The Epidemiology of *Borrelia burgdorferi* in Horses in Ontario and Assessment of Serologic Testing Methods

by

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ABSTRACT

The Epidemiology of *Borrelia burgdorferi* in Horses in Ontario and Assessment of Serologic Testing Methods

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The objectives of this thesis were to determine the seroprevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* antibodies in horses in Ontario, identify demographic and management factors associated with *B. burgdorferi* seropositivity and compare the ability of a point-of-care ELISA to a Multiplex ELISA to detect *B. burgdorferi* antibodies over time. Serum samples from 551 horses from 76 veterinary clinics were submitted along with 473 accompanying questionnaires filled out by the owners of the horses. The overall prevalence of *B. burgdorferi* seropositivity for horses on at least one test was 17% (91/551). The odds of being seropositive were increased by the presence of oak trees near the horses' pastures. A high-risk cluster was also identified in Eastern Ontario. Performing regular checks for ticks was associated with decreased odds of seropositivity. Agreement between the two ELISA tests was fair (kappa=0.23) and horses remained seropositive one year after initial testing on both tests.

DEDICATION

This thesis is dedicated to my parents, for their unconditional support I could not have done this thesis without.

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STATEMENT OF WORK

The preparation of this thesis was the sole responsibility of Megan Neely.

Assistance with serological testing of samples was provided by Michelle Beaudoin-Kimble, research technician in Clinical Studies at the Ontario Veterinary College. Serum samples were submitted to the Cornell Animal Health Diagnostic Center for laboratory testing in Ithaca, New York.

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LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

AIC	Akaike information criterion
A. phagocytophilum	*Anaplasma phagocytophilum*
BLUP	Best linear unbiased predictor
B. burgdorferi	*Borrelia burgdorferi*
CI	Confidence interval
ELISA	Enzyme-linked immunosorbent assay
GIS	Geographic information system
ICC	Intraclass correlation coefficient
OR	Odds ratio
Osp	Outer surface protein

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CHAPTER 1: LITERATURE REVIEW AND RESEARCH OBJECTIVES

1.1 *BORRELIA BURGDORFERI*

1.1.1 Life cycle

Lyme disease is caused by a bacterial pathogen, *Borrelia burgdorferi*, and is transmitted by ticks; the primary tick vector in eastern North America is *Ixodes scapularis* (Burdorfer et al., 1982). *Ixodes scapularis* tick has four life stages; egg, larva, nymph and adult. These stages are maintained in a two-year enzootic life cycle (Berger et al., 1995). Tick larvae are uninfected when they hatch, as there is no transovarial transmission of *B. burgdorferi* (Piesman et al. 1986; Magnarelli et al. 1987). Ticks become infected as larvae or nymphs when they feed on infected reservoir hosts (small rodents, squirrels, birds) and acquire the *B. burgdorferi* bacterium (Diuk-Wasser et al., 2006; Mather and Mather, 1990). The acquired spirochetes are retained throughout the successive development stage or moult (Kurtenbach et al., 2006; Piesman and Schwan, 2010) and can be transmitted to the host of their next blood meal (Mather and Mather, 1990). Ticks in the nymphal stage feed on a similar range of hosts as larvae, which perpetuates the enzootic transmission cycle of spirochetes into hosts to become reservoirs for the next generation of ticks (Kurtenbach et al., 2006).

Reservoir species commonly include migratory birds and rodents (Kurtenbach et al., 2006; Piesman and Schwan, 2010). Migratory birds are largely responsible for carrying the bacterium across distances to new regions (Kurtenbach et al., 2006; Piesman and Schwan, 2010). The majority of adult ticks feed on large mammals such as deer, dogs, horses and humans which are not generally responsible for perpetuating the bacterial life cycle and are considered

incidental, dead-end hosts and are not a part of the enzootic cycle of *Ixodes* ticks (Kurtenbach et al., 2006; Piesman and Schwan, 2010).

1.1.2 **Microbiology**

Ticks can harbour and transmit a wide range of pathogens simultaneously, some of which have the ability to produce long term infections if untreated or can establish new infections with re-exposure (Beall et al., 2008; Hodzic et al., 1999; Krause et al., 2006; Otranto et al., 2009). Burgdorfer et al. (1982) discovered a spirochete in *I. scapularis* and proved it to be the etiological agent of Lyme disease, which was subsequently named *Borrelia burgdorferi* (Burgdorfer et al.,1982; Johnson et al., 1984). *Borrelia burgdorferi* belongs to the eubacterial phylum of spirochetes (Burgdorfer et al.,1982). Members of this phylum share a distinct morphology that includes corkscrew-shaped bacteria with flagella between the inner and outer membranes to increase motility (Burgdorfer et al., 1982; Fraser et al., 1997; Zhang and Norris, 1998). The bacterial cell wall has a cytoplasmic membrane with many sequence-encoded lipoproteins which includes the outer surface proteins (Osp's) named A through F, which are differentially expressed (Fraser et al., 1997; Zhang and Norris, 1998). *Borrelia burgdorferi* possesses one of the most complex genomes of known bacteria (Casjens et al., 2010; Fraser et al., 1997), with a genome size of 910,725 linear chromosomal base pairs (Fraser et al., 1997). The bacterium lacks mechanisms to synthesize amino acids, nucleotides and fatty acids necessitating acquisition of these from a host. It also does not seem to require iron, an unusual feature in bacteria (Gheradini et al., 2010; Posey and Gherardini, 2000; von Lackum and Stevenson, 2005). A unique feature of the outer membrane of *B. burgdorferi* is the large number and variety of the lipoproteins on the outer surface of the spirochete and their differential

expression (Bergstrom and Zuckert, 2010). The translocation and pathways for directing lipoproteins across the outer membrane to the outer surface are not well understood (Bergstrom and Zuckert, 2010). The spirochete also undergoes extensive transcriptional changes throughout the enzootic cycle (Samuels, 2011; Skare et al., 2010). The influx of blood during the larvae blood meal triggers the spirochetes to replicate; a process that continues for weeks during the moult (Pal and Fikrig, 2010). During the nymphal blood meal, bacterial replication and distribution reach their peak at 48 hours (de Silva and Fikrig 1995; Dunham-Erms et al., 2009; Ohnishi et al., 2001; Pal and Fikrig, 2010; Piesman et al., 2001). Host factors in the tick saliva that improve the survival of *B. burgdorferi* in the vertebrae are transmitted into the tick bite including the bacterial protein OspC, which is crucial for the establishment of an early *B. burgdorferi* infection (Pal et al., 2004; Grimm et al., 2004; Tilly et al., 2006).

The bacterium can adapt to changing host environments and evade the defense mechanisms of the mammalian host (Radolf et al., 2012). The innate and acquired immune responses are imperative for controlling infection and the inflammatory response, and development of a humoral response is required to clear *B. burgdorferi* in the host (Connolly and Benach, 2005). Antigenic variation is one system utilized by *B. burgdorferi* to evade antibodies as well as employing mechanisms to suppress host inflammatory responses and limit tissue damage to increase the chances of the infection to persist in the host, though little is understood about the mechanisms and processes that occur in the mammalian host during late infection (Kraiczy et al., 2004; Norris, 2006; Petnicki-Ocwieja et al., 2011; Zhang et al., 1997).

1.1.3 Transmission

Although *B. burgdorferi* spirochetes have also been demonstrated in vectors such as deerflies, horseflies, and mosquitoes, transmission of the bacterium into the mammalian host occurs from the bite of an infected tick (Burgdorfer, 1984). *Borrelia burgdorferi* spirochetes are deposited into the bite wound through the tick saliva during feeding (Riberio et al., 1987). In contrast to nymphal ticks, larvae acquire spirochetes from reservoir hosts rapidly; bacteria can be detected in the midgut of larval ticks within 24 hours and are in high numbers by 48 hours (Schwan and Piesman, 2000). However, when the nymphal tick attaches to a new host, transmission to the host is less rapid as the tick must be attached for 48 hours for replication of the bacteria to occur and increase the ability to transmit the spirochetes (Piesman et al., 1987). If the nymph has only been attached for 24 hours, infection is less likely to occur (Piesman et al., 1987). The transmission process from the tick occurs when the tick attaches to a susceptible host, the *B. burgdorferi* spirochetes replicate and penetrate the tick gut wall and then migrate to the salivary glands (Ribeiro et al., 1987). This process prior to dissemination takes approximately 48 hours, at which time the pathogens are acutely susceptible to damage and destruction by antibody mediated mechanisms of the host (Ackerman et al., 1981; Piesman et al., 1987). Once tick feeding begins, the bacterium regulates certain genes up or down to allow transmission and survival by enhancing their ability to evade immune responses (Pal et al. 2001; Kraiczy et al. 2002).

There is a major change in expression of the outer surface proteins of *B. burgdorferi* from OspA to OspC during the change in location from the tick midgut to the salivary glands (de Silva et al., 1996; Schwan et al., 1995). This shift in protein expression facilitates the migration of the

bacterium to the salivary glands, so that the spirochetes can be deposited into the host (Pal et al., 2000; Schwan and Piesman, 2000). During the blood meal, aspects of the tick gut change such as temperature and pH, which influences the expression of OspC (Carroll et al., 1999; Ojaimi et al., 2003; Revel et al., 2002; Schwan et al., 1995).

1.1.4 Changes in tick populations

The existence of endemic regions of *Ixodes* tick species, and the establishment of new populations, is constrained by factors including habitat and climate (Gray, 2002). These factors affect not only tick survival rates but also mating and feeding rates as temperature and humidity influence tick activity (Vall and Smith, 1998). In the early 1990's, Long Point, Ontario was the only known endemic region of *I. scapularis* (Barker et al., 1992). Passive surveillance and detection of human cases by clinicians has subsequently identified *I. scapularis* in multiple regions of Ontario, and in Nova Scotia, Manitoba, and New Brunswick, and *I. pacificus*, the main vector for *B. burgdorferi* in western North America, has been identified in British Columbia (Holden et al., 2006; Nelder et al., 2014; Ogden et al., 2005). The emergence of Lyme disease risk follows a progressive pattern, which is consistent with warming temperatures, as tick population establishment is dependent on temperature suitability (Ogden et al., 2008b; Sperling, et al., 2012). Climate change has been predicted to alter the geographic distribution of Lyme disease in Canada as statistical modelling has suggested that the geographic distribution of *I. scapularis* is extending north as the climate changes (Brownstein et al., 2005; Ogden et al., 2008).

Lyme disease in the USA occurs in highest incidence in the northeastern states, that border highly populated areas in Canada. Birds migrating northward in the spring carry ticks across into Canada (CDC, 1999; Scott et al., 2001). *Ixodes scapularis* follows a northward expansion trend, which may lead to increased transmission risk for *B. burgdorferi* and other tick-borne pathogens such as *Anaplasma phagocytophilum* (Root et al., 2003; Thompson et al., 2001). Other factors that may further the spread of *I. scapularis* into Ontario and Canada are the number and range of habitats with high densities of suitable reservoirs and hosts throughout southeastern Canada, in addition to the increasing temperatures that could potentially allow establishment of tick populations (Banfield, 1977; Gallivan et al., 1998; Ogden et al., 2005). In model simulations, temperature has been determined to be the main factor in tick development rates in colder climates, rather than day-length as previously thought (Ogden et al., 2004). The average annual days above $0^{\circ C}$ has been used as the index to determine suitable temperature conditions to map the potential geographic range of tick establishment in Canada (Ogden et al., 2005). As the number of days per month above $0^{\circ C}$ has been increasing throughout the winter months in Ontario, it is expected that tick activity will be increasing in those months as well (Ogden et al., 2005).

As mentioned above, to become successfully established in new regions, ticks require hosts for feeding and suitable habitats. Preferred habitats include deciduous forests, particularly containing oak and maples trees with significant leaf litter (Curtis, 1959), as well as soils with good drainage such as sand (Curtis, 1959; Hole, 1976; Kitron et al., 1992). The main host for adult ticks are white-tailed deer (*Odocoileus virginianus*) and for larvae are white-footed mice (*Peromyscus leucopus*) (Lane et al., 1991). The movement of adult *I. scapularis* ticks primarily

by deer and birds increases the dispersal of the ticks (Ogden et al., 2008a; Piesman et al., 1979). Birds expand the range of infected ticks, especially during spring and fall migrations (Ogden et al., 2008a). As temperatures are now increasing earlier in the spring than previous years, larva-to-nymph development happens earlier in the year. Warmer temperatures mean larval ticks are able to feed and moult into host-seeking nymphs by early autumn of the same year they hatch (Ogden et al., 2006).

In Ontario, the prevalence of *B. burgdorferi* infected ticks has been found to be increasing over recent years (Clow et al., 2016; Nelder et al., 2014). Passive surveillance of ticks submitted by the public from 2008-2012 found that the prevalence of infected *I. scapularis* ticks increased from 8.4% in 2008 to 19.2% in 2012 (Nelder et al., 2014). The largest increases were seen in the central west region followed by the eastern and south-west regions of Ontario. As well, there was markedly more infected tick submissions from two health units in eastern Ontario in comparison to the other health units in the province (Nelder et al., 2014). More recently, research by Clow et al. (2016) identified eastern Ontario as a "hot spot" for *B. burgdorferi* infected *I. scapularis* ticks, likely due to significant spread of the tick by wildlife and the amount of preferred habitat for survival in the region (Clow et al., 2016). *Anaplasma phagocytophilum* has also been found in *I. scapularis* in the province, but at a much lower prevalence than *B. burgdorferi.* Passive surveillance found only 0.3% of ticks were infected with *A. phagocytophilum* in Ontario (Nelder et al., 2014).

1.2 EPIDEMIOLOGY OF LYME DISEASE

1.2.1 In Humans

Lyme disease is the most commonly diagnosed vector-borne disease in both the United States and Canada (Lane et al., 1991). Due to the increased transmission risk and the improved awareness of Lyme disease, there are close to 300,000 human cases of Lyme disease that are reported in the USA each year, of which 93% are reported from 10 northeastern and midwestern states (Bacon et al., 2007; Bacon et al., 2008; Hinckley et al., 2014). The risk of Lyme disease for dogs and humans varies by region and is related to the presence of infected ticks (Eng et al., 1988; Kitron and Kazmierczak 1997; Walker et al., 1998). Early *B. burgdorferi* infections in humans can be recognized by an expanding erythema migrans skin lesions, which develops at the site of the tick bite in approximately 70-80% of cases (Bacon et al., 2008). The presentation of erythema migrans usually occurs before seroconversion, and early serologic testing can be unreliable and insensitive as antibodies can take several weeks or months to develop, making diagnosis more difficult (Mikkila et al., 1999; Oksi et al., 1995). Persistent infections can progress to randomly disseminated infections, with varying clinical signs. Lyme disease is associated with polyarthritis, carditis and neurologic disease (Imai et al., 2011). Serologic testing tends to be more sensitive and specific for humans with later stage Lyme disease (Bacon et al., 2003; Steere et al., 2008).

Diagnosis of Lyme disease requires examining each patient's clinical picture which may include if the patient presents with erythema migrans, and if they have been exposed to ticks where the disease is endemic, as well as serological testing (Ogden et al., 2009). Two-tiered

serologic testing is recommended by public health agencies in North America and Europe (CDC, 1995 & 2007; CMAJ, 1991; Canadian Public Health Laboratory Network, 2007). This process includes using an enzyme-linked immunosorbent assay (ELISA) followed by Western blot which increases the specificity of using an ELISA alone (CDC, 1995). A C6-based assay is also approved as a one-tier alternative to whole-cell antigen-based assays, as studies show this approach has superior sensitivity and specificity and eliminates the need for additional serologic testing, though is used less frequently than the two-tiered approach (Weinstein, 2008).

Currently there is no vaccine available for humans; however, proper prevention against tick attachment such as wearing long sleeves and pants, applying N,N-diethyl-meta-toluamide (DEET) repellents and permethrin-treated clothing can reduce the risk of exposure (Stafford III, 2007). The duration of tick attachment increases the risk of infection so prompt removal of the tick remains one of the most pragmatic disease prevention methods in all species (Stafford III, 2007).

1.2.2 **In dogs**

In tick-endemic regions, serological estimates of *B. burgdorferi* exposure in dogs range from 40-89% (Burgess, 1986; Cohen et al., 1990; Levy and Magnarelli, 1992). Canine exposure rates may be used as an indicator of the risk for human exposure as the likelihood of a dog becoming exposed is high in endemic regions and canine exposure has been found to mimic the geographic distribution reports of illness in humans (Bowman et al., 2009; Duncan et al., 2004; Hinrichsen et al., 2001). In Lyme endemic regions, 70-90% of all dogs, clinically healthy or ill, are seropositive, making diagnosis difficult (Frank et al., 1989; Magnarelli et al., 1987; 1990).

Clinical signs develop in approximately only 5% of exposed dogs; most infected dogs are clinically normal (Cohen et al., 1990; Levy and Magnarelli, 1992). Shifting or single limb lameness, swollen joints, and fever are the most common clinical signs (Levy et al., 1992). A study by Levy et al. (1992) found less than 5% of seropositive dogs developed lameness during a 20-month observation period. Genetic susceptibility, immune reactions, or repeated exposures to the bacterium may all be factors that determine which dogs will develop clinical signs (Greene et al., 1998). The incidence rates of less common clinical signs including myocarditis and renal failure (Lyme nephropathy) are unknown, due to the difficulty of determining a definitive diagnosis (Roush et al., 1989; Sigal, 1992). Diagnosing Lyme disease continues to be a challenge as a positive antibody result to *B. burgdorferi* cannot diagnose an active infection or predict onset of clinical disease (Littman et al., 2006). Veterinarians must consider many variables for diagnosis including evidence of exposure to ticks, presentation of clinical signs, serology confirmed antibody presence, response to antibiotic treatment and a decline in antibody concentrations in repeat testing (Littman et al., 2006). Other tick-borne pathogens such as *A. phagocytophilum* can have similar clinical presentations and should be considered as a differential diagnosis (Littman et al., 2006).

Naturally infected dogs develop antibodies against OspA and B as well as many other proteins as shown with Western blot analysis (Levy and Magnarelli, 1992). The banding patterns seen on Western blot cannot distinguish between natural infections and vaccination response due to OspA antibodies being temporally expressed in some chronically infected dogs (Conlon et al., 2000; Levy et al., 1993; Shin et al., 1993). In a study by Appel et al. (1993) experimentally infected dogs seroconverted by 4-6 weeks, developed intermittent lameness by 2-4 months after

injection, and antibodies persisted for more than a year post infection. This model is predicted to closely follow trends seen in naturally infected dogs (Appel et al., 1993). Several canine vaccines exist, with estimated efficacies ranging from 50-85%, with a preventable fraction of >90% in a field trial by Levy (2000). The antibodies the vaccines induce do not interact with the C6-peptide used in serologic testing, however, whole-cell vaccines induce antibodies detectable with whole cell ELISA and IFA (Green and Straubinger 2006; O'Connor et al., 2004).

Two canine vaccines are available in Canada for use in dogs. The first is a purified OspA vaccine that is designed to prevent mobilization of *B. burgdorferi* to the salivary gland within the tick vector and preventing infection to the host (Sadziene and Barbour, 1996; Conlon et al., 2000). Research by Eschner and Mugnai (2015) found this vaccination reduced the incidence of seroconversion of *B. burgdorferi* in dogs living in an endemic region in Maine, USA (Eschner and Mugnai, 2015). The second type is a multivalent vaccine which uses a combination of OspA, OspB, and/or OspC which act both inside the tick to prevent migration of *B. burgdorferi* and within the canine host by inducing bactericidal antibody (Earnhart and Marconi, 2007; LaFleur et al., 2009).

Monitoring the distribution of infected ticks and detecting antibody responses in dogs can assist with surveillance for detection of movement and establishment of new endemic tick regions in previously non-endemic areas (Little et al., 2010). Risk factors include the amount of area/range the dog covers outside, especially if used for hunting or herding. Pet dogs with a limited range outside have a lower risk of exposure, regardless of time spent outside (Guerra et al 2001). As well, tick exposure has been reported to be higher in forested and urban areas with trees and leaf litter, than agricultural grasslands (Guerra, 2001). Knowing these environmental

risk factors and the associated geographic distribution as well as obtaining information from dogs as a surveillance tool may help to develop prevention and control methods for humans (Guerra, 2001).

1.3 LYME DISEASE IN HORSES:

1.3.1 Epidemiology

The increasing incidence and geographic distribution of human and canine Lyme disease has raised concerns among horse owners and equine practitioners about the risk of exposure to *B. burgdorferi* for horses (Chang, 2000). As grazing animals, horses are continuously at risk for exposure to tick bites (Laus, 2012). Horses provide a plentiful supply of nutrients to adult *I. scapularis* ticks and the occasional nymph (Laus, 2012). Horses from endemic areas may have serologic evidence of exposure without clinical signs. Seroprevalence ranging from 0.2-45% has been reported in the United States and from 6-29% in various European countries (Cohen et al., 1992; Magnarelli et al., 2000; Evengall et al., 2001; Bhide et al., 2008; Laus et al., 2013; Stefanciková et al., 2008; Ebani et al., 2012; Hansen et al., 2010). Prevalence in Ontario has not been reported but is expected to follow canine and human trends; however, seroprevalence in horses may be higher than in humans due to the increased likelihood that a tick will remain attached to a horse for greater than 48 hours, and that horses spend more time outside than people (Chandrashekar, 2008). Simultaneous infection with multiple tick-borne pathogens may account for some of the clinical variations seen in comparison to infections with a single pathogen; however, further research is necessary to understand co-infections in horses.

1.3.2 **Clinical Signs/Diagnosis**

In horses, Lyme disease can be particularly difficult to diagnose which can result in potential misdiagnoses and health risks including inappropriate antibiotic treatment (Chandrashekar, 2008; Manion et al., 2001). A variety of non-specific clinical signs can mimic other equine diseases and reflects the disseminated nature of the bacteria (Hagstoz, 2001). Additionally, clinical signs are often sparse, and many infected horses do not show obvious clinical signs (Divers, 2007). Commonly associated clinical signs of equine Lyme disease are poor performance, shifting lameness, fever, and general discomfort/inability to work or perform (Manion et al., 2001). Clinical data implicating lameness as a syndrome of Lyme disease though is currently lacking in horses (Divers, 2007). Musculoskeletal injuries and diseases resulting in lameness are highly prevalent in equines due to their athletic purposes and anatomical predispositions. Therefore, diagnosing Lyme disease based solely off a clinical presentation of lameness is difficult and requires extensive examinations to eliminate other sources of injury or soreness (Bushmich, 1994).

Clinical manifestations of *B. burgdorferi* infection in horses also include neuroborreliosis and uveitis (Imai et al., 2011; Priest et al., 2012). Equine uveitis, one of the most common causes of blindness, has also been associated with *B. burgdorferi* infections (Priest, 2012). Polymerase chain reaction (PCR) confirms identification of spirochetes within aqueous and vitreous humor in horses suspected of clinical Lyme disease with ocular lesions (Priest, 2012). Uveitis can present as primary (single episodes) or recurrent and is the most common extraneural manifestation of Lyme disease in horses (Burgess et al., 1986; Hahn, et al., 1996; Hollingsworth, 2011; Imai et al., 2012; Priest et al., 2012;).

Borrelia burgdorferi infections have also been reported to impact the equine central nervous system. Cases of both encephalitis and neuroborreliosis have been studied (Burgess et al., 1987, Imai et al., 2012). Equine neuroborreliosis is difficult to diagnose, however if histological patterns associated with meningitis, myocarditis and radiculoneuritis are recognized, neuroborreliosis should be considered as a possible diagnosis (Imai et al., 2011). However, the reliability of diagnostic testing is poor, adding to the difficulty of making a definitive diagnosis for neuroborreliosis. Horses that present with weight loss, ataxia, cranial nerve deficits and a history of uveitis should be considered as possible cases of neuroborreliosis (Imai et al., 2011).

Horses can be both a dead-end host and chronic carrier of spirochete, but the presence of antibodies does not necessarily indicate disease, rather that exposure has occurred (Hagstoz, 2001). Subclinical infections appear to occur in the equine population within high incidence rates in endemic areas for Lyme disease (Manion et al., 2001). Clinical diagnosis of Lyme disease in horses relies on physical examinations, often including radiographs and nerve blocking to eliminate other sources of lameness, as well as extensive neurological examinations (Bushmich, 1994; Manion et al., 2001). Positive serological test results can aid in confirmation of *B. burgdorferi* infections, however, seropositivity may not differentiate between past or current exposure and diagnosis of an active infection remains enigmatic (Egenvall et al., 2001; Bushmich; 1994).

1.3.3 **Treatment**

Diagnostic testing for Lyme disease can be costly and is often a factor in a veterinarian and owner's decision making for disease confirmation and treatment options (Johnson et al.,

2008). Veterinarians therefore may use response to antibiotic therapy as part of the diagnostic work up, marking clinical improvements post-antibiotic treatment as an indicator of disease status. This approach is not reliable as common antibiotics used for treatment of *B. burgdorferi* infections have anti-inflammatory effects (Johnson et al., 2008; Sapadin and Fleischmajer, 2006). Antibiotics commonly used are drugs in the tetracycline and penicillin families, including doxycycline and oxytetracycline (Divers, 2007; Levy and Magnarelli, 1992). As a structural isomer of tetracycline, with greater lipid solubility doxycycline is a broad spectrum, antimicrobial agent that is a common choice when treating equine cases (Riond and Riviere, 1988; Walker, 1992; Wilson et al, 1988). Doxycycline inhibits protein synthesis and has been found to have an increased and rapid absorption and distribution rate after oral administration and slow elimination from the body (Aronson, 1980; Jha et al., 1989; Olemapenay and Mitema, 1995; Riond et al., 1990 Riond and Riviere, 1988; Wilson et al., 1988; Ziv and Sulman, 1974). Intravenously administered oxytetracycline has been found to be more efficacious in comparison to orally administered doxycycline (Chang et al., 2005). The practicality of owners being able to treat their horse with intravenous antibiotics is questionable and it may be more feasible for an oral antibiotic such as doxycycline to be used. Oral trimethoprim-sulpha antibiotics have also previously been used as possible treatment, however, studies have shown antibiotic resistance in *B. burgdorferi* to this agent (Sambri et al., 1990).

1.3.4 **Prevention**

Duration of tick attachment increases the risk of transmission of *B. burgdorferi* spirochetes; therefore, regular checks for ticks as well as prompt removal is a practical way to reduce the risk of contracting an infection (Piesman et al., 1986). Use of antibiotics a

prophylactic treatment in response to an attached tick being found on a horse is not recommended (Divers et al., 2007). Prophylactic treatment in humans with doxycycline (single 200mg dose given after recognizing tick bite) has been found to be highly effective (87%) at preventing the development of Lyme disease in humans (Nadelman, 2001). Environmental modifications, such as clearing brush and shrubs, keeping grass short and restricting the horse's access to forest and wooded areas, may decrease the risk of tick exposure (Divers et al., 2009).

Currently, no vaccine to prevent Lyme disease exists for horses. In a study by Chang et al, (1999), ponies were vaccinated with an aluminum adjuvanted recombinant outer-surface protein A (rOspA) vaccine and challenged with *B. burgdorferi* infected adult ticks. All four ponies vaccinated showed protection against infection (Chang et al., 1999). Another study evaluating horse response to 3 different canine Lyme vaccines (rOspA antigen and 2 whole cell bacterins) showed OspA antibody responses were not influenced by injection route but only transient antibody responses were produced (Guarino et al., 2017). It is cautioned that protection should not be automatically assumed after vaccinating horses with Lyme vaccines for dogs (Guarino et al., 2017). Other prevention methods including acaricides such as permethrin have been found to be effective in humans but efficacy in horses is unproven (Bushmich, 1999; Chang et al., 2000).

1.4 *BORRELIA BURGDORFERI* TESTING METHODS

1.4.1 Antibody targets

The antigenic composition of *B. burgdorferi* is complex and often varies depending on the host and stage of infection (Johnson, 2011). Spirochetes have evolved mechanisms to sense and respond accordingly to different host environments to ensure survival in its hosts (Jonsson

and Bergstrom, 1995). These antigenic shifts appear to be part of the immune evasion strategies of the spirochetes (de Silva et al., 1998). In the midgut of the tick, the *B. burgdorferi* bacterium produces an outer surface protein (OspA) that is present before the ticks have a blood meal (Barbour et al., 1983). OspA is selectively retained in the tick gut, as OspA is important for adhesion and binds to the epithelial cells. The bacteria that produce OspA rarely enter the salivary glands and are important for retaining spirochetes in the midgut until transmission, at which time OspA production is down-regulated (Ohnishi et al., 2001; Schwan et al., 1995; de Silva et al., 1997; Pal U et al., 2000; Yang et al., 2004). In the canine vaccine, OspA is commonly used due to its immunogenicity, so in dogs, a positive titer value for OspA is often interpreted as a response to the vaccine. In contrast, long-term cases of Lyme disease in humans has shown documented expression of OspA antibodies in late infections and are often associated with arthritis that is non-response to antibiotic treatment (Akin et al., 1999; Johnson, 2011; Kalish et al., 1993; Kalish et al., 1995). Further investigation of a positive antibody response to OspA in an unvaccinated horse may be warranted (Johnson, 2011).

After attachment to the mammalian host an essential switch from OspA to OspC occurs in the tick midgut during the tick blood meal (de Silva et al., 1996; Montgomery et al., 1996; Schwan et al., 1995). Change in temperature has been proposed as partially responsible for the differential switch in expression. OspC is induced at 32-37°C after the tick begins feeding on warm blood, but not at 24°C (Ramamoorthy and Philipp, 1998; Tilly et al., 1997). During tick feeding the spirochetes multiply and migrate through the midgut wall and invade the salivary glands. OspC presence in the saliva is essential for the infectivity of *B. burgdorferi* when tick saliva containing spirochetes is transmitted into the host (Benach et al., 1987; Gern et al., 1990;

Piesman et al., 1987; Piesman et al., 1991; Ribiero et al., 1987; Zung et al., 1989). Spirochetes in the tick gut are homogenous, with most producing OspA and display little genetic heterogeneity prior to tick attachment. OspA producing spirochetes begin to produce OspC during the blood meal, creating a heterogenous population of proteins present on the surface of the spirochetes in the gut (Ohnishi et al., 2001).

Synthesis of OspC peaks at 48hrs after initial tick attachment and occurs just before the spirochetes invade the host (Piesman 1995). The production of OspC is maintained for the first weeks after transmission into the host but is not required for persistence after the bacteria are established in the host (Eicken et al., 2001; Kumaran et al., 2001; Liang et al., 2004; Schwan et al., 1995). While the molecular function of the OspC protein is undefined, it appears to be a virulence factor, required at the initial stage of infection (Grimm, 2004).

Outer surface protein F (OspF) has been found to occur later in infection (Akin et al., 1999; Grimm et al., 2004). In dogs, it has been previously reported that OspF is a valuable diagnostic indicator of infection (Magnarelli et al., 1997, 2001). Antibodies to OspF may be more important indicators of infection than OspC, based on research on dog with clinical signs of lameness or joint disorders (Magnarelli et al., 2001). Using antibody production patterns for OspC and OspF may give an indication of when exposure occurred. Antibodies to OspC are a marker of early infection, while antibodies to OspF are an indication of a later infection (Wagner et al., 2011).

Persistent mammalian *B. burgdorferi* infection requires the Vlse (Vmp-like sequence, expressed) protein, which begins being synthesized when OspC production decreases (Bankhead

and Chaconas et al., 2007; Crother et al., 2004; Glockner et al., 2006; Purser et al., 2003; O'Connor et al., 2004). This lipoprotein contributes to the resistance of acquired immune response (Tilly et al., 2008). Incorporating the surface-exposed Vlse has been found to be effective in confirming current or past *B. burgdorferi* infections, as it is only expressed in active infections in mammalian hosts (Magnarelli et al., 2000; Wagner et al., 2013). The surface protein contains an antigen (C6) that contains the sequence of a conserved region (IR6) that is immunodominant and is expressed only during natural *B. burgdorferi* infection (O'Connor et al., 2004; Chandrashekar et al., 2008).

1.4.2 **Commercially available tests**

Enzyme-linked immunosorbent assays (ELISA) are commonly used to detect class-specific or total immunoglobulins (Ig) of the *B. burgdorferi* bacterium (Fikrig et al., 1993; Greene et al., 1988; Magnarelli and Anderson, 1989; Shin et al., 1993). ELISA testing allows for quantification of antibody concentrations. Recently produced ELISA's contain more specific and purified recombinant antigens of *B. burgdorferi* and have improved the ability to confirm an infection (Gerber et al., 1995; Magnarelli et al., 1996; Padula et al., 1994). The use of whole cell lysates for ELISA has been found to result in false-positives due to antibody reactivity with flagellin and other antigens such as heat-shock proteins (Hagstoz, 2001). Serum analysis using the recombinant antigens of *B. burgdorferi* has been found to reduce the number of false-positives and improves the specificity of the ELISA when screening equine sera for antibodies (Hagstoz, 2001; Magnarelli et al., 2000). In the United States, re-testing samples with Western blot after a positive test on ELISA has been found to verify results in horses (Magnarelli et al., 2000). In humans and dogs, incorporating Vsle lipoprotein is effective in confirming *B.*

burgdorferi infections detected with an ELISA (Liang et al., 2000). Additionally, ELISA with C6 peptide has been found to have high specificity and sensitivity when used for dogs and people (Bacon et al., 2003). An advantage of using the C6 peptide-based ELISA is that it can provide accurate results for vaccinated animals, as the C6 peptide is non-reactive to the OspA used in canine vaccines (O'Connor et al., 2004).

The C6 ELISA uses the synthetic C6 peptide derived from the IR6 region of the Vlse protein (O'Connor et al., 2004). This new testing method is marketed for use in dogs, as it detects antibodies to *B. burgdorferi*, *A. phagocytophilum*, *Ehrlichia canis*, and detects *Dirofilaria immitis* (heartworm) antigen (Johnson et al. 2008). The test is not species specific and allows the identification of animals that are infected with both *B. burgdorferi* and *A. phagocytophilum* (Chandrashekar et al., 2008). A previous study in Minnesota determined that 24 of 164 canine samples showed evidence of previous exposure to both pathogens (Beal et al., 2008). The high specificity of the C6 peptide minimizes false positives seen with whole-cell ELISA and eliminates the need for confirmatory Western blot (Chandrashekar, 2008). An advantage of this test is that it can be used in-clinic and provides results within 8 minutes giving veterinarians the ability to test for *B. burgdorferi* exposure in-clinic (Chandrashekar, 2008).

In a study by Johnson et al. (2008) experimentally infected ponies were tested with the C6 ELISA. The test was found to have specificity of 100% and sensitivity of 63%. The specificity when testing naturally infected horses may be lower as false-positive results can occur. The sensitivity was stated to be lower than predicted, and possible reasoning included the assay not be optimized for equine serum, a difference in the C6 antibody response occurred, or that the samples were collected too soon after experimental infection (Johnson et al., 2008). The

ponies in the study tested negative for *B. burgdorferi* with the C6 ELISA test after receiving a course of antibiotics. The results of this study provide evidence for the use of the C6 ELISA for horses, and the possibility of determining successful treatment of a *B. burgdorferi* infection in horses (Johnson et al., 2008).

The recommended Lyme antibody testing procedure in humans by the Centers for Disease Control and Prevention (CDC) is an ELISA or IFA followed by Western blot (Wagner et al., 2011). A new testing method, a Lyme Multiplex assay, has been developed at the Animal Health Diagnostic Center at Cornell University for detecting Lyme disease in horses and dogs (Wagner et al., 2011). The assay uses simultaneous detection of soluble analytes in biological samples (Morgan et al., 2004; Prabhakar et al., 2005). Fluorescent beads bind to individual antigens including OspA, OspC and OspF, as markers for vaccination, acute and late stage infections respectively (Wagner et al., 2011b). The Multiplex requires a smaller quantity of sample fluid in comparison to ELISA and Western blot, giving the Multiplex assay the advantage when serum volume is low. This would occur in situations such as very early infections in cerebral spinal fluid samples from horses with neurological clinical signs (Wagner et al., 2011a). In a study by Wagner et al., (2011a) the Multiplex assay proved to be an improved diagnostic tool for detection of antibodies in CSF, as it out-performed the conventional ELISA and Western blot tests. Similar to a Western blot and the C6 ELISA, the Multiplex assay can detect OspA when it has been used as a vaccine (Wagner et al., 2011b). Test results from the Multiplex with a positive OspA titer (>2000-28,000) would typically be seen in vaccinated animals, or if there is a positive titer for OspC or OspF as well, the horse would be considered

infected with *B. burgdorferi*. Positive OspC titer (> 1000-10,000) is indicative of early infection, and positive OspF (>1250-26,000) indicates chronic infection (Schvartz et al., 2015).

1.4.3 **Western blot**

Currently, a "Gold Standard" test does not exist, though Western blot is commonly used, often paired with an ELISA (Jacobson et al., 1996). The use of antigens separated by molecular size has aided in determining which *B. burgdorferi* antigens are immunodominant at different stages of Lyme disease (Dressler et al., 1993; Engstrom et al., 1995). *Borrelia burgdorferi* antigens elicit either IgM or Ig G antibodies, depending on the stage and manifestation of the Lyme infection. In early infections, IgM and IgG antibodies are directed toward OspC and flagellar antigens but only IgG can be used after the first 4 weeks after initial infection occurs (Aguero-Rosenfeld et al., 1996; Dressler et al., 1993). This is because as antibody response increases over time, Western blot sensitivity increases as well (Aguero-Rosenfeld et al., 2005; Dressler et al., 1993; Engstrom et al., 1995). Western blot performs similarly to ELISA in late stage infections, however Western blot has a slightly higher specificity due to less cross-reactivity and performs better in acute-stage infections (Aguero-Rosenfeld et al., 1993, 1996). Limitations of Western blot include the cost and inconsistencies of antibody responses in patients with the same clinical presentation of Lyme disease (Dressler et al., 1993; Engstrom et al., 1995). These immunoblots are commonly used as part of a two-tiered testing system, in which Western blot confirms a result obtained by ELISA or immunofluorescence assays (O'Conner et al., 2004).

1.4.4 **Immunofluorescence assays**

Immunofluorescence assays (IFA) were the first diagnostic tool used to isolate antibodies to confirm *B. burgdorferi* infections in humans (Steere et al., 1983). IFA uses cultured organisms fixed onto glass slides and fluorescence microscopy to detect antibodies (Magnarelli et al., 1984; Russel et al., 1984). However, isolating the *B. burgdorferi* spirochete from mammalian species can be difficult. Steere et al., (1983) used class-specific conjugates to show that human patients with Lyme disease produce IgM during the first weeks of infection after the erythema migrans rash has presented. It was also found that IgG is produced slower than IgM, and elevated antibody levels persist for months or years on IFA. A high titer for IgM would be expected in patients with erythema migrans rash, while an IgG titer would be expected in patients with Lyme arthritis (Steere et al., 1983). In a study by Russell et al., (1984) it was found that IFA performs very similarly to ELISA however, ELISA results were found to be more precise and easier to interpret. In horses and dogs, whole-cell IFA is usually performed with a follow-up Western blot test to confirm diagnosis (Shvartz et al., 2015). When compared to the Western blot, IFA and ELISA perform similarly and were found to both have a specificity of ~92%. The ELISA had a stronger test sensitivity of 85%, whereas the sensitivity of IFA was 67% (Lindenmayer et al., 1990). This may account for some preference of using ELISA over IFA for Lyme disease confirmation.

1.4.5 **Polymerase chain reaction**

Polymerase chain reaction (PCR) has been used to detect *B. burgdorferi* DNA from blood, synovial fluid and other samples with varying results (Chang, 2000). PCR can detect both

viable and non-viable organisms, intact and fragmented spirochete DNA and has been used to detect DNA in the synovial fluid of a joint with chronic Lyme arthritis (Bradley et al., 1994; Nocton et al., 1994). PCR testing has also been used to detect the presence of *B. burgdorferi* spirochetes within ocular fluids of two horses (Priest, 2012) as well as detecting *B. burgdorferi* infection in the equine central nervous system (Burgess and Mattison, 1987; Hahn et al., 1996). In humans, PCR has the highest sensitivity for *B. burgdorferi* DNA in skin biopsy samples in patients with erythema migrans. The low sensitivities in other systems other than the skin render PCR as a sub-optimal choice for Lyme disease detection (Dumler, 2001; Molloy et al., 2001). In horses, PCR testing of cerebral spinal fluid (CSF) in suspected cases of Lyme neuroborreliosis is recommended although the sensitivity is low possibly because the organism does not reside in the CSF (James et al., 2010; Johnstone et al., 2016; Wagner et al., 2011).

1.5 *ANAPLASMA PHAGOCYTOPHILUM*

1.5.1 Microbiology

Another tick-borne pathogen transmitted by *I. scapularis* is *Anaplasma phagocytophilum*, which is the etiological agent of granulocytic anaplasmosis. This disease notably affects humans, horses, and dogs (Dumler et al., 2001). The exact pathogenesis of *A. phagocytophilum* infection has yet to be elucidated and primary bacterial replication sites are unknown after the bacteria enter the skin during a tick bite (Woldehiwet, 2006). The bacterium is found in vacuoles within the cytoplasm and infects eosinophils and neutrophils in the blood (Gokce and Woldehiwet, 1999; Pusterla and Madigan, 2013; Woldehiwet, 1987). It has been suggested that *A. phagocytophilum* infection may lead to immunosuppression in humans that are infected (Lepidi

et al., 2000). Granulocytic anaplasmosis can be fatal in humans, but this is largely due to secondary infections and organ failures. This may occur due to a suppressed immune system however it is difficult to confirm (Walker and Dumler, 1997). *Anaplasma phagocytophilum* is able to infect bone marrow-derived cell lines, so it has been hypothesized that the immunosuppression may be caused by the bacterium restricting the production of these cells and/or affecting their functions (Archambault et al., 2000; Dines et al., 2004; Drumler et al., 2001). While several bacteria can survive within macrophages, *A. phagocytophilum* is one of only two bacterial pathogens that can survive and multiply in neutrophils, though the mechanisms for this are not fully understood (DeLeo, 2004). Clinical manifestations of anaplasmosis seem to be, at least in part, dependent on macrophage activation (Drumler et al., 2007). The inflammatory response seen in mammalian hosts is not due to the bacterial load, but rather that the infection affects neutrophil function to promote inflammation and impair neutrophil antimicrobial defenses (Carlyon and Fikrig, 2006; Choi et al., 2007; Drumler et al., 2005). Similar to *B. burgdorferi*, this bacterium uses antigenic variations of surface proteins to evade detection and elimination by the host. The major surface protein of *A. phagocytophilum* is P44, which is thought to be responsible for the majority of the antigenic variations by the differential transcription of the p44 genes (Zhi et al., 1999, 2002).

1.5.2 **Transmission**

Equine granulocytic anaplasmosis is transmitted by *Ixodes* tick species. In Europe, the main vector is *I. ricinus*, while the main tick species are *I. scapularis* in eastern North America and in western North America, *I. pacificus* are the main tick species (Bown et al., 2003). The incidence of the disease varies by region, as well as the severity of the disease, which is

primarily dictated by the strain of *A. phagocytophilum* in the reservoir. A reservoir animal is also required for the bacterium persisting in nature as transvarial transmission does not occur (Woldehiwet, 2006). Rodents, namely the white-footed mice (*Peromyscus leucopus*), are the main reservoir for the ticks; however, ticks also transmit the bacteria to ruminants, as well as humans, horses, and dogs (Ogden et al., 1998a; Liz et al., 2000; Bown et al., 2003, 2006). As *A. phagocytophilum* and *B. burgdorferi* are maintained in almost the same tick vector and reservoir system, the geographic distribution is considered to be very similar. However, the host range for *A. phagocytophilum* is significantly wider (Bown et al., 2003; Daniels et al., 1998; Rikihisa, 1991).

Anaplasma phagocytophilum infects granulocytic cells, primarily neutrophils (de la Fuente, 2016). Ticks acquire the bacterium as larvae feeding on infected reservoir animals within 2 days of attachment (Hodzic et al., 1998). Feeding after development to the subsequent life-stage of the tick stimulates the replication and migration of the bacteria (Hodzic et al., 1998). The bacteria initially infect the midgut of the tick before traveling to the salivary glands, for transmission into a susceptible host after 24-48 hours of attachment (des Vignes et al., 2001; Hodzic et al., 1998; Katavolos et al., 1998). The obligate intracellular Gram-negative bacterium multiplies in the cytoplasm of cells in the tick and vertebrate hosts and has adapted to use many strategies to infect the tick and host including inhibition of cell apoptosis, modifying the cytoskeleton and manipulating the host immune response and infection proteins (de la Fuente, 2016).

1.5.3 **Epidemiology in horses**

Infection with *A. phagocytophilum* occurs generally in spring, summer and fall when ticks are active (Madigan et al., 1990). Horses are not considered to be effective reservoirs as it has been found that the organism is generally only present during the acute phase of the disease (Madigan and Gribble, 1987). As would be expected, the seroprevalence of antibodies against *A. phagocytophilum* are higher in endemic vs. non-endemic regions, and most horses that are exposed likely do not develop signs of disease (Madigan et al., 1990). Equine granulocytic anaplasmosis is not transmissible between horses and persistent infections do not occur. The first case of the disease was reported in California in 1969 and since then it has been well described in the United States, Brazil, and Europe (Foley et al., 2004; Madigan et al., 1996; Pusterla and Madigan, 2013; Uehlinger et al., 2011). In Canada, anaplasmosis has been reported in British Columbia, Nova Scotia, Saskatchewan, New Brunswick and Ontario; however, the overall risk in Canada for exposure to *A. phagocytophilum* is considered to be low (Berrington et al., 1996; Bourque and Goltz, 2012; Burgess et al., 2012; Uehlinger et al., 2011).

In experimental exposure of horses to infected ticks, the incubation period is 8-12 days. The incubation from a natural infection is believed to be less than 14 days (Pusterla and Madigan; 2013). Clinical signs of equine anaplasmosis include fever, anorexia lethargy, ataxia, reluctance to move and distal limb edema (Madigan, 1993; Madigan and Gribble, 1968). However, in horses under the age of 4, clinical signs are milder and include low-grade fever, depression, and moderate limb edema (Pusterla and Madigan, 2013). The clinical signs in older adult horses can be severe; horses with ataxia and weakness have been found to sustain fractures from falling (Pusterla and Madigan, 2013). Laboratory tests have found that hematological

abnormalities can also occur in *A. phagocytophilum* infections, such as anemia, leukopenia and thrombocytopenia (Madigan and Gribble, 1968; Pusterla and Madigan, 2013; Sellon, 2003). Tetracyclines are known to be an effective treatment; however, experimental infection in horses has demonstrated that spontaneous recovery without treatment can occur (Frazen et al., 2009). Currently, there is no vaccine, so prevention is limited to tick control and use of acaracides (Blagburn et al., 2004).

1.5.4 **Diagnostic testing**

Diagnosis is based on geographic region, clinical signs, and laboratory findings. Serologic testing can determine if an exposure to the bacterium has occurred (Schvartz et al., 2015). Differential diagnosis of the disease are commonly Lyme disease, equine infectious anemia, or equine viral arteritis (Madigan, 1993; Lepidi et al., 2000; Silaghi et al., 2011). Microscopic analysis of blood smears from infected animals can detect the presence of *A. phagocytophilum* in neutrophils and sometimes eosinophils (Sells et al., 1976; Pusterla et al., 1998). The recommended testing method to detect antibodies against *A. phagocytophilum* is IFA (Chapman et al., 2006). IFA is considered to be the "gold standard" test for detection of *A. phagocytophilum* antibodies and is commonly used to evaluate the performances of other diagnostic methods (Pusterla and Madigan 2007). This immunoassay uses whole-cell antigens consisting of neutrophilic granulocytic infected cells, however, these tests are limited in the ability to detect infection in the acute stage, and there may be cross-reaction against other bacteria (Chandrashekar et al., 2008; Magnarelli et al., 2000, 2001). Additionally, this test is costly and time-consuming, and is not always a practical choice for veterinarians (Veronesi et al., 2014).

Another common diagnostic test is PCR which is performed using specific primers to isolate DNA in full blood, leukocytes, bone marrow or spleen biopsies (Adaszek et al., 2009, 2013). PCR can also distinguish between strains of *A. phagocytophilum* (Łukaszewska et al., 2008). PCR may be the most sensitive technique to detect *A. phagocytophilum*, as a study by Barlough et al., (1995) was able to confirm exposure in the blood of a horse 9 days after infection, and only 1 day after fever occurred. PCR, however, is also time-consuming and costly, and may not be reliable depending on the stage of infection (Barlough et al, 1995).

The C6 ELISA test also detects antibodies to *A. phagocytophilum* using a peptide derived from the P44 protein, major surface protein 2 (MSP2) (Chandrashekar et al., 2008). In a study of dogs by Chandrashekar et al., (2008), the C6 ELISA performed very well in comparison to IFA, with a sensitivity and specificity of 100% for the samples tested. Similarly, in dogs, the C6 ELISA was found to have a sensitivity of 99.1% and 100% specificity compared to IFA for canine samples (Chandrashekar et al., 2007). Further investigative studies are required to determine the in-clinic reliability of the C6 ELISA, as developing a rapid and accurate diagnostic tool for monitoring and detecting *A. phagocytophilum* exposure and infection is imperative for horses.

1.6 CONCLUSION

With the range of established tick populations expanding in Ontario, it is imperative to determine prevalence and incidence rates of *B. burgdorferi* exposure and infections in horses. Determining the relationship between seropositivity and disease is vital for assessing the relevance of diagnostic testing as well as the epidemiology of the disease. The goal of this

research is to provide valuable information for equine practitioners while improving horse health and contributing to the global field of Lyme disease.

1.7 THESIS HYPOTHESES AND OBJECTIVES

The prevalence of Lyme disease in humans and dogs in Ontario has increased in recent years. A similar trend of prevalence is expected to occur in horses, however, very little is known about equine Lyme disease. Predictive models indicate continued rapid expansion of ticks and infectious pathogens including *Borrelia burgdorferi* and *Anaplasma phagocytophilum*. Therefore, determining the prevalence of *B. burgdorferi* exposure in horses, as well as further understanding the etiology of the disease is imperative as this will be a continuing and increasing problem in upcoming years.

1.7.1 Thesis Hypotheses:

Following the predictions of tick and climate change patterns, it is hypothesized that the highest rates of *B. burgdorferi* infections will be found in regions near eastern Ontario, around the north shores of Lake Ontario and the St. Lawrence river. It is also hypothesized that geographic region will be the main factor associated with seropositivity.

Two serologic tests are clinically available for detecting *B. burgdorferi*. The Lyme multiplex assay detects outer surface proteins to *B. burgdorferi* antigens and is a commonly used testing method by equine practitioners. The C6 ELISA, marketed for dogs, detects antibodies to both *B. burgdorferi* and *A. phagocytophilum* and recently has been evaluated for use in horses; however, further investigation about the clinical usefulness is required. Therefore, we have

hypothesized that the C6 ELISA will perform similarly to the multiplex assay, and OspF, the marker for chronic infection, will be the most common antibody titer found.

1.7.2 **Thesis Objectives:**

Objective I: To determine the seroprevalence of *B. burgdorferi* and *A. phagocytophilum* in Ontario horses

Currently, the incidence of Lyme disease and prevalence rates of exposure do not exist for horses in Ontario. Equine exposure rates are expected to mimic human and canine trends. *Anaplasma phagocytophilum* is a less common bacterium and only a single case of granulocytic anaplasmosis has been reported in an Ontario horses. The prevalence is expected to be lower for *A. phagocytophilum* infections. The seroprevalence will be described for each test.

Objective II: To identify risk factors for seropositivity

Studies of *B. burgdorferi* exposure in dogs has found that the amount of area a dog can roam to be a major factor in exposure to the bacteria. Another factor is the presence of forests, particularly with oak and maple trees. Risk factors for *B. burgdorferi* exposure have not been evaluated for horses. Demographic and management risk factors will be analyzed for each test, using any positive test as an indication of seropositivity and both univariate and multivariate analysis will be conducted.

Objective III: To compare performance of in-clinic C6 ELISA to Lyme multiplex assay

A “Gold Standard” test does not exist in order to determine sensitivity and specificity of other tests. Two-way comparison between the tests will be analyzed as well as the agreement level between the C6 ELISA and multiplex using Kappa will be described (Landis and Koch, 1977).

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CHAPTER 2: SEROPREVALENCE AND EVALUATION OF RISK FACTORS ASSOCIATED WITH SEROPOSITIVITY FOR *BORRELIA BURGDORFERI* IN ONTARIO HORSES

2.1 ABSTRACT:

The blacklegged tick, *Ixodes scapularis,* has undergone a rapid range expansion within Ontario in the past two decades. This tick can transmit the bacterium that causes Lyme disease, *Borrelia burgdorferi* as well as *Anaplasma phagocytophilum,* the cause of equine granulocytic anaplasmosis. Currently, there is no baseline data on the level of exposure or disease prevalence in the equine population and therefore we have limited understanding of the risk these pathogens may pose. The objectives of this study were to a) assess the seroprevalence of *B. burgdorferi* and *A. phagocytophilum* in horses in Ontario, b) evaluate risk factors associated with seropositivity, and c) compare the performance of in-clinic ELISA to a Multiplex ELISA. Serum samples were obtained from 551 clinically healthy horses across Ontario. Horse owners filled out a questionnaire that collected information on demographics, clinical history, and farm management. Sera were tested with a Multiplex ELISA targeting outer surface proteins A, C, and F (Animal Health Diagnostic Center, Cornell University, New York, USA) and a point of care ELISA targeting the C6 antigen (IDEXX SNAP® 4Dx® Plus test, IDEXX Laboratories). The overall prevalence of *B. burgdorferi* seropositivity for horses on at least one test was 17% (91/551). The individual test prevalence rates were as follows: C6 ELISA 5.1% (28/551), OspA 3.4% (19/551), OspC 1.8% (10/551) and OspF 9.6% (53/551). The agreement between the C6 ELISA and the Multiplex was fair (kappa=0.23). A spatial cluster of high prevalence (i.e., hot spot) was detected in Eastern Ontario. *Anaplasma phagocytophilum* antibodies were detected in 1% (6/551) of horses, 3 of which were also positive for *B. burgdorferi* (0.005%). Based on mixed effect multivariable logistic regression, the odds of being seropositive for *B. burgdorferi*

on the C6 ELISA were significantly increased by the presence of oak trees by pastures while the odds were significantly decreased by performing regular tick checks. The continued expansion of *I. scapularis* populations in various regions poses ongoing risk for horses, although the clinical relevance of *B. burgdorferi* seroprevalence remains unclear. Our findings illustrate that certain ecological factors may increase the risk of pathogen exposure, but further investigation is needed. This information may be used to propose changes in farm management to decrease the risk of exposure to blacklegged ticks.

2.2 INTRODUCTION

Over the past two decades, there has been a significant increase in populations of the blacklegged tick, *Ixodes scapularis* (Ogden et al., 2005). Northward spread from the United States has resulted in the establishment of *I. scapularis* in multiple regions of Ontario, particularly in Eastern Ontario and areas along the north shores of Lake Ontario and Lake Erie (Clow et al., 2016). Climate change and other ecological changes have contributed to the range expansion of *I. scapularis* and this pattern is predicted to continue due to the rise in temperature and increasing humidity creating more suitable habitats throughout the province (Ogden et al., 2005). This tick species poses a potential health risk to humans as well as horses and pets, as it can transmit multiple pathogens. Of particular interest are the bacteria *Borrelia burgdorferi* and *Anaplasma phagocytophilum* which cause Lyme disease and equine granulocytic anaplasmosis, respectively (Burgdorfer et al., 1982; Madigan, 1993). The ongoing expansion of this tick species throughout the province has raised concerns about the risk of tick-borne diseases for horses in Ontario.

The clinical aspects of Lyme disease in horses are poorly described. The best-documented clinical manifestations of *B. burgdorferi* infection in horses are neuroborreliosis and uveitis, with cutaneous pseudolymphoma being possible but rare (Burgess & Mattison, 1987; Hahn et al., 1996; Imai et al., 2011; James et al., 2010; Johnstone et al., 2016; Priest et al., 2012; Wagner et al., 2011). Although clinical signs often attributed to equine Lyme disease including shifting lameness, fatigue, arthritis and joint pain, these are common signs of Lyme disease in other species, and data implicating Lyme disease as a cause of these in horses is currently lacking (Divers, 2007). Experimental infection of ponies with *B. burgdorferi* resulted in no observed clinical signs, and although sample size was small, the connection between clinical signs and clinical disease remains uncertain (Chang et al., 2000). Conversely, clinical signs of equine granulocytic anaplasmosis have been well elucidated, the disease often being self-limiting and horses responding well to treatment (Barlough et al., 1995; Madigan, 1993; Pusterla & Madigan, 2013).

Definitive diagnosis of Lyme disease in horses is challenging. Serological testing can identify if a horse has been exposed to the bacterium, but it does not confirm Lyme disease. The potential widespread exposure (and seroconversion) to *B. burgdorferi* in endemic areas and the potential ability of these antibodies to persist at high levels even after treatment limits the positive predictive value of serological testing. Better understanding of both the baseline seroprevalence rates in healthy horses and the kinetics of serum antibodies over time is required. Different testing options are available for horses. A multiple antigen ELISA assay (Multiplex[1])

[1] Animal Health Diagnostic Center, Cornell University, New York, USA

detects and quantifies serum antibodies to individual antigens of three outer surface proteins (Osps) of *B. burgdorferi*: OspA, OspC and OspF (Morgan et al., 2004; Prabhakar et al., 2005). They are reported to be associated mainly with vaccination, acute and late stage infections, respectively (Wagner et al., 2011b). An alternative test is a point-of-care ELISA that has been widely used in dogs that detects antibodies against the C6 antigen of *B. burgdorferi*, and the p44 antigen of *A. phagocytophilum* (IDEXX SNAP® 4Dx®[2]) (Johnson et al., 1984; Piantedosi et al., 2017). Two-tiered antibody testing involving an ELISA test followed by Western blot is considered to be the recommended approach in humans; however, there is no gold standard for horses and there is limited consensus of a recommended test for diagnosis (Divers et al., 2018).

Serological surveys conducted in the United States have found that seroprevalence in clinically normal horses ranges from 0.2% to 45% and that seroprevalence rates and geographic range of seropositive horses are increasing (Cohen et al., 1992; Metcalf et al., 2008; Magnarelli et al., 2000; Funk et al., 2016). In Europe, seropositivity rates of 6-29% have been reported in various countries (Evengall et al., 2001; Bhide et al., 2008; Laus et al., 2013; Stefanciková et al., 2008; Ebani et al., 2012; Hansen et al., 2010). Prevalence rates for *A. phagocytophilum* antibodies in North America are lower than that of *B. burgdorferi*, though the geographic distribution of the two agents has been found to coincide as both are maintained in the same tick and reservoirs (Baneth, 2014; Massung et al., 2005; Nelder et al., 2014; Ogden et al., 2008).

Currently, there is a lack of baseline data on the seroprevalence in the equine population in Ontario and a limited understanding of the risk these pathogens may pose. The objectives of

[2] SNAP® 4Dx® Plus ELISA, IDEXX Laboratories Inc., Westbrook, ME.

this study were to a) assess the seroprevalence of *B. burgdorferi* and *A. phagocytophilum* in horses in Ontario, b) evaluate risk factors associated with seropositivity, and c) compare the performance of in-clinic ELISA to a Multiplex ELISA.

2.3 METHODS

Sample population: In the summer 2016, 302 veterinary clinics who provide services to equine owners in Ontario, Canada were contacted by email and asked to participate in an equine Lyme disease surveillance study. Each participating clinic was requested to submit serum samples from 10 clinically healthy horses, each residing at different farms in the veterinarian's area. Horses that had travelled outside of Ontario (ever) or that were younger than 3 years of age were excluded.

Questionnaire: Each participating horse owner was requested to complete a questionnaire (Appendix 2.1) that evaluated demographics, clinical history and farm management for each horse. Demographics included the horse's age, sex, breed, discipline (i.e., breeding, companion only, trail riding, local competition, riding school, or hunting) and how long they had resided on that property. Questions about farm/owner management included the amount of time spent outside on pasture for each season, if regular checks for ticks were performed, had a tick ever been found on the horse, if the horse had been vaccinated with the canine Lyme vaccine, and if any tick repellent was regularly applied to their horses. Environmental exposure questions included if horses were prevented from grazing in forested areas, if oak trees were in/bordering the paddock, and with what frequency were wildlife (i.e., coyotes, skunks, deer, opossums, fox) seen on the property. As well owners were asked to select any habitats the horse had been

exposed to in the previous 12 months (grassland, shrubland, wetland, forested, or urban/recreation). Travel history was not asked for due to the selection criteria for the study, though horse owners were asked if the horse had left the property but stayed in Ontario, for various lengths of time (<24 hours to > 14 days) in the past 12 months.

C6 ELISA: Sera were tested for the presence of antibodies to *B. burgdorferi* and *A. phagocytophilum* using the IDEXX SNAP® 4Dx® Plus (IDEXX Laboratories, Westbrook, Maine, United States), according to manufacturer's instructions. In brief, 3 drops of serum were added to the sample tube followed by 4 drops of conjugate. The sample was mixed by inverting the tube 4 times before pouring entire contents on to the 'sample well' of the SNAP® device. Results were read 8 minutes after test activation. Sera was then aliquoted into cryovials and stored at -80°C.

Equine Lyme Multiplex Assay: A serum sample for each horse was shipped on dry ice to the Animal Health Diagnostic Center (AHDC) at Cornell University, Ithaca, New York. Samples were tested with the equine Lyme multiplex assay to determine the presence of antibodies to *B. burgdorferi* antigens (ADHC, 2011). In brief, the multiplex ELISA uses fluorescent bead-based multiple antigens to detect and quantify serum antibodies to three antigens, OspA, OspC, and OspF which serve as markers of exposure.

Statistical analysis:

Seroprevalence estimates were calculated for each test (positive on any test; positive on C6 ELISA; positive on Multiplex; positive on each outer surface protein on the Multiplex) along with 95% confidence intervals.

Cohen's kappa test with 95% confidence intervals was employed to determine the agreement between the C6 ELISA and each outer surface protein (OspA, OspC, OspF) antibody on the Multiplex (Dohoo et al., 2009). The kappa value was interpreted using the guidelines established by Landis and Koch (1977).

Breed was categorized into 8 breed groups (heavy, heavy cross, light, light cross, pony, sport horse, warmblood, not specified). Sex was categorized into 3 groups (gelding, mare, stallion). Responses were reviewed using descriptive statistics and categories were combined for questions if there was a low response rate, as long as combined categories still provided relevant data.

Linearity of the continuous variable (age) was assessed graphically relative to the log odds of each test outcome using locally weighted regression (lowess curve). The assumption of linearity was considered to be met if the lowess curve formed a straight line. If the variable violated the linearity assumption, the quadratic term was included in the model and tested for significance. If the non-linear relationship could not be modeled as a quadratic relationship the variable was categorized based on quartiles.

Separate univariable mixed effect logistic regressions with the random effect of county were conducted to examine the influence of demographics, clinical history, and farm management factors on the presence of a positive result for *B. burgdorferi* (outcome variable) for any positive on either test and individually for the C6 ELISA, Multiplex, OspF on the Multiplex ELISA. Due to the limited number of positive results, analysis was not conducted for OspA and OspC outcome variables. All significant variables with a liberal $\alpha \leq 0.2$ were considered for

inclusion in the final multivariable model. Causal diagrams were constructed to examine confounding and intervening relationships between explanatory variables and each test outcome. A variable was considered a confounder if removal of the variable resulted in a ≥30% change in the coefficient of the explanatory variable of interest. The correlation between explanatory variables was assessed using Pearson's correlation analysis with a cut off of > 0.8. Any variables that were highly correlated were not put in the same model together. Potential intervening variables for each variable of interest were also identified from the casual diagrams and were not included in model building when exploring the variable of interest. Variables that were not statistically significant and not suspected of confounding based on the casual diagrams or confounding analysis were excluded from multivariable analysis. Two-way interactions were explored for all significant main effects for each test outcome from the univariable analyses with a significance level of $\alpha = 0.05$.

Mixed multivariable logistic regression models with random effect for county was fit by manual backward elimination followed by selected forward addition using variables that were statistically significant on univariable analysis. Explanatory variables were left in the model if they were statistically significant at $\alpha=0.05$ or showed evidence of confounding. Each variable was assessed for significance with a partial F-test before final removal from the model.

If there was more than one model possible, the final models were compared using the Akaike Information Criterion (AIC); a lower AIC value indicated superior model fit (Dohoo et al. 2009).

Outliers were identified by graphically examining the Pearson residuals. Model fit was assessed by examining the homoscedasticity and normality of the Best Linear Unbiased Predictors (BLUPs).

All statistical analysis was conducted using STATA version 14 (STATACorp, College Station, TX; 2015).

Spatial analysis:

Spatial data was projected using ArcGIS 10.3.1 (Esri, Redlands, CA; 2015). Location of veterinary clinic was geocoded by 6-digit postal code and plotted as longitude and latitude. The base map layer consisted of World Topographic Map Esri database. Point locations were plotted as a layer based on attribute-value for seropositivity. The spatial scan statistic was conducted using SaTScan 9.4 (www.satscan.org, 2015) using purely spatial analysis with the Bernoulli probability model to assess regions of high prevalence. The spatial window was set to default the maximum spatial cluster size to 50% of the population at risk. Monte Carlo replications were set to a limit of 999. Only statistically significant clusters with no geographical overlap were reported. A significance level of $\alpha=0.05$ was used for spatial analyses.

2.4 RESULTS

A total of 76 veterinary clinics from 33 counties from across Ontario participated in the study with sera from 563 horses submitted. Twelve samples were excluded as questionnaire responses indicated the horses were less than 3 years of age or had traveled outside of the province. The questionnaire response rate was 82% (451/551). The mean age was 13 (range 3-

33). The sex ratio was 55% geldings (304/551), 42% mares (231/551), 1.5% stallions (8/551), and 8 unspecified. Most of the horses had been exposed to grassland (89%), followed by shrubland (48%), forested land (41%), wetlands (34%) and least commonly, urban/recreational areas (18%). Only 13% of owners reported that they had found a tick on their horse, though only 50% of owners reported that they checked regularly for ticks, and only 8% reported that they used insect repellent. Deer were the most frequent wildlife reported, followed by coyotes, skunks, and foxes, and least frequently, opossums. Clinical histories from the past 12 months were non-specific.

The prevalence of *B. burgdorferi*, defined as a positive test result on either test was 17% (95% CI: 13.8%- 20.3%) as shown in Table 2.1. The prevalence rate on the C6 ELISA was 5.1% (3.4%-7.3%). *Anaplasma phagocytophilum* antibodies were detected in 1% (0.4%-2.4%) of horses, of which 3 were also positive for *B. burgdorferi*. On the Multiplex ELISA 14.2% (11.4%-17.3%) of the samples had a positive titer. The most frequently detected antibodies were for the OspF antigen with 9.6% (7.3%-12.4%) of the samples having a positive titer, followed by OspA at 3.4% (2.1%-5.3%), and OspC at 1.8% (0.9%-3.3%) (Table 2.1).

The two-way comparison between the C6 ELISA and the Multiplex showed evidence that the cross-positivity varied between tests (Figure 2.1). The kappa value assessing the agreement between a positive test result for *B. burgdorferi* on the C6 ELISA and any outer surface protein (Osp) on the Multiplex was 0.23, while the kappa value assessing the agreement between having a positive OspF titer and being seropositive on the C6 ELISA was 0.27 (Table 2.2). Both kappa values represent fair agreement between tests (Landis & Koch, 1977).

The variables brought forward for multivariable modeling for the outcome of being seropositive on either test were: if deer were seen 'occasionally' on the property when compared to the referent of 'never' (OR= 2.22 (0.74-6.67), p-value=0.157), horses that had insect repellent applied regularly (OR= 1.94 (0.88-4.29), p-value=0.103) and horses that spent 6-12 hours per day outside in the winter (OR= 0.69 (0.31-1.23), p-value=0.17) and age groups 13-18 years old (OR= 2.21(1.14-4.29) p-value=0.019) and 19-33 years old (OR=2.68 (1.33-5.40) p-value=0.006) (Appendix 2.1).

Analysis of seropositive results from the Multiplex ELISA found the following variables significant and were used in multivariable modeling: deer being seen on the property 'occasionally' (OR= 2.67 (0.77-9.31), p-value=0.122) and 'frequently' (OR= 2.48 (0.69-8.95), p-value=0.165), horses that were turned out in the fall for 6-12 hours (OR= 0.52 (0.22-1.22), p-value=0.133) and 24 hours/day (OR= 0.57 (0.26-1.28), p-value=0.173), prevention from accessing forest by fencing (OR= 0.64 (0.34-1.21), p-value=0.168) and age groups 13-18 years old (OR= 2.28 (1.12- 4.64) p-value= 0.023) and 19-33 years old (OR=2.91 (1.14-6.08) p-value= 0.004) (Appendix 2.2). Univariable analysis was also conducted for the test outcome of a positive OspF titer (Appendix 2.3) but was not conducted for OspA or OspC due to the small number of positive results.

The variables brought forward for multivariable modeling for the outcome of having a positive result for *B. burgdorferi* on the C6 ELISA included oak trees in or bordering the pastures (OR=6.80 (1.74-26.66), p-value=0.006), being exposed to forested habitats in the past 12 months (OR=2.37 (0.71-7.83), p-value=0.159), trail riding (OR=2.10 (0.70-6.30) p-value=0.184), having previously found a tick on the horse (OR=0.32 (0.09-1.15) p-value=0.081),

regularly checking for ticks (OR=4.86 (1.26-18.85) p-value =0.022) and applying tick repellent to the horses (OR=3.27 (0.89-12.01) p-value=0.075) , prevention from forest by fencing (OR=0.30 (0.10-0.92) p-value=0.034) (Appendix 2.4). Univariable analysis was not conducted for *A. phagocytophilum* results due to the low number of positive results.

The final mixed multivariable logistic regression model with an outcome of a positive result on the C6 ELISA included statistically significant associations between the presence of oak trees and performing regular checks for ticks. The presence of oak trees bordering or in the horse's pasture had significantly higher odds of the horse being seropositive on the C6 ELISA than horses that did not have oak trees near the pasture (OR=7.305 (1.831-29.148), p-value=0.005). Performing regular tick checks significantly reduced the odds of the horses being seropositive (OR=0.087 (0.011-0.718), p-value=0.023). Access to forested habitats in the past 12 months showed evidence of confounding with performing regular tick checks and was included in the final model (OR=1.11 (0.309-3.993), p-value=0.872). No statistically significant associations were found with multivariable modeling for any other test outcomes.

A high-risk cluster was identified among seropositive horses for a positive result on any test (Figure 2.2). When using the data from horses that were seropositive on the C6 ELISA, the same high rate cluster was identified (Figure 2.3); however, when the seropositive results from the Multiplex were used (including all outer surface proteins), a smaller cluster was identified within the previous cluster (Figure 2.3).

2.5 DISCUSSION

As the geographic range of *I. scapularis* and associated pathogens continues to expand northward in Ontario, the risk of horses being bitten and becoming seropositive increases (Durrani et al., 2011). The seroprevalence of *B. burgdorferi* and *A. phagocytophilum* in clinically healthy horses in our study was 17% and 1%, respectively and these findings establish a baseline for the seroprevalence of both *B. burgdorferi* and *A. phagocytophilum* in Ontario horses for future monitoring of these pathogens in the province.

Based on results from seroprevalence studies conducted in the United States where the blacklegged tick is also endemic, we had hypothesized that horses in high-risk regions would be more likely to have antibodies for *B. burgdorferi.* Research has shown that antibodies consistent with previous exposure to *B. burgdorferi* are frequently detected in horses that live in endemic regions (Divers et al., 2012; Funk et al., 2016; Magnarelli et al., 2000). Cluster analysis of our equine data identified a significant spatial cluster of *B. burgdorferi* seropositive horses in Eastern Ontario which corresponds with surveillance data of high-risk areas in the province (Clow et al., 2016; Nelder et al., 2014).

Recently, Clow et al., (2016) identified a hot spot of *I. scapularis* in Eastern Ontario in 2014. Passive surveillance data from 2008 through 2012 found the prevalence of *B. burgdorferi* infected *I. scapularis* ticks increased yearly throughout Ontario, with the highest increases found in the Central West, followed by Eastern and South West regions of Ontario (Nelder et al., 2014). It would be expected that horses, humans and dogs in these areas will be at a greater risk for becoming infected by these ticks. A study by Magnarelli et al., in 2000, was conducted to

determine if horses living in endemic regions in the northeastern United States had detectable antibody levels for *B. burgdorferi*. The seroprevalence of *B. burgdorferi* antibodies in the 82 horses sampled was 45% and it was concluded that horses may have detectable antibodies when living in tick-infested areas and may exhibit associated clinical signs. More recently, in 2013, samples from horses presumed to be healthy were submitted for routine equine infectious anemia testing in Southwest Virginia and were also tested for presence of antibodies to *B. burgdorferi* using the Multiplex ELISA from Cornell University. Of the 250 samples tested, 33% (83/250) were seropositive for at least one outer surface protein (Funk et al., 2016). These findings were consistent with tick data from Southwestern Virginia, which found the prevalence of *B. burgdorferi* infected ticks to be 33% in that area (Herrin et al., 2014). The prevalence we found in our equine samples is also consistent with tick data from the province. Using passive surveillance and testing ticks submitted by the public, Nelder et al., (2014) found that the infection prevalence of *I. scapularis* with *B. burgdorferi* in Ontario was 19.1% in 2012. Additionally, the researchers found an overall prevalence of *A. phagocytophilum* to be 0.3% in Ontario which confirms that while present, the risk of infection is low in Ontario. This is consistent with the low seroprevalence (1%) we observed in horses (Nelder et al., 2014).

An important exclusion criterion for our study was that horses had not traveled outside of Ontario. This was to limit the likelihood that horses included in our study had been exposed to the bacteria outside of the province. The risk of Lyme disease varies geographically, and it remains the most commonly reported vector-borne disease in humans in the United States (Schwartz et al., 2017). In horses, seroprevalence from the United States has ranged from 0.2% in Texas to 14.8% in the Pacific Northwest and 45% in the Northeastern United States (Cohen et

al., 1992; Magnarelli et al., 2000; Metcalf et al., 2008). In Canada, human reports of Lyme disease occur most often from Ontario, Nova Scotia and Quebec (Gasmi et al., 2016). It is currently unknown if it is possible that horses could be predictors of future infections for humans (Wagner & Erb, 2012).

Serological testing provides information about the exposure status for horses that may have been exposed to *B. burgdorferi* bacteria at some point in time. However, distinguishing between previous exposure or an active infection and confirming clinical disease can be difficult. The Multiplex ELISA detects antibodies to three outer surface proteins of *B. burgdorferi* that can aid in determining the stage of infection (Wagner et al., 2011; Wagner & Freer, 2009). In our study, we found OspF to be most frequently detected and OspA was detected more frequently than OspC. Outer surface protein A is usually considered to be a vaccination-induced antibody. There is not a licensed *B. burgdorferi* vaccine for horses in Canada and anecdotally, off-label use of available canine vaccines is rare. Here, a history of *B. burgdorferi* vaccination was reported for two horses, neither of which had detectable OspA levels. None of 19 horses that had a positive titer for OspA had a history of vaccination.

Outer surface protein A is mainly expressed by *B. burgdorferi* in the midgut of the tick (Pal et al., 2000). OspA is down regulated when the tick takes a bloodmeal, and OspC is upregulated during transmission of the bacteria resulting in mainly OspC being expressed in the host initially (Schwan & Piesman, 2000). It has been suggested that OspA can induce a weak, transient antibody response in early infection of dogs and horses (Chang et al., 2000; Wagner et al., 2012). Therefore, positive horses in this study could represent very recent exposure. In humans, OspA has been associated with chronic, and often severe, arthritis (Akin et al., 1999).

Research in dogs has found that OspA may increase after infection in some non-vaccinated animals and can maintain high titer values for prolonged time periods (Greene et al., 1988; Guerra et al., 2000; Littman, 2003).

Our study detected OspC in 10 of the 551 samples tested. It has been shown in both humans and canines that OspC is an early marker of infection and is detectable in infections as early as 3 weeks, generally persisting for 4-5 months. (Grimm et al., 2004; Wagner et al., 2011; Wagner et al., 2013). As our sample collection occurred between the months of July and August, it is likely that horses with positive OspC titres, were infected during the year of sampling based on the current interpretation (ADHC, 2011). Outer surface protein F becomes detectable on the Multiplex 5-8 weeks after infection and can remain detectable for months (Funk et al., 2016; Wagner et al., 2011; Wagner et al., 2013). There was a greater likelihood that the horses in this sstudy would have a positive OspF result as they may still be maintaining a detectable antibody level from exposure in previous years. Two horses had positive titres for both OspC and OspF which suggests infection 3-5 months prior to sample collection based on interpretation of the literature (Wagner et al., 2012). However, interpretation of the timing of exposure in horses cannot be performed with a great degree of confidence at this time because there is limited field data available.

In this study, of the 91 positive samples, only 15 (16%) tested positive on both the Multiplex ELISA and C6 ELISA and agreement between the two tests was only fair (kappa=0.23) (Landis & Koch, 1977). It would be expected that agreement between the C6 ELISA and OspF would be highest, and while there was some agreement, it was only fair (kappa=0.27). The agreement between OspA and OspC and the C6 ELISA were much lower, as

both agreements were considered to be slight (kappa=0.05 and kappa=0.02 respectively) (Landis & Koch, 1977). These results show a low level of agreement between the two tests which is inconsistent with literature on these testing methods in endemic regions in United States (Wagner et al., 2013) but is more consistent with research from Canada in low prevalence regions (Schvartz et al., 2015). Discordant testing complicates diagnosis of Lyme disease even further and highlights the need for more study.

The C6 ELISA was validated for its usefulness in horses in a study with experimentally infected ponies, which found a fair sensitivity (63%) and excellent specificity (100%) on samples that were confirmed positive or negative by skin tissue culture, PCR, and serology (ELISA and Western blot) (Johnson et al., 2008). Chandrashekar et al., (2008) also evaluated the C6 ELISA and its ability to detect antibodies to *B. burgdorferi* and *A. phagocytophilum* using 164 equine serum samples. They found a sensitivity of 100% and specificity of 95% for detecting *B. burgdorferi* antibodies relative to Western Blot and 100% specificity and sensitivity for detecting *A. phagocytophilum* antibodies when compared to an immunofluorescence assay (Chandrashekar et al., 2008). However; in another study conducted in Canada that compared the C6 test and its ability to detect both antibodies in horses, agreement between the C6 test and indirect fluorescent antibody test for *A. phagocytophilum* was limited (kappa = 0.1) as well as for an ELISA-Western blot for detecting *B. burgdorferi* and the C6 test (kappa=0.23) (Schvartz et al., 2015).

One of the highlighted features of the C6 peptide used in the C6 ELISA is that it is nonreactive to serum from animals vaccinated with an OspA-based vaccine and allows for distinction of antibodies due to natural infections from vaccination (Liang et al., 2000; O'Connor et al., 2004). Subsequently, if a horse was positive for OspA, but also positive on the C6, it

should indicate a natural OspA antibody response rather than a vaccine-induced antibody response. Interestingly in this study, two horses were positive for OspA and *B. burgdorferi* on the C6 with no history of being vaccinated against Lyme disease. While numbers are small, this highlights the need for further scrutiny of tests, something that is complicated by lack of a clear case definition and Gold Standard diagnostic test.

Only one horse that was seropositive for OspC also tested positive on the C6 test and this horse was also positive for OspF. A study from 2012 found that antibodies to OspF are highly correlated with C6 antibodies when both are used as markers of infection on the Multiplex test (Wagner et al., 2012). They also found that there was strong agreement between OspC/OspF positives with C6, but the agreement between OspC/C6 without OspF was limited. Our findings support the minimal agreement between C6 and OspC. Antibodies to the C6 antigen are known to be present as early as 5 weeks post-experimental infection in dogs, which is 1-2 weeks after OspC antibodies are said to develop (Liang et al., 2000; Wagner et al., 2012). Overall, the agreement found in our study is lower than the agreement found by Wagner et al., (2012) between the C6 test and the C6 antigen with OspF. However; in a Canadian study from a low prevalence region, poor agreement was found between the C6 test and the Multiplex test (kappa=-0.04) (Schvartz et al., 2015). Their research suggested that the Multiplex test may have produced a false-negative result by their case definition and cautioned against use of serologic testing as a diagnostic tool in low-prevalence regions (Schvartz et al., 2015).

Due to the lack of agreement between the testing methods, each test was used individually for risk-factor analysis as well as combined to determine if there were differences in factors associated with positivity for each test. The results of the mixed-effect univariable

logistic regression for each test outcome found varying factors to be significant for one or more outcome variables.

Multivariable modeling found that having oak trees in or bordering the pastures significantly increased the odds of being seropositive for *B. burgdorferi* on the C6 ELISA. The presence of oak trees is a well-documented phenomenon that is associated with increased populations of *Ixodes scapularis* ticks (Elias et al., 2013; Ostfeld et al., 2001; Ostfeld et al., 2006). Each lifestage of *I. scapularis* has preferred hosts on which it will take a bloodmeal (Burgdorfer et al., 1982). Larval and nymph ticks often get their blood meal from small rodents, particularly sthe white-footed mice (*Peromyscus leucopus*), while adult ticks will often feed on white-tailed deer (*Odocoileus virginianus*)(Anderson & Magnarelli, 1984; Bosler et al., 1984; Lane, 1991; Main et al., 1982). Acorns from oak trees serve as a critical food source for both mice and deer (Ostfeld et al., 1996; Ostfeld et al., 2001, 2006). Research has shown a strong positive correlation between the abundance of acorns and the density of *I. scapularis* (Jones et al., 1998; Ostfeld et al., 2001). Currently the relationship between horses and their proximity to oak trees and the association with increased risk of Lyme disease has not been studied. Our research provides new information about the possibility that the proximity of oak trees may increase the risk of horses encountering *I. scapularis* and being exposed to *B. burgdorferi.* Identification of a biologically plausible risk factor may provide some additional support for the accuracy of this test.

As our results showed, regular tick checks were significantly negatively associated with seropositivity on the C6 although a true causal (protective) relationship cannot be established with this type of study. As transmission of the bacteria to the host occurs within 24-48 hours of tick attachment prompt removal of ticks reduces the risk of infection. Therefore, performing daily

checks for ticks and properly removing ticks could decrease the risk of transmission (Piesman et al., 1987).

Access to forested habitat in the past 12 months showed evidence of confounding with performing regular tick checks. Previous research has shown that *I. scapularis* prefers to inhabit forested areas rather than fields or grassy areas (Bertrand & Wilson, 1996, 1997; Lindsay et al., 1998; Schulze et al., 1998). This is largely due to the ability of the tick to survive throughout all seasons in forested habitats as the soil and leaf litter act as protection from harsh weather (Guerra, 2002). Reducing the risk of infection requires not only checking horses regularly for ticks but also management practices for preventing tick exposures. Reduction of leaf litter, access to forested areas and decreased proximity or removal of oak trees can reduce the number of potential hosts and limit the suitable habitat space (Stafford III, 2007).

2.6 CONCLUSIONS

Diagnosis of Lyme disease in horses remains challenging and depends on appropriate clinical signs, elimination of differential diagnoses, probability of exposure to infected ticks, and serological testing (Divers, 2007). We found a lack of agreement between the two serological testing methods and their ability to detect *B. burgdorferi* exposure in horses that requires further investigation with repeated sampling to determine how these tests perform and how the antibodies may change over time. Our study suggests that limiting horse's exposure to forests and oak trees may reduce the risk of being seropositive. A combination of managing environmental factors and regular checks with subsequent prompt removal of ticks is crucial for controlling tick populations and possible exposure for horses. Continued surveillance studies in the areas of North America

where *I. scapularis* inhabits will help determine if there are patterns in the spread of Lyme disease and anaplasmosis in horses. This seroprevalence data and risk factor analysis, will help to inform equine practitioners when assessing the risk of potential *B. burgdorferi* exposure.

Table 2.1: Results from serological testing of serum samples from clinically healthy horses (n=551) using the C6 ELISA and the Lyme Multiplex ELISA.

Test	# of positive observations	Prevalence	95% Confidence Interval
C6 ELISA			
Borrelia burgdorferi	28	0.051	0.034-0.073
Anaplasma phagocytophilum	6	0.011	0.004-0.024
Co-exposure	3	0.005	4.0e-3 -0.000
Multiplex ELISA			
OspA (titer > 2000)	19	0.034	0.021-0.053
OspC (titer > 1000)	10	0.018	0.009-0.033
OspF (titer > 1250)	53	0.096	0.073-0.124
Positive on any test (C6 or Multiplex)	91	0.165	0.138- 0.203
Positive on any Multiplex Osp	78	0.142	0.114-0.173

Table 2.2: The level of agreement between a positive *B. burgdorferi* (*Bb*) on the C6 ELISA and the individual outer surface proteins (Osp's) on the Multiplex ELISA as shown by the kappa value and expected/actual agreement

Tests	Kappa	Expected agreement	Actual Agreement
C6 ELISA (*Bb*) + Any Osp	0.225	82.20%	86.21%
C6 ELISA (*Bb*) + OspA	0.046	91.82%	92.20%
C6 ELISA (*Bb*) + OspC	0.027	93.29%	93.47%
C6 ELISA (*Bb*) + OspF	0.273	86.28%	90.02%

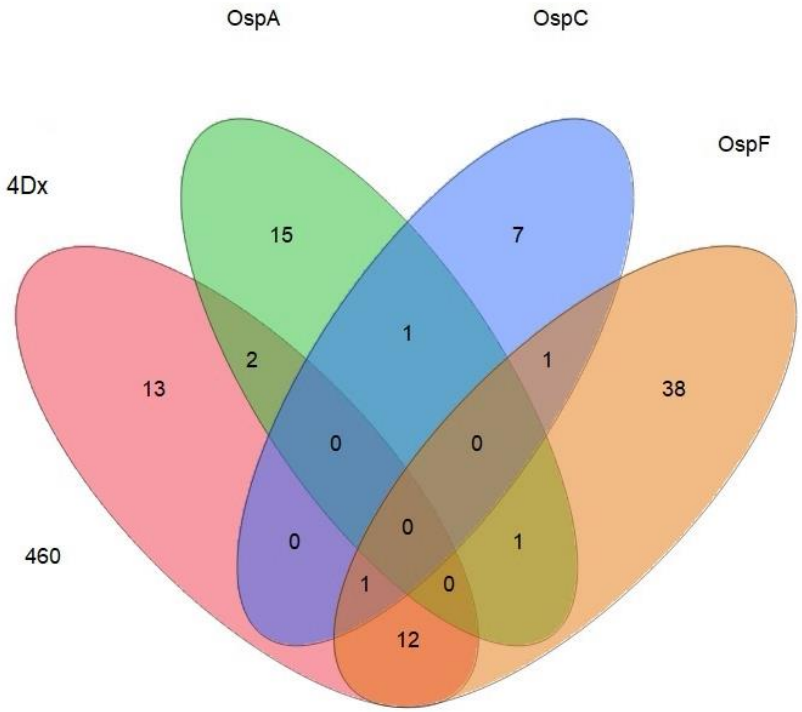

Figure 2.1: Venn diagram illustrating cross-positivity of *B. burgdorferi* positive test results for the C6 ELISA and the individual Osp's from the Multiplex ELISA. 460 horses were negative on both tests.

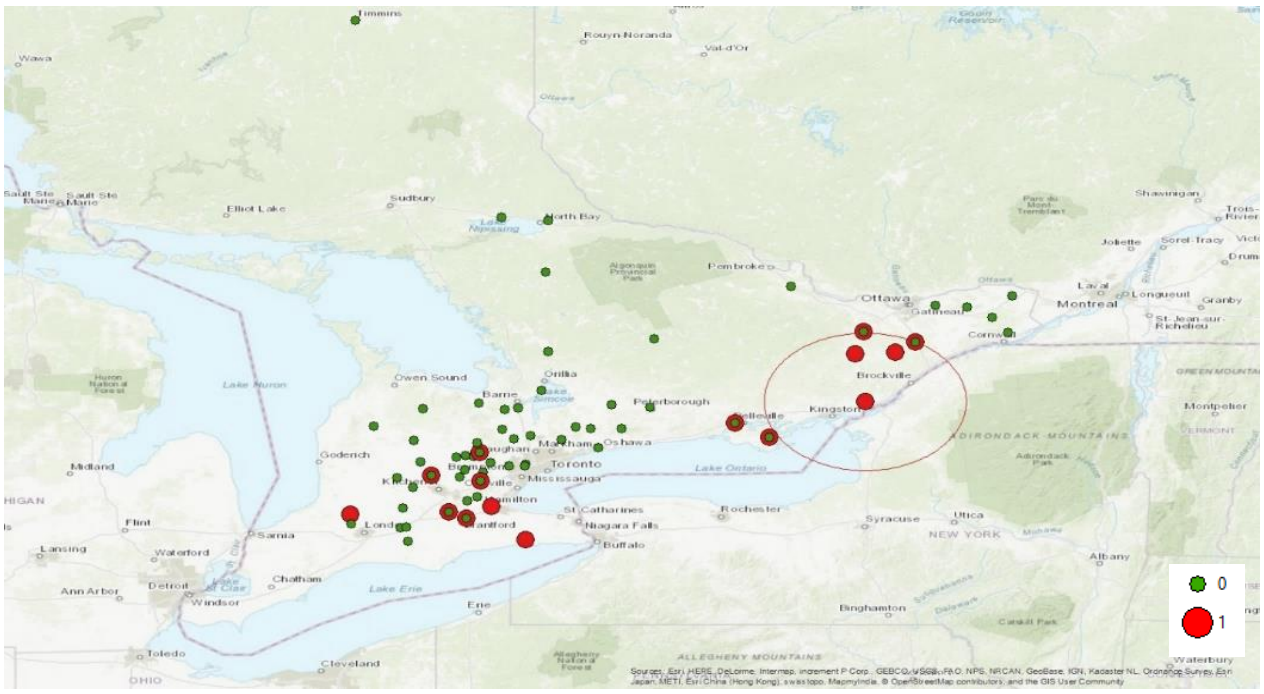

Figure 2.2: The dot density of veterinary clinics showing the prevalence of *B. burgdorferi* positive (red) and negative (green) samples in Ontario horses. A significant spatial cluster of four veterinary clinics with seropositive samples from either test was detected in Eastern Ontario (hollow red circle).

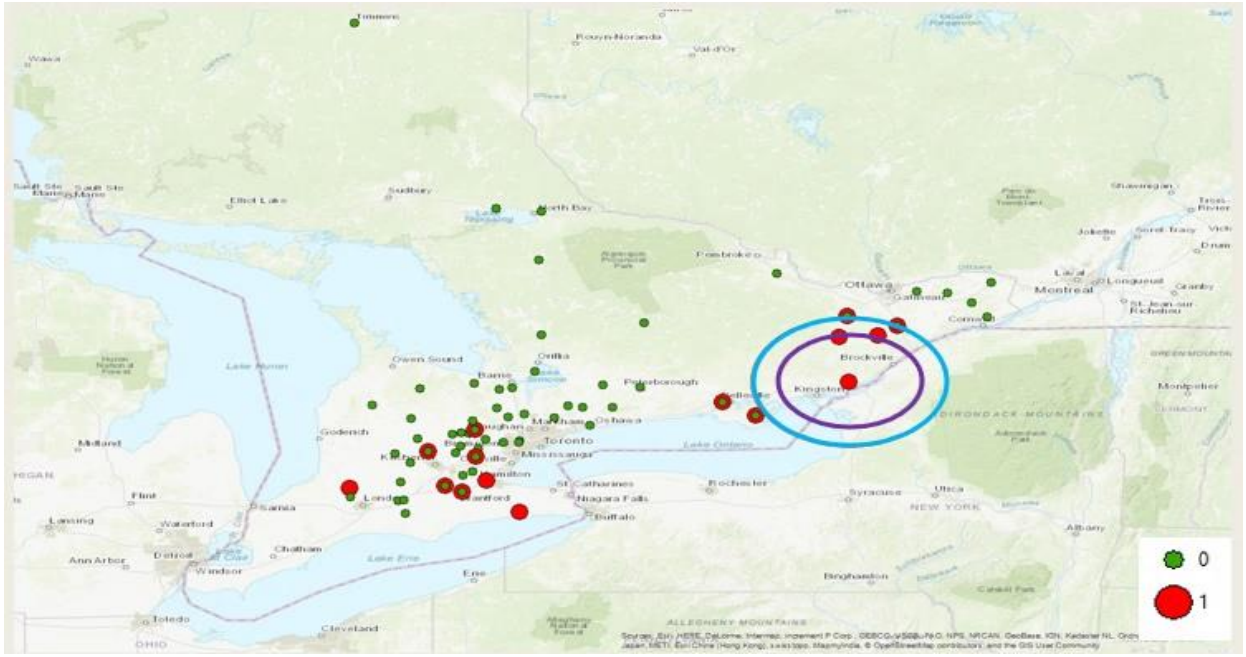

Figure 2.3: The dot density of the prevalence of *B. burgdorferi* positive (red) and negative (green) samples in Ontario horses. The hollow blue circle represents a significant spatial cluster of C6 ELISA seropositive samples from four veterinary clinics in Eastern Ontario, and the purple hollow circle represents the significant spatial cluster of Multiplex seropositive samples that contained two of the four veterinary clinics.

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2.8 APPENDICES

Appendix 2.1: Results of mixed-effect univariable logistic regression analysis with county as random effect for all explanatory variables associated with any positive test result

Explanatory variable		Number of responses	% positive	Odds Ratio	Confidence Interval (95%)	P-value	Wald Chi	ICC
Length of time horse has left the property in the past 12 months								
Less than 24 hours (Y, N=referent)	N	284/425	16.5% (12.4%-21.4%)	Referent			0.060	0.148 (0.059-0.326)
	Y	141/425	14.9% (9.5%-21.9%)	1.062	0.659-1.713	0.805		
1-7 days (Y, N=referent)	N	323/389	15.5% (11.7%-19.9%)	Referent			0.710	0.148 (0.059-0.326)
	Y	66/389	13.6% (6.4%-24.3%)	1.231	0.760-1.992	0.398		
8-14 days (Y, N=referent)	N	356/374	15.4% (11.9%-19.6%)	Referent			1.240	0.150 (0.059-0.329)
	Y	18/374	16.7% (3.5%-41.4%)	1.33	0.805-2.198	0.266		
More than 14 days (Y, N=referent)	N	336/384	16.1% (12.3%-20.4%)	Referent			0.260	0.148 (0.059-0.325)
	Y	48/384	14.6% (6.1%-27.8%)	1.138	0.695-1.865	0.607		
Hours spent outside, based on season								
Spring								
Less than 5 hours (including 0)		71/449	18.3% (10.1%-29.3%)	Referent			0.649	0.064 (0.010-0.324)
6-12 hours		169/449	14.8% (9.8%-21.1%)	0.694	0.320-1.502	0.354		
24 hours		209/449	15.3% (10.7%-20.9%)	0.795	0.380-1.666	0.544		
Summer								
Less than 5 hours (including 0)		72/449	18.1% (10.0%-28.9%)	referent			0.677	0.060 (0.008-0.326)
6-12 hours		140/449	14.3% (8.9%-21.2%)	0.703	0.318-1.555	0.384		
24 hours		237/449	15.6% (11.2-20.9%)	0.832	0.403-1.718	0.619		
Fall								
Less than 5 hours (including 0)		66/449	18.2% (9.8%-29.6%)	Referent			0.619	0.067 (0.011-0.324)
6-12 hours		168/449	14.9% (9.9%-21.2%)	0.667	0.296-1.504	0.329		
24 hours		215/449	15.3% (10.8-20.9%)	0.765	0.352-1.663	0.499		
Winter								
Less than 5 hours (including 0)		111/445	18.9% (12.1-27.5%)	Referent			0.388	0.074 (0.130-0.322)
6-12 hours		170/445	14.1% (9.3%-20.3%)	0.618	0.311-1.229	**0.170**		
24 hours		164/445	15.2% (10.1-21.7%)	0.787	0.405-1.527	0.479		
Horse is prevented from grazing in forested areas by fencing (Y, N=referent)	N	93/423	20.4% (12.8-30.1%)	Referent			0.328	0.059 (0.009-0.308)
	Y	330/423	15.8% (12.0%-20.1%)	0.737	0.400-1.359	0.328		
Presence of oak trees bordering or in the pasture (Y, N=referent)	N	251/370	14.7% (10.6%-19.7%)	Referent			0.265	1.23e-30
	Y	119/370	19.3% (12.7%-27.6%)	1.386	0.781-2.459	0.265		
Habitats horse has spent any time in during past 12 months								
Shrubland (Y, N=referent)	N	208/430	15.4% (10.8-21.0%)	Referent			0.794	0.013 (4.7e-5-0.782)
	Y	222/430	16.2% (11.6%-21.7%)	0.932	0.551-1.578	0.794		
Forested (Y, N=referent)	N	174/419	15.5% (10.5-21.8%)	Referent			0.728	0.012 (3.1e-5-0.832)
	Y	245/419	16.7% (12.3-22.0%)	0.909	0.531-1.557	0.728		
Grassland (Y, N=referent)	N	395/442	16.5% (12.9-20.5%)	Referent			0.397	0.057 (0.007-0.327)
	Y	47/442	12.8% (4.8-25.7%)	1.506	0.584-3.883	0.397		
Wetland (Y, N=referent)	N	144/419	13.2% (8.1-19.8%)	Referent			0.302	0.022 (5.0e-4-0.507)
	Y	275/419	17.1% (12.8-22.1%)	0.736	0.411-1.317	0.302		
Urban/Recreational (Y, N=referent)	N	79/422	13.9% (7.2-23.5%)	Referent			0.529	0.018 (2.2e-4-0.595)

	Y	343/422	16.6% (12.8-21.0%)	0.797	0.393-1.615	0.529		
Animals seen on the property								
Coyotes								
Never		68/448	16.2% (8.4-27.1%)	Referent			0.890	0.044 (0.004-0.353)
Occasionally		289/448	17.0% (12.8-21.8%)	1.094	0.525-2.277	0.811		
Frequently		91/448	14.3% (7.8%-23.2%)	0.933	0.377-2.308	0.881		
Deer								
Never		44/448	9.1% (2.5-21.7%)	Referent			0.367	0.055 (0.008-0.308)
Occasionally		251/448	17.1% (12.7-22.4%)	2.216	0.736-6.673	**0.157**		
Frequently		153/448	16.3% (10.9-23.2%)	2.058	0.66-6.419	0.213		
Foxes								
Never		118/442	18.6% (12.1-26.9%)	Referent			0.635	0.063 (0.009-0.345)
Occasionally		275/442	15.3% (11.2-20.1%)	0.747	0.409-1.362	0.341		
Frequently		49/442	16.3% (7.3-29.7%)	0.839	0.335-2.101	0.708		
Opossums								
Never		276/439	15.9% (11.8-20.8%)	Referent			0.660	0.044 (0.003-0.393)
Occasionally		149/439	17.4% (11.7-24.5%)	1.134	0.647-1.987	0.660		
Frequently		14/439	0	1				
Skunks								
Never		108/442	15.7% (9.4-24.0%)	Referent			0.966	0.048 (0.005-0.339)
Occasionally		265/442	16.6% (12.3-21.6%)	1.088	0.58-2.042	0.792		
Frequently		69/442	15.9% (8.2-26.7%)	1.07	0.456-2.512	0.876		
Tick has previously been found on horse	N	386/443	15.3% (11.8-19.3%)	Referent			0.275	0.054 (0.006-0.342)
(Y, N=referent)	Y	57/443	22.8% (12.7-35.8%)	1.509	0.721-3.158	0.275		
Horse is checked regularly for ticks	N	225/452	16.9% (12.2-22.4%)	Referent			0.600	0.054 (0.007-0.320)
(Y, N=referent)	Y	227/452	15.4% (11.0-20.8%)	1.150	0.682-1.942	0.600		
Insect repellent is applied to horse	N	38/449	26.3% (13.4-43.1%)	Referent			0.103	0.046 (0.005-0.330)
(Y, N=referent)	Y	411/449	15.3% (12.0-19.2%)	1.937	0.875-4.291	**0.103**		
Horse is used for any of these activities								
Companion only (Y, N=referent)	N	256/451	14.8% (10.7-19.8%)	Referent			0.511	0.049 (0.005-0.331)
	Y	195/451	17.4% (12.4-23.5%)	1.191	0.708-2.003	0.511		
Trail riding (Y, N=referent)	N	286/451	16.1% (12.0-20.9%)	Referent			0.839	0.052 (0.006-0.323)
	Y	165/451	15.8% (10.6-22.2%)	0.946	0.551-1.623	0.839		
Local competition (Y, N=referent)	N	326/451	16.3% (12.4-20.7%)	Referent			0.874	0.050 (0.006-0.328)
	Y	125/451	15.2% (9.4-22.7%)	0.953	0.529-1.719	0.873		
Breeding (Y, N=referent)	N	413/451	15.5% (12.1-19.4%)	Referent			0.319	0.054 (0.007-0.319)
	Y	38/451	21.1% (9.6-37.3%)	1.558	0.652-3.726	0.319		
Riding school (Y, N=referent)	N	398/451	16.1% (12.6-20.1%)	Referent			0.830	0.051 (0.006-0.325)
	Y	53/451	15.1% (6.7-27.6%)	0.914	0.402-2.077	0.830		
Hunting (Y, N=referent)	N	448/451	16.1% (12.8-19.8%)	Referent				
	Y	3/451	0	1				
Age of the horse								
3-9 years old		163/528	11.0% (6.7-16.9%)	Referent			0.008	0.183 (0.076-0.377)
10-12 years old		102/528	10.8% (5.5-18.5%)	0.939	0.408-2.162	0.883		
13-18 years old		148/528	20.9% (14.7-28.4%)	2.208	1.136-4.293	**0.019**		
19-33 years old		115/528	23.5% (16.1-32.3%)	2.674	1.326-5.396	**0.006**		

Appendix 2.2: Results of mixed-effect univariable logistic regression analysis with county as random effect for all explanatory variables associated with a positive titer on the Multiplex ELISA

Explanatory variable		Number of responses	% positive	Odds Ratio	Confidence Interval (95%)	P-value	Wald Chi	ICC
Length of time horse has left the property in the past 12 months								
Less than 24 hours (Y, N=referent)	N	284/425	16.1% (11.8-21.3%)	Referent				0.066 (0.013-0.272)
	Y	141/425	13.5% (8.3-20.2%)	1.044	0.632-1.724	0.868	0.030	
1-7 days (Y, N=referent)	N	323/389	13.3% (9.8-17.5%)	Referent				0.066 (0.013-0.270)
	Y	66/389	13.6% (6.4-24.3%)	1.18	0.713-1.953	0.520	0.410	
8-14 days (Y, N=referent)	N	356/374	13.5% (10.1-17.5%)	Referent				0.066 (0.013-0.270)
	Y	18/374	16.7% (3.6-41.4%)	1.163	0.69-1.959	0.572	0.320	
More than 14 days (Y, N=referent)	N	336/384	14.3% (10.7-18.5%)	Referent				0.065 (0.013-0.271)
	Y	48/384	12.5% (4.7-25.2%)	0.969	0.577-1.627	0.905	0.010	
Amount of time horse spends outside in each season								
Spring								
Less than 5 hours (including 0)		71/449	15.5% (8.0-26.0%)	Referent				0.059 (0.008-337)
6-12 hours		169/449	13.6% (8.8-19.7%)	0.775	0.344-1.75	0.540	0.758	
24 hours		209/449	12.9% (8.7-18.2%)	0.743	0.336-1.642	0.463		
Summer								
Less than 5 hours (including 0)		72/449	16.7% (8.9-27.3%)	Referent				0.065 (0.009-0.333)
6-12 hours		140/449	12.1% (7.2-18.7%)	0.614	0.265-1.424	0.256	0.515	
24 hours		237/449	13.5% (9.4-18.5%)	0.699	0.325-1.502	0.359		
Fall								
Less than 5 hours (including 0)		66/449	18.2 (9.8-29.6%)	Referent				0.075 (0.013-0.334)
6-12 hours		168/449	12.5% (7.9-18.5%)	0.523	0.224-1.219	**0.133**	0.298	
24 hours		215/449	13.0% (8.8-18.3%)	0.572	2.555-1.279	**0.173**		
Winter								
Less than 5 hours (including 0)		111/445	16.2% (9.9-24.4%)	Referent				0.072 (0.012-0.338)
6-12 hours		170/445	12.9% (8.3-18.9%)	0.660	0.319-1.366	0.263	0.497	
24 hours		164/445	12.8% (8.1-18.9%)	0.706	0.345-1.443	0.339		
Horse is prevented from grazing in forested areas by fencing (Y, N=referent)	N	93/423	19.4% (11.9-28.9%)	Referent				0.065 (0.011-0.301)
	Y	330/423	13.3% (9.9-17.5%)	0.640	0.339-1.207	**0.168**	0.168	
Oak trees are bordering or in the pasture (Y, N=referent)	N	251/370	13.1% (9.2-18.0%)	Referent				6.4e-31
	Y	119/370	15.1% (9.2-22.8%)	1.177	0.633-2.190	0.606	0.606	
Habitats horse has spent any time in during past 12 months								
Shrubland (Y, N=referent)	N	208/430	13.5% (9.1-18.9%)	Referent				0.018 (3.0e-4-0.522)
	Y	222/430	14.0% (9.7-19.2%)	0.954	0.546-1.667	0.869	0.869	
Forested (Y, N=referent)	N	174/419	12.6% (8.1%-18.5%)	Referent				0.021 (4.7e-4-0.494)
	Y	245/419	15.1% (10.9-20.2%)	0.802	0.448-1.435	0.458	0.458	
Grassland (Y, N=referent)	N	395/442	14.4% (11.1-18.3%)	Referent				0.064 (0.01-0.323)
	Y	47/442	10.6% (3.5-23.1%)	1.571	0.570-4.330	0.382	0.382	
Wetland (Y, N=referent)	N	144/419	13.2% (8.1-19.8%)	Referent				0.028 (0.001-0.395)
	Y	275/419	13.8% (10.0-18.5%)	0.948	0.521-1.725	0.861	0.861	
Urban/Recreational (Y, N=referent)	N	79/422	11.4% (5.3-20.5%)	Referent				0.024 (7.8e-4-0.428)
	Y	343/422	14.6% (11.0-18.8%)	0.733	0.340-1.578	0.427	0.427	

Animals seen on the property								
Coyotes								
Never		68/448	14.7% (7.3-25.4%)	Referent				0.057 (0.007-0.332)
Occasionally		289/448	14.2% (10.4-18.8%)	0.998	0.462-2.156	0.995	0.988	
Frequently		91/448	14.3% (7.8-23.2%)	1.054	0.417-2.67	0.911		
Deer								
Never		44/448	6.8% (1.4-18.7%)	Referent				0.065 (0.011-0.306)
Occasionally		251/448	15.1% (10.9-20.2%)	2.673	0.768-9.306	**0.122**	0.303	
Frequently		153/448	14.4% (9.2-21.0%)	2.483	0.689-8.951	**0.165**		
Foxes								
Never		118/442	13.6% (8.0-21.1%)	Referent				0.068 (0.01-0.34)
Occasionally		275/442	14.2% (10.3-18.9%)	1.015	0.526-1.958	0.965	0.889	
Frequently		49/442	16.3% (7.3-30.0%)	1.244	0.479-3.233	0.654		
Opossums								
Never		276/439	14.5% (10.6-19.2%)	Referent				0.062 (0.008-0.344)
Occasionally		149/439	14.1% (8.9-20.7%)	1.006	0.547-1.849	0.986	0.986	
Frequently		14/439	0	1				
Skunks								
Never		108/442	13.9% (8.0-21.9%)	Referent				0.0584 (0.008-0.326)
Occasionally		265/442	14.3% (10.4-19.1%)	1.08	0.556-2.112	0.814	0.963	
Frequently		69/442	14.5% (7.2-25.0%)	1.118	0.457-2.731	0.807		
Tick has previously been found on horse	N	386/443	13.7% (10.5-17.6%)	Referent				0.070 (0.011-0.338)
(Y, N=referent)	Y	57/443	17.5% (8.7-29.9%)	1.231	0.548-2.769	0.615	0.615	
Horse is checked regularly for ticks	N	225/452	13.8% (9.6-19.0%)	Referent				0.06 (0.009-0.324)
(Y, N=referent)	Y	227/452	14.5% (10.2-19.8%)	0.975	0.561-1.693	0.928	0.928	
Insect repellent is applied to horse	N	38/449	21.1% (9.6-37.3%)	Referent				0.056 (0.008-0.318)
(Y, N=referent)	Y	411/449	13.6% (10.5-17.3%)	1.677	0.711-3.957	0.237	0.238	
Horse is used for any of these activities								
Companion only (Y, N=referent)	N	256/451	12.5% (8.7-17.2%)	Referent				0.054 (0.006-0.334)
	Y	195/451	15.9% (11.1-21.8%)	1.287	0.743-2.229	0.368	0.368	
Trail riding (Y, N=referent)	N	286/451	15.0% (11.1-19.7%)	Referent				0.062 (0.009-0.321)
	Y	165/451	12.1% (7.6-18.1%)	0.743	0.413-1.338	0.322	0.322	
Local competition (Y, N=referent)	N	326/451	13.5% (10.0-17.7%)	Referent				0.060 (0.008-0.325)
	Y	125/451	15.2% (9.4-22.7%)	1.185	0.647-2.168	0.583	0.583	
Breeding (Y, N=referent)	N	413/451	13.6% (10.4-17.2%)	Referent				0.062 (0.009-0.321)
	Y	38/451	18.4% (7.7-34.3%)	1.578	0.628-3.956	0.332	0.332	
Riding school (Y, N=referent)	N	398/451	13.8% (10.6-17.6%)	Referent				0.058 (0.008-0.322)
	Y	53/451	15.1% (6.7-27.6%)	1.136	0.496-2.602	0.762	0.762	
Hunting (Y, N=referent)	N	448/451	14.1% (11.0-17.6%)	Referent				
	Y	3/451		1				
Age of the horse								
3-9 years old		163/528	8.6 (4.8-14.0%)	Referent			0.005	0.107 (0.029-0.325)
10-12 years old		102/528	7.8% (3.4-14.9%)	0.879	0.349-2.215	0.785		
13-18 years old		148/528	17.6% (11.8-24.7%)	2.280	1.121-4.640	**0.023**		
19-33 years old		115/528	20.9 (13.9-29.4%)	2.912	1.395-6.080	**0.004**		

Appendix 2.3: Results of mixed-effect univariable logistic regression analysis with county as random effect for all explanatory variables associated with a positive OspF titer on the Multiplex ELISA

Explanatory variable		Number of responses	% positive	Odds Ratio	Confidence Interval (95%)	P-value	Wald Chi	ICC
Length of time horse has left the property in the past 12 months								
Less than 24 hours (Y, N=referent)	N	284/425	9.9% (6.7-13.9%)	Referent			0.030	0.035 (0.001-0.497)
	Y	141/425	9.9% (5.5-16.1%)	0.947	0.532-1.686	0.854		
1-7 days (Y, N=referent)	N	323/389	9.3% (6.4-13.0%)	Referent			0.110	0.035 (0.001-0.486)
	Y	66/389	9.1% (3.4-18.7%)	1.104	0.617-1.973	0.739		
8-14 days (Y, N=referent)	N	356/374	9.3% (6.5-12.8%)	Referent			0.160	0.035 (0.001-0.482)
	Y	18/374	16.7 (3.6-41.4%)	1.132	0.623-2.057)	0.685		
More than 14 days (Y, N=referent)	N	336/384	10.1% (7.1-13.9%)	Referent			0.210	0.0.34 (0.001-0.514)
	Y	48/384	8.3% (2.3-20.0%)	0.869	0.477-1.585)	0.648		
Amount of time horse spends outside in each season								
Spring								
Less than 5 hours (including 0)		71/449	8.5% (3.2-17.5%)	Referent			0.919	0.005 (5.74e-13, 1)
6-12 hours		169/449	10.1% (6.0-15.6%)	1.202	0.446-3.24	0.716		
24 hours		209/449	9.1% (5.6-13.8%)	1.076	0.407-2.847	0.882		
Summer								
Less than 5 hours (including 0)		72/449	9.7% (4.0-19.0%)	Referent			0.928	0.009 (2.45e-08, 1)
6-12 hours		140/449	8.6% (4.5-14.5%)	0.859	0.318-2.321	0.765		
24 hours		237/449	9.7% (6.3-14.2%)	0.983		0.971		
Fall								
Less than 5 hours (including 0)		66/449	10.6% (4.4-20.6%)	Referent			0.902	0.015 (4.78e-06, 0.979)
6-12 hours		168/449	8.9% (5.1-14.3%)	0.795	0.295-2.145			
24 hours		215/449	9.3% (5.8-14.0%)	0.838	0.326-2.154			
Winter								
Less than 5 hours (including 0)		111/445	9.9% (5.1-17.0%)	Referent			0.971	0.014 (2.65e-06, 0.987)
6-12 hours		170/445	9.4% (5.5-14.8%)	0.92	0.398-2.127	0.845		
24 hours		164/445	9.1 (5.2-14.6%)	0.906	0.396-2.073	0.814		
Horse is prevented from grazing in forested areas by fencing (Y, N=referent)	N	93/423	14.0% (7.7-22.7%)	Referent			0.204	0.030 (7.0e-4-0.574)
	Y	330/423	9.4% (6.5-13.0%)	0.632	0.312-1.283	**0.204**		
Oak trees are bordering or in the pasture (Y, N=referent)	N	251/370	8.4% (5.3-12.5%)	Referent			0.428	4.96e-32
	Y	119/370	10.9% (5.4-18.0%)	0.744	0.359-1.543	0.427		
Habitats horse has spent any time in during past 12 months								
Shrubland (Y, N=referent)	N	208/430	9.1% (5.6-13.9%)	Referent			0.785	7.28e-33
	Y	222/430	9.9% (6.3-14.6%)	0.914	0.479-1.742	0.784		
Forested (Y, N=referent)	N	174/419	8.0% (4.5-13.1%)	Referent			0.315	5.16e-31
	Y	245/419	11.0% (7.4-15.6%)	0.707	0.359-1.390	0.315		
Grassland (Y, N=referent)	N	395/442	9.9% (7.1-13.2%)	Referent			0.899	0.016 (2.3e-5, 0.924)
	Y	47/442	10.6% (3.5-23.1%)	0.937	0.345-2.550	0.899		
Wetland (Y, N=referent)	N	144/419	9.7% (5.4-15.8%)	Referent			0.831	0.007 (9.85e-09,1)
	Y	275/419	9.1% (6.0-13.1%)	1.078	0.541-2.148	0.831		
Urban/Recreational (Y, N=referent)	N	79/422	8.9% (3.6-17.4%)	Referent			0.776	3.98e-29
	Y	343/422	9.9% (7.0-13.6%)	0.884	0.377-2.074	0.776		

Animals seen on the property								
Coyotes								
Never		68/448	11.8% (5.2-219%)	Referent			0.728	0.014 (5.67e-06, 0.971)
Occasionally		289/448	9.0% (6.0-12.9%)	0.752	0.321-1.76	0.511		
Frequently		91/448	11.0% (5.4-19.3%)	0.951	0.345-2.617	0.922		
Deer								
Never		44/448	2.3% (0.06-12.0%)	Referent			0.253	0.033 (0.001-0.563)
Occasionally		251/448	10.4% (6.9-14.8%)	5.344	0.694-41.157	**0.108**		
Frequently		153/448	11.1% (6.6-17.2%)	5.69	0.725-44.645	**0.098**		
Foxes								
Never		118/442	9.3% (4.7-16.1%)	Referent			0.500	0.034 (0.001-0.631)
Occasionally		275/442	9.1% (6.0-13.1%)	0.946	0.441-2.031	0.887		
Frequently		49/442	14.3 (5.9-27.2%)	1.652	0.589-4.631	0.34		
Opossums								
Never		276/439	9.8% (6.5-13.9%)	Referent			0.738	0.016 (1.0e-4 -0.961)
Occasionally		149/439	10.7 (6.3-16.9%)	1.121	0.576-2.181	0.738		
Frequently		14/439	0	1				
Skunks								
Never		108/442	10.2% (5.2-17.5%)	Referent			0.850	0.018 (4.0e-3 -0.898)
Occasionally		265/442	9.4% (6.2-13.6%)	0.929	0.436-1.978	0.848		
Frequently		69/442	11.6% (5.1-21.6%)	1.193	0.444-3.204	0.727		
Tick has previously been found on horse (Y, N=referent)	N	386/443	8.8% (6.2-12.1%)	Referent			0.118	0.024 (2.4e-4 -0.718)
	Y	57/443	15.8% (7.5-27.9%)	0.512	0.230-1.180	**0.118**		
Horse is checked regularly for ticks (Y, N=referent)	N	225/452	10.2% (6.6-14.9%)	Referent			0.698	0.020 (9.3e-5 -0.824)
	Y	227/452	9.3% (5.8-13.8%)	1.134	0.601-2.136	0.698		
Insect repellent is applied to horse (Y, N=referent)	N	38/449	18.4% (7.7-34.3%)	Referent			0.070	0.016 (3.3e-5-0.896)
	Y	411/449	9.0% (6.4-12.2%)	2.287	0.933-5.604	**0.070**		
Horse is used for any of these activities								
Companion only (Y, N=referent)	N	256/451	9.0% (5.8-13.2%)	Referent			0.545	0.016 (1.6e-5 -0.939)
	Y	195/451	10.8% (6.8-16.0%)	1.215	0.648-2.277	0.544		
Trail riding (Y, N=referent)	N	286/451	10.5% (7.2-14.6%)	Referent			0.456	0.24 (1.7e-3 -0.772)
	Y	165/451	8.5% (4.7-13.8%)	0.773	0.392-1.523	0.456		
Local competition (Y, N=referent)	N	326/451	9.5% (6.6-13.2%)	Referent			0.762	0.019 (5.3e-5 -0.872)
	Y	125/451	10.4% (5.1-16.2%)	1.113	0.558-2.220	0.762		
Breeding (Y, N=referent)	N	413/451	9.0% (6.4-12.1%)	Referent			0.062	0.032 (5.9e-4-0.643)
	Y	38/451	18.4% (7.7-34.3%)	2.443	0.958-6.230	**0.062**		
Riding school (Y, N=referent)	N	398/451	9.8% (7.1-13.2%)	Referent			0.938	0.018 (4.1e-5 -0.890)
	Y	53/451	9.4% (3.1-20.7%)	0.961	0.359-2.576	0.938		
Hunting (Y, N=referent)	N	448/451	9.8% (7.2-13.0%)	Referent				
	Y	3/451	0	1				
Age of the horse								
3-9 years old		163/528	6.7% (3.4-11.8%)	Referent			0.073	0.046 (0.003-0.467)
10-12 years old		102/528	4.9% (1.6-11.1%)	0.713	0.239-2.130	0.545		
13-18 years old		148/528	12.8% (7.9-19.3%)	2.025	0.923-4.446	**0.079**		
19-33 years old		115/528	13.0% (7.5-20.6%)	2.071	0.905-4.742	**0.085**		

Appendix 2.4: Results of mixed-effect univariable logistic regression analysis with county as random effect for all explanatory variables associated with a *Borrelia burgdorferi* positive result on the C6 ELISA

Explanatory variable		Number of responses	% positive	Odds Ratio	Confidence Interval (95%)	P-value	Wald Chi	ICC
Length of time horse has left the property in the past 12 months								
Less than 24 hours (Y, N=referent)	N	284/425	4.9% (2.7-8.1%)	Referent			0.120	0.437 (0.205-0.699)
	Y	141/425	1.4% (0.2-5.0%)	0.852	0.348-2.083	0.725		
1-7 days (Y, N=referent)	N	323/389	4.6% (2.6-7.5%)	Referent			0.160	0.438 (0.207-0.670)
	Y	66/389	0	0.831	(0.335-2.062)	0.690		
8-14 days (Y, N=referent)	N	356/374	3.9% (2.2%-6.5%)	Referent			0.600	0.435 (0.202-0.701)
	Y	18/374	5.6% (0.1-27.3%)	1.443	0.573-3.634	0.437		
More than 14 days (Y, N=referent)	N	336/384	3.9% (2.1-6.5%)	Referent			0.500	0.432 (0.200-0.697)
	Y	48/384	4.2% (0.5-1.4%)	1.383	0.563-3.40	0.479		
Amount of time horse spends outside in each season								
Spring								
Less than 5 hours (including 0)		71/449	5.6% (1.6-13.8%)	Referent			0.538	0.320 (0.096-0.677)
6-12 hours		169/449	3.0% (0.9-6.8%)	0.439	0.102-1.893	0.270		
24 hours		209/449	3.3% (1.4-6.8%)	0.570	0.144-2.62	0.424		
Summer								
Less than 5 hours (including 0)		72/449	5.6% (1.5-13.6%)	Referent			0.726	0.300 (0.086-0.66)
6-12 hours		140/449	3.6% (1.2-8.1%)	0.644	0.152-2.723	0.549		
24 hours		237/449	3.0% (1.2-6.0%)	0.580	0.149-2.258	0.432		
Fall								
Less than 5 hours (including 0)		66/449	4.5% (0.9-12.7%)	Referent			0.901	0.307 (0.904-0.665)
6-12 hours		168/449	3.6% (1.3-7.6%)	0.765	0.165-3.555	0.733		
24 hours		215/449	3.3% (1.3-6.6%)	0.703	0.155-3.193	0.648		
Winter								
Less than 5 hours (including 0)		111/445	3.6% (1.0-9.0%)	Referent			0.733	0.329 (0.101-0.683)
6-12 hours		170/445	2.9% (1.0-6.7%)	0.754	0.174-3.261	0.706		
24 hours		164/445	4.3% (1.7-8.6%)	1.252	0.317-4.941	0.748		
Horse is prevented from grazing in forested areas by fencing (Y, N=referent)	N	93/423	8.6% (3.8-16.2%)	Referent			0.034	0.257 (0.059-0.657)
	Y	330/423	2.4% (1.1%-4.7%)	0.304	0.101-0.916	**0.034**		
Oak trees are bordering or in the pasture (Y, N=referent)	N	251/370	1.2% (2.5-3.5%)	Referent			0.006	0.217 (0.025-0.747)
	Y	119/370	8.4% (4.1-14.9%)	6.804	1.736-26.662	**0.006**		
Habitats horse has spent any time in during past 12 months								
Shrubland (Y, N=referent)	N	208/430	3.8% (1.7-7.4%)	Referent			0.547	0.124 (0.004-0.817)
	Y	222/430	2.7% (1.0-5.8%)	1.404	0.465-4.233	0.547		
Forested (Y, N=referent)	N	174/419	4.6% (0.2-8.9%)	Referent			0.159	0.179 (0.015-0.764)
	Y	245/419	2.0% (0.7-4.7%)	2.365	0.714-7.829	**0.159**		
Grassland (Y, N=referent)	N	395/442	3.8% (2.1-6.2%)	Referent			0.528	0.316 (0.094-0.673)
	Y	47/442	2.1% (0.05-11.3%)	2.043	0.223-18.754	0.528		
Wetland (Y, N=referent)	N	144/419	2.1% (0.4-6.0%)	Referent			0.342	0.213 (0.024-0.750)
	Y	275/419	3.6% (1.8-6.6%)	0.517	0.133-2.015	0.342		
Urban/Recreational (Y, N=referent)	N	79/422	1.3% (0.03-6.9%)	Referent			0.365	0.148 (0.008-0.785)
	Y	343/422	3.5% (1.8-6.0%)	0.381	0.047-3.079	0.365		

Animals seen on the property								
Coyotes								
Never		68/448	4.4% (0.92-12.4%)	Referent			0.894	0.314 (0.095-0.674)
Occasionally		289/448	3.5% (1.8-6.3%)	0.725	0.175-2.997	0.656		
Frequently		91/448	3.3% (0.7-9.3%)	0.871	0.148-5.109	0.878		
Deer								
Never		44/448	2.3% (0.06-12.0%)	Referent			0.782	0.306 (0.089-0.666)
Occasionally		251/448	3.2% (1.4-6.2%)	1.215	0.132-11.142	0.863		
Frequently		153/448	4.6% (1.9-9.2%)	1.732	0.186-16.097	0.629		
Foxes								
Never		118/442	4.2% (1.4-9.6%)	Referent			0.541	0.349 (0.110-0.699)
Occasionally		275/442	3.3% (1.5-6.1%)	0.473	0.125-1.79	0.27		
Frequently		49/442	4.1% (0.5-14%)	0.702	0.115-4.298	0.702		
Opossums								
Never		276/439	4.0% (2.0-7.0%)	Referent			0.997	0.294 (0.081-0.664)
Occasionally		149/439	3.4% (1.1-7.7%)	0.998	0.281-3.548	0.997		
Frequently		14/439	0	1				
Skunks								
Never		108/442	4.6% (1.5-10.5%)	Referent			0.878	0.311 (0.089- 0.674)
Occasionally		265/442	3.4% (1.6-6.3%)	0.732	0.219-2.451	0.613		
Frequently		69/442	2.9% (3.5-10.1%)	0.861	0.138-5.391	0.873		
Tick has previously been found on horse	N	386/443	2.6% (1.2-4.7%)	Referent			0.081	0.199 (0.030-0.669)
(Y, N=referent)	Y	57/443	10.5% (4.0-21.5%)	0.317	0.088-1.150	**0.081**		
Horse is checked regularly for ticks	N	225/452	5.8% (3.1-9.7%)	Referent			0.022	0.302 (0.088-0.660)
(Y, N=referent)	Y	227/452	1.3% (0.3-3.8%)	4.863	1.255-18.845	**0.022**		
Insect repellent is applied to horse	N	38/449	10.5% (2.9-24.8%)	Referent			0.075	0.281 (0.072-0.661)
(Y, N=referent)	Y	411/449	2.9% (1.5-5.0%)	3.266	0.888-12.012	**0.075**		
Horse is used for any of these activities								
Companion only (Y, N=referent)	N	256/451	3.9% (1.9-7.1%)	Referent			0.675	0.307 (0.091-0.662)
	Y	195/451	3.1 (1.1-6.6%)	0.790	0.264- 2.370	0.675		
Trail riding (Y, N=referent)	N	286/451	2.8% (1.2-5.4%)	Referent			0.184	0.333 (0.103-0.686)
	Y	165/451	4.8% (2.1-9.3%)	2.103	0.702-6.302	**0.184**		
Local competition (Y, N=referent)	N	326/451	3.7% (1.9-6.3%)	Referent			0.882	0.308 (0.091-0.665)
	Y	125/451	3.2% (0.9-8.0%)	0.911	0.265-3.131	0.882		
Breeding (Y, N=referent)	N	413/451	3.4% (1.9-5.6%)	Referent			0.386	0.328 (0.099-0.683)
	Y	38/451	5.3% (0.6-17.7%)	2.169	0.376-12.491	0.386		
Riding school (Y, N=referent)	N	398/451	3.8% (2.1-6.1%)	Referent			0.292	0.344 (0.108-0.696)
	Y	53/451	1.9% (0.05-10.1%)	0.309	0.035-2.752	0.292		
Hunting (Y, N=referent)	N	448/451	3.6% (2.1-5.7%)	Referent			0.675	0.307 (0.091-0.662)
	Y	3/451	0	1.265	0.422- 3.794	0.675		
Age of the horse								
3-9 years old		163/528	4.9% (2.1-9.4%)	Referent			0.756	0.445 (0.213-0.703)
10-12 years old		102/528	3.9% (1.1-9.7%)	0.670	0.173-2.603	0.563		
13-18 years old		148/528	6.1% (2.8-11.2%)	1.348	0.449-4.046	0.595		
19-33 years old		115/528	6.1% (2.5-12.1%)	1.269	0.385-4.186	0.695		

CHAPTER 3: EVALUATION OF *BORRELIA BURGDORFERI* SEROPOSITIVITY AND TEST REPEATABILITY IN HORSES OVER A 12-MONTH PERIOD

3.1 ABSTRACT

The blacklegged tick (*Ixodes scapularis*), which transmits *Borrelia burgdorferi*, the causative agent of Lyme disease, has recently undergone rapid geographic range expansion. In horses, Lyme disease remains an enigmatic disease, with limited understanding of the clinical aspects of the disease and many issues pertaining to selection and interpretation of diagnostic tests. The objectives of this study were to evaluate *B. burgdorferi* seropositivity in naturally exposed horses over a 12-month period and to determine the repeatability of two common serological testing methods.

Serum samples were collected from a cohort of 22 seropositive horses previously identified in a seroprevalence study approximately one year later after initial testing. Samples were tested using a C6 ELISA and multiplex ELISA targeting outer surface proteins A, C and F.

One year after initial testing, 14/22 horses remained seropositive; 7 were positive on the multiplex ELISA, 2 on C6 ELISA and 5 on both tests. Repeatability on the C6 ELISA was 100%, and 95% on the multiplex, with no significant difference between paired sample Multiplex titer values. These results indicate strong intra-test reliability though further investigation is required to determine the clinical significance of serological testing.

3.2 INTRODUCTION

Borrelia burgdorferi is one pathogen transmitted by the *Ixodes scapularis* tick in Ontario, Canada, and is becoming a growing concern as tick populations are increasing in Canada. Ths has corresponded with increases in tick-borne diseases (Clow et al., 2017; Nelder et al., 2014; Ogden et al., 2005, 2006). Lyme disease is caused by the spirochete bacterium *B. burgdorferi* and can produce complex, debilitating infections although the clinical manifestations of equine Lyme disease are not fully understood. Neuroborreliosis, uveitis and subcutaneous pseudolymphoma are uncommon but proven manifestations of *B. burgdorferi* infection (Burgdorfer et al., 1982; Imai et al., 2011; Priest et al., 2012; Sears et al., 2012). Lyme disease is most commonly implicated in other manifestations such as shifting lameness, although confirmatory evidence is currently limited.

Serological testing is commonly used to determine if horses have been exposed to *B. burgdorferi*. Two main testing approaches are used in North America. One is a multiplex ELISA that targets three outer surface proteins (Osps), OspA, OspC and OspF. It has been suggested that this test can aid in determining the infection stage or vaccination status of the horse based on three antibodies (Wagner et al., 2011) since the outer surface proteins are expressed transiently and develop at different stages of infection (Tilly et al., 2008; Liang et al., 2004). Antibodies to OspA are considered to be detectable in horses that have been vaccinated with a canine *B. burgdorferi* vaccine or in very early infections, and are often undetectable by the time of clinical testing (Wagner et al., 2011). OspA is found in the midgut of ticks prior to attachment to the host and is downregulated when the tick takes a bloodmeal thus being less likely to be transmitted into the host (Schwan et al., 1995). OspC is upregulated during tick feeding and is readily

transmitted into the host with sufficient attachment time (Schwan et al., 1995). Therefore, OspC seropositivity has been considered to indicate acute infection, and may not be detected on follow-up testing as it is downregulated after infection (Wagner et al. 2011). In contrast, OspF seropositivity has been reported to develop 5-8 weeks after infection and can persist for many years (Funk et al., 2013; Wagner et al., 2011).

Another commonly used test is an ELISA which detects antibody response to the synthetic peptide C6, corresponding to a highly-conserved region (IR6) of the surface protein, VlsE (Vmp-like sequence, expressed) (Liang et al., 2000). This method has shown greater sensitivity and specificity as a single-tier option to the common two-tier (ELISA and WB) in humans (Lipsett et al., 2016). The C6 ELISA offers point-of-care testing that is not influenced by vaccination (O'Connor et al., 2004).

Horses living in regions where *B. burgdorferi* is endemic may have antibodies due to previous exposure but may never exhibit clinical disease (Divers et al., 2018; Magnarelli et al., 2000). Additionally, a gold standard testing method does not exist for horses, which complicates clinical testing and assessment of available diagnostic tests.

The agreement between these serologic tests and their ability to detect *B. burgdorferi* infections can vary and few studies have analyzed *B. burgdorferi* antibodies in horses at more than one time-point. Therefore, the objectives of this study were to evaluate *B. burgdorferi* seropositivity in naturally exposed horses over a 12-month period and determine the repeatability of two common serologic testing methods.

3.3 METHODS

Horses that were identified as positive on a Multiplex ELISA or C6 ELISA during a prevalence study in Ontario, Canada (Neely et al., unpublished data) were eligible for enrolment. 22 of 91 (24%) seropositive horses were available for re-testing one year after the initial test.

Serum was separated and aliquoted into 4 replicates per horse. Two replicates from each horse were tested simultaneously for the presence of *B. burgdorferi* antibodies using a C6 ELISA (IDEXX SNAP® 4Dx® Plus, IDEXX Laboratories, Westbrook, Maine, United States), according to manufacturer's instructions in which the presence of antibodies is determined by development of colour on the sample spot for *B. burgdorferi.* The other two replicates were tested for antibodies against *B. burgdorferi* Osps A, C and F via multiplex ELISA (Animal Health Diagnostic Center, Cornell University, Ithaca, New York). The multiplex is reported with titer values for antibodies to each outer surface protein antigen. A sample may have an 'equivocal' titer result due to very early infection or can be induced by non-specific serum reactions.

Cohen's kappa test with 95% confidence intervals was employed to determine the agreement between the two tests. Paired t-tests were used to compare initial and follow-up testing titres. A significance level of α=0.05 was used for all statistical analysis. Statistical analysis was conducted using STATA version 14 (STATACorp, College Station, TX; 2015).

3.4 RESULTS

Of the 22 horses sampled, there were 14 geldings and 8 mares with a mean age of 14 years old (range 6-23 years old). No horses had clinical signs suspicious for Lyme disease at the initial time of sampling and did not develop signs of Lyme disease during the one-year time period between samplings. The initial sample collection was completed between July-August 2016 and the follow-up testing was completed in August-September 2017.

One year after initial testing, 14 of the 22 (64%) initially positive horses were positive on at least one of the *B. burgdorferi* tests. Five of those 14 (36%) were positive on both tests, seven (50%) horses were positive to one or more of Osps A, C or F and two (14%) were positive on the C6 ELISA only (Table 3.1). Nine (45%) horses had the same test results as the previous year. Five of the ten horses that had a positive OspF titre in 2016 changed to an equivocal OspF titre value (Figure 3.1), and one horse that was OspA positive initially had an equivocal result one year later (Figure 3.2) while another previously negative became positive. There were no significant differences between OspF and OspA titres from initial testing in 2016 and follow-up testing in 2017.

Paired testing the second samples with the C6 ELISA tested identified complete agreement, with 8 paired samples testing positive for *B. burgdorferi.* Eleven of twelve samples tested using the multiplex ELISA yielded the same qualitative (positive, negative, equivocal) result (kappa=0.91, Table 2). The sample with discordant findings was positive for OspF (titer=1548) on one replicate but equivocal (titer=1095) on the other. There was no significant difference found in the magnitude of the OspF titres between the two replicates (p=0.7813).

3.5 DISCUSSION

Duration of seropositivity has been minimally assessed in horses but is important for understanding the clinical presentation of Lyme disease and for assessment of diagnostic testing results. Though re-exposure cannot be ruled out our results show that naturally exposed horses may remain seropositive for at least 12 months post-initial testing on both serological tests. This information is particularly important when interpreting serologic test results and determining if treating for a *B. burgdorferi* infection is appropriate.

While re-exposure cannot be ruled out, no horses had positive OspC titres at the time of follow-up testing, suggesting that there was no exposure in the previous 3-5 months (Wagner et al., 2011). In this study, this timeframe equates to spring or early summer, when horses would be at higher risk of re-exposure due to higher activity of adult and nymphal ticks, respectively (Clow et al., 2018). Horses can be exposed to the pathogen any time infected ticks are present and questing, with the risk increasing at certain times of the year based on tick activity patterns. Specifically, in eastern Canada, adult ticks are active in the spring and nymphal ticks are active in early summer (Clow et al., 2018). Horses in this study were sampled between July to August in 2016, and between August-September in 2017, and may have been at higher risk for re-exposure in the fall or spring prior to resampling. Therefore, while re-exposure cannot be ruled out as a cause for persistent seropositivity, it is considered to be more likely that persistence of antibodies is from previous exposure.

As expected, OspF was the most commonly detected antibody response both years of testing. Interestingly though, all horses that were previously OspF positive that remained positive

had a numerical decline in their titres upon retesting. OspF titres are thought to be indicative of historical exposure and remain above the positive threshold for years (Funk et al., 2013; Wagner et al., 2011). The decrease is also supportive of these being residual titres from earlier exposure, rather than re-exposure. Antibodies against the OspF have been shown to develop later in the infection and increase in concentration up to 3-5 months post-infection (Akin et al., 1999; Appel et al., 1997; McDowell et al., 2001; Wagner et al, 2012b). OspF has previously been described to be a useful serologic marker for *B. burgdorferi* infections (Magnarelli et al., 1997). Canine studies have found OspF antibodies 18 months post-infection showing that OspF may be a reliable marker for late-stage infections (Wagner et al., 2011). Our study found horses remained seropositive for OspF one year after initial testing, which could indicate that antibody concentrations remain chronically elevated.

It has been assumed that a positive OspA titer can be from vaccination or may be an early indicator that the horse has been infected (Wagner et al., 2011), yet none of the horses in this study that had positive OspA titres had been vaccinated against Lyme disease. Further, three horses that had positive OspA titres initially remained OspA positive, with only one of those subsequently developing antibodies against OspF and C6. Whether this horse remained seropositive for OspA, or was recently re-exposed prior to the second sampling, cannot be answered. Repeated detection of OspA without progression to OspC or OspF seropositivity could be indicative of separate very recent exposures, without subsequent development of an immune response to other Osps after the first exposure. This may be the case for one horse previously only C6 positive that remained positive but also became positive for OspA, which would be consistent with recent exposure. These findings raise questions about the recommended

interpretation of OspA titres and indicates a need to better assess the dynamics of these Osp titres and their relevance for clinical diagnosis. In humans, OspA was found to be directly related to the severity and duration of arthritis in infected patients (Akin et al., 1999). At this time, the clinical relevance of an OspA titer in the horse is not well understood but these data suggest that it cannot be assumed that OspA titres simply reflect vaccination or early exposure. Further, it is plausible that prompt antimicrobial treatment could abort progression of Lyme disease and corresponding titres (Chang et al., 2005; Divers et al., 2012). However, none of these horses were known to have been treated with antimicrobials during the period shortly after initial sampling and results from the initial sample were not reported to owners and veterinarians until months later. Therefore, antimicrobial-associated impacts on Osp titres were unlikely to play a role.

Few studies have determined if a horse may test positive on a C6 ELISA months or years after initially testing positive (Johnson et al., 2008). As previously described, the VlsE antigen of which the synthetic C6 peptide is derived from, is not expressed in the tick, but it is expressed when the bacterium is transmitted into the host (Liang et al., 2000). In a study with experimentally *B. burgdorferi* infected dogs, serum antibodies were detected as early as 3 weeks with a C6 ELISA test and were still detectable up to 69 weeks (Liang et al., 2000). Previous research has found that C6 antibodies are robust infection markers that develop after OspC antibodies but before OspF antibodies and can remain detectable over 18 months post infection in dogs (Wagner et al., 2012a). Comparable data are not available for horses, but this study provides evidence that there may be similar persistence of C6 antibodies in horses.

Reproducibility of results is important for any diagnostic test. While agreement between the two tests was limited, intra-sample repeatability was high with both tests. The only discordant results were one horse that had a positive OspF titer (1548) and an equivocal OspF titer (1095) on the same sample. Currently, the likelihood of developing Lyme disease has not been correlated to the magnitude of the titer (Divers et al., 2018).

The small sample size is a limitation of this study, as only a subset of positive horses were available for retesting. Studies of larger populations will provide more precise information. The potential for re-exposure is an ever-present risk with endemic diseases and avoiding this would be impossible without moving these horses to a Lyme disease free region, something that is impractical.

3.6 CONCLUSION

Equine Lyme disease is of growing concern in North America, which emphasizes the need for improved testing in horses. This current study provides further information about two serological testing methods and their ability to detect *B. burgdorferi* antibodies over time. The findings from this study provide valuable information that will aid in determining if there is clinical relevance to *B. burgdorferi* antibody testing in horses and illustrates how crucial it is to not rely on serologic testing alone for diagnosis of Lyme disease. In the future, studies should be conducted with more frequent serological sampling and detailed history gathering including tick exposure, clinical signs and treatment history, as this may allow for better understanding and interpretation of each outer surface protein and the relevance of a positive C6 ELISA test result.

Table 3. 1: Serum antibody titers against *Borrelia burgdorferi* in 22 horses tested in July-August 2016 and in August-September 2017.

Initial Results		Retest Results							
		C6	OspA	OspC	OspF	C6/ OspA	C6/ OspF	C6/OspA/ OspF	Negative on all tests
C6	6	2	-	-	-	1	1	-	2
Osp A	4	-	2	-	-	-	1	1	1
Osp C	-	-	-	-	-	-	-	-	-
Osp F	10	-	-	-	5	-	-	-	5
C6/OspF	1	-	-	-	-	-	1	-	-
C6/OspC/OspF	1	-	-	-	-	-	1	-	-

Table 3.2: Intra-test kappa agreement between the C6 ELISA and the Multiplex ELISA for serum antibody titers in horse for *Borrelia burgdorferi*.

Test	# positive replicate 1	# positive replicate 2	Kappa	Expected agreement	Actual Agreement
C6 ELISA	7	7	1.000	56.61%	100.00%
Multiplex	11	12	0.909	50.00%	95.45%

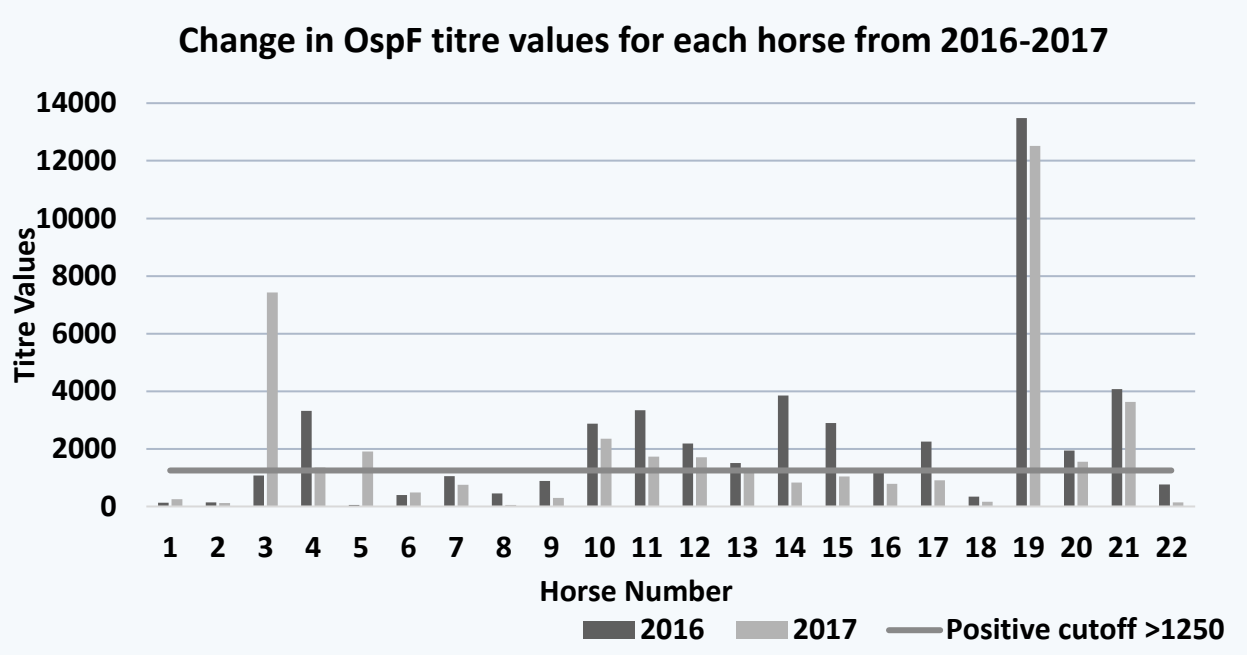

Figure 3.2: Outer surface protein F titer values for each horse on the Multiplex test with the positive titer cut-off of > 1250 for 2016 and 2017 results

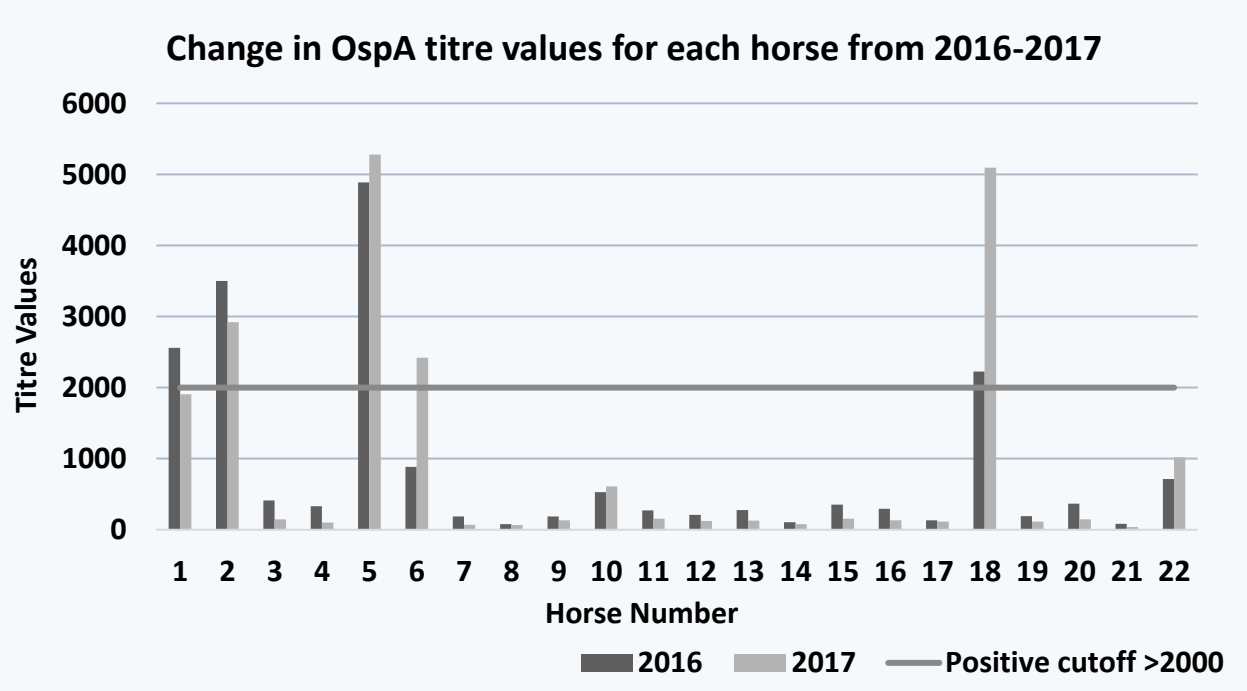

Figure 3.3: Outer surface protein A titer values for each horse on the Multiplex test with the positive titer cut-off of > 2000 for 2016 and 2017 results

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CHAPTER 4: GENERAL DISCUSSION

4.1 SUMMARY OF SIGNIFICANCE

Over the past decade, an increase in the range of blacklegged tick populations throughout the province of Ontario has dramatically increased the risk of being exposed to tick-borne pathogens (Ogden et al., 2006). Our research project aimed to investigate the seroprevalence of *B. burgdorferi* and *A. phagocytophilum* in clinically healthy horses to provide baseline information on the risk of exposure for horses in Ontario. As well, we examined demographic and management factors that are associated with the presence of antibodies to these pathogens to increase our understanding of what may contribute to increased or decreased risk of exposure. We utilized two common serologic testing methods, a C6 ELISA and a multiplex ELISA, to determine each test's ability to detect antibodies to *B. burgdorferi* and compare the performance of both tests.

Using the results from either test, the seroprevalence of *B. burgdorferi* in Ontario horses was 17% (91/551). These results are remarkable as it shows horses are being exposed to *B. burgdorferi* in Ontario, which could have significant health impacts. The seroprevalence of *B. burgdorferi* and *Anaplasma phagocytophilum* in this study (Chapter 2) are similar to the prevalence of these pathogens found in other passive surveillance studies in Ontario and elsewhere in Canada (Dibernardo et al., 2014; Nelder et al., 2014). The similarity in seroprevalence in horses and ticks infected with *B. burgdorferi* could be due to horses living in close proximity to areas where tick exposure occurs; however, currently it is unknown if the seroprevalence data are useful as marker of prevalence in ticks or vice versa. The high-risk cluster of seropositive horses identified in eastern Ontario was consistent with the one risk areas

noted by Public Health Ontario and corresponds with a significant cluster identified in Ontario by active tick surveillance (Public Health Ontario, 2018; Clow et al., 2016). Previously, equine practitioners have had to rely on human Lyme disease data to determine the geographic risk and likelihood of horses being infected. Extra precaution for tick prevention is particularly important in this high-risk area and highlights the need for a commercially available and effective vaccine for horses to have protection from exposure.

Anaplasma phagocytophilum has been identified in *I. scapularis* ticks in Thousands Islands, Ontario at an infection prevalence of 3.4% (Werden et al., 2015). This study found that 1% (6/551) of horses have been previously exposed to the pathogen. Anaplasmosis is an emerging disease in Ontario though has not posed a significant health threat to horses in Ontario (Werden et al., 2015). As the climate continues to change and become more hospitable to *I. scapularis*, the risk of being bitten by ticks in Ontario increases (Ogden et al., 2005). With this study as a baseline of seroprevalence, we know that horses are being periodically exposed, and although anaplasmosis is rare, it is important for veterinarians consider as a differential if corresponding clinical signs are present. Continued monitoring and surveillance should be conducted as the pathogen becomes more established in the province.

Risk factors have not been previously identified for *B. burgdorferi* exposure in horses in North America. In this study, risk factor analyses were conducted using responses from a questionnaire with county as a random effect to account for the spatial clustering in eastern Ontario. The presence of oak trees bordering or in the horse's pasture significantly increased the odds of the horse being seropositive for *B. burgdorferi* on the C6 ELISA and performing regular tick checks significantly reduced the odds of the horses being seropositive. This information can

be used by horse owners and those directly responsible for the care of horses to reduce the risk of exposure to these pathogens. Based on the results of the current study, interventions that could be considered include limiting the number of oak trees near horses' paddocks. Oak trees have been associated with increased tick populations as the acorns attract rodents and deer which serve as reservoirs and hosts for *B. burgdorferi* and *I. scapularis*, respectively (Ostfeld et al., 2001). The significant association between performing tick checks and decreased risk of exposure shows how critical it is for owners to be diligent and promptly remove any ticks, though this may be easier said than done. Performing tick checks on horses can be a futile process as the size and hair coat of horses can lead to ticks being missed in even a scrutinous exam and therefore is somewhat subjective in nature. There is a possibility that people who regularly check for ticks may also take other protective measures, making tick checks a proxy for another effective measure such as possibly habitat limitations or topical preventatives such as insect repellent or blanketing. It is important to note that access to forested habitat confounds this relationship, so it must be taken into consideration. Performing regular tick checks when controlling for the confounder is still protective, but less so, meaning tick checks may be a good method of protection, but less effective when exposure risk is really high (having access to forested habitat). These preliminary but novel risk factors for horses are important for veterinarians to consider when making recommendations for tick bite and Lyme disease prevention, as well continuing to educate owners on the risks of *B. burgdorferi* infections, can help owners take necessary precautions against tick bites.

Lyme disease in horses remains enigmatic, as there is still little understanding or consensus of the clinical signs and manifestations which hinders the interpretation of serologic

test results (Divers, 2007). This study confirms that horses in Ontario are being exposed to *B. burgdorferi* but provides limited clinical relevance as no horses were suspected to have Lyme disease or had pertinent clinical signs. Although *B. burgdorferi* positive horses were identified with both serological testing methods, there was only slight agreement between the two tests (kappa=0.23); only 13 horses were positive on both the C6 ELISA and the Multiplex. It cannot be determined with these preliminary results which test provided more accurate results of the true number of horses exposed without a gold standard as reference method. As the two tests used in this study utilize different *B. burgdorferi* antigens, the question remains if the discrepancies are related to the different antibodies being detected or if it's related to their inherit sensitivity and specificity. The Multiplex was designed in hopes of more accurate diagnosis of horses that had previously been infected and quantifying the antibody levels (Wagner et al., 2012). However, the magnitude of antibody titer levels has not been correlated to increased likelihood of disease or clinical signs (Divers et al., 2018). Intuitively, it would be expected that an older aged horse would be more likely to have been previously exposed. Univariable analyses showed that age was only an important factor for the Multiplex test, and specifically OspF antibodies, but age was not a significant factor for the C6 ELISA. The interpretation of a positive OspF titer supports the association with increased age, since OspF indicates a chronic or persistent antibody concentration from previous exposure or infection whereas C6 antibodies are generally more transient than OspF (Funk et al., 2013; Wagner et al., 2012). However, the dynamics of different antibodies against *B. burgdorferi* are poorly understood.

This lack of agreement between testing methods and the limited literature on *B. burgdorferi* antibodies over time prompted the second study for this thesis, in which 22 horses

that were seropositive for *B. burgdorferi* in 2016 were resampled. One year later, 14 horses remained seropositive; 9 horses had the same test results, 2 remained positive on the C6 ELISA only and 3 horses remained seropositive for OspA. Though the re-exposure history for the horses is unknown, the results show that antibodies in horses may still be detectable on both the C6 and Multiplex one year later. Our study findings do not contradict the interpretations of the OspC and OspF antibodies on the Multiplex, but it does raise questions about the OspA interpretation (Wagner et al.,2012). Though re-exposure cannot be ruled out, OspA antibodies appear to persist up to 12 months after exposure. Lyme disease in humans can result in chronic arthritis that has been associated with persistent OspA antibodies. In our study, there were no clinical signs associated with the OspA antibodies, nor any vaccination history (Akin et al., 1999). Results from this study showed that horses can have antibodies from previous exposure up to one year later while remaining clinically normal. These findings highlight how diagnosis of Lyme disease in horses based solely on serologic test results could lead to misdiagnosis and inappropriate antibiotic treatment (Chang et al., 2005; Divers et al., 2012). Presence of antibodies alone does not indicate disease, and without clinical signs, treating with antibiotics based solely on serological test results would potentially lead to costly, unnecessary use of antimicrobials (Divers et al., 2007). This is an important area of contention. A gold standard testing method for determining if a horse is infected with *B. burgdorferi* currently does not exist (Divers et al., 2018). This study illustrates discrepancies between the ability of serological tests to detect antibodies. These results demonstrate the need for an improved testing method, particularly one that may be able to detect an active infection.

4.2 LIMITATIONS

Our study involved the participation of equine practitioners and client-owned horses and therefore excluded horses that do not access veterinary care; however, this was a practical and economical design choice that also garnered public interest in the study. This may have a socioeconomic or regional bias for owners who may struggle to afford veterinary care or live in remote regions without regular veterinary care. As well there was an intentional geographical bias that placed the focus on known areas that were higher risk and had larger densities of horses which limits how the data can be extrapolated to the province level. Additionally, the questionnaire provided to owners did not collect a history of travel outside of the province. We accounted for travel by providing participating veterinarians with the sample criteria, including that horses were to not have travelled outside of the province. Therefore, it was under the discretion of each veterinarian to ensure the horses selected for participation in the study met the criteria outlined for inclusion in the study.

Horses for each clinic were analyzed based on the postal code of the veterinary clinic they were submitted under, which may not reflect the actual location of where the tick exposure occurred or where the horses resided. As equine veterinarians travel to their clients, there is a radius around the clinic in which the horses may reside. Horses may also have traveled to other parts of the province, which may have changed the likelihood of exposure that cannot be controlled for. This limits the resolution that is available for identifying regional risks. Different risk regions were pooled for risk factor analysis which could result in a relevant risk factor being lost if it only occurred in one area. Similarly, inclusion of low prevalence areas could reduce the

ability to find significant risk factors as those risk factors might be present in low risk areas, complicating assessment of them analytically.

Recall bias is a limitation of using questionnaire data which could lead to misclassification of the data (Chapter 2). The questionnaire also contained an open-ended question regarding clinical history, which resulted in a plethora of responses too vague to code for analyses. Other questions had very poor response rates which resulted in categories being combined or questions being removed completely from analysis. Future studies with more concise questions may reduce confusion on how to respond. In addition, a follow-up survey was provided to veterinarians approximately six months after the initial testing in 2016 to determine if the horses in the study had developed any clinical signs suggestive of Lyme disease after sample collection. However, no completed surveys were returned so clinical signs were not evaluated for this study.

The small sample size was a significant limitation of the second study (Chapter 3). The 22 horses sampled were selected based on convenience sampling of previously positive horses. An inherent risk based on the small sample size is an increased likelihood of a Type II error (i.e., lack of power), therefore, drawing conclusions must be done with caution (Dohoo et al., 2009). The sample collection was performed by equine practitioners on client-owned horses, which limited participation on the cooperation of the equine practitioners and the horse owners. While most owners were incentivized by free antibody testing, veterinarians were limited by time and convenience of sampling horses that may not be seen regularly. However, as this study analyzed trends in antibodies over a 12-month time period, statistical power was not required for all aspects of the study.

4.3 FUTURE DIRECTIONS

The geographic range of ticks is expanding rapidly northward into Canada and the incidence of tick-borne diseases is correspondingly increasing (Clow et al., 2016; Nelder et al., 2014; Ogden et al., 2006). Based on the findings from the second study, it would be ideal to conduct a large-scale longitudinal study with more frequent sample testing and documentation of clinical signs to determine if trends exist for seroconversion of *B. burgdorferi* antibodies over time in horses. It would also be interesting to determine if differences exist for horses living in endemic and non-endemic regions where re-exposure risks differ. Our study showed that horses in Ontario are not only being exposed to the Lyme disease bacteria, but the antibodies can remain detectable months later. Regardless of the sample size, the follow-up testing showed outer surface protein antibody trends that were inconsistent with previous literature on the interpretation of these antibodies. These results, along with the lack of agreement between the tests and the preliminary risk factors found provide a strong basis for future studies. Continued comparison and utilization of various serologic testing methods may help to determine if a specific test is superior though without a Gold Standard, the validation will be a gradual process. Studies conducted over a longer-time period may also provide insight into clinical signs of the disease and allow for additional evaluations of available serologic tests and associations between clinical signs and antibody titers.

It has been predicted that with ongoing climate change, habitats in more northern areas will become climatically suitable for the blacklegged tick, facilitating spread throughout Ontario (Clow et al., 2016; Ogden et al., 2005). Future surveillance studies will need to be conducted to monitor changes in endemic and emerging high-risk regions in Ontario and surrounding

provinces (Bouchard et al., 2015). Horses and humans can be exposed to the same pathogens, but because horses spend more time outdoors their chances of being exposed to tick-borne pathogens may be higher. It may be useful to use equine data to compare to human data in order to determine if horses may act as sentinels for disease. Horses may serve as sentinels to determine if risks change over time and if there are significant associations between geographic factors and the incidence of *B. burgdorferi* infections. This could be advantageous for additional tick-borne diseases that may pose risks in the future. Seroprevalence of tick-borne pathogens in the horse can provide insight into different tick species' distribution presently and can be used as an indirect indication of tick spread when monitored over time.

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