Neosporosis in Ontario Broodmares: Seroprevalence and Risk Factors for Exposure to *Neospora caninum* and *Neospora hughesi*

by

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ABSTRACT

NEOSPOROSIS IN ONTARIO BROODMARES: SEROPREVALENCE AND RISK FACTORS FOR EXPOSURE TO NEOSPORA CANINUM AND NEOSPORA HUGHESI

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Advisor:

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Dr. Tracey Chenier

The seroprevalence and risk factors for exposure to *Neospora caninum* and *Neospora hughesi* in Ontario broodmares were investigated. The prevalence estimates for *N. caninum* and *N. hughesi* were 27.4% (60/219) and 29.7% (65/219), respectively. The IFAT cut-off titre for *N. caninum* and *N. hughesi* was 1:40 and 1:160, respectively. Risk factors for *N. caninum* included presence of farm dogs (OR = 6.70; 95% CI = 2.14 – 20.97; p = 0.001), and high stocking density (OR = 2.83; 95% CI = 1.27 – 6.30; p = 0.011). Presence of livestock, excluding cattle, was associated with reduced risk of exposure (OR = 0.17; 95% CI = 0.05 – 0.53; p = 0.002). The only risk factor for exposure to *N. hughesi* was feeding hay on the ground in the paddock (OR = 4.31; 95% CI = 1.65 – 11.22; p = 0.003). This study demonstrated significant exposure to *Neospora* spp. in Ontario broodmares.
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for the encouragement during the COVID-19 lockdowns, when my research was delayed, and on-campus experience was no longer possible.

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DECLARATION OF WORK

I declare that I performed all the work described in this thesis with the exception of the following:

Blood collection from broodmares across Ontario was performed by licensed veterinarians.

IFAT analysis of serum samples was performed by the Conrad Lab\(^1\) at the University of California, Davis.

The preparation and staining of fetal tissues for histological evaluation were performed by the Animal Health Laboratory at the University of Guelph.

Histological evaluation of fetal tissues was performed by Dr. Rob Foster in the Department of Pathobiology at the University of Guelph.

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<th>Glossary</th>
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<tr>
<td>AHL</td>
<td>Animal Health Laboratory</td>
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<tr>
<td>AUP</td>
<td>Animal Utilization Protocol</td>
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<td>BCS</td>
<td>Body condition score</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<td>EIA</td>
<td>Equine infectious anemia</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>EPM</td>
<td>Equine protozoal myeloencephalitis</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>IFAT</td>
<td>Immunofluorescent antibody testing</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td><em>N. caninum</em></td>
<td><em>Neospora caninum</em></td>
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<td><em>N. hughesi</em></td>
<td><em>Neospora hughesi</em></td>
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<td>REB</td>
<td>Research Ethics Board</td>
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<td>Sn</td>
<td>Sensitivity</td>
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<td><em>S. neurona</em></td>
<td><em>Sarcocystis neurona</em></td>
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<td>Sp</td>
<td>Specificity</td>
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Chapter One: Literature Review

Introduction

Neosporosis is a disease caused by a cyst-forming coccidian parasite of the phylum Apicomplexa. *Neospora caninum* is one of several cyst-forming protozoal parasites of animals with morphological similarities to *Toxoplasma gondii*. *Neospora caninum* was differentiated as its own genus and species in 1988 (Dubey *et al.*, 1988). This new classification arose following studies examining the ultrastructural characteristics of tissue cysts, and immunohistochemistry (IHC) confirmed that the two were distinct (Bjerkas and Prethus, 1988). Identification of *N. caninum* as a newly recognized tissue coccidium distinct from *T. gondii* explained the etiology of certain diseases and reproductive failure in cattle worldwide. Multiple seroprevalence and morbidity studies were conducted on *N. caninum* in cattle; however, significantly less is known about its impact on horses. *Neospora caninum* has a major economic impact on the cattle industry. This review will compare and contrast the role of *N. caninum* as an etiologic agent of disease in domestic host species.

Due to its worldwide presence, the economic impact of *N. caninum* is significant. In a systemic review, Schmidt *et al.* (2018) documented the impact of *N. caninum* infection and abortion on cattle. They calculated the annual cost of *N. caninum* infection and abortion to the New Zealand beef industry was $1.1 million USD. The estimated median total cost per annum in the cattle industry due to *N. caninum* related losses exceeded $1.298 billion USD, with nearly two-thirds of the losses occurring in the dairy industry. A systematic review from January 2012 estimated that the total average losses related to *N. caninum* surpassed $1.298 billion USD per annum globally, reaching as high as $2.380 billion USD. The median annual losses on individual
dairy farms were $1,600 USD, while on beef farms these costs amounted to only $150 USD (Reichel et al., 2013). A better understanding of the lifecycle, prevalence, and pathogenesis of *N. caninum* within various intermediate species such as cattle and horses, will enable control of the risk factors for exposure in order to minimize economic losses.

**History of *Neospora caninum***

*Neospora caninum* is a coccidian protozoan parasite belonging to the Phylum Apicomplexa. Initially, it was often misidentified as another apicomplexan parasite, *Toxoplasma gondii* since it has a similar structure and life cycle (Dubey et al., 2007), with some minor distinguishing differences. Apicomplexan protists are a group of morphologically and ecologically diverse obligate parasites (Votýpka et al., 2017). The life cycle of coccidian members of the Apicomplexa involves sexual and asexual multiplication in their hosts and an environmentally resistant cyst form. Transmission strategies within the group are diverse, which range from direct transmission to more complex cycles between predators and their prey or other vectors (Votýpka et al., 2017).

Neosporosis is primarily a disease of cattle (Dubey, 2011; Dubey and Lindsay, 1996; Dubey et al., 2017) and dogs (Dubey, 1999; Dubey et al., 2017; Dubey et al., 1988; McAllister et al., 1998), with canid species identified as the definitive hosts (Dubey et al., 2011; Gondim et al., 2004; McAllister et al., 1998). Toxoplasmosis can occur in a wider range of intermediate hosts and is an important disease of humans and small ruminants with felids identified as the definitive hosts (Lindsay and Dubey, 2020; Khan et al., 2020; Dubey and Ferguson, 2015; Dubey, 2006). Domestic dogs were first found to be a definitive host and also excrete oocysts of
*Neospora caninum* in 1988 (Lindsay *et al*., 1999; McAllister *et al*., 1998). In later years, coyotes (*Canis latrans*) and wolves (*Canis lupis*) were also confirmed to be definitive hosts (Dubey *et al*., 2017; Gondim *et al*., 2004).

**Life Cycle of *Neospora caninum***

The life cycle of *N. caninum* was elucidated relatively recently (Dubey, 2004; Dubey and Beattie, 1988; Dubey and Schares, 2007; Khan *et al*., 2020; Lindsay and Dubey, 2020). It includes three infectious stages: oocysts, tachyzoites, and tissue cysts (Lindsay and Dubey, 2020). Oocysts can be either unsporulated or sporulated. Unsporulated oocysts are excreted in the feces of definitive hosts, such as dogs and coyotes. Unsporulated oocysts measure 10 – 12 µm in diameter and then sporulate outside of the host (McAllister *et al*., 1998). Presently, the frequency of shedding of *N. caninum* oocysts, the survival of oocysts in the environment, and the sporulation that occurs outside of the host is not understood (Lindsay and Dubey, 2020). Oocysts have smooth cell walls that measure 0.6 – 0.8 µm thick (Lindsay *et al*., 1999). The unsporulated oocyst is diploid and resistant to freezing and drying (Khan *et al*., 2020). The environmental resistance of *N. caninum* oocysts is thought to be similar to those of *T. gondii*, with previous reviews suggesting they are viable in “moderate environmental conditions” for long periods of time (Dubey, 2004), and within moist soil conditions for months to years (Dubey and Beattie, 1988). It is thought that *T. gondii* oocysts are more resistant overall than *N. caninum*, due to the poor understanding of *N. caninum* oocysts (Lindsay and Dubey, 2020). The majority of oocysts contain two sporocysts (Lindsay *et al*., 1999). Sporulation of oocysts begins within 24 hours after excretion (Dubey, 2004). Sporulated oocysts may be subspherical or spherical in shape and their size can be as large as 11.7 x 11.3 µm (Lindsay *et al*., 1999). The other stages, tachyzoites
and tissue cysts, are found within the intermediate host. Tachyzoites are approximately 6 by 2 µm and are typically found in the central nervous system. Tachyzoites eventually produce bradyzoites by endodyogeny after receiving a signal from the host to begin stage conversion to produce the dormant thick-walled tissue cyst stage (Lindsay and Dubey, 2020). Tissue cysts are oval in shape and can be up to 107 µm in length, with walls that are up to 4 µm thick in the brain and the enclosed oval bradyzoites are typically 7 – 8 µm by 2 µm. Tissue cysts are also found in extraneural tissues, especially skeletal muscle (Dubey et al., 2007).

Definitive hosts, such as dogs and other canids, become infected with *N. caninum* by ingesting tissues containing tachyzoites and tissue cysts from intermediate host species (Figure 1.1; Dubey and Schares, 2011). Intermediate hosts, including cattle, goats, sheep, and horses, are infected through the ingestion of food and water sources contaminated with canid feces and thus with *N. caninum* oocysts. *Neospora caninum* is also effectively transmitted trans-placentally in cattle (Williams et al., 2009), with previous studies reporting that 37 – 61% of seropositive cows give birth to a seropositive calf (Bartels et al., 2007; Dijkstra et al., 2008; More et al., 2009). Abortion in ruminants occurs following bloodborne infection of the maternal caruncular septum where the parasite crosses to the fetal placental villi, causing damage to that maternal-fetal connection (Dubey and Schares, 2011). Placental infection elicits a variety of responses in both the maternal and fetal unit that ultimately leads to abortion. Innes (2007) proposed that the immuno-compromise of pregnancy may cause reactivation of a dormant infection in the dam. The parasite differentiates from tissue cyst into a tachyzoite that can then cross the placenta and infect the fetus.
Figure 1.0.1: *Neospora caninum* life cycle highlighting the three infectious life stages: oocysts, tachyzoites, and tissue cysts. Vertical and horizontal transmission is displayed. Adapted from Dubey *et al.* (2007).

**Diagnosis of *Neospora caninum* infection in cattle**

Diagnostic methods to identify exposure to *N. caninum* in various host species include the serological methods of a modified agglutination test for *Neospora* specifically (N-MAT, NAT), a direct agglutination test (DAT), an immunofluorescent antibody test (IFAT), and an enzyme-linked immunosorbent assay (ELISA), with both competitive (cELISA) and indirect (iELISA) assay types reported (Bildfell *et al.*, 1994; Duffield *et al.*, 2001; Fioetti *et al.*, 2003;
The N-MAT test measures the direct agglutination of the protozoa by *N. caninum*-species specific antibodies in serum and therefore eliminating the need for secondary host-specific anti-isotype serum (Packham *et al*., 1998). Most studies on *Neospora* to date used an ELISA test, with fewer reporting use of IFAT (Table 1.1). However, the IFAT is considered the gold standard diagnostic test for *N. caninum* due to its higher sensitivity (Sn) and specificity (Sp).

Previous studies estimating seroprevalence of *N. caninum* exposure in cattle vary greatly by geographical location, and a lack of consistency in testing methods makes comparisons across studies difficult. Wapenaar *et al.* (2007) investigated the performance and agreement of various commercially available and in-house (ELISA, cELISA, iELISA, IFAT, and NAT) *N. caninum* antibody assays in North American dairy cattle. All of the test cut-off points were determined by the manufacturers’ recommendations. Of the 397 samples, IFAT had 11% seropositivity with both sensitivity and specificity reported to be 97%. The sensitivity of the other tests were over 89% except for NAT which was 66%. The Sp was high (>94%) for all tests except for the iELISA which was 52%.

Routine histopathological evaluation of aborted tissues also aids in the diagnosis of *N. caninum* infection. Degenerative to inflammatory lesions may be found throughout fetal tissues but are frequently found in the central nervous system (CNS), heart, and liver. Pale, white foci are seen in skeletal muscle and heart tissue of some fetuses (Dubey *et al*., 1998). Often fetuses are autolysed or mummified, and areas of discolouration may be present in cotyledons (Fioetti *et al*., 2003).
Abortion due to *N. caninum* can be confirmed by a variety of methods. *Neospora caninum* has typical lesions that can be identified, in the heart, brain, liver, and lungs. Immunohistochemical staining is more reliable than H&E staining for the identification of *N. caninum* organisms (Boger and Hattel, 2003). Polyclonal and monoclonal antibodies for IHC (Cole *et al.*, 1994; Lindsay and Dubey, 1989) that are specific to *N. caninum*, are commercially available. Cross-reactivity in IHC between *N. caninum* antibodies to the related apicomplexan parasites *T. gondii* and *Sarcocystis* spp. is possible, however abortion due to related organisms especially *Sarcocystis* is rare (Anderson *et al.*, 1991; Canada, 2002). Tachyzoites are often indistinguishable from released bradyzoites based on IHC staining, unless bradyzoite-specific antibodies are used (McAllister *et al.*, 1996). A diagnosis of *N. caninum* should not be made unless the zoites are visible, to ensure staining is specific (Dubey and Schares, 2006). While histology and IHC are helpful in identifying changes in tissues due to infection, molecular detection using PCR for *N. caninum* is superior due to increased sensitivity and specificity (Barry *et al.*, 2017; Baszler *et al.*, 1999; Jenkins *et al.*, 2002). PCR is highly sensitive and precise process used for detection of genetic material from pathogens in small amounts (Barry *et al.*, 2019). Baszler *et al.* (1999) compared the detection of *N. caninum* using PCR on formalin fixed and fresh tissues of spontaneously aborted bovine fetuses. They found that PCR had a sensitivity of 100% and a specificity of 94% for formalin fixed brain tissues. The sensitivity and specificity of fresh brain tissues were 77% and 100% respectively. The agreement between *N. caninum* PCR detection and true status was 97% and 88% for formalin fixed and fresh tissues, respectively.
Transmission of *Neospora caninum* in Cattle

Vertical transmission from dam to fetus and post-natal ingestion of oocysts are the reasons *N. caninum* persists within a herd (Bartel *et al.*, 2007; de Aquino Diniz *et al.*, 2019; De Meerschman *et al.*, 2002; Moore *et al.*, 2009; Nascimento *et al.*, 2014; Pereyra *et al.*, 2019). The risk of vertical transmission varies among herds. Bartel *et al.* (2007) reported a vertical transmission risk of 61.8% in Dutch dairy cows, while a study in Argentina found a 39.9% vertical transmission risk in dairy cows (Moore *et al.*, 2009). In a study of dairy (n = 76) and beef (n = 148) aborted fetuses, De Meerschman *et al.* (2002) found a significant association (p = 0.004; OR not reported) between a high level of antibodies in a dam and the occurrence of histopathological lesions of *N. caninum* infection in her aborted fetus. Sero-positive fetuses were never born to sero-negative cows. In some herds, virtually all calves are born infected with *N. caninum* but since they often present asymptptomatically, the true seropositivity within a herd due to vertical transmission may be underestimated (Bergeron *et al.*, 2000; Björkman *et al.*, 1996; Frössling *et al.*, 2005; Hall *et al.*, 2005; Schares *et al.*, 1998). Horizontal transmission of *N. caninum* only occurs through the ingestion of sporulated *N. caninum* oocysts from the environment (Anderson *et al.*, 1997; Gondim *et al.*, 2004; Khan *et al.*, 2020; Lindsay and Dubey, 2020; Trees *et al.*, 2002); cow-to-cow transmission has not been documented. *Neospora caninum* organisms are most often observed in the brain and heart of cattle fetuses, and rarely in other organs. Wouda *et al.* (1997) reported that *N. caninum* was found in the brain (71/80; 89%), heart (11/80; 14%), and liver (21/80; 26%) of bovine aborted fetuses.

Due to concerns about infection of calves by feeding pooled milk, studies examined whether *N. caninum* was found in the milk of seropositive cows. *Neospora caninum* DNA was
identified in milk, including colostrum (Moskwa et al., 2006). However, the study did not examine milk for the presence of tachyzoites but used colostrum sediment to amplify genomic regions with PCR testing. Davison et al. (2001), however, examined milk for tachyzoites. They first dosed six calves with *N. caninum* tachyzoites via colostrum and/or milk replacer on four separate occasions and then tested for seroconversion. All 6 calves seroconverted to *N. caninum* post-inoculation. Next, seven uninfected calves were fostered onto *N. caninum* infected dams, but no evidence of *N. caninum* infection was found in the calves. No statistical results were reported in this study, but this confirms that calves can be infected with *N. caninum* by the oral route with experimentally infected colostrum or milk replacer. However, natural infection by *N. caninum* of calves via lactogenic transmission has yet to be reported (Dijkstra et al., 2001).

Seroprevalence of *Neospora caninum* in Cattle

The presence of *N. caninum* was investigated in many countries and regions, including Canada (Bildfell et al., 1994; Duffield et al., 2001; Keefe and Van Leeuwen., 2000; Paré et al., 1998; VanLeeuwen et al., 2002; Waldner et al., 2001), the United States of America (McAllister et al., 2000; Rodriguez et al., 2002), Brazil (Minervino et al., 2008; Schmit et al., 2018), New Zealand (Okeoma et al., 2004; Tennent-Brown et al., 2000), and China (Yu et al., 2007). The percentage of animals positive for *N. caninum* is location dependent presumably due to lifecycle factors such as the presence of intermediate hosts, type of production system (i.e., dairy farms vs beef farms), and management practices. Study design and diagnostic test differences also account for variations in seroprevalence estimates across studies. Table 1.1 presents a summary of seroprevalence studies by geographical location. Thurmund et al. (1997) reported a prevalence of up to 70% in dairy cattle which is significantly higher than other seroprevalence studies in the
USA. Hoar et al. (2007) found a seroprevalence of 16.7% in 900 tested beef cattle, using a kinetic ELISA test, while Sanderson et al. (2000) found a seroprevalence of 23.0% from 2585 beef cattle on 55 different farms, also using an ELISA test.

Several Canadian studies examined the status of *N. caninum* in cattle. Hobson et al. (2002) found a seroprevalence of 22.1% (264/1196 cows) in their study of 28 Ontario dairy farms. Bulk milk samples were tested for the presence of *N. caninum* antibodies via ELISA to determine herd-level prevalence in cows from Prince Edward Island (PEI) from 2004 and 2005. The positivity of samples ranged from 6.4 – 10.2% depending on year, month, and herd (Wapenaar et al., 2007). Each herd in this study was known to have a herd seroprevalence of ≥ 15.0%. The proportion of bulk milk positive farms in PEI from 2004 to 2005 increased (p = 0.11). Paré et al. (1998) investigated the seroprevalence in case-positive herds in Quebec, Canada. The mean seropositivity in herds that experienced abortion due to *N. caninum* was higher (22.5%) than herds not experiencing abortion (7.5%). Additional details regarding specific titre concentration and statistical analyses were not presented in their manuscript.

Additional investigations of *N. caninum* in Canadian cattle are needed to elucidate the true prevalence. Knowledge of the seroprevalence of *N. caninum* provides insight on the impact of infections on the cattle industry and the success of intervention measures. However, little is known about the prevalence of *N. caninum* in other livestock species including horses.

Table 1.1: The seroprevalence of Neospora caninum in cattle. Adapted from Dubey and Schares 2007.

<table>
<thead>
<tr>
<th>Location</th>
<th>Test Type</th>
<th>Sample Size</th>
<th>Seroprevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algeria</td>
<td>ELISA</td>
<td>102</td>
<td>3.9%</td>
<td>Ghamli et al., 2009</td>
</tr>
<tr>
<td>Argentina</td>
<td>IFAT</td>
<td>4190</td>
<td>14.2%</td>
<td>Moore et al., 2009</td>
</tr>
<tr>
<td>Country</td>
<td>Test</td>
<td>Total</td>
<td>Percentage</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>------</td>
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<td>------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>Argentina</td>
<td>IFAT</td>
<td>1042</td>
<td>25.7%</td>
<td>Moore et al., 2008</td>
</tr>
<tr>
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<td>173</td>
<td>80.9%</td>
<td>More et al., 2009</td>
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<tr>
<td>Argentina</td>
<td>IFAT</td>
<td>90</td>
<td>73.0%</td>
<td>More et al., 2008</td>
</tr>
<tr>
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<td>93</td>
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<tr>
<td>Brazil</td>
<td>IFAT</td>
<td>2452</td>
<td>25.4%</td>
<td>Schmit et al., 2018</td>
</tr>
<tr>
<td>Brazil</td>
<td>ELISA</td>
<td>159</td>
<td>15.1%</td>
<td>Marques et al., 2011</td>
</tr>
<tr>
<td>Brazil</td>
<td>IFAT</td>
<td>1098</td>
<td>62.5%</td>
<td>Anreotti et al., 2010</td>
</tr>
<tr>
<td>Canada</td>
<td>ELISA*</td>
<td>659</td>
<td>15.0%</td>
<td>Wapenaar et al., 2007</td>
</tr>
<tr>
<td>Canada</td>
<td>ELISA</td>
<td>5080</td>
<td>12.0%</td>
<td>Peregrine et al., 2006</td>
</tr>
<tr>
<td>Canada</td>
<td>ELISA</td>
<td>900</td>
<td>22.1%</td>
<td>Keefe et al., 2000;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VanLeeuwen et al., 2002</td>
</tr>
<tr>
<td>New Brunswick</td>
<td>ELISA</td>
<td>439</td>
<td>25.5%</td>
<td></td>
</tr>
<tr>
<td>Nova Scotia</td>
<td>ELISA</td>
<td>3531</td>
<td>21.3%</td>
<td></td>
</tr>
<tr>
<td>P.E.I</td>
<td>ELISA</td>
<td>439</td>
<td>10.4%</td>
<td></td>
</tr>
<tr>
<td>Ontario</td>
<td>ELISA</td>
<td>1204</td>
<td>8.2%</td>
<td></td>
</tr>
<tr>
<td>Manitoba</td>
<td>ELISA</td>
<td>1530</td>
<td>7.0%</td>
<td></td>
</tr>
<tr>
<td>Saskatchewan</td>
<td>ELISA</td>
<td>238</td>
<td>5.6%</td>
<td></td>
</tr>
<tr>
<td>Colombia</td>
<td>ELISA</td>
<td>238</td>
<td>76.9%</td>
<td>Ceneno and Benavides et al., 2013</td>
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<td>Colombia</td>
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<td>357</td>
<td>54.1%</td>
<td>Zambrano et al., 2001</td>
</tr>
<tr>
<td>Germany</td>
<td>ELISA</td>
<td>1950</td>
<td>1.0%</td>
<td>Schares et al., 2009</td>
</tr>
<tr>
<td>Greece</td>
<td>ELISA</td>
<td>1573</td>
<td>15.2%</td>
<td>Sotiraki et al., 2008</td>
</tr>
<tr>
<td>Iran</td>
<td>ELISA</td>
<td>237</td>
<td>32.0%</td>
<td>Youseffi et al., 2009</td>
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<tr>
<td>Mexico</td>
<td>ELISA</td>
<td>543</td>
<td>44.8%</td>
<td>Salguero-Romero et al., 2021</td>
</tr>
<tr>
<td>Mexico</td>
<td>ELISA</td>
<td>813</td>
<td>11.6%</td>
<td>Segura-Correa et al., 2010</td>
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<tr>
<td>Mexico</td>
<td>ELISA</td>
<td>863</td>
<td>26.0%</td>
<td>Romero-Salas et al., 2010</td>
</tr>
<tr>
<td>Mexico</td>
<td>ELISA</td>
<td>596</td>
<td>11.6%</td>
<td>Garcia-Vazquez et al., 2009</td>
</tr>
</tbody>
</table>
Effects on Reproductive Systems

Studies on seroprevalence in livestock add to our understanding of how widespread *N. caninum* infections are on farms and their implications for animal health. Various effects of exposure to *N. caninum* on reproductive performance and milk production were studied. Waldner et al. (1998) reported that 30% of beef cows (n = 419 cows) were seropositive during their 4-year study in Alberta, Canada and found that seropositive cows had higher odds of abortion (OR = 5.7) and stillbirths (OR = 2.8) than their seronegative counterparts and were at significantly greater odds of being culled than seronegative cows (OR = 1.9; no confidence limits or p values reported). The majority of economic losses due to *N. caninum* can be attributed to abortion, however *N. caninum* was also associated with early embryonic death (Waldner et al., 1998; Waldner et al., 2001; Waldner, 2005). Waldner et al. (2001) studied a 350 cow-calf beef herd before and after a *N. caninum* abortion epidemic. The first breeding season after the outbreak, 13.5% of heifers and 22.2% of the cows were not pregnant. The seropositive animals in the spring were more likely to be found open in the fall (OR = 2.0; 95% CI = 1.1 – 3.7; p < 0.05). Early embryonic death was proposed to occur when pro-inflammatory cytokines produced by T helper 1 cells are present at the maternal interface and damage the placental connection.

<table>
<thead>
<tr>
<th>Country</th>
<th>Test</th>
<th>Number</th>
<th>Prevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pakistan</td>
<td>ELISA</td>
<td>237</td>
<td>32.0%</td>
<td>Shabbir et al., 2011</td>
</tr>
<tr>
<td>Spain</td>
<td>IFAT</td>
<td>5196</td>
<td>15.7%</td>
<td>Gonzalez-Warleta et al., 2008</td>
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<tr>
<td>Spain</td>
<td>ELISA</td>
<td>37,090</td>
<td>22.5%</td>
<td>Eiras et al., 2011</td>
</tr>
<tr>
<td>USA</td>
<td>ELISA</td>
<td>900</td>
<td>16.7%</td>
<td>Hoar et al., 2007</td>
</tr>
<tr>
<td>USA</td>
<td>ELISA</td>
<td>2,585</td>
<td>23.0%</td>
<td>Sanderson et al., 2000</td>
</tr>
</tbody>
</table>

*milk samples*
(Haddad et al., 2005; Innes et al., 2002). The damage is exacerbated by the maternal immune system developing and responding with a strong cell proliferation response and the production of IFN-gamma in response to parasite antigens (Haddad et al., 2005; Innes et al., 2002). A large study of 140 dairy herds (n = 6,864 cows) investigated the relationship between seropositivity, abortion status and milk production. Abortion status and seropositivity did not affect milk production in the observational study (p = 0.1; Hobson et al., 2002). While N. caninum positive and negative cows produced the same amount of milk overall, milk production for seropositive cows as a group was significantly less (p = 0.04) than seronegative cows at a projected 305 days of production (Hobson et al., 2002). However, in the same study, a case-control study was also conducted and found that seropositive cows had significantly (p=0.04) less 305-day milk production than their seronegative counterparts.

The reported incidence of abortions due to N. caninum varies depending on geographical location. Twelve percent of abortions in English and Welsh dairy cattle were attributed to N. caninum infection (Davison et al., 1999), compared to 38.7% of abortions in dairy cattle in North-Western Spain (Mainar-Jaime et al., 1999). Abortion due to N. caninum was investigated in many provinces across Canada, including Ontario (Hobson et al., 2005; Keefe et al., 2000; Wilson et al., 2016). In Ontario, Hobson et al. (2005) noted that 21.7% (362/1669) of aborting dairy cows were seropositive for N. caninum. In a British Columbia retrospective study of 236 aborted fetus submissions from 2007 to 2014, 18.2% were diagnosed with N. caninum infection (Wilson et al., 2016). This same study also reported significantly more abortions were diagnosed with active surveillance that recruited dairy farmers would submit all of their aborted fetuses, placenta, milk and serum from the dam (41% of abortions) than passive surveillance (13.3% of
abortions; p < 0.001). Abortion due to *Neospora caninum* may begin as an epidemic in previously unexposed herds but becomes endemic over time due to vertical transmission of *N. caninum*. Abortions are deemed to be epidemic if more than 10% or 12.5% of cows at risk abort within 6 - 8 weeks (Schares *et al.*, 2002; Wouda *et al.*, 1999a). In some *N. caninum* herd outbreaks, as many as 33% of dairy cows abort over a few months (McAllister *et al.*, 1996; Schares *et al.*, 2002; Thilsted and Dubey, 1989). Cows in herds endemically affected by *N. caninum* have 3 times the risk of abortion in comparison to cows in seronegative herds (Davison *et al.*, 1999; Paré *et al.*, 1997; Wouda *et al.*, 1998).

Anderson *et al.* (1995) recruited 19,798 cows from 26 California dairy herds to study abortion. Fourteen of the herds had a history of abortion due to neosporosis. Over a one-year period, all available aborted fetuses (n = 266) were submitted to a diagnostic laboratory to determine the cause of abortion. The diagnostic procedures conducted on the fetuses included post-mortem, aerobic bacterial culturing of abomasal fluid, and histologic evaluation of the brain, lung, heart, spleen, liver, kidney, thymus, small intestine, abomasum, lymph node, skeletal muscle, and placenta if available. If gross lesions were identified, further analysis was done for fungi and bacteria. If protozoal infection was suspected, IHC with anti-*Neospora* rabbit antisera was used to identify tachyzoites or tissue cysts depending on the tissue. Abortions were attributed to *Neospora* when typical histological lesions were present, and protozoa were positively identified with the antisera. They found that *N. caninum* infection was the most commonly identified cause of abortion (113/266; 42.5%) in that study, and 87.2% of abortions (232/266) were from herds with a previous history of neosporosis. Neosporosis was also determined to be a cause of abortion in 6 of the 12 control herds with no previous history of the
disease. These findings suggest that abortion due to *N. caninum* is present within dairy herds that are known to experience neosporosis as well as herds that have a history of sporadic abortion only. In a British Columbia study, 18.2% of the 236 aborted bovine fetuses examined were diagnosed with *N. caninum* as the causative agent using histopathology, PCR and IHC testing (Wilson *et al*., 2016). The prevalence of abortions commonly occurred at 3-6 months of gestation (26.9%) The majority of *N. caninum* positive aborted fetuses had myocardial lesions, epicarditis, and lesions in the brain and liver (Wilson *et al*., 2016). Dubey and Schares (2011) had previously concluded that *N. caninum* lesions were most commonly found in the brain and pulmonary tissue of affected bovine fetuses.

Venereal transmission of *N. caninum* may also occur. One group investigated the presence of *N. caninum* in fresh and frozen bull semen (Ortega-Mora *et al*., 2003). Five of the 8 bulls examined were seropositive for *N. caninum*, with IFAT titers greater than or equal to 1:250. PCR testing found *N. caninum* DNA in fresh non-extended semen samples (4/5 seropositive bulls) and in extended frozen semen samples (3/5 seropositive bulls). However, the study also noted that although *N. caninum* DNA was found in the semen, the study methodology did not attempt to determine whether infectious stages of the parasite were present. The possibility of venereal transmission of the parasite through semen remains unclear, but worth further investigation.

Risk Factors for Exposure to *Neospora caninum* in Cattle

An understanding of risk factors for exposure to *N. caninum* infection and *N. caninum* abortion is necessary to minimize losses. Seroprevalence studies have demonstrated large
differences in prevalence based on country, region, and type of cattle such as dairy or beef (Bartels et al., 2006; Carvalho-Patrício et al., 2013; Koiwai et al., 2005; Moore et al., 2002; Nazir et al., 2013; Quintanilla-Gozalo et al., 1999; Zárate-Martínez et al., 2020). Differences in diagnostic method, study design, and sample size complicate comparisons across studies, making it difficult to determine which factors are of the greatest concern for exposure and infection.

Dubey et al. (2007) summarized a variety of risk factors in cows for seropositivity to *N. caninum*. Age, presence of the definitive host (canids), or other intermediate hosts, food and water sources, administration of colostrum/milk to neonates, calving management, cattle stocking density and acreage, herd size, replacement heifers, climate, vegetation index, human population density, other infectious agents, and breed were all found to be important risk factors.

Table 1.2 summaries of several different risk factors for exposure to *N. caninum* that have been investigated.

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Cattle</th>
<th>Horses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Bartels et al., 2006; Davison et al., 1999; Dyer et al., 2000; Sanderson et al., 2000; Salguero-Romero et al., 2021</td>
<td>Not consistently identified as a risk factor or not investigated Yeargan et al., 2013; Kliger et al., 2007; Ciaramella et al., 2004</td>
</tr>
<tr>
<td>Definitive hosts</td>
<td>Pare et al., 1998; von Blumröder et al., 2006; Mainar-Jaime et al., 1999; Corbellini et al., 2006</td>
<td>Abreu et al., 2014</td>
</tr>
</tbody>
</table>
### Intermediate hosts


### Grazing and feed

- Sanderson *et al.*, 2000; Otranto *et al.*, 2003; Barling *et al.*, 2001

### Water source

- Ould-Amrouche *et al.*, 1999

### Herd Characteristics


### Breed


### Farm location

- Hassig and Gottstein, 2002

---

**Age:** Several studies have investigated the effect of age (Bartels *et al.*, 2006; Davison *et al.*, 1999; Dryer *et al.*, 2000; Waldner *et al.*, 1998; Wilson *et al.*, 2016). It is unclear if increasing age is a risk factor itself or whether age is a biased risk factor related to the culling practices of the cattle industry. The effect of age as a risk factor for *N. caninum* has varied with study location. For example, a study investigating seroprevalence in Germany, the Netherlands, Spain and Sweden determined that older cattle in Spain had a higher probability of being seropositive, but in Sweden the probability of testing positive decreased with increasing age (Bartels *et al.*, 2006). The researchers hypothesized that the higher probability in Spain with age was due to horizontal transmission since their cattle were on average 1.7-2.4 years older than the other countries,
meaning Spanish cattle had a longer period of time for exposure to the parasite (Bartels et al., 2006). Wilson et al. (2016) found that the breed of the dam was significantly associated with *N. caninum* infection, and also found a higher prevalence in dairy versus beef breeds. However, other factors associated with breed such as management style, feed, calving seasons, and herd sizes may act as a proxy for breed and thereby affect the apparent prevalence of disease.

**Presence of definitive hosts:** The definitive hosts of *N. caninum* are canine species such as dogs and coyotes (Dubey and Schar, 2011). It is common for farmers to have farm dogs, so the presence of farm dogs was investigated as a risk factor. In most risk factor studies, the presence of farm dogs (currently or in the past 10 years) was significant (Paré et al., 1998; von Blumröder et al., 2006). The risk for seropositivity appears to increase with the presence of dogs on the farm (Corbellini et al., 2006; Mainar-Jaime et al., 1999; Paré et al., 1998; Schar et al., 2004; Wouda et al. 1999). Cedeño and Benavides (2013) found that farms that fed dogs leftovers were 15 times more likely to experience seropositivity than farms that did not (OR = 15.44; 95% CI = 1.94-123.22; p = 0.006). However, the authors did not define the meaning of leftovers, how the practice was measured, or a hypothesis as to why it may be contributing to increased seropositivity (Cedeño and Benavides, 2013). Dijkstra et al. (2002) described the transmission route of *N. caninum* from farm dog to cattle. It is believed that the dogs defecate in the feeding alleys, stored grass, or corn silage, thus contaminating these food sources. Dogs defecating in feed sources was reported more often by farmers with herds with evidence of infection (Dijkstra et al., 2002).
**Breed:** Several studies investigated if breed was a risk factor for seropositivity (Akca et al., 2005; Armengol et al., 2007; Bartels et al., 2006; Munhoz et al., 2009; Nazir et al., 2013). Bartels et al. (2006) reported that breed was a significant risk factor \( (p < 0.001) \). For example, in Sweden, Swedish Red and White breed were 2.5 \( (95\% \text{ CI} = 1.3 – 4.8; \ p = 0.001) \) times more likely to be seropositive for *N. caninum* than other breeds such as the Swedish Fresian. Breed comparisons should be interpreted with caution since there are many differences in production systems and uses for the various breeds. Bartels et al. (2006) explained that the differences between native Spanish breeds and Holstein Friesian, Rubia Gallega, or mixed breeds was due to management intensity.

**Reproduction:** Cedeño and Benavides (2013) found that farms that have tissue residues from abortions (e.g., placentas) that were left outside and not buried were 3.8 times more likely to be seropositive than farms that remove these tissues \( (\text{OR} = 3.8; \ 95\% \text{ CI} = 1.5-9.6; \ p = 0.003) \) from their study that included 10 different dairy farms. They also found that farms using natural breeding with bulls were 19.7 times more likely to have seropositive bulls than the bulls on farms using artificial insemination \( (\text{OR} = 19.68; \ 95\% \text{ CI} = 2.34-165.52; \ p = 0.012) \), however the sample size was small \( (3 \text{ bulls}) \). It is possible that there was another unidentified management factor in the study acting as a proxy for breeding management method, affecting the results.

**Herd size:** Schares et al. (2004) reported that the risk of bulk milk testing positive for *N. caninum* antibodies using ELISA, increased with increasing herd size. A herd size effect might be due to a higher demand for replacement heifers (Dubey and Schares, 2007), stocking density, or farm hygiene practices (Schares et al., 2004).
Stocking density: High stocking density may result in a greater proportion of cattle being exposed to an infected *N. caninum* source. Two different Texas based studies reported that a high stocking density was a potential risk factor for seropositivity (Barling *et al*., 2000; Barling *et al*., 2001). These studies also suggested that higher stocking were associated with a greater use of supplemental feeding practices. Supplemental feed requires properly maintained and cleaned storage facilities to avoid attracting rodents that in turn attract the definitive hosts of *N. caninum*.

Intermediate hosts: The presence of other potential intermediate hosts, such as horses, rabbits, ducks and other poultry could increase the probability of exposure and infection. In one study, the presence of horses on the farm increased the odds of cattle abortion due to *N. caninum* on the farm (Hobson *et al*., 2005). Bartels *et al*. (1999) reported that the presence of poultry on the farm was associated with *N. caninum* abortions and proposed that poultry may be a vector for *N. caninum* oocysts. These results warrant further investigation.

Food and water sources: The life cycle of *N. caninum* requires unsporulated oocysts to enter the environment and sporulate; they contaminate food and water that the cattle consume (Dubey and Schares, 2007). Investigating food and water sources, along with management practices (e.g. how often are they cleaned), provides insights into exposure. A study conducted in France reported that the use of ponds instead of a well or public water supply was a risk factor for exposure in dairy cattle (Ould-Amrouche *et al*., 1999). A Texas based study in beef cattle, investigated how use of different feeder types might be associated with exposure. Use of a hay ring for feeding round bales was associated with an increased risk of seropositivity (OR = 3.04;
p<0.05; 95% CI = 1.19 – 8.27), whereas a self-contained feeder had a protective effect (OR = 0.43; p<0.01; 95% CI = 0.22 – 0.81); self-contained feeders prevented exposure of hay to the weather and limited access of other animals that could contaminate the feed. Cows often calve, abort, or expel placentas near hay feeders and because these feeders are rarely moved, feces of definitive hosts consuming the expelled placentas will be highly concentrated in this area (Barling et al., 2001). Alternatively, if placentas infected with *N. caninum* tachyzoites are expelled in the feeding area, there would be a higher chance of the grazing animals consuming infected feed. Two separate studies conducted in the northwestern United States and in Italy, demonstrated that cattle grazing on the range during the summer was associated with reduced risk of seropositivity (Otranto et al., 2003; Sanderson et al., 2000). The results of these studies suggest livestock feeding management practices play a significant role in risk of exposure to *N. caninum*.

Understanding risk factors for exposure to *N. caninum* in cattle provides valuable information about the life cycle and where the greatest risk for infection lies on farms. As cattle are intermediate hosts, information may be extrapolated to other intermediate hosts, such as horses, for further investigation. Identifying risk factors for exposure provides insight as to where contamination of feed and water sources by oocysts may happen and therefore provides information to help producers prevent infection in their animals.

*Neospora caninum* in Equids

Neosporosis is an important disease of cattle worldwide, however little is known about its pathogenicity in horses (Dubey et al., 1999; Lindsay and Dubey, 2020; Llano et al., 2021;
Moreira *et al.*, 2019; Nazir *et al.*, 2018; Pitel *et al.*, 2003). Two species of this coccidian parasite, *N. caninum* and *Neospora hughesi*, are known to infect horses. *Neospora hughesi* causes neurological disease, while *N. caninum* causes fetal loss (Dubey and Porterfield, 1990; Lindsay and Dubey, 2020; Pitel *et al.*, 2003; Villalobos *et al.*, 2006). In 2015, the total incidence of all abortions per known pregnancy was 8.5% for broodmare farms in Ontario, Canada (Cooper *et al.*, 2021). Recent studies linking reproductive failure and abortions to *N. caninum* in horses suggest that neosporosis may be an unrecognized cause of equine abortion (Abreu *et al.*, 2014; Kliger *et al.*, 2007; Mazuz *et al.*, 2020). *Neospora caninum* was found to cause equine protozoal myeloencephalitis (EPM) in a 6-year-old captive zebra (*Equus zebra*) based on IHC and DNA-analysis (Ruppert *et al.*, 2021). Little is known about the epidemiology and risk factors of *N. caninum* or *N. hughesi* in horses, and more research is needed to understand the pathogenic role of *Neospora spp.* infection in horses. Studies identifying the epidemiology and risk factors could help horse breeders prevent disease and potential economic losses from pregnancy failure.

**Seroprevalence of Neospora caninum in Horses**

Several studies report the seroprevalence of *N. caninum* in horses in various countries around the world (Table 1.3); the seroprevalence in Canada is unknown. Most studies suffer from low sample numbers, and as seen with cattle, little consistency exists across studies in the tests and cut-off titers used. A slaughterhouse study conducted in Nebraska and Texas, USA found that 69 of 296 horses (23.3%) were seropositive for *N. caninum* with the *Neospora* agglutination test (NAT) (Dubey *et al.*, 1999). A subsequent study using the same NAT test found 86 of 276 (31.1%) wild horses in Wyoming, USA had antibodies to *N. caninum* (Dubey *et al.*, 2003). The prevalence estimates of *N. caninum* in males and females in that study were
similar, although they did not specifically report the seroprevalence estimates or if the difference was tested statistically. The same study found high antibody titres (≥1:400) in 18.6% of the 86 positive horses, with the highest titre reported as 1:12,800 (Dubey et al., 2003), suggesting active infection.

Seroprevalence estimates in other countries range from 11.9 – 70.9% in Israel (Kliger et al., 2007; Mazuz et al., 2020), 20.6% in Texas (Dubey et al., 1999), 23% in France (Pitel et al., 2001), 28% in Italy (Ciaramella et al., 2004), and 58% in Brazil (Oliveira Kock et al., 2019). Other equids can also be infected with Neospora species. A Brazilian study of 500 serum samples from 500 Brazilian donkeys (Equus asinus) in 30 different municipalities across Brazil were tested using IFAT and immunoblot. Two of 500 (0.4%) were positive for N. caninum with a titre of 1:100 and had a 37kDa antigen specific to Neospora identified using an immunoblot. Approximately 22% of the 500 donkey samples showed a strong optical reaction and/or incomplete fluorescence, which may interfere with the IFAT interpretation (Galvão et al., 2015). Seropositivity rates similar to cattle and reports of reproductive and neurological disease in infected horses, have resulted in further investigation into neosporosis in horses.

Table 1.3: The seroprevalence of Neospora caninum in horses. Adapted from Dubey and Schares 2007.

<table>
<thead>
<tr>
<th>Location, USA</th>
<th>Test Type</th>
<th>Cut-off / Inhibition</th>
<th>Sample Size</th>
<th>Seroprevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Texas, USA</td>
<td>NAT</td>
<td>¥</td>
<td>296</td>
<td>23.0%</td>
<td>Dubey et al., 1999</td>
</tr>
<tr>
<td>Wyoming, USA</td>
<td>NAT</td>
<td>¥</td>
<td>276</td>
<td>31.1%</td>
<td>Dubey et al., 2003</td>
</tr>
<tr>
<td>Country</td>
<td>Test Type</td>
<td>Dilution</td>
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<td>Positive Rate</td>
<td>Reference</td>
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<td>------------------------------------------------</td>
</tr>
<tr>
<td>Italy</td>
<td>IFAT</td>
<td>1:50</td>
<td>150</td>
<td>28%</td>
<td>Ciaramella et al., 2004</td>
</tr>
<tr>
<td>Panama, Brazil</td>
<td>IFAT</td>
<td>1:50</td>
<td>100</td>
<td>58%</td>
<td>Oliveira Koch et al., 2019</td>
</tr>
<tr>
<td>Nigeria, West Africa</td>
<td>IFAT</td>
<td>1:50</td>
<td>144</td>
<td>8%</td>
<td>Bartova et al., 2017</td>
</tr>
<tr>
<td>Curitiba, Brazil</td>
<td>IFAT</td>
<td>1:50</td>
<td>97</td>
<td>14.4%</td>
<td>Villalobos et al., 2011</td>
</tr>
<tr>
<td>Iran</td>
<td>N-MAT</td>
<td>1:40</td>
<td>235</td>
<td>20%</td>
<td>Tavalla et al., 2015</td>
</tr>
<tr>
<td>Italy</td>
<td>IFAT</td>
<td>1:50</td>
<td>643</td>
<td>2.3%</td>
<td>Bartova et al., 2015</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>cELISA</td>
<td>&gt;50%</td>
<td>552</td>
<td>24%</td>
<td>Bartova et al., 2010</td>
</tr>
<tr>
<td>Jordan</td>
<td>ELISA</td>
<td>¥</td>
<td>227</td>
<td>3.0%</td>
<td>Talafha et al., 2015</td>
</tr>
<tr>
<td>France</td>
<td>DAT</td>
<td>1:40, 1:80, 1:100</td>
<td>434</td>
<td></td>
<td>Pitel et al., 2001</td>
</tr>
<tr>
<td>Israel</td>
<td>IFAT</td>
<td>1:50</td>
<td>800</td>
<td>11.9%</td>
<td>Kliger et al., 2007</td>
</tr>
<tr>
<td>Israel</td>
<td>IFAT</td>
<td>1:50</td>
<td>31</td>
<td>70.9%</td>
<td>Mazuz et al., 2020</td>
</tr>
<tr>
<td>Portugal</td>
<td>IFAT</td>
<td>1:50</td>
<td>385</td>
<td>9.1%</td>
<td>Waap et al., 2020</td>
</tr>
</tbody>
</table>

¥ value not available.
Effects on Reproductive Systems

The economic implications of *N. caninum* infection could be significant due to reproductive failure and abortion. Dubey and Porterfield (1990) were the first to report *N. caninum* in a fetus aborted at 273 days of gestation from an 8-year-old cross-bred mare. *Toxoplasma gondii* was originally suspected as the cause due to the presence of a *Toxoplasma gondii*–like organism in the lung of the fetus. However, the mare was seronegative for *Toxoplasma gondii* antibodies. Immunohistochemistry demonstrated numerous *N. caninum* tachyzoites in alveolar parenchyma with groups up to 50μm in diameter, and individual organisms ranging from 3 x 3 to 5 x 2 μm in size. No tissue cysts were identified in this fetus. In 2018, *N. caninum* was identified as the cause of an equine abortion in Montana USA. Anderson *et al.* (2019) were the first to histologically, ultra-structurally, immunohistochemically, and molecularly confirm that equine abortion was caused by *N. caninum*. Samples of the heart, lung, liver, skeletal muscle, brain, tongue, and placenta were taken from the 280 gestational-day fetus. Pneumonia and myocarditis were found to be the predominant histopathological lesions in the affected fetus. This contrasts with the predominant lesions in cattle, which are typically found in the brain (Dubey *et al.*, 2017). An Israeli study suggests that *N. caninum* may be a common cause of abortion in that country (Mazuz *et al.*, 2020). A total of 31 paired samples collected from aborting mares and fetuses were tested with IFAT and PCR analysis. The researchers collected brain, liver, kidney, lung, and heart tissues, as well as thoracic fluid, from the aborted fetuses. The PCR analysis on the fetal samples focused on the identification of *N. caninum*-specific target loci ITS1 and Nc5. The abortions occurred primarily in the second and third trimesters (30/31). The seroprevalence of the aborting mares was 70.9% (22/31) and the
prevalence of *N. caninum* DNA in the aborted fetuses was 41.9% (13/31). The anti-*Neospora* antibody titres from seropositive mares ranged from 1:50 to 1:6,400. Transplacental transmission of *N. caninum* from positive mares to their aborted fetuses occurred in 45.4% (10/22) of seropositive mares (Mazuz *et al.*, 2020). These findings suggest that *Neospora caninum* may be an unrecognized cause of abortion in horses.

In addition to abortion, several studies report an association between seropositivity to *N. caninum* and other reproductive failure outcomes in broodmares. (Abreu *et al.*, 2014; Kliger *et al.*, 2007; McDole and Gay, 2002; Pitel *et al.*, 2003; Villalobos *et al.*, 2006). A Brazilian study of 112 mares on 5 farms used IFAT with a cut-off titre of 1:50 to investigate the relationship between seropositivity and reproductive issues (Abreu *et al.*, 2014). The seroprevalence of *N. caninum* in mares with a known history of reproductive issues (e.g., late term abortion, embryonic mortality, and stillbirths) was significantly higher (25.71%) than mares without reproductive issues (6.49%; p=0.01). They also reported a positive association between seropositivity and reproductive issues using the Fisher’s exact test (p = 0.010). They also found a significantly higher seroprevalence for *N. caninum* in mares located on farms that had seropositive dogs on premises (p = 0.018). Kliger *et al.* (2007) used IFAT testing with a cut-off of 1:50 and reported a significantly higher (p < 0.001) seroprevalence of *N. caninum* in aborted mares (52/140; 37.5%) compared to the other clinically normal horses (95/800; 11.9%). Another study examined the association between seropositivity and fetal loss in horses from Brazil (Villalobos *et al.*, 2006). Using IFAT with a cut-off titre of 1:50, they reported that mares considered reproductively diseased with either abortion or neonatal mortality (n=483) had higher seroprevalence to *N. caninum* (15.1%) than the reproductively healthy (n=325) group (5.8%).
Further analysis showed a significant association (p < 0.001) with clinical signs of reproductive failure and *Neospora* spp. infection. The findings from Villalobos *et al.* (2006) contradict the findings of McDole and Gay (2002) and Pitel *et al.* (2003), who also investigated the association between seropositivity and fetal loss and found no statistically significant associations.

Risk Factors for Exposure to *Neospora caninum* in Horses & Other Equids

Although *N. caninum* and *T. gondii* are similar in structure and were once thought to be the same parasite, the risk factors for exposure are different. The risk factors for exposure to *N. caninum* are thought to be similar between horses and cattle, although few studies have examined risk factors specifically in horses. One study of donkeys showed a significant association between age (p=0.01) and breed (p = 0.01) with seropositivity (Machacova *et al.*, 2013). The same study also reported no association between the risk of infection and sex, as well as the use of the donkeys (i.e., milk, pet, breeding, and protection; p > 0.05). As noted previously, studies on cattle incidentally found a relationship between the seropositivity and the presence of horses on farm (Coelho *et al.*, 2011; Hobson *et al.*, 2005; Reichel *et al.*, 2020), including an Ontario study (Hobson *et al.*, 2005). Talafha *et al.* (2015) investigated the risk factors for *Neospora* spp. in horses in Jordan. The risk factors traditionally associated with seropositivity in cattle such as age, sex, breed, usage, body condition score, grazing type, mixing with horses on the same property, and a history of previous diseases, were not significantly associated with the seroprevalence to *Neospora* spp. in these horses. Seropositivity was low, with only 7 (3%) of 227 sera demonstrating antibodies for *Neospora* spp. on ELISA. Interestingly, there was a significant regional difference (p =0.018) between the 5 climatic regions in Jordan. Seropositive horses were located in Amman and Irbid, while the other regions (Zarqa, Jordan
Valley, and Wadi Mousa) had zero prevalence (Talafha et al., 2015). This study did however only use chi square test of association and did not report a statistical inclusion of a random intercept for location or any type of regression model, likely due to the small sample size. In a study from Israel (Mazuz et al., 2020), geographic location of the farm was the only significant factor associated with positive serology (p < 0.05), similar to Talafha et al. (2015) in Amman. However, an earlier Israeli study found geographic location (Coastal Plain, Galilee and the Golan Heights, Jerusalem and Mount Judea, Negev and Arava) was not a significant factor for seropositivity (p = 0.157; Kliger et al., 2007). Mazuz et al. (2020) divided geographic location into north, centre, and south Israel, which likely has overlap in study location with the regions in studies from Talafha et al. (2015) and Kliger et al. (2007). It is apparent that more research is needed to identify risk factors for exposure to *N. caninum* in horses and to educate owners and breeders about ways to reduce exposure.

**Neospora caninum** and Non-Equid Species

Investigations into the presence and effect of *N. caninum* are reported for common domesticated farm animals such as cattle and horses. Several studies investigated other intermediate hosts and potential definitive hosts.

**Intermediate hosts:**

Several studies examined different intermediate host species including water buffalo (Neveraukas et al., 2015; Salguero-Romano et al., 2021), opossums (Gondim et al., 2017), yaks (Meng et al., 2017), sparrows (Gondim et al., 2010), and raccoons (Kornacka et al., 2018). A seroprevalence study in water buffalo (*Bubalus bubalis*) based in Central and Southern Mexico found that 243/543 (44.8%) animals were seropositive based on a commercial ELISA test. The
same study reported that buffalos that were in close proximity to cattle were significantly more likely to be seropositive compared to buffalos not exposed to cattle (p<0.01; Salguero-Romano et al., 2021). Meng et al., (2017) found a 10.4% seroprevalence for the semi-wild yak (Bos grunniens) in China using a competitive-inhibition ELISA (Meng et al., 2017).

Tissue samples of heart, lung and brain from 7/44 (15.9%) wild racoons (Procyon lotor) in the Czech Republic, Germany, and Poland were positive on ELISA for *N. caninum* (Kornacka et al., 2018). Unfortunately, no histopathology was conducted and therefore, the presence of tissue cysts could not be determined. Sparrows (Passer domesticus) are known to be intermediate hosts for many parasites and can spread oocysts across vast areas very efficiently. A Brazilian study found 3 of 40 (7.5%) sparrows tested positive for *N. caninum* DNA using PCR (Gondim et al., 2010). A study in Bahia, Brazil found 50 of 400 chickens (Gallus domesticus) to be seropositive by PCR (Costa et al., 2008). In addition, chickens kept in outdoor housing had a significantly higher (p < 0.001) seropositivity (47/200; 23.5%) compared to indoor-housed chickens (3/200; 1.5%).

Investigation into potential other *N. caninum* definitive hosts

Few studies have examined the possibility of other definitive hosts aside from canids. The role of opossums as a definitive host has been investigated (Gondim et al., 2017). The opossum (Didelphis albiventris and Didelphis virginiana) is of particular interest because they are known definitive hosts for *Sarcocystis neurona*. Intestinal scrapings were positive for *N. caninum* sporocysts from 3 of 39 (7.7%) opossum carcasses from Bahia state, Brazil (Gondim et al., 2017). Yai et al. (2003) also reported finding seropositive opossums (Didelphis marsupialis) in Sao Paulo, Brazil. They reported that 84 of 396 opossums (21.2%) were seropositive by IFAT
using a cut-off titre of 1:25. In contrast, a recent study (Zitelli et al., 2021) also in Brazil, found none of the 76 opossums (Didelphis albiventris and Philander frenatus) sampled were seropositive for *N. caninum* based on a cELISA test. Houk et al. (2010) reported a 0% seroprevalence among North American opossums (Didelphis virginianna) in Southern Louisiana using an IFAT with a cut-off titre of 1:100. These findings suggest that although the results are mixed, opossums may potentially be a definitive host for *N. caninum* and a potential source of infection to other animals; additional studies are needed.

*Neospora hughesi*

*Neospora hughesi* is closely related to *N. caninum* as they are both Apicomplexan protozoa in the same genus. *Neospora hughesi* was originally isolated from a horse with myeloencephalitis (Marsh et al., 1996). It was confirmed to be a previously unrecognized species based on molecular, antigenic, and structural differences to *N. caninum* (Marsh et al., 1998). The life cycle of *N. hughesi* is currently unknown. It has only been documented to naturally infect horses (Gondim et al., 2009). The dominant tachyzoite antigens of *N. caninum* and *N. hughesi* (SAG1 and SRS2) are different and these polymorphisms allow for the two species to be distinguished (Marsh et al., 1999).

Diagnosis of *Neospora hughesi*

Equine protozoal myeloencephalitis (EPM) is commonly caused by *Sarcocystis neurona*. An ante-mortem diagnosis of EPM is made in horses and ponies through a thorough neurologic examination and the collection of serum and cerebrospinal fluid (CSF) to identify antibodies to *S. neurona* (Finno et al., 2007; Fur et al., 2002). The University of California Davis tests for
EPM caused by *N. hughesi* after a negative serology for *S. neurona* in a horse that has clinical signs of EPM (Packham *et al.*, 2002). Multiple different testing platforms have been investigated and used for the diagnosis of *N. hughesi*. These include IFAT, DAT/NAT, and ELISA. Renier *et al.* (2016) investigated two different serologic tests to support a diagnosis of EPM with serum and CSF using IFAT and ELISA for *S. neurona* and *N. hughesi*. They reported that both IFAT and ELISA serum/CSF samples titer ratio showed similar performance in the detection of specific antibodies to both *S. neurona* and *N. hughesi*, with the ELISA test being slightly more specific. The cut-off for a positive IFAT result was >100. One study found that the IFAT for *N. hughesi* antibodies in horses could be used to screen negative samples; however, low titer (≤ 1:100) positive samples require additional dilutions or ideally, should be tested by immunoblot analysis to determine specific antibody–antigen reactions (Vardeleon *et al.*, 2001). Similar results were obtained with low titer positive sera when the *Neospora* IFAT was used for detecting serum antibody in cattle (Conrad *et al.*, 1993).

Packham *et al.* (2002) tested multiple different serologic techniques (ELISA, IFAT, and DAT) with sera from experimentally infected *N. hughesi* horses and concluded that IFAT was the most reliable for determining a positive case. When the cut-off was 1:640, the IFAT had a sensitivity and specificity of 100% (95% CI = 65.2 – 100) up to 120 days post infection in experimentally infected animals. Screening IFAT titre results at 1:320 was reported to have 100% sensitivity for identifying all seropositive cases of *N. hughesi*, however the specificity decreased to 71.4% (95% CI = 30.3 – 94.9). A lower cut-off titre decreases the specificity, resulting in more false positives, risking falsely identifying a horse as positive (Vardeleon *et al.*, 2001). Gondim *et al.* (2009) reported that *N. caninum* antibody titres were higher than *N. hughesi*
titres. The negative aspect of being falsely diagnosed based principally on antibody analysis is that the breeder can be stigmatized resulting in a lower profitability for a breeding operation or the horse unnecessarily receiving antiprotozoal drug treatment (Vardeleon et al., 2001).

Clinical signs of *Neospora hughesi*

The primary protozoan parasite linked to equine protozoal myeloencepalitis (EPM) is *Sarcocystis neurona*. A role for *N. hughesi* in cases of clinical EPM negative for *S. neurona* was suggested; it also appears to cause an infection of the central nervous system (Cheadle et al., 1999; Daft et al., 1997; Dubey et al., 2001; Finno et al., 2007; Hamir et al., 1998; Marsh et al., 1996; Marsh et al., 1998). *Neospora hughesi* infection was studied in mice, gerbils, and dogs (Walsh et al., 2000). Clinical signs of EPM include ataxia, weakness, muscle wasting, incoordination, behavioural changes, loss of proprioception, and death (Dubey & Lindsay et al., 2001; Finno et al., 2007; Mackay et al., 1997). In 2007, a 10-year-old Canadian born Arabian x Quarter horse gelding in Saskatoon, Saskatchewan presented with neurologic disease and PCR analysis showed a correspondence to *N. hughesi*. This appears to be the first *N. hughesi* induced EPM in a horse outside of the United States (Wobeser et al., 2009). Interestingly, Saskatoon, Saskatchewan is located in Western Canada, which does not have opossums. Opossums are the definitive hosts of *S. neurona* and widely accepted as an animal that will spread the organism of EPM. This case suggests *N. hughesi* is transmitted by an animal that has not yet been discovered as a definitive host. A recently completed seroprevalence study in Alberta, Canada found a 29% seropositivity in horses to *N. hughesi* (unpublished data - personal communication A. Whitehead). *Neospora hughesi* was identified in horses that were *S. neurona* positive on western blots (James et al., 2017). Thus, some animals diagnosed with EPM due to *S. neurona* using this
methodology may be misdiagnosed, and the prevalence of EPM caused by \textit{N. hughesi} may be underestimated. Finally, serum antibody reacting to \textit{Neospora} spp. or \textit{S. neurona} antigens may only indicate exposure to the parasite or a very closely related parasite with similar antigens and not necessarily indicate an active infection is causing clinical signs (Vardeleon \textit{et al.}, 2001). There is thus likely to be another definitive host of \textit{N. hughesi}.

Seroprevalence

Vardeleon \textit{et al.} (2001) investigated seroprevalence of \textit{N. hughesi} in geographic regions known to have and not to have the North American opossum (\textit{Didelphis virginiana}), since the North American opossum serves as the definitive host for \textit{S. neurona}. The geographic range of the study included U.S. States (California, Florida, Missouri, and Montana) and New Zealand; Montana and New Zealand were selected as regions devoid of \textit{Didelphis virginiana}. The total seroprevalence in the U.S. was 57.7\% (108/187), with the highest recorded seroprevalence in Missouri at 84.6\% (33/39), while New Zealand had 23.8\% (5/21). The reported overall seropositivity for horses in both countries was 17\% (36/208) for \textit{N. hughesi} based on immunoblot analysis at a 1:100 cut-off titre. Pusterla \textit{et al.} (2014) examined 3,123 serum samples using IFAT with a cut-off titre of 1:320. Samples were collected from horses across the USA and divided into 4 groups based on IFAT results. The groups were \textit{N. hughesi} seropositive only, \textit{S. neurona} seropositive only, both \textit{N. hughesi} and \textit{S. neurona} seropositive, and both \textit{N. hughesi} and \textit{S. neurona} seronegative. The study found a significant association between state/location and seropositivity (p < 0.0001). This study is the largest \textit{N. hughesi} seroprevalence study with positive animals from 25 states (Pusterla \textit{et al.}, 2014). Another study investigated the seroprevalence of \textit{N. hughesi} in healthy equids in 18 states of the United States.
blood samples, they found 34% (1785/5250) of healthy equids were seropositive for *N. hughesi* antibodies (James *et al.*, 2017), indicating significant exposure to this parasite in the population.

**Effects on Reproductive Systems**

*Neospora hughesi* is primarily known as a rare cause of neurological disease in horses. Pusterla *et al.* (2011) investigated the possibility of endogenous transplacental transmission of *N. hughesi* during a 2-year time period on a single study farm in California. The study used 74 mare and foal pairs following a clinical diagnosis of neurological neosporosis in a weanling foal on the farm. Pre- and post-suckle serum of each foal was collected and submitted for IFAT analysis. Ninety-six (96%) of colostral samples in 2006, and 11% of colostral samples in 2007, were positive for *N. hughesi*. Only three of 74 foals were born seropositive. Passive transfer of colostral antibodies to *N. hughesi* was identified in 15 of the foals. In 10 foals and 9 mares, seroconversion from negative to positive occurred over the two years of the study.

In a follow up study, Pusterla *et al.* (2014) determined that transplacental infection with *N. hughesi* occurs from latently infected mares to their foals, which is similar to how *N. caninum* remains within a cattle herd. A total of eight healthy foals were born from 3 seropositive mares across a 7-year time period. Whole blood was collected from both the mare and foal shortly after birth (prior to colostrum ingestion) and again at 24 and 48 hours postpartum. Using IFAT testing, with a cut-off titre of 1:160 to identify *N. hughesi* positives (Pusterla *et al.*, 2014), all 8 foals prior to colostrum ingestion were found to be seropositive with titres between 1:640 and 1:20,480. Duarte *et al.* (2004) investigated the transplacental transmission of *N. hughesi* on California farms that sourced horses from California and Kentucky. The study reported that
mares who were positive for *N. hughesi* were 1.7 times more likely to have experienced a previous abortion than negative mares (95% CI = 0.5 – 5.6) when adjusted for age and state of birth, however this is not statistically significant as the confidence interval contains 1.

**Risk Factors for Exposure to *Neospora hughesi***

The life cycle and definitive host for *N. hughesi* is unknown. It is thought to be similar to *N. caninum*. Risk factors for exposure to *N. hughesi* are not established. Duarte *et al.* (2004) investigated the risk of exposure, age of first exposure, and the patterns of decay of maternally derived antibodies in a cohort of foals from farms (n=4) in California with a history of horses with EPM. A total of 484 foals were enrolled in the study at the time of their birth from 2000–2002, with serum samples obtained from newborn foals ≤ 4 days after the initial ingestion of colostrum. Each subsequent sample was obtained at approximately 3-month intervals from June 2000 to June 2002. The reported risk of exposure to *N. hughesi* was 3.1% over the two years, and the median age at exposure was 0.8 years. There was no statistically significant difference in the risk of exposure to *N. hughesi* among farms (p = 0.83). The median time for maternal antibody decay for *N. hughesi* was 91 days. Duarte *et al.* (2004) concluded that exposure to *N. hughesi* was low in foals between birth to 2.5 years of age and that the maternally derived antibodies could potentially cause false-positive results for 3 to 4 months after birth. James *et al.* (2017) investigated several different risk factors for *N. hughesi* to healthy equids from the United States. They found that breed, sex, use, and age (p<0.01) were all significantly associated with seropositivity using an IFAT titer cut off of 1:160. Warmbloods (OR = 1.53; 95% CI = 1.16 – 2.03; p < 0.05) were more likely to be seropositive than quarter horses. Male equids (OR = 0.85; 95% CI = 0.73 – 0.99; p < 0.05) were less likely to be seropositive than females. Breeding stock
(OR = 1.37; 95% CI = 1.04 – 1.82; p < 0.05) were more likely to be seropositive than competition stock (referent group). Increasing age was significantly associated with a higher risk of seropositivity (OR = 1.04; 95% CI = 1.02 – 1.04; p < 0.01). There was no significant difference in seropositivity among the four regions examined in 2013 (i.e., no difference between the Northeast region such as New York and Pennsylvania, and the South region such as Florida and Mississippi).

Rationale for Study

Understanding how *Neospora caninum* infects livestock, such as cattle and horses, allows producers to better manage their exposure by changing their farm management practices to reduce contact to this parasite. The potential economic loss due to *N. caninum* should incentivize further studies into understanding the epidemiology of infection.

Existing evidence supports a role for *N. caninum* as a cause of equine reproductive loss, including abortion. Up to 20% of equine abortions have no diagnosis assigned, and testing for *Neospora* spp. is not routine, therefore it could be missed. A clearer picture of vertical transmission in horses is also needed, to better understand the impact of this parasite on a horse's reproductive success.

Additional studies are needed to better differentiate *Neospora* subspecies, and to have more sensitive and specific diagnostic methods. The existing literature commonly groups different *Neospora* spp. together (*Neospora caninum* and *N. hughesi*) as a neosporosis infection, as cross-reactivity may be high for some tests, such as ELISA. Co-infection may also occur, particularly if both parasites share similar life cycles and risk factors.
In Canada, no previous studies have reported the seroprevalence of *N. caninum* in horses, and only one unpublished study exists on *N. hughesi* in horses based in Alberta. Such a study would provide valuable insight into the prevalence of *Neospora*, and what role it may have in equine disease. Understanding risk factors for exposure to *N. caninum* in cattle provides valuable information about the life cycle and where the greatest risk for infection lies on farms. As cattle are intermediate hosts, information may be extrapolated to other intermediate hosts, such as horses, for further investigation. Identifying risk factors for exposure provides insight as to where contamination of feed and water sources by oocysts may happen and therefore provides information to help farmers protect their animals from infection. Risk factors in cattle have been widely investigated. Previous studies suggest that age (Bartels *et al.*, 2006; Wilson *et al.*, 2016), disposal of aborted tissues (Cedeno & Benavides, 2015), high stocking density (Barling *et al.*, 2001; Barling *et al.*, 2000), food/water sources (Barling *et al.*, 2001; Dubey & Schares, 2007; Ould-Amrouche *et al.*, 1999), and the presence of horses on the farm (Coelho *et al.*, 2011; Hobson *et al.*, 2005, Reichel *et al.*, 2020) are risk factors for exposure in cattle. Little is known about risk factors for *N. caninum* in horses. Similar to cattle, age (Machacova *et al.*, 2013) and geographic location of the farm are reported to be risk factors for horses (Mazuz *et al.*, 2020; Talafha *et al.*, 2015). Additional studies are needed, as elucidating the risk factors for exposure is an important step in understanding the disease and allow for the prevention and control of *N. caninum* on equine farms.

The objectives of our study were to determine the seroprevalence of *N. caninum* and *N. hughesi*, as well as the risk factors for exposure in Ontario broodmares (addressed in Chapter 2). We also sought to determine the rate of *N. caninum* abortions from equine fetuses submitted to one pathology laboratory in Ontario, Canada (addressed in Chapter 3). We hypothesized that
seroprevalence of *N. caninum* would be similar to that found for Canadian cattle. We hypothesized that the risk factors for exposure and disease due to *N. caninum* would include close proximity to canids, presence of cattle, pasturing, contaminated feed, and stocking density.
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Chapter Two: Neosporosis in Ontario broodmares: Seroprevalence and risk factors for exposure to *Neospora caninum* and *Neospora hughesi*

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Abstract

*Neospora caninum* is an important cause of abortion in cattle worldwide. Recent confirmed cases suggest it is an unrecognized cause of equine abortion. Horses with antibodies to *Neospora caninum* exhibit higher pregnancy failure rates than unexposed horses. The objective of this research was to describe the seroprevalence of *N. caninum* in Ontario broodmares and identify risk factors for exposure. We hypothesized that the seroprevalence to *N. caninum* in Ontario broodmares would be similar to Ontario cattle (~20%). In this cross-sectional study, serum samples from 219 Ontario broodmares from 63 farms were tested for antibodies to *N. caninum* and *N. hughesi* using an indirect immunofluorescence antibody test (IFAT). Using mixed logistic regression models with a random intercept for farm, the associations between mare and environmental factors and seroprevalence were examined. Thirty-one of the 63 participating farms (49.20%) had at least 1 broodmare seropositive for *N. caninum*. Antibodies (titre ≥1:40) for *N. caninum* were found in a total of 60/219 (27.40%) broodmares. The prevalence estimates of titres of 1:40, 1:80, and 1:160 were 17.81% (39/219), 3.65% (8/219), and 5.94% (13/219), respectively. In this study, the seroprevalence of *N. caninum* antibodies in Ontario broodmares was similar to Ontario cattle. Risk factors for exposure to *N. caninum* (titre ≥ 1:40) include the presence of farm dogs (OR = 6.70; 95% CI = 2.14 – 20.97; p = 0.001) and high stocking density (OR = 2.83; 95% CI = 1.27 – 6.30; p = 0.011). The presence of livestock,
excluding cattle, was associated with reduced risk of exposure (OR = 0.17; 95% CI = 0.06 – 0.53; p = 0.002). Thirty-three of the 63 participating farms (52.4%) had at least 1 broodmare seropositive for \( N. hughesi \). Antibodies (titre ≥1:160) for \( N. hughesi \) were found in 65/219 (29.7%) broodmares. Feeding hay on the ground of the paddock significantly increased the odds of exposure to \( N. hughesi \) (OR = 4.31; 95% CI = 1.65 – 11.22; p = 0.003).

2.1 Introduction

Neosporosis is a significant cause of reproductive failure in cattle worldwide. The two agents of concern for neosporosis are \( N. caninum \) and \( N. hughesi \). \( Neospora caninum \) is a protozoal parasite of both 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) and is a cause of abortion in bovine species around the world (Dubey, 2003). This cyst-forming protozoal parasite was not identified as its own genus and species until 1988 (Dubey et al., 1988). Before 1988, \( N. caninum \) was incorrectly classified as \( Toxoplasma gondii \) due to their morphological similarities. The differences between \( N. caninum \) and \( T. gondii \) were identified through immunohistochemistry (IHC) and examination of ultrastructural characteristics of cysts (Bjerkas and Prethus, 1988). \( Neospora hughesi \) is closely related to \( N. caninum \) and was originally isolated from a horse with myeloencephalitis (Marsh et al., 1996). \( Neospora hughesi \) was classified as a new species based on molecular, antigenic, and structural differences to \( N. caninum \) (Marsh et al., 1998). The life cycle of \( Neospora hughesi \) is unknown and has only been documented to naturally infect horses (Gondim et al., 2009). Differences between two immunodominant tachyzoite antigens (i.e., SAG1 and SRS2 and additional peptide differences can distinguish \( N. hughesi \) from \( N. caninum \) (Marsh et al., 1999).
Considerable epidemiological evidence supports an association between dogs and *N. caninum* infection and abortion in cattle, and dogs were eventually discovered to be definitive hosts 10 years after the official classification (McAllister et al., 1998). Coyotes were identified as hosts a few years later (Gondim et al., 2004). It is known that *N. caninum* has the ability to infect fetuses via transplacental transmission and this accounts for up to 95% of seropositive animals in endemically infected cattle herds (Anderson et al., 1995, 1997; Barr et al., 1993; Dubey et al., 2007). Horizontal transmission can also occur. In cattle, the potential sources of exposure include colostrum or milk from infected animals, infected placentae, and fetal fluids (Davison et al., 2001; Ho et al., 1998; Moskwa et al., 2006). The ability of *N. caninum* to cause abortion in cattle has major economic impacts on farm economics and cattle management practices. Many management practices are similar between cattle and horses which suggests that risk factors for *N. caninum* infection may be similar between the two species. A reported risk factor for abortion due to *N. caninum* in cattle is the presence of horses on the same farm (Hobson et al., 2005). The reason for this association is unknown but warrants further study. Seropositivity to *N. caninum* within horse populations can range from 8% to 71%, depending on study geographic location (Bartova et al., 2017; Mazuz et al., 2020). Hobson et al. (2005) reported that 22.1% of Ontario cattle were seropositive for *N. caninum*.

There are several reports of an association between neosporosis and neurologic disease in horses (Cheadle et al., 2000; Daft et al., 1996; Hamir et al., 1998; Lindsay et al., 1996; Marsh et al., 1996; Pronost et al., 2000). *Sarcocystis neurona* is the most commonly identified cause of equine protozoal myeloencephalitis (EPM), however *N. hughesi* was identified in rare cases (Duarte et al., 2004; Wobeser et al., 2009). *Neospora hughesi* is challenging to treat because
cysts in the tissue have the ability to re-infect the host post-treatment (Vardeleon et al., 2001). Vardeleon et al. (2001) investigated the seroprevalence of *N. hughesi* by geographic region using immunoblot analysis at a 1:100 cut-off titre. Total seroprevalence in the U.S. was 57.7% (108/187), and the highest recorded seroprevalence was in Missouri at 84.6% (33/39); in New Zealand, the seroprevalence was reported to be 23.8% (5/21). James et al. (2017) found that 34% (1785/5250) of healthy equids in the United States were seropositive for *N. hughesi* antibodies, indicating significant exposure to this parasite in the population. Few studies have investigated neosporosis in Canadian horses. In 2007, a 10-year-old Canadian born Arabian x Quarter horse gelding in Saskatoon, Saskatchewan diagnosed with neurologic disease was positive for *N. hughesi* on PCR analysis but negative for *S. neuroni* (Wobeser et al., 2009); this was the first *N. hughesi* induced EPM in a horse outside of the United States. A recent study in Calgary, Alberta found 29% of serum samples from hospitalized horses were positive for *N. hughesi* (unpublished results, A. Whitehead). No previous studies have examined seroprevalence to *N. caninum* in Canadian horses. The objective of this study was to determine the seroprevalence of *N. caninum* and *N. hughesi* in Ontario broodmares, and the risk factors associated with exposure to each parasite.

2.2 Materials and Methods

2.2.1 Study Population

A total of 63 Ontario farms were enrolled in the study. These farms had a total of 219 broodmares, and were located across Ontario, Canada (Table 2.1). In total, 167 survey responses were provided for individual broodmares.
Table 2.1: Ontario regions * where farms with broodmares were enrolled (n = 63).

<table>
<thead>
<tr>
<th>Regions in Ontario</th>
<th># of Farms</th>
<th># of Horses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Central</td>
<td>22</td>
<td>93</td>
</tr>
<tr>
<td>Western</td>
<td>27</td>
<td>91</td>
</tr>
<tr>
<td>North-Eastern</td>
<td>6</td>
<td>27</td>
</tr>
</tbody>
</table>

*Regions were classified by Government of Ontario (2017).

2.2.2 Study design and data collection

In this cross-sectional study, convenience sampling was used to select farms in 2019 - 2020. Farms were included in the study based on having at least one broodmare that resided in Ontario for at least 12 consecutive months and were willing to participate. Farms without broodmares were excluded from the study. Only mares that remained in Ontario for the previous consecutive 12 months were sampled. This excluded any mares that foaled in Ontario but then travelled to Kentucky for breeding. The project enlisted the help of licenced veterinarians across Ontario to increase the geographical range of participating farms. Veterinary recruitment was initiated with personal communications with veterinarians and horse owners. All animal procedures and human participation were approved by the University of Guelph Animal Care Committee and Research Ethics Board, respectively (AUP# 4290 and REB#19-10-033). Signed consent forms that included owner/farm name, contact information, and broodmare information such as age, breeding status, previous number of foals, and reproductive history, were completed prior to collection of blood samples.

Blood samples were collected from the jugular vein as per routine aseptic procedure. Blood was collected into a 10mL BD Vacutainer serum tube labelled with mare name and farm name. All samples were packaged with ice packs and transported to the Theriogenology Laboratory at
the University of Guelph. If the samples were submitted by veterinarians, the consent form was included in the package. Blood samples were centrifuged at 2500 rpm for 10 minutes (Becton Dickson, USA). The resulting serum was collected and transferred into a 1mL cryotube. Samples were stored in a -80°C freezer until serological analysis.

Consent and broodmare information was uploaded into an Excel spreadsheet to organize the data (Microsoft Excel, 2018). Serum samples were transported to the Conrad Packham laboratory\(^2\) at the University of California, Davis on dry ice, in an appropriate dry ice shipping container, for indirect immunofluorescent antibody testing (IFAT). The species-specific secondary antibody used was anti-horse fluorescein isothiocyanate (FITC). Serum samples were tested for antibodies against *N. caninum* and *N. hughesi* using IFAT as previously described by Conrad *et al.* (1993). Antigen slides (Cell Line Associated, Newfield, New Jersey) were prepared with tachyzoites of EN-1 isolate and to an initial serum screening dilution of 1:40. The fluorescein-labeled, affinity-purified antibodies directed against species-specific horse IgG (Jackson ImmunoResearch) were diluted 1:1,000 in PBS and added in 10-ul aliquots to each well. The endpoint titer was the last serum dilution showing distinct, whole parasite. Fluorescent slides were read by two independent, blinded readers to verify and compare results (Packham *et al.*, 2002). The sensitivity and specificity of the IFAT at the cut-off of 1:40 titre as reported by the lab were 98\% and 99\%, respectively.

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Participating farms were asked to complete a comprehensive online survey using the Qualtrics SE (Qualtrics, Provo, Utah) software, consisting of questions about farm management practices to assess risk factors for exposure. This included questions such as total farm acreage, total number of horses, total number of broodmares, paddock characteristics, presence of cattle, presence of rodents (not defined in questionnaire), presence of other livestock (excluding cattle), presence of farm dogs and wild canids, and general cleaning/management such as how feed is stored, and how often water sources are cleaned. Prior to implementation, the survey was reviewed by study supervisors and pre-tested by 3 independent farm managers.

2.2.3 Statistical Analysis

Data from the survey and results of IFAT testing for *N. caninum* and *N. hughesi* were compiled into a spreadsheet (Excel, Microsoft Co., Redmond, Washington) to assess the quantity and quality of the data. Once the data were assessed for recording errors and organized, the file was uploaded into StataSE software (StatCorp, College Station, Texas). A causal diagram (Figure 1) was created to assess potential causal relationships among independent variables and testing seropositive for *N. caninum* and *N. hughesi*. A horse was considered seropositive if it had a titre $\geq 1:40$ for *N. caninum* and $\geq 1:160$ for *N. hughesi*. The choice of cut-off titer for *N. caninum* was based on recent studies using IFAT and titers of 1:40 or 1:50. The choice of a higher cut-off titer for *N. hughesi* than *N. caninum* in our study was at the recommendation of the testing laboratory based upon ongoing research and confirmed positive cases of *N. hughesi*. Extensive testing in the laboratory using positive tissues from confirmed cases has established 1:160 as the optimal titer to reduce false positives (Personal communication; Pusterla, 2014; Vardeleon *et al.*, 2001).
To avoid issues with collinearity, the correlation among independent variables were assessed using various correlation coefficients (i.e., Pearson, Spearman rank, and Phi correlation) depending on the variables. If the correlation was greater than $|0.7|$, the more biologically plausible variable was selected. For continuous variables, the linearity with the log odds of testing positive for *N. caninum* and *N. hughesi* were assessed using locally weighted regression (lowess). If the relationship was not linear, the variable was categorized or modeled as a quadratic relationship if appropriate. Initially, mixed univariable logistic regression models were fit, with a random intercept for farm, using a liberal significance level ($\alpha = 0.2$). Variables significant on univariable analysis were considered for subsequent multivariable modelling. A variable remained in the model if it was statistically significant ($\alpha = 0.05$) or acted as an explanatory antecedent (i.e., confounding variable). A variable was defined as an explanatory antecedent if it was a non-intervening variable and its removal resulted in a 20% or greater change of a statistically significant coefficient. Models were fit with a random intercept for farm. The fit of mixed models was assessed by determining if the best linear unbiased predictors (BLUPs) met the assumptions of normality and homoscedastity. For ordinary logistic regression models, fit was assessed using Hosmer-Lemeshow or Pearson goodness-of-fit tests depending on whether the data were binary or binomial, respectively. Pearson residuals were examined for potential outliers. Cohen’s Kappa test was performed to assess the agreement beyond chance in test results for *N. caninum* and *N. hughesi* to determine if cross-reactivity between the two species might occur with the IFAT tests (Kappa = 0.05).
Figure 2.1: Causal diagram of the potential causal relationships between predictor variables and testing positive for *N. caninum* and *N. hughesi*. 
2.2.4 Independent Variables

Independent variables were obtained from the questionnaire. Each question had an independent variable associated with it (e.g. how many times paddocks are cleaned, water sources available in the paddock). Each question was assessed individually, unless the following question was in direct relation with the previous (e.g. Do you have farm dogs? If yes, how many?).

2.2.4.1 Breeds The reported breeds of horses were divided into four breed categories: Thoroughbred, Standardbred, Quarter Horse, and other (Table 2.2). Horses reported as crossbred were included in the main breed category reported by the owner (e.g. Thoroughbred X was listed as a Thoroughbred). The Other category combined all other breeds, such as ponies, miniatures, Arabians, draft breeds, and Appaloosas.

Table 2.2: Breakdown of the breeds of broodmares that were enrolled in the study.

<table>
<thead>
<tr>
<th>Horse Breeds</th>
<th># of horses</th>
<th>% of sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thoroughbred</td>
<td>78</td>
<td>35.9</td>
</tr>
<tr>
<td>Standardbred</td>
<td>47</td>
<td>21.7</td>
</tr>
<tr>
<td>Quarter horse</td>
<td>31</td>
<td>14.3</td>
</tr>
<tr>
<td>Other</td>
<td>61</td>
<td>28.1</td>
</tr>
</tbody>
</table>

2.2.4.2 Age Broodmare age was categorized into 3 groups of horses: 4 – 8, 9 – 13, and 14+ years of age.
2.2.4.3 Stocking density  Individual farm stocking density was calculated based on the USDA definition of stocking density as pounds (lbs) of livestock per acre, and the stocking density (SD) variable was created based on this calculation. To calculate stocking density on each farm, weights were assigned as follows: ponies and miniature horses: 500 lbs.; light horses: 1000 lbs.; draft horses: 1800 lbs. Total pounds of horses on a farm was calculated by multiplying animals in each weight category by assigned weight, which was then divided by the reported farm acres to obtain the stocking density for each specific farm.

2.2.4.4 Geographic location  Farm locations were first assessed using the town/city reported in the survey (17 different locations). To increase sample size for statistical analysis, farms were then grouped by county (23 different counties) and then subsequently grouped by the region in Ontario (4 regions) in which the farm resided. Farms were grouped based on the Government of Ontario Regions and Offices (2017). Due to a small sample size, the North and Eastern regions were grouped together, resulting in 3 final regional categories for statistical analysis.

2.2.4.5 Reproductive issues  A wide range of reproductive issues were reported. They included endometritis, endometrial cysts, low fertility, embryonic death, abortion, stillborn, dystocia, uterine trauma, congenital anomalies, and allergy. Those that were deemed unrelated to the study were removed from the analysis; this included bacterial endometritis, allergy, dystocia, uterine trauma, and cleft palate in a foal. The remaining issues were collapsed into a dichotomous variable (i.e., reproductive issues present versus absence) since specific issues had small sample sizes.
2.2.4.6 Presence of cattle  Of the 63 participating farms, 9 farms reported having cattle for a total of 37 animals. The uses of cattle included dairy and beef. Due to sample size cattle was initially grouped in one “yes” category and investigated further.

2.2.4.7 Presence of farm dogs  Farm dogs was explored as a dichotomous variable (yes/no) and as a categorical variable based on the number of dogs (none, 1 dog, 2 dogs, 3+ dogs). Due to the extrapolation of the number of dogs from the original yes/no category, both variables could not be included in the same model due to collinearity. Therefore, the number of dogs were kept as a univariate analysis.

2.3 Results

2.3.1 Descriptive Statistics – *N. caninum* and *N. hughesi*

A total of 219 broodmares from 63 farms across Ontario participated in the study. Survey response rate was 79.4%, with 50 of 63 farms completing the questionnaire to varying degrees; 46 farms answered all of the questions. The seroprevalence for *N. caninum* in 219 broodmares at titre cut-offs of 1:40, 1:80, and 1:160 were 27.4% (n = 60), 9.6% (n = 21), and 5.9% (n = 13), respectively (Table 2.3). Thirty-one of the 63 participating farms (49.2%) had at least 1 broodmare seropositive for *N. caninum* (Table 2.6). The seroprevalence for *N. hughesi* in 219 broodmares at titre cut-offs of 1:160, 1:320, 1:640, and 1:1280 were 29.7% (n = 65), 16.0% (n = 35), 7.3% (n = 16), and 1.8% (n = 4), respectively (Table 2.4). The within broodmare herd seroprevalence was calculated for each farm when the information was available (Table 2.5). On farms that were positive, the lowest within broodmare herd seroprevalence was 2.1% and 3.9%
for *N. caninum* and *N. hughesi*, respectively. The highest for both *N. caninum* and *N. hughesi* was 66.7% and 80.0%, respectively. Thirty-three of 63 farms (52.4%) had at least one broodmare seropositive for *N. hughesi* (Table 2.6). Breeds represented in the study were: Thoroughbred (78/219; 35.6%), Standardbred (47/219; 21.7%), Quarter horse (31/219; 14.3%), and other (61/219; 28.1%). The reproductive status of the broodmares was defined as open (52/197; 26.4%), pregnant (98/197; 49.8%), lactating (20/197; 10.2%), maiden (16/197; 8.1%), and barren (11/197; 5.6%). Broodmares in the study ranged from 4 to 35 years of age. The distribution of mares across the age categories was: n=66 for ages 4 – 8; n=67 for ages 9 – 13; and n=62 for ages 14 – 35. The distribution of the horses across Ontario by region was Central (93/211; 44.1%), Western (91/211;43.1%), and Northern-Eastern (27/211; 12.8%).

Table 2.3: Seroprevalence of Neospora caninum by IFAT titre in Ontario broodmares (n=219).

<table>
<thead>
<tr>
<th><em>N. caninum</em> Titre</th>
<th># of seropositive broodmares (% of sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 1:40</td>
<td>60 (27.4)</td>
</tr>
<tr>
<td>≥1:80</td>
<td>21 (9.6)</td>
</tr>
<tr>
<td>≥1:160</td>
<td>13 (5.9)</td>
</tr>
</tbody>
</table>

Table 2.4: Seroprevalence of Neospora hughesi by IFAT titre in Ontario broodmares (n = 219).

<table>
<thead>
<tr>
<th><em>N. hughesi</em> Titre</th>
<th># of broodmares (% of sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥1:160</td>
<td>65 (29.7)</td>
</tr>
<tr>
<td>≥1:320</td>
<td>35 (16.0)</td>
</tr>
<tr>
<td>≥1:640</td>
<td>16 (7.3)</td>
</tr>
<tr>
<td>≥1:1280</td>
<td>4 (1.8)</td>
</tr>
</tbody>
</table>
Table 2.5: The within herd seroprevalence of (i) N. caninum and (ii) N. hughesi for Ontario broodmares.

<table>
<thead>
<tr>
<th></th>
<th>Within broodmare herd seroprevalence (%)</th>
<th># of farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) N. caninum</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>1 – 20</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>20 – 40</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>40 – 67</td>
<td>6</td>
</tr>
<tr>
<td>ii) N. hughesi</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>1 – 20</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>20 – 40</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>40 – 80</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2.6: Summary of farms and broodmares positive for N. caninum, N. hughesi, and dual infection (N. caninum and N. hughesi).

<table>
<thead>
<tr>
<th>At least 1 Positive Broodmare</th>
<th>N. caninum positive (%)</th>
<th>N. hughesi positive (%)</th>
<th>N. caninum and N. hughesi positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Farms</td>
<td>31/63 (49.2)</td>
<td>33/63 (52.4)</td>
<td>12/63 (19.0)</td>
</tr>
<tr>
<td>Number of Broodmares</td>
<td>60/219 (27.4)</td>
<td>65/219 (29.7)</td>
<td>20/219 (9.1)</td>
</tr>
</tbody>
</table>
2.3.2 Risk Factors for *N. caninum*

The presence of farm dogs (OR = 6.70; 95% CI = 2.140 – 20.971; p = 0.001), and high stocking density (OR = 2.83; 95% CI = 1.27 – 6.30; p = 0.011) increased the odds of being seropositive (Table 2.5). The presence of livestock, excluding cattle, decreased the odds of being seropositive (Table 2.5); OR = 0.17; 95% CI = 0.06 – 0.53; p = 0.002). A total of 34/46 (73.9%) of reporting farms said they have dogs. Of these farms, 16 (47.1%) also had seropositivity for *N. caninum*. When assessing if the number of dogs was a risk factor in a univariable model, it was slightly above significance with three or more farm dogs (p = 0.051). See Table 2.4 for number of dogs by farm and univariable results. Thirty percent (14/46) of farms reported having livestock on their farm on the questionnaire. See Table 2.7 for breakdown of type of livestock reported. Age, breed, status, geographic location (Figures 2.1 – 2.2), reproductive issues, the presence of wild canids, management practices, and the presence of cattle were not statistically significant risk factors for *N. caninum* exposure in Ontario broodmares.

Table 2.7: Results of mixed univariable logistic regression models examining risk factors for (i) *N. caninum* and (ii) *N. hughesi* seropositivity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>i) Neospora caninum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of dogs (yes/no)</td>
<td>5.20</td>
<td>1.34 – 20.19</td>
<td>0.017</td>
</tr>
<tr>
<td>Number of dogs</td>
<td>0.16</td>
<td>0.03 – 1.01</td>
<td>0.051</td>
</tr>
<tr>
<td>Presence of <em>livestock</em> (yes/no)</td>
<td>0.19</td>
<td>0.05 – 0.74</td>
<td>0.017</td>
</tr>
<tr>
<td>High stocking density (yes/no)</td>
<td>4.05</td>
<td>1.27 – 12.89</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Probability</td>
<td>Confidence Interval</td>
<td>p-Value</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------</td>
<td>---------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Presence of cattle (yes/no)</td>
<td>0.84</td>
<td>0.15 – 5.84</td>
<td>0.843</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 - 13 years</td>
<td>0.91</td>
<td>0.30 – 2.76</td>
<td>0.861</td>
</tr>
<tr>
<td>14+ years</td>
<td>1.86</td>
<td>0.59 – 5.84</td>
<td>0.288</td>
</tr>
<tr>
<td>Breed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standardbred</td>
<td>0.67</td>
<td>0.15 – 2.96</td>
<td>0.599</td>
</tr>
<tr>
<td>Quarter Horse</td>
<td>0.88</td>
<td>0.23 – 3.35</td>
<td>0.853</td>
</tr>
<tr>
<td>Other</td>
<td>0.47</td>
<td>0.14 – 1.54</td>
<td>0.212</td>
</tr>
<tr>
<td>Geographic location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>North + East</td>
<td>1.16</td>
<td>0.23 – 5.94</td>
<td>0.859</td>
</tr>
<tr>
<td>West</td>
<td>1.32</td>
<td>0.48 – 3.65</td>
<td>0.588</td>
</tr>
<tr>
<td>Pregnant</td>
<td>1.11</td>
<td>0.44 – 2.83</td>
<td>0.826</td>
</tr>
</tbody>
</table>

**ii) Neospora hughesi**

<table>
<thead>
<tr>
<th></th>
<th>Probability</th>
<th>Confidence Interval</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feeding hay on ground (yes/no)</td>
<td>4.31</td>
<td>1.65 – 11.22</td>
<td>0.003</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 – 13 years</td>
<td>0.75</td>
<td>0.27 – 4.49</td>
<td>0.583</td>
</tr>
<tr>
<td>14+ years</td>
<td>0.62</td>
<td>0.21 – 1.81</td>
<td>0.385</td>
</tr>
</tbody>
</table>
Table 2.8: Results of mixed multivariable logistic regression models examining risk factors for (i) *N. caninum* and (ii) *N. hughesi* seropositivity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Odds Ratio (95% CI)</th>
<th>P value</th>
<th>Farm Intercept (Standard Error)</th>
<th>ICC (Standard Error)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>i) Neospora caninum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence of Dogs (yes/no)</td>
<td>6.70 (2.13 – 20.97)</td>
<td>0.001</td>
<td>1.26e-34 (3.22e-17)</td>
<td>3.84e-35 (9.77e-18)</td>
</tr>
<tr>
<td>Presence of Livestock* (yes/no)</td>
<td>0.17 (0.06 – 0.53)</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Stocking Density (yes/no)</td>
<td>2.83 (1.27 – 6.30)</td>
<td>0.011</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ii) Neospora hughesi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parameter</td>
<td>Odds Ratio 95% CI</td>
<td>P value</td>
<td>Farm Intercept 95% CI</td>
<td>ICC 95% CI</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-----------------------</td>
<td>---------</td>
<td>------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Feeding Hay on the Ground (yes/no)</td>
<td>4.31 (1.65 – 11.22)</td>
<td>0.003</td>
<td>0.71 (0.14 – 3.64)</td>
<td>0.18 (0.04 – 0.52)</td>
</tr>
</tbody>
</table>

*Not including cattle

Table 2.9: Number of farms with 0-3 farm dogs present among Ontario broodmare farms studied (2019 – 2020).

<table>
<thead>
<tr>
<th>Number of dogs</th>
<th>Number of farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2.10: Number of farms with livestock excluding cattle present among Ontario broodmare farms studied (2019 – 2020).

<table>
<thead>
<tr>
<th>Type of livestock</th>
<th>Total number of livestock</th>
<th>Number of farms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry *</td>
<td>90</td>
<td>5</td>
</tr>
<tr>
<td>Swine</td>
<td>29</td>
<td>3</td>
</tr>
<tr>
<td>Goats</td>
<td>21</td>
<td>2</td>
</tr>
<tr>
<td>Sheep</td>
<td>60</td>
<td>1</td>
</tr>
<tr>
<td>Rabbits</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>Llamas &amp; Alpacas</td>
<td>N/A</td>
<td>1</td>
</tr>
<tr>
<td>Donkeys</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

* Two farms reported "lots", and were excluded from the grouping
Figure 2.2: Spatial distribution of *N. caninum* seropositive farms in Ontario, Canada.

Figure 2.3: Spatial distribution of *N. hughesi* seropositive farms in Ontario, Canada.
2.3.4. Risk Factors for *N. hughesi*

The only significant variable found to be a risk factor for exposure to *N. hughesi* was feeding hay on the ground in the paddock (OR = 4.31; 95% CI = 1.65 – 11.22; p = 0.003). A total of 22/46 (47.8%) farms reported that they feed hay on the ground in the paddock. Age, breed, status, geographic location, reproductive issues, the presence of wild canids, management practices, high stocking density, presence of farm dogs, presence of livestock, and the presence of cattle were not statistically significant risk factors for *N. hughesi* exposure in Ontario broodmares.

2.4 Discussion

The seroprevalence of *N. caninum* antibodies in the Ontario broodmares tested in this study using a titer cut-off value of 1:40 was 27.4%. This seropositivity prevalence is similar to the 20.1% previously reported for Ontario cattle (Hobson 2005). Seropositivity in horses in previous studies from different geographic locations have varied from 6% to 78% in Brazil and France, respectively (Abreu et al., 2014; Pitel et al., 2003). Seropositivity studies based in the United States fall within that range with Wyoming at 31.1% (Dubey et al., 2003) and Texas at 23.3% (Dubey et al., 1999). However, both studies used NAT, whereas the present study used IFAT. Comparison across studies is difficult due to different methodologies and variations in titer cut-off values used to determine positivity. Some previous studies used an IFAT cut-off value of 1:50 (Mazuz et al., 2020; Kligler et al., 2007), while others used NAT with a 1:40 cut-off (Tavallan et al., 2015), DAT with a 1:50 cut-off (Pitel et al., 2001), and ELISAs with >50% response (Bartova et al., 2010).
The seroprevalence of *N. hughesi* antibodies in the Ontario broodmares tested using a titer cut-off value of 1:160 was 29.2% and increased to 71.8% with the same cut-off titre for *N. caninum*, 1:40. Few previous studies have examined the seroprevalence of *N. hughesi* in horses (Pusterla, 2014; Vardeleon *et al.*, 2001). Pusterla (2014) found an overall (*N. hughesi* seropositive and *N. hughesi* seropositive with *S. neurona* horses) seroprevalence of 2% in a study with 3123 horses from across 49 states. Texas reported the highest seroprevalence to *N. hughesi* 18.4%. A recent study in Alberta, Canada found a 29% seroprevalence rate in local horses (A. Whitehead, personal communication). When considering dual infection with *N. caninum* and *N. hughesi* on farms and in broodmares, the percent positives on farm for both dropped nearly 32%, and the percent positives for broodmares dropped approximately 19.5%.

**Age:** In this study, age was not significantly associated with risk of seropositivity for either *N. caninum* or *N. hughesi*. This is in agreement with Kliger *et al.* (2007), who found that age was not a significant factor for *N. hughesi*. However, this finding is in disagreement with James *et al.* (2017) who found that mares five years of age and older were more likely to test positive for *N. hughesi* than younger horses with the 1:160 titre used in this study. Advancing age is expected to provide more opportunity for exposure to *N. caninum* and *N. hughesi* due to more lifetime exposure to infection. Alternatively, foals infected transplacentally by positive mares are expected to remain seropositive for life. This could contribute to age not being a risk factor since infection and seroconversion occurred prior to birth. Comparisons across studies are difficult due to differences in testing methods, sample sizes, horse uses, and geographic location. Age has been investigated several times in cattle (Salguero-Romero *et al.*, 2021; Wilson *et al.*, 2016; Bartels *et al.*, 2006; Dyer *et al.*, 2000; Sanderson *et al.*, 2000; Davison *et al.*, 1999), with
conflicting results reported. Comparing risk factors, especially age, between equine and cattle can be difficult due to the differences in the production life of each species and farm management practices. However, seroconversion over time has been documented for *N. hughesi* (Pusterla *et al.*, 2011). That study found that 10 foals and 9 mares over a two-year study period seroconverted for *N. hughesi*. Exposure likely occurred through the ingestion of sporulated oocysts from the environment.

**Stocking density:** Broodmares from farms with a high stocking density were significantly more likely to be seropositive for *N. caninum* compared to mares from farms with low stocking density. High stocking density was a significant risk factor in cattle (Barling *et al.*, 2001; Barling *et al.*, 2000). The researchers suggested that higher densities meant a greater use of supplemental feeding practices, contributing to exposure risk. Supplemental feed must be properly stored in clean facilities, to avoid attracting rodents that in turn attract definitive hosts of *N. caninum*. In contrast, supplemental feeding of grain to mares in the present study was not found to be a significant risk factor for exposure. Caution is needed when comparing supplemental feeding strategies between equine and cattle. Supplemental feed such as silage consumed by cattle is mostly stored in silos or piles, not in the individual feed bags that are typically used for feeding horses. Having a high stocking density and crowding of horses may result in over-grazing of pastures and a tendency for horses to consume canid feces present in the environment. In cattle, a high stocking density could also expose animals to infected fetal membranes, as cows may calve, abort, or expel placentas in close proximity to hay feeders. This increases seropositivity within the herd as the concentration of placentas or contamination increases (Barling *et al.*, 2001).
Farms with high stocking density may also have poor cleaning and management practices that lead to contamination of feed with rodent and canid feces.

**Presence of livestock:** Mares from farms with livestock other than cattle were at significantly lower odds of testing positive for *N. caninum*. Study farms reported a variety of livestock including sheep, goats, pigs, rabbits, donkeys, miniature donkeys, wild turkeys, llamas, alpacas, and ducks. Livestock may act as a “sponge” due to grazing preferences, consuming feed or grass contaminated with canid feces in the environment, and thus reducing the risk to horses on the premises (Grev *et al*., 2017; Yayota *et al*., 2015). It is also possible that better biosecurity measures are in place on a horse farm that houses other species, leading to reduced contamination and risk of exposure.

**Presence of cattle:** The presence of cattle on the same farm as broodmares was not a significant risk factor for seropositivity in the present study. This is in contrast to Hobson *et al.* (2005), who found that the presence of horses on Ontario cattle farms significantly increased the risk of abortion due to *N. caninum* (Hobson *et al*., 2005). The relationship between horses and cows for risk of exposure to *N. caninum* is unknown, as both species are intermediate hosts for the parasite. Grazing habits of the two species are different, with cattle often grazing tall grasses that horses find over-mature and bitter (Grev *et al*., 2017; Yayota *et al*., 2015), and horses are often managed differently than cattle, even when kept on the same farm. There may be management differences between a cattle farm that keeps a few horses as pets compared to a horse breeding farm that has no or few cows. More investigation is needed to understand the relationship between horses and cattle when considering risk factors for neosporosis.
**Presence of farm dogs:** Farms that reported having dogs were significantly more likely to have seropositive mares than those that did not have dogs. This is in agreement with Abreu *et al.* (2014), who found that seroprevalence in mares for *N. caninum* was significantly higher on farms that had seropositive dogs. This suggested that the presence of farm dogs, particularly seropositive dogs, is a risk factor for horses being exposed to *N. caninum*. Several studies in cattle also confirmed the role of farm dogs in exposing cattle to *N. caninum* (Corbellini *et al.*, 2006; Mainar-Jaime *et al.*, 1999; Pare *et al.*, 1998; von Blumröder *et al.*, 2006). It is unlikely that horse owners will remove dogs from their farms, however the importance of this risk factor cannot be understated. Horse owners should be educated about the lifecycle of *N. caninum* and how dogs transmit the parasite to horses. Implementation of biosecurity measures such as limiting the access of dogs to paddocks, ensuring the timely clean-up of dog feces, and the implementation of technology or fences to limit the access of wild canids to horse paddocks, could reduce the risk of exposure of horses to this parasite.

**Breed:** The risk of *N. caninum* and *N. hughesi* exposure was not significantly different among horse breeds. Studies in cattle have conflicting results regarding a breed association with risk of exposure to *N. caninum* (Bartels *et al.*, 2006; Lopez *et al.*, 2007). In horses, James *et al.* (2017) reported that breed, specifically warmbloods, had significantly greater odds of being seropositive for *N. hughesi* than other breeds such as Quarter Horses, Thoroughbreds, and draft horses. Bartels (2006) suggested any breed differences in risk seen in cattle were likely a reflection of different management practices. Many of the horse farms in our study housed multiple breeds
together, potentially eliminating any breed effect associated with farm management.

**Location:** Geographic region was not associated with risk of seropositivity for either *N. caninum* or *N. hughesi*. A few other studies found that the risk of seropositivity varied by geographic location. Reported seroprevalence from different areas of the world vary from 11.9 – 70.9% in Israel (Kliger *et al*., 2007; Mazuz *et al*., 2020), 20.6% in Texas (Dubey *et al*., 1999), 23% in France (Pitel *et al*., 2001), 28% in Italy (Ciaramella *et al*., 2004), and 58% in Brazil (Oliveira Kock *et al*., 2019). Some studies did not explicitly investigate geographic differences. However, when comparing different studies from different locations within the same country, they report widely different point estimates for seroprevalence. There is not enough information on *N. caninum* infection in horses in Canada (excluding Alberta) to be able to compare Ontario’s seropositivity to Canada as whole and most of the other provinces in Canada.

**Cleaning/management practices:** Cleaning and management practices were not associated with risk of seropositivity to *N. caninum* and *N. hughesi*. This was particularly interesting as several grazing, feed, water source, and cleaning practices were reported as risk factors in cattle studies (Barling *et al*., 2001; Otranto *et al*., 2003; Ould-Amrouche *et al*., 1999; Sanderson *et al*., 2000). Few previous studies in horses evaluated cleaning and management practices. Talfha *et al*. (2015) reported that age, sex, breed, usage, body condition score, grazing type, presence of other horses, and disease history were not significant risk factors for *N. caninum* in horses. These findings were in direct contrast to previous studies in cattle. The response rates for individual management practices self-reported in our survey may not have provided enough power to be able to detect an association. It is also possible that the survey did not capture the cleaning and
management practices that are important to seropositivity in Ontario broodmares. Additional studies on specific farm management practices on study farms may help identify important risk factors missed with our survey.

**Feeding practices:** Broodmares on farms that fed hay on the ground compared to those that did not were at a significantly greater odds of being seropositive for *N. hughesi*. Barling *et al.* (2001) found that beef cattle fed round bales using a simple hay ring were at higher risk of exposure to *N. caninum* compared to cattle fed hay in enclosed feeders. Feeding hay off the ground in contained feeders should reduce exposure to oocysts in the environment. Interestingly, feeding hay on the ground was found to be a risk factor for exposure to *N. hughesi*, but not *N. caninum*, in our study. This could imply that the definitive host of *N. hughesi* is more likely to interact with hay than canids currently do. Other exposure areas or modes of transmission may be more important for *N. caninum* in horses than method of feeding hay.

**Summary and Conclusions**

In this study, the seroprevalence estimates for *N. caninum* and *N. hughesi* were 27.4% (60/219) and 29.7% (65/219), respectively. The risk factors for *N. caninum* exposure included the presence of dogs, presence of livestock, and having a high stocking density. The only risk factor identified for *N. hughesi* was feeding hay on the ground in the paddock. Education of horse owners to these risk factors is needed to reduce exposure to these parasites. None of the variables found significant for exposure to *N. caninum* were significant risk factors for exposure to *N. hughesi*. This suggests that the epidemiology of exposure and specific lifecycles may be more
different than originally thought. Further investigation is needed into the definitive host and life cycle of *N. hughesi* to improve our understanding of the role of this parasite in equine disease.
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Chapter Three – Equine Abortion due to *Neospora caninum* in Ontario broodmares

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Abstract

Neosporosis is a common cause of abortion in cattle worldwide, however its role in equine abortions is unknown. Thirteen aborted equine fetuses were submitted to the study for routine postmortem examination and investigation of possible *N. caninum* infection. Samples of lung, heart, liver, kidney, brain, thymus, skeletal muscle, intestine, thoracic fluid, brain, and placenta were submitted for bacteriology, mycoplasmodiology/molecular biology, virology, and histology on selected tissues. All fetuses were late gestation abortions. None of the aborted fetuses or placenta were positive for *N. caninum* on PCR testing. The most common cause of abortion was bacterial placentitis (3/13; 23.1%) and idiopathic abortion (3/13; 23.1%), followed by EHV-1 and umbilical cord torsion (2/13; 15.4% for each diagnosis), flexural anomaly, hydrocephalus, and placental mineralization (1/13; 7.7% for each diagnosis).

3.1 Introduction

Neosporosis is an important disease of cattle worldwide, however its pathogenicity in horses is poorly understood (Dubey *et al.*, 1999; Lindsay and Dubey, 2020; Llano *et al.*, 2021; Moreira *et al.*, 2019; Nazir *et al.*, 2018). *Neospora caninum* and *N. hughesi* are two species known to infect horses. *Neospora hughesi* is a rare cause of neurological disease in horses, while *N. caninum* is best recognized for fetal loss in cattle (Dubey and Porterfield, 1990; Lindsay and Dubey, 2020; Pitel *et al.*, 2003; Villalobos *et al.*, 2006). *Neospora caninum* can infect a variety of both
Neospora caninum is one of the most efficient transplacentally transmitted parasites (Dubey & Schares, 2011). Transplacental infection allows N. caninum to infect a fetus causing either abortion or its persistence within a population (Mazuz et al., 2020; Paré et al., 1998; Schares et al., 1998). There is evidence of transplacental transmission of Neospora spp. in horses (Pitel et al., 2003; Pusterla et al., 2011). The epidemiology and pathology of N. caninum infection in equine abortions and transmission is not understood. Mares with a history of reproductive failure, such as abortion, are more likely to be seropositive for N. caninum than mares without a history of abortion (Abreu et al., 2014; Kliger et al., 2007 and Villalobos et al., 2006). Anderson et al. (2019) attributed an equine abortion in 2018 to N. caninum infection. In that case report, lesions in the fetus were found in the lungs and heart, in contrast to typical findings of meningoencephalitis in aborted calves (Anderson et al., 2000; Peters et al., 2001; Soler et al., 2022). Based on findings in Israel, Mazuz et al. (2020) suggested that N. caninum may be a significant cause of equine abortions (Mazuz et al., 2020). They reported finding evidence of N. caninum in 13 of 31 aborted equine fetuses in Israel using IHC screening of tissues with an anti-Neospora antibody and PCR. They also reported that 22 of 31 aborting mares were seropositive
for *N. caninum*, suggesting that *N. caninum* could be a cause of previously idiopathic equine abortion. Therefore, understanding the differences of *Neospora* infection between horses and cattle would provide valuable information regarding the epidemiology and role of *Neospora* in equine abortions. The objective of this study was to determine if *N. caninum* was present in equine abortions in Ontario.

3.2 Materials and Methods
This study examined equine fetuses and placentas in the study population (see Chapter 2) submitted during 2019, 2020, and 2021 seasons to the Animal Health Laboratory (AHL), Guelph, ON. Additional aborted fetuses were sought from the Ontario Veterinary College Large Animal Clinic, with client consent. A post-mortem examination of each aborted fetus and placenta (when available), was conducted to determine the cause of the abortion. Standard postmortem evaluation included measurement of the fetal weight, fetal sex, crown-rump and umbilical cord length, and any visible abnormalities, including congenital defects, were noted. The placenta was examined to determine the pregnant and non-pregnant horn, and the cervical star was located. Placental abnormalities and lesions were recorded. The fetal thoracic cavity was opened, and thoracic fluid collected in a sterile manner. The oral cavity and trachea were opened, and examined for lesions, including presence of a fibrin cast. The abdomen was opened, and a sample of stomach contents obtained. Appropriate samples were collected and submitted for bacteriology, mycoplasmaology/molecular biology, virology, and histology (Table 3.1) at the AHL. Additional tissue samples from the brain, liver, spleen, thymus, kidney, thoracic fluid and stomach contents were collected into whirl pack bags and stored in a -80°C freezer in case further tests were required. Samples collected for bacteriology, mycoplasmaology/molecular
biology, and virology were placed into Whirl pack bags labelled with the case ID, date, and intended testing (e.g., bacteriology). These Whirl pack bags were then chilled in a standard refrigerator and immediately sent to the intended laboratories. The samples collected for histology were immediately placed into a formalin filled bottle labelled with case ID, pathologist initials, date, and species. The bottle was sealed and placed on an agitator for 48 hours prior to histological preparation and examination.

Table 3.1: The types of tests applied to tissues from the aborted equine fetal submissions conducted by the AHL.

<table>
<thead>
<tr>
<th>Type</th>
<th>Post-Mortem Tissue Test</th>
<th>Tissues Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriology</td>
<td>Bacterial culture</td>
<td>Lung, stomach, and/or placenta</td>
</tr>
<tr>
<td>Mycoplasmology/molecular biology</td>
<td><em>Borrelia burgdorferi</em> and <em>Anaplasma phagocytophilum</em> PCR</td>
<td>Kidney and placenta</td>
</tr>
<tr>
<td>Mycoplasmology/molecular biology</td>
<td>Potomac fever PCR</td>
<td>Pooled placenta, lung, liver, and spleen</td>
</tr>
<tr>
<td>Virology</td>
<td><em>Equid alphaherpes virus</em></td>
<td>Pooled lung, thymus, liver, spleen, adrenal, placenta, thoracic fluid, kidney and brain</td>
</tr>
<tr>
<td>Virology</td>
<td><em>Neospora caninum</em> and <em>Leptospira interrogans</em></td>
<td></td>
</tr>
<tr>
<td>Virology</td>
<td><em>Alphaarterivirus equid</em> (Equine arteritis virus)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>NA</td>
<td>Formalin-fixed placenta including samples of cervical star and fetal tissues including eyelid, skeletal muscle, thyroid gland, thymus, lung, heart, liver, kidney, adrenal gland, spleen, jejunum, colon (with meconium), brain.</td>
</tr>
</tbody>
</table>

*PCR also conducted; NA is not applicable.
3.3 Results

A total of 13 fetuses were examined. Three of the aborting mares were from the study population and the remaining 10 were from other locations in Ontario, Canada. None of the submissions had lesions attributable to N. caninum infection. The diagnosis of the abortions included bacterial abortion, equine herpes virus – 1 (EHV-1), hydrocephalus, umbilical cord torsion, placental mineralization, congenital anomaly, and idiopathic abortion (Table 3.2). Idiopathic/undiagnosed abortions accounted for 25% of this group. Gestational stage information was only provided for 8 of the 13 submissions. All eight of the mares for which gestational stage was provided, aborted in late gestation (>320 days). The breeds of the aborting mares were Thoroughbred (6/13; 46.2%), Standardbreds(2/13; 15.4%), Belgian (1/13; 7.7%), and not reported (4/13; 30.8%). Serostatus to N. caninum and N. hughesi was only available for three mares; titers for all three mares were seronegative (<1:40) on IFAT for N. caninum.

Table 3.2: Summary of the diagnosis for 13 submitted aborted equine fetuses from 2019 – 2022.

<table>
<thead>
<tr>
<th>Cause of Abortion</th>
<th># of abortions (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial abortion*</td>
<td>3 (23.1)</td>
</tr>
<tr>
<td>Congenital anomaly†</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>EHV-1</td>
<td>2 (15.4)</td>
</tr>
<tr>
<td>Hydrocephalus</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Idiopathic/no cause identified</td>
<td>3 (23.1)</td>
</tr>
<tr>
<td>Placenta mineralization</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Umbilical cord torsion</td>
<td>2 (15.4)</td>
</tr>
</tbody>
</table>

*One abortion was due to Streptococcus equi zooepidemicus placentitis, another had streptococci in the lung and stomach but had no signs of placentitis, and the third was due to Staphylococcus schleiferi subspecies coagulans placentitis.
3.4 Discussion

Neosporosis was not identified as a cause of abortion in this limited case series, and no evidence of *N. caninum* was found in any of the fetuses. The PCR test used to detect *N. caninum* in the fetal tissues is one utilized in a bovine abortion diagnosis panel and is not equine-specific. However, the parasite infecting horses is presumed to be the same species infecting cattle. *Neospora caninum* causes abortion in cattle through transplacental infection of the fetus with tachyzoites (Mazuz et al., 2014; Paré et al., 1996; Schares et al., 1998). There is also evidence of transplacental transmission of *Neospora* spp. in horses (Pitel et al., 2003; Pusterla et al., 2011). However, the role of *N. caninum* infection in equine abortions and transmission is not well understood and no evidence was identified here.

A cause could not be identified in 23.1% (3/13) of the abortions. Other studies reported similar percentages of idiopathic/undiagnosed equine abortions (Agerholm et al., 2021; Macleay et al., 2022; Roach et al., 2021). The proportions of idiopathic abortions have been reported to be 11.2% (Roach et al., 2021), 18.8% (Denmark; Agerholm et al., 2021), 28.8% (Macleay et al., 2022), and 40.9% (Macleay et al., 2022) of cases depending on stage of gestation. Similar to other equine abortion studies, EHV-1 infection and umbilical cord torsion were commonly identified causes of abortion in our study (Foote et al., 2012; Laugier et al., 2011; Lunn et al., 2009; Schulman et al., 2015; Smith et al., 2003; Snider, 2007).

The present study was limited due to the low number of abortion submissions during the COVID-19 pandemic years. *Neospora caninum* was recently documented to cause abortions in horses (Abreu et al., 2014; Kliger et al., 2007; Mazuz et al., 2020). Mazuz et al. (2020) found *N. caninum* DNA in 13 of 31 (41.9%) aborted equine fetuses in Israel using IHC and PCR screening of tissues with an anti-*Neospora* antibody. They also reported that 22 of 31 (70.9%) aborting
mares were seropositive for *N. caninum*. The seroprevalence in Israel has recently been reported to be as high as 70.9% (Mazuz et al., 2020), while the seroprevalence of horses in Ontario, Canada was found to be 27.4% as mentioned above. Anderson et al. (2019) also documented that *N. caninum* infection in aborted equine fetuses resulted in lesions different from those in aborted bovine fetuses. Pneumonia and myocarditis were the predominant histopathological lesions in the equine fetuses found positive for *N. caninum*. This contrasts with cattle, in which lesions are typically found in the fetal brain and heart (Dubey et al., 2017). An awareness of the differences between species in presentation will assist pathologists in recognizing potential cases of *N. caninum* abortion in horses.

The current study did not detect *N. caninum* by PCR from any of the aborted equine fetuses, and no histological lesions consistent with neosporosis were identified in any tissues. In agreement with previous studies, the cause of a large proportion (25%) of abortion cases were undiagnosed, confirming the need to investigate more potential causes of equine abortions. Ongoing screening of all equine abortions submitted to diagnostic laboratories for the presence of *N. caninum* DNA is suggested, to determine the prevalence of *Neospora* in equine abortions.
References


https://doi.org/10.1016/j.vetpar.2007.06.002


https://doi.org/10.1016/j.cvfa.2019.11.004

https://doi.org/10.1016/j.actatropica.2021.105970


https://doi.org/10.1016/j.vetpar.2016.01.006
Chapter Four: Summary and Future Directions

The objective of this study to investigate the seroprevalence and risk factors for exposure to \( N. caninum \) and \( N. hughesi \) was accomplished. Establishing the presence of \( N. caninum \) and \( N. hughesi \) in this population of Ontario broodmares provided important information regarding the epidemiology of both protozoans. In this study, about half of the farms had at least one seropositive broodmare, and almost one-third of broodmares were seropositive for Neospora sp. This suggests widespread exposure to this parasite in the Ontario horse population. Based on the limited number of fetuses examined, neosporosis was not identified as the cause of abortion in this study population of Ontario broodmares. Similar to cattle, presence of farm dogs, high stocking density, and feeding practices were identified as significant risk factors for exposure. Educating horse owners about the parasite and management practices to reduce their horse’s exposure is needed.

The limitations of this study include the use of a self-reported owner questionnaire and a limited number of abortion submissions. The responses from self-reported questionnaires are impacted by misclassification bias. Participants may not accurately recall how often something on the farm is done or occurs such as seeing wild canids on the property. Owners may also under-report the presence of rodents out of embarrassment. There is also concern with the questionnaire respondents, as their involvement in the day-to-day operations on the farm varied. One respondent was the daughter of the farm owners, who in recent years had reduced her involvement in the farm operations. The questionnaire was offered online, by phone, and in-person with a print-out. Telephone or in-person questionnaires may result in respondents feeling pressured to answer management questions with a perceived desired response, rather than the
true cleaning/management protocols of the farm (e.g., misclassification bias). This could cause the results to be biased towards the null (i.e., underestimates the true effect). In addition, non-response bias (i.e., selection bias) should also be considered. For instance, individuals who experience abortions and have poorer management practices might be less willing to participate in the study. However, if this scenario occurred our results would be biased toward the null (i.e., underestimates of the true effect).

Surveys are limited in the ability to capture all possible responses which could potentially introduce confounding bias with variables and/or could lead us to miss analysing an important independent variable. Participants of the survey were encouraged to leave additional comments, which highlighted some areas of confusion. For example, one question asked participants where they store their feed, with the intention of asking about their hay. However, feed could have been interpreted as supplemental grain, grain as the only source of feed given, or hay. It was also noted that some farms reported that they house horses differently according to season, which the survey did not account for. Some farms reported bringing horses in at night and feeding grain twice a day. Participants also additionally noted the presence of cats, wild rabbits, and wild turkeys. One farm was in proximity of a popular walking trail where people frequently let their dogs off leash and can be seen near the horses or in the pens. This study was not able to investigate the association between seropositivity and having a pond in the paddock due to small sample size as only one farm reported having a pond. The questionnaire also did not ask about the disposal protocols of placentas following abortions or foaling, or the foaling environments. Future studies examining risk factors for exposure should consider these factors when designing questionnaires.
Additional studies should investigate the seroprevalence and risk factors for exposure to both species of *Neospora* in the general horse population within Canada. Further investigation into the role of *N. caninum* in equine abortion is also warranted, as this study was limited by the low number of aborted fetuses submitted. Future studies examining aborted fetuses from known seropositive mares would provide additional evidence of the role of *N. caninum* in abortions and the characteristics of vertical transmission in horses. Collection of a serum sample from any aborting mare could be valuable in understanding infection and titres of *N. caninum* during an active abortion. The development and use of an equine specific *N. caninum* PCR test to screen all equine abortions for the parasite may aid in the diagnosis of cases. Development of specific IHC testing of tissues would provide further evidence of *N. caninum* in equine abortions. A geographical investigation with reference to the location of opossums in Ontario could be beneficial in understanding definitive hosts of *N. caninum*, as some areas of Ontario do not have this potential host species. Investigating the efficacy and relationship of cattle farms, who vaccinate for *N. caninum*, and the relationship with horses and cattle herds being seropositive would advance the knowledge of the interaction of this protozoa with both animal populations. Seroprevalence and abortion studies of *N. caninum* should also consider testing the farm dogs for infection. Active screening of canids to determine their serostatus for *N. hughesi* is recommended to rule out canids as a definitive host. This is supported from the dual infection information from this study, with positive farms dropping from 49.2% and 52.4% for *N. caninum* and *N. hughesi* respectively, to 19.0% dual infection. For total broodmares positive, it dropped from 27.4% and 29.7% for *N. caninum* and *N. hughesi* respectively, to 9.1% dual infection. This suggests that the lifecycle and definitive host for *N. hughesi* is more different from *N. caninum* than originally thought. Further studies should target other carnivore domestic species to
potentially identify *N. hughesi*'s definitive host. There is a need to establish the definitive host of *N. hughesi*, as it remains elusive.
Appendix A

Appendix 1: Locations of Ontario farms enrolled in the study (n=63).

<table>
<thead>
<tr>
<th>Reported Location (ON)</th>
<th># of Farms</th>
<th># of Horses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addison</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Arva</td>
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<td>1</td>
</tr>
<tr>
<td>Aurora</td>
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<td>1</td>
</tr>
<tr>
<td>Brantford</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Burford</td>
<td>2</td>
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</tr>
<tr>
<td>Burgessville</td>
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<td>1</td>
</tr>
<tr>
<td>Caledon</td>
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<tr>
<td>Caledonia</td>
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<td>6</td>
</tr>
<tr>
<td>Cayuga</td>
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<td>2</td>
</tr>
<tr>
<td>Dorchester</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Erin</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Forest City</td>
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<td>7</td>
</tr>
<tr>
<td>Gananoque</td>
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<td>5</td>
</tr>
<tr>
<td>Grimsby</td>
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<td>1</td>
</tr>
<tr>
<td>Guelph</td>
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</tr>
<tr>
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<td>3</td>
</tr>
<tr>
<td>Ingersoll</td>
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<td>1</td>
</tr>
<tr>
<td>Kendal</td>
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<td>4</td>
</tr>
<tr>
<td>Kettleby</td>
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</tr>
<tr>
<td>King</td>
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<tr>
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<tr>
<td>Paris</td>
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<tr>
<td>Port Perry</td>
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<td>6</td>
</tr>
<tr>
<td>Puslinch</td>
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 Appendix 2: Counties of Ontario farms enrolled in the study across Ontario (n=63).

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<tr>
<th>County in Ontario</th>
<th># of farms</th>
<th># of Horses</th>
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<tbody>
<tr>
<td>Brant</td>
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<tr>
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<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Haldimand</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Halton</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Lambton</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Leeds and Grenville</td>
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<td>11</td>
</tr>
<tr>
<td>Lincoln</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Middlesex</td>
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<td>2</td>
</tr>
<tr>
<td>Norwich</td>
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<td>1</td>
</tr>
<tr>
<td>Region</td>
<td>Value 1</td>
<td>Value 2</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Ontario</td>
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<tr>
<td>Oxford</td>
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<td>3</td>
</tr>
<tr>
<td>Parry Sound</td>
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<td>6</td>
</tr>
<tr>
<td>Prescott and Russell</td>
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<td>6</td>
</tr>
<tr>
<td>Simcoe</td>
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<td>9</td>
</tr>
<tr>
<td>Wentworth</td>
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<td>1</td>
</tr>
<tr>
<td>York-Durham</td>
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<td>3</td>
</tr>
<tr>
<td>Peel</td>
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<td>21</td>
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<td>Wellington</td>
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<td>31</td>
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<tr>
<td>York</td>
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