Parasitol Res (2000) 86: 165–168

RAPID COMMUNICATION

J.P. Dubey · D.S. Lindsay

Gerbils (*Meriones unguiculatus*) are highly susceptible to oral infection with *Neospora caninum* oocysts

Received: 10 September 1999 / Accepted: 10 September 1999

Abstract Laboratory-reared gerbils (*Meriones unguiculatus*) were found to be highly susceptible to oral infection with *Neospora caninum* (NC-Liv strain) oocysts. Gerbils fed ~1000 oocysts became sick or died at 6–13 days post feeding of oocysts (PFO). *N. caninum* was isolated in cell culture and from γ-interferon-knockout mice inoculated with homogenates of mesenteric lymph nodes of gerbils examined as early as 1 day PFO. Numerous *N. caninum* tachyzoites were found in ulcerative lesions in the intestines of gerbils examined at 7–9 days PFO. In a gerbil fed 10 oocysts, *N. caninum* tachyzoites were found in lesions in the brain. Gerbils fed 10 oocysts developed antibodies to *N. caninum* by 18 days PFO as determined by the *Neospora* agglutination test (titers ≥1:500). All gerbils remained negative for antibodies to *Toxoplasma gondii* as determined by the *Toxoplasma* agglutination test.

Introduction

The coccidian *Neospora caninum* is a major cause of abortion in cattle and also causes crippling diseases in companion animals (Dubey and Lindsay 1996; Dubey 1999). Until recently, passage from the dam to the fetus was the only known mode of transmission. In 1998 the domestic dog was found to be a definitive host for *N. caninum* (McAllister et al. 1998). However, the role of the *N. caninum* oocyst in the natural epidemiology of neosporosis is unknown. There are several unresolved problems in this area:

1. *N. caninum* oocysts have not been found in the feces of naturally infected dogs.
2. Are there other definitive hosts in the life cycle of *N. caninum*?
3. Dogs fed tissue cysts shed relatively few oocysts (McAllister et al. 1998; Lindsay et al. 1999a).
4. There is no sensitive and efficient method for identification of *N. caninum* oocysts in canine feces. *N. caninum* oocysts are morphologically identical to *Hammondia heydorni* oocysts (Lindsay et al. 1999b), and they cannot be identified without bioassay.
5. There is no good bioassay for *N. caninum* oocysts because even immunosuppressed mice are resistant to oral feeding of oocysts. For example, in the study reported by Lindsay et al. (1999a), *N. caninum* oocysts were infective to only one of eight γ-interferon-knockout (KO) mice. Moreover, the KO mice are very expensive and often in short supply.

In the present report, we demonstrate the high susceptibility of gerbils (*Meriones unguiculatus*) to oral infection with *N. caninum* oocysts.

Materials and methods

Two mixed-breed littermate dogs were individually fed eight mouse brains containing tissue cysts of the NC-Liverpool isolate of *Neospora caninum* (Barber et al. 1995). Both dogs were negative (titer < 1:25) for antibodies to *N. caninum* as determined by the indirect fluorescent antibody test (IFAT; Cole et al. 1995) and the *N. caninum* agglutination test (Romand et al. 1998) prior to feeding infected mouse brains. Feces containing *N. caninum* oocysts were collected from each dog on days 5–10 postfeeding, mixed in 2% (v/v) sulfuric acid, strained with a tea strainer, and placed in 2-l plastic beverage bottles, and the suspension was aerated from an aquarium pump for 48–72 h (Lindsay et al. 1999a). Oocysts were concentrated by flotation in Sheather's sugar solution and were stored at

J.P. Dubey (✉)
United States Department of Agriculture,
Agricultural Research Service,
Livestock and Poultry Sciences Institute,
Parasite Biology and Epidemiology Laboratory,
Beltsville, MD 20705, USA
e-mail: jdubey@lpsi.barc.usda.gov; Fax: +1-301-5049222

D.S. Lindsay
Center for Molecular Medicine and Infectious Diseases,
Department of Biomedical Sciences and Pathobiology,
Virginia-Maryland Regional College of Veterinary Medicine,
Virginia Tech, 1410 Prices Fork Road,
Blacksburg, VA 24061-0342, USA

4 °C in 2% sulfuric acid solution. After 28–30 days of storage the oocysts were treated in 5.25% (v/v) sodium hypochlorite (SH) solution at 4 °C for 10 min. The SH was washed off the oocysts by repeated centrifugation in sterile Hanks' balanced salt solution (HBSS). Oocysts were then stored in sterile HBSS at 4 °C for less than 2 weeks before being fed to gerbils. The numbers of oocysts in the inocula were estimated by the performance of four counts in a hemacytometer. No oocyst was observed in these counts, and a value of 1000 oocysts/ml was assumed because some oocysts were present in wet mounts.

Female gerbils (30–40 g; Charles River Lakeview, Neufield, N.J., USA) were used in the present study. Oocysts were diluted 10-fold (10^{-1} to 10^{-5}), and groups of 2–8 gerbils were fed oocysts with an animal-feeding needle (Table 1). All gerbils were examined at necropsy.

Four gerbils (numbers 6136–6139) were fed ~1000 oocysts and were killed on days 1, 2, 3, and 4 post feeding of oocysts (PFO; Table 1). Their entire small intestines were flushed with 10% neutral buffered formalin (NBF) solution. Samples taken at about 2-cm intervals were blocked in paraffin for histologic examination. The other gerbils were examined at 7–32 days PFO (Table 1).

Portions of all major organs, including the brain, heart, liver, kidneys, eyes, spleen, adrenals, kidneys, stomach, small and large intestine, tongue, and skeletal muscle from the thigh, were also fixed in NBF solution and processed for histologic examination. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and examined microscopically. Selected tissues were embedded in methylacralate, and 3-μm sections were stained with H&E for microscopic examination. For immunohistochemical testing, paraffin-embedded sections were subjected to examination using polyclonal rabbit anti-*N. caninum* serum according to the techniques and reagents described by Lindsay and Dubey (1989a) or by monoclonal antibodies as described by Cole et al. (1993). For isolation of *N. caninum* in cell culture, portions of mesenteric lymph nodes or lung were homogenized and inoculated onto M617 or equine kidney cells (Dubey et al. 1999). For bioassay, mesenteric lymph nodes from the gerbils killed on days 1 and 2 PFO were homogenized and inoculated subcutaneously into γ-interferon-KO mice as described elsewhere (Dubey et al. 1998).

Table 1 Neosporosis in gerbils fed *Neospora caninum* oocysts

Dilution fed	Gerbil number	Day of necropsy[a]	*N. caninum* in tissues[b]	NAT[c]
Neat	6138	K 1	Not seen	ND
	6137	K 2	Not seen	ND
	6139	K 3	I	ND
	6136	K 4	I, Li[d]	ND
	6135	KI 7	H[d], I, Li[d], Lu, M.L.	ND
	6134	KI 9	B, I, Lu, Li[d], M.L.	ND
	6132	KI 9	B, I, Lu, Li[d], M.L., S	ND
	6133	D 11	B, I, Lu, M.L.	ND
10^{-1}	6130	KI 13	B, I, Lu, M, M.L.	50
	6131	K 18	B, Lu, Li[d]	> 500
10^{-2}	6128	K 21	B, Lu, Li[d], M.L.[d]	5000
	6129	K 25	Not seen	50
10^{-3}	6126	KI 16	A, B, H[d], K, Li[d], Lu, M, T[d]	> 500
	6127	K 28	Not seen	< 25
10^{-4}	6144	K 31	Not seen	< 25
	6145	K 31	Not seen	< 25
10^{-5}	6124	K 32	Not seen	25
	6125	K 32	Not seen	< 25

[a] *D* Died, *K* killed, *KI* killed when ill
[b] *B* Brain, *H* heart, *I* intestine, *Li* liver, *Lu* lung, *K* kidney, *M* muscles, *T* tongue, *A* adrenal, *M.L.* mesenteric lymph nodes, *S* stomach
[c] *NAT* Reciprocal *Neospora* agglutination-test titers, *ND* not determined
[d] Lesions; organisms were not seen

Sera from gerbils were examined for antibodies to *N. caninum* and *Toxoplasma gondii* using the modified direct agglutination test for each parasite (Dubey and Desmonts 1987; Romand et al. 1998).

Results

The gerbils killed at 1–4 days PFO were clinically normal. Gerbils fed undiluted inocula (Table 1) first became dull at 6 days PFO and were euthanized on days 7 and 9 PFO. As visualized grossly, the small intestines were hyperemic and edematous and Peyer's patches appeared enlarged. The mesenteric lymph nodes were edematous and had pale areas indicative of necrosis. Gerbils examined on days 7 and 9 PFO exhibited peritonitis with 1–3 ml of exudate; *Neospora caninum* was not observed in the exudate. Lesions and *N. caninum* stages were not observed in histology sections of tissues taken from gerbils at 1 and 2 days PFO. The γ-interferon-KO mice injected with mesenteric lymph-node preparations from gerbils killed at 1, 2, and 4 days PFO died of neosporosis at 10, 11, and 7 days after infection, respectively, and tachyzoites were seen in their tissues. *N. caninum* was isolated in cell cultures inoculated with mesenteric lymph-node preparations from gerbils killed at 1 and 7 days PFO and from the lungs of the gerbil killed at 13 days PFO.

N. caninum tachyzoites and lesions were seen in Peyer's patches of gerbils at 3 and 4 days PFO. At day 3 PFO, a few tachyzoites were seen in the epithelium lining a Peyer's patch and were associated with individual cell necrosis.

At day 4 PFO, several groups of tachyzoites were associated with necrosis of Peyer's patches (Fig. 1). At 7 and 9 days PFO there was focal ulcerative transmural enteritis (Figs. 2, 3). Tachyzoites were seen in lesions in

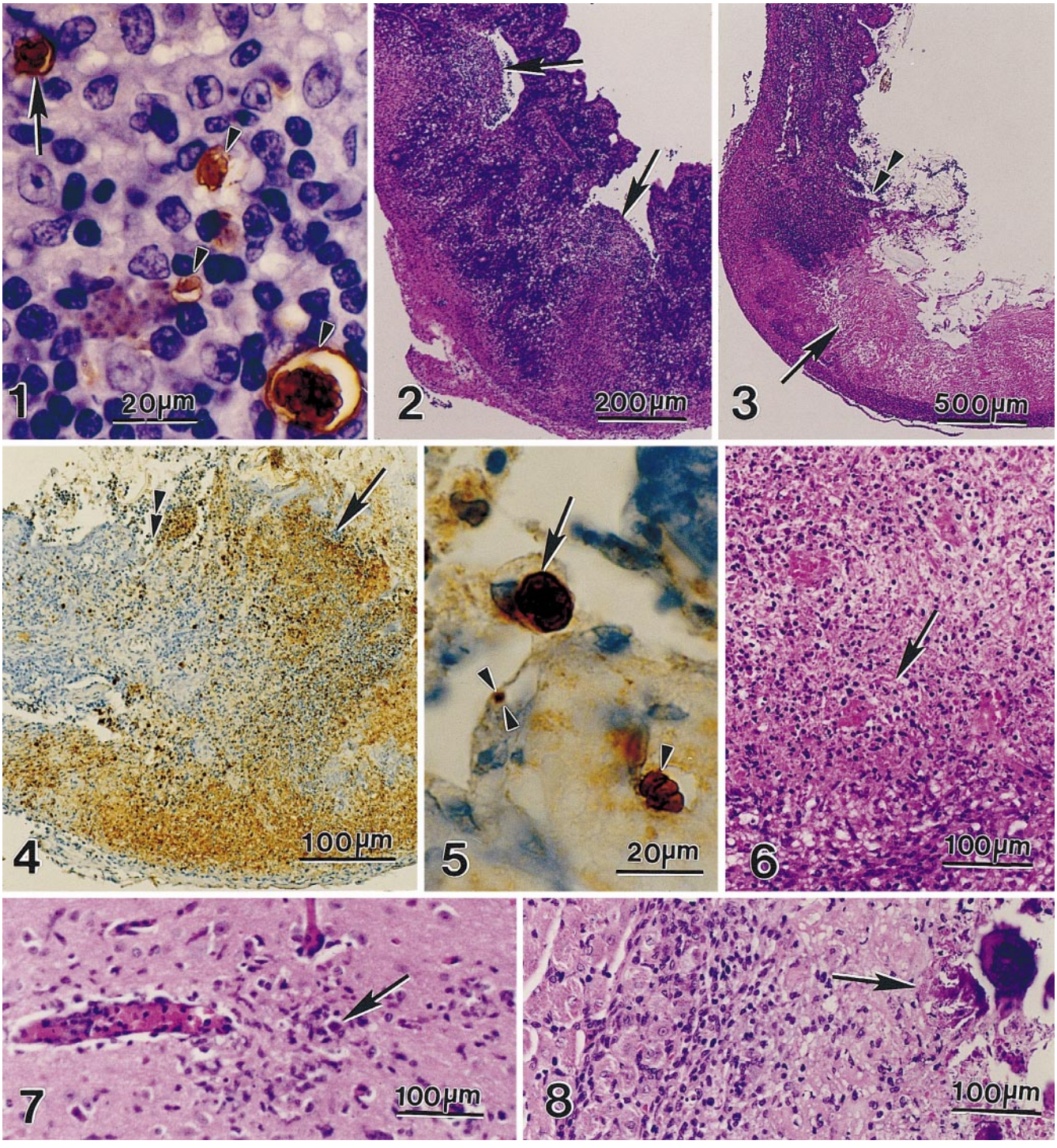
►

Figs. 1–8 Lesions and *Neospora caninum* tachyzoites detected in sections of tissues taken from gerbils fed *N. caninum* oocysts
Fig. 1 Peyer's patch in small intestine at 4 days PFO. Note the tachyzoites in the epithelium (*arrow*) and in the lamina propria (*arrowheads*). Immunohistochemical staining with anti-*N. caninum* antibodies
Fig. 2 Peyer's patch in small intestine at 7 days PFO. Note the necrosis (*arrows*) of lymphoid tissue. H&E
Fig. 3 Necrosis and ulceration (*arrow*) of a Peyer's patch in small intestine at 9 days PFO. *Double arrowheads* point to the edge of an ulcer. H&E
Fig. 4 Higher magnification of the area of the ulcer (*arrow*) shown in Fig. 3. Note the edge of the ulcer (*double arrowheads*). The *brown material* represents *N. caninum* antigen. Immunohistochemical staining with anti-*N. caninum* antibodies
Fig. 5 Higher magnification of Fig. 4, showing one intracellular group of tachyzoites in a denuded epithelial cell in the lumen (*arrow*), one group of tachyzoites in the lamina propria (*arrowhead*), and one tachyzoite in the epithelium (*opposing arrowheads*). Immunohistochemical staining with anti-*N. caninum* antibodies
Fig. 6 Necrosis (*arrow*) of a mesenteric lymph node at 9 days PFO. H&E
Fig. 7 Cerebrum at 9 days PFO, showing vasculitis and an inflammatory focus. H&E
Fig. 8 Skeletal muscle at 16 days PFO, showing an inflammatory focus along with necrosis and mineralization (*arrow*). H&E

the intestines and mesenteric lymph nodes (Figs. 4–6). By day 9 PFO, *N. caninum* had spread to the heart and brain (Figs. 7, 8). In the gerbil killed on day 16 PFO, multiple inflammatory foci along with necrosis and mineralization were present in skeletal muscles (Fig. 8). Antibodies to *Toxoplasma gondii* were not found in a 1:25 dilution of sera from any gerbil. Gerbils seroconverted to *N. caninum* after 13 days of infection (Table 1).

Discussion

Until now, there has been no satisfactory rodent model for neosporosis. *Neospora caninum* is not pathogenic for outbred mice (Lindsay and Dubey 1989b, 1990). Although certain inbred strains of immunodeficient mice and chemically immunosuppressed mice are susceptible to parenteral inoculation with *N. caninum* tachyzoites



(Lindsay et al. 1995; Dubey et al. 1998), none seems to be highly susceptible to oral inoculation with *N. caninum* oocysts (Lindsay et al. 1999a). Although gerbils are susceptible to parenteral inoculation with tachyzoites of the NC-3 strain and the NC-JPA-1 strain, results obtained in gerbils have been variable (Cuddon et al. 1992; Gondim et al. 1999). The NC-3 strain of *N. caninum* has not been recovered after the first passage in gerbils (Cuddon et al. 1992), whereas the NC-JPA-1 strain can be maintained by serial passage in gerbils (Gondim et al. 1999). The oral oocyst model of *N. caninum* in gerbils should be useful for the study of host parasite responses in an immunocompetent host. The intestinal lesions seen in the present study are similar to those reported by Gray et al. (1996) in a horse with visceral neosporosis.

The results of the present study demonstrate that *N. caninum* oocysts can remain viable for at least 14 days after treatment with 5.25% SH solution. Procedures used to obtain clean, viable oocysts of *N. caninum* have not previously been addressed because oocysts have only recently been discovered. Treatment with SH sterilizes oocyst preparations and reduces levels of fecal matter. However, fecal debris remained a problem in the present study, and the actual numbers of oocysts present in the inocula were impossible to determine by hemacytometer counts. Because oocysts were not visible in hemacytometer chambers, their actual numbers were probably lower than the 1000 estimated. Therefore, the values listed in Table 1 represent crude estimates, and further research is needed to determine the relationships between oocyst counts and actual infective doses. In the present study, oocysts of only one strain (NC-Liv) were tested. Further research is needed to study the pathogenicity of different strains of oocysts in gerbils.

Acknowledgements We thank O.C.H. Kwok, S.K. Shen, and Diane Hawkins-Cooper for their technical assistance. A portion of this research was supported in part by a grant from the Virginia-Maryland Regional College of Veterinary Medicine (to D.S.L.).

References

Barber JS, Holmdahl OJM, Owen MR, Guy F, Uggla A, Trees AJ (1995) Characterization of the first European isolate of *Neospora caninum* (Dubey, Carpenter, Speer, Topper, and Uggla). Parasitology 111: 563–568

Cole RA, Lindsay DS, Dubey JP, Blagburn BL (1993) Detection of *Neospora caninum* in tissue sections using a murine monoclonal antibody. J Vet Diagn Invest 5: 579–584

Cole RA, Lindsay DS, Blagburn BL, Sorjonen DC, Dubey JP (1995) Vertical transmission of *Neospora caninum* in dogs. J Parasitol 81: 208–211

Cuddon P, Lin DS, Bowman DD, Lindsay DS, Miller TK, Duncan ID, Lahunta A de, Cummings J, Suter M, Cooper B, King JM, Dubey JP (1992) *Neospora caninum* infection in English springer spaniel littermates: diagnostic evaluation and organism isolation. J Vet Intern Med 6: 325–332

Dubey JP (1999) Recent advances in *Neospora caninum* and neosporosis. Vet Parasitol 84: 349–367

Dubey JP, Desmonts G (1987) Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet J 19: 337–339

Dubey JP, Lindsay DS (1996) A review of *Neospora caninum* and neosporosis. Vet Parasitol 67: 1–59

Dubey JP, Dorough KR, Jenkins MC, Liddell S, Speer CA, Kwok OCH, Shen SK (1998) Canine neosporosis: clinical signs, diagnosis, treatment and isolation of *Neospora caninum* in mice and cell cultures. Int J Parasitol 28: 1293–1304

Dubey JP, Mattson DE, Speer CA, Baker RJ, Mulrooney DM, Tornquist SJ, Hamir AN, Gerros TC (1999) Characterization of a *Sarcocystis neurona* isolate (SN6) from a naturally infected horse from Oregon. J Eukaryot Microbiol 46: 500–506

Gondim LFP, Saeki S, Onaga H, Haritani M, Yamane I (1999) Maintenance of *Neospora caninum* tachyzoites using Mongolian gerbils (*Meriones unguiculatus*). NZ Vet J 47: 36

Gray ML, Harmon BG, Sales L, Dubey JP (1996) Visceral neosporosis in a 10-year-old horse. J Vet Diagn Invest 8: 130–133

Lindsay DS, Dubey JP (1989a) Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. Am J Vet Res 50: 1981–1983

Lindsay DS, Dubey JP (1989b) *Neospora caninum* (Protozoa: Apicomplexa) infections in mice. J Parasitol 75: 772–779

Lindsay DS, Dubey JP (1990) Infections in mice with tachyzoites and bradyzoites of *Neospora caninum* (Protozoa: Apicomplexa). J Parasitol 76: 410–413

Lindsay DS, Lenz SD, Cole RA, Dubey JP, Blagburn BL (1995) Mouse model for central nervous system *Neospora caninum* infections. J Parasitol 81: 313–315

Lindsay DS, Dubey JP, Duncan RB (1999a) Confirmation that dogs are a definitive host for *Neospora caninum*. Vet Parasitol 82: 327–333

Lindsay DS, Upton SJ, Dubey JP (1999b) A structural study of the *Neospora caninum* oocyst. Int J Parasitol 29: 1521–1523

McAllister MM, Dubey JP, Lindsay DS, Jolley WR, Wills RA, McGuire AM (1998) Dogs are definitive hosts of *Neospora caninum*. Int J Parasitol 28: 1473–1478

Romand S, Thulliez P, Dubey JP (1998) Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitol Res 84: 50–53