were more comparable to those seen in samples taken at the time of abortion from naturally infected cows.

At present, bradyzoites within tissue cysts and tachyzoites are the only stages of *Neospora* that have been identified in cattle or other host species, and the complete life cycle of *Neospora* is unknown. However, if this parasite cycle is similar to those of closely related *Toxoplasma* or *Sarcocystis* species, cattle are most likely to be naturally infected by ingestion of sporulated oocysts, which may be passed in the feces of a carnivorous definitive host. Efforts are being made to identify the definitive host for the bovine *Neospora* parasites and to isolate the infective oocyst stage. However, the results of the present study indicate that regardless of the mode of natural infection, culture-derived tachyzoites are able to elicit seroconversion in infected heifers and the production of appreciable antibody levels in both the heifers and their progeny. Epidemiologic studies are currently under way to evaluate seroconversion of naturally infected calves and adult cattle to *Neospora* on dairies in California.

Although the present study was designed primarily to determine if Neospora-infected cattle developed a detectable antibody response to the parasite, we also evaluated the potential usefulness of the IFA test for serodiagnosis of neosporosis in cows. Strict criteria were established for selecting infected animals, which required the positive identification of tachyzoites and/or tissue cysts that reacted immunohistologically with *Neospora* antisera in the aborted fetus or calf. However, the identification of animals that were unquestionably free of *Neospora* was more problematic. In addition, the potential for false-positive reactions arising from antigenic cross-reactivity with other closely related protozoa, such as *Toxoplasma gondii,* had to be considered because this problem has arisen with IFA tests for other apicomplexan parasites.[26]

The results of this initial evaluation showed that some of the cattle that were selected as presumably low-risk animals had titers of 160 or 320 to *Neospora* and/or *Toxoplasma* antigens. Careful examination of the pattern of tachyzoite fluorescence produced in the IFA test revealed that in some cases sera from cattle maintained in the feedlot only reacted with the apical end of the parasites. This reactivity may be due to the presence of common antigens associated with the apical complex structures of *Neospora* and *Toxoplasma,* which might be highly conserved or cross-reactive with apical complex antigens of other closely related coc-

cidia, such as *Sarcocystis, Cryptosporidium,* or *Eimeria.* Proper interpretation of the bovine *Neospora* IFA test relies upon differentiation of apical fluorescence from whole parasite fluorescence, because only the latter is a true positive reaction. Antigenic cross-reactivity does not appear to be a major problem for the interpretation of the *Neospora* IFA test. However, this possibility should be examined more closely and efforts should be made to develop a more specific serodiagnostic test that will utilize antigen(s) from bovine Neospora isolates that are reliably reactive with sera from infected cattle.

The second major challenge to the serodiagnosis of bovine *Neospora* infections will be the establishment of a reliable cutoff titer for distinguishing infected and uninfected animals. Although most infected cattle had titers ≥ 1,280 and the titers of uninfected cattle were generally ≤ 80, a high proportion of infected dams (22%) had titers (320-640) that were within 2 dilutions of the titers (160-320) of many apparently uninfected cattle (16%). In addition, the serial samples taken from 6 cows showed that in all cases their titers decreased to 160-640 within the first 5 months postabortion. Since the antibodies that are detected in this assay are directed against tachyzoite antigens, this decline may occur either because the cow has eliminated the parasite or because there is a decrease in antigenic stimulation once the parasites have encysted in the dam's tissues. If this pattern of titer fluctuation after abortion is representative, then differentiation of Neospora-infected cows with low titers from uninfected cows could be difficult using the IFA test.

The results of these and other studies indicate that caution must be taken in the interpretation of serologic results if they are to be utilized in designing strategies for the control of neosporosis in cattle. Many questions remain unanswered. What does a serologic titer tell us about the outcome of pregnancy? Are cows that are seropositive to *Neospora* more likely to abort or produce a congenitally infected calf? Are heifer calves that are born with or acquire *Neospora* infections going to be more or less likely to reproduce successfully? Of particular importance to the producer is whether seropositive cattle should be maintained in the herd or culled. Further studies on the biology and pathogenesis of this newly recognized protozoa1 parasite should provide the answers to these important questions.

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