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


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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 × 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 at 2000 rpm (1171 × g). After discarding the supernatant, the
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).
ized water to remove H2SO4, and suspended in buffer.

2.5. DNA extraction and PCR amplification

DNA extraction and PCR amplification (Sambrook et al., 1989), allowed to air-dry, and the DNA was further concentrated by ethanol precipitation, using a QIAamp Mini DNA kit and instructions provided by the manufacturer (Qiagen). After spin-column elution, beaded glass bead on a Mini-Bead Beater (Bio-Spec Products, AL (Qiagen, Inc., Valencia, CA, USA). The oocysts were purified with the manufacturer (Qiagen). After spin-column elution, 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.

2.4. In vitro cultivation

Lung tissue from KO mouse 49 (Table 2) with demonstrable tachyzoites was homogenized in RPMI-1640 medium supplemented with 1-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 twice in deionized water to remove H2SO4, 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 1
Details of the wolves positive for Neospora-like oocysts.

<table>
<thead>
<tr>
<th>Wolf I.D.</th>
<th>Date</th>
<th>NAT</th>
<th>PCR</th>
<th>Bioassay in KO mice</th>
<th>N. caninum strain designation</th>
<th>Nc 5 sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>W072210LDM2</td>
<td>8-4-2010</td>
<td>&lt;25</td>
<td>+</td>
<td>K(109)-Neg.Ks(29)-Pos.</td>
<td>NcWolfUS1</td>
<td>JF827721</td>
</tr>
<tr>
<td>W082010ADW1</td>
<td>8-24-2010</td>
<td>&lt;25</td>
<td>+</td>
<td>Neg.</td>
<td>NcWolfUS2</td>
<td>No</td>
</tr>
<tr>
<td>W082510JPG1</td>
<td>8-31-2010</td>
<td>&lt;25</td>
<td>+</td>
<td>Neg.</td>
<td>NcWolfUS3</td>
<td>No</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>KO mice</th>
<th>Swiss Webster mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td>48</td>
<td>49</td>
</tr>
<tr>
<td>Day p.i.</td>
<td>Smear</td>
<td>Cell culture</td>
</tr>
<tr>
<td>KS 17</td>
<td>Pos.</td>
<td>ND</td>
</tr>
<tr>
<td>KS 13</td>
<td>KS 17</td>
<td>ND</td>
</tr>
</tbody>
</table>

D: died; K: killed apparently healthy; ks: killed when sick; ND: no data; Neg.: not detected. Pos.: detected. NAT: Neospora agglutination test.

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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 myelitis 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. 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.](image)

![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.](image)
3.2. Genetic characterization

Nc5 amplification products (~350 bp) were identified by 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, primary Nc5 PCR on mouse lung 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 buffalos 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.

Acknowledgements

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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


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