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Rapid communication

Gray wolf (*Canis lupus*) is a natural definitive host for *Neospora caninum*

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ABSTRACT

The gray wolf (*Canis lupus*) was found to be a new natural definitive host for *Neospora caninum*. *Neospora*-like oocysts were found microscopically in the feces of three of 73 wolves from Minnesota examined at necropsy. *N. caninum*-specific DNA was amplified from the oocysts of all three wolves. Oocysts from one wolf were infective for the gamma interferon gene knock out (KO) mice. Viable *N. caninum* (designated NcWolfUS1) was isolated in cell cultures seeded with tissue homogenate from the infected mouse. Typical thick walled tissue cysts were found in outbred mice inoculated with the parasite from the KO mouse. Tissue stages in mice stained positively with *N. caninum*-specific polyclonal antibodies. Our observation suggests that wolves may be an important link in the sylvatic cycle of *N. caninum*.

1. Introduction

The protozoan *Neospora caninum* infects many species of warm blooded animals and is a major cause of bovine abortion worldwide (Dubey, 2003; Dubey et al., 2007). Its life cycle involves canids as definitive hosts and ruminants as intermediate hosts.

The domestic dog (*Canis domesticus*) is both a natural and experimental definitive host for *N. caninum* (McAllister et al., 1998; Basso et al., 2001). Two other canids, the coyote (*Canis latrans)* and the Australian dingo (*C. domesticus*) have been demonstrated as its experimental definitive hosts (Gondim et al., 2004a; King et al., 2010). We report here shedding of viable *N. caninum* oocysts in feces of naturally infected gray wolf (*Canis lupus*).

2. Materials and methods

2.1. Examination of wolf feces for N. caninum oocysts

Wolf samples were provided by the Minnesota Department of Natural Resources, which included wolves provided by the USDA-APHIS-Wildlife Service, vehicle-killed or found dead of other causes. Feces (1–10 g) were collected from the rectum of 73 wolves examined at necropsy. Feces were refrigerated, and shipped without preservation to the Animal Parasitic Diseases Laboratory (APDL), Beltsville, MD. At APDL, feces were emulsified in water, filtered through gauge, and centrifuged in a 50 ml tube at 1000 rpm ($292 \times g$) for 10 min. After discarding the supernatant, the sediment was mixed with approximately 50 ml of 33% aqueous sucrose solution (sp. gr. 1.15) and centrifuged for 10 min. A drop of the float from the very top of the meniscus was examined microscopically between a glass slide and coverslip for oocysts. Five ml of the liquid from the very top of the float were mixed with 45 ml of water and the suspension was centrifuged for 10 min at 2000 rpm ($1171 \times g$). After discarding the supernatant, the

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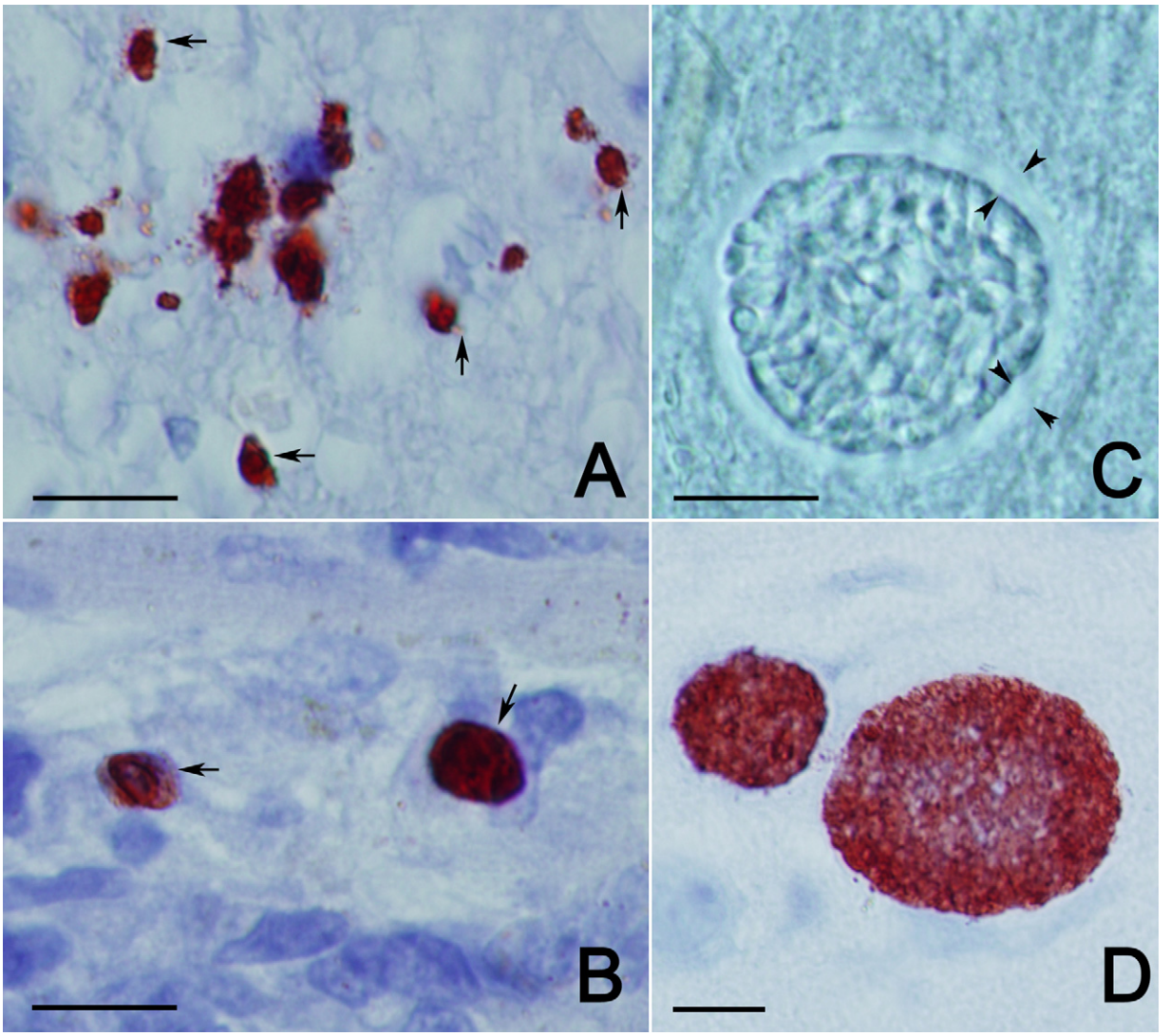

Fig. 1. *Neospora caninum* in tissues of mice inoculated with isolate from the wolf. ADW1 A, B, D, immunohistochemical staining with polyclonal *N. caninum* specific rabbit antibody. C = unstained. (A) Section of spinal cord of SW mouse 334, 70 day p.i. This mouse had paralysis in hind limbs. Several tachyzoites (arrows) are present in a focus of myeltis. (B) Section of the heart of KO mouse 103, 70 days p.i. Tachyzoites (arrows) are present in an inflammatory focus. (C) Tissue cyst in SW mouse 70 days p.i. Note thick cyst wall (arrows). (D) Two tissue cysts in section of the cerebrum of the same SW mouse as in C. Bar = 10 μm.

sediment was mixed with 10 ml of 2% sulfuric acid, and incubated on a shaker at room temperature for 7 days.

2.2. Bioassays of oocysts in mice for N. caninum

For bioassay, the sulfuric acid of incubated oocysts was neutralized with 3.3% NaOH, centrifuged for 10 min at 2000 rpm. The supernatant was discarded, and the sediment was mixed with antibiotic saline, and inoculated orally and subcutaneously into gamma gene knock out (KO) mice (McAllister et al., 1998). The KO mice that died or were euthanized when ill were examined for *Neospora*. Survivors were bled 2 months later and their sera were examined for antibodies to *N. caninum* and *Toxoplasma gondii* using respective agglutination tests as described previously by Romand et al. (1998) for *Neospora* (*Neospora* agglutination test, NAT) and by Dubey and Desmonts (1987) for *T. gondii* (modified agglutination test, MAT). Sera were screened at 1:25 serum dilution.

2.3. Necropsy and immunohistological examination

Samples of all major organs of mice were fixed in 10% buffered formalin. Paraffin-embedded tissues were sectioned at 5 μm and examined after staining with hematoxylin and eosin (H and E). Immunohistochemistry was performed on paraffin-embedded sections at APDL using reagents and methods described previously by Lindsay and Dubey (1989). *N. caninum* tachyzoites and tissue cysts (Fig. 1A–D) were recognized in smears and sections using previously defined structural features (Dubey et al., 2002).

Table 1
Details of the wolves positive for *Neospora*-like oocysts.

Wolf I.D.	Date	NAT	PCR	Bioassay in KO mice	*N. caninum* strain designation	Nc 5 sequence
W072210LDM2	8-4-2010	<25	+	K(109)-Neg.Ks (29)-Pos.	NcWolfUS1	JF827721
W082010ADW1	8-24-2010	<25	+	Neg.	NcWolfUS2	No
W082510JPG1	8-31-2010	<25	+	Neg.	NcWolfUS3	No

K: killed apparently healthy; ks: killed when sick on day indicated in parenthesis; NAT: *Neospora* agglutination test; PCR: polymerase chain reaction.

2.4. In vitro cultivation

Lung tissue from KO mouse 49 (Table 2) with demonstrable tachyzoites was homogenized in RPMI-1640 medium supplemented with L-glutamine, and seeded on to CV1 cells. The brain of SW mouse 334 containing tissue cysts was homogenized and trypsinized (0.5%) for 10 min at 37 °C and centrifuged at 2000 rpm for 10 min (Dubey and Schares, 2006). After removing trypsin by centrifugation, the homogenate was seeded on to two flasks of CV1 cells. The cell cultures were observed microscopically for growth of *N. caninum* for 3 months.

2.5. DNA extraction and PCR amplification

N. caninum oocysts from three wolf fecal samples (ADW1, JPG1, LDM2) were washed two times in deionized water to remove H_2SO_4, and suspended in buffer AL (Qiagen, Inc., Valencia, CA, USA). The oocysts were broken open by two-2 min extractions with 0.5 mm sterile glass bead on a Mini-Bead Beater (Bio-Spec Products, Inc., Bartlesville, OK, USA). The oocyst DNA was purified using a QIAamp Mini DNA kit and instructions provided by the manufacturer (Qiagen). After spin-column elution, the DNA was further concentrated by ethanol precipitation (Sambrook et al., 1989), allowed to air-dry, and suspended in 20 μl TE (1 mM Tris–HCl, pH 6.8, 0.1 mM EDTA). DNA from mouse lung and from cell cultures inoculated with *N. caninum* isolated from mouse lung were extracted using the QIAamp Mini DNA kit, but without bead-beating. Oocysts, mouse lung, and cell culture DNA were subjected to PCR amplification of the Nc5 gene sequence (Kaufmann et al., 1996; Müller et al., 1996; Liddell et al., 1999) and analyzed by acrylamide gel electrophoresis followed by EtBr staining, and visualization and capture on a GelLogic 200 Imaging System (Kodak). For oocysts samples (JPG1) that generated a low amount of amplification product in primary PCR, a nested PCR amplification using NP7/NP10 primers was conducted (Wapenaar et al., 2006). Primary and nested amplification products were excised directly from polyacrylamide gels and separated from the gel slice by incubation for 16 h at 37 °C in elution buffer (Sambrook et al., 1989). The eluted amplification products were removed to a new microcentrifuge tube, concentrated by ethanol precipitation, dried at room temperature, suspended in sterile water, and then inserted into pGEM-T Easy cloning vector (Novagen, San Diego, CA) using T4 DNA ligase (NEB, Ipswich, MA, USA). The ligation mixtures were introduced into *Escherichia coli* DH5 using standard procedures (Hanahan, 1983), followed by colony PCR analysis using standard procedures (Güssow and Clackson, 1989). At least two separate PCR amplifications were conducted for each DNA sample, and a minimum of three recombinant clones were evaluated for each amplification. Colony amplification reactions exhibiting the expected size product by polyacrylamide gel electrophoresis were then subjected to sequence analysis using M13 forward and reverse primers, and a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). Sequencing reactions were run on an ABI373 sequencer and analyzed using Sequencher 4.9 software (GeneCodes Corp., Ann Arbor, MI, USA). Sequences for each oocyst, mouse lung, and cell culture DNA amplification products were first aligned (Altschul et al., 1990) using Sequencher 4.9 software to produce a consensus sequence (GenBank Accession No. JF827721), which was then aligned to Nc5 sequences deposited in GenBank using the ClustalX software (Thompson et al., 1997). Nc5 sequences included in the analysis were AF190701 (bovine), X84238 (dog), AY459289 (bovine), FJ464412 (bovine), EF202082 (rodent), HM031965 (bison), FR823382 (dog), DQ132435 (fox), EF463098 (bovine), EF581827 (bovine), and DQ132440 (coyote). Phylogenetic trees were reconstructed from these alignments using the neighbor joining method (Saitou and Nei, 1987). The stability of the branching order was confirmed by performing 1000 bootstrap replicates.

Table 2
Infectivity of *N. caninum* tachyzoites of the NcWolfUS1 strain to mice.

Data	KO mice				Swiss Webster mice								
Mouse no.	46	48	49	104	326	327	328	329	330	331	332	333	334
Day p.i.	Ks 17	Ks 17	Ks 13	Ks 17	D 26	K 70	K 70	K 70	K 70	K 70	K 70	K 70	Ks 70
Smear	Pos.[a]	Pos.[a]	Pos.[a]	Pos.[a]	NE	Neg.	Neg.	Pos.[b]	Neg.	Neg.	Neg.	Neg.	Pos.[b]
Cell culture	ND	ND	Pos.	ND	ND	ND	ND	ND	ND	ND	ND	ND	Pos.
Sections	ND	ND	Pos.	ND	ND	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Pos.
NAT	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.

D: died; K: killed apparently healthy; ks: killed when sick; ND: no data; Neg.: not detected. Pos.: detected. NAT: *Neospora* agglutination test.
[a] Tachyzoites in lungs.
[b] Tissue cysts in brain.

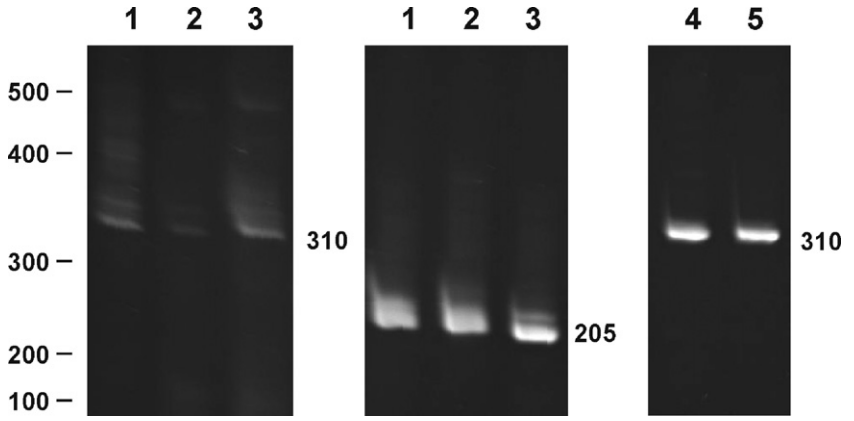

Fig. 2. Amplification of *N. caninum* Nc5 gene sequence using primary (Np6+/Np21+, panels 1 and 3) or nested (Np7/Np10, panel 2) PCR on DNA extracted from oocysts recovered from wolf feces (1, wolf ADM; 2, wolf JPG; 3, wolf LDM) or from lung tissue (4) of KO mouse inoculated with oocysts from wolf LDM2, or from cell cultures (5) inoculated with tachyzoites recovered from mouse lung. Primary PCR product ~310 nt; nested PCR product ~205 nt.

3. Results

3.1. Isolation of Neospora from wolf feces

Unsporulated *Neospora*-like oocysts (approximately 12 μm in diameter) were seen microscopically in fecal floats of three of 73 wolves. These oocysts sporulated at room temperature. Oocysts were not counted but estimated to be approximately 10,000 in total sample (approximately 10 g feces). Oocysts were not measured because of the small numbers.

One of the two KO mice inoculated with oocysts from wolf ADW1 became ill and was euthanized day 29 p.i., and protozoal tachyzoites were found in smears of its lung (Table 1). Homogenate of lung of the mouse was inoculated into four KO mice and nine SW mice; the SW mice were medicated with dexamethasone phosphate in drinking water (10 μg/ml) from day 0 to 46 day p.i. (Table 2). Tachyzoites were found in smears of lungs of all four KO mice. Tachyzoites (Fig. 1A and B) were found in tissues of SW or KO mice and tissue cysts were found in SW mice (Fig. 1C and D). The SW mouse no. 334 became paretic in both hind limbs and was euthanized day 70 p.i. Histologically, this mouse had focal myeltis and polyradiculoneuritis associated with tachyzoites (Fig. 1A). Antibodies to *N. caninum* were found in 11 of the 13 mice inoculated with the wolf strain NcWolfUS1 (Table 2). All sera were negative for *T. gondii* antibodies in 1:25 dilution of mouse serum (data not shown).

Protozoal tachyzoites were observed within 1 week of inoculation of tachyzoites from KO mouse 49, and the culture was cryopreserved when cells started to lyse on 61 days p.i. In the two flasks seeded with trypsinized brain of mouse 334, *N. caninum* grew slowly, and cultures were cryopreserved on days 91 and 129 days.

Neither antibodies nor *N. caninum* tissue stages were seen in KO mice fed oocysts from wolves ADW1, and JPG1 (Table 1).

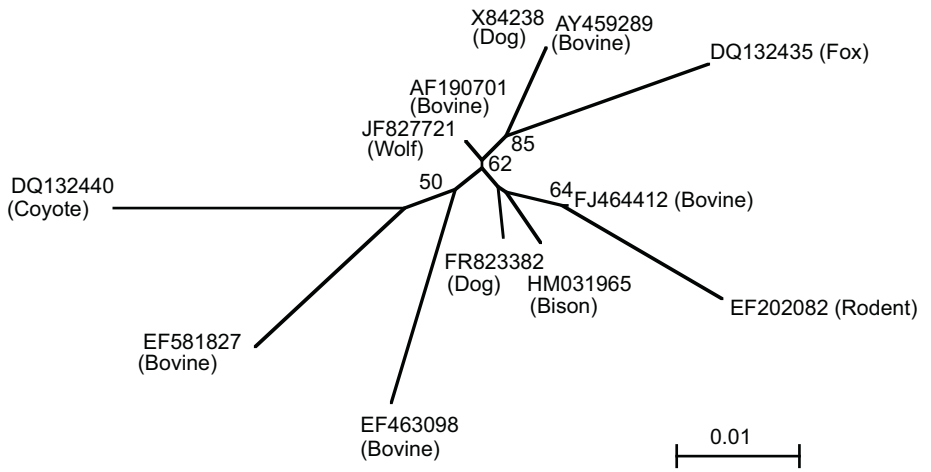

Fig. 3. Unrooted phylogenetic tree constructed using neighbor joining, and Nc5 sequences AF190701 (bovine), X84238 (dog), AY459289 (bovine), FJ464412 (bovine), EF202082 (rodent), HM031965 (bison), FR823382 (dog), DQ132435 (fox), EF463098 (bovine), EF581827 (bovine), DQ132440 (coyote). Bootstrap values over 50% are shown at nodes.

3.2. Genetic characterization

Nc5 amplification products (~350 bp) were identified by primary PCR using NP6+/NP21+ primers on all three oocysts DNA samples (Table 1). However, Nc5 clones were obtained only with ADM1 and LDM2 possibly because the amplification signal in primary PCR with JPG oocysts was low (Fig. 2). A strong signal was observed in nested PCR on all three oocysts samples from wolves (Fig. 2). Also, positive amplification reactions were observed in primary Nc5 PCR on mouse lung tissue and cell culture DNA (Fig. 2). Alignment of Nc5 sequences from all oocysts, mouse tissue, and cell culture DNA samples revealed nearly complete agreement with each other, with only minor clone-specific differences that were probably due to Taq polymerase errors during amplification (data not shown). BLAST-N searching using the *N. caninum* wolf Nc5 consensus sequence revealed a 96–99% identity with *N. caninum* Nc5 sequences in GenBank (Supplement Fig. S1). Neighbor joining method was used to produce an unrooted tree (Fig. 3). The sequences from the wolf were mostly closely related to *N. caninum* isolate from a bovid calf in Austria (AF190701).

4. Discussion

The number of *N. caninum* oocysts shed by naturally and experimentally infected dogs is low, yet infection is common in cattle worldwide (Dubey et al., 2007). *N. caninum* is one of the most efficiently transplacentally transmitted parasites among all known microbes of cattle. Once infection is introduced into a herd, up to 90% of progeny could be transplacentally infected (Dubey et al., 2007). Therefore, only a few oocysts may be needed for transmission of the parasite to cattle. Gondim et al. (2004a) proposed a sylvatic cycle of *N. caninum* in the US. *N. caninum* antibodies have been found in gray wolf in several countries including the US (Gondim et al., 2004b; Dubey and Thulliez, 2005; Steinman et al., 2006; Sobrino et al., 2008; Almberg et al., 2009; Björkman et al., 2010; Stieve et al., 2010; Dubey and Schares, in press). Almberg et al. (2009) found *N. caninum* antibodies in 50% of 220 wolves from the Yellowstone National Park, indicating efficient sylvatic cycle of *N. caninum* proposed by Gondim et al. (2004b).

In the present study, the infectivity of *N. caninum* oocysts to KO mice was low, probably because rodents and other small mammals are not good hosts for *N. caninum*, including the KO mice (McAllister et al., 1998). Although in one study, *N. caninum* oocysts were lethal for gerbils (*Meriones unguiculatus*), the results were inconsistent (Dubey and Lindsay, 2000). Viable *N. caninum* could not be isolated or demonstrated histologically in gerbils fed oocysts derived in dogs by feeding naturally infected buffalo tissues (Neto et al., 2011). Additionally, not all isolates of *N. caninum* can be grown in cell culture. Therefore, wolves join dogs as the only documented sources of viable oocysts of *N. caninum*. Although *N. caninum* DNA has been reported in feces of naturally infected coyotes and foxes (Wapenaar et al., 2006), viable *N. caninum* has not been isolated from tissues or feces of these hosts. The definitive diagnosis of *N. caninum* oocysts in wolf feces was made based on the recovery of characteristic thick walled tissue cysts of the parasite (Dubey et al., 2002). We are aware that oocysts of felid coccidians *T. gondii*, and *Hammondia hammondi* were detected in dog feces, resulting from coprophagia (Schares et al., 2005), and thus wolves could have been a transport rather than a true definitive host. However, unlike *T. gondii* and *H. hammondi*, only a few *N. caninum* oocysts have been found in naturally infected dogs, and there is a very remote possibility of wolves ingesting dog feces in the wilderness.

The Nc5 sequence data also supported that observed tachyzoites were *N. caninum* revealing greater than 95% similarity with Nc5 sequences in the GenBank database. Cladistic analysis of the Nc5 sequences obtained by PCR amplification of DNA from *N. caninum* oocysts recovered from wolves showed no obvious grouping in the phylogenetic tree. This evolutionary analysis suggests that sequences from isolates of *N. caninum* from the intermediate hosts are interspersed with sequences derived from the definitive hosts, and points to an exchange of *N. caninum* between domestic (dog) and sylvatic (wolf) definitive hosts. Understanding this cycle further will require additional *N. caninum* samples from definitive hosts, and wild intermediate hosts, such as deer.

Conflict of interest

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2011.05.018.

References

Almberg, E.S., Mech, L.D., Smith, D.W., Sheldon, J.W., Crabtree, R.L., 2009. A serological survey of infectious disease in Yellowstone National Park's canid community. PLoS ONE 4, e7042–e7050.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Basso, W., Venturini, L., Venturini, M.C., Hill, D.E., Kwok, O.C.H., Shen, S.K., Dubey, J.P., 2001. First isolation of *Neospora caninum* from the feces of a naturally infected dog. J. Parasitol. 87, 612–618.

Björkman, C., Jakubek, E.B., Arnemo, J.M., Malmsten, J., 2010. Seroprevalence of *Neospora caninum* in gray wolves in Scandinavia. Vet. Parasitol. 173, 139–142.

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

Dubey, J.P., Lindsay, D.S., 2000. Gerbils (*Meriones unguiculatus*) are highly susceptible to oral infection with *Neospora caninum* oocysts. Parasitol. Res. 86, 165–168.

Dubey, J.P., Barr, B.C., Barta, J.R., Bjerkås, I., Björkman, C., Blagburn, B.L., Bowman, D.D., Buxton, D., Ellis, J.T., Gottstein, B., Hemphill, A., Hill, D.E., Howe, D.K., Jenkins, M.C., Kobayashi, Y., Koudela, B., Marsh, A.E., Mattsson, J.G., McAllister, M.M., Modrý, D., Omata, Y., Sibley, L.D., Speer, C.A., Trees, A.J., Uggla, A., Upton, S.J., Williams, D.J.L., Lindsay, D.S., 2002. Redescription of *Neospora caninum* and its differentiation from related coccidia. Int. J. Parasitol. 32, 929–946.

Dubey, J.P., 2003. Neosporosis in cattle. J. Parasitol. 89 (Suppl.), S42–S56.

Dubey, J.P., Thulliez, P., 2005. Prevalence of antibodies to *Neospora caninum* in wild animals. J. Parasitol. 91, 1217–1218.
Dubey, J.P., Schares, G., 2006. Diagnosis of bovine neosporosis. Vet. Parasitol. 140, 1–34.
Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemology and control of neosporosis and *Neospora caninum*. Clin. Microbiol. Rev. 20, 323–367.
Dubey, J.P., Schares, G. Neosporosis in animals – the last five years. Vet. Parasitol., in press.
Gondim, L.F.P., McAllister, M.M., Pitt, W.C., Zemlicka, D.E., 2004a. Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 34, 159–161.
Gondim, L.F.P., McAllister, M.M., Mateus-Pinilla, N.E., Pitt, W.C., Mech, L.D., Nelson, M.E., 2004b. Transmission of *Neospora caninum* between wild and domestic animals. J. Parasitol. 90, 1361–1365.
Güssow, D., Clackson, T., 1989. Direct clone characterization from plaques and colonies by the polymerase chain reaction. Nucl. Acids Res. 17, 4000.
Hanahan, D., 1983. Studies on the transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166, 557–580.
Kaufmann, H., Yamage, M., Roditi, I., Dobbelaere, D., Dubey, J.P., Holmdahl, O.J., Trees, A., Gottstein, B., 1996. Discrimination of *Neospora caninum* from *Toxoplasma gondii* and other apicomplexan parasites by hybridization and PCR. Mol. Cell. Probes 10, 289–297.
King, J.S., Slapeta, J., Jenkins, D.J., Al-Qassab, S.E., Ellis, J.T., Windsor, P.A., 2010. Australian dingoes are definitive host of *Neospora caninum*. Int. J. Parasitol. 40, 945–950.
Liddell, S., Jenkins, M.C., Dubey, J.P., 1999. A competitive PCR assay for quantitative detection of *Neospora caninum*. Int. J. Parasitol. 29, 1583–1587.
Lindsay, D.S., Dubey, J.P., 1989. Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. Am. J. Vet. Res. 50, 1981–1983.
McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A., McGuire, A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. Int. J. Parasitol. 28, 1473–1478.
Müller, N., Zimmermann, V., Hentrich, B., Gottstein, B., 1996. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. J. Clin. Micro. 34, 2850–2852.
Neto, A.F.A., Bandini, L.A., Nishi, S.M., Soares, R.M., Driemeier, D., Antoniassi, N.A.B., Schares, G., Gennari, S.M., 2011. Viability of sporulated oocysts of *Neospora caninum* after exposure to different physical and chemical treatments. J. Parasitol. 97, 135–139.
Romand, S., Thulliez, P., Dubey, J.P., 1998. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitol. Res. 84, 50–53.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Sambrook, J., Fritsch, F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.
Sobrino, R., Dubey, J.P., Pabón, M., Linarez, N., Kwok, O.C., Millán, J., Arnal, M.C., Luco, D.F., López-Gatius, F., Thulliez, P., Gortázar, C., Almería, S., 2008. *Neospora caninum* antibodies in wild carnivores from Spain. Vet. Parasitol. 155, 190–197.
Schares, G., Pantchev, N., Barutzki, D., Heyddorn, A.O., Bauer, C., Conraths, F., 2005. Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int. J. Parasitol., 1525–1537.
Steinman, A., Shpigel, N.Y., Mazar, S., King, R., Baneth, G., Savitsky, I., Shkap, V., 2006. Low seroprevalence of antibodies to *Neospora caninum* in wild canids in Israel. Vet. Parasitol. 137, 155–158.
Stieve, E., Beckmen, K., Kania, S.A., Widner, A., Patton, S., 2010. *Neospora caninum* and *Toxoplasma gondii* antibody prevalence in Alaska wildlife. J. Wildlife Dis. 46, 348–355.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 24, 4876–5482.
Wapenaar, W., Jenkins, M.C., O'Handley, R.M., Barkema, H.W., 2006. *Neospora caninum*-like oocysts observed in feces of free-ranging red foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*). J. Parasitol. 92, 1270–1274.