ABSTRACT

NEOSPORA CANINUM EXPOSURE IN WISCONSIN WILDLIFE

By Todd C. Anderson

The parasitic protozoan *Neospora caninum* is a major cause of abortion in cattle worldwide. Transmission occurs by fecal-oral contamination from infected dog feces or by congenital route in infected cattle. Wolves, coyotes, cattle, and deer have been implicated in transmission, but the host range of the parasite is still poorly defined. Many studies have demonstrated seroprevalence within a single mammalian species in an area, but few have examined the interaction of different potential host species in the same geographical area. To further define the host range of the parasite and to characterize the sylvatic wildlife transmission cycle, we determined seroprevalence and molecular prevalence of *N. caninum* in several central Wisconsin wildlife species. Western blot analysis was used to demonstrate the presence of circulating antibodies against *N. caninum*. Seroprevalence in wildlife species varied from 19.0% in white-tailed deer, 14.7% in coyotes, 11.1% in foxes to 0% in raccoons and opossums. PCR specific for the Nc5 locus of *N. caninum* was performed on brain tissue of 54 raccoons and 53 opossums, however no positive samples were found. Fecal analysis was then performed in an effort to find *N. caninum* and to characterize the intestinal parasite loads of wildlife species. Although the fecal analysis revealed no active shedding of *N. caninum* oocysts, several other parasite species were found in the samples. This study suggests that parasite life cycle is occurring in Wisconsin between coyote and deer.

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by

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CHAPTER I

INTRODUCTION

Biology

Neospora caninum is an apicomplexan parasite in the Class Coccidea (Order Eimerida, Family Sarcocystidae) found predominately in canids and ruminants. Clinical neosporosis in cattle was historically misdiagnosed as toxoplasmosis caused by *Toxoplasma gondii*, until closer examination revealed that these two species have distinct morphological, immunological, and host range differences (Dubey et al. 1988). Neosporosis is a major cause of abortion in cattle throughout the world (Dubey, 2003). Serologic evidence suggests that a wide range of mammalian species in the United States are exposed to this parasite, including cattle, raccoons, deer, coyotes and domestic dogs (Dubey and Lindsey, 1996).

Life cycle

Neospora caninum has a heteroxenous life cycle, requiring both a definitive host and an intermediate host. Sexual reproduction occurs in the intestine of dogs (McAllister et al., 1998) and coyotes (Gondim et al., 2004). Ruminant animal species, including cattle and white-tailed deer, act as intermediate hosts for *Neospora caninum.* They become infected with *N. caninum* by ingesting canid feces containing sporulated parasite oocysts. The oocyst of *N. caninum* is approximately 10 μm in size, with a colorless wall between 0.6-0.8 μm in thickness (Figure 1). Within a sporulated (infective) oocyst, two 8.4 x 6.1 μm sporocysts contain four 6.5 x 2.0 μm sporozoites (Lindsay et al. 1998; Dubey et al. 1996). Once in the intestine, oocysts and sporocysts break, releasing the

sporozoites. The sporozoites penetrate intestinal epithelial cells and asexually reproduce to form tachyzoites. Tachyzoites are oval in shape with an approximate size of 3-7 x 1-5 μm. Asexual reproduction occurs by endodyogeny, which is the formation of 2 daughter cells inside the mother cell (Dubey et al. 2002). Tachyzoites reside within the parasitophorous vacuole of the host cell cytoplasm (Dubey et al. 2002). When the mother cell bursts, tachyzoites are released and can penetrate adjacent cells and invade extraintestinal sites. Tachyzoites invade many different cell types and host cells, including muscular, nervous, and uterine cells. Cyst formation in extraintestinal tissues occurs when the host's immune response slows down tachyzoite production at those sites. Tissue cysts are composed of many bradyzoites enclosed in a thick, cyst wall. Bradyzoites are the infective stage for the definitive host (canids), and are found encysted in the central nervous system and skeletal muscle (Peters et al. 2001).

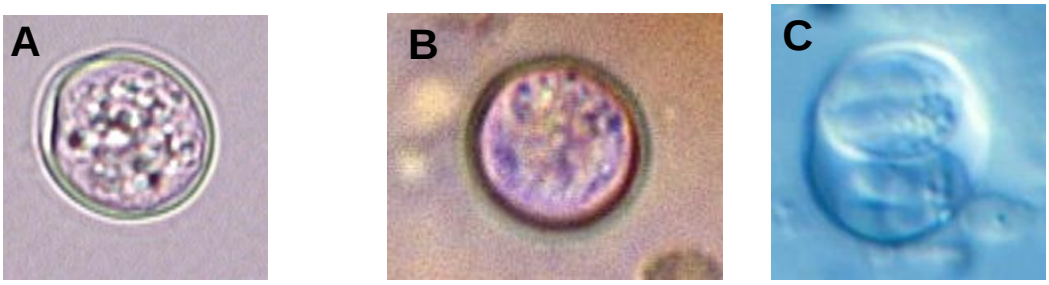

Figure 1. *Neospora caninum* oocysts from dog feces, 100X. Unsporulated oocysts in fresh feces are not infective for the intermediate host. Sporulation (nuclear division) occurs in the environment and takes approximately 1-3 days. Panel A. A 10 μm unsporulated oocyst. Though the oocyst wall can be defined, it is very difficult to differentiate the clear oocyst from the background debris found in a typical fecal preparation (not shown). Panel B. Unsporulated oocyst, phase contrast, from McAllister. With this type of lighting oocysts glow peach or pink, and are easier to pick out of the background of fecal matter. Panel C. Sporulated oocyst, Normarski DIC, from from McAllister. The internal morphology of the oocyst, demonstrating two sporocysts each containing four sporozoites.

Canids become infected by ingesting tissue containing bradyzoites or tachyzoites, or by ingesting feces containing oocysts (Figure 2). Canids infected with bradyzoites act as definitive hosts, while those infected with oocysts act as intermediate hosts (McAllister et al., 1998). Sexual reproduction of the parasite occurs when bradyzoites enter the intestinal epithelial cells. In coccidia, differentiation of dividing bradyzoites into micro (♂) and macro (♀) gametes is followed by fertilization and oocyst production. Oocysts rupture their host cells and enter the lumen of the gut where they are excreted in the feces. The duration of oocyst shedding in canids can range from 1 to several days, and the frequency of these shedding periods is also highly variable (Dubey et al., 2007). The total number of oocysts that are shed in the feces can be as high as a 100,000 oocysts but are generally much lower in wild canids (Lindsay et al., 1999). Due to the high cost and low success rate of oocyst production in the laboratory, the natural factors affecting shedding rates remain unknown.

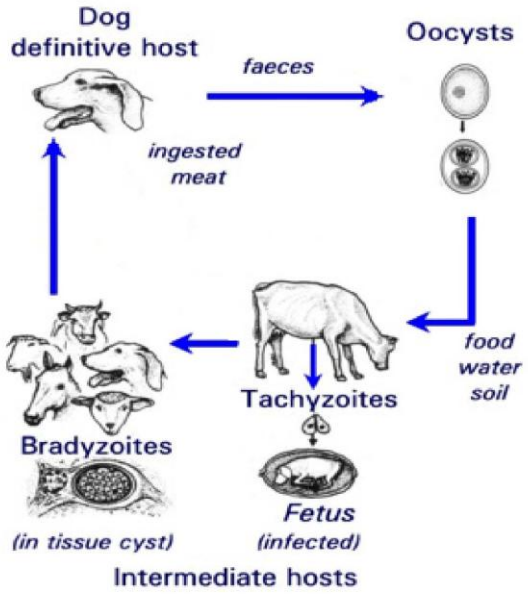

Figure 2. Life cycle of *Neospora caninum.* The prepatent period in the dog is 5 days (Lindsay, et al, 1999). Modified from Dubey et al., 2004.

Transmission

Domestic and sylvatic life cycles of *N. caninum* appear to significantly overlap (Gondim 2004). The domestic cycle occurs when cattle are the intermediate host, and dogs consume aborted cattle fetuses or dead cattle that contain tissue cysts. The sylvatic cycle is composed of deer as the intermediate host and coyotes (and possibly wolves) as the definitive host (Figure 3). Coyotes ingest tissue cysts when they feed on deer carcasses, aborted fawns, and the eviscerated guts of deer during hunting season.

Horizontal and vertical transmission of *N. caninum* has been demonstrated experimentally. Vertical transmission occurs when tachyzoites are transferred from a mother to her fetus. The rate of transmission was reported by Davidson et al. (1999) to be as high as 95%. Key factors influencing the rate include age of the mother and number of offspring (Dijkstra, 2003). This method of transmission can also be observed

in successive generations, leading to the persistence of the parasite on farms (Bjorkman et al. 1996). While the vertical transmission of this parasite occurs often, it apparently does not lead to high rates of abortion. In a study by Bjorkman et al. (2003), a cattle herd that had 76-78% seropositivity for *N. caninum* had an abortion rate of only 2.5-5.5%. The majority of calves born to infected mothers are seropositive and asymptomatic. The high rate of vertical transmission precludes interaction with a definitive host in this type of situation.

Horizontal transmission occurs when an animal ingests food contaminated by oocysts or muscle tissue containing tachyzoites or tissue cysts, and is highly correlated with acute Neosporosis. Domestic transmission in ruminants often results from eating mixed feed contaminated with infective dog feces. Dogs become infected by predation on cattle or consumption of aborted cattle fetuses. Horizontal transmission is apparently necessary for maintenance of the parasite in a single location. In the absence of definitive hosts, the parasite infection rate eventually decreases to a point where the parasite is lost from an area or herd (French et al. 1999).

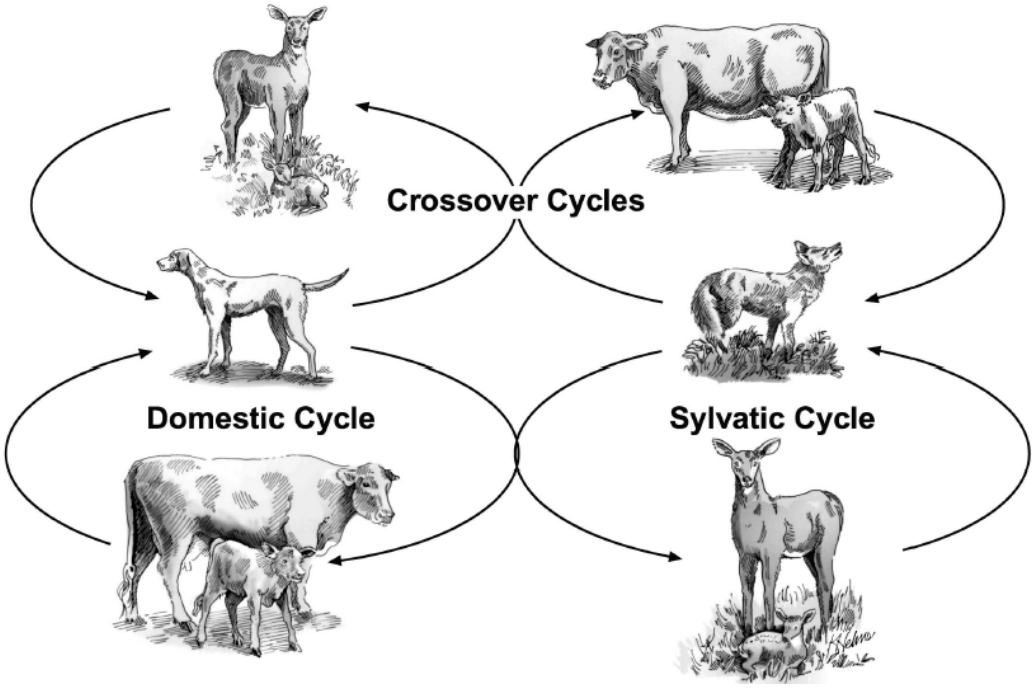

Figure 3. Sylvatic and domestic transmission of *N. caninum*. All routes have been demonstrated experimentally, except deer to coyote transmission. (Gondim, et al. 2004a).

Pathogenesis and Diagnosis

The clinical symptoms of neosporosis in dogs can range from none to severe neuromuscular disease. It is interesting that most asymptomatic, seropositive dogs have minor cell damage to the intestinal lining, indicating subclinical damage (Dubey and Lindsay, 1996). In older individuals, the clinical symptoms can range from general stiffness to organ failure. The stiffness is attributed to tissue cysts in the central nervous system. Neonatal pups that show symptoms of neosporosis typically have hind leg paralysis and neurological problems caused by tachyzoite invasion of the brain and resulting immune response *in utero* (Dubey and Lindsay, 1996) Pups that are born with severe neosporosis die very quickly.

Seropositive adult ruminants are typically asymptomatic, but a seropositive pregnant cow can transmit the parasite to the fetus, causing congenital birth defects or abortion. The outcome of the infection depends on fetal age and the size of the inoculum (Gondim 2004). In the first trimester of pregnancy, a fetus is most susceptible to *Neospora caninum* because its immune system is developing and cannot control parasite replication (Dubey et al. 2006). Tachyzoites can elicit an *in utero* immune response in older fetuses; however, they survive to become asymptomatic seropositive calves. The majority of detected abortions occur in the middle third of the gestation period; however, this may be misleading because most abortions in the first trimester are simply reabsorbed by the mother and never detected (Williams et al. 2000).

Damage to the fetus is caused by invading tachyzoites and is exacerbated by the interplay with maternal and fetal immune systems. A cow typically secretes beneficial cytokines such as IL-10 and TGFα, while destructive cytokines such as IL-2, IL-12, IFN-γ and TNF-α are kept in check to protected the fetus while *in utero* (Buxton et al. 2003). When the fetus is infected congenitally with *N. caninum*, the mother's immune system shifts the balance toward the parasite-destroying cytokines and IgG, delivering excessive amounts to the fetus. Because of this disruption, newly exposed dams are more likely to abort and have lower circulating anti-Neospora IgG. It is thought that persisting *Neospora caninum* IgG confers protective immunity for successive offspring (Bjorkman et al. 2003). The abortion rate is inversely proportional to the IgG antibody titer of the mother (McAllister et al., 2000).

Seroprevalence

Neospora caninum is a cosmopolitan parasite, and evidence for exposure has been reported from many countries. A study conducted Dubey et al. (1996) demonstrated 0.25-16.6% seroprevalence in dogs in Sweden. In the United States, the percentage of seropositive dogs was 7% (Cheadle et al, 1999), while in New Zealand they had a substantially higher prevalence of 76% (Antony and Williamson, 2003). In the southeastern United States, 48% of white-tailed deer had antibodies for *N. caninum* (Lindsay et al. 2002), and in northeastern Illinois 41% of white-tailed deer were seropositive (Dubey et al. 1999) (Table 1). Cattle worldwide are seropositive, and outbreaks (or 'abortion storms') cause the number of positively infected cattle in an area to increase greatly. During a neosporosis outbreak in Sweden, 76-78% of the cattle tested positive for the parasite (Bjorkman et al. 2003). *N. caninum* exposure does not appear to affect fertility in the cow. According to Bjorkman et al. (2003), 85% of seropositive cows transmitted the parasite to their offspring before they were born, and 22% of seronegative calves were infected with *N. caninum* within the first year.

Table 1. Seroprevalence of *Neospora caninum* antibodies in wildlife. Indirect fluorescent antibody test (IFAT). *Neospora* agglutination test (NAT)

Animal species	Region	Test	% Positive	Reference
White-tailed deer	Illinois	NAT	41%	Dubey et al 1999
Raccoons	Southeastern US	NAT	10%	Lindsay et al.2001
Grey wolf	Minnesota	IFAT	39%	Gondim et al. 2004
Coyotes	Illinois	IFAT	15%	Gondim et al. 2004
Moose	Minesota	IFAT	13%	Gondim et al. 2004

Economic importance

Neosporosis has a significant economic impact on cattle production. In the United States, neosporosis causes between 2.5- 24.4% of all abortions (Anderson et al 1995: Thurmond et al. 1995). In Mexico, the abortion rate due to neosporosis was as high as 77% (Morales et al. 2001). Not only is the loss due to abortion economically important, but infection exacts indirect costs as well, including diagnosis, rebreeding, milk decrease, culling of infected cattle, and lower reproductive output (Dubey, 2003). There are conflicting reports about the potential milk production losses, but Hernandez et al. (2001) reported a 3-4 % decrease in seropositive cows. Meanwhile, Hobson et al. (2002) report that there was no difference in milk production between positive and negative cows. The monetary losses caused by *N. caninum* are difficult to compute but was estimated that California alone loses nearly 35 million dollars to neosporosis annually (Dubey et al. 2003).

Control

There are several control methods for *Neospora caninum*, but the most effective is management. This method involves keeping detailed records of births, deaths, incoming individuals, and test results. Cattle should be tested periodically to monitor them for evidence of a point source outbreak Seropositive cows should be replaced by uninfected cows or culled to reduce the potential reservoir of the parasite on the farm. The most important aspect of cattle management is keeping the feeding and watering areas free of wild animals and domestic dogs. Contamination of feed has the potential to infect the entire herd. Adequate barriers must be erected around the cattle to minimize contact from wild animals, and feed storage areas should be kept clean and secure.

Another method of control is the preventive use of vaccines. Exposure to *N. caninum* ensures life-long seropositivity. There are two types of vaccines for *N. caninum.* The first type of vaccine is made from killed tachyzoites and is manufactured by Neoguard®. A field trial in Costa Rica tested this vaccine on 25 cattle herds. This study vaccinated half of each herd, with equal numbers of *N. caninum* seropositive cattle in each experimental group. Of the 25 herds, 15 experienced lower abortion rates in their vaccinated groups than in their unvaccinated groups (Romero et al., 2004). Conversely, 6 out of the 25 herds had a negative prevention effect associated with the vaccine, rendering this study's results controversial. The other type of vaccine is still in trials, and is comprised of live, attenuated tachyzoites. Although both vaccine types may be effective, once given the vaccine, the cow will always test seropositive for *N. caninum* antibodies. In short, the most efficacious control of this parasite is prevention.

Rationale of Project

Neospora caninum is an economically important, recently described parasite that affects cattle and dairy farms worldwide. Domestic transmission of this parasite has been repeatedly demonstrated, but the sylvatic cycle is largely uncharacterized. There are many animal species in the wild that have antibodies to this parasite, so the possibility exists that they could play a role in crossing over of the two cycles. We are investigating the possibility that non-ruminant intermediate hosts exist. Further defining the host range of the parasite will allow better control methods to be implemented by farmers.

The goal of this project was to characterize the sylvatic transmission cycle of *Neospora caninum* in Central Wisconsin. To accomplish this goal, several different animal species from the same geographical area were studied to establish evidence that the life cycle naturally occurs (Figure 4). White-tailed deer are a known host of this parasite, so as a first step the seroprevalence in deer was established and reported.

Figure 4. The Chronic Wasting Disease Eradication Zone was the site of animal collection by the Wisconsin Wildlife Cooperative Unit at the University of Wisconsin-Madison. At necropsy, biological samples were preserved and sent to the University of Wisconsin–Oshkosh as part of a collaborative research effort. (Figure provided by the WDNR).

Specific Aims

- Determine seroprevalence in white-tailed deer (*Odocoileus virginianus*), coyotes (*Canis latrans*), and small mammalian scavengers.
- Correlate seroprevalence with molecular prevalence in small scavengers.
- Use parasitological methods to demonstrate the role of coyotes as definitive hosts, and potentially identify additional definitive hosts.
- Conduct an intestinal parasite survey of mammalian scavengers to quantify parasite loads.

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CHAPTER II

SEROLOGICAL EVIDENCE OF NEOSPORA CANINUM EXPOSURE IN WISCONSIN WHITE-TAILED DEER

Introduction

The sylvatic life cycle of *N. caninum* involves interactions between ruminants (white-tailed deer) and canids (coyotes). White-tailed deer are abundant in our study area, and we began our studies by serologically estimating the level of *N. caninum* exposure in deer. The following publication details the methodology associated with using enzyme-linked immunosorbent assay (ELISA) and Western blot on degraded (field-collected) blood samples and provides indirect evidence that the *N. caninum* sylvatic life cycle exists in our study site.

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Short communication

Neospora caninum antibodies detected in Midwestern white-tailed deer (*Odocoileus virginianus*) by Western blot and ELISA

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Abstract

White-tailed deer (*Odocoileus virginianus*) serve to maintain the *Neospora caninum* life cycle in the wild. Sera from white-tailed deer from south central Wisconsin and southeastern Missouri, USA were tested for antibodies to *N. caninum* by Western blot analyses and two indirect ELISAs. Seroreactivity against *N. caninum* surface antigens was observed in 30 of 147 (20%) of WI deer and 11 of 23 (48%) of MO deer using Western blot analysis. Compared to Western blot, the two indirect ELISAs were found to be uninformative due to degradation of the field-collected samples. The results indicate the existence of *N. caninum* antibodies in MO and WI deer, and that Western blot is superior to ELISA for serologic testing when using degraded blood samples collected from deer carcasses.

Keywords: *Neospora caninum*; White-tailed deer (*Odocoileus virginianus*); Seropositivity; Western blot; ELISA; Missouri; Wisconsin

1. Introduction

Neosporosis caused by the apicomplexan parasite *Neospora caninum* is a major cause of bovine abortion worldwide (Dubey, 2003). The life cycle involves canids as definitive hosts and ruminants as intermediate hosts. Sylvatic transmission occurs between canids (such as coyotes) and ruminants (such as white-tailed deer) (Gondim et al., 2004a,b; Vianna et al., 2005). We are currently investigating the Midwestern sylvatic cycle, including the possibility that other mammals can act as hosts for *N. caninum*. Here we report *N. caninum* seropositivity in white-tailed deer (*Odocoileus virginianus*) from two Midwestern sites (WI and MO) based on Western blot analyses, and the evaluation of two indirect enzyme-linked immunosorbent assays (ELISAs) for detection of circulating anti-*Neospora* antibodies in hunted deer.

2. Materials and methods

Whole blood samples were collected from the thoracic cavities of 147 hunter killed white-tailed deer at the Wisconsin Department of Natural Resource hunter check station in Sauk City, WI (November 2004, 2005), and from 23 deer at a Missouri Department of Natural Resource hunter check station in Ste. Genevieve County, MO (November 2001). Samples were separated by centrifugation immediately after collection and the serum stored at −20 °C. Two *Neospora* agglutination

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test (NAT, Romand et al., 1998) positive and two NAT negative white-tailed deer (Dubey et al., 1999) were used as controls. WB analysis was used to confirm the presence or absence of circulating antibodies against *N. caninum*. A defined WB protocol (Howe et al., 1998) was modified by using 1:1000-diluted rabbit anti-deer antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as a secondary reagent. Samples were considered seropositive if antibody binding was observed for the Ncp29 (SAG1) and Ncp 35 (SRS2) antigens (Howe et al., 1998). Seropositivity was calculated by dividing the number of seropositive deer by the total number of deer screened and multiplied by 100.

A Ncp29 cattle ELISA (Howe et al., 2002) was modified for use with deer serum by using horseradish peroxidase-conjugated rabbit anti-deer IgG. The assay was optimized by varying the following conditions: (a) test of bovine serum albumin (BSA), nonfat dry milk, fetal bovine serum and rabbit serum in coating and antibody diluent solutions, (b) temperature at which assay was conducted (room temperature versus 37 °C), (c) phosphate buffered saline [PBS] versus polyethylene glycol blocking solutions, and (d) type of microtiter plate used (Immulon 4, Thermo Electron, Milford, MA; EIA/RIA Corning, Corning, NY). The assay was conducted at room temperature using Corning plates blocked with BSA in PBS, deer serum and horseradish peroxidase-conjugated rabbit anti-deer IgG at 1:1000, and washing plates four times prior to addition of chromogenic *O*-phenylenediamine. Absorbance at 492 nm (A_{492}) was determined using a SPECTRAmax Plus 384 ELISA plate reader (Molecular Devices, Sunnyvale, CA). All samples were tested in triplicate. Absorbance values were normalized by subtraction of the mean absorbance of two wells that had been treated in the absence of primary antibody.

An indirect ELISA using whole *N. caninum* tachyzoite lysate was developed as an alternative to the recombinant antigen ELISA. Whole cell-culture derived NC-1 strain tachyzoites (Dubey et al., 1988) were boiled for 5 min in 1× non-reducing Laemmli buffer (0.100 mM Tris pH 6.8, 4% SDS, 10 % glycerol). Protein concentration was estimated using the bicinchoninic acid protein assay (Sigma Chemical, St. Louis, MO). Microtiter plate wells were coated with 10 μg/mL tachyzoite lysate in PBS for 4 °C overnight (Howe et al., 2002). The remainder of the assay was conducted as for the optimized recombinant antigen assay, except that the assay was evaluated at 4 and 37 °C. Missouri deer samples were tested in triplicate, Wisconsin samples in duplicate. For both ELISAs, the percent positivity of each sample was calculated using the following equation: PP = [(mean sample A_{492} − mean negative control A_{492})/(mean positive control A_{492} − mean negative control A_{492})] × 100 (Wright et al., 1993). The sensitivity and specificity of each assay were calculated with PP cutoff values of 0, 10 and 20.

3. Results

Western blot analysis revealed seroreactivity against immunodominant *N. caninum* surface antigens in 30 of 147 (20%) of WI deer and 11 of 23 (48%) of MO deer (Fig. 1). Twenty-five WI samples (4 of which were WB positive) and 23 MO samples (11 of which were WB positive) were selected to assess the performance of the NcP29 ELISA. Mean negative control A_{492} values ranged from 0.212 to 0.468, while A_{492} values for positive controls ranged from 0.680 to 2.491. Percent positivity of WB-negative test samples ranged from 27.8 to 631.2, and from 23.6 to 183.1 for Western blot positives (Fig. 2A). The highest combined sensitivity (91%) and specificity (70%) was attained using a positive cutoff of 0 PP, which was deemed unacceptable for a high throughput screening tool. To determine if the presence of EDTA could improve the accuracy of the assay, additional samples were collected in EDTA vacuum tubes. However the resulting plasma was completely uninformative in the recombinant antigen ELISA (data not shown).

In an effort to design an assay with higher specificity that could be used for screening numerous samples, we developed an alternate ELISA using whole parasite

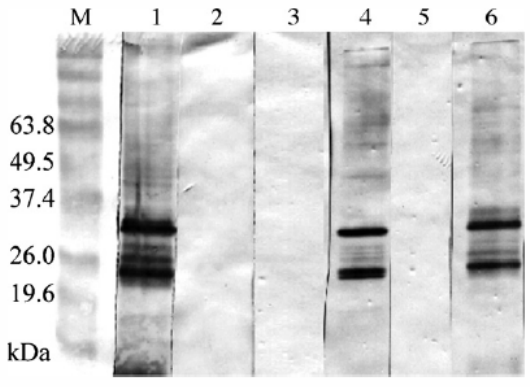

Fig. 1. Western blot analysis of deer serums samples using rabbit anti-deer secondary antibody. Immunodominant proteins of 35 and 25 kDa were bound by the agglutination positive deer (lane 1), WI (lane 4), and MO (lane 6) deer; but not by the agglutination negative deer (lane 2), WI (lane 3), and MO (lane 5) samples. Molecular weights were estimated by comparison to Kaleidoscope prestained standard (M) (Bio-Rad, Hercules, CA).

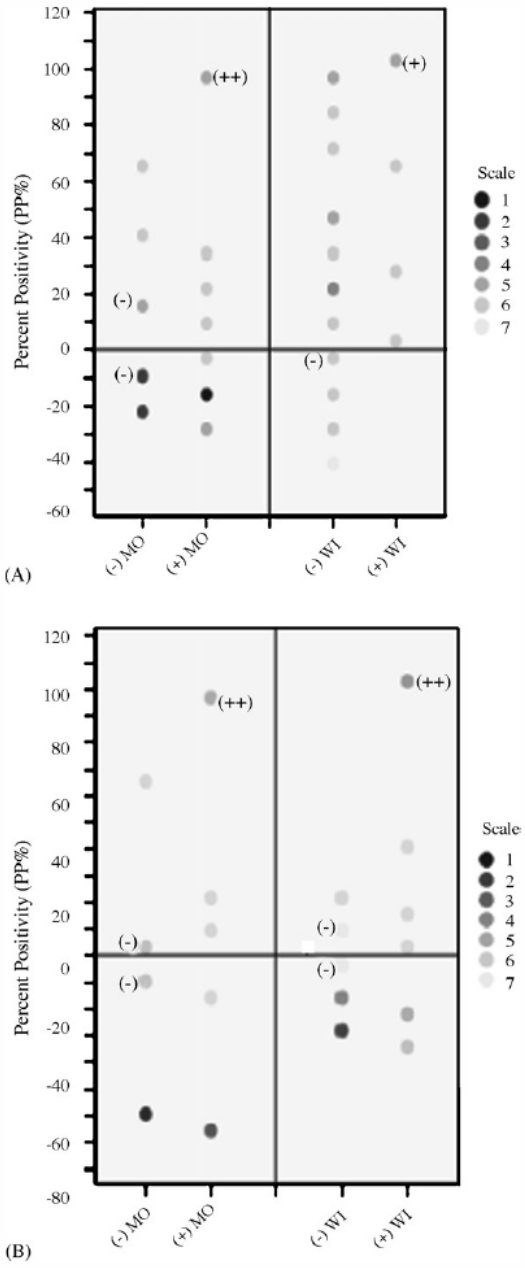

Fig. 2. Performance of indirect ELISAs with field-collected serum samples. Panel A: recombinant antigen (Ncp29) assay. Panel B: whole tachyzoite antigen assay. Values are expressed as percent positivity, the number of samples in each data point is indicated by the shade scale. Shown are test samples from Western blot negative (−MO, −WI) and positive (+MO, +WI) deer from Missouri and Wisconsin, as well as NAT-positive and negative control samples from Illinois (indicated in chart by (+, −) symbols). The presence of ++ indicates that two positive control samples overlap on one data point.

lysate as the target antigen. Forty-two WI samples (10 of which were WB positive) and the 23 MO samples were selected to test the validity of the assay. The groups were tested separately, with Wisconsin samples run in duplicate, MO in triplicate. Negative control values ranged from 0.131 to 0.245, and positive control values ranged from 0.340 to 1.023. Percent positivity of WB-negative test samples ranged from −65.45 to 26.4, while WB positive test values ranged from 65.5 and 169.0 (Fig. 2B). The assay performed with high sensitivity (up to 91% at a cutoff of 0 PP), however the specificity of the assay ranged between 52 and 71%. The performance of the assay also differed between sample groups, with higher sensitivity and lower specificity observed in the WI samples (data not shown). To determine if serum samples had degraded due to poor collection conditions, the total IgG concentration of the WI and MO samples were examined. Binding of anti-deer secondary antibody to serum dilutions indicated that the total IgG concentration in the control sera was at least two orders of magnitude greater than the MO and WI samples (data not shown).

4. Discussion

The overall purpose of our research is to obtain evidence of the sylvatic *N. caninum* life cycle in central Wisconsin, with which to qualify future studies on transmission in regional wildlife species. Importantly, our data demonstrated that WB is a much better technique for screening deer serum samples because it is more informative and less sensitive to sample degradation than ELISA. Seropositivity of 20 and 48% was observed in the two Midwestern sites, which agrees with previous report of approximately 40% in neighboring Illinois deer (Dubey et al., 1999). Mounting evidence points to a role of white-tailed deer as natural intermediate hosts in the sylvatic life cycle of *N. caninum* (Gondim et al., 2004a; Lindsay et al., 2002; Vianna et al., 2005) and to linkage of the domestic and sylvatic cycles in some areas (Tiemann et al., 2005). It is possible that management programs aimed at prevention and control of neosporosis may need to factor in situations in which cattle have extensive contact with wildlife.

N. caninum tissue cysts are not histologically apparent in adult cattle and, presumably, deer; hence serological methods such as direct agglutination test (Packham et al., 1998), indirect fluorescent antibody test (Conrad et al., 1993), ELISA (for review see Dubey and Schares, 2006), immunoblot (Bjerkas et al., 1994),

and rapid immunochromatographic test (Liao et al., 2005) are undertaken to screen populations for exposure. Evaluation of serological results from wildlife serum samples is often difficult because of the degradation of antibodies after death. This is particularly more applicable to serologic tests that use color as an indicator, e.g. the ELISA tests. In contrast to ELISA, Western blot analysis gives a direct visual confirmation of antibody bound to specific diagnostic antigens (i.e., NcP29 and NcP35), thereby providing greater confidence in the results. Although the NAT test is simple and can be used with autolysed samples, NAT is no longer available commercially (J.P. Dubey, personal information). Therefore, we evaluated ELISA and WB in the present study.

Collection of deer blood samples for biological research is often performed at hunter check stations, from animals that have been eviscerated in the field. Many factors contribute to the degradation of serum proteins, such as length of time since death of the animal, ambient temperature in the field, presence or absence of ice in the body cavity, contamination of the cavity with debris, and sample storage conditions. It is difficult to collect high quality blood samples in this situation, and the researcher must be prepared to evaluate them using an assay that performs well under these circumstances (Greiner and Gardner, 2000). In this case, WB was superior to ELISA for establishing evidence of *N. caninum* exposure in Midwestern deer.

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CHAPTER III

SURVEY OF WILD MAMMALS FOR *NEOSPORA CANINUM* EXPOSURE

Introduction

Our preliminary studies established 20% seroprevalence in white-tailed deer from the CWD eradication zone in Central Wisconsin (Anderson et al., 2007). We initiated a serological and molecular survey of scavenging mammals (coyote, opossum, and raccoon) from the area to search for evidence of their participation in parasite transmission. Little work has been conducted on the potential role of non-ruminant hosts for *N. caninum,* and we hypothesized that because they are abundant in the study area and scavenge deer carcasses, they are naturally exposed to the parasite. Here we report the results of Western blot analyses and polymerase chain reaction (PCR) on scavenging mammals.

Materials and Methods

Sample collection - As a part of a Chronic Wasting Disease (CWD) epidemiological study in Dane County, WI, the Wisconsin Cooperative Wildlife Research Unit (WCWRU) at the University of Wisconsin Madison used live traps to collect scavengers of deer. The scavengers were euthanized upon collection. The study extended from 2003 to 2005, and approximately 800 animals were collected, mainly coyotes (*Canis latrans*), raccoons (*Procyon lotor*), and opossums (*Didelphus virginianus*) (Mike Samuel, personal communication). Carcasses were refrigerated or frozen, and subsequently necropsied to isolate brain tissue for CWD testing, blood for serology and brain tissue for DNA analysis, all of which were stored at -20ºC. Upon arrival to the

University of Wisconsin Oshkosh, blood samples were thawed, and serum was separated by centrifugation. All samples were stored at -20ºC.

Seroprevalence of *N. caninum* – Western blot analysis confirmed the presence or absence of circulating antibodies against *N. caninum* NC-1 tachyzoite (Dan Howe, University of Kentucky-Lexington). A cattle Western blot protocol (Howe et al., 1998) was modified for each of the scavenger species by using species-specific secondary reagents, specifically a 1:1,000 dilution of goat anti-dog antibody conjugated to horseradish peroxidase for fox and coyote samples, 1:1,000 dilution of goat anti-raccoon antibody conjugated to horseradish peroxidase for raccoon samples, and 1:1,000 dilution of goat anti-opossum antibodies conjugated to horseradish peroxidase for opossum (KPL, Gaithersburg, MD). Samples were considered seropositive if antibody binding was observed for the Ncp29 (SAG1) and Ncp35 (SRS2) antigens (Howe et al., 1998). Controls consisted of preimmune and immune dog serum samples that were generously provided by M. McAllister at the University of Illinois-School of Veterinary Medicine. Dot blot analysis was conducted to ensure binding of the primary antibodies (serum) by the secondary reagents.

DNA extraction - DNA was extracted from the frozen brain tissue of test animals by organic extraction followed by ethanol precipitation. Approximately 500 mg of each frozen brain tissue sample were homogenized using a sterile wooden rod and suspended in 600 mL digestion buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA pH 8, and 0.5% SDS) and 20 µL of 20 mg/mL proteinase K (Ausubel et al., 1997). Samples were incubated overnight at 50º C. After centrifugation at 14,000 rpm for 5 min, 600 µL of the

sample supernatant were extracted with 600 µL of 25:24:1 phenol/chloroform/isoamyl alcohol. DNA was stored at -20º C while in 800 µL 100% ethanol and 40 µL 3M sodium acetate. Once the precipitate formed, the sample was pelleted at 10,000 rpm for 5 min, the resulting pellet was washed twice with cold 70% ethanol, and resuspended in 50 µL of water. Samples were characterized by agarose gel electrophoresis to verify integrity (data not shown) and quantified spectrophotometrically.

DNA quality – A one hundred nanogram sample of each coyote and fox brain DNA was tested for viability using primer pair FH2079-1

(5'CAGCCGAGCACATGGTTT3') and FH2079-2

(5'ATTGATTCTGATATGCCCAGC3'), which amplify a 240-296 nucleotide canid-specific microsatellite region from chromosomal DNA (Sacks, 2004). Positive controls consisted of coyote DNA samples obtained from B. Sacks, University of California Davis; negative controls were performed by substituting water for DNA in the reaction. The amplified samples were visualized on a 1.0% agarose gel by comparing to 100 bp ladder (data not shown) (Fisher Scientific, Waltham, MA). Amplified products were stained with ethidium bromide, visualized via UV transillumination, and images captured using the Fotodyne Mini Visionary system. Only those samples that yielded products from microsatellite PCR were screened for *N. caninum* DNA.

Raccoon and opossum brain DNA was tested for viability using random primer OPA19 (5'CAAACGTCGG3') that amplifies random eukaryotic DNA polymorphisms (Yu, 2004). A positive control consisted of coyote DNA samples obtained from B. Sacks, University of California Davis, and negative controls were performed by

substituting water for DNA in the reaction. The amplified products were electrophoresed, visualized, and photographed, as for canid-specific microsatellite PCR. DNA samples that were not amplified by either OPA19 or FH2079-1 andFH2079-2 were subjected to the extraction process again to purify the DNA further and were retested. Only those samples that yielded products from random primer PCR were screened for *N. caninum* DNA.

Detection of *N. caninum* by PCR –Amplifiable DNA from coyote, fox, opossum, and raccoon brains was screened for the presence of *N. caninum* DNA using the NP21/NP6 primer pair (Yamage et al., 1996). The PCR conditions consisted of an initial denaturing step at 95°C for 5 min followed by 40 cycles at 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min, with a final extension step at 72°C for 10 min. This assay amplifies the Nc5 region of *N. caninum* chromosomal DNA, which is unique to this parasite. One hundred nanograms DNA were used as template in each reaction. *Neospora caninum* NC-1 strain tachyzoite DNA was used as a positive control, and sterile water was used as a negative control. Amplified products were visualized as above.

Results

Seroprevalence of *N. caninum* – Western blot assays were conducted to examine the seroprevalence of *N. caninum* in wildlife serum samples. Blood samples were classified as positive when antibodies against the parasite bound to immunodominant bands at 29 and 17 kDa (Figure 1). Seropositive samples had varying band intensity, indicating variance in the amount of circulating anti-*Neospora* IgG (Figure 1). In experiments performed by Jeff Seidling, antibodies to *N. caninum* were found in 5 out of the 34 (14.7%) coyote serum samples and in 1 out of 9 fox serum samples (11.1%) (Table 1). I found that 0 out of 42 opossums and 0 out 45 raccoons tested positive for *N. caninum* antibodies in their serum. A large number of raccoon and opossum samples were tested and no antibody binding was observed. This could be attributed to true seronegativity, or it could be a result of low assay sensitivity. We could not generate or obtain control sera from raccoon and opossum, to evaluate the assay, and were limited to performing dot blots to confirm that the species-specific secondary reagents indeed bound serum IgG from the target species (data not shown),

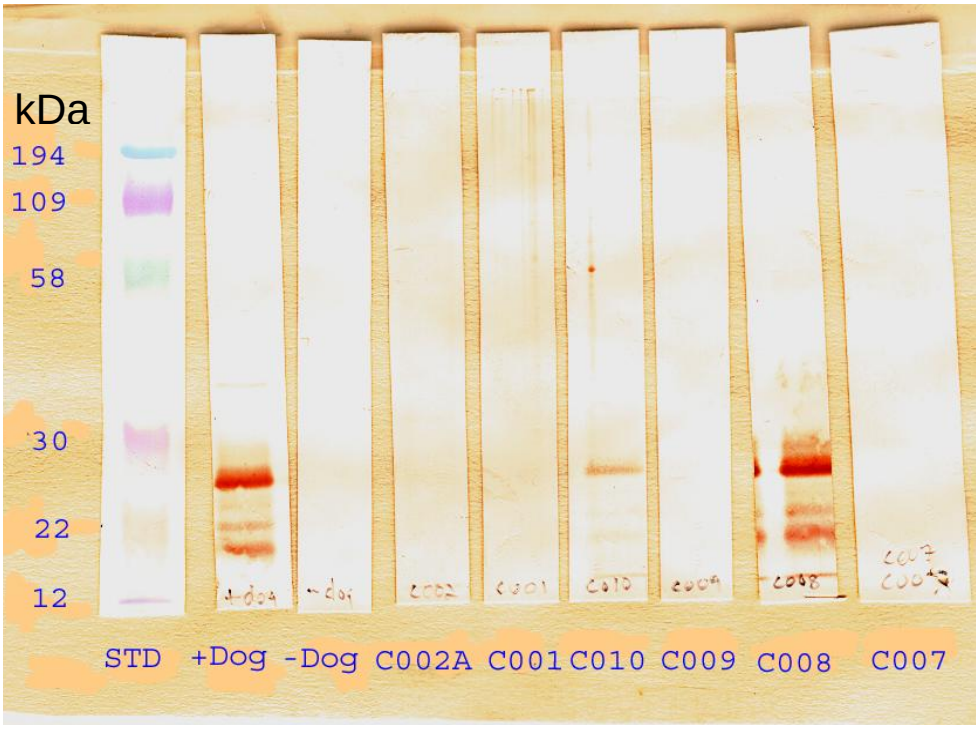

Figure 1. A representative Western blot demonstrating seroreactivity to *N. caninum* antigens in coyotes (Jeff Seidling). Immunodominant proteins of 29 and 17 kDa were bound by positive control dog serum, and by test samples from coyote 010, and coyote 007. The negative dog control and coyotes C002A, C001, C009, and C007 had no seroreactivity to the antigen.

Table 1. Seroprevalence of *N. caninum* in Wisconsin scavenger mammals by Western blot. Positive serum samples had immunodominant bands at 17kDA and 29kDa. Seroprevalence for each species was expressed as number of positive samples divided by total number of samples for that species.

Species	Number tested	Number positive	Seroprevalence
Coyote	34	5	14.7%
Fox	9	1	11.1%
Opossum	42	0	0%
Raccoon	45	0	0%

Molecular Screen for *N. caninum* – Microsatellite regions of coyote and fox DNA were amplified by PCR to establish DNA integrity by J. Seidling. All 8 coyote and 4 fox samples contained DNA that was amplifiable by PCR (Figure 2). Random primed PCR revealed that 53 of 113 opossum (46.9%) and 52 of 110 (47.3%) raccoon brain DNA samples were amplifiable by PCR (Table 1). An additional round of purification allowed previously unamplifiable samples to be used. If after two

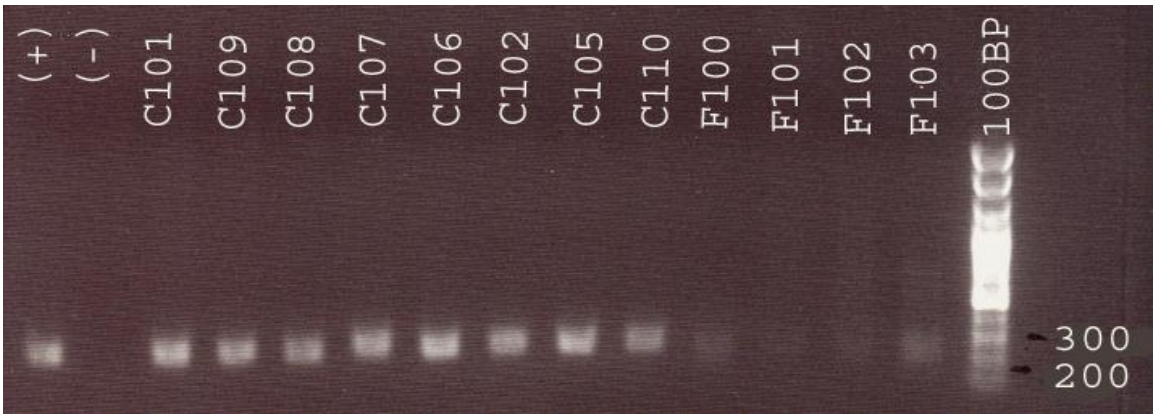

Figure 2. Microsatellite PCR for canids demonstrates DNA integrity. Both coyote and fox samples showed banding at the expected size, between 200-300bp.

DNA extractions a sample was still unamplifiable, it was omitted from the parasite-specific molecular screen. The parasite-specific molecular screen revealed that no coyote or fox (J. Seidling), and no raccoon or opossum brain DNA samples contained parasite DNA (Table 2).

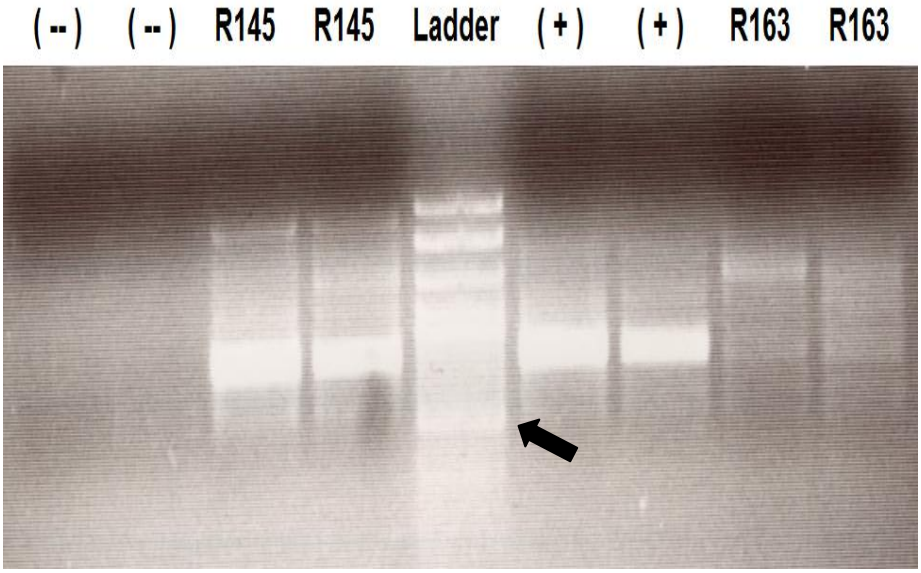

Figure 3. Random primer PCR on raccoon DNA samples. A 100 base pair ladder was used for comparison (the arrow indicates the 500 bp band). The positive sample was coyote DNA. Banding indicates amplifiable DNA.

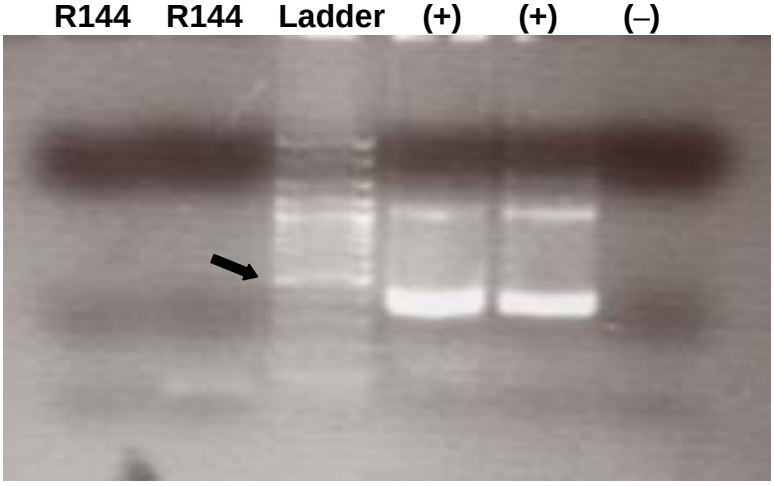

Figure 4. *N. caninum*-specific PCR on Central Wisconsin raccoon brain DNA samples. The arrow indicates the 500bp band. The positive control, *N. caninum* NC-1 strain DNA, yields a 328 bp product.

Table 2. Results of DNA extraction and parasite screen.

Species	# of extracted brain DNA sample	# of samples w/ amplifiable DNA	# of *N. caninum* positive samples
Coyote	8	8	0
Fox	4	4	0
Opossum	113	53	0
Raccoon	110	54	0

Discussion

Central Wisconsin coyotes are seropositive for *N. caninum*, suggesting that they may play a role in the sylvatic cycle in our study area. This correlates with other reports (Gondim 2004b, Lindsay 2006) that implicate coyotes in parasite transmission to deer. We found an 11% seroprevalence in foxes, slightly higher than previously reported seroprevalences in foxes of 6% in Spain (Simpson et al. 1997). The prevalence of *N. caninum* antibodies in coyotes indicates that there are definitive hosts of *N. caninum* in the study area. While coyotes are a known definitive host for this parasite, foxes have only been implicated as intermediate hosts (Gondim et al., 2004, Almería et al. 2002). A seropositive reaction by Western blot indicated that the animal was exposed to *N. caninum* at some point in its life, and the antibodies against the parasite are a long-term artifact of that exposure.

No molecular evidence of *N. caninum* tachyzoites or bradyzoites was found in the brain tissue samples. The Nc5 assay can repeatedly amplify as little as a single tachyzoite in 2 mg of brain tissue (Yamage et al. 1996). In an active infection, tachyzoites are concentrated within the central nervous system and typically form tissue cysts that contain thousands of bradyzoites. The lack of *N. caninum* positive DNA

samples can be attributed to animals that did not have a current parasitic infection or that the parasite infection was relatively new and the tachyzoites did not have time to migrate to the brain tissue. A possible source of error for this molecular method was in the actual sample collection. The primary focus of the Wisconsin Wildlife Cooperative Unit study was to find CWD prions in brain tissues, and they provided us with remnants of brain samples. As a result, sample quality is unknown, as is the part of the brain collected.

The lack of seroreactivity in opossum and raccoon serum differs greatly from reported seroprevalence findings of 21.2% of opossums (Yai et al., 2003) and 9.9% of raccoons (Lindsay et al. 2001). There are three possible reasons that no antibodies to *N. caninum* were detected: 1.) Scavenging mammals such as raccoons and opossums do not consume contaminated foods. This scenario is unlikely because they feed on deer carcasses, which have a *N. caninum* seroprevalence of 20-48% (Anderson et al., 2007). 2.) Our assay was not sensitive enough to detect low levels of antibodies. Although the dot blot demonstrated that the raccoon and opossum serum samples bound to their respective secondary conjugate, there was no way to know that serum samples could bind to the NC-1 parasite antigen. Other researchers use indirect fluorescent-antibody test (IFAT) because it has a higher sensitivity and specificity to antibodies, and additionally the antibody titers can be quantified (Duarte et al, 2003). *Neospora* agglutination test (NAT) is another serologic assay often used when surveying wildlife species for *N. caninum* seroprevalence, because secondary antibodies are not needed, but it is no longer commercially available. 3.) Animals were truly seronegative and had not feed on infected deer carcasses, or are refractory to the infection. This hypothesis is supported by

the observation that a random sample of 20 tested raccoon and opossum serum samples were also seronegative by *Neospora* Agglutination Test (NAT) (J.P. Dubey, personal communication).

This serological and molecular survey of Wisconsin wildlife for *N. caninum* has allowed us to determine reliable estimates of seroprevalence in coyotes and foxes. The role that coyotes play in the transmission cycle is known, but the role of raccoons and opossums needs to be further studied. Lemberger et al. (2005) confirmed by PCR that raccoons are intermediate hosts for *N. caninum,* but the definitive host status of this species is still unknown. Because technical difficulties were encountered for serological testing, fecal examination is an alternative to finding new definitive hosts for this parasite.

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CHAPTER IV

INTESTINAL PARASITE SURVEY OF WISCONSIN SCAVENGING MAMMALS

Introduction

The only known definitive hosts for the protozoan parasite *Neospora caninum* are domestic dogs and coyotes, though the role of wolves in transmission has been inferred (McAllister et al., 1998, Gondim et al., 2004). Many wild animals such as raccoons and opossums have antibodies against *N. caninum*, but the potential role that they play in the *N. caninum* life cycle is not known (Lindsay et al., 2001). For a species to be a definitive host, it must support sexual development of the parasite and shed parasite oocysts in its feces. Here we report a fecal survey of scavenging WI wildlife species for *N. caninum* oocysts, and other intestinal parasites.

Materials and Methods

Carcasses collected by the Wisconsin Cooperative Wildlife Research Unit (WCWRU) were refrigerated or frozen, and necropsied to isolate brain tissue for CWD testing, blood for serology, and fecal samples for parasitological examination. The fecal samples were obtained directly from the large intestine and then frozen for storage.

We screened fecal samples from 38 coyotes, 20 opossums, and 35 raccoons. Samples were thawed to room temperature, and approximately 2g of feces processed using Sheather's centrifugation concentration method. The Sheather's sugar solution was made from 7.1 M $C_6H_{12}O_6$ sugar, 0.22 M H_2CO adjusted to a specific gravity of 1.27 (Bowman, 1999). Slides were microscopically examined for parasite oocysts, eggs and larvae, which were identified based on morphological characteristics, including

dimensions measured by a stage micrometer. Aleja Carvajal helped in the fecal parasite identification by examining approximately 30 percent of the fecal samples. The intensity of each parasite species in the samples was calculated and normalized to eggs per gram measurement. Parasite prevalence in each of the three animal species was calculated as the ratio of infected samples to the total number of fecal samples for that animal. Variation among the different parasite counts was represented by the standard error of the mean.

Results

Thirty-five raccoon fecal samples were tested for intestinal parasites, and none of the samples had oocysts that were consistent with those of *N. caninum*, while eight of the thirty-five samples were free of any parasites. Seven species of parasites were identified from raccoon fecal samples (Table 1), four of which were nematodes (*Baylisascaris procyonis, Capillaria* sp., *Uncinaria* sp. and *Physaloptera sp*), two apicomplexan parasite of raccoons (*Sarcocystis* sp., and *Isospora* sp.), and one apicomplexan parasite of earthworms (*Monocystis agilis*) that is commonly found in the intestinal tracts of scavenging animals that eat earthworms. Each individual raccoon was infected with a mean of 2 parasite species.

Table 1. Intestinal parasites of raccoon fecal samples from Dane County, WI. Prevalence values are based on the total sample size of 35 raccoons. Intensity is a ratio of eggs per gram. The presence of *Isospora* sp. was classified as positive or negative.

Parasite taxon	Prevalence # infected (%)	Intensity range	Mean intensity (± SE)
B. procyonis	19 (54%)	0.5-929.4	142.9 (±51.0)
Capillaria sp.	18 (51%)	0.5-56.6	10.76 (±3.6)
Isospora sp.	1 (3)	-	-
Sarcocystis sp.	3(1%)	1.7-23.5	12.6 (±7.07)
Uncinaria sp.	1 (3)	-	-

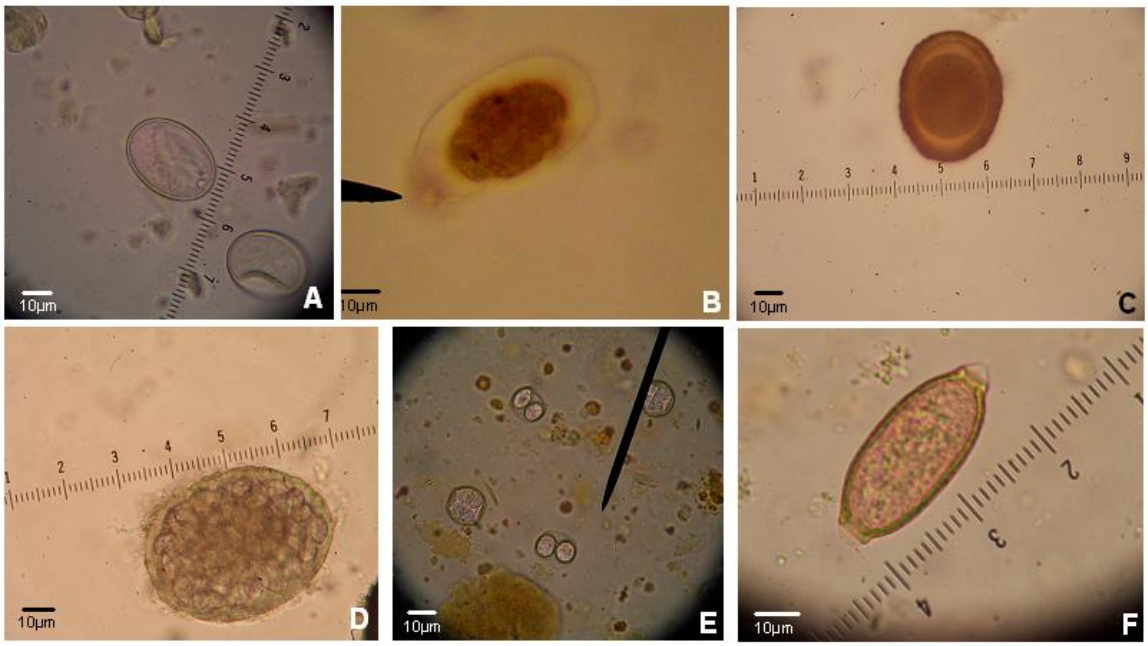

Fig. 1. A-F. Intestinal parasites found in raccoon feces. (A) *Physaloptera* sp. (B) *Uncinaria* sp. (C) *Baylisascaris procyonis* (D) *Monocystis* sp. (E) *Isospora* sp. (F) *Capillaria* sp.

Twenty opossum fecal samples were examined for intestinal parasites, and none of the samples had oocysts that were consistent with *N. caninum* oocysts while six species of parasites were identified from 20 opossum fecal samples (Table 2). Parasites were found in the fecal samples of all but 3 individuals. The parasites found included five nematode species (genera *Trichuris*, *Physolaptera*, *Cruzia*, *Toxascaris* and *Uncinaria*), and one apicomplexan (*Sarcocystis* sp.). In addition, a single trematode-type egg with the dimensions of 47.5μm by 30μm was unable to be identified to genus due to the poor sample quality. Opossums were infected with a mean of 1.5 parasite species per animal. Sixty percent of the opossum fecal samples contained *Monocystis* sp., which are harmless to mammals.

Table 2. Intestinal parasites of opossum fecal samples from Dane County, WI. Prevalence values are based on the total sample size of 20 animals. Intensity is the ratio of eggs per gram.

Parasite taxon	Prevalence # infected (%)	Intensity Range	Mean intensity (± SE)
Trichuris sp.	1 (5%)	8.7	8.7
Cruzia sp.	1 (5%)	-	-
Physaloptera sp.	2 (10%)	0.5-2.7	1.6 (±0.8)
Sarcocystis sp.	1 (5%)	37.7	37.7
Toxascaris sp.	9 (45%)	0.5-79.8	13.3 (±8.1)
Trematode sp.	1 (5%)	-	-
Uncinaria sp.	4 (20%)	7.5-214.2	65.9 (±43.4)

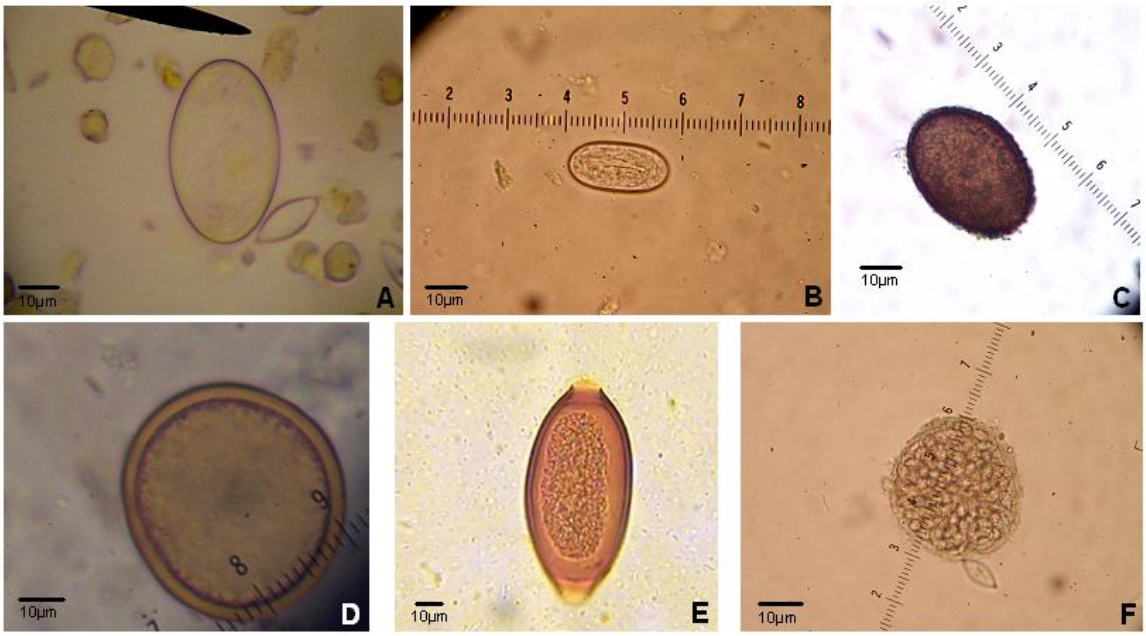

Fig. 2. A-F. Intestinal parasites found in opossum feces. (A) Unidentified trematode egg (B) *Physaloptera* sp. (C) *Cruzia americana*. (D) *Toxascaris* sp. (E) *Trichuris* sp. (F) *Monocystis* sp.

Thirty-eight coyote fecal samples were tested for intestinal parasites, and none of the samples had oocysts that were consistent with *N. caninum* oocysts, while no parasites were observed in 9 of the fecal samples. Seven species of parasites were identified, including three nematode genera (*Capillaria, Toxocaris, Uncinaria*), taeniid cestodes, and two apicomplexans (*Isospora, Sarcocystis*). When coyotes were infected with a parasite there was a mean of 1.2 parasite species per individual. Eight percent of the coyote fecal samples contained *Monocystis* sp.

Table 3. Intestinal parasites of coyote fecal samples from Dane County, WI. Prevalence values are based on a total sample size of 38 coyotes. Intensity is a ratio of eggs per gram. No parasites were found in 23.7% of the coyote samples.

Parasite taxon	Prevalence # infected (%)	Intensity range	Mean intensity (± SE)
Capillaria sp.	6 (16%)	0.5-25.4	8.0 (±3.6)
Isospora sp.	6 (16%)	0.5-61.6	16.0 (±8.9)
Sarcocystis sp.	16 (42%)	10.5-908.7	131.1 (±53.0)
Taenia sp.	8 (21%)	0.5-57.6	18.7 (±7.7)
Toxocara leonina	7 (18%)	0.5-26	9.5 (±3.6)
Uncinaria sp.	1 (3%)	0.5	0.5

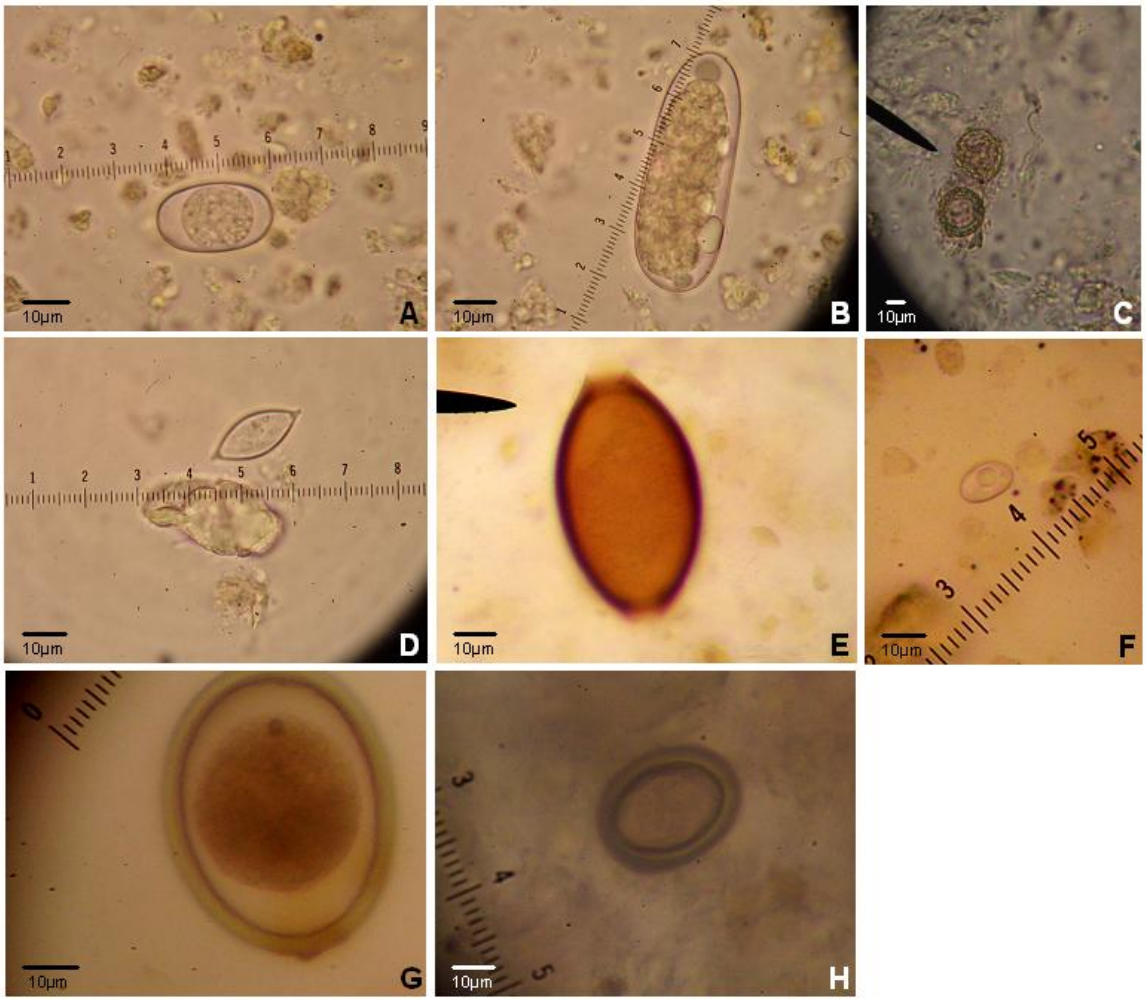

Fig. 2. A-F. Intestinal parasites found in coyote feces. (A) *Isospora* sp. (B) *Uncinaria* sp. (C) *Taenia* sp. (D) *Monocystis* sp. (E) *Capillaria* sp. (F) *Sarcocystis* sp. (G) *Toxascaris leonina* (H) *Taenia* sp.

In addition, four fecal samples from skunks were examined for parasites. Two out of the four samples lacked parasites. The parasite species found in parasite positive fecal samples were *Baylisascaris* sp. The skunk fecal samples contained a large concentration of coleopteran wing parts.

Discussion

The purpose of this study was to obtain evidence for *N. caninum* oocyst shedding by coyotes and other native mammals, and to document the other naturally occurring intestinal parasites in these animals. We found no evidence of *N. caninum* oocyst shedding in the species we sampled, even in known definitive hosts (coyotes). This could be due to several factors. First, the samples had been frozen and thawed a minimum of one time, and often two to three times prior to examination. It is not known if *N. caninum* oocysts maintain structural integrity in those conditions. Second, the window of oocyst shedding by the definitive host is quite short, only lasting 1-7 days in experimentally infected dogs (Dubey et al., 2007). It is quite possible that animals could have been infected but were not actively shedding oocysts at the time of sample collection. Finally, it is possible that the animals we sampled had never been exposed to the parasite and were truly negative. We cannot make solid conclusions at this time, except to state that we have succeeded in collecting preliminary data supporting future null hypotheses regarding the roles of opossums and raccoons in the sylvatic *N. caninum* cycle.

There is a notable lack of published data on the diversity of intestinal parasites in Wisconsin wildlife. To assist us in parasite identification, we relied on surveys from other regions of the United States including Kentucky (Cole et al., 1987) and Connecticut (Richardson et al., 2005), and on published lists of parasite species known to infect different species (Hawkins, 1949). Many of the parasites can only be identified to the genus level, as further identification requires morphological study of the adult worms.

We intend to submit this data to The Journal of Wildlife Diseases, by combining information from an ongoing survey of adult worms from the intestines of representative animals from this site, to establish a record of naturally occurring intestinal parasites in Wisconsin wildlife.

An understanding of the present parasite fauna in these wildlife species is essential in determining the potential risk of disease transmission. In coyote fecal samples, the intestinal parasite fauna can vary greatly between study sites even within a state, depending on coyote ecology (Gompper et al., 2003). Our data will will contribute to an understanding of the ecology of WI wildlife diseases upon publication of a temporal and spatial analysis of disease prevalence (West Nile Virus, CWD, paratuberculosis, heartworm) that will be coauthored by all investigators that cooperated in the UW Madison study.

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CHAPTER V

SUMMARY

The goal of this project was to characterize the sylvatic transmission cycle of *Neospora caninum* in Central Wisconsin. Seroprevalence of white-tailed deer (*Odocoileus virginianus*), coyotes (*Canis latrans*), and other small scavenging mammals was determined by ELISA and Western blot analysis of serum samples. Coyotes and white-tailed deer were seropositive for *N. caninum*, suggesting that they play a role in transmission of the parasite. Serological studies of other small mammals (raccoon, opossum, mink) refute the hypothesis that alternate hosts exist.

Results of seroprevalence were correlated with molecular prevalence of *N. caninum* in scavenging mammals. Detection of this parasite by molecular techniques would indicate that the host animal had an active infection of *N. caninum*. A correlation between serological and molecular evidence would have helped determine the scavenger's host status (intermediate or definitive). No molecular evidence of *N. caninum* was found in any of the brain tissue tested, suggesting that none of the mammals were actually infected with *N. caninum* at the time of capture.

The role of coyotes and potentially new definitive hosts was investigated by fecal survey. No *N. caninum* oocysts were observed in any of the coyote or scavenger mammal fecal samples. The lack of positive fecal samples does not exclude any of the scavenger species from being a potential definitive host. Definitive hosts for this parasite have a very brief window during which they shed the oocysts, so with the small number of samples used in this study, the window could have been missed. An intestinal parasite

survey of mammalian scavengers was conducted to quantify parasite loads by fecal floatation. Mean intensities and prevalence of the various intestinal parasites were calculated for each animal species.

Despite the lack of *N. caninum* infection in scavenger mammals, future studies are necessary to determine the role these scavengers may play in the transmission cycle. Reliable positive controls for Western blot analysis, a larger sample size, and better quality samples need to be established to thoroughly test these hypotheses. In addition, the possible role of birds and rodents as hosts for *N. caninum* needs consideration. With the potentially large host range of this parasite, prevention is the best control method, but a new live attenuated vaccine is being developed.