Sensitive and Specific Identification of *Neospora caninum* Infection of Cattle Based on Detection of Serum Antibodies to Recombinant Ncp29

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Neosporosis is an economically important disease of dairy cattle caused by the protozoan *Neospora caninum*. Diagnostic tests for neosporosis are complicated by the potential for cross-reaction of antibodies to antigens that are similar between *N. caninum* and closely related parasites *Toxoplasma gondii* and *Sarcocystis cruzi*. To provide a sensitive and specific assay for detecting antibodies to *N. caninum* in the serum of infected animals, we have investigated a recombinant form of the antigen known as Ncp29 (rNcp29), which is a major surface protein of the parasite. Ncp29 is encoded by a gene that is homologous to the SAG1 gene previously characterized from *T. gondii*. An enzyme-linked immunosorbent assay (ELISA) was used to screen animals for the presence of serum antibodies specific to rNcp29. The rNcp29 ELISA readily distinguished between cattle known to be infected with *N. caninum* (optical density [OD] > 1.2 at 1:500 or greater dilution) and negative controls (OD < 0.5 at 1:500). Additionally, sera from animals that were infected with *T. gondii* or *S. cruzi* were negative. The rNcp29 ELISA developed here provides a specific and sensitive assay for detecting neosporosis in cattle.

*Neospora caninum* is a coccidian parasite that causes abortions and neonatal morbidity and mortality in cattle, sheep, goats, deer, and horses (15). Neosporosis is an economically important disease in cattle, where it causes abortion and paralysis of newborn animals. Outbreaks of neosporosis are sporadic but have been reported in the United States, Sweden, The Netherlands, the United Kingdom, South Africa, Japan, Australia, and New Zealand (16). Neosporosis is also an important cause of paralysis in dogs (12), and it has been recently discovered that the dog is capable of oral-fecal transmission of this parasite (33). Herbivorous mammals, including dairy cows, presumably become infected by ingesting the resistant oocysts (a spore-like stage), which are passed in dog feces and which presumably become infected by ingesting the resistant oocysts (a spore-like stage), which are passed in dog feces and which contamination of feed, range, and water sources. Additionally, vertical transmission from mother to offspring is probably responsible for much of the infection burden in animals (16). Thus, control of neosporosis will ultimately rely on detection of infections in the dog and the various intermediate hosts such as cattle.

*N. caninum* is closely related to *Toxoplasma gondii* based on ribosomal small-subunit RNA sequences (31), and they have significant morphological, life cycle, and molecular similarities. Due to the close similarity between these two organisms, serological diagnosis is complicated by the potential for false-positive results due to antigenic cross-reactivity (6, 20). Their close similarity is evident from the many homologous genes that also exist in *N. caninum* (3, 9, 13). While an extremely important development for establishing the etiology of abortion in cattle due to protozoans, this assay requires experienced and trained personnel and is subjective in nature, making results from different laboratories difficult to compare. Due to the reliance on whole antigens, the IFAT also increases the likelihood of cross-reaction with antibodies to conserved antigens that may be found in other related parasites. One example of this is that polyclonal sera from animals immunized or infected with *N. caninum*...
often recognize cross-reacting proteins in lysates of *T. gondii* (6, 20). While these cross-reacting bands are typically weaker than the homologous antigen preparation and thus can be factored out by using appropriate dilutions, cross-reactions still complicate the development of standardized diagnostic tests. Consequently, there is a need for development of standardized diagnostic test that offers reliable, sensitive, and specific detection based on defined *Neospora*-specific molecules.

Despite a high degree of molecular similarity between *T. gondii* and *N. caninum*, we have observed that a majority of the monoclonal antibodies to *T. gondii* that are generated do not cross-react to *N. caninum* (23) and that, correspondingly, monoclonal antibodies to cell surface proteins Ncp29 and Ncp35 do not cross-react to *T. gondii* (21). Surface protein Ncp35 has recently been purified in a native form from the parasite and used in an enzyme-linked immunosorbent assay (ELISA) to detect antibodies in infected cattle (35). However, the use of native antigens requires the propagation of the parasite in mammalian cell culture, an expensive and time-consuming process. Recombinant antigens have the added benefit that they are easily produced in large quantities and can be readily standardized for diagnostic assays. This led us to explore the possibility of using recombinant antigens to develop a species-specific diagnostic assay for neosporosis.

We have designed an ELISA that utilizes recombinant Ncp29 (rNcp29), an immunodominant surface antigen of *N. caninum* tachyzoites. This assay provides a sensitive and specific detection of antibodies in sera of infected animals and is not complicated by cross-reactions to antisera against related parasites such as *T. gondii*.

### MATERIALS AND METHODS

**Recombinant-protein production.** The open reading frame of the gene encoding Ncp29 (GenBank accession no. AF132217) was amplified with KlenTaq-LA polymerase (Clontech) from genomic DNA of the Nc-1 strain of *N. caninum*. The sequence of the forward primer was 5′-GATCCATATGTGAAAATACTACCTCTA3′. The sequence of the reverse primer was 5′-GATCTTCGAGCGCTATCGAGGACTACG3′. The PCR-amplified fragment was ligated into an NdeI- and XhoI-digested pET-22b expression vector (Novagen) by using NdeI and XhoI restriction sites that were incorporated into the amplification primers. The resulting recombinant protein lacks the predicted 30-amino-acid signal pepti- 

de at the N terminus and the last 4 amino acids of the carboxyl terminus of the native protein and contains a C-terminal histidine tag. Plasmid prNcp29 was transformed into *Escherichia coli*, BL21, and a clone that expressed high levels of rNcp29 was selected for use. Following induction with IPTG, rNcp29 was extracted from inclusion bodies with 6 M urea, and the protein was purified by nickel column chromatography according to the manufacturer’s protocol (Novagen). The purified protein was concentrated with Centricon-10 columns (Amicon), and the protein concentration was determined by a colorimetric protein assay (Pierce) prior to storage at −80°C until use.

**SDS-PAGE and Western blotting.** Tachyzoites of the Nc-1 strain of *N. caninum* were propagated in human fibroblasts and purified as described previously (21). Parasite cell pellets and lysates of bacteria expressing recombinant Ncp29 were resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and separated on 10% acrylamide gels (25). Proteins were transferred to nitrocellulose by semidry electrophoresis in Tris-glycine, pH 8.3, and Western blotted by using horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Labs) and chemiluminescence (Pierce) as described previously (21). Primary antisera consisted of sera from mice that were chronically infected with *N. caninum* and previously described monoclonal antibod- 

eyd6C11, which reacts to Ncp29 (21).

**ELISA.** Purified rNcp29 was diluted to 10 μg/ml in phosphate-buffered saline (PBS), 100 μl of the mixture was added to each well in high-binding-capacity ELISA plates (Corning), and wells were incubated for 1 h at 37°C or overnight at 4°C. The plates were rinsed three times with PBS containing 0.05% Tween 20 and blocked for 1 h with PBS containing 0.05% Tween 20 and 1% gamma globulin-free bovine serum albumin (BSA; Sigma, St. Louis, Mo.). Antisera were diluted in PBS containing 0.05% Tween 20 and 0.1% BSA, 100 μl of the mixture was added to each of duplicate wells of the ELISA plate, and the wells were incubated for 2 h at room temperature (RT). The wells were rinsed three times with PBS containing 0.05% Tween 20 and incubated for 2 h at RT with 100 μl of protein G-horseradish peroxidase conjugate (Pierce) diluted to 1:2,500 in PBS containing 0.05% Tween 20 and 0.1% BSA. Finally the plate was rinsed four times with PBS containing 0.05% Tween 20. Chromogenic substrate o-phenyl-

enediamine dihydrochloride (Sigma) was dissolved in 0.05 M phosphate-citrate buffer to a concentration of 0.4 mg/ml and 200 μl of substrate was added to each well. After a 10-min incubation, the reaction was stopped with 50 μl of 2 M H2SO4, and the A590 was measured in an ELISA plate reader. Samples were performed in duplicate, and mean values are reported.

**Bovine sera.** Samples were obtained from three uninfected cows and again following experimental infection with tachyzoites of the BPA-1 isolate of *N. caninum* (1, 8). Serum samples were also obtained from 10 known positive animals and 10 known negative animals, as determined by IFAT (9). A complete description of the sera and the clinical histories for the animals can be found in Table 1. To assess the specificity of the rNcp29 ELISA, we also obtained serum samples from bovine animals that were experimentally infected with either *Sarcocystis cruzi* or *T. gondii* (17).

### RESULTS AND DISCUSSION

To assess the immunoreactivity of rNcp29, the recombinant protein was expressed in *E. coli* as a His-tagged fusion protein and purified by nickel chromatography. rNcp29 was readily recognized in Western blots by antisera from *Neospora*-in- 

fected mice and by murine monoclonal antibody 6C11, which had been previously selected for its reactivity to native Ncp29

| Samplea | Animal Type | Diagnosisa | Titerb | IFAT | rNcp29 ELISA |
| --- | --- | --- | --- | --- | --- |
| N1 25Z | Adult | Negative | <80 | <100 |
| N2 6674 | Adult | Negative | <80 | <100 |
| N3 369 | Adult | Negative | <80 | <100 |
| N4 370 | Adult | Negative | <80 | <100 |
| N5 405 | Adult | Negative | <80 | <100 |
| N6 2290 | Adult | Negative | <80 | <100 |
| N7 354 | Adult | Negative | <80 | <100 |
| N8 374 | Adult | Negative | <80 | <100 |
| N9 398 | Adult | Negative | <80 | <100 |
| N10 364 | Adult | Negative | <80 | <100 |
| P1 CS1 | Calf | Positive | 20,480 | 16,000 |
| P2 CS2 | Calf | Positive | 20,480 | 32,000 |
| P3 1088 | Adult | Positive | 20,480 | 32,000 |
| P4 2744 | Adult | Positive | 1,280 | 16,000 |
| P5 48291 | Calf | Positive | 1,280 | 16,000 |
| P6 5629 | Adult | Positive | 10,240 | 32,000 |
| P7 CS3 | Adult | Positive | 640 | 8,000 |
| P8 522 | Adult | Positive | 160 | 8,000 |
| P9 927 | Adult | Positive | 640 | 8,000 |
| P10 3923 | Adult | Positive | 640 | 8,000 |
| Pre1 A114 | Adult | Preinoculation | <80 | ND |
| Post1 A114 | Adult | Postinoculation | 10,240 | ND |
| Pre2 7 | Adult | Preinoculation | <80 | ND |
| Post2 7 | Adult | Retest | 10,240 | ND |
| Pre3 A114 | Adult | Preinoculation | <80 | ND |
| Post3 A114 | Adult | Postinoculation | 10,240 | ND |

a Pre, sample from clinically normal cow taken before experimental infection; Post, sample from the same cow taken after infection (1, 8).
b Positive, positive immunoperoxidase result and detectable parasite clusters, with free tachyzoites detected in tissues of infected or aborted offspring; negative, healthy, normal control.
c Reciprocal titer, where the OD was twofold greater than that for the pooled negative sera. ND, not done.
d Calves were infected congenitally and were sampled postoclomost.
rNcp29 assay was more sensitive, detecting higher titers in animals P7, P8, P9, and P10 than the IFAT. The positive cutoff for the IFAT is considered to be 1:320; therefore animal P8 would have been missed by this assay yet was clearly positive by the rNcp29 ELISA. There was no significant increase in ELISA titers for the negative animals relative to the IFAT titers.

Previous serological assays have been hampered by the potential for cross-reaction of *Neospora* antigens with those of closely related parasites such as *T. gondii* (6, 20). Therefore, an additional 23 bovine antisera from animals that had been experimentally infected with either *T. gondii* or *S. cruzi* were also tested by the rNcp29 ELISA. With the exception of a single animal that had been experimentally infected with *Sarcocystis* (sample S9), all of the control antisera exhibited an OD<sub>490</sub> of <0.5, while, by comparison, the known positive animals listed in Table 1 exhibited OD<sub>490</sub> values of >1.5 (Fig. 3). Further examination of the sample from animal S9 using a variety of assays revealed that this animal had an elevated anti-*Neospora* titer, thus indicating that it had previously been naturally infected with *N. caninum* (17).

Despite the similarity with related gene product SAG1, which occurs in *T. gondii*, we did not detect antigenic cross-reaction between *N. caninum* and *T. gondii* by using rNcp29. Animals infected with *N. caninum* reacted strongly to Ncp29 while those infected with *T. gondii* showed negative ELISA titers. Proteins Ncp29 and SAG1 (also known as p30) from *T. gondii* have approximately 50% identity at the amino acid level, and both contain 12 cysteine residues that are predicted to be involved in the conformational folding of the protein, a property that is likely important for the highly antigenic nature of SAG1 (5, 7, 24). Following infection with *T. gondii*, antibodies to SAG1 appear very early and include immunoglobulin M (IgM) and IgA, as well as IgG classes (10, 34), making this
antigen ideally suited for detection of acute infection. Due to its unique antigenicity, surface location, and abundant expression in *N. caninum*, Ncp29 will likely prove to be an excellent antigen for detection of early infection during outbreaks of neosporosis.

Isolation of recombinant parasite antigens that react to sera from *N. caninum*-infected animals has previously been used to identify several other candidate antigens for serodiagnosis. Two such clones, initially called Nc4.1 and Nc14.1, have been used in an ELISA to distinguish between infected and noninfected cows by detecting antibodies in the serum (26). Completion of the sequences originally identified by Nc14.1 and Nc4.1 revealed they are homologous to products of the GRA6 and GRA7 genes, respectively, of *T. gondii*, which encode proteins found in secretory dense granules (22, 26, 27). In separate studies, clones N54 and N57 were isolated based on screening a recombinant library with serum from a naturally infected cow (29). Antigen N54 corresponds to clone Nc4.1 described above, while N57 is a portion of a subtilisin-like protease (28). These were utilized as recombinant proteins for development of an ELISA for screening herd serum based on the presence of specific antibodies to *N. caninum* (29). The recombinant antigens did not show cross-reactivity to *T. gondii* and performed significantly better in the ELISA than whole tachyzoite antigens. However, in both cases, a wide range of positive values are observed in infected animals, and hence the difference between negative and positive sera is often slight, a problem also encountered by using the Nc41 and Nc14.1 antigens described above (leading to overlapping values or differences of less than 0.1 OD unit). In contrast, the rNcp29 ELISA described here detected a large difference between positive (OD ≥ 1.2) and negative (OD ≤ 0.5) sera. Whether this is due to a higher degree of sensitivity remains to be established by further testing of additional samples, for example, from field surveys of naturally infected herds. Additionally, it will be important to evaluate the assay on longitudinal samples from animals pre- and postabortion to determine the positive predictive value of specific titers.

Although we have only evaluated the rNcp29 ELISA with bovine samples, the assay may also be useful for diagnosis of neosporosis in dogs. Canids are a definitive host for *N. caninum* (33), and detection of infections in dogs could be of practical value in two ways. First, adequate control of neosporosis on cattle farms will ultimately depend on disrupting transmission. Good management practices to prevent infection will be aided by determining which animals are infected. Secondly, neosporosis is a cause of neurological disease in dogs (11, 14, 16). Early detection of these infections, combined with adequate treatment, could prevent recurrent episodes of congenital infection and paralysis that have been known to occur in dogs (11, 12).

We have recently reported that the gene encoding the p29 antigen is entirely conserved among a variety of isolates of *N. caninum* from bovine and canine hosts from North America and Europe (GenBank accession no. AF141960, AF141961, AF141962, and AF141963) (32). Such a high degree of conservation indicates that the rNcp29 ELISA will be widely applicable to a range of different isolates that could potentially infect different hosts species. Experimental infections of nonhuman primates indicate a capacity of *N. caninum* to cause congenital infection and disease in rhesus monkeys (4). While *N. caninum* is generally not considered an agent of human infection, exposure of humans by consumption of animal tissues and close contact with infected animals may pose a risk of infection. Indeed, a recent survey indicated positive serological titers in several humans suggesting that additional testing is warranted (36). The interest in emerging pathogens, particularly those which may be food borne, supports such screening, and it is likely that the rNcp29 ELISA described here will provide a sensitive assay to conduct such serological surveys in the future.

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