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On the Efficacy and Safety of Vaccination with Live Tachyzoites of *Neospora caninum* for Prevention of *Neospora*-Associated Fetal Loss in Cattle

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Infection of cattle with *Neospora caninum* may result in abortion or the birth of a congenitally infected calf. Vaccination with live *N. caninum* protects against experimental infection of cattle and mice, and the naturally attenuated Nc-Nowra strain of *N. caninum* is of particular interest as a potential vaccine candidate. Vaccination of heifers prior to breeding with live Nc-Nowra tachyzoites by either the subcutaneous or the intravenous route reduced the rate of abortion and the presence of the parasite in calves as determined by PCR and serology after infection of cows with a virulent isolate. Protected fractions were 55.6% to 85.2% depending on the route of vaccination and growth conditions of the vaccine strain, with cryopreserved Nc-Nowra tachyzoites being less effective, with a 25.9% protected fraction. Vaccination appeared to reduce the rate of pregnancy after artificial insemination in some groups compared to nonvaccinated, nonchallenged controls. One animal that was vaccinated but not challenged experienced an abortion, but Nc-Nowra could not be detected in any of the cows in this group or their progeny. This study confirms that live vaccination can be an effective method of preventing neosporosis in cattle and yet highlights the technical hurdle of preservation of live parasites that must be overcome for a vaccine to be commercially successful.

Neospora caninum is a protozoan parasite first identified in dogs but now mostly recognized as being associated with fetal death and abortion in midterm pregnant cattle (1). Although canids such as dogs and dingoes are known to be definitive hosts for this parasite (2), the main form of transmission among cattle is transplacental *in utero* from the dam to its fetus. Abortion and fetal loss are the clinical manifestation of this route of transmission, as a consequence of a recrudescent parasitemia occurring during pregnancy (3). Chronic, long-term infection by *N. caninum* of cattle is widespread, occurring in many cattle-producing countries of the world.

Abortion and fetal loss in cattle are one area of considerable concern in the livestock industries, and a major proportion of the losses can be attributed to infectious diseases, including neosporosis (4). In Australia, for example, 1 to 5% of pregnant cattle typically abort, with the major proportion of these being attributed to neosporosis. Mathematical modeling, including decision tree analysis, has evaluated the potential for control of *N. caninum*-associated abortion in cattle, suggesting that vaccination is the only realistic economical form of control (5). Test-and-cull, where infected cattle are identified by serology and then removed from breeding programs, is used infrequently on farms when veterinary investigations are conducted into causes of abortion (6). Despite the high cost of implementing test-and-cull programs, and in the absence of an efficacious vaccine, it currently remains the only option available to cattle producers.

The development of vaccines against neosporosis has been discussed previously (7). A killed vaccine (NeoGuard; Intervet/Schering Plough), based on inactivated tachyzoites, was developed previously (8, 9) but did not gain widespread acceptance due to doubts on efficacy. An alternative approach being considered, which is based on the injection of live tachyzoites before pregnancy, relies on the observation that protective immunity that prevents fetal death and abortion can be induced in cattle (10, 11). Such immunity is thought to be mediated by cell-mediated immune mechanisms such as those associated with gamma interferon (IFN). Vaccines based on recombinant proteins may also be possible. One emerging formulation uses cyclophilin as the immunogen, giving rise to increased levels of gamma interferon and protective immunity in a nonpregnant mouse model (12).

The study described here aimed to investigate further the practicalities of live vaccination against neosporosis in cattle. Using the Nc-Nowra isolate of *N. caninum*, the effect of the route of administration of a live formulation was investigated in a "vaccinate and challenge model of fetal death" similar to the one described by Williams et al. (11). Subcutaneous and intravenous routes of injection of live Nc-Nowra tachyzoites were compared for their ability to protect against a fetopathic challenge given during pregnancy. A frozen and thawed preparation of tachyzoites was also included in the study to assess the potential for preservation of a live parasite vaccine. The results provide further evidence on the efficacy of a live vaccine approach for the prevention of *Neospora*-associated abortion and fetal death in cattle. The limitations of a live vaccine against neosporosis in cattle are also discussed.

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TABLE 1 Study design and vaccination protocol[a]

Treatment group	Vaccination culture and regimen.	Challenged
1	Saline placebo, day 0	Y
2	Nc-Nowra, i.v., RPMI medium, day 0	N
3	Nc-Nowra, i.v., RPMI medium, day 0	Y
4	Nc-Nowra, s.c., RPMI medium, day 0	Y
5	Nc-Nowra, s.c., RPMI medium, frozen/thawed, day 1	Y
6	Nc-Nowra, i.v., PMEM, day 0	Y
NTX	None	N

[a] Abbreviations: i.v., intravenously; s.c., subcutaneously; Y, yes; N, no.

MATERIALS AND METHODS

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Thomas D. Morris, Inc., where the trial was conducted. One hundred ten beef heifers approximately 16 months of age were located and purchased by Thomas D. Morris, Inc., and screened for antibodies to *N. caninum* by competitive enzyme-linked immunosorbent assay (cELISA) conducted at the Washington Animal Disease Diagnostic Laboratory at the College of Veterinary Medicine at Washington State University in Pullman, WA.

Animals and trial design. Ninety-six animals that were never vaccinated against *N. caninum* and demonstrated <30% inhibition in the cELISA were chosen for inclusion in the study. On day 0, all heifers were randomly allocated into 7 groups (6 groups of 15 animals and 1 group of 6 sentinel animals). All animals except those allotted to groups 1 and 5 and the sentinels (NTX) were vaccinated with 1×10^7 *N. caninum* Nc-Nowra tachyzoites either subcutaneously or intravenously as described in the study design (Table 1). Heifers in group 5 were vaccinated on day 1 using a frozen and thawed parasite suspension containing 1×10^7 tachyzoites/ml. All animals received 1 ml of vaccine or placebo. Heifers were vaccinated once against infectious bovine rhinotracheitis (IBR) virus, parainfluenza virus type 3 (PI3), bovine respiratory syncytial virus (BRSV), and bovine viral diarrhea (BVD) virus types 1 and 2 with Bovi-Shield Gold FP5 (Pfizer Animal Health) 21 days prior to vaccination with *N. caninum*. The animals were maintained on pasture until just prior to challenge, at which time they were transported to a biosafety level 2 facility. At 327 days postvaccination, animals were moved to a separate necropsy facility.

Estrus was synchronized by intramuscular injection of 6 ml of Lutalyse on day 49 after vaccination, followed by a second dose on day 60 postvaccination. Heifers were bred by artificial insemination approximately 72 and 80 h following the second Lutalyse injection, were monitored for signs of standing estrus for up to 10 days, and were bred again if estrus appeared. Breeding occurred from days 63 through 75 postvaccination, with some animals bred up to 5 times. Heifers were examined by ultrasound at 120 days postvaccination (approximately 57 days of gestation) to determine pregnancy status by detection of fetal heartbeat and/or movement. Heifers were reexamined for pregnancy status on days 138, 167, 195, and 223 postvaccination.

On day 139 postvaccination, confirmed pregnant heifers, except for those in group 2 and NTX, were challenged by intravenous administration of 1 ml of parasite suspension containing 1.2×10^6 *N. caninum* Nc-S197 tachyzoites/ml. Twenty-five heifers that never became pregnant were euthanized on day 140. In addition, one heifer from each of groups 1 and 2 which were pregnant initially but found to be open after day 121 and two pregnant heifers from group 2 were euthanized on day 140. The reproductive tracts were removed and examined grossly for evidence of resorbed pregnancies. Uterine and oviduct samples from each heifer were placed in formalin for histopathological examination. Specimens were submitted to the Borgess Research Institute, Medical Device Research Laboratory, Portage, MI, for processing to slides. Tissues were stained with hematoxylin and eosin, and sections were examined by light microscopy. Blood was collected for measurement of antibodies on days 138, 167, and 195 postvaccination. After challenge, animals were observed at least once daily for signs of estrus or abortion. Aborted fetuses and/or placentas; spleen, heart, lung, and liver; and rostral, temporal, and occipital sections of the cerebellum, cerebrum, brain stem, and spinal cord were collected where possible and frozen at −80°C. Blood was collected from any heifer that aborted at the time of abortion and again 2 to 3 weeks later. On days 334 and 336 postvaccination, all remaining pregnant heifers were euthanized. Fetuses were removed, a blood sample was collected from each fetus, and the fetuses were euthanized. Samples of spleen, heart, lung, and liver and rostral, temporal, and occipital sections of the cerebellum, cerebrum, and brain stem were collected from each fetus and frozen at −80°C. In addition, brain tissue from eight heifers in each of group 1 (nonvaccinated, challenged) and group 2 (vaccinated, nonchallenged) was collected for mouse bioassay and PCR for detection of *Neospora caninum*. Sampling of heifers comprised 5 g of tissue from each of the left and right rostral, temporal, and occipital lobes of the cerebrum, cerebellum, and brain stem/spinal cord.

Nc-Nowra cell culture. *Neospora caninum* Nc-Nowra was maintained on Vero cells grown in either RPMI medium (Sigma catalog no. R5885) with 2% equine serum (Quad Five catalog no. 262-500) and 1% L-glutamine (Gibco catalog no. 25030) and antibiotics (penicillin-streptomycin solution; Gibco catalog no. 15140-122) or a proprietary minimal essential medium (PMEM) supplemented with 1% bovine serum (JRH Biosciences; catalog no. 12107-1000 M) and antibiotics. To prepare the vaccine culture, frozen seed stock of *N. caninum* Nc-Nowra was thawed at 37°C and added to monolayers of host cells grown in tissue culture flasks. The culture medium was removed and replaced with fresh medium 6 to 8 h after infection. The medium was replaced every 3 to 4 days if needed until tachyzoites were observed breaking out of the host cells. The cells and tachyzoites were then harvested by removing the cell layer with a sterile cell scraper and centrifuging the resulting suspension of cells and tachyzoites for 15 min at 1,000 × *g*. The pellet was resuspended in 5 ml of Hanks' balanced salts solution (Gibco catalog no. 14170-112) supplemented with 20 mM HEPES buffer (Gibco 15630-080) (HBSS-HEPES), and remaining host cells were disrupted with a stomacher (Seward model 80). The number of tachyzoites was determined by direct count. The suspension was then used to further passage the parasite culture for amplification. The vaccine was prepared from a pooled suspension from multiple flasks after two passages, adjusted to contain 1×10^7 tachyzoites/ml as determined by direct count.

Parasites for vaccination of group 5 were frozen at −80°C at 1×10^8 tachyzoites/ml in HBSS-HEPES with 3.5% bovine serum albumin and 15% dimethyl sulfoxide (DMSO). This formulation was demonstrated to yield 50 to 90% viability after a −80°C freeze-thaw cycle (results not shown), although the viability of the vaccine preparation used in this study was not determined. After harvest, counting, and centrifugation of tachyzoites, the pellet was resuspended in 2.5 ml of cryopreservative solution, held at 4°C for 1 h and −10°C for 2 h, and then transferred to −80°C and held overnight. The frozen preparation was thawed in a 37°C water bath and diluted to 25 ml with HBSS-HEPES to obtain a final concentration of 1×10^7 tachyzoites/ml. The concentration of tachyzoites was confirmed by direct count, and the vaccine culture was administered to animals within 2 h of thawing.

Challenge strain. The challenge strain *Neospora caninum* Nc-S197 was isolated from the brain of a congenitally infected calf from a U.S. beef herd suffering from an outbreak of neosporosis in 1997. The calf died shortly after birth; brain tissue was removed, homogenized, and injected intraperitoneally into *scid/beige* mice; and the parasite was subsequently isolated from the lung into culture. The organism was propagated for 10 *in vitro* passages and then frozen as working stock. Parasites were cultivated on MA104 cells growing in Opti-MEM medium (Gibco catalog no. 31985-070) supplemented with 5% fetal bovine serum (JRH Biosciences catalog no. 12107-1000 M) and antibiotics (penicillin-streptomycin solution; Gibco catalog no. 15140-122). Parasites were passaged and harvested

TABLE 2 Primers used for diagnostic and microsatellite PCR

Target sequence	Forward	Reverse	Expected product size (bp)
NC5 outer	5′-GGT TGT GTT TGG CTG TGA AGG ACA-3	5′-CCA TTC ATC ACT GCC ACC ACG AAT-3	1,035
NC5 inner	5′-CAG GGT GAG GAC AGT GTG TAC AT-3′	5′-GTC CGC TTG CTC CCT ATG AA-3′	110
MS10 outer	5′-TCG TCG GAC ACA AAA GTG AG-3	5′-CCT TTC TCT CTT CCT ATA GC-3′	460
MS10 inner	5′-CCC TCG TGT CGT ACT CGT AG-3	5′-CCC TGT TTG ACG TAG ATT GA-3	148 (Nc-Nowra), 139 (S197)

as described above. The challenge culture was pooled from multiple flasks and adjusted to 1.2×10^6 tachyzoites/ml in HBSS-HEPES after 4 passages *in vitro*.

ELISA. Antibodies to *N. caninum* in serum samples taken from heifers during the trial were assayed by a direct ELISA. Soluble antigen was prepared by subjecting tachyzoites of *N. caninum* strain PB-1-2c to three freeze-thaw cycles followed by sonication and centrifugation to remove cell debris. The solution was adjusted to contain 1 mg protein/ml, and protease inhibitors were added. Nunc Maxisorp plates were coated with 200 ng protein per well after dilution in 0.01 M sodium tetraborate buffer. Plates were blocked for 2 h at 37°C with 1% casein in Dulbecco's phosphate-buffered saline (DPBS). Serum samples were diluted in 1% casein-DPBS and added to the plates, and then the plates were incubated for 1 h at 37°C. After three washes with DPBS-0.05% Tween 20, peroxidase-conjugated goat anti-bovine IgG (KPL catalog no. 14-12-06 or Jackson Laboratories catalog no. 101-035-003) diluted 1:3,000 in 1% casein-DPBS was added to each well, and the plates were incubated for 1 h at 37°C. Plates were washed again, and 100 μl of peroxidase substrate (KPL catalog no. 50-62-00) was added to each well. Plates were then incubated at room temperature, and the optical density (OD) of each well was read at 405 nm. A sample was considered positive if the OD was greater than that of the average OD plus 3 standard deviations of the negative-control serum. Pretrial serum samples and samples of fetal fluids were assayed by competitive ELISA (cELISA) at the Washington Animal Disease Diagnostic Laboratory at the College of Veterinary Medicine at Washington State University in Pullman, WA.

Molecular assays. To detect parasites in tissues by PCR, frozen fetal tissue and heifer brain samples were thawed and diced with scissors, and a 5-g sample was added to 20 ml of saline. The mixture was homogenized in a stomacher (Seward model 80) to yield a 20% suspension of tissue. Total DNA was extracted from 100 μl of the suspension using Qiagen DNeasy extraction kits. The extracted DNA was subjected to a nested PCR diagnostic assay for detection of *N. caninum* DNA. Primers were designed to detect the NC5 diagnostic gene sequence (13) and are provided in Table 2. For the outer reaction, 1 unit of Platinum *Taq* DNA polymerase (Invitrogen catalog no. 10966-026), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs) (Invitrogen catalog no. 18427-103), 0.4 μM (each) primer, and 2 μl of template DNA were combined in a total volume of 50 μl. Reactions were performed in 96-well plates sealed with a pierceable aluminum seal (AlumaSeal II pierceable sealing film for PCR; Excel Scientific catalog no. AF100), and reaction products were diluted 1:10 in a second 96-well tray before being transferred into a third 96-well tray containing the reaction mix for the inner PCR. The inner reaction mixture contained 25 μl of PCR Master Mix (Applied Biosystems catalog no. 4304437), 0.6 μM (each) primer, and 5 μl of 10-fold-diluted product from the first reaction in a total volume of 50 μl. The reaction conditions are provided in Table 3. The products of the second reaction were examined on 2% agarose 48-well e-gels (Invitrogen catalog no. A10571) for the presence of bands of the expected size. In order to differentiate between the vaccine and challenge strains, a PCR assay based on the MS10 microsatellite sequence (GenBank accession number CF659321) was used for selected samples. This sequence exhibits a high degree of length polymorphism between *Neospora* strains (14). A nested PCR assay for detection of the sequence (15) was adapted for use with the Nc-Nowra and S197 strains by using a pair of primers described in reference 16. The inner and outer primer pairs are listed in Table 2. The reaction mixture used for the outer reaction was the same as that used for the outer reaction of the NC5 assay. The reaction mixture for the inner reaction was identical with the exception of the use of 2 mM $MgCl_2$ instead of 1.5 mM. Using 4% agarose e-gels (Invitrogen catalog no. G5018-04), the products of the nested PCR from the two strains could be readily distinguished based on the difference in their size. All PCRs were carried out in a Bio-Rad C1000 thermal cycler.

In order to determine whether vaccination with live *N. caninum* Nc-Nowra resulted in active infection in the brains of vaccinated animals, brain tissue samples from the vaccinated, nonchallenged group (group 2) were examined for the presence of parasite DNA by diagnostic PCR and for the presence of viable parasites by mouse bioassay (see below). Brain tissue samples from the nonvaccinated, challenged group (group 1) were also examined in these assays. In addition, serum samples from selected heifers in groups 1 and 2 were examined by diagnostic PCR to detect the presence of parasite DNA. If parasite DNA was detected by diagnostic PCR in any of the samples, the sample was also tested in the microsatellite nested PCR assay to confirm the identity of the parasite strain.

Bioassay. A bioassay using interferon-knockout (IFN-KO) mice (Jackson Laboratories; B6.129S7-IFNg^{tm1ts}/J) was used to detect *Neospora caninum* in brain tissue of six nonvaccinated, challenged cows (group 1) and eight vaccinated, nonchallenged cows (group 2). Brain tissue was collected and homogenized as described above, and samples of homogenates from the different brain regions (left and right rostral, temporal, and occipital lobes of the cerebrum, the cerebellum, and the brain stem/spinal cord) were pooled to provide a single sample from each animal. The pooled samples were treated with trypsin (0.05% final concentration; Invitrogen catalog no. 15400054) at 37°C for 30 min. The suspension was centrifuged and washed twice in DPBS, and the pellet was resuspended in a volume of saline containing antibiotics (1,000 IU of penicillin and 100 μg streptomycin per ml; Gibco catalog no. 15140-122) resulting in a 10× concentration of the original homogenate. The particle size of the suspension was reduced by repeated aspiration through successively smaller needles, starting with 18 or 20 gauge and working down to 23 to 25 gauge. After acclimation, mice were weighed and injected intraperitoneally with 1 ml of concentrated brain homogenate, saline, or 1×10^4 Nc-Nowra or Nc-S197 tachyzoites grown in cell culture as described above. Each preparation was given to 3 mice. Mice were observed twice daily for clinical signs of neosporosis (tetraplegia, ataxia, failure to groom, and abnormal behavior consistent with neurological damage) and weighed once weekly to monitor weight gain/loss. Mice that exhibited symptoms of neosporosis or loss of ≥10% of their body weight from the previous week were

TABLE 3 Reaction conditions for PCR

	NC5		MS10	
Step	Outer	Inner	Outer	Inner
1	94°C, 1 min	94°C, 1 min	94°C, 1 min	95°C, 1 min
2	94°C, 30 s	94°C, 30 s	94°C, 30 s	95°C, 30 s
3	60°C, 30 s	55°C, 30 s	55°C, 30 s	60°C, 30 s
4	68°C, 1 min	68°C, 30 s	68°C, 30 s	72°C, 30 s
5	Repeat steps 2 to 4 34 times	Repeat steps 2 to 4 34 times	Repeat steps 2 to 4 34 times	Repeat steps 2 to 4 34 times
6	68°C, 5 min	68°C, 5 min	68°C, 5 min	72°C, 5 min
7	Hold 4°C	Hold 4°C	Hold 4°C	Hold 4°C

TABLE 4 Pregnancy and prechallenge seroconversion rates[a]

Treatment group	Vaccination	Challenged	No. pregnant/total (%)[b]	No. seropos./total no. sampled prechallenge (D139)	No. seropos./total samples postchallenge (study termination)
1	Saline	Y	14/15 (93.3) A	0/13[d]	5/5
2	i.v., RPMI medium	N	11/13[c] (84.6) A, C	8/10[d]	8/8
3	i.v., RPMI medium	Y	12/16[c] (74) A, D	14/14	9/9
4	s.c., RPMI medium	Y	9/15 (60) B, C, D, E	10/15	7/8
5	s.c., RPMI medium, frozen	Y	9/15 (60) B, C, D, E	1/15	4/5
6	i.v., PMEM	Y	7/15 (46.7) B, D	15/15	6/6
NTX	None	N	6/6 (100) A, E	0/6	0/6

[a] Seroconversion was determined by cELISA. Abbreviations: i.v., intravenous; s.c., subcutaneous; Y, yes; N, no; seropos., seropositive.
[b] Values followed by dissimilar capital letters are significantly different ($P \leq 0.10$).
[c] One animal in group 2 was inadvertently challenged and moved into group 3; a second heifer in group 2 died shortly after vaccination.
[d] One pregnant heifer was euthanized for histopathology of the reproductive tract.

euthanized, and brain, lung, heart, and liver samples were collected and frozen for analysis by PCR to detect the presence of *Neospora*. The study was terminated 8 weeks after injection, at which time all remaining mice were euthanized and blood, brain, lung, and liver samples were collected. Because of their small size, heart and lung were combined into a single sample. Tissue samples were processed, and DNA was extracted as described for bovine tissue. Blood was processed to serum and assayed for the presence of antibodies to *Neospora* by direct ELISA. The bovine brain homogenates given to mice were also examined for the presence of parasites by nested PCR using the NC5 diagnostic assay described above.

Statistical analysis. The 10% level of significance ($P \leq 0.10$) was used in statistical comparisons. Since the study used a relatively small number of animals, the slightly higher level of risk associated with the 10% level of significance compared to 5% was deemed acceptable. The percentage of animals with *Neospora*-induced abortion and/or infection was the variable of primary interest. The incidence of pregnancy rates was analyzed using Fisher's exact test to compare treatment differences. The incidence of animals with fetuses infected with *N. caninum* or aborted was also analyzed using Fisher's exact test. The procedure "Proc Binomial" from StatExact was used to calculate the prevented fractions and 90% confidence intervals for each vaccinated treatment group compared to the nonvaccinated, challenged control group. For categorical laboratory assay data, pregnancy data, and fetal protection data, the number and percentage of animals in each categorical class were summarized by treatment and time point as well as by treatment across time points.

RESULTS

Pregnancy, seroconversion, and histopathology. Although each group initially comprised 15 vaccinated, pregnant animals, the number of animals in each group changed as the study progressed. One animal in group 2 was found dead the morning following vaccination. This animal had acute cardiopulmonary congestion that was attributed to anaphylaxis, which likely played a role in the death of the animal. A second group 2 heifer was inadvertently challenged and moved to group 3. Thus, on the day of challenge there were 13 animals in group 2 and 16 animals in group 3 (Table 4).

Differences in pregnancy rates were observed at 75 days of gestation (Table 4). Both groups receiving subcutaneous vaccine grown in RPMI medium (groups 4 and 5) and the group receiving the vaccine grown in PMEM intravenously (group 6) had significantly lower pregnancy rates than did the saline control group. Of the 39 animals vaccinated intravenously with freshly prepared tachyzoites, 29 (groups 2, 3, and 6) had seroconverted by day 139 postvaccination (75 days of gestation) based on direct ELISA results (Table 4). Ten of 15 animals vaccinated with fresh material by the subcutaneous route seroconverted, but only one of the 15 animals receiving the frozen and thawed vaccine seroconverted. Unvaccinated animals (group 1 and sentinels) were seronegative prior to challenge on day 139.

Histopathology conducted on samples collected on day 140 postvaccination from nonpregnant animals revealed lesions of potential clinical significance in two of them. One animal from group 5 had subacute inflammation and formation of lymphoid nodules within the lamina propria of the oviduct, and one from group 6 had granulomatous inflammation of the uterus; however, no fetal remains or microorganisms were observed. No significant lesions were observed in any other samples from nonpregnant animals. The two pregnant heifers from group 2 that were euthanized prior to challenge had no significant lesions in the oviduct or uterus. One of the two heifers lost a fetus prior to sampling and had returned to estrus. No significant gross lesions were found in the fetus from the remaining heifer. No significant lesions were seen in any other heifers that were sampled for histopathology.

Abortion and fetal infection. A fetus was considered infected if it was either aborted or resorbed during the study, as indicated by a previously confirmed pregnant heifer being open (nonpregnant) at the end of the study. A fetus was also considered infected if it was recovered and was positive for *in utero* exposure to *N. caninum* indicated by the presence of parasite DNA in fetal tissues as determined by PCR or the presence of antibodies to *N. caninum* in body fluids as determined by cELISA. Although 16 fetuses were missing at the end of the study and presumably aborted/resorbed, only 5 were recovered for diagnostic evaluation. All 5 were negative for *Leptospira*, *Brucella abortus*, and IBR virus. Two more fetuses were recovered from a heifer in group 6 that was carrying twins, one of which was stillborn. This fetus was positive for BVD virus in the liver by fluorescent-antibody (FA) test, but its surviving twin was negative. All other fetal serum and fluid samples were negative for BVD virus.

Of the 14 pregnant cows in the saline control group (group 1), two were euthanized prior to challenge to collect samples for histopathology of the uterus. Of the remaining 12 pregnant animals that were challenged, nine aborted their fetuses (Table 5). Of the two aborted fetuses that were recovered, one was seropositive and both had PCR-positive brain tissue. The three surviving fetuses were negative in both assays. Two group 2 animals (vaccinated, not challenged) were euthanized prior to challenge for collection of histopathology samples, reducing the number challenged to 9. Eight of the 9 fetuses in this group remained viable throughout the study, and all were negative for *N. caninum* exposure by serology.

TABLE 5 Postchallenge fetal viability, infection, and seroconversion[a]

Treatment group	Vaccination	Challenged	No. viable fetuses/total pregnant at study termination (%)	No. aborted fetuses recovered	No. seropos. calves/total	Total fetal infection (%)[b,c]	Prevented fraction (90% CI)
1	Saline	Y	3/12 (25)	2	1/4[d]	9/12 (75) A	NA
2	i.v., RPMI medium	N	8/9 (88.9)	0	0/8	1/9 (11.1) B, C	85.2 (44.8–99.0)
3	i.v., RPMI medium	Y	10/12 (83.3)	1	0/9	2/12 (16.7) B, C	77.8 (39.1–96.3)
4	s.c., RPMI medium	Y	8/9 (88.9)	0	0/8	3/9 (33.3) B, C	55.6 (5.0–85.2)
5	s.c., RPMI medium, frozen	Y	6/9 (66.7)	2	0/5	5/9 (55.6) A, C	25.9 (−31.5–68.3)
6	i.v., PMEM	Y	8/8 (100)[e]	2 (twins)	1/8[f]	1/8 (12.5) B, C	83.3 (28.9–98.8)
NTX	None	N	6/6 (100)	0	0/6	0/6 (0)[g]	NA

[a] Abbreviations: i.v., intravenous; s.c., subcutaneous; Y, yes; N, no; CI, confidence interval; NA, not applicable.
[b] Values followed by dissimilar capital letters are significantly different ($P \leq 0.10$).
[c] Number of abortions or open heifers plus number of calves PCR positive and/or seropositive.
[d] One nonviable calf was sampled at necropsy.
[e] One cow had two fetuses.
[f] Both twins were seropositive but were counted as a single event because the cow was the experimental unit.
[g] NTX results were not included in the statistical analysis.

Parasite DNA was found in spleen from one fetus, but this was later determined to originate from the challenge strain Nc-S197 rather than the Nc-Nowra vaccine strain. The single aborted fetus was not recovered, precluding any diagnostic testing. Ten of the 12 viable fetuses in the group vaccinated intravenously with Nc-Nowra tachyzoites prepared from cells grown in RPMI medium (group 3) survived challenge, contained no detectable antibodies to *Neospora*, and were PCR negative. The single recovered aborted fetus contained parasite DNA in lung, brain, and placenta. Among the group vaccinated with this preparation subcutaneously (group 4), eight of nine fetuses survived, with two of the eight survivors containing parasite DNA in placenta, brain, and heart. Six of the nine viable fetuses in the group receiving the frozen vaccine (group 5) survived to study termination. Parasite DNA was detected in the lung of one of them and the placenta associated with a second fetus, but all were seronegative. The two recovered aborted fetuses contained parasite DNA in the placenta, one also contained parasite DNA in the brain, and the other was PCR positive in liver, heart, lung, and spinal cord. All fetuses in the group vaccinated with tachyzoites grown in PMEM (group 6) remained viable, and one heifer contained two fetuses, both of which were seropositive (Table 5). The remaining fetuses showed no evidence of exposure *in utero*. The fraction of animals that were protected by vaccination ranged from 55.6% to 85.2% for groups receiving freshly prepared vaccine and 25.9% for the group receiving the frozen vaccine (Table 5). All fetuses in the sentinel group (NTX) remained viable throughout the study, and no evidence of exposure to *N. caninum* was found in the fetuses.

Strain differentiation by PCR. Titration of genomic DNA from Nc-S197 and Nc-Nowra strains in the microsatellite PCR assay revealed a small but detectable difference in product size, with the Nc-Nowra product being slightly larger than the S197 product (Fig. 1). Parasite DNA could be detected for both strains down to 0.4 ng per reaction mixture, and as little as 0.04 ng per reaction mixture could be detected for Nc-S197. This amount of DNA is equivalent to approximately 400 tachyzoites (16). Two vaccinated, nonchallenged heifers had PCR-positive brain tissue samples; however, the size of the product obtained was consistent with that of the Nc-S197 challenge strain (Fig. 2A). No PCR products of a size consistent with Nc-Nowra were obtained from any heifer brain tissue or serum sample (Fig. 2A and B).

Parasite detection by bioassay. Within 1 h after intraperitoneal injection of brain tissue homogenates into IFN-KO mice, all mice exhibited signs of distress, including a hunched appearance and lethargy. The mice recovered from these initial symptoms within 24 h, however. Two mice that received tissue from a nonvaccinated, challenged heifer died at 2 and 5 days after injection of brain homogenate, respectively, without apparent clinical signs. Two additional mice given tissue homogenate from two other heifers in this group were found dead within 6 days after injection of brain homogenates, also with no apparent clinical signs. Two mice receiving brain homogenate from two separate vaccinated, nonchallenged heifers died within 9 days of injection. One of the mice injected with Nc-S197 exhibited lethargy and tremors at day 9 postinjection, and all three mice in this group were euthanized for tissue collection. All mice in this group had positive livers, one mouse also had positive heart-lung samples, and parasites were detected in the brain of another mouse. One mouse injected with Nc-Nowra was euthanized at 7 days postinjection as a result of loss of 10% of its body weight from the initial weight on day 0. A second mouse in this group was found dead the following day, and the third mouse was euthanized because it was lethargic and ataxic and was exhibiting tremors. The liver, heart-lung, and brain samples of all mice infected with the Nc-Nowra strain were positive for

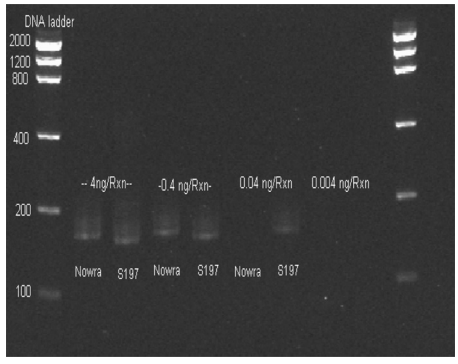

FIG 1 Detection and differentiation of Nc-S197 and Nc-Nowra genomic DNA by MS10 nested PCR with a 4% agarose gel and ethidium bromide staining.

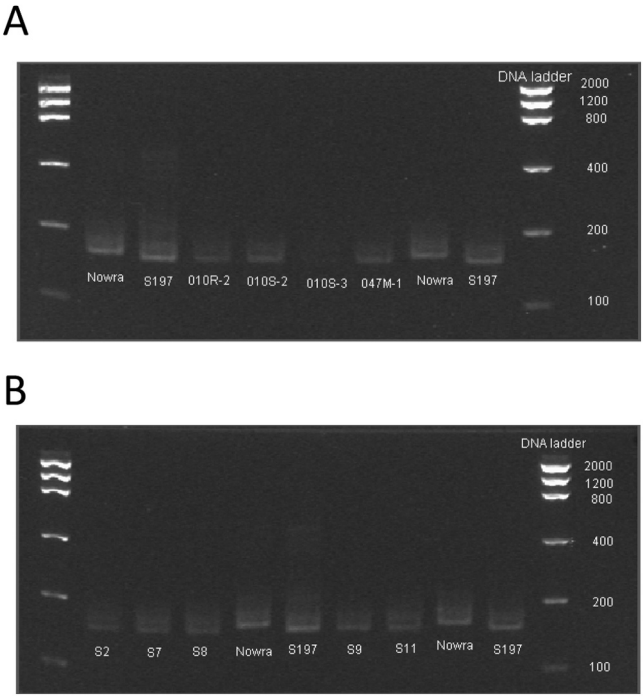

FIG 2 Microsatellite PCR products run on 4% agarose gels to aid in strain identification. (A) Nc-S197 (lanes 3 and 9) and Nc-Nowra (lanes 2 and 8) genomic DNA standards and samples from group 2 heifer 121 brain rostral lobe (010R, lane 4); brain stem (010S, lanes 5 and 6); fetal brain from calf of NTX heifer 108 (047M, lane 7). (B) S2, serum from uninfected sentinel heifer (lane 2); S7, S8, and S9 (lanes 3, 4, and 7, respectively), serum from a group 1 heifer that aborted, collected 0, 30, and 60 days postchallenge, respectively; S197 (lanes 6 and 10) and Nc-Nowra (lanes 5 and 9) genomic DNA; S11 (lane 8), serum collected 30 days postchallenge from a group 2 heifer containing a fetal calf with a PCR-positive spleen.

Neospora by NC5 diagnostic PCR. The remaining mice were all PCR negative for all tissues.

DISCUSSION

Vaccination of heifers with 10^7 tachyzoites of the Nc-Nowra strain of *N. caninum* by either the intravenous or the subcutaneous route provided a degree of protection from subsequent challenge with the Nc-S197 strain. The incidence of infection in the offspring of vaccinated cows as measured by abortion, serology, or detection of parasite DNA in tissues was significantly reduced in all vaccinated groups with the exception of the frozen and thawed vaccine. In addition to reducing the incidence of abortions, vaccination also reduced transplacental transmission of the parasite. Most of the calves from vaccinated cows were healthy and showed no evidence of infection at the end of the trial. This result is consistent with that of Williams et al. (11), who demonstrated complete protection of cows from abortion after challenge with the virulent Nc-Liverpool strain among animals vaccinated with the Nc-Nowra strain by the intravenous route. In the present study, intravenous and subcutaneous routes provided roughly equivalent protection against challenge in that there was no significant difference in the degree of protection. Since most cattle vaccines are administered subcutaneously, this result suggests that a vaccine based on the Nc-Nowra strain could be administered in a manner consistent with current vaccination practices.

The single abortion observed in the group that was vaccinated but not challenged raises the possibility that the Nc-Nowra strain retains some degree of virulence. No evidence of infection with Nc-Nowra was found in any of the remaining calves or in the brains of the vaccinated, unchallenged cows. Since no evidence of infection was found in the brains of the nonvaccinated, challenged cows, however, this result may reflect limitations of the methods of detection rather than absence of infection. The results are further complicated by the presence of DNA from the challenge strain Nc-S197 in calf and cow tissues from the vaccinated, nonchallenged group and in a serum sample from a nonvaccinated, noninfected sentinel heifer. Although this might suggest some horizontal transfer of the challenge strain, a more likely explanation is cross-contamination of samples during either necropsy, sample processing, or conduct of PCRs. Although steps to avoid such cross-contamination were taken, the high sensitivity of nested PCRs enables the method to detect very small amounts of contaminating DNA. In any case, further studies on the degree of attenuation and safety in pregnant cattle of the Nc-Nowra strain will be required before it can begin to be commercialized.

Tachyzoites preserved by freezing at −80°C in DMSO did not induce the same degree of protection as did live tachyzoites that were not frozen. Although the viability of the frozen vaccine preparation used in this study was not determined due to logistical constraints, results in our laboratory demonstrated that a high degree of viability (50 to 90%) could be retained using the freezing procedure described. Viability assessments were based on an *in vitro* cell invasion assay, and the ability of such an assay to predict parasite survival, replication, and immunogenicity in the cow is uncertain. Preservation of the parasite in a form robust enough to be infective and/or immunogenic is a major technical hurdle for an effective live vaccine to prevent neosporosis in cattle.

An interesting observation in this study was the reduced prechallenge pregnancy rates in certain groups compared with the nonvaccinated control. Both groups receiving subcutaneous vaccine prepared in RPMI medium, either frozen or nonfrozen, had reduced pregnancy rates. The group receiving the RPMI medium vaccine by the intravenous route had a pregnancy rate similar to that of the nonvaccinated control group. This observation may lead to speculation that the effect was route dependent, with the intravenous route being tolerated better than the subcutaneous route. The group vaccinated intravenously with vaccine prepared in a different medium also exhibited a reduced rate of pregnancy, however. Unless the medium used to prepare the vaccine also had an impact on pregnancy rate independent of the effect of route of administration, it is difficult to reconcile these results. A larger study will be required to determine if there is an interaction between route of administration, growth medium, and conception rate. The presence of antibody to BVD virus in one tissue of one aborted fetus that was part of a set of twins is a somewhat anomalous observation. It would be expected that in the presence of a BVD infection, there would be other positive samples, especially from the twin of the fetus with the BVD-positive liver. The possibility of a false-positive laboratory result cannot be excluded. It is well established that vaccines do not generally provide 100% protection, and so it is conceivable that there was a low level of infection in one animal. If the animals were exposed to BVD virus during the trial, however, the minimal incidence of positive samples would suggest that the animals were well protected by vaccination. Given the high incidence of neosporosis detected in the

fetuses and the minimal incidence of BVD virus-positive samples, it is unlikely that BVD virus played a role in the abortions seen in this study. It has been suggested based on mouse studies with *Leishmania major* (17) and *Toxoplasma gondii* (18) that induction of Th1 cytokine responses might interfere with fetal implantation and development of normal pregnancy. Since vaccination of cattle with live *Neospora* tachyzoites is expected to induce such cytokine responses (11, 19, 20), it is possible that this response to the vaccine affected the pregnancy rates in this study. Although it would be expected that the cytokine response to vaccination would have faded by the time that the animals were inseminated 2 months later, it is conceivable that in some cases there was an active disseminated infection in the early stages of pregnancy that affected pregnancy rates. This could potentially induce an increase in proinflammatory cytokines which could negatively impact embryo implantation. The differences between treatment groups could be a result of the robustness of the secondary cytokine response, which could be route dependent and may also vary depending on the medium used to prepare the vaccine, depending on how these factors influenced the parasite burden resulting from the initial infection. The effect of vaccination with live *Neospora* on subsequent pregnancy will need to be examined further.

This study confirms other results demonstrating that a live attenuated vaccine against bovine neosporosis can be very effective. The results also serve to highlight some of the technical difficulties associated with the practical use of such a vaccine. Tachyzoites preserved by freezing at −80°C did not appear to retain the infectivity and immunogenicity of unfrozen tachyzoites, even though tachyzoites were still viable after freezing by this method. Preservation of the parasite using more commercially acceptable methods such as lyophilization is a key hurdle in the development of a vaccine for bovine neosporosis. Two potential safety issues were seen in this study, one being the possibility of residual virulence of this naturally attenuated strain and the other being the potential for negative effects of vaccination on pregnancy, possibly as a result of induction of Th1-type cytokine responses by vaccination with live tachyzoites. Another hurdle to live vaccine development not addressed in this study is the potential for reversion to virulence of an attenuated strain. This could occur during parasite proliferation if recrudescence of the infection takes place during pregnancy, should the attenuated strain not be completely eliminated from the vaccinated animal. Passage of the parasite from dam to offspring in the case of an incompletely attenuated strain could also provide an opportunity for reversion to virulence. Development of a completely avirulent, stable strain of *N. caninum* and ensuring that the immune response to vaccination does not interfere with pregnancy are two additional areas of research that will be required to obtain a practical live vaccine. Although the results presented here suggest that such a vaccine could be effective, it remains to be determined whether it would prevent endogenous transplacental transmission from chronically infected dams to their offspring in the case where cows became infected prior to vaccination.

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