Identification of Bovine *Neospora* Parasites by PCR Amplification and Specific Small-Subunit rRNA Sequence Probe Hybridization

MICHAEL S. Y. HO,¹ BRADD C. BARR,¹ ANTOINETTE E. MARSH,¹ MARK L. ANDERSON,¹
JOAN D. ROWE,² ALICE F. TARANTAL,³ ANDREW G. HENDRICKX,³ KAREN SVERLOW,¹
J. P. DUBEY,⁴ AND PATRICIA A. CONRAD^{1*}

Department of Pathology, Microbiology, and Immunology,¹ and Department of Population Health and Reproduction,²
School of Veterinary Medicine, University of California, and California Regional Primate Research Center,³ Davis,
California 95616, and Parasite Biology and Epidemiology Laboratory, Livestock and Poultry Sciences Institute,
Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705⁴

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***Neospora* is a newly recognized genus of pathogenic coccidia, closely related to *Toxoplasma gondii*, that can cause abortion or congenital disease in a variety of domestic animal hosts. On the basis of the small-subunit rRNA gene sequences of *Neospora* spp. and other apicomplexa coccidia, oligonucleotide primers COC-1 and COC-2 were used for PCR amplification of conserved sequences of approximately 300 bp in size. A *Neospora*-specific chemiluminescent probe hybridized to Southern blots of amplification products from *Neospora* DNA but not to Southern blots with amplified DNA from the other coccidian parasites tested. A *Toxoplasma*-specific probe whose sequence differed from that of the probe for *Neospora* spp. by a single base pair was used to distinguish these parasites by specific Southern blot hybridization. The PCR system detected as few as one *Neospora* tachyzoite in the culture medium or five tachyzoites in samples of whole blood or amniotic fluid spiked with *Neospora* parasites. In addition, *Neospora* PCR products were successfully amplified from whole blood and amniotic fluid samples of experimentally infected bovine and rhesus macaque fetuses. These results indicate that this PCR and probe hybridization system could be a valuable adjunct to serology and immunohistochemistry for the diagnosis of *Neospora* infections in bovine or primate fetuses.**

Neospora is a genus of tissue-cyst-forming protozoa identified first in dogs (13, 22) and cattle (6, 47) but subsequently shown to be naturally infective for sheep (23, 25), goats (8, 21), horses (26), and deer (50). To date, cases of neosporosis in humans have not been reported. However, the experimental infection of pregnant rhesus macaques with a bovine *Neospora* isolate resulted in transplacental transmission and fetal infections comparable to those seen in human fetuses with *Toxoplasma gondii* infection (11).

Neospora caninum and *T. gondii* are phylogenetically closely related on the basis of nuclear small-subunit (nss)-rRNA gene sequence analysis (28, 32, 34). Marsh et al. (38) showed that the nss-rRNAs of the bovine *Neospora* isolate BPA-1 and *N. caninum* were homologous and differed from the nss-rRNA of *T. gondii* in only four base pairs. This gene sequence information was utilized in our study to develop a PCR-based system for the amplification of a conserved region of the coccidial nss-rRNA gene and to design a specific DNA probe for the detection of *Neospora* spp. in the blood and amniotic fluids of cattle and nonhuman primates.

MATERIALS AND METHODS

Parasite cultivation. Bovine *Neospora* isolates BPA-1 and BPA-2 were obtained from aborted bovine fetuses as previously described (15). *Neospora* sp. isolates BPA-3 and BPA-4 were collected from congenitally infected dairy calves that were born with neurological signs of disease (9). The *N. caninum* isolate, NC-1, was obtained from a naturally infected puppy (24). The *T. gondii* RH strain (kindly provided by John Boothroyd, Stanford University, Palo Alto, Calif.) was

isolated from a child with toxoplasmosis (43). The other two isolates of *T. gondii* were obtained from tissues of a naturally infected goat (GT-1) and a cow (CT-1) (19, 20). Tachyzoites of these isolates were propagated continuously in vitro in stationary monolayer cultures of bovine cardiopulmonary aortic endothelial cells (CPAE; ATCC CCL209) as described previously (15).

Preparation of DNA samples for PCR. (i) **Parasite specimens.** To test the specificity of the PCR detection system with a *Neospora*-specific probe, tachyzoites of bovine *Neospora* isolates BPA-1 to -4, *N. caninum* (NC-1 isolate), and *T. gondii* (RH, CT-1, and GT-1 isolates) was harvested from cultures as previously described (38). The number of tachyzoites was determined by counting culture aliquots diluted with phosphate-buffered saline (PBS; pH 7) in a hemocytometer with a light microscope ($\times 100$ magnification). Tachyzoites (10^6) were washed once with PBS and resuspended in 100 μ l of sample buffer solution provided with an IsoQuick Nucleic Acid Extraction kit (MicroProbe Corp., Garden Grove, Calif.). The tachyzoite suspension was mixed with 100 μ l of lysis solution, 700 μ l of extraction matrix, and 400 μ l of extraction buffer. Each sample was vortexed for 10 s and centrifuged at $12,000 \times g$ for 5 min. The aqueous phase was removed, and a volume of ice-cold isopropanol equal to that of the aqueous phase and 0.1 volume of sodium acetate were mixed with the aqueous phase to precipitate DNA at -20°C for 2 h or overnight. The DNA pellet obtained after centrifugation ($12,000 \times g$ for 10 min at 4°C) was rinsed with 70% (vol/vol) ethanol, air dried, and dissolved in RNase-free water (MicroProbe Corp.) for PCR.

Sarcocystis cruzi, *Sarcocystis tenella*, and *Sarcocystis capracanis* bradyzoites were isolated from experimentally infected cattle, sheep, and goats, respectively (27, 42). Total genomic DNA was isolated by the rapid DNA extraction protocol with an IsoQuick Nucleic Acid Extraction kit as described above.

Cryptosporidium parvum and *Eimeria bovis* DNAs were extracted from oocysts (kindly provided by David Lindsay, Auburn University, Auburn, Ala.). Oocyst DNA was isolated with a Mini-beadbeater machine (Biospec Products, Bartlesville, Okla.) and chemical reagents from a IsoQuick Nucleic Acid Extraction kit. Briefly, 600 μ l of sample buffer solution containing 10^7 oocysts was mixed with 600 μ l of lysis solution containing guanidine thiocyanate (MicroProbe Corp.). Two-milliliter screw-cap vials were filled to 85% capacity with 0.5-mm-diameter sterile glass beads (Biospec Products), and the remaining volume was filled with oocysts in lysis solution. The vials were screwed tightly, inserted into the arm assembly of the Mini-beadbeater, and vibrated at 5,000 rpm for 90 s. The vials were removed, and the top and bottom portions of the vials were carefully sliced open with a sterile razor blade. Oocyst lysate was then removed from the vials by centrifugation at $400 \times g$ for 5 min. A wet mount of the lysate (10 μ l) was

* Corresponding author. Mailing address: Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616. Phone: (916) 752-7210. Fax: (916) 752-3349.

prepared to confirm complete oocyst disruption. The oocyst DNA was then isolated with an IsoQuick Nucleic Acid Extraction kit.

(ii) **Bacterial samples.** Pathogenic and nonpathogenic bacteria which inhabit the bovine reproductive tract, including *Acinetobacter calcoaceticus*, *Campylobacter fetus* subsp. *venerealis*, *Corynebacterium pyogenes*, *Enterobacter aerogenes*, *Escherichia coli*, *Haemophilus somnus*, *Moraxella bovis*, *Pasteurella multocida*, *Staphylococcus epidermidis*, and *Streptococcus lactis* (kindly provided by the Veterinary Medicine Teaching Hospital Diagnostic Laboratory, University of California, Davis) were cultivated by standard methods (18, 49). Cells were harvested in log growth phase, and genomic DNAs were prepared as previously described (29).

(iii) **Bovine and rhesus monkey samples.** Pregnant cows 3 and 413 were experimentally infected with live BPA-1 *Neospora* tachyzoites as described by Barr et al. (12). The negative control cow 4 was inoculated with uninfected CPAE monolayer culture that was processed in the same way as the infected culture. In addition, four pregnant rhesus macaques were experimentally infected with BPA-1 *Neospora* tachyzoites at 65 (monkeys 1A and 1B) and 43 (monkeys 2A and 2B) days of gestation and two control pregnant macaques (monkeys 1C and 2C) were inoculated with uninfected culture material as previously described (11). Experimental procedures for group 3 rhesus macaques were the same as those used previously (11), except that the pregnant monkeys were inoculated with BPA-1 *Neospora* tachyzoites (monkeys 3A, 3B, and 3D) or uninfected culture material (monkey 3C) at 25 days of gestation and the fetuses were not removed by cesarean section so as to determine the natural outcome of the infection. Bovine and monkey whole blood samples were collected from gravid adults by venipuncture into EDTA vacutainer tubes (Becton Dickinson, Rutherford, N.J.) before infection and when fetuses were surgically removed by cesarean section (cows 3 and 413; monkeys 1A, 1B, 1C, 2A, 2B, and 2C) or throughout pregnancy (monkeys 3A, 3B, 3C, and 3D) as previously described (46). DNAs from the whole blood samples (100 μ l) were isolated with an IsoQuick Nucleic Acid Extraction kit as noted above.

Amniotic fluid samples were collected during cesarean sections prior to delivery of the newborn. Specimens were processed immediately for DNA isolation by the boiling method, a procedure similar to that described by Grover et al. (30). Briefly, fresh amniotic fluid samples (10 ml) were centrifuged at $4,000 \times g$ for 10 min at room temperature. The supernatant was carefully removed, and the remaining cells at the bottom of the tubes were washed with 1 ml of sterile PBS. The samples were then transferred to clean microfuge tubes and centrifuged at $4,000 \times g$ for 10 min. The supernatant was carefully removed and discarded. The cells were resuspended and mixed well with 500 μ l of sterile PBS. Samples were heated at 95°C for 10 min and then vortexed for 5 s. After another 10 min of heating and 5 s of vortexing, the samples were centrifuged at $16,000 \times g$ for 10 min to precipitate all cell debris. The aqueous phase was very carefully pipetted and mixed with 0.1 volume of sodium acetate and a volume of ice-cold isopropanol equal to that of the aqueous phase to precipitate DNA at -20°C for 2 h or overnight. The DNA pellet obtained after centrifugation ($12,000 \times g$ for 10 min at 4°C) was rinsed with 70% (vol/vol) ethanol, air dried, and dissolved in RNase-free water for PCR.

In addition, DNA was prepared as described previously (16) from uninfected bovine thymocytes (kindly provided by Jeffery Stott, University of California, Davis) and CPAE monolayer cells for use as uninfected bovine control DNA.

(iv) **Spiked whole blood and amniotic fluid samples.** To test the sensitivity of the PCR system, various numbers (1, 5, 50, and 500) of in vitro-cultivated *Neospora* tachyzoites (BPA-1 isolate) were mixed with 100 μ l of whole blood or amniotic fluid samples from negative control cows or rhesus macaques. DNA was isolated with an IsoQuick Nucleic Acid Extraction kit or by the boiling method, as described above. To investigate the effects of whole blood or amniotic fluid on the sensitivity of the system, cultivated tachyzoites were mixed with 100 μ l of culture medium and DNA was isolated as described above.

Selection of PCR primers and specific internal probes. Oligonucleotide PCR primers (COC-1, 5'-AAGTATAAGCTTTTATACGGCT-3', and COC-2, 5'-CACTGCCACGGTAGTCCAATAC-3') were designed by comparing nsrRNA gene sequences of bovine *Neospora* isolates with those of *N. caninum*, *T. gondii*, *Sarcocystis* spp., and *Cryptosporidium parvum* as previously described (38). Four nucleotide variations located at nucleotides 167, 222, 254, and 689 were consistently detected when sequences of *Neospora* isolates were compared with those of *T. gondii* (38). Probes specific for *Neospora* spp. (5'-AGTCAAACGCG-3') and *Toxoplasma* spp. (5'-AAGTCAACGCG-3') were selected on the basis of nucleotide 254, which has a thymine base in *Neospora* isolates and which is absent in *T. gondii*.

Amplification by PCR and product detection. DNA amplification was carried out in a total volume of 50 μ l with approximately 50 to 100 ng of DNA mixed with a solution containing 10 mM Tris-HCl (pH 9.0), 50 mM potassium chloride, 0.1% Triton X-100, 1.0 mM magnesium chloride, 200 μ M each deoxynucleoside triphosphate, 0.424 μ M bovine *Neospora* PCR primer (COC-1), and 0.384 μ M bovine *Neospora* PCR primer (COC-2). Bovine serum albumin (50 μ g) (Gibco/BRL, Gaithersburg, Md.) and glycerol (5%) (Gibco/BRL) were added to the oocyst DNA reaction mixture to prevent the inhibition of PCR amplification (31, 35) that may result from the presence of PCR inhibitors in fecal material (35, 48). All PCR samples underwent precycle denaturation at 94°C for 4 min to reduce nonspecific amplification (39). Then, 2.5 U of *Taq* DNA polymerase (Promega Corp., Madison, Wis.) was added and the mixture was overlaid with 50 μ l of

mineral oil. Amplification was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus Corp., Norwalk, Conn.) for 51 cycles with denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 2 min. The last cycle was given a prolonged extension period of 7 min. Negative reagent controls (no DNA) were included with each batch of test samples. To avoid carryover of contaminating nucleic acids, which may result in false-positive reactions, the steps of pre-PCR sample preparation, the mixing of PCR reagents with the samples, and the final PCR thermal cycle reactions were performed in separate laboratories (37, 40).

After amplification, 5 μ l of each sample or the BioMarker Low DNA size standard (BioVentures, Inc., Murfreesboro, Tenn.) was electrophoresed on a 3% Nusieve 3:1 agarose gel (FMC Bioproducts, Rockland, Maine), stained with 0.5 μ g of ethidium bromide per ml, and examined under UV illumination. Amplification products were transferred to nylon membranes (Hybond-N⁺; Amersham Corp., Arlington Heights, Ill.) by the Southern blotting method (44) and cross-linked with a Stratalinker UV cross-linker (Stratagene, La Jolla, Calif.). Prehybridization, preparation of the labeled internal probe, and hybridization were performed as recommended by the manufacturer for the enhanced chemiluminescence 3'-oligolabeling and detection systems (Amersham Corp.). Labeled (*Neospora*-specific or *Toxoplasma*-specific) internal probe was added to a final concentration of 10 ng of hybridization solution per ml and incubated overnight at 30°C with gentle agitation. After hybridization, the membranes were washed twice for 5 min each time at room temperature in $5\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-sodium dodecyl sulfate (SDS) and then twice for 5 min each time at room temperature in $0.5\times$ SSC-0.1% (wt/vol) SDS. Membrane blocking, antibody incubations, signal generation, and detection were performed as recommended by the manufacturer. Membranes were exposed to Kodak X-Omat film for 3 to 10 min.

RESULTS

Specificities of *Neospora* detection by PCR and Southern blot hybridization. Products of 294 bp were amplified from DNAs of bovine *Neospora* isolates (Fig. 1A, lanes 2 to 5, and 1B, lane 13), *N. caninum* (Fig. 1A, lane 6), *T. gondii* (Fig. 1A, lanes 7 to 9), and *Eimeria bovis* (Fig. 1B, lane 3) with PCR primers COC-1 and COC-2. In addition, an approximately 350-bp product was amplified from *Sarcocystis cruzi*, *Sarcocystis tenella*, and *Sarcocystis capracanis* DNAs (Fig. 1A, lanes 10 to 12) and an amplification product of approximately 280 bp was produced with *Cryptosporidium parvum* DNA (Fig. 1B, lane 2). No PCR products were produced with DNAs from bovine thymocytes (Fig. 1A, lane 13), CPAE monolayer cells (Fig. 1A, lane 14), or bacteria (*A. calcoaceticus*, *Campylobacter fetus* subsp. *venerealis*, *Corynebacterium pyogenes*, *Enterobacter aerogenes*, *E. coli*, *H. somnus*, *M. bovis*, *Staphylococcus epidermidis*, and *Streptococcus lactis*) (Fig. 1B, lanes 4 to 12). In repeated experiments, amplification products were not produced with any of the negative reagent controls (Fig. 1B, lane 14). Only the amplification products produced with DNAs of bovine *Neospora* isolates BPA-1 to -4 and *N. caninum* (Fig. 1C, lanes 2 to 6, and 1D, lane 13) showed hybridization signals with the *Neospora*-specific chemiluminescent internal probe. Moreover, the *Toxoplasma*-specific internal probe hybridized to PCR products from all of the *T. gondii* isolates tested (Fig. 1E, lanes 7 to 9) but not to amplification products from any of the *Neospora* isolates or other protozoa tested.

Sensitivity of *Neospora* tachyzoite detection by PCR. To test the sensitivity of the PCR detection system, 1:10 serial dilutions of cultivated bovine *Neospora* (BPA-1 isolate) tachyzoites were added to whole blood or amniotic fluid samples from a total of six negative control cows and monkeys. All samples were repeated at least three times to confirm consistent results. PCR amplification products from bovine whole blood or amniotic fluid samples containing 500, 50, and 5 tachyzoites in each PCR mixture were observed in ethidium bromide-stained gels (Fig. 2A and B, lanes 7 to 9). No amplification product was produced from bovine whole blood or amniotic fluid sample controls (Fig. 2A and B, lane 11). To determine the effect of whole blood or amniotic fluid samples on the sensitivity of the PCR detection system, serial dilutions of bovine *Neospora*

FIG. 1. Specificities of *Neospora* detection by PCR and Southern blot hybridization. (A and B) Amplification products were observed after electrophoresis under UV light. (C to F) Southern blot of the same gel hybridized to *Neospora*-specific (C and D) or *Toxoplasma*-specific (E and F) chemiluminescent internal probes. In panels A, C, and E, the indicated lanes contain the following: lanes 2 to 5, bovine *Neospora* isolates BPA-1 to -4, respectively; lanes 6, *N. caninum*; lanes 7 to 9, *T. gondii* RH, CT-1, and GT-1, respectively; lanes 10, *Sarcocystis cruzi*; lanes 11, *Sarcocystis tenella*; lanes 12, *Sarcocystis capracanis*; lanes 13, bovine thymocytes; and lanes 14, CPAE monolayer cells. In panels B, D, and F, the indicated lanes contain the following: lanes 2, *Cryptosporidium parvum*; lanes 3, *Eimeria bovis*; lanes 4, *A. calcoaceticus*; lanes 5, *Campylobacter fetus* subsp. *venerealis*; lanes 6, *Corynebacterium pyogenes*; lanes 7, *Enterobacter aerogenes*; lanes 8, *E. coli*; lanes 9, *H. somnus*; lanes 10, *M. bovis*; lanes 11, *Staphylococcus epidermidis*; lanes 12, *Streptococcus lactis*; lanes 13, bovine *Neospora* (BPA-1 isolate) DNA controls; and lanes 14, negative reagent control. Lanes 1 in panels A to F contain the BioMarker Low DNA size standard, whose base pair designations appear on the left.

tachyzoites were added to culture medium. The result showed that in the absence of whole blood or amniotic fluid samples, amplification products could be produced from one tachyzoite (Fig. 2A and B, lane 5). Uninfected monkey blood or amniotic fluid samples that were spiked with *Neospora* tachyzoites produced results identical to those described above.

Detection of *Neospora* in fetal bovine and rhesus monkey samples. Whole blood and amniotic fluid samples from experimentally infected bovine or rhesus monkey dams and fetuses were used to evaluate the PCR and the probe hybridization system for the ability to detect *Neospora* infection. *Neospora* PCR products were amplified from whole blood and amniotic fluid samples from infected bovine fetuses of cows 3 and 413 (Fig. 3A, lanes 7, 8, 10, and 11) and from infected monkey fetuses from rhesus dams 1A, 1B, 2A, and 2B (Fig. 4A and B, lanes 7, 8, 10, and 11). No PCR product was amplified from blood and amniotic fluid samples of uninfected control bovine or monkey dams and fetuses (Fig. 3A, 4A, and 4B, lanes 6, 9, and 12). In addition, whole blood samples collected during cesarean sections from the infected bovine and monkey dams did not produce detectable PCR amplification products (Fig. 3A, 4A, and 4B, lanes 4 and 5).

Detection of *Neospora* in whole blood samples from rhesus monkey dams. *Neospora* PCR amplification products were detected in the blood of infected monkey dam 3A from week 4 to week 8 after infection (Table 1). At week 8, the fetus was sonographically identified as nonviable and the dam was subsequently euthanized. No PCR product was amplified from the blood of infected dam 3B or the uninfected control dam 3C. PCR amplification products were detected only at weeks 3 and 10 for infected dam 3D (Table 1).

DISCUSSION

Currently, the diagnosis of *Neospora* infections in cattle requires postmortem examinations of infected animals, primarily

of aborted fetuses or infected calves. A presumptive diagnosis of protozoal infection can be made on the basis of characteristic histological lesions (2). Definitive diagnosis is then based on specific identification of parasites by immunohistochemistry with *Neospora*-specific antisera (3, 10, 12). Serological evaluation of *Neospora* infection by an indirect fluorescent antibody test (17) or enzyme-linked immunoassay (41) is useful for the

FIG. 2. Sensitivity of *Neospora* detection by PCR of tachyzoites in culture medium, bovine whole blood, or amniotic fluid. (A and B) Amplification products were observed after electrophoresis under UV light. (C and D) Southern blot of the same gel hybridized to the *Neospora*-specific chemiluminescent internal probe. In panels A to D, the indicated lanes contain the following: lanes 2 to 5, 500, 50, 5, and 1 *Neospora* tachyzoite, respectively, in culture medium; lanes 6, culture medium-negative control; lanes 12, bovine *Neospora* (BPA-1 isolate) DNA controls; and lanes 13 to 14, negative reagent controls. In panels A and C, lanes 7 to 10 contain 500, 50, 5, and 1 *Neospora* tachyzoite, respectively, added to bovine whole blood samples and lanes 11 contain whole blood-negative control. In panels B and D, lanes 7 to 10 contain 500, 50, 5, and 1 *Neospora* tachyzoite, respectively, added to bovine amniotic fluid samples and lanes 11 contain amniotic fluid sample-negative control. Lanes 1 in panels A to D contain the BioMarker Low DNA size standard, whose base pair designations appear on the left.

FIG. 3. Detection of *Neospora* spp. by PCR amplification of whole blood and amniotic fluid samples from experimentally infected cattle. (A) Amplification products were observed after electrophoresis under UV light. (B) Southern blot of the same gel hybridized to the *Neospora*-specific chemiluminescent internal probe. In panel A, the indicated lanes contain the following: lanes 2 and 3, preinfection whole blood samples from cows 3 and 413, respectively; lanes 4 and 5, whole blood samples collected during cesarean sections from experimentally infected cows 3 and 413, respectively; lane 6, whole blood sample collected from uninfected control cow 4 during cesarean section; lanes 7 and 8, whole blood samples collected from the fetuses removed by cesarean section from cows 3 and 413, respectively, after 48 days of inoculation with *Neospora* isolate BPA-1 tachyzoites; lane 9, whole blood sample collected from uninfected control fetus from cow 4; lanes 10 and 11, amniotic fluid samples collected from fetuses of cows 3 and 413, respectively; lane 12, amniotic fluid sample collected from the uninfected control fetus from cow 4; lane 13, bovine *Neospora* (BPA-1 isolate) DNA control; and lane 14, negative reagent control. Lanes 1 in panels A and B contain the BioMarker Low DNA size standard, whose base pair designations appear on the left.

detection of antibodies to *Neospora* spp. However, a negative *Neospora* indirect fluorescent antibody titer does not rule out the possibility of infection, particularly in young fetuses (7). The objective of this study was to develop a highly sensitive and specific method for the detection of *Neospora* parasites in the blood and amniotic fluids of infected animals.

Development of a PCR-based system to amplify a conserved region of the nss-rRNA gene allows the detection of several apicomplexa coccidia, which can then be specifically identified

TABLE 1. Detection of *Neospora* spp. by PCR in whole blood samples from rhesus monkey dams

Collection date (wk postinfection)	Presence of <i>Neospora</i> DNA in infected dam ^a :		
	3A	3B	3D
1	—	—	—
2	—	—	—
3	—	—	+
4	+	—	—
5	+	—	—
6	+	—	—
7	+	—	NC ^b
8	+	NC	NC
9	—	—	—
10	—	—	+

^a —, no amplification product; +, amplification of *Neospora* DNA. Monkey dam 3A was infected with live tachyzoites at 25 days of gestation. The fetus died in utero during week 8, and the dam was euthanized. Monkey dams 3B and 3D were infected with live tachyzoites at 26 days of gestation, and the fetuses remained viable to parturition. (Monkey dam 3C was a control animal inoculated with uninfected monolayer cell culture; no *Neospora* DNA was detected in samples from this animal.)

^b NC, not collected.

FIG. 4. Detection of *Neospora* spp. by PCR amplification of whole blood and amniotic fluid samples from experimentally infected rhesus macaques. (A and B) Amplification products were observed after electrophoresis under UV light. (C and D) Southern blot of the same gel hybridized to the *Neospora*-specific chemiluminescent internal probe. Panels A and C show the results with samples from monkey dams 1A, 1B, and 1C, and panels B and D show results with samples from monkey dams 2A, 2B, and 2C. In panels A to D, the indicated lanes contain the following: lanes 2 and 3, preinfection whole blood samples from monkey dams 1A and 1B (panels A and C) and dams 2A and 2B (panels B and D), respectively; lanes 4 and 5, whole blood samples collected from dams 1A and 1B (panels A and C) and dams 2A and 2B (panels B and D), respectively, at the times of their cesarean sections with inoculation of *Neospora* isolate BPA-1 tachyzoites; lanes 6, whole blood samples collected from control dam 1C (panels A and C) and dam 2C (panels B and D), respectively, at the times of their cesarean sections with inoculation of uninfected culture material; lanes 7 and 8, whole blood samples collected from fetuses of monkey dams 1A and 1B (panels A and C) and dams 2A and 2B (panels B and D), respectively; lanes 9, whole blood samples collected from uninfected control fetuses from dam 1C (panels A and C) and dam 2C (panels B and D), respectively; lanes 10 and 11, amniotic fluid samples collected from fetuses of monkey dams 1A and 1B (panels A and C) and dams 2A and 2B (panels B and D), respectively; lanes 12, amniotic fluid samples collected from uninfected control fetuses from dam 1C (panels A and C) and dam 2C (panels B and D), respectively; lanes 13, *Neospora* (BPA-1 isolate) DNA control; and lanes 14, negative reagent control. Lanes 1 in panels A to D contain the BioMarker Low DNA size standard, whose base pair designations appear on the left.

by hybridization with the appropriate probes, such as the *Neospora*- and *Toxoplasma*-specific probes used in this study. The PCR primers COC-1 and COC-2 were specifically selected by comparing nss-rRNA gene sequences of bovine *Neospora* isolates available from GenBank. Because of the similarity of nss-rRNA gene sequences among various coccidian parasites, PCR amplification products of various sizes, from 280 to 350 bp, were amplified from the DNAs of *Neospora* spp., *Toxoplasma* spp., *Sarcocystis* spp., *Cryptosporidium parvum*, and *Eimeria bovis* with these primers. However, no PCR products were produced with DNAs from bacteria, bovine thymocytes, or CPAE monolayer cells, as predicted from gene sequence comparisons. In order to distinguish PCR products of *Neospora* spp. from those of other coccidia, a very short *Neospora*-specific probe (5'-AGTCAAACGCG-3') that has a single base pair difference from the *Toxoplasma*-specific probe, *Toxoplasma* spp. being the most closely related coccidia (28, 32, 34, 38), was designed to hybridize to *Neospora* PCR products but not to those of other coccidian parasites. The critically selected *Toxoplasma*-specific probe (5'-AAGTCAAACGCG-3'), which has an adenine base deletion in its middle compared with the *Neospora*-specific probe, hybridized to PCR products of *Toxoplasma* spp. but not to those of *Neospora* spp. or *Eimeria* spp. In addition, *Eimeria* spp. are intestinal parasites that are not found in amniotic fluid or fetal blood samples.

PCR products were consistently amplified from whole blood

or amniotic fluid samples of *Neospora*-infected bovine and rhesus monkey fetuses, and the identities of these products were easily confirmed with the chemiluminescent *Neospora*-specific internal probe. The effectiveness of this method to detect infection in naturally infected and aborted bovine fetuses is currently being evaluated.

In addition to detecting *Neospora* spp. in fetal samples, we wanted to determine the applicability of this test for the detection of parasites in the blood of experimentally infected dams. PCR has been employed successfully for the detection of hemoparasites (4, 5, 14). However, only 53% of the patients with acute toxoplasmic lymphadenopathy were PCR positive when the initial blood samples were collected within the first 5 weeks of illness (33). Amplification of samples collected at various time points throughout these pregnancies revealed that *Neospora* PCR products were detected inconsistently in the maternal blood. For example, the blood samples of monkey dam 3A tested positive for *Neospora* infection from week 4 to week 8 postinfection. A *Neospora* sp. was detected intermittently in the blood samples of infected dam 3D at weeks 3 and 10 only, and no PCR products were amplified from the blood of infected dam 3B (Table 1). Shibata and colleagues (45) found that *Toxoplasma* spp. were detected by PCR sporadically at 9 and 12 days postinfection in the blood samples of experimentally infected mice. The lack of consistent results by PCR to determine the presence of both *Neospora* spp. and *Toxoplasma* spp. may be attributable to low numbers of tachyzoites in the blood of infected adult hosts and/or the fact that the parasites circulate in the peripheral blood for only a limited period of time and for various lengths of time depending on the individual hosts.

The PCR probe detection system that we have developed could also be a valuable tool for studies focused on the identification of the definitive host for *Neospora* spp. Presently, the life cycle of the bovine *Neospora* parasite is still unknown; however, its close biological and ultrastructural resemblance to *T. gondii* (10) suggests that these protozoa may have similar biological life cycles in which cattle serve as an intermediate host. It is thought, then, that cattle are infected by ingesting food or water contaminated with coccidian oocysts previously shed in the feces of an unidentified carnivorous definitive host. With the PCR probe system that we have developed, it may now be possible to screen intestinal coccidia from domestic and wildlife species so as to identify the definitive host(s).

Coccidian parasite oocysts have very strong oocyst walls that can resist repeated freeze-thaw, boiling, chemical digestion, and enzymatic digestion. Traditionally, oocysts were frozen in liquid nitrogen and ground to fine powder and DNA was isolated with extraction buffers (36). Recently, Abrahamsen et al. (1) described a new method of oocyst RNA isolation which involved the disruption of oocysts in a French pressure cell and then RNA isolation by standard methods. Both procedures were tedious and time consuming, and it was very difficult to prevent the formation of amplicon or cross-contamination of equipment and samples. In this paper, we describe a fast and safe method for oocyst DNA isolation by placing oocysts in a sterile vial containing guanidine thiocyanate and then disrupting them mechanically with a Mini-beadbeater machine (Biospec Products) filled with sterile glass beads. All reagents and supplies were kept sterile, and the oocyst disruption process was conducted in an entirely closed system to prevent cross-contamination of DNA samples. The short processing time enables multiple samples of oocysts from different animals to be examined in a relatively short period, hence facilitating the search for the unidentified *Neospora* definitive host(s).

In conclusion, the PCR and probe hybridization detection

system described here may be usefully employed in epidemiological studies and provide a powerful supplementary method for the diagnosis of *Neospora* infection in the aborted fetuses of bovines and primates. Although human neosporosis has not been reported, this test may become a valuable tool for determining whether such infections occur in patients who are reported to be diagnosed with toxoplasmosis.

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REFERENCES

- Abrahamsen, M. S., T. G. Clark, and M. W. White. 1995. An improved method for isolating RNA from coccidian oocysts. *J. Parasitol.* **81**:107-109.
- Anderson, M. L., B. C. Barr, and P. A. Conrad. 1994. Protozoal causes of reproductive failure in domestic ruminants. *Vet. Clin. North Am. Food Anim. Pract.* **10**:439-461.
- Anderson, M. L., P. C. Blanchard, B. C. Barr, J. P. Dubey, R. L. Hoffman, and P. A. Conrad. 1991. *Neospora*-like protozoan infection as a major cause of abortion in California dairy cattle. *J. Am. Vet. Med. Assoc.* **198**:241-244.
- Arai, M., C. Mizukoshi, F. Kubochi, T. Kakutani, and Y. Wataya. 1994. Detection of *Plasmodium falciparum* in human blood by a nested polymerase chain reaction. *Am. J. Trop. Med. Hyg.* **51**:617-626.
- Barker, R. H., T. Banchongaksorn, J. M. Courval, W. Suwonkerd, K. Rimwungtragoon, and D. E. Wirth. 1992. A simple method to detect *Plasmodium falciparum* directly from blood samples using the polymerase chain reaction. *Am. J. Trop. Med. Hyg.* **46**:416-426.
- Barr, B. C., M. L. Anderson, J. P. Dubey, and P. A. Conrad. 1991. *Neospora*-like protozoal infections associated with bovine abortions. *Vet. Pathol.* **28**:110-116.
- Barr, B. C., M. L. Anderson, K. W. Sverlow, and P. A. Conrad. 1995. Diagnosis of bovine fetal *Neospora* infection with an indirect fluorescent antibody test (IFAT). *Vet. Rec.* **37**:611-613.
- Barr, B. C., M. L. Anderson, L. W. Woods, J. P. Dubey, and P. A. Conrad. 1992. *Neospora*-like protozoal infections associated with abortion in goats. *J. Vet. Diagn. Invest.* **4**:365-367.
- Barr, B. C., P. A. Conrad, R. Breitmeyer, K. Sverlow, M. L. Anderson, J. Reynolds, A. E. Chauvet, J. P. Dubey, and A. A. Ardans. 1993. Congenital *Neospora* infection in calves born from cows that had previously aborted *Neospora*-infected fetuses: four cases (1990-1992). *J. Am. Vet. Med. Assoc.* **202**:113-117.
- Barr, B. C., P. A. Conrad, J. P. Dubey, and M. L. Anderson. 1991. *Neospora*-like encephalomyelitis in a calf: pathology, ultrastructure, and immunoreactivity. *J. Vet. Diagn. Invest.* **3**:39-46.
- Barr, B. C., P. A. Conrad, K. W. Sverlow, A. F. Tarantal, and A. G. Hendrickx. 1994. Experimental fetal and transplacental *Neospora* infection in the nonhuman primate. *Lab. Invest.* **71**:236-242.
- Barr, B. C., J. D. Rowe, K. W. Sverlow, R. H. BonDurant, A. A. Ardans, M. N. Oliver, and P. A. Conrad. 1994. Experimental reproduction of bovine fetal *Neospora* infection and death with a bovine *Neospora* isolate. *J. Vet. Diagn. Invest.* **6**:207-215.
- Bjerkas, I., S. F. Mohn, and J. Presthus. 1984. Unidentified cystforming sporozoan causing encephalomyelitis and myositis in dogs. *Z. Parasitenkd.* **70**:271-274.
- Britto, C., M. A. Cardoso, C. M. Vanni, A. Hasslocher-Moreno, S. S. Xavier, W. Oelemann, A. Santoro, C. Pirmez, C. M. Morel, and P. Wincker. 1995. Polymerase chain reaction detection of *Trypanosoma cruzi* in human blood samples as a tool for diagnosis and treatment evaluation. *Parasitology* **110**:241-247.
- Conrad, P. A., B. C. Barr, K. W. Sverlow, M. Anderson, B. Daft, H. Kinde, J. P. Dubey, L. Munson, and A. Ardans. 1993. *In vitro* isolation and characterization of a *Neospora* sp. from aborted bovine foetuses. *Parasitology* **106**:239-249.
- Conrad, P. A., K. Iams, W. C. Brown, B. Sohanpal, and O. K. ole-MoiYoi. 1987. DNA probes detect genomic diversity in *Theileria parva* stocks. *Mol. Biochem. Parasitol.* **25**:213-226.
- Conrad, P. A., K. Sverlow, M. Anderson, J. Rowe, R. BonDurant, G. Tuter, R. Breitmeyer, C. Palmer, M. Thurmond, A. Ardans, J. P. Dubey, G. Duhamel, and B. Barr. 1993. Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. *J. Vet. Diagn. Invest.* **5**:572-578.
- Corbeil, L. B., W. Woodward, A. C. S. Ward, W. D. Mickelsen, and L. Paisley.

1985. Bacterial interactions in bovine respiratory and reproductive infections. *J. Clin. Microbiol.* **21**:803–807.
19. Dubey, J. P. 1980. Mouse pathogenicity of *Toxoplasma gondii* isolated from a goat. *Am. J. Vet. Res.* **41**:427–429.
20. Dubey, J. P. 1992. Isolation of *Toxoplasma gondii* from a naturally infected beef cow. *J. Parasitol.* **78**:151–153.
21. Dubey, J. P., H. M. Acland, and A. N. Hamir. 1992. *Neospora caninum* (Apicomplexa) in a stillborn goat. *J. Parasitol.* **78**:532–534.
22. Dubey, J. P., J. L. Carpenter, C. A. Speer, M. J. Topper, and A. Uggla. 1988. Newly recognized fatal protozoan disease of dogs. *J. Am. Vet. Med. Assoc.* **192**:1269–1285.
23. Dubey, J. P., W. J. Hartley, D. S. Lindsay, and M. J. Topper. 1990. Fatal congenital *Neospora caninum* infection in a lamb. *J. Parasitol.* **76**:127–130.
24. Dubey, J. P., A. L. Hattel, D. S. Lindsay, and M. J. Topper. 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. *J. Am. Vet. Med. Assoc.* **193**:1259–1263.
25. Dubey, J. P., and D. S. Lindsay. 1990. *Neospora caninum* induced abortion in sheep. *J. Vet. Diagn. Invest.* **2**:230–233.
26. Dubey, J. P., and M. L. Porterfield. 1990. *Neospora caninum* (Apicomplexa) in an aborted equine fetus. *J. Parasitol.* **76**:732–734.
27. Dubey, J. P., C. A. Speer, G. Callis, and J. A. Blixt. 1982. Development of the sheep-canid cycle of *Sarcocystis tenella*. *Can. J. Zool.* **60**:2464–2477.
28. Ellis, J., K. Luton, P. R. Baverstock, P. J. Brindley, K. A. Nimmo, and A. M. Johnson. 1994. The phylogeny of *Neospora caninum*. *Mol. Biochem. Parasitol.* **64**:303–311.
29. Ferrari, F. A., E. Ferrari, and J. A. Hoch. 1982. Chromosomal location of a *Bacillus subtilis* DNA fragment uniquely transcribed by sigma-28-containing RNA polymerase. *J. Bacteriol.* **152**:780–785.
30. Grover, C. M., P. Thulliez, J. S. Remington, and J. C. Boothroyd. 1990. Rapid prenatal diagnosis of congenital *Toxoplasma* infection by using polymerase chain reaction and amniotic fluid. *J. Clin. Microbiol.* **28**:2297–2301.
31. Gumerlock, P. H., Y. J. Tang, and J. Silva. 1993. PCR detection of toxigenic *Clostridium difficile*, p. 288–293. In D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), *Diagnostic molecular microbiology principles and applications*. American Society for Microbiology, Washington, D.C.
32. Guo, Z. G., and A. M. Johnson. 1995. Genetic comparison of *Neospora caninum* with *Toxoplasma* and *Sarcocystis* by random amplified polymorphic DNA-polymerase chain reaction. *Parasitol. Res.* **81**:365–370.
33. Guy, E. C., and D. H. Joynton. 1995. Potential of the polymerase chain reaction in the diagnosis of active *Toxoplasma* infection by detection of parasite in blood. *J. Infect. Dis.* **172**:319–322.
34. Holmdahl, O. J., J. G. Mattsson, A. Uggla, and K. E. Johansson. 1994. The phylogeny of *Neospora caninum* and *Toxoplasma gondii* based on ribosomal RNA sequences. *FEMS Microbiol. Lett.* **119**:187–192.
35. Hoss, M., M. Kohn, S. Paabo, F. Knauer, and W. Schroder. 1992. Excrement analysis by PCR. *Nature (London)* **359**:199.
36. Jenkins, M. C., R. Fayer, M. Tilley, and S. J. Upton. 1993. Cloning and expression of a cDNA encoding epitopes shared by 15- and 60-kilodalton proteins of *Cryptosporidium parvum* sporozoites. *Infect. Immun.* **61**:2377–2382.
37. Kwok, S. 1990. Procedures to minimize PCR-product carryover, p. 142–158. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), *PCR protocols: a guide to methods and applications*. Academic Press, Inc., San Diego, Calif.
38. Marsh, A. E., B. C. Barr, K. Sverlow, M. Ho, J. P. Dubey, and P. A. Conrad. 1995. Sequence analysis and comparison of ribosomal DNA from bovine *Neospora* to similar coccidial parasites. *J. Parasitol.* **81**:530–535.
39. Nuovo, G. J., F. Gallery, P. MacConnell, J. Becker, and W. Bloch. 1991. An improved technique for the *in situ* detection of DNA after polymerase chain reaction amplification. *Am. J. Pathol.* **139**:1239–1244.
40. Orrege, C. 1990. Organizing a laboratory for PCR work, p. 447–454. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), *PCR protocols: a guide to methods and applications*. Academic Press, Inc., San Diego, Calif.
41. Paré, J., S. K. Hietala, and M. C. Thurmond. 1995. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. *J. Vet. Diagn. Invest.* **7**:352–359.
42. Powell, E. C., G. Pezeshkpour, J. P. Dubey, and R. Fayer. 1986. Types of myofibers parasitized in experimentally induced infections with *Sarcocystis cruzi* and *Sarcocystis capracanis*. *Am. J. Vet. Res.* **47**:514–517.
43. Sabin, A. 1941. Toxoplasmic encephalitis in children. *JAMA* **116**:801–814.
44. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. Shibata, H., S. K. Rai, M. Satoh, K. Murakoso, K. Sumi, S. Uga, T. Matsumura, and A. Matsuoka. 1995. The use of PCR in detecting *Toxoplasma* parasites in the blood and brains of mice experimentally infected with *Toxoplasma gondii*. *Kansenshogaku Zasshi* **69**:158–163.
46. Tarantal, A. F. 1993. Hematologic reference values for the fetal long-tailed macaque (*Macaca fascicularis*). *Am. J. Primatol.* **29**:209–219.
47. Thilsted, J. P., and J. P. Dubey. 1989. Neosporosis-like abortions in a herd of dairy cattle. *J. Vet. Diagn. Invest.* **1**:205–209.
48. Tsai, Y. L., C. J. Palmer, and L. R. Sangermano. 1993. Detection of *Escherichia coli* in sewage and sludge by polymerase chain reaction. *Appl. Environ. Microbiol.* **59**:353–357.
49. Ward, A. C. S., L. R. Stephens, B. J. Winslow, R. P. Gogolewski, D. C. Schaefer, S. K. Wasson, and B. L. Williams. 1986. Isolation of *Haemophilus somnus*: a comparative study of selective media, p. 479–486. In American Association of Veterinary Laboratory Diagnosticians. Proceedings of the 29th Annual Meeting. The Association Press, Madison, Wis.
50. Woods, L. W., M. L. Anderson, P. K. Swift, and K. W. Sverlow. 1994. Systemic neosporosis in a California black-tailed deer (*Odocoileus hemionus columbianus*). *J. Vet. Diagn. Invest.* **6**:508–510.