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Canine neosporosis: clinical signs, diagnosis, treatment and isolation of *Neospora caninum* in mice and cell culture

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Abstract

Clinical signs, diagnosis, treatment and isolation of *Neospora caninum* from two littermate dogs are described. Three of six pups from a Labrador bitch developed paralysis. Neosporosis was diagnosed ante mortem by serological examination in two of the affected pups. At necropsy, tissue cysts were seen in unstained smears and in histologic sections of their brains. Tissue cysts were often thin-walled ($\sim 1\ \mu$m) but antigenically and ultrastructurally identified as *N. caninum*. Furthermore, *N. caninum* (isolates NC-4, NC-5) was isolated in mice and in cell cultures inoculated with neural tissues of these two dogs. Serological diagnosis of neosporosis using a variety of tests is discussed. © 1998 Australian Society for Parasitology. Published by Elsevier Science Ltd.

Keywords: Neosporosis; *Neospora caninum*; Clinical signs; Isolation; Mice; Cell culture; Treatment; Clindamycin

1. Introduction

Neosporosis, caused by the cyst-forming apicomplexan, *Neospora caninum*, is now recognised as an important cause of mortality in dogs throughout the world [1, 2]. In most instances, diagnosis of canine neosporosis has been made post mortem. In four instances, diagnosis was confirmed by isolation of *N. caninum* from tissues of affected dogs [3–6]. Of these four isolates of *N. caninum*, the NC-1 isolate, originally obtained from neural tissues of a paralysed dog [3], is widely used as a source of antigen for serological diagnosis of neosporosis. In addition to canine isolates, a few other isolates of *N. caninum* have been obtained from cattle [7–9], and a horse [10]. However, the complete life-cycle of *N. caninum* and sources of infection are unknown.

In the present paper, we report antemortem diagnosis of neosporosis, treatment, and successful isolation of *N. caninum* in cell culture and in mice from tissues of two littermate dogs.

2. Materials and methods

2.1. Case history

Six pups were born on 19 December 1996 to an 8-year-old Labrador Retriever bitch. The pups appeared normal at birth and were dewormed at 23 days of age. Eleven days after deworming (pups were 34 days old), one male pup (dog 1) was brought to the Warner Robins Animal Hospital

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(WRAH), Warner Robins, GA, because it suddenly became lame. Palpation demonstrated laxity in cranial cruciate ligaments in the right stifle joint, with a pronounced cranial drawer sign. A diagnosis of strained/ruptured cranial cruciate ligament was made and a splint was applied. Re-examination of the dog, 5 days later, revealed anisocoria (dilation of left pupil), muscle atrophy of the right front leg, and a drooping of the right side of the face and lip. The owner elected euthanasia and the dog was buried; necropsy was not permitted.

At 40 days of age, the remaining pups were brought to the WRAH for distemper vaccination. During physical examination, one pup (dog 2) was found to have a limp of the right rear leg. Five days later, the limp of the right rear limb was found to be more pronounced, with laxity of the stifle and slight stiffness of both rear limbs. At the same time, a third pup (dog 3) was found to have a non-painful mild limp of the right rear leg. At this time, neosporosis was suspected and sera of affected and unaffected pups were submitted to the USDA Laboratory in Beltsville, MD for serological diagnosis. The diagnosis of neosporosis was confirmed (see Results section). The owner elected to have dog 2 euthanised. At necropsy, selected tissues were fixed in 10% buffered neutral formalin. Unfixed samples of brain and spinal cord were shipped refrigerated to Beltsville for parasite isolation.

After establishment of the diagnosis of neosporosis, the remaining pups and bitch were administered clindamycin (Antirobe, UpJohn Pharmaceuticals; 12.5–18.5 mg kg^{-1} body weight orally, twice daily for 10 days). Clinical signs in dog 3 did not worsen and the remaining pups remained clinically normal. Dog 3 was killed and necropsied at 131 days of age because it was still lame. Tissues and serum samples were submitted to the Beltsville laboratory for further evaluation. Two pups (dogs 4 and 5) were sold in good health. Serum samples from the bitch and the remaining pup (dog 6) were obtained periodically for *N. caninum* antibody evaluation (see Tab. 1).

2.2. *Detection of* N.caninum *by direct microscopic examination of dog brain*

For direct microscopic examination, 2–3-mm^3 pieces of the cerebrum of dogs 2 and 3 were crushed on glass slides under coverslips, and examined microscopically for tissue cysts.

2.3. *Attempted isolation of* N.caninum *from dogs*

2.3.1. *Dog* 2

Coverslips from two smears of the brain of dog 2 with demonstrable tissue cysts were removed and washed along with glass slides in a bottle with 50 ml of 0.85% NaCl solution (saline). To this mixture, 50 ml 0.5% trypsin solution in PBS, pH 7.2 was added and the mixture was incubated at 37°C for 10 min in a shaking water bath to dislodge brain tissue from glass and to free bradyzoites from tissue cysts. The mixture was centrifuged at 1200 *g* for 10 min and the pellet was suspended in 1-ml antibiotic saline (1000 units of penicillin and 100 μg of streptomycin per ml of saline) and inoculated into two γ-interferon knockout (KO) mice, 0.5 ml i.p. into one mouse and 0.5 ml s.c. into the other mouse.

Brain and spinal cord (total 25 g) of the dog were homogenised in 125 ml of saline, centrifuged at 1200 *g*, and the sediment was suspended in 25 ml of saline. Six millilitres of the homogenate were mixed with 6 ml of 0.5% trypsin solution in PBS and incubated at 37°C in a shaker bath. After 30-min incubation, the homogenate was centrifuged twice, suspended in 6 ml of antibiotic solution, and inoculated s.c. into 12 20–25-g Swiss Webster mice, eight of which were cortisonised [methyl prednisolone acetate (MPA), 2.5 mg i.m. on day 0 and 21 days later].

For isolation of *N. caninum* in cell culture, 5 g of dog brain that had been kept at 4°C for 3 days after necropsy were homogenised in HBSS and 15 ml of the homogenate were incubated with 15 ml of 0.5% trypsin solution for 30 min at 37°C, centrifuged, washed twice with HBSS, and then inoculated onto HS68 cells in a tissue culture flask as described [3]. After 1-h incubation at 37°C, the inoculum was removed, the cells rinsed with RPMI 1640 culture medium, and incubated in fresh RPMI 1640 medium at 37°C in 5% CO_2, 95% air.

2.3.2. *Dog 3*

Homogenates (20% w/v) of brain and spinal cord in saline were trypsinised, washed, and suspended in antibiotic saline as described for dog 2. The

Table 1
Antibody titres to *Neospora caninum* in dogs

	Antibodies to *N. caninum*							
		IFAT					CI ELISA[c]	
Dog no.	Day bled[a]	IgG[b]	IgM[c]	NAT[d]	r-ELISA[e]	Iscom-ELISA[f]	O.D.[g]	%Inhibition
2	50	50	<10	80	<100	0.181	0.328	67.1%
	53	100	<10	40	<100	0.270	0.322	67.7%
3	50	400	<10	200	200	0.426	0.157	84.2%
	131	3200	<10	≥6400	200	1.474	0.128	87.1%
4	50	50	<10	80	<100	0.111	0.435	56.3%
6	49	200	<10	40	200	0.377	0.239	76.0%
	138	100	<10	40	400	0.870	0.116	88.4%
	182	200	<10	80	200	1.169	0.178	82.1%
Bitch	50	3200	<10	≥6400	1600	1.280	0.214	78.5%
	138	1600	<10	≥6400	800	1.433	0.149	85.0%
	182	1600	<10	≥6400	800	1.466	0.191	80.8%

[a]Age or day after parturition. [b]Cut-off 1:50. [c]Cut-off 1:10. [d]Cut-off 1:20. [e]Cut-off 1:100. [f]Absorbance values <0.2 were considered negative; 0.2–0.4 as questionable, and ≥0.4 as positive [16]. [g]Cut-off value 0.39 = negative [18].

homogenates were inoculated i.p. into a KO mouse and s.c. into 10 mice (five BALB/c and five Swiss Webster).

The brain homogenate was also inoculated i.p. into four mice (two Swiss Webster and two BALB/c) and s.c. into six mice (three Swiss Webster and three BALB/c). All 10 mice were injected i.m. with 2.5 mg of MPA on the day of inoculation with canine tissues. In addition, the Swiss Webster mice were each given 2.5 mg of MPA 3 days before inoculation with canine tissues. Each mouse received 0.5 ml of the homogenate.

For isolation in cell culture, two flasks of HS68 cells were inoculated with 1 ml of the trypsin-digested brain homogenate. After 1 h the RPMI 1640 medium was removed, and the cells were rinsed with fresh RPMI 1640 medium and incubated at 37°C in 5% CO_2, 95% air.

2.4. Preparation of DNA samples and PCRs

DNA was prepared from canine tissues (samples of brain and right rear limb muscle tissue from dog 3, and brain tissue from dog 2) and cell culture-derived tachyzoites according to standard procedures [11]. Two *N. caninum*-specific PCR assays were used to detect the presence of *N. caninum* DNA; the *N. caninum* 14-3-3 gene nested PCR assay [11] and the Nc5 PCR assay [12] using the improved primers Np21+ and Np6+ [13]. As a control, the B1 gene PCR assay was used to test for the presence of *Toxoplasma gondii* [14]. The components and conditions of the amplifications were essentially as described by the authors. Briefly, reactions contained 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 μM (for Nc14-3-3)–0.5 μM (for Nc5) each primer, 0.2 mM dNTPs and 25 units ml^{-1} Taq polymerase (Gibco BRL). Thermocycling consisted of 30 cycles of the following: denaturation, 1 min at 94°C; annealing, 1 min at 58°C (for Nc14-3-3) or 62°C (for Nc5); extension, 72°C for 1.5 min. In the case of the nested Nc14-3-3 assay, after the primary amplification of 30 cycles, the reactions were diluted 1:10 in sterile distilled water and 2 μl of this were added to the secondary reaction mixture and a further 30 cycles of amplification were then carried out. Amplification products were analysed by electrophoresis through 6% polyacrylamide gels and stained with ethidium bromide. Sizes of specific products generated in the PCR assays are: Nc14-3-3, 614 bp; Nc5: 337 bp; B1, 195 bp.

2.5. *Western blot analysis of* N.caninum *isolates*

Culture-derived NC-1, NC-4 and NC-5 tachyzoites were harvested and purified as described [2], pelleted by centrifugation, resuspended in sonication buffer containing protease inhibitors, and extracted for total protein [15]. An equal amount (25 μg) of total protein was diluted in SDS–PAGE sample buffer, heated in a boiling water bath for 3 min, and pelleted by centrifugation at 13 000 *g* for 5 min. Soluble parasite antigen in the supernatant was electrophoresed on SDS–PAGE followed by electroblotting to Immobilon membrane (Millipore). After blotting, the membranes were treated with PBS containing 2% non-fat dry milk for 30 min to block non-specific antibody binding in subsequent steps. After blocking, the membranes were probed with rabbit sera against NC-1 tachyzoites [15] for 2 h at room temperature followed by biotinylated anti-rabbit IgG (Vector Laboratories) for 1 h and then avidin-peroxidase (Sigma) for 1 h. The immunoblots were washed three times between each incubation step. Antibody binding was assessed by treating the membranes with peroxidase substrate (0.06% 4-chloro-1-naphthol, 0.02% H_2O_2 [15].

2.6. *Serological examination*

Sera from dogs were examined for antibodies to *N. caninum* using the IFAT [3], iscom-ELISA [16, 18], competitive inhibition ELISA [17], direct *Neospora* agglutination test (NAT) [19], and recombinant (r)-ELISA [20]. For IFAT, *N. caninum* tachyzoite smears and anti-dog conjugates were obtained commercially (VMRD, Pullman). For IgM determination, anti-IgM-specific canine conjugate and IFAT were used with a 1:10 dilution of canine serum; the test was performed by Dr D. Scott Adams, VMRD, Pullman. For iscom-ELISA, sera were diluted 1:100 and tested by Dr Camilla Björkman, Swedish University of Agricultural Sciences, Uppsala, as described [16]. The r-ELISA was performed using a pool of two proteins (NCDG1 and NCDG2) as described [20]. Sera from dogs were also examined for antibodies to *T. gondii* using the modified agglutination test (MAT) as described [21].

Sera from all dogs from all bleedings were examined in each test at the same time to avoid day to day variation.

2.7. *Examination of mice inoculated with canine tissues*

Mice inoculated with canine tissues were examined for *N. caninum* and *T. gondii* parasites. Impression smears of lungs and brains of mice that died were fixed in methanol and examined after staining with Giemsa. Survivors were bled from the orbital sinus 8 weeks p.i. and 1:25 dilutions of their sera were examined for *T. gondii* antibodies by the MAT. For *N. caninum* antibodies, sera were diluted 1:100 and then assayed by r-ELISA [20]. A *Neospora*-positive control sample was obtained by pooling sera from several mice that had been inoculated with *N. caninum* tachyzoites.

Female Swiss Webster and BALB/c mice were obtained from Taconic Farms, New York and the KO mice were provided by Dr J.F. Urban, USDA, Beltsville.

2.8. *Histological and immunohistochemical examination of canine and mouse tissues*

For histological examination, tissues were fixed in 10% buffered neutral formalin. From dogs 2 and 3 specimens of brain, heart, skeletal muscle, liver and spleen were fixed for histological examination. In addition, small and large intestines, pancreas and kidneys of dog 2 were also fixed in formalin. Paraffin wax-embedded sections were cut at 5–6 μm thickness and examined after staining with H & E. For immunohistochemical examination, deparaffinised sections were stained with anti-*N. caninum* and *T. gondii* antibodies using polyclonal rabbit serum [22], and mAb *N. caninum* antibodies [23].

2.9. *Transmission EM*

For TEM, tissue removed from brain smears was fixed in 3% glutaraldehyde in Millonings' buffer, post-fixed in 1% (w/v) osmium tetroxide and prepared as described previously [24], except that it was stained with 1% (w/v) phosphotungstic acid

and 1% uranyl acetate in 70% (v/v) ethanol. Ultrathin sections were stained with lead citrate and examined in a JEOL 100 CX EM.

3. Results

3.1. Histological examination of dog tissues

Tissue cysts were seen in unstained squashes made from the brains of both dogs. In dog 2, only a few tissue cysts were seen in five of 30 smears of cerebrum. Most tissue cysts were small ($\sim 25\,\mu$m) and thin walled ($\sim 1\,\mu$m). More tissue cysts were seen in the brain of dog 3. Both thin- and thick-walled tissue cysts were present (Figure 1).

In dog 2, microscopic lesions were seen in the heart, skeletal muscles and brain. Tachyzoites were seen associated with lesions. There were multifocal areas of non-suppurative myositis. The myositis was more severe in the heart than in skeletal muscles. The encephalitis was characterised by perivascular cuffs and few glial nodules. Only a few tissue cysts were seen.

In dog 3, lesions were seen only in the brain. The encephalitis was characterised by multifocal areas of malacia, perivascular cuffs and mononuclear cell infiltrates in the neural parenchyma (Figure 1(A)). Few groups of tachyzoites were present in inflammatory areas and some tissue cysts were degenerating (Figure 1(A, B)). Tissue cysts were unevenly distributed; some sections had up to 12 tissue cysts per section, whereas others had none. Tissue cysts in sections (Figure 1(C, D)) were oval to circular, $10–42 \times 10–40\,\mu$m ($n = 15$) in size. The cyst wall was ~ 1-μm thick.

Ultrastructurally, four tissue cysts examined were oval in shape. The cyst wall was irregular in outline, and consisted of a parasitophorous vacuolar membrane and a thick underlying granular layer, approximately 1-μm thick. The parasitophorous vacuolar membrane had minute indentations (Figure 2).

Organisms in tissues (muscles of dog 2 and brain of dog 3) reacted positively to *N. caninum* polyclonal serum [22] and with mAb [23]. Tissue cysts from both dogs reacted weakly with *T. gondii* antibodies.

3.2. Isolation of N.caninum in mice and cell culture

Neospora caninum was isolated in mice and cell culture inoculated with neural tissues of both dogs. The KO mouse inoculated i.p. with washings from the brain squash of dog 2 became ill 14 days p.i. and tachyzoites were found in its peritoneal exudate (pex) at 15 days p.i. The mouse died of systemic neosporosis at 21 days p.i. Tachyzoites from pex were infective to cell culture as well as to other KO mice. The KO mouse inoculated s.c. with canine brain died 28 days p.i. and tachyzoites were found in the pex and the lungs.

Of the cortisonised Swiss Webster mice inoculated with canine tissues, four mice died at 23, 33, 34 and 39 days p.i. *Neospora caninum* tachyzoites were demonstrable in tissues of mice that died 34 and 39 days p.i.; tissues of two other mice were autolysed. The remaining four mice were killed 62 days p.i.; all mice had *N. caninum* antibodies in r-ELISA at a titre of $\geqslant$1:3200. Thick-walled tissue cysts of *N. caninum* were seen in brain squashes of two (one tissue cyst in one and three tissue cysts in the other) of the four mice. All four Swiss Webster non-cortisonised mice inoculated with canine brain remained clinically normal and were killed 62 days p.i. Antibodies to *N. caninum* were present in three mice (ELISA titres $<$1:100, 1:100, 1:100 and 1:800) but tissue cysts were not seen in any of the four mice. Antibodies to *T. gondii* were not found in 1:25 dilutions of serum in the MAT.

In the original flask inoculated with brain tissue of dog 2, parasites were not seen until day 58 p.i. The original flask still had some infected cells on day 102 p.i. when the flask was discarded. In follow-up experiments, the organism had been subcultured several times and infected cells were destroyed faster in subcultured flasks than in the original flask.

Neospora caninum tachyzoites obtained from pex of the KO mouse inoculated i.p. with brain of dog 2 were inoculated onto HS68 cells in a tissue-culture flask. After 90-min incubation, the flask was rinsed twice and fresh culture medium was added. Cells infected with tachyzoites were seen the next day. However, infection spread slowly to the adjoining cells and most host cells were not infected. The *N. caninum* isolate from dog 2 was designated NC-4 and frozen in liquid nitrogen.

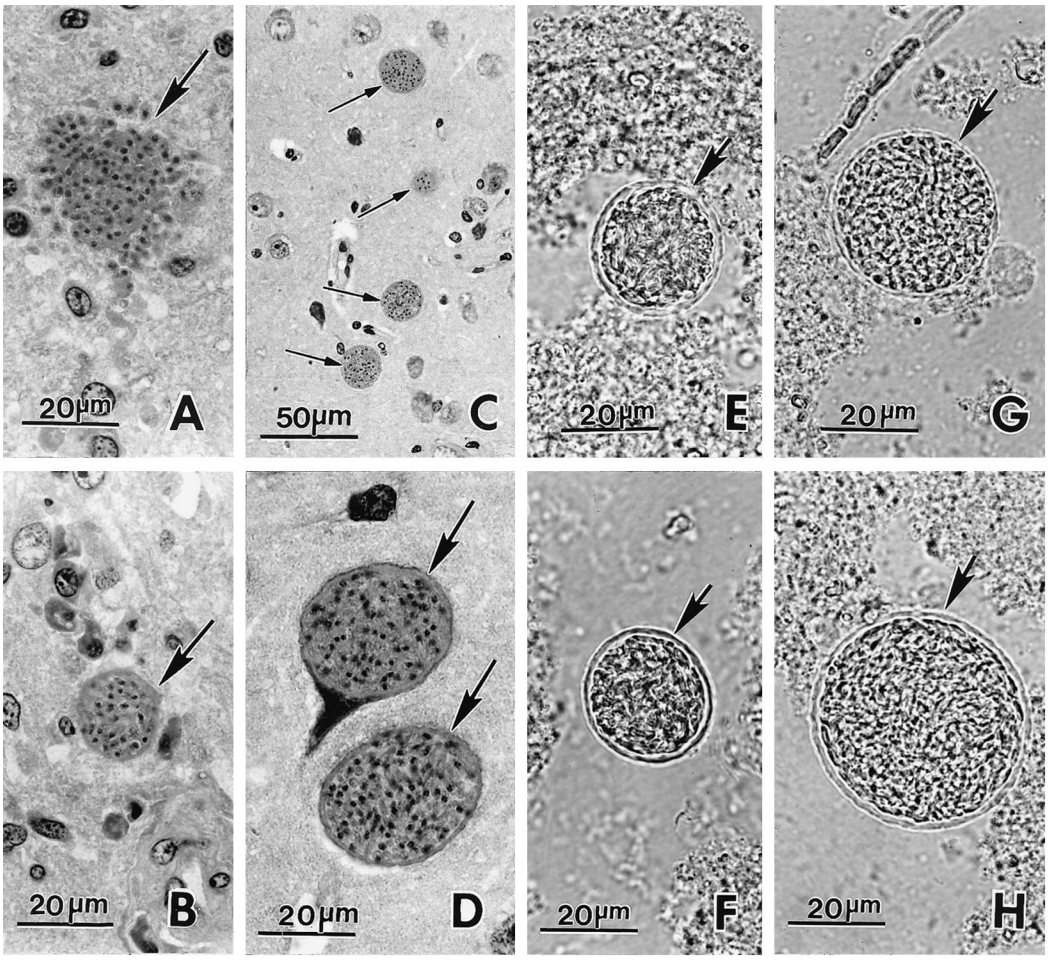

Fig. 1. *Neospora caninum* and lesions in cerebrum of dog 3. (A) A group of tachyzoites (arrow). H & E stain. (B) Degenerating tissue cyst (arrow) surrounded by mononuclear cells. H & E stain. (C) Four tissue cysts (arrows) without any host reaction. H & E stain. (D) Two tissue cysts with wavy cyst walls (arrows). H & E stain. (E–H) Tissue cysts in brain homogenate. Unstained. Note the varying thicknesses of the cyst walls (arrows).

The KO mouse inoculated i.p. with neural tissue of dog 3 became ill 10 days p.i. and was killed on day 12 p.i. Numerous *N. caninum* tachyzoites were seen in pex, lungs and liver, and tachyzoites obtained from the pex were infective to cultured HS68 cells.

Both Swiss Webster mice inoculated i.p. with tissues of dog 3 died of bacterial peritonitis 7 and 8 days p.i., and *N. caninum* was not seen in their pex. The remaining three mice inoculated s.c. were killed 49 days p.i. and two tissue cysts were seen in histologic sections of the brain of one mouse (Figure 3); tissue cysts were small (20 μm) and had 1-μm thick cyst walls. Of the two BALB/c mice inoculated i.p. with canine brain, one died 8 days p.i. and its tissues were not examined because of autolysis. The other mouse inoculated i.p. was killed 9 days p.i. and numerous *N. caninum* tachyzoites were seen in its pex and liver. One BALB/c mouse inoculated s.c. died 14 days p.i. and was not examined because of autolysis. The remaining two BALB/c mice inoculated s.c. were killed 49 days p.i. and degenerating *N. caninum* tissue cysts were seen in sections of their brain Fig. 3.

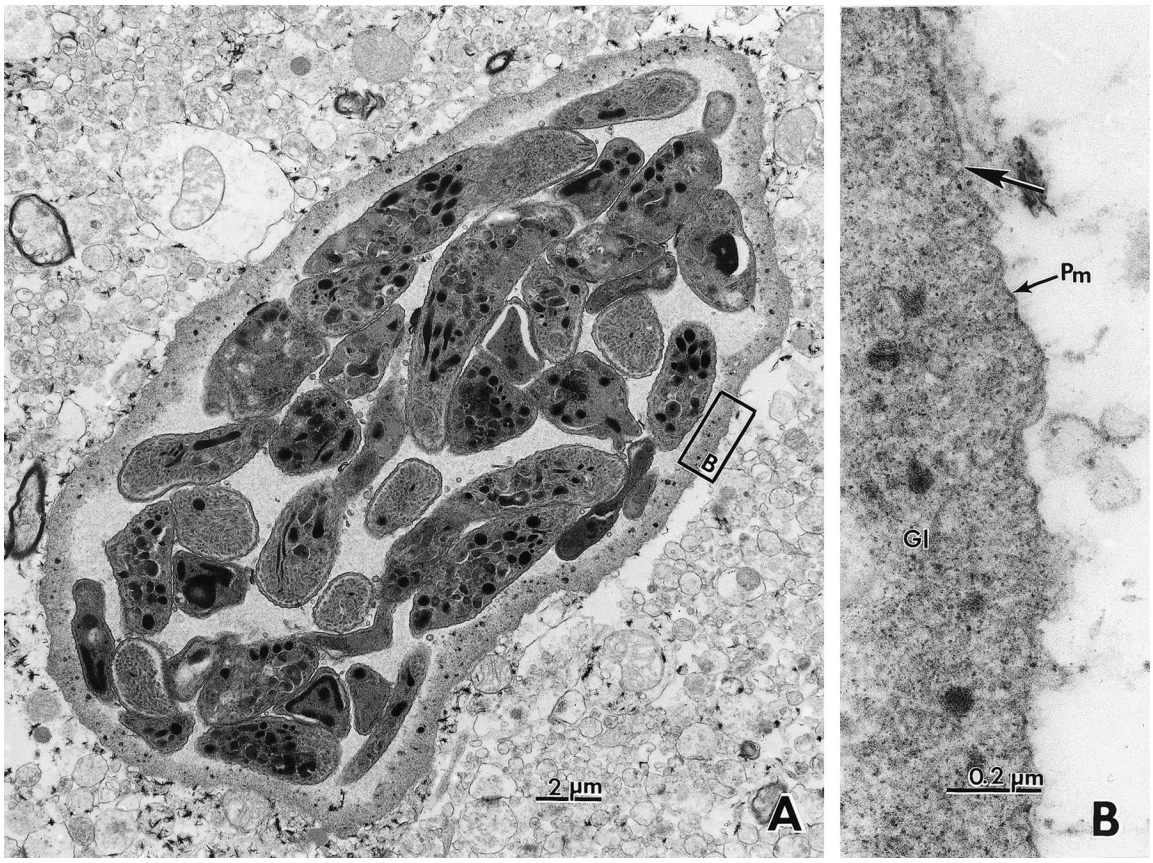

Fig. 2. Transmission EM of *N. caninum* tissue cysts in the brain of dog 3. (A) Brain-tissue cyst showing approximately 35 bradyzoites and an irregularly shaped cyst wall. (B) Higher magnification of the portion of cyst wall shown in (A); note that the parasitophorous vacuolar membrane (Pm) has minute indentations (arrow); GL = granular layer of cyst wall.

Neospora caninum tachyzoites were seen 13 days p.i. of the tissue culture flask with brain homogenate of dog 3. On day 29 p.i. some infected cells died, leaving clear areas. Of the remaining cells in the flask, approximately 50% were infected. The cells were scraped, suspended in 10% foetal bovine serum in culture medium and frozen in liquid nitrogen after adding dimethyl sulphoxide. This *N. caninum* isolate was designated NC-5.

3.3. Serological responses of dogs

Antibodies to *N. caninum* were detected in all dogs, but antibody levels varied with the serological test Table 1. The bitch had consistently high levels of antibodies in all tests. IgM antibodies were not detected in any dog. Antibodies to *T. gondii* were not detected in a 1:25 dilution of serum in MAT of any of the pups. The bitch had *T. gondii* MAT titres of $\geq$1:500, 1:400 and 1:400 in three bleeds.

3.4. Western blots

Immunostaining blots of NC-1, NC-4 and NC-5 tachyzoite antigen showed no differences in the banding pattern among the isolates (Figure 4). The minor differences observed were due to slightly different amounts of total antigen loaded onto the gel.

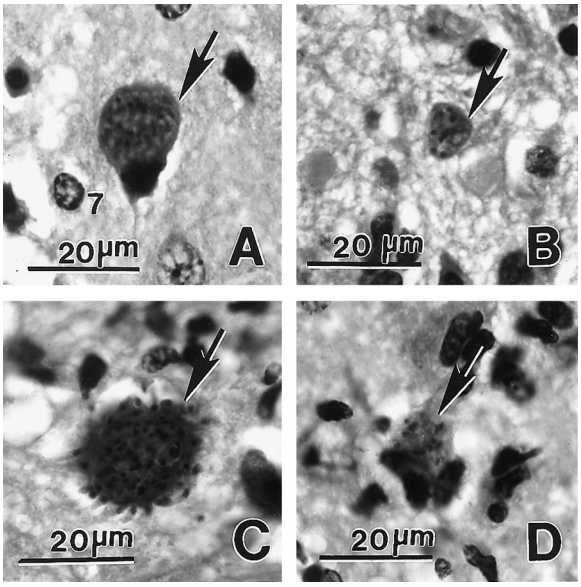

Fig. 3. Lesions and tissue cysts in sections of brain of mice 49 days after inoculation with neural tissues of dog 3. H & E stain. (A, B) Swiss Webster mice. Note intact small tissue cysts (arrows) without host reaction. (C, D) BALB/c mice with ruptured (C) and degenerating (D) tissue cysts (arrows). Note glial cells surrounding *N. caninum*.

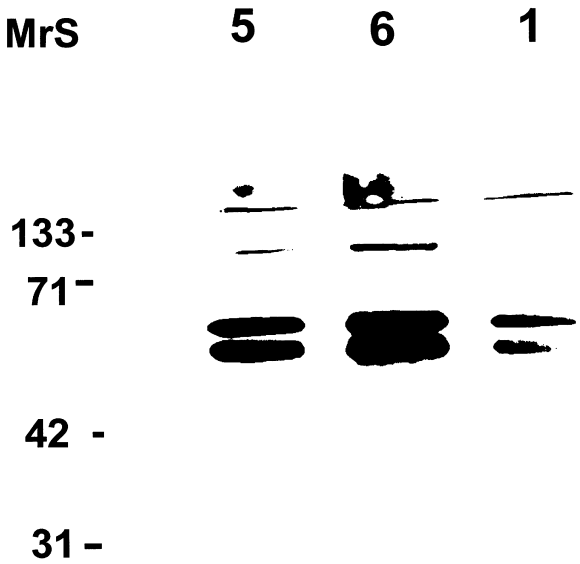

Fig. 4. Immunostaining of *N. caninum* tachyzoite antigen impregnated on Immobilon membrane with sera from rabbits immunised with NC-1 isolate tachyzoite antigens. 5 = NC-4 tachyzoite antigen; 6 = NC-5 tachyzoite antigen; 1 = NC-1 tachyzoite antigen; MrS = molecular weight markers.

3.5. *PCR detection of* N. caninum

Figure 5 shows the results of the *N. caninum*-specific PCR assays performed using DNA extracted from the cell-culture-derived tachyzoites of the NC-4 and NC-5 isolates. The NC-1, NC-4 and NC-5 isolates were positive in the *N. caninum*-specific Nc14-3-3 and Nc5 PCR assays. The NC-1, Nc-4 and Nc-5 isolates were negative in the *T. gondii* B1 PCR assay (not shown).

Neospora caninum DNA was detected in the brain tissue from dog 2 (note: muscle tissue was not available to be tested for dog 2) and in the brain and muscle tissue from dog 3 (not shown), using the Nc-5 and the Nc14-3-3 PCR assays (Figure 5B). The *T. gondii* B1 gene PCR was negative for the canine DNA samples (Figure 5B).

4. Discussion

Little is known of the significance of serological data in the diagnosis of canine neosporosis, except that IgG IFAT titres are generally $\geqslant$1:400 in dogs with confirmed clinical neosporosis [3–5, 25, 26]. There are few, if any, sequential serological data available from dogs with clinical neosporosis. In the present study, two infected litter mates, presumably infected at the same time, differed in their antibody response. At 50 days of age, dog 2 had an IFAT titre of 1:50, whereas dog 3 had an IFAT titre of 1:400. The presence of relatively low antibody titres in dogs 2 and 3 with confirmed neosporosis at 50 days of age may be due to the recency of infection.

A rising IgG antibody titre is usually indicative of acute infection. Dog 3 had a considerable rise in IgG level by IFAT, NAT and iscom-ELISA over a 12-week period, but this was not reflected by other tests used. The presence of IgM antibodies is also indicative of acute infection. In the present study, IgM antibodies were not detected in either of the two young dogs with confirmed neosporosis. This may be related to suppression of antibody synthesis associated with the presence of maternally transferred IgG antibodies as reported with *T. gondii* infections [27]. Although the antibody levels in colostrum were not determined, the dam had a high

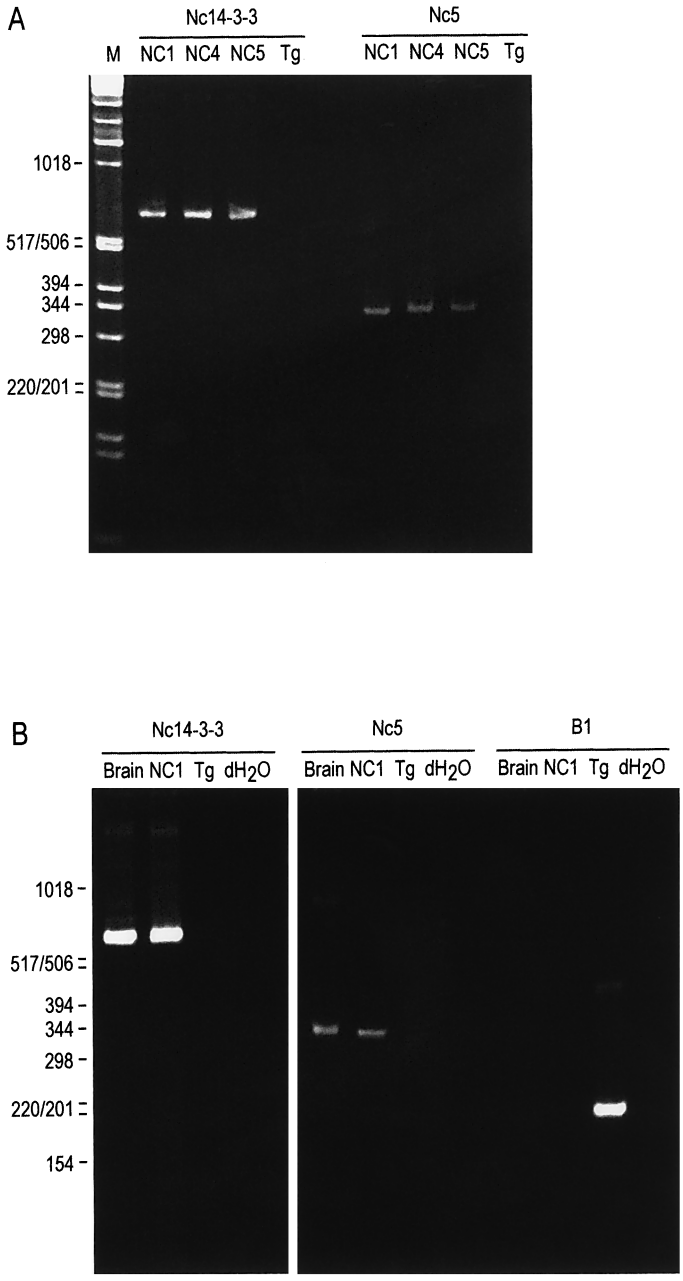

Fig. 5. Polyacrylamide gel electrophoresis of PCR products. (A) The Nc14-3-3 PCR assay products from DNA extracted from cell-culture-derived tachyzoites of the NC-1, NC-4 and NC-5 isolates, along with negative control *T. gondii* DNA (Tg), are shown on the left side of the gel. The products of the Nc5 PCR assay on the same DNA samples are shown on the right side of the gel. Molecular weight markers (1 kb ladder, GIBCO) are on the left, with relevant markers indicated (in bp). (B) PCRs from DNA prepared from brain sample from dog 2. Products of the *N. caninum*-specific Nc14-3-3 and Nc5 assays, and the *T. gondii*-specific B1 PCR assay, are shown. Samples were DNA from dog 2 brain tissue, NC-1 tachyzoites and *T. gondii*. A water sample (dH_2O) was included as a negative control. Molecular weight markers (in bp) (1 kb ladder, GIBCO BRL) are indicated on the left.

level of serum IgG *N. caninum* antibodies in all tests. Antibody titres in the IFAT paralleled titres obtained by the newly described NAT and by the iscom-ELISA. Whole tachyzoites are used as antigen in both IFAT and NAT, and in the iscom-ELISA tachyzoite membrane antigens are used.

Antibody titres were also sought by three types of ELISA. Little is reported on the use of ELISA for the diagnosis of clinical neosporosis. One of the problems with serological assays is setting the cut-off values, and there are no ELISA data from dogs experimentally infected with *N. caninum*. With both iscom-ELISA and r-ELISA, serum from dog 2 gave negative results, but this may have been due to the cut-off values being set too high. The results indicate that there is a need for further evaluation of the ELISA for the diagnosis of canine neosporosis.

Neosporosis is not a new disease, but until 1988 it was misdiagnosed as toxoplasmosis because *N. caninum* structurally resembles *T. gondii* [1]. Tachyzoites of both parasites are structurally similar by light microscopy. However, they can be distinguished ultrastructurally by their rhoptries; rhoptries of *N. caninum* are electron-dense whereas those of *T. gondii* are electron-lucent [28]. By light microscopy, tissue cysts of *N. caninum* can often be distinguished from tissue cysts of *T. gondii*, because *N. caninum* tissue cysts are thick walled ($\geqslant 1\ \mu$m) compared with thin-walled ($<0.5\ \mu$m) tissue cysts of *T. gondii*. However, this distinction is not always possible, as demonstrated in the present paper. The thickness of the cyst wall may be age-related. Several tissue cysts in dogs in the present report were thin walled and indistinguishable from *T. gondii* tissue cysts. However, tissue cysts in both dogs were *N. caninum* and not *T. gondii*, because dogs 2 and 3 were serologically negative to *T. gondii*. Furthermore, *T. gondii* was not isolated in mice and cell culture, and the PCR analysis detected only *N. caninum*.

Little is known of the formation of *N. caninum* tissue cysts in any host. In the original description of *N. caninum* [1], four large (55–107-μm long) tissue cysts were seen in the cerebellum of a 2-year-old Poodle. Since that report such large *N. caninum* tissue cysts have not been reported in dogs. Bjerkås and Presthus [29] found tissue cysts measuring 20–60 μm in diameter in seven dogs from Norway. Jardine [30] made a detailed study of tissue cysts of *N. caninum* in three dogs from South Africa. Tissue cysts were up to 31.2-μm long, but mostly $17 \times 14.5\ \mu$m [30]. In the present study, several tissue cysts seen in a 131-day-old dog (dog 3) were small and thin walled. The relatively large and thick-walled tissue cysts observed in the 2-year-old Poodle may be related to the age of the dog or the strain of the parasite.

Tissue cysts seen in the present report were structurally similar to those described previously [29–32]. However, Jardine [30] observed tissue cysts from one dog as having branching septa separating bradyzoites in groups. Such septa were not seen in the four tissue cysts we examined and, in our opinion, the septa observed by Jardine [30] may either be artifacts or belong to another parasite, as *N. caninum* tissue cysts have been reported as having no septa [2].

In the present study, diagnosis of neosporosis was made by direct demonstration of tissue cysts in the brains of dogs, by serological examination, by demonstration of *N. caninum* DNA in dog brain, by histology, by TEM, and by isolation of the parasite in cell culture and mice. All of these methods can aid in the diagnosis of neosporosis. The simplest and quickest method is by demonstration of *N. caninum* in biopsy (e.g., muscular tissue) or in CSF. The use of PCR can also provide a quick and specific diagnosis.

Antiprotozoal drugs are beneficial to dogs with clinical neosporosis. Clindamycin, sulfonamides and pyrimethamine have been used with encouraging results [25, 33–35]. Of 16 neosporosis-confirmed dogs with hind-limb paralysis, treatment was considered partially effective in 10 [25]. Viable *N. caninum* was not demonstrable in an adult dog with histologically proven neosporosis-associated dermatitis after prolonged clindamycin therapy [35]. Although the effect of clindamycin therapy on tissue cysts of *N. caninum* is unknown, clindamycin has no effect on tissue cysts of *T. gondii* [36]. In the present study, both tachyzoites and tissue cysts of *N. caninum* were demonstrable in the brain of dog 3, suggesting that short-term treatment may not kill all tachyzoites.

The full life-cycle of *N. caninum* is unknown. A carnivore host is suspected in the life-cycle, but

preliminary attempts to find the *N. caninum* oocyst have been unsuccessful [2]. Unfortunately, most of this work was done using the NC-1 isolate of *N. caninum* which does not produce many tissue cysts (Dubey, unpublished observations). Experimental production of viable tissue cysts of *N. caninum* is a problem. As yet, tissue cysts have not been seen in cell cultures inoculated with *N. caninum*. *Neospora caninum* is non-pathogenic and rarely infective to outbred Swiss Webster mice. Although tissue cysts can be found in cortisonised outbred mice infected with *N. caninum*, the numbers of tissue cysts produced are variable [37–40]. In addition, tissue cysts from cortisonised mice were not infective to cats by the oral route [41]. Although *N. caninum* can be fatal to inbred BALB/c mice the results are variable [42, 43], but histologically confirmed structurally intact tissue cysts are rare in BALB/c mice [42]. In BALB/c mice inoculated with *N. caninum*, clinical neural neosporosis has been seen as long as 18 months p.i. in association with *N. caninum* tachyzoites (Dubey, unpublished). Finding of small, degenerating or rupturing tissue cysts in BALB/c mice in the present study may explain the pathogenesis of chronic neosporosis associated with tissue-cyst rupture. In the present study KO mice inoculated with *N. caninum* died of overwhelming neosporosis but tissue cysts were not seen. Therefore, efforts should continue to produce tissue cysts in non-cortisonised mice.

Although *N. caninum* is structurally similar to *T. gondii*, the isolation of *N. caninum* in mice or cell culture is more difficult than the isolation of *T. gondii*. *Neospora caninum* grows more slowly than *T. gondii* in cell culture. This was evident with the NC-4 isolate of *N. caninum* from dog 2, where organisms were not detected until 58 days p.i. Therefore, cell culture should not be discarded until 2 months after inoculation with suspected material. Of the three types of mice (cortisconised outbred, BALB/c and KO) used to isolate *N. caninum* in the present study, KO mice were the most susceptible. Similar results were obtained with the *N. caninum* isolate from a calf by Stenlund et al. [8].

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