Neonatal *Neospora caninum* infection in dogs: Isolation of the causative agent and experimental transmission

P. Dubey, MVSc, PhD; A. L. Hattel, DVM; D. S. Lindsay, PhD; M. J. Topper, DVM

Summary: Neospora caninum *infection was diagnosed in 5 young dogs from 2 litters with a common parentage. The pups were born healthy, but developed hind limb paresis 5 to 8 weeks after birth. The predominant lesions were polyradiculoneuritis and granulomatous polymyositis.* Neospora caninum *was seen microscopically in sections of naturally infected pups, and was isolated in cell cultures, mice, and dogs inoculated with infected canine tissues. Antibodies to* N caninum *were detected in sera of infected dogs by indirect fluorescent antibody test.*

Neospora caninum is a recently recognized protozoan parasite that can cause fatal disease in young as well as old dogs.[1] In 1988, Cummings et al[2] described a 5-year-old female Labrador Retriever (dog 1) that whelped a litter of 8 pups in 1987 (litter 1), 5 of which were determined to have severe polyradiculoneuritis in association with a *Neospora*-like organism. Here we report clinical neonatal *Neospora* infections in 2 other litters of dogs with a common parentage and report the isolation of the etiologic organism.

Dog 1 was mated again with the same sire (dog 2) and whelped 7 pups (litter 2) that progressed normally until 5 to 6 weeks of age, when they all developed rigid hind limb paresis. The disease in each pup progressed clinically at different rates. Three pups (dogs 3, 4, and 5) were euthanatized and necropsied. Three pups were killed by the owner and were not necropsied. One mildly affected pup (dog 6) is still alive, seems to be recovering, and has minimal ataxia.

A 3-year-old female Labrador Retriever (dog 7), not related to dog 1, was mated with dog 2 and whelped 7 pups (litter 3). One pup died at 3 weeks of age, probably from pneumonia, and was not necropsied. Three pups developed hind limb paresis at approximately 8 weeks of age. Two of these 3 pups (dogs 8 and 9) were euthanatized at 12 weeks of age and necropsied; the third was euthanatized by the owner and was not necropsied. Three healthy pups were sold.

Clotted and unclotted blood samples (EDTA tubes) from dogs 3, 4, 5, 6, 8, and 9 were analyzed for hematologic values. Sera were stored at −70 C until used for serologic examinations.

From each of the 5 dogs that were necropsied, portions of skin, eyes, cerebrum, cerebellum, pons, medulla, spinal cord, lungs, heart, liver, spleen, mesenteric lymph nodes, kidneys, adrenal glands, small and large intestines, stomach, urinary bladder, spinal nerves, and skeletal muscles from all 4 limbs including the vastus lateralis, semimembranosus, gluteus medius, biceps femoris, supraspinatus, subscapularis, and triceps brachii were fixed in neutral buffered 10% formalin. Paraffin-embedded tissues were cut at 5 μm, stained with hematoxylin and eosin, and examined.

All 5 dogs necropsied were mildly anemic, were hypoproteinemic, and had high serum creatine kinase values. Gross lesions were seen in dogs 5, 8, and 9. The limb muscles were atrophic and diffusely pale. Pinpoint to long foci of extreme pallor also were seen scattered throughout the limb muscles and in the diaphragm. Pinpoint hemorrhages were seen throughout the lungs. Dog 8 had decubitus ulcers on the hind limbs.

Microscopically, the predominant lesions in all 5 dogs were polyradiculoneuritis (Fig 1) and myositis. Organisms were scant, even in areas with extensive inflammation (Fig 2). Skeletal myositis characterized by mild necrosis, vasculitis, basophilia, infiltrations of mononuclear cells and in some instances, neutrophils, involved all limbs, but particularly the hind limbs. Few tachyzoites were seen in limb muscles of dogs 4 and 9. Myositis similar to that seen in limb muscles were seen in the tongue of dogs 3 and 4 and in the diaphragm of dogs 3, 4, and 5. Myocarditis characterized by foci of infiltra-

From the Zoonotic Diseases Laboratory, Livestock and Poultry Sciences Institute, USDA, ARS, BARC-East, Building 1040, Room 100, Beltsville, MD 20705 (Dubey, Lindsay), the Animal Disease Diagnostic Laboratory, Department of Veterinary Science, The Pennsylvania State University, University Park, PA 16802 (Hattel), and the Division of Veterinary Pathology, Armed Forces Institute of Pathology, Washington, DC 20306 (Topper).

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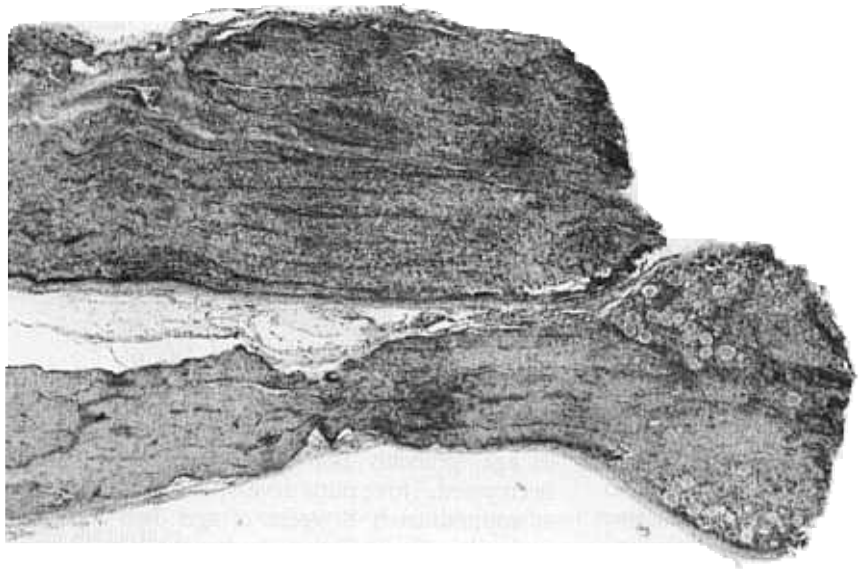

Figure 1—Photomicrograph of section of lumbar spinal nerve of dog 3. Notice severe inflammation. H&E stain; ×25.

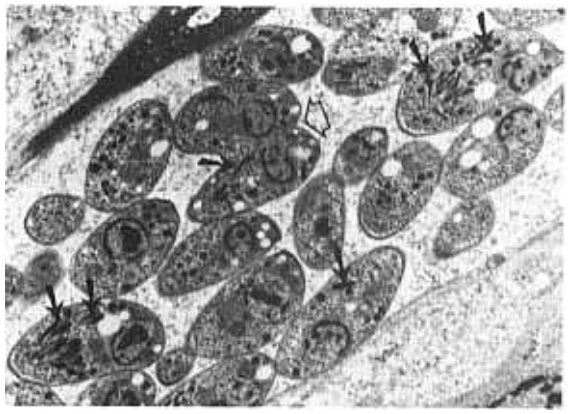

Figure 2—Electronmicrograph showing Neospora caninum *tachyzoites in a neural cell in lumbar spinal nerve of dog 3. Notice numerous rhoptries (solid arrows). One organism (open arrow) is dividing into 2 by endodyogeny. Whether vacuoles surrounding tachyzoites are real or artifact was not ascertained because the preparation was made from formalin-fixed deparaffinized material; ×4,000.*

tions of mononuclear cells was evident in dogs 3, 4, and 5. A few *N caninum* tachyzoites were seen in the myocardium of dog 3.

Encephalomyelitis characterized by gliosis and perivascular cuffings was observed in the brain and spinal cord of all 5 necropsied dogs. Although multiple foci of inflammation were seen throughout the brains of dogs in litter 2, organisms were not found. A few tissue cysts and a few *N caninum* tachyzoites were seen in the brain of dogs 8 and 9. The most severe lesions were in spinal nerves and consisted of perivascular cuffings and infiltrations of mononuclear cells, eosinophils, and, in some instances, neutrophils (Fig 1). Lesions were seen at the base as well as in branches of spinal nerves. This polyradiculoneuritis was identical to that observed in litter 1 described by Cummings et al.[2]

Having found *N caninum*-like organisms in dogs, the objectives of the investigation reported here were to attempt to isolate *N caninum* in cell cultures and in experimentally infected dogs and mice, to attempt to reproduce the disease in dogs, and to develop a serologic test to detect *N caninum*-specific antibodies.

Materials and methods

Animals—Tissues from dogs 3, 4, 5, 8, and 9 were obtained for conducting experimental studies. Swiss white mice (25 eight-week-old females) and 3 clinically normal female Beagle pups (dogs 10, 11, 12; 8-, 14-, and 16-week-old littermates [litter 4], respectively) were used in inoculation studies. Dog 13, the dam of litter 4, was housed with her pups and was used as an uninfected control (Table 1).

Tissues—Portions of major internal organs, especially spinal nerves, from each necropsied dog (dogs 3, 4, 5, 8, and 9) were disrupted in saline (0.9% NaCl) solution by use of a blender, filtered through gauze, and centrifuged at 400 × g for 10 minutes. The supernatant was discarded, and the sediment was suspended in approximately 10 ml of saline solution containing antimicrobials (1,000 IU of penicillin and 100 μg of streptomycin/ml of saline solution). Homogenates were prepared from tissues of each dog.

Cell cultures—Bovine monocytes[a] or cardiopulmonary arterial endothelial cells were grown in 25-cm² tissue culture flasks. Growth medium was RPMI 1640[b] plus 10% fetal bovine serum,[b] 2 m*M* L-glutamine, 50 μg of dihydrostreptomycin/ml, 50 U of penicillin G/ml, and 5×10^{-2} m*M*

[a]Speer CA, Montana State University, Bozeman, Mont: Personal communication, 1988.

[b]GIBCO Laboratories, Grand Island, NY.

Table 1—Results of indirect immunofluorescent antibody (IFA) and direct agglutination (DAG) tests for Neospora caninum *and* Toxoplasma gondii *in dogs*

Dog No.	Infection	*N caninum* IFA titer	*T gondii*	
			IFA titer	DAG titer
1 (bitch)	Natural	200	50	>250
2 (sire)	Natural	N	50	250
3*	Natural	800	N	25
4*	Natural	200	N	250
5*	Natural	800	N	N
6*	Natural	50	N	N
7 (bitch)	Natural	400	50	25
8†	Natural	100	N	N
9†	Natural	1,600	N	N
10‡	Experimental (*N caninum*)	400	N	25
11‡	Experimental (*N caninum*)	N	N	N
12‡	Experimental (*N caninum*)	400	N	N
13 (bitch)	Noninfected control	N	N	25
14	Experimental	N	400	>250

*Litter 2. †Litter 3. ‡Litter 4.
N = no titer detected.

2-mercaptoethanol/ml. Cell cultures inoculated with tissues obtained from naturally (dogs 3, 4, 5, 8, and 9) and experimentally infected pups (dogs 10, 11, and 12; see *Tissue inoculation* and *Tachyzoite inoculation*) as well as from an uninfected dog (dog 13) were maintained in identical media in which the fetal bovine serum was decreased to 2%. Cell cultures were incubated at 37 C in a 5% CO_2-95% air environment.

One to 6 flasks were used in each isolation attempt. Flasks were observed daily, using an inverted microscope[c] equipped with phase contrast optics. For ultrastructural examinations, monolayers were scraped with a rubber policeman, fixed in glutaraldehyde, and processed for transmission electron microscopy, as described.[3] Cell culture-grown tachyzoites (30,000/spot) of *N caninum* and the RH strain tachyzoites of *Toxoplasma gondii*[4] were air-dried onto multispot slides[d] and stored at −70 C until used as antigen in an indirect fluorescent antibody (IFA) test (see *Serologic examination*).

Tissue inoculation—Homogenate of tissues from dogs 3, 4, 5, 8, and 9 was inoculated SC into 5 mice (1 ml/mouse). The mice were observed for at least 6 weeks and were examined for *T gondii* and *N caninum* parasites and antibodies, as described.[4] Briefly, blood was obtained from each mouse 4 weeks after inoculation, and their sera were examined for antibodies to *T gondii* in the modified agglutination test.[5] Mice were euthanatized 6 to 12 weeks after inoculation, and their brains were examined microscopically for *T gondii* cysts.[4]

Tissue homogenates (2.5 ml each) from dogs 3 and 4 were inoculated SC into dog 10. Dog 11 was inoculated SC with tissue homogenate (5 ml) from dog 5. Dog 10 was euthanatized on postinoculation day (PID) 63, and dog 11 was euthanatized on PID 38. Tissues obtained from dogs 10 and 11 at necropsy were used for inoculation of cell cultures.

Tachyzoite inoculation—Dog 12 was inoculated SC with 100,000 *N caninum* tachyzoites obtained from cell cultures inoculated with tissues from dogs 3 and 4. Dog 12 also was given 200 mg of methylprednisolone acetate[e] 22 and 29 days after inoculation with *N caninum*.

Serologic examination—Blood samples had been obtained from the 5 naturally infected dogs (dogs 3, 4, 5, 8, and 9) at the time of euthanasia and from their dams and the sire about 2 weeks later, and serum was prepared. Blood samples from dog 6 were obtained at approximately 6 months of age. Pre- and postinoculation (see *Tissue inoculation* and *Tachyzoite inoculation*) blood samples were obtained from dogs 10, 11, and 12 and from uninfected control dog 13. Canine sera were examined for antibodies to *T gondii* in the modified agglutination test, as described.[5]

For the determination of antibodies to *N caninum*, sera were examined in the IFA test, using the culture-derived tachyzoites. Sera were diluted in phosphate-buffered saline solution (PBSS). Slides were incubated for 30 minutes with dog sera at doubling dilutions of 1:50 to 1:6,400 and then were washed 3 times (5 minutes each) in PBSS, incubated in fluorescein isothiocyanate-labeled goat anti-dog IgG[f] for 30 minutes, washed 3 times (5 minutes each) in PBSS, mounted in 90% glycerol in PBSS, and examined with an epifluorescent microscope. Serum from dog 13 (no exposure to *N caninum* or *T gondii*) was used as a negative control (1:100 dilution) in

[c]Zeiss IM 35, Baltimore Instrument Co Inc, Baltimore, Md.
[d]Eire Scientific Inc, Cleveland, Ohio.
[e]TechAmerica Group Inc, Elwood, Kan.
[f]Kirkegaard & Perry Laboratories Inc, Gaithersburg, Md.

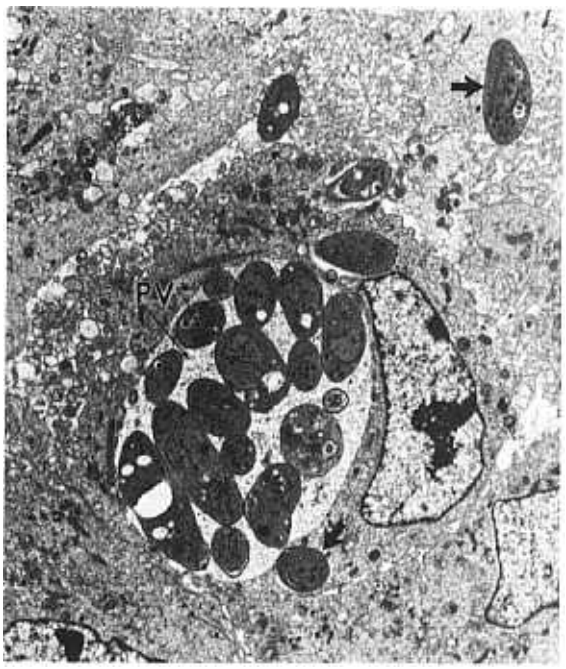

Figure 3—Electronmicrograph showing Neospora caninum *tachyzoites in cardiopulmonary arterial endothelial cells 4 days after inoculation of dog 10. Notice tachyzoites in parasitophorous vacuole (PV) and free (arrows) in the host cell cytoplasm; ×3,136.*

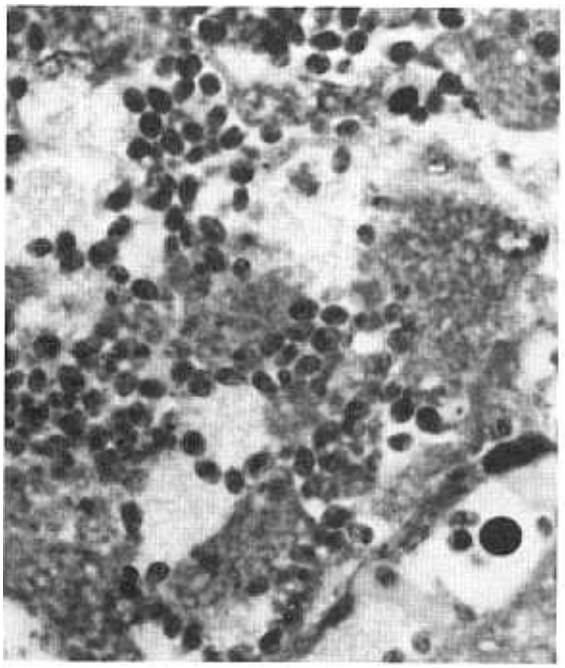

Figure 4—Photomicrograph of section of liver of dog 12. Notice numerous Neospora caninum *tachyzoites. Giemsa stain; ×1,125.*

the IFA test. Serum from a dog (dog 14) inoculated with *T gondii* oocysts[6] was used as a *T gondii*-positive control (1:100 dilution) in the IFA test. Serum from dog 12 (inoculated with 100,000 *N caninum* tachyzoites) was used as a *N caninum*-positive control (1:100 dilution) in the IFA test.

The IFA test used to examine mouse sera was performed similarly, except sera were screened at a 1:100 dilution and fluorescein isothiocyanate-labeled rabbit anti-mouse IgG[s] was the second antibody. Sera from mice experimentally infected with *N caninum* or with the RH strain of *T gondii* (1:100 dilution) were used as positive controls, and sera from mice with no exposure to *N caninum* or *T gondii* were used as negative controls (1:100 dilution).

Results

In flasks inoculated with tissues from dogs 3 and 4, tachyzoites of *N caninum* were first seen on PID 19. Five of 6 flasks eventually contained tachyzoites by PID 25 (Fig 3). In flasks inoculated with tissues from dog 5, tachyzoites of *N caninum* were first seen on PID 5, and 6 of 6 flasks contained tachyzoites by PID 8. None of 4 flasks inoculated with tissues from dog 8 contained tachyzoites during the 60-day examination period. Flasks inoculated with tissues from dog 9 were contaminated with bacteria, and the cells died on PID 2 to 7.

Tachyzoites of *N caninum* were not seen in flasks inoculated with tissues from experimentally infected dogs 10 and 11 or from their dam (dog 13) during the 60-day observation period. Tachyzoites of *N caninum* were seen on PID 2 and 3 in the 2 flasks inoculated with tissues from experimentally infected dog 12.

Dogs 10 and 11, inoculated with infected canine tissues, and the uninoculated dam (dog 13) remained clinically normal; neither lesions nor organisms were found in histologic sections of their tissues.

Dog 12, inoculated with tachyzoites obtained from cell cultures, remained clinically normal for 4 weeks after inoculation. Two days before death, she appeared depressed, but otherwise appeared normal. At necropsy, gross lesions were seen in lungs and liver. Epistaxis was observed, and the lungs were hemorrhagic. The liver was dark and friable and had areas of pale discoloration. Microscopically, the liver had multifocal areas of coagulative necrosis containing numerous *N caninum* tachyzoites (Fig 4). More than half of the liver was necrotic. The myocardium contained a few focal infiltrations of mononuclear cells and a few *N caninum* tachyzoites. Several long groups of tachyzoites were seen in limb muscles, but there was little host reaction. A group of tachyzoites was seen in ocular muscles. Small foci of gliosis were in the brain and the spinal cord.

Neither antibodies to *T gondii* nor *T gondii* organisms were found in mice inoculated with tissues from the 5 naturally infected dogs, from 3 experimentally infected dogs and their dam, and from the *N caninum*-infected cell cultures. *Neospora caninum* tissue cysts were in the brains of 3 of 25 mice inoculated with tissues from confirmed *N caninum*-infected dogs (Fig 5).

[s]ICN ImmunoBiologicals, Lisle, Ill.

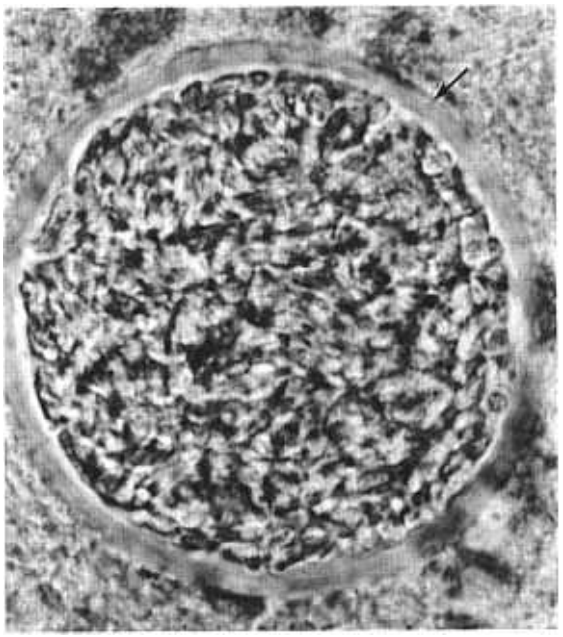

Figure 5—Photomicrograph of Neospora caninum *tissue cyst in the brain of a mouse inoculated with tissues of dogs 2 and 3. Notice thick cyst wall (arrow). Unstained; ×1,000.*

Neospora caninum IFA titers (>100) were found in sera of all mice inoculated with tissues from the 5 naturally infected dogs and from dog 12. Results of serologic examinations of naturally infected and experimentally infected dogs for antibodies to *N caninum* and *T gondii* are shown in Table 1. Antibodies to *N caninum* were not found in dogs 10, 11, 12, and 13 before inoculations.

Discussion

The 5 naturally infected dogs were considered to be infected with *N caninum* and not with *T gondii*, because *T gondii* was not isolated in mice or in cell cultures. Antibodies to *N caninum* were detected in infected dogs. Antibodies to *T gondii* were not detected in 4 dogs, and the presence of low concentrations of agglutinating *T gondii* antibodies in 2 dogs (dogs 3 and 4) might have been from the seropositive dam (dog 1). Organisms in tissues of naturally infected dogs, in experimentally infected dogs, in cell cultures, and in mice were structurally similar to *N caninum* and not to *T gondii*, with one exception. In cell cultures infected with *N caninum*, tachyzoites were located within the parasitophorous vacuoles as well as in direct contact with host cell cytoplasm, whereas parasitophorous vacuoles were not found in dogs naturally infected with *N caninum*.[1]

The findings of hind limb paresis of littermates and infection in young dogs suggested congenital infection. The results of the present study also indicated that *N caninum* does not affect all dogs with the same severity, and infection is not always fatal. Infection in 2 successive litters from the same bitch suggested reactivation of subclinical infection and transmission during the neonatal period. Although 3 litters had a common sire, the lack of antibodies to *N caninum* in the sire suggested that *N caninum* infection was not sexually transmitted.

The source of infection and the life cycle of *N caninum* are unknown,[1,7,8] making epidemiologic determinations difficult or impossible. However, the results of the present study, indicating that *N caninum* can infect noncanine cells and mice, provide tools that can be used for diagnosis and future epidemiologic investigations.

References

1. Dubey JP, Carpenter JL, Speer CA, et al. Newly recognized fatal protozoan disease of dogs. *J Am Vet Med Assoc* 1988;192:1269–1285.
2. Cummings JF, de Lahunta A, Suter MM, et al. Canine protozoan polyradiculoneuritis. *Acta Neuropathol* 1988;76:46–54.
3. Speer CA, Dubey JP. Scanning and electron microscopy of ovine mesenteric arteries infected wih first generation meronts of *Sarcocystis tenella*. *Can J Zool* 1982;60:203–209.
4. Dubey JP, Beattie CP. *Toxoplasmosis of animals and man*. Boca Raton, Fla: CRC Press Inc, 1988;1–220.
5. Dubey JP, Desmonts G. Serological responses of equids fed *Toxoplasma gondii* oocysts. *Equine Vet J* 1987;19:337–339.
6. Dubey JP. Toxoplasmosis in dogs. *Canine Pract* 1985;12:7–28.
7. Bjerkås I, Mohn SF, Presthus J. Unidentified cyst-forming sporozoan causing encephalomyelitis and myositis in dogs. *Z Parasitenkd* 1984;70:271–274.
8. Bjerkås I, Presthus J. Immuno-histochemical and ultrastructural characteristics of a cyst-forming sporozoon associated with encephalomyelitis and myositis in dogs. *Acta Pathol Microbiol Immunol Scand* 1988;96:445–454.