Canine and Bovine *Neospora caninum* Control Sera Examined for Cross-Reactivity Using *Neospora caninum* and *Neospora hughesi* Indirect Fluorescent Antibody Tests

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Neospora caninum is a well known protozoan parasite of domestic and wild animals. Neospora hughesi is a closely related protozoan with an unknown life cycle, host range, and infection prevalence. Many serologic surveys of N. caninum have been performed without consideration of potential cross-reactions with N. hughesi, which could confound results. The aim of this study was to investigate whether postexposure sera from animals experimentally infected with N. caninum exhibit significant reactivity differences when tested using N. caninum and N. hughesi Immunofluorescent Antibody Tests (IFAT). Pre- and post-infection serum samples from 10 dogs, 20 calves, and 17 cows were tested by dual IFATs. All pre-exposure samples for N. caninum tested seronegative for both organisms. All postexposure samples that were seropositive for N. caninum were also positive for N. hughesi, although N. hughesi antibody titers were usually 1 dilution lower ($P < 0.02$). Serologic surveys for N. caninum may be confounded by cross-reacting titers with N. hughesi, but true positive N. caninum antibody titers are greater than, or equal to, cross-reacting N. hughesi antibody titers.

Neospora caninum is a well recognized protozoan parasite of domestic and wild animals (Anderson et al., 1991; Woods et al., 1994; Barber and Trees, 1996; Gondim, McAllister, Mateus-Pinilla et al., 2004; Huang et al., 2004) that is frequently associated with bovine abortion worldwide (Dubey, 2003). It was classified in 1988 as the first species in the Neospora genus, based on structural and antigenic characteristics of asexual stages of the parasite (tachyzoites and encysted bradyzoites) observed in infected dogs (Dubey et al., 1988). Ten years after its classification, dogs were discovered to be definitive hosts of the parasite (Gondim, McAllister, Pitt, and Zemlicka, 2004).

A similar organism isolated from a horse with myeloencephalitis (Marsh et al., 1996) was proposed as a new species, Neospora hughesi, based on molecular, antigenic, and structural differences when compared to N. caninum (Marsh et al., 1998). In other reports, major antigens of N. caninum and N. hughesi, i.e., SAG1, SRS2, GRA6, and GRA7, were compared and shown to be different (Marsh et al., 1999; Dubey et al., 2001; Walsh et al., 2001). Mice, horses, and a rabbit infected with N. hughesi have shown serologic reactivity with N. caninum (Marsh et al., 1998; Dubey et al., 2001; Packham et al., 2002; Hoane et al., 2005), and a rabbit infected with N. caninum had serologic reactivity with N. hughesi (Packham et al., 2002).

Serological studies for detection of animals with Neospora spp. have been reported by several authors (Cheadle et al., 1999; Vardeleon et al., 2001; Gupta et al., 2002; Packham et al., 2002; Dubey et al., 2003; Piel et al., 2003). In these studies, it is not possible to know the actual number of N. hughesi- or N. caninum-infected animals. Recombinant ELISAs based on 29-kDa major surface antigens of N. caninum (tNc-SAG1) (Howe et al., 2002) and N. hughesi (tNh-SAG1) (Hoane et al., 2005) have been developed. These ELISAs exhibited high specificities and sensitivities; however, cross-reactivity between N. caninum and N. hughesi was still observed (Hoane et al., 2005).

The aim of the present study was to investigate whether domestic animal hosts (dogs, calves, and cows) experimentally infected with N. caninum exhibit significant reactivity differences when tested using N. hughesi or N. caninum Immunofluorescent Antibody Tests (IFAT).

MATERIALS AND METHODS

Animal sera

Serum samples were obtained from calves, cows, and dogs that had been experimentally infected with N. caninum between 2000 and 2004 at the College of Veterinary Medicine at the University of Illinois at Urbana-Champaign, Illinois. Forty serum samples, including 20 pre-infection and 20 postinfection sera, were obtained from newborn calves, which were intravenously infected with cultured tachyzoites or orally inoculated with sporulated oocysts of N. caninum (Gondim et al., 2002; Gondim, McAllister, Pitt, and Zemlicka, 2004). Twenty serum samples from dogs (10 pre-infection and 10 postinfection sera) were collected from dogs that consumed N. caninum-infected tissues (Gondim et al., 2002, 2005). Thirty-four cow sera, consisting of 17 pre-infection sera and 17 postinfection sera, were obtained from cows that were orally infected with N. caninum oocysts (Gondim, McAllister, Anderson-Sprecher et al., 2004).

Parasites

Tachyzoites of N. caninum (NC-beef strain) (McAllister et al., 2000) and N. hughesi (Nh-A1 strain) (Walsh et al., 2001) were cultured in Vero cells with RPMI containing L-glutamine, supplemented with 5% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Tachyzoites were harvested when approximately 80% of the cell monolayers were infected. The infected cell suspensions were forced through 26-gauge syringe needles, suspended in phosphate buffered saline, and filtered using 5-μm syringe filters. The filtrate was washed 3 times by centrifugation (1,200 g for 10 min) in PBS, and the sediment was suspended in PBS to a final concentration of 500 to 1,000 tachyzoites/μl. The purified tachyzoites were distributed on the wells of Teflon-coated glass slides, dried at room temperature, and fixed for 5 min in methanol. Antigen slides were stored at ~20 C until further used.

Immunofluorescent Antibody Test

Serum samples were tested simultaneously for N. caninum and N. hughesi using antigen slides that were stored at ~20 C for 1 to 5 wk. Dog and calf sera were screened at 1:50, and cow sera were screened at 1:200. Maximum antibody titers for both parasites were determined for all positive samples using doubling dilutions. Fluorescein-labeled
Table I. Cross-reactivity of sera from *Neospora caninum*-infected animals with *N. hughesi* tachyzoites, as determined by Immunofluorescent Antibody Tests.

<table>
<thead>
<tr>
<th>Antibody titers</th>
<th>For <em>N. caninum</em></th>
<th>For <em>N. hughesi</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dog</td>
<td>Calf</td>
</tr>
<tr>
<td>50</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>*</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>0</td>
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<tr>
<td>400</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>800</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>1,600</td>
<td>2</td>
<td>*</td>
</tr>
<tr>
<td>3,200</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6,400</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>12,800</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>25,600</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

* Not tested at the referred serum dilution.

The present study highlights the potential for cross-reaction between *N. caninum* and *N. hughesi* serologic tests, which complicates the interpretation and confidence in the results of serological surveys for only 1 organism. To our knowledge, this is the first time that sera from dogs and cattle experimentally infected with *N. caninum* have been tested using both *N. caninum* and *N. hughesi* tachyzoites as antigens. Serology for another closely related organism, *Hammondia heydorni*, was not included in the experiment because there is no test available to detect antibodies against this parasite.

The present study also shows that *N. caninum* postexposure sera from dogs and cattle that were tested in parallel by IFAT using *N. caninum* and *N. hughesi* tachyzoites exhibited higher titers when *N. caninum* was employed as antigen. Although *N. caninum* titers were higher than *N. hughesi* titers in *N. caninum*-infected animals (*P < 0.02*), at the same time there was significant cross-reactivity with *N. hughesi* (*P < 0.001*). Therefore, serological surveys for *N. hughesi* may be confounded by titers to *N. caninum*. Although we were not able to compare the opposite effect (*N. caninum* titers in *N. hughesi*-infected animals), it seems probable that there is the same possibility for cross-reacting titers and false-positive results with *N. hughesi* serology.

Packham et al. (2002) compared different serologic techniques (IFAT, ELISA, and DAT) using sera from horses experimentally infected with *N. hughesi* and concluded that IFAT was the only test, among the studied ones, that allowed discrimination between infected and noninfected horses. Recently, an ELISA test using a recombinant *N. hughesi* SAG1 antigen has been developed to detect *N. hughesi* antibodies in horses (Hoane et al., 2005); this test was shown to be more specific than the previous ones. However, it failed to discriminate between *N. caninum* and *N. hughesi*-infected animals.

Natural infection with *N. hughesi* has not been reported in animals other than horses. This fact does not exclude the possibility that *N. hughesi* infects different domestic and wild animals. It is also expected that the definitive host of *N. hughesi*, which is unknown so far, is some widely distributed carnivore.

It is reasonable to wonder if numerous serologic investigations of *N. caninum*-infected animals might have been confounded due to cross-reactions with *N. hughesi*. Another potentially confounding factor is infection with the closely related parasite *Hammondia heydorni* (Ellis et al., 1999), because there is no...
serologic technique available for detection of *H. heydorni*-infected animals. Therefore, at this moment, it is not possible to serologically differentiate *N. hughesi*, *N. caninum*, and *H. heydorni* infections. Certain differentiation of the 3 parasites is only possible when parasite DNA is obtained (Marsh et al., 1998; Ellis et al., 1999; Slapeta et al., 2002). Further studies are needed to develop specific serologic techniques for *N. hughesi*, *N. caninum*, and *H. heydorni*.

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LITERATURE CITED


