Prevalence of equine leptospiral shedding using urine polymerase chain reaction and serum microscopic agglutination testing

by

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B.S., Dickinson College, 2009
B.V.M.S., University of Glasgow, 2014

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Clinical Science
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2018

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Abstract

Leptospirosis is a worldwide veterinary and public health concern, and emerging infectious disease of horses. The spirochete can be directly transmitted by contaminated urine, placental fluids, semen, infected tissues, reservoir hosts, or flood waters. Seroprevalence and infecting serovar vary with geography, yet diagnosis using the gold standard microscopic agglutination test (MAT) merely confirms a high exposure rate. Subclinical infection can complicate diagnosis. The aims of this study were to use semi-nested PCR on urine from apparently healthy horses to determine period prevalence of leptospiral shedding and to correlate these findings with MAT results to establish associations with client based survey data regarding horse management and environment.

Serum and free-catch urine were collected from 204 healthy horses between May 2016-December 2017. Serum was used to determine GGT, creatinine concentrations, and six serovar MAT (Canicola, Hardjo, Icterhemorrhaegiae, Pomona, Grippotyphosa, Bratislava). Urine samples were submitted for PCR testing of leptospiral 23S rRNA. Client consent and survey data were collected for all subjects. Potential risk factors included drinking water source, exposure to livestock and dogs, geographical location, season, and precipitation.

Two horses were positive on urine PCR for leptospirosis (shedding prevalence 1%), yet only one had a high reciprocal MAT titer of ≥ 800. Both horses were negative on urine PCR one month later without treatment. Approximately 77% of horses (157/204) were seroreactive (MAT reciprocal titer ≥ 100) for at least one serogroup, and Bratislava was detected more frequently than others (47.5%; (97/204)).

Apparently healthy horses infrequently shed *Leptospira* spp. in urine, yet seroreactivity in clinically normal horses is high (77%), confirming high exposure rates to *Leptospira* spp. in the

Central Midwest. Further studies should target serovar specific PCR tests and incorporate PCR testing in horses clinically affected with leptospirosis.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I would like to express my appreciation to my major professor, Dr. Elizabeth Davis, for her continued support and guidance throughout this project, my graduate school experience, and my internal medicine residency program at Kansas State University. I would like to thank my committee members, Dr. Laurie Beard, Dr. Christopher Blevins, and Dr. Ram Raghavan for their support of this project. I would like to thank my fellow authors on the manuscript, entitled "**Prevalence of Equine Leptospiral Shedding using Urine Polymerase Chain Reaction and Serum Microscopic Agglutination Testing**" that is currently under review, including Dr. Laurie Beard, Dr. Christopher Blevins, Ashley Deforno, and Dr. Ram Raghavan. I would also like to thank the Kansas State University Veterinary Diagnostic Lab for sample processing; Ashley Deforno for helping to collect samples; Dr. Kenneth Harkin for consultation on leptospirosis; and Kara Smith for technical assistance. I would like to thank Dr. Robert Larson and Jiena Gu McLellen for help with statistical analysis. I would like to thank Zoetis, Inc., the Gish Foundation, and the Department of clinical sciences for funding for this project. Without many people's support and guidance, this project would not have been possible.

Chapter 1 - Introduction

Leptospirosis is considered to be a worldwide veterinary and public health concern. It is caused by a pathogenic species of *Leptospira* spp., most commonly *Leptospira interrogans sensu lato.* It causes clinical disease in many mammalian species including, but not limited to, dogs, cats, horses, rodents, livestock, and humans. Specific serovars affecting different species tend to be distributed geographically. *Leptospira* spp. are a gram negative, motile, obligate aerobic, tightly coiled spirochete which can persist in warm, moist environments for up to six months (Levett 2001). Leptospirosis is maintained in the environment by subclinically infected maintenance hosts. The reported prevalence of maintenance hosts within a population tends to be between 30 and 50%. (Hamond & Lilenbaum 2012; Langoni et al. 2009). Such a high prevalence of disease is maintained as the bacteria colonize the proximal renal tubules, causing a chronic kidney infection and a carrier state. Virulent *Leptospira* are then shed in the urine (Divers & Chang 2009; Hamond & Lilenbaum 2012). They can also invade the female reproductive tract and fetal membranes (Divers & Chang 2006; Divers & Chang 2009; Genovez et al. 2004). Infection between these hosts is usually by direct contact, and incidental hosts may become infected when they ingest or come in contact with urine or fetal membranes in the contaminated environment, feed, or water. Natural hosts/reservoirs of the bacteria tend to not exhibit any clinical signs. Areas experiencing flooding and hurricanes, as well as humans who work closely with animals or waste products tend to hold a higher risk for becoming infected (Levett 2001).

Horses are largely asymptomatic carriers or subclinically infected, but when clinical disease is present, there are reports of horses presenting with non-specific signs including anorexia, lethargy, fever, and icterus (Divers & Chang 2006; Divers & Chang 2009). Further, leptospirosis in horses has been associated with a higher incidence of equine recurrent uveitis

(ERU), acute renal failure, sporadic abortions, placentitis, stillbirths, and, more recently, pulmonary hemorrhage and hemolysis (Delph et al. 2018; Divers & Chang 2006; Erol et al. 2015; Frellstedt 2009; Gerding & Gilger 2016; Niedermaier et al. 2006; Rohrbach et al. 2005; Verma et al. 2013). As clinical signs associated with leptospirosis are non-specific, disease in horses may occur more frequently than is diagnosed, and exposure to *Leptospira* spp. may be more prevalent than was previously thought. The incidence and importance of equine leptospirosis has not been extensively studied to date.

Traditionally, ante-mortem diagnosis of *Leptospira* spp. has relied on serological tests, such as the microscopic agglutination titer test (MAT) and identification of the organism. Culture remains the gold standard for the serovar specific diagnosis of leptospirosis, yet this requires careful handling of specimens, a high cost, and a slow result turnaround (4-6 weeks) (Harkin et al. 2003; Hamond & Lilenbaum 2012; Hamond et al. 2014; Trap et al. 1992). Furthermore, in a 2003 canine study, no dogs that tested positive on serology or using urine polymerase chain reaction (PCR), had a positive culture result, indicating a low sensitivity. In that same study, even a dog showing signs of severe clinical leptospirosis, was positive on both PCR and serology but was culture negative (Harkin et al. 2003).

Epidemiological studies have relied on seroprevalence using MAT, which is limited in its ability to determine serovar, and, only reports exposure, not carrier status or active infection (Ye et al. 2014). Rising paired titers or extremely high titers (≥800 (OIE 2012); ≥6400 (Harkin et al. 2003)) only raise suspicion that a horse may be infected. Furthermore, in a study detecting canine leptospirosis, the MAT testing for predicted shedding only had 22% sensitivity and 79% specificity, reiterating the fact that it is not the ideal test to determine bacterial shedding (Harkin & Hays 2016). In a recent study by Zoetis LLC., the reported seroprevalence of leptospirosis in

horses was 76.2% in the Midwestern United States, prompting the development of a commercially available vaccine specifically for horses. This study further showed that 75% of healthy horses have been exposed to at least one leptospiral serogroup (Zoetis LLC 2015).

Recent developments with PCR to detect *Leptospira* spp. in bodily fluids or tissue have shown it is the most sensitive test to determine active infection and carrier status. Urine PCR (as well as PCR on fetal membranes and aqueous/vitreous humor) of leptospiral DNA has been reported in horses and many other species to definitively diagnose leptospiral shedding (Erol et al. 2015; Fang et al. 2015; Finger et al. 2014; Genovez et al. 2004; Hamond & Lilenbaum 2012; Hamond et al. 2014). South American studies, specifically conducted in Brazil, revealed that 66% of horses were seropositive (reciprocal titers ≥200) for either serovar Bratislava or Copenhagen, and urine PCR detecting the pathogenic *lipL*32 gene was positive in 62% of the 142 samples (Hamond et al. 2014). Interestingly, some horses who were seronegative were positive on urine PCR, and vice versa, suggesting that serology tests may overlook infected animals, and that horses diagnosed with leptospirosis based on serology alone, may not be shedding. This result may also be due to the PCR methodology used, resulting in a false positive result from other contaminating spirochetes, however the *LipL*32 gene is thought to be specific to pathogenic *Leptospira* spp. (Stoddard et al. 2009). In the previously mentioned 2014 study by Hamond et al., urine PCR was shown to be an accurate diagnostic tool for leptospirosis. Forty-two percent of horses were MAT positive, and comparably, 36% were positive on urine PCR. Furthermore, 96 animals were MAT negative, but PCR positive, therefore shedding bacteria into the environment even though serology was normal. Compared to other species, in a study on 132 suspect and 13 healthy dogs, urine PCR had a 100% sensitivity, and 88.3% specificity when compared to MAT, clearly demonstrating how beneficial its clinical applications could be to all

leptospirosis cases (Harkin et al. 2003). PCR is much more sensitive for detection of leptospirosis than culture, with 41 out of 500 dogs being positive on urinary PCR (semi-nested 23S rRNA) in the same study. PCR has the added benefits of rapidly available results (<24 hours) and has an increased sensitivity compared to the MAT.

With regard to human health and exposure, leptospirosis can cause severe clinical disease, which may result in hospitalization. Higher prevalence rates in humans are seen based on occupation, with higher rates seen in abattoir workers and farmers (Barwick et al. 1998). In a New Zealand study, 74% of the reported cases of leptospirosis were from farmers (Erol et al. 2015). Furthermore, recent climate change has led to increased hurricane and flooding activity worldwide. As farming and livestock work constitutes a significant portion of the Midwest's workforce, and with the growing evidence of climate change, a further look into the shedding prevalence of horses and livestock and human exposure must be explored.

To our knowledge, an investigation of the equine shedding of *Leptospira* spp. by asymptomatic horses in the Central Midwest using urine PCR (23S rRNA based) has not been performed and would be of practical use for determining carrier prevalence in a specific geographical area, as well as increasing awareness of the potential for infectious and zoonotic spread by horses in the environment and to their owners.

Objective

The primary objectives of this study were to evaluate the frequency of leptospiral shedding in urine of asymptomatic, apparently healthy horses in Kansas, Missouri, and Nebraska using semi-nested PCR and to compare these findings to seroprevalence in this region among horses living in various environments. A secondary objective was to determine the potential risk of zoonotic/interspecies exposure to *Leptospira* spp. bacteria from horses who appear healthy.

Hypothesis

Our hypotheses were that, based on previous seroprevalence surveys and the fact that we are not in an endemic area, the frequency of *Leptospira* spp. DNA shedding in urine from healthy horses will be lower than the reported seroprevalence, but some horses would be positive and asymptomatic. Further, we hypothesized that horses stabled outside, living near fresh water sources such as ponds, and living in close proximity to dogs and / or livestock would be at greater risk to serve as asymptomatic shedders compared with horses who were predominantly stabled and have limited exposure to *Leptospira* spp. reservoir hosts.

Chapter 2 - Literature Review

Leptospirosis

Etiology

Leptospirosis is caused by a gram negative, motile, obligate aerobic, tightly coiled spirochete, and over 300 different serovars exist globally. It is caused by infection with a pathogenic *Leptospira* spp. that maintains itself in nature by colonizing the renal tubules of reservoir or maintenance host(s) and are shed into the environment via urine (Delaude et al. 2017; Levett 2001). Maintenance hosts/carriers for host-adapted strains typically shed the bacteria into the environment during urination, but display no or minimal clinical signs. The infection usually results from direct transmission via a contaminated environment and food/water source with urine, placental fluids, and tissues of affected animals (Divers & Chang 2009; Hamond et al. 2013; Levett 2001). Infected aborted fluid and tissue may also be shed into the environment, depositing the bacteria into feed and water. Leptospires can also gain exposure to the incidental host through mucous membranes or skin abrasions (Hamond et al. 2013). For the horse, potential reservoir hosts of concern are wildlife, rodents, livestock, dogs, and other horses (Fang et al. 2015).

Zoonotic Potential

Leptospirosis is considered a zoonotic pathogen resulting in human leptospirosis being a major public health issue, mainly in developing countries located in South America and Asia. It is currently considered an emerging disease within developed countries, however, with global warming and climate change, disease frequency is anticipated to increase. Outbreaks have been linked to natural disasters and flooding, as well as there being an increased occupational risk for professions such as farmers, slaughterhouse workers, sewage workers and veterinarians (Delaude

et al. 2017; Fang et al. 2015). The disease is highly endemic in Brazil, with over 10,000 human cases reported between 2009-2011, and an overall mortality rate of 9.3% (Finger et al. 2014). Between 2008 and 2011 in New Zealand, a human infection rate of 2.0 per 100,000 population was reported, and almost half were admitted to hospitals, indicating that disease can have serious zoonotic implications, even in developed countries (Fang et al. 2015)

Epidemiology and Seroprevalence

Leptospirosis is a worldwide emerging pathogen. While this disease is typically reported in tropical and subtropical regions, the over 300 different serovars tend to be regionally distributed. Many seroprevalence surveys have been performed, however variations in diagnostic testing protocols and MAT titer cut-offs make it slightly more complicated to compare this reactivity results between populations. In the United States, a recent survey by Zoetis LLC found that between 69-77.5% of horses are seroreactive (reciprocal MAT titer ≥ 100) to at least one serovar of *Leptospira* spp. (2015). This study looked at 5,261 healthy horses who tested positive for at least one leptospiral serovar in an investigation that included 53 veterinary clinics in 18 states. Knowledge of which host species maintain which serovars and in which geographic locations is important to enhance control and understanding of the disease. After infection, antibodies can persist for years or even during the entire lifespan of an animal (Levett 2001). Many studies have also raised the question as to what influence the biotope where the infected or exposed animals live has on survey results. In other words, is the infection geographically specific (Houwers et al. 2011)? The answers to these questions could have serious implications on the worldwide distribution of the bacteria, as well as its diagnosis and control. Risk factors for clinical infection as well as associations of seroreactivity with environmental or climatic

factors have been reported from the majority of the major continents, with sometimes contradicting results.

Worldwide, seroprevalence rates of leptospirosis in horses from different areas have been between 12.8% and 79% (Arent & Kezierska-Mieszkowska 2013; Barwick et al. 1998; Hamond et al. 2013; Loueiro et al. 2013). In a South African study, there was a reported apparent prevalence of *Leptospira* spp. (reciprocal titers of ≥100) between 32-43% (Hamond et al. 2013). In a large multispecies serological study from Italy which analyzed 43,935 animal specimens, an 8 serogroup MAT panel was used (Australis, Ballum, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pomona, Sejroe, Tarassovi), with a positive cut-off reciprocal titer of ≥100 (Tagliabue et al. 2016). Of these samples, 6,279 sera showed positive titers, with bovine being the most frequently positive samples (bovine-46.9%, swine-27.5%, ovine and goat -7.4%, dog -6.9%, and wild boar-4.5%). Interestingly, in this study, equine samples were not frequently submitted, but had one of the highest seroreactivity rates after rodents (Tagliabue et al. 2016). In a study from Brazil in 2014, seroprevalence rates among a variety of farm species were all quite high, indicating a high rate of exposure; From the 512 serum samples tested, 43.5 % were seroreactive (cattle: 45.6 %, horses: 41.3 %, goats: 34 % and pigs: 60 %) (Hamond et al. 2014).

In an equine and canine study conducted in the Netherlands, a total of 89 of 112 healthy horses (79%) had agglutinating antibodies against one or more serovars with Copenhagen and Patoc being predominant, followed by Bratislava and Grippotyphosa, while healthy dogs in the same region during the same time period had a seropositivity rate of 72%, with the most commonly agglutinated serovar being Copenhagen, closely followed by Patoc and to a much lesser degree Icterohaemorrhagiae (Houwers et al. 2011). As neither of those sample populations

were vaccinated against serovar Copenhagen, it can be postulated that these animals had been exposed to the bacteria or had experienced subclinical infection.

In Poland in 2013, a total of 620 horses were surveyed and 17 different serogroups typed. Of the 620 horses, 39% had reciprocal MAT titers of ≥100 (positive) to at least one of the 17 serovars. Grippotyphosa was the most prevalent serovar in that region, and Sejroe, was the second most common (Arent 2013). A retrospective, cross-sectional study (using MAT) performed in Switzerland in clinically healthy horses found 58.5 % seropositivity (≥100) against one or more of the 15 different serovars tested. The most prevalent serovar was Pyrogenes (22.6 %), followed by serovars Canicola (22.1 %) and Australis (19.2 %), and 20.3% of horses had a reciprocal MAT titer of greater than or equal to 400 (Blatti et al. 2011). In a North American diagnostic laboratory study consisting of 29 states and one Canadian province, a 45% seroprevalence was noted (Carter et al. 2012).

Serogroup Pomona (serovars Pomona or Kennewicki) is mainly responsible for clinical leptospirosis in horses in North America (Yan et al. 2010, Timoney et al. 2011). But, while other members of the serogroup Pomona have been reported in western Europe, Kennewicki has not been reported specifically. Reported clinical infections associated with the pathogenic serogroup Pomona include hemorrhagic, acute febrile syndromes, renal failure, jaundice, hepatic failure, hemolytic anemia, and reproductive disease (Cutle et al. 2017). Experimental infection in horses using the serovar Pomona and Kennewicki demonstrated leptospiremia 2-6 days after infection and leptospiruria four weeks after infection, thus, indicating that horses are able to harbor and spread the disease in addition to other species (Finger et al. 2014).

Bratislava relevance is quite controversial within the literature. Horses are considered to be an important maintenance host for Bratislava, but in some studies it is considered pathogenic (Arent et al. 2015). Further, it has been suspected that this serovar can also be carried by asymptomatic dogs, which may in turn act as a source of infection for other domestic and wild animals and for humans (Ellis 2010). In another study, dogs had 4.7 times higher odds of being seropositive to Bratislava (MAT ≥100) if the owner also owned a horse, indicating that intra-species transmission may be more common than we previously thought (Delaude et al. 2017). The Australis serogroup (contains serovar Bratislava) has the highest reported serological prevalence rate in the United Kingdom, Portugal, Sweden and Italy (Arent et al. 2015; Baverud et al. 2009). Interestingly, in a study by Arent and others from 2015, it was concluded that there are differences in DNA profiles between Australis strains recovered from pig and wildlife species compared with equine isolates. By using restriction endonuclease analysis, they argued that there are adaptations of some serogroups within the Australis strain (particularly REA type B1 and M1) in horses, increasing maintenance status, and that this is consistent with the hypothesis that horses living near cities are more likely to be infected with Bratislava than horses living in the country due to exposure to other horses and wildlife (Bauverud et al. 2009). In a large serological study from Italy looking at 8 serogroups, serogroup Australis (represented by Bratislava) was present in dogs, wild boars, horses, hares, swine, foxes, and rodents; Sejroe was found in cattle, sheep, goats, and buffalo; Icterohaemorrhagiae was observed in dogs, goats, and foxes; Pomona was detected in swine, cattle, and wild species; and Grippotyphosa was only reported in hares, indicating that there are many serovars found in many apparently host and non-host adapted species (Tagliabue et al. 2016).

Risk Factors

The high seroprevalence in healthy horses indicates that they are often exposed to or infected with *Leptospira* spp. without developing clinical disease. This could be a consequence of an exposure to host-adapted *Leptospira* spp., leading to an inapparent infection in their hosts, or due to subclinical, non-host-adapted infections. A canine meta-analysis from 2016 identified the following major risk factors: male sex, mixed-breed, young dogs (<1 year), working dogs, habitat flooding, as well as an urban environment (Azocar-Aedo & Monti 2016). Risk factors for increased seroprevalence or infection in horses that have been reported are increasing age, being a mare, being a pony, increasing duration spent on pasture per day, as well as seasonal variation (elevated in the fall and autumn), flooding and hurricanes, proximity to livestock and wildlife, as well as to contaminated sources (Blatti et al. 2011). Further, other favorable environmental conditions such as warm temperatures, moisture, neutral soil pH, and standing surface water are considered risk factors for increased infectivity of leptospirosis (Hamond et al. 2013; Loureiro et al. 2013). In a study in Australia, Wangdi and others (2013) found that the odds that horses in an area with an average annual rainfall of >2000 mm becoming seropositive was 6.1 times higher compared to horses in areas with an average annual rainfall of <1000 mm. In a study by Hamond and others (2013), the presence of other animals on the same pasture with or nearby to horses, such as pigs, had a significant influence on the likelihood of the horses being seropositive to Bratislava and Pomona (maintenance host is the pig). Climate change, global warming, and increased risk of hurricanes and flooding could make this a potentially devastating emerging disease.

Clinical Presentations

Clinical manifestations of disease in incidental hosts are typically mild, but may include fever, renal and hepatic injury, pulmonary hemorrhage, and reproductive failure (Adler 2015; Yan et al. 2010). Interestingly, infected maintenance hosts show leptospiruria of higher intensity and duration compared to incidental hosts (Delaude et al. 2017). Host adapted serovars typically cause chronic or subclinical infections, which is why those infected act as potential undetectable reservoirs for other species (Hamond et al. 2013).

Horses are believed to be largely asymptomatic carriers, but, when clinical disease is present, there are reports of horses presenting with non-specific signs including anorexia, lethargy, fever, and icterus (Divers & Chang 2006; Divers & Chang 2009). Further, leptospirosis in horses has been associated with a higher incidence of equine recurrent uveitis (ERU), acute renal failure, sporadic abortions, placentitis, stillbirths, and, more recently, pulmonary hemorrhage (Delph et al. 2018; Divers & Chang 2006; Erol et al. 2015; Frellstedt 2009; Gerding & Gilger 2016; Niedermaier et al. 2006; Rohrbach et al. 2005; Verma et al. 2013). As clinical signs associated with leptospirosis are typically non-specific, disease in horses may occur more frequently than is diagnosed, and exposure to *Leptospira* spp. may be more prevalent than was previously thought.

Abortions, stillbirths, or early neonatal fatalities are of great concern to the equine industry, particularly in what are considered endemic areas such as Kentucky. An estimated $102 million in losses was reported secondary to *Leptospira*-associated abortions in Thoroughbred horses in Kentucky from 1993-2012 (Carter et al. 2012). The dominant reported serogroup/serovar causing equine abortion in the USA is Pomona type Kennewicki (Erol et al. 2015). Significant pathological changes can be found in both the placental and fetal tissues from

these abortions which may include edema, necrosis and infiltration of suballantoic stroma by neutrophils and mononuclear cells indicating placentitis and, perivascular and transmural inflammation and necrosis composed of macrophages, neutrophils, lymphocytes and plasma cells in umbilical arteries indicating funisitis (Erol et al. 2015). Fetal kidneys and livers may also be affected, which is the reason for icterus being more commonly reported in foals (Erol et al. 2015; Verma et al. 2013). Reports indicate that infected mares may shed leptospires in the urine for up to 14 weeks postpartum and act as reservoirs for in-contact animals (Donahue et al. 1995).

Equine recurrent uveitis is a multi-etiological syndrome, yet leptospirosis has consistently been implicated in its etiology. ERU is characterized by recurrent or persistent uveitis in one or both eyes secondary to a T-helper type 1 (Th1) cell response and is a leading cause of vision loss in horses. Many hypotheses about the role of leptospirosis in ERU exist. These include the release of enzymes and toxins, such as a glycoprotein toxin, LPS, and hemolysin which have direct damaging effects on the uvea or that the presence of *Leptospira* in the eye activates an inflammatory cascade and immune mediated response (Polle et al. 2014). In the United States, Pomona is the most commonly detected strain in horses with ERU, whereas Grippotyphosa is the most common in Europe (Gerding & Gilger 2016; Polle et al. 2014). The overall prevalence of ERU varies widely with geographic location, but, in the United States, there is an observed prevalence of between 1%–15% (Polle et al. 2014). The Appaloosa breed is 8x as likely to develop ERU, and, in addition, Pomona-seropositive Appaloosas with ERU reportedly have the poorest prognosis for vision when compared with other breeds, likely indicating a genetic predisposition in addition to infectious causes (Dwyer et al. 1995; Gerding & Gilger 2016). The most common clinical signs of ERU are keratic precipitates, aqueous flare, fibrin within the anterior chamber, iridal hyperpigmentation, corpora nigra atrophy, posterior synechia, cataract

formation, vitreal cellular infiltrate, lens luxation, phthisis bulbi and blindness (Gerding & Gilger 2016; Polle et al. 2014). A major aim of the Gerding and Gilger 2016 study was to determine the impact that leptospirosis has on ERU and blindness in horses. They found that 26.0% of all blind horses (28.4% of ERU eyes) on initial evaluation had positive leptospirosis titers in the serum and/or aqueous humor. Interestingly, ERU horses in this study that had positive serum titers to *Leptospira* spp. (25.0%), were significantly less than horses that had positive aqueous humor titers (43.3%), indicating that samples from predilection sites may be far superior for diagnosing leptospirosis than serum (Gerding & Gilger 2016). However, it must be noted that cross-reactivity between serovars exists and that MAT is not serovar specific. In a study by Polle and others from 2014, 16/21 (76%) control and 23/27 (85%) affected horses were positive for at least one serovar on serum MAT (≥100), with Bratislava most commonly identified. In that study, horses with antibody titers in ocular fluid that were higher than antibody titers in serum were defined as having intraocular antibody production and they used two different criteria: A ratio of 1 if ocular titer and/or serum titer >1 and ratio of 4 if ocular titer and/or serum titer >4 (Polle et al. 2014). With such high variability of serology, PCR of aqueous humor may be far superior to detecting the spirochete in quiescent ERU patients than MAT on serum or ocular fluids.

Respiratory manifestations of leptospirosis have been reported in humans, dogs, and more recently, foals. In the human literature, a prevalence rate of 20-85% of respiratory manifestations of leptospirosis have been reported, mainly intra-alveolar hemorrhage (Dohlnikhoff et al. 2007; Trevajo et al. 1998). In the same reports, not all patients with radiographic changes displayed outward signs of pulmonary hemorrhage. In dogs, upwards of 70% of patients with clinical leptospirosis have pulmonary complications, and, in one study, resulted in humane euthanasia of 43% of all patients (Harkin & Gartell 1996). In humans and

dogs, there does not appear to be an age predilection, however among necropsy and clinical cases reported in horses, the majority of cases are less than six months of age (Broux et al. 2012). Again, pulmonary hemorrhage as a clinical manifestation of leptospirosis in horses is quite rare. Yet, there is increasing evidence that horses may be similar to humans and canines with subclinical respiratory manifestations. In 2012, Hamond & Lilenbaum reported that pulmonary hemorrhage was present on endoscopy in 34% of training racehorses that were seropositive. The most likely pathogenesis of pulmonary hemorrhage in leptospirosis is secondary to systemic vasculitis and resultant tissue damage. It has also been suggested that it may be a result of immunoglobulin deposition and reaction, similar to proposed mechanisms of ERU (Broux et al. 2012). It is possible that PCR could be performed on pulmonary tissue obtained during endoscopy in order to diagnose leptospirosis in such cases. Acute renal failure and hepatic failure are also reported equine conditions (Yan et al. 2010).

Subclinical infection of horses is probably the most common form of disease, ultimately leading to under diagnosis, however this can be difficult to document. In a study by Hamond & Lilenbaum from 2012, the athletic performance of 119 racing Thoroughbred horses from Brazil was analyzed. Seventy-one percent of horses showed reactive titers (≥100), and almost half had what the group considered high titers (≥400). Even with those high titers, no clinical signs associated with leptospirosis were observed. Ninety percent of the horses with substandard racing performance were seroreactive with high titers, indicating that subclinical infection may have a major effect on horses' abilities, however, due to confounding factors, it is difficult to rule out other causes of poor performance besides leptospirosis from this study (Hamond & Lilenbaum 2012). Another major limitation of that study was that no attempts were made to

directly isolate *Leptospira* spp. using culture or PCR, so only previous exposure can really be considered. Further studies of subclinical infections and their effect on performance are lacking.

Prevention and Control

Treatment of leptospirosis in horses has mostly been based on therapies in other species such as cattle and dogs. Streptomycin and/or penicillin, or tetracyclines are the most commonly used antibiotics, yet streptomycin is no longer considered a first choice treatment based on increased risk of adverse side effects (Verma et al. 2013). If a mare has a high reciprocal titer of leptospiral antibodies (≥800), a higher dose of twice daily penicillin G (20 million units) has been suggested for preventing the fetus from becoming infected in utero (Verma et al. 2013).

Since both seronegative and seroreactive animals can shed bacteria, direct detection of leptospires in urine (by culturing or PCR) is an important tool for a successful control program, and may better guide vaccination protocols. Obviously environmental contamination control is ideal, but with climate change and the high degree of subclinical infections and inapparent urine shedding, these practices can be complicated.

Vaccines

Leptospira spp. have a variety of different virulence factors allowing them to be effective pathogens. They also have a number of different mechanisms allowing them to evade the host immune system, particularly pathogenic strains. These bacteria can rapidly translocate between mammalian cells, which is why bacteremia is so short and they can quickly disseminate to multiple organs, eventually leading to an induced programmed cell death (Wang & Wegrzyn 2007). For these reasons, the development of an effective leptospirosis vaccine has faced numerous challenges. To date, vaccines have been designed to target bacterial motility

mechanisms, lipopolysaccharides (LPS), lipoproteins, outer-membrane proteins (OMPs) and other virulence factors. Vaccine design has included recombinant proteins, lipopolysaccharide (LPS), inactivated and attenuated bacterial strains, and DNA backbones (Wang & Wegrzyn 2007). Commonly targeted virulence factors include outer membrane proteins LAg42, Loa22, and Lk73.5, lipoproteins LipL32, LipL45 and LipL21, periplasmic flagella FlaA and FlaB, and LPS (Klassen et al. 2003; Wang & Wegrzyn 2007).

The most commonly used veterinary leptospiral vaccines are bacterins and subunit based, with some demonstrated efficacy in cattle and dogs. In a study by Klassen and others from 2003, when dogs were vaccinated with two doses of vaccines and subsequently challenged with pathogenic leptospirosis, a high rate of protection was observed and duration of immunity was at least one year. In a study of a commercial inactivated leptospirosis vaccine (with adjuvant) in cattle, a marked protective response was induced after booster vaccination (Samina et al. 1997). Some downsides to leptospirosis vaccines include elevated or positive MAT titers that complicate titer interpretation, serovar specific vaccines being used in species where that serovar is either host adaptive or not a regional concern, as well as not being able to prevent carrier/shedding status, even though they are effective at preventing disease in the vaccinated animal (Miller et al. 2011).

Equine vaccination, until recently, has mainly been "off label." Cattle vaccines have occasionally been administered to horses, though because of its "off label use," potential for adverse side effects, lack of important equine serovars, and the resultant little to no serovar cross-protection in currently available vaccines, this is not recommended (Verma et al. 2013). In 2015, Zoetis LLC developed an equine specific vaccine to with serogroup Pomona. LEPTO EQ INNOVATOR with a Metastim adjuvant is labeled to help prevent leptospiremia caused

by serogroup Pomona, though has not yet been proven, thus does not have a full USDA-licensed label claim. Through preventing leptospiremia, the aim of the vaccine is to help reduce the potential risk of equine recurrent uveitis (ERU) infections, abortions or acute renal failure caused by Pomona (2015). It is whole-cell inactivated vaccine that is produced using an equine isolate of Pomona that undergoes an intensive purification to have low reactivity. It is recommended that horses receive this vaccine prior to exposure to leptospirosis, or beginning at 6 months of age as maternal antibody begins to wane. A variety of product studies have been performed through Zoetis LLC. These studies demonstrated that vaccinated horses, 6 months of age or older, challenged with serovar Pomona demonstrated 0% urinary shedding. Further, 99.9% of the horses were reaction-free, and the vaccine is safe for use in pregnant mares for all trimesters of pregnancy (Zoetis LLC 2015). A potential downside to this vaccine is that it is made from serovar Pomona, which may not be the most pathogenic equine serovar seen in every area, and as previously discussed, very little cross protection between serogroups exists. A potential solution to this problem would be to develop a multivalent vaccine composed of multiple serovars to provide greater protection over a larger geographic distribution.

Diagnosis

Improvements to diagnostic testing have led to an increased awareness of incidence of leptospirosis worldwide, however further developments need to be explored. The incidence in canine patients has apparently increased in recent years, however this has not been well documented in human or equine patients (Delaude et al. 2017). The previously mentioned virulence factors have been used extensively as molecular targets in the diagnosis of leptospirosis.

Culture and Advanced Molecular Testing

Definitive diagnosis of leptospiral infections is made by culture of urine or infected tissue. Culture, however, has a variety of limitations that make it a less practical method of diagnosis. These limitations include, but are not limited to, the complexity of reagents, several weeks of growth time, and contamination problems in culture media (Donahue et al. 1995; Erol et al. 2015; Harkin et al. 2003; Hamond & Lilenbaum 2012; Hamond et al. 2014; Trap et al. 1992). Furthermore, in a 2003 canine study, no dogs that tested positive on serology or using urine polymerase chain reaction (PCR), had a positive culture result, indicating a low sensitivity for detecting leptospiruria. In that same study, a dog showing signs of severe clinical leptospirosis was positive on both PCR and serology, but did not culture positive (Harkin et al. 2003). In an equine ocular study by Polle and others from 2014, the isolation rate of *Leptospira* spp. via culture of vitreous from horses with ERU and that were seroreactive was low at 21.4%. Similarly, Faber and others found an isolation rate of leptospires in aqueous of 22% from seroreactive horses (2000). This low yield and complicated methodology make culture less than desirable as the gold standard for diagnosis.

Other testing methods previously explored have included fluorescent antibody test (FAT), immunohistochemistry (IHC), and dark-field examination of fetal fluids, but sensitivity and specificity of these methods is questionable (Erol et al. 2015). The utility of dual antigen ELISA has also been explored recently to distinguish pathogenic strain infected horses versus host adapted or vaccine strain exposed horses. Leptospiral immunogenic proteins, specifically Sph1, LigA, Hsp15 and *LipL*45 (Qlp42), are up-regulated in infected horses but are undetectable in cultured organisms. This is in contrast to *LipL*32, which is abundant in culture and elicits a

profound antibody response (Velineni & Timoney 2016). In a recent study, serum was collected from horses pre and post vaccination and was compared to horses that were naturally infected with serovar Pomona. Infection, but not vaccine, sera reacted strongly with Sph1, LigA and Lk90. Further, *LipL*45 and Hsp15 reacted moderately with infection sera and weakly with vaccine sera, thereby confirming that dual antigen ELISA based on immunologic proteins or *LipL*32 combined with host-induced Sph1 and Lk90 may be useful in the future differentiating infection from vaccine responses (Velineni & Timoney 2016). Another study by Ye and others from 2014 assessed the use of a four recombinant protein (r*LipL*21, rLoa22, r*LipL*32, and rLigACon4-8) ELISA for *Leptospira interrogans*. The sensitivity and specificity of ELISA compared to MAT were 82.39% and 86.15%, respectively (Ye et al. 2014). Unfortunately, these tests are not widely available, and the effects of cross reactivity or infection with serovars other than Pomona have not been explored.

Microscopic Agglutination Titers (MAT)

MAT titers for the diagnosis of leptospirosis are commonly used to determine previous exposure to *Leptospira* spp. in domestic and wild animals, as well as in humans (Adler, 2015). In brief, this test determines the presence of anti-leptospiral IgG and/or IgM antibodies. These results are based on agglutination of live *Leptospira* spp. after incubation with patient serum (or vitreous) at various dilutions. This test has a very high degree of subjectivity, which explains the significant variability reported between laboratories (Delaude et al. 2017; Limmathurotsakul et al. 2012; Miller et al. 2011). There is also risk for cross-contamination between serovars or potentially to humans, as the test uses live bacteria (Polle et al. 2014). The difference in prevalence between studies may be due to the region, study population, sampling techniques,

environmental conditions, serovars chosen for the specific MAT panel, and the interpretation of results in relation to the reciprocal titer cut-off used.

In addition to the high degree of subjectivity, MAT has limited specificity in identifying the infecting serogroup/serovar. This in mainly due to the fact that in acute infection the patient IgM, which predominates in early infection, has lower binding specificity when compared to IgG, as well as the fact that typically, a single serovar from a specific serogroup is used as a representative for the entire serogroup in MAT, such as using serovar Bratislava for serogroup Australis. (Delaude et al. 2017). In addition, MAT titers are better diagnostic tools for determining previous leptospirosis exposure, as it is assumed that IgG predominates in sera of patients that have previously been exposed. This potentially increases the specificity in identifying the MAT serogroup specific reactivity patterns when compared to acutely infected patient sera (Delaude et. al 2017). Unfortunately, MAT titers are further complicated by cross-reactivity between different serogroups which can lead to MAT reactivity at lower dilutions (Barwick et al. 1998; Erol et al. 2015). Further, when performing MAT on serum in patients with ERU or localized infections, leptospires are surrounded by an osmophilic protein coat, which may impair recognition of the bacteria by phagocytes, and result in a negative serology result, despite being infected (Polle et al. 2014). A further problem with using serum MAT titers to aid in the etiologic diagnosis of ERU is that because ERU usually occurs months to years after acute infection, and there are periods of quiescence, antibody concentrations decline below a detectable level making culture or serology detection difficult (Gerding & Gilger 2016; Polle et al. 2014).

Specific titer levels of the MAT are quite difficult to interpret and there are conflicting opinions on which levels indicate active infection versus exposure when using a single point

result **(Figure 1**). Most accept that when using the MAT for definitive diagnosis of active infection, a four-fold increase in paired titers is diagnostic of active infection (Erol et al.; Limmathurotsakul et al. 2012; OIE 2012). However, when using a single point result, this is much more complicated. It is unknown how to interpret these results in animals without clinical signs of leptospirosis, however. Furthermore, finding data to evaluate the sensitivity and specificity of the MAT in detecting subclinical infection or exposure is limited, which makes defining seropositivity difficult, as well as calculating seroprevalence.

In 2012, Otaka and others concluded that serology is an acceptable screening tool for leptospirosis on a herd basis, but that it is not a good predictor for detecting bovine carriers as an individual. This conclusion was based on the fact that half of the cattle shedding *Leptospira* spp. in their urine as detected by PCR were seronegative on serology (Otaka et al. 2012). Similarly, in a canine study (Harkin et al. 2003a), serologic diagnosis of leptospirosis alone was questioned, as many dogs that were seronegative were PCR positive (using 23S rDNA). Those dogs may have been infected by a serovar (eg, Autumnalis) that was not detected on that specific MAT, they were potentially immunosuppressed and unable to mount an antibody response, or died prior to seroconversions. Some apparently healthy dogs in this study were actively shedding leptospires in their urine, indicating that PCR may be suited for detecting active shedding, as well as in the early diagnosis of leptospirosis prior to seroconversion. When the 8 dogs in this study who were believed to be false negatives were placed into the true positive group, the PCR test had a sensitivity of 100%, specificity of 94%, positive predictive value of 67%, and negative predictive value of 100%., demonstrating a test with high value for determining carriers and infected animals (Harkin et al. 2003a).

In a recent report looking at 1652 human patients with suspected leptospirosis from three observational studies and one randomized control trial, the use of MAT as the reference standard came into question. This report compared culture, MAT, immunofluorescence assay (IFA), lateral flow (LF) and/or PCR targeting the 16S rRNA gene using Bayesian latent class models and random-effects meta-analysis. They found that, when compared with culture plus MAT, PCR had a sensitivity and specificity of 55.5% and 82.5%, respectively; But when recalculated using Bayesian latent class modeling, the sensitivity and specificity of PCR were 52.7% and 97.2%. They concluded that culture plus MAT represents a relatively poor gold standard against which to compare alternative diagnostic tests for leptospirosis, and that care must be taken when evaluating reference methods compared to point of care analysis (Limmathurotsakul et al. 2012).

Figure 2-1: Leptospirosis MAT titer interpretation; Adapted from: https://ahdc.vet.cornell.edu/docs/LeptoMat_Fact_Sheet.pdf

Result	Lepto MAT Interpretation
Acute Serum Sample	
< 100	Seronegative
>/= 800	OIE (2012): Previous or active infection; vaccination
>/= 6,400	Highly suggestive of active infection
Convalescent Serum (Paired) Samples	
4 fold increase from baseline	Definitive diagnosis

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a highly sensitive and rapid diagnostic tool that can be used for the detection of leptospirosis in a variety of different samples such as urine, aqueous

humor, allantoic fluid, as well as hepatic, pulmonary, placental, and fetal tissues. Reported sensitivity is higher than that found in culture, with a detection limit around $10\text{-}10^2$ leptospires per milliliter of sample (Stoddard et al. 2009; Hamond et al. 2014). When compared to indirect serological methods, PCR is better suited to detect carriers, as it is directly related to the detection of animals that are shedding, which can better equip veterinarians and owners for developing successful prevention and control programs.

Another point to consider with PCR is methodology. There are a variety of different targets, but ideally they should be able to differentiate pathogenic from non-pathogenic strains of *Leptospira* spp.. Recently, differences between the types of PCR assays and their respective targets has come into question, particularly when considering other spirochetes or bacterial and fungal contaminants causing false positive results. In a study by Fink and others from 2015, the performance of 3 real time PCR (qPCR) assays was assessed, 1 targeting the 16S ribosomal RNA (rRNA) gene and the other 2 targeting the *lipL*32 gene. When using DNA extracted from laboratory-cultured pathogenic *Leptospira* spp., all 3 assays demonstrated 100% specificity and had identical lower limits of detection when tested on urine samples collected aseptically from 30 dogs suspected for leptospirosis. However, when tested on 30 urine samples that were collected by the free-catch method, the 16S rRNA–based assay falsely detected 13.3% of the samples as positive for pathogenic *Leptospira* spp., yet nucleotide sequence analysis of the amplified DNA fragments showed that these were false positives due to unrelated bacteria (Fink et al. 2015). These results highlight the importance of validated, sample-specific PCR-based diagnostic assays, that the use of 16S rRNA based assays may lead to increased false positive results, and that sampling method (free-catch vs aseptic) may have an effect on diagnosis.

One and a half to eight percent of dogs not suspected to have leptospirosis have been reported to be shedding pathogenic strains of leptospirosis in their urine, however this has not been as well documented in horses as in other species (Harkin et al. 2003; Rojas et al. 2010; Llewellyn et al. 2016). In a recent study (Fang et al. 2015) looking at sheep and cattle from abattoirs in New Zealand assessing potential human exposure, 330/542 (60.9%) of the sampled animals had antibodies against Hardjobovis and/or Pomona, with an overall seroprevalence of 61% (95% CI: 48–73). A reciprocal MAT titer of ≥48 was considered positive for both serovars. In the same abattoir with such a high seroprevalence, almost half of the kidneys/urine tested were positive on qPCR (DNA gyrase subunit B (gyrB) gene) for leptospirosis. As a result, the application of urine qPCR to detect shedding status of livestock was implemented as a surveillance technique and to decrease potential zoonotic risk of exposure (Fang et al. 2015). In a study similar to that done on cattle performed by Otaka and others in 2012, Hamond and others (2012a) looked at four herds of unvaccinated horses in Brazil living on known endemic farms for leptospirosis. Out of 144 horses, 45.8% were seroreactive (reciprocal titer ≥100), while 61.8% were positive on urine PCR (*LipL*32 gene). Furthermore, of the horses positive on PCR, 41 horses were seroreactive and 48 horses were not (Hamond et al. 2012a). This study helps to confirm that not all horses that are shedding leptospirosis are seroreactive, and that if only a MAT is performed, potential carriers or infections may be missed. Furthermore, in a multispecies study from 2014 in Brazil, it was demonstrated that out of 512 animals, almost half were seroreactive on MAT (≥200). Yet on urine PCR (*LipL*32 gene), 36.2 % of horses, 21.6 % of cattle, 77.4 % of goats, and 33.3 % of pigs were positive, and not all of these PCR positive animals were also seroreactive, indicating that PCR is a much more sensitive diagnostic tool for the individual animal and detecting carriers (Hamond et al. 2014). It should be stated that in

these 3 studies from Hamond and others in Brazil, PCR was conducted for both pathogenic and non-pathogenic *Leptospira* spp. strains as controls. Additionally, since these are free-catch samples with the potential for other bacterial or fungal contamination, PCR was also conducted for other bacteria (coliforms, *Streptococci* spp. and *Staphylococci* spp.) and fungi (*Candida* spp.) in order to confirm specificity of the reactions for detection of pathogenic *Leptospira* spp. (Hamond & Lilenbaum 2012; Hamond et al. 2012a; Hamond et al. 2014).

In a 2015 study by Erol and others on leptospiral infected aborted fetuses, results indicated that real time PCR directed at the *LipL*32 gene was an effective method for the diagnosis on both placenta and fetal kidney (and liver when available). This study also determined that PCR had a higher sensitivity than the fluorescent antibody test, which was only able to detect only 18/21 (85.7%) cases (Erol et al. 2015). This study also emphasized that perhaps this discrepancy may be a result of the FAT being unable to detect the spirochete once the placenta has autolyzed, yet the PCR can detect the genetic material regardless of contamination. Further, since the described PCR is not serovar specific, MAT should continue to be used in clinical cases for serogroup/serovar determination if advanced typing methods are not available. In a study by Polle and others from 2014, *Leptospira* spp. were cultured from the eyes of six out of 31 (46 eyes) ERU affected horses (serovars Grippotyphosa and Pomona), and 45% were positive on PCR, indicating a high sensitivity for detection. Although obtaining an intraocular sample is much more invasive, the prevalence of *Leptospira* spp. infection based on PCR and MAT results from intraocular fluids compared with control horses is much higher (Polle at al. 2014). The diagnosis of intraocular infections does not appear to be aided by serology and requires sampling of ocular fluid for superior diagnosis. In studies using vitreous samples, MAT could detect anti-Leptospira antibodies in 90% of eyes with ERU and, using

PCR, DNA was detected in 71% of vitreous samples from eyes with ERU in Germany and 70% in California (Wollanke et al. 2004; Faber et al. 2000). One potential problem, aside from its highly invasive nature, with sampling ocular fluid for PCR is false negative results due to the small amount of fluid being sampled and the potential of missing detection because of the low overall amplification rate (Polle et al. 2014).

In a Brazilian study of local cart horses at three different sampling time points (Finger et al. 2014)), an overall seropositivity of 75.8% was found, with Icterohaemorrhagiae in 80.8% of the horses (reciprocal MAT titer ≥100). In the same study, blood and urine were qPCR (*LipL*32) negative. These results demonstrate that horses may be constantly exposed to *Leptospira* spp. in the environment which can be determined by single point MAT titers; However, in the absence leptospiremia or leptospiruria, it is difficult to say these horses are actively infected or are shedding the bacteria (Finger et al. 2014). Therefore, PCR seems better suited for determining individual leptospirosis status, whereas MAT may be better for determining if a herd has been exposed to certain serovars.

During the 1980s, restriction endonuclease digestion (RED) gained popularity in the identification of leptospiral serovars. Briefly, RED consists of DNA extraction of DNA from a homogeneous population of organisms, digestion of the DNA with a restriction endonuclease, and electrophoresis of the digested DNA in an agarose gel to create a fragment and a characteristic "fingerprint" for any particular DNA based on molecular weight (Marshall et al. 1981). Polymerase chain reaction-restriction endonuclease analysis has been used to determine leptospiral serovars and species. In a study by Brown & Levett (1997) this method was compared to arbitrarily primed PCR (AP-PCR) and low stringency PCR (LS-PCR). The study concluded that based on 11 randomized leptospiral strains, 36 clinical isolates from human patients and

dogs, and 12 survey isolates from trapped rats, that these methods agreed with those from serological identification. Recently, the usefulness of RED has been questioned given recent data from Arent and others (2017). Methods such as pulse field gel electrophoresis, multilocus sequence typing, multispacer sequence typing, or variable number tandem repeat analysis (VNTR), offer greater transportability of data between laboratories, have good sensitivity, and require significantly less DNA, which may make them more highly discriminatory compared to RED (Cutler 2017; Harkin et al. 2016). In a study by Harkin and others from 2016, VNTR utility was assessed in urine PCR samples from dogs. VNTR was able to identify 14 distinct VNTR patterns, potentially indicating that 14 unique serovars had infected the group of dogs. Further, they demonstrated MAT had poor sensitivity, but high specificity for identifying serogroup Grippotyphosa when compared with VNTR, suggesting that newer described molecular techniques for leptospiral serovar identification should be utilized (Harkin et al. 2016).

Chapter 3 - Materials and Methods

Ethical Approval

The study complied with all IACUC regulations (IACUC #3727).

Subject Selection

The study was designed as a cross-sectional prevalence study representing horses of mixed breeds and ages, owned by Kansas State University, the Animal Science Unit Equine herds, and clients of the Kansas State University Veterinary Health Center (KS, NE, MO). This study was performed over 19 consecutive months to account for temporal bias. Apparently healthy horses presented for pre-purchase exams, dentals, and annual vaccines, as well as volunteered animals, were evaluated by physical examination by a licensed veterinarian.

Power analysis was performed prior to sample collection to determine appropriate sample size (126-153 total needed), based on true prevalence estimates (3-5% prevalence), with all other assumptions held constant, a desired confidence of 0.95, and a precision of 0.05 (Arent et al. 2013; Humphry et al. 2004; KR Harkin-Personal Communication).

Inclusion Criteria and Sample Collection

Horses were excluded if there was any history of renal disease, ERU, recent abortions (in the last six months), liver failure, pulmonary hemorrhage or they had ever received a leptospirosis (on or off label) vaccine. Samples (one serum sample via aseptic venipuncture and one free catch urine sample) were collected on the same day either at the VHC or at the horse's home environment. All samples were either submitted to the Kansas State University Diagnostic lab directly, or submitted within 48 hours post collection after being stored at 4ºC. Blood was collected into tubes without anticoagulant. Blood was either sent to the laboratory uncentrifuged or left to clot for 30 min before centrifugation at 2000 × g for 10 min and subsequent serum

separation. Serum samples were submitted for creatinine, gamma glutanyl transferase (GGT), and the six serovar MAT, while the urine sample was submitted for PCR. Urine samples were collected using a free catch method. If the horse did not void with the use of alpha-2 agonists or during the exam, a single dose of furosemide 5% was given intravenously at a dose of 0.5mg/kg bwt.

Enrollment criteria included permission and written consent. Using an owner reported survey, we recorded environment and management practices for each horse including: street address (to allow for geographical grouping), stable vs. pasture environment, proximity to water sources, where drinking water is obtained (city, rural, well etc.), recent rainfall on the property, and proximity and contact with any livestock or dogs. All positive results were reported to the owners and recommendations on management and handling of these horses was given on an as needed basis. Maps were generated using https://maps.google.com. Rainfall data was determined using https://www.wunderground.com/history.

Creatinine and GGT

Serum creatinine and GGT were determined using the Cobas 6000 analyzer series, Roche diagnostics USA.

Microscopic Agglutination Titers

Serum samples were collected from each horse and submitted to the Kansas State Veterinary Diagnostic Lab-Serology Lab for the previously validated six serovar MAT based on the National Veterinary Services Laboratory's (NVSL) protocol (Ames, Iowa, USA). In brief, spectrophotometry is used to detect leptospirosis cell counts to the six serovars (representative of serogroups): Canicola, Pomona, Grippotyphosa, Icterohemorrhagiae, Hardjo, and Bratislava. The

strains selected are grown in liquid leptospiral culture medium and used as leptospiral antigens to do a transmittance percentage. The number of antigens used is determined and a screening test may be performed with a 1/50 serum dilution. Quality control is performed. A volume of each antigen, equal to the diluted serum volume, is added to each well, making the final serum dilution 1/100 in the screening test and these microtitration plates are incubated for 1.5 hours. End-point titers are determined. The plates are examined with dark-field microscopy. MAT positivity was defined as positive reaction to at least one serovar included in the 6 serovar panel at reciprocal of ≥100. Similar to a study by Delaude and others in 2017, based on a lack of a consensus of what represents a positive MAT titer, this study opted to use reciprocal titers of ≥100 and ≥800 to calculate seroprevalence.

Polymerase Chain Reaction

Free catch urine samples were collected and stored in a sterile plastic container. Urine was refrigerated for a maximum of 48 hours prior to submission to the Kansas State University Diagnostic Lab for processing. A semi-nested polymerase chain reaction (PCR) was performed, using the previously described primers and probe, to amplify a *Leptospira* genus conserved region of the 23S rRNA (Harkin et al. 2014). In brief, 1.8 ml of urine was centrifuged at 14,500rpm for 10 min and the supernatant was discarded. The pellet was resuspended in a phosphate-buffered saline solution and this suspension was used for DNA isolation. The DNA isolation was performed by using a commercial kit and by following the manufacturer's instructions (Qiagen, Valencia, CA). All PCR reactions included positive and negative amplification controls for each run. Positive and negative samples were defined by following the PCR data analysis recommendations as described previously (Harkin et al. 2014).

Statistical Analysis

All data was collected into Microsoft Excel. The prevalence of positive urine PCR and MAT seropositivity were calculated. In order to examine the different reported titer levels indicating active infection on MAT seropositivity, seroprevalence was defined at both reciprocal titers of ≥100 and ≥800. Risk factor analysis was restricted to MAT seropositivity as outcome measure because only two horses tested positive on PCR. Risk factor analysis was performed using 95% confidence intervals (95 % CI) by the modified Wald method. Logistic regression models (R, Commercial Statistical Software) were performed. The Chi-square test and odds ratios were used to assess associations between seroreactivity and potential risk factors using reciprocal MAT values of ≥100 and ≥800 with a multivariate model. Values of $p \leq 0.05$ were considered statistically significant.

Chapter 4 - Results

Samples were collected from total of 204 horses of mixed age, breed, and sex between May 2016 and December 2017. Sixty-nine mares, 103 geldings, and 32 stallions were represented. Age ranged from 1-40 years, with the majority of horses being between the ages of 1-20 years **(Figure 4-1)**. Quarter horse type breeds were overrepresented in this population (124/204; 61%), followed by Thoroughbreds (16/204;7.8%), Warmbloods (15/204;7.4%), and 15 other breeds and crossbreeds (**Figure 4-2).** Horses were located in 54 different zip codes spread between Kansas, Nebraska, and western Missouri (Kansas State University-VHC service area) (**Figure 4-3**).

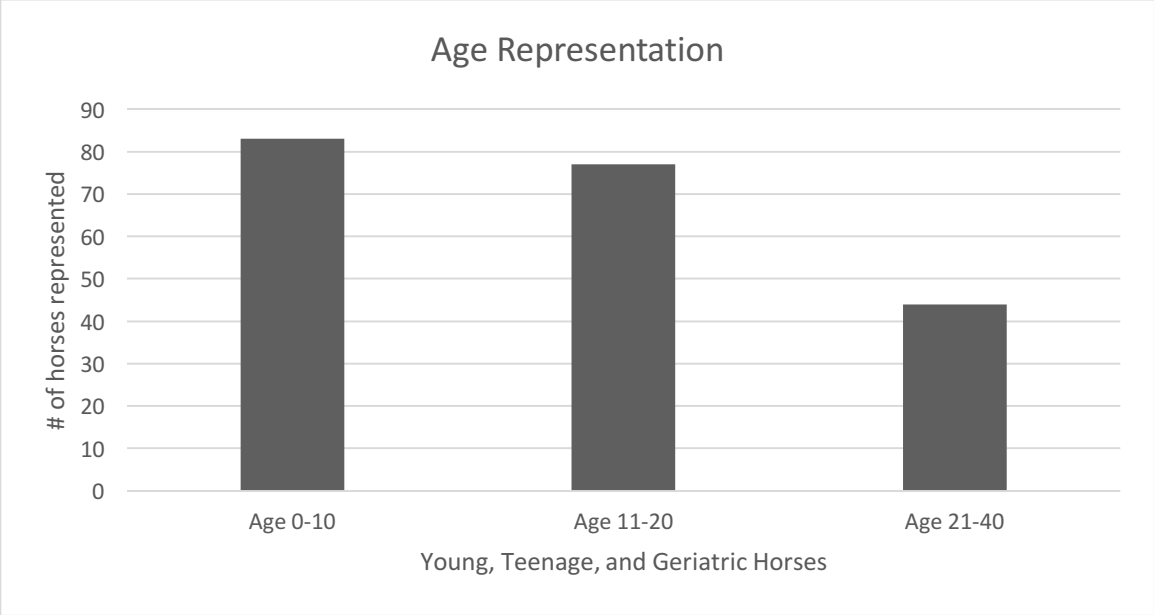

Figure 4-1: Signalment. Age of horses ranged from 1-40 years. When divided into intervals (young (0-10yrs), teenage (11-20yrs), geriatric(21-40yrs)), 83/204 (40.6%), 77/204 (37.7%), and 44/204 (21.5%) horses represented each age range respectively.

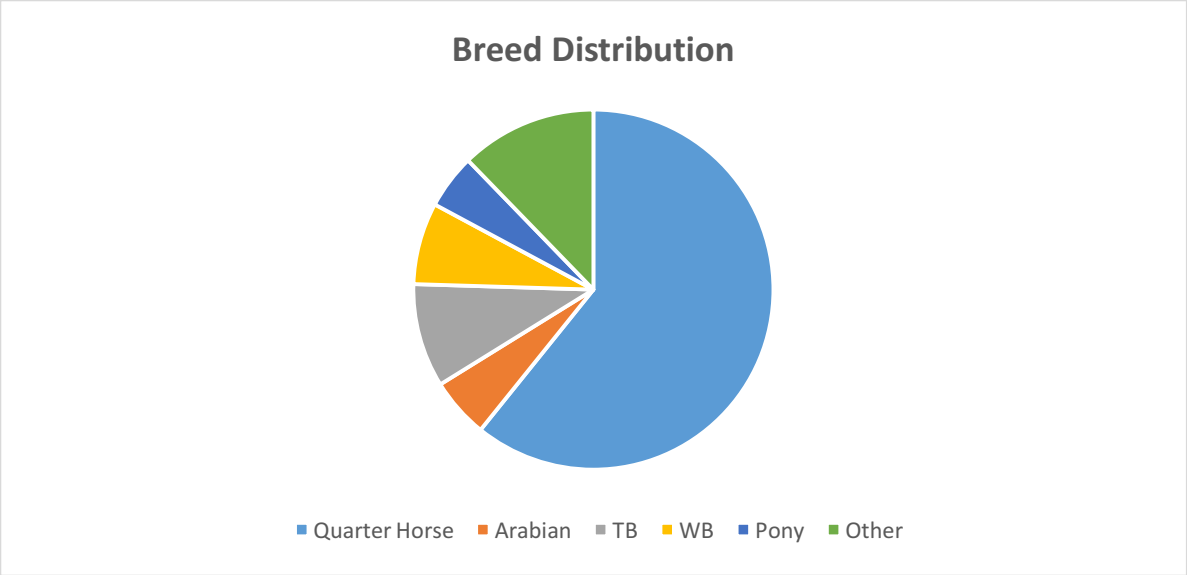

Figure 4-2: Breed representation. Quarter horse type breeds were overrepresented (61%). "Other" composed of Appaloosa, Draft, Miniature, Paso Fino, Spotted Mountain, Mustang, Missouri Fox Trotter, Morgan, Tennessee Walking Horse, Gypsy Vanner, and Palamino breeds.

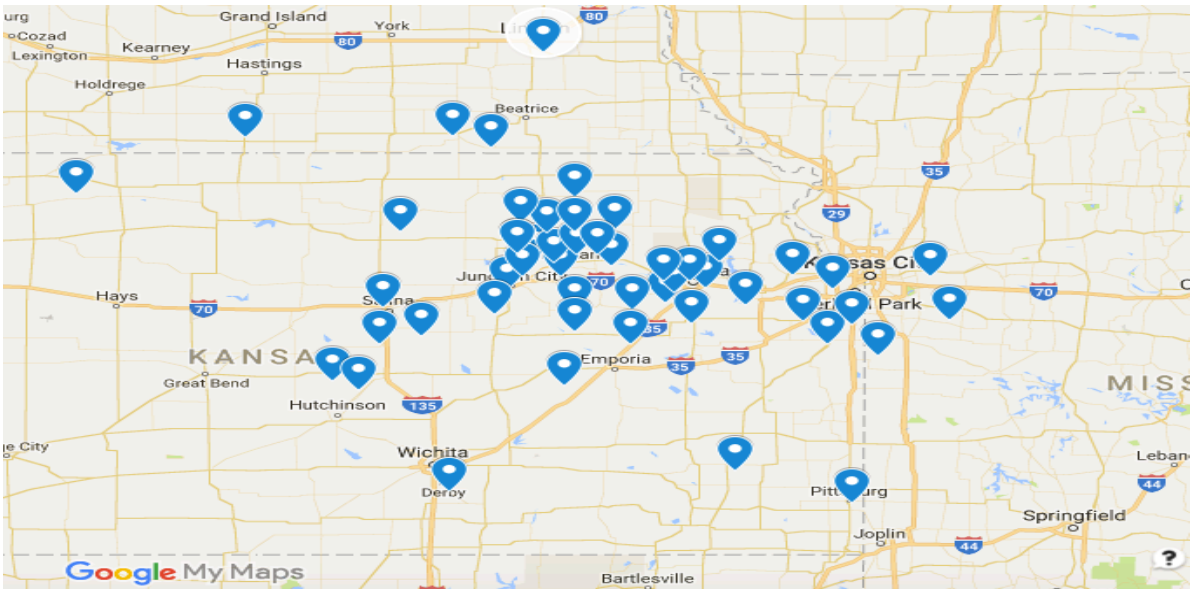

Figure 4-3: 54 zip codes in Kansas, Missouri, and Nebraska were represented.

Based on the client survey, the majority of horses were maintained on pasture either full time (36%) or part of the time (32%). The primary water source for most horses in this population was treated rural or city water (70%), as opposed to well water or other water sources.

Pond water as the primary water source was low at 1.5%, but 31.8% of the horses had creeks, ponds, or streams on the farm that they lived. Approximately 75% of the study population had contact with either sheep, cattle, or dogs (reservoir species). The majority of owners (86%) reported rain where their horse was located in the previous week. Despite efforts to collect equal numbers of samples from each season to account for temporal bias, the majority of samples were collected in the fall (85/204; 41.6%), however the 3 other seasons were equally represented. (**Table 4-1).**

Table 4-1: Client based survey categorical responses concerning environmental conditions and management practices of the 204 horses most likely to affect leptospirosis carrier and exposure status based on its biological nature.

	Number of Horses (n/204)	**Percentage (%)**
HOUSING		
Stable	21	10.29
Pasture	74	36.27
Mixed	67	32.84
Dry Lot	42	20.59
PRIMARY WATER SOURCE		
Treated (City + Rural)	143	70.00
Well	56	27.45
Pond	3	1.47
Other	2	0.98
CONTACT W CATTLE, SHEEP, DOGS		
Yes	151	74.02
No	53	25.98
RAIN PAST 7 DAYS		
Yes	177	86.76
No	27	13.24
SEASON		
Spring	44	21.57
Summer	39	19.12
Fall	85	41.67
Winter	36	17.65

The average creatinine was approximately 1.4 mg/dL and the average GGT was 15 U/L. No horses had evidence of renal or hepatic compromise, and physical examinations were all deemed normal by the veterinarians performing the clinical examinations.

Two of 204 horses were positive on urine PCR for leptospirosis, which is an overall shedding prevalence of 1% for this study population. The PCR-positive horses were sampled on June 30th, 2016 (Horse 1), and on October 28th, 2016 (Horse 2). Both horses were from the same zip code in Kansas, but were from different farms. Neither horse had traveled out of state in the previous 6 months, they were both maintained on pasture full time, they both drank from rural water sources, and they both had access to cattle and/or dogs. The horse sampled on June 30 lived with 3 other horses, had a creatinine of 0.8 mg/dL, a GGT of 7 U/L, and there had been 2.43 inches of rainfall in the area in the preceding week. The PCR cycle threshold (Ct) for this horse was 15.45. The horse sampled on October 28 lived on a property with 25 other horses, had a creatinine of 1.1 mg/dL, and GGT of 18 U/L, and there had been 0.79 inches of rainfall in the area in the last 7 days. The PCR Ct for this horse was 12.55. While the horse sampled on June 30 was seroreactive to more than one serovar on initial sampling, one month later without therapy being administered, the MAT revealed only seroreactivity to Bratislava. Interestingly, the horse sampled on October 28 was highly seroreactive (1: 25,600) to serogroup Icterohemorrhagiae at initial sampling, and one month later following no therapy, the horse was sampled again and was still highly reactive to that serovar as well as Bratislava (**Table 4-2**). While some would consider these titers for horse initially sampled on October 28 to be consistent with active infection, the horse displayed no outward clinical signs of leptospirosis, nor renal, hepatic, pulmonary, or other organ compromise.

Table 4-2: Initial and one month re-check examination MAT titers on the two urine PCR positive horses. All values should be read as 1: x.

Horse 1	Canicola	Pomona	Grippotyphosa	Icterohaemorrhagiae	Hardjo	Bratislava
6/30/16	Neg	100	Neg	200	100	Neg
7/28/16	Neg	Neg	Neg	Neg	Neg	200
Horse 2						
10/28/16	800	100	100	25,600	Neg	3,200
11/28/16	400	Neg	Neg	6,400	Neg	3,200

Overall seroprevalence (horses who had at least one reciprocal MAT titer of ≥100 to one serogroup) was approximately 77%. When using the reciprocal MAT titer of ≥800 to one serogroup, the seroprevalence was 14.7%. The maximum titer for MAT was 1:25,600 for the serogroup Icterohaemorrhagiae, followed by serovar Pomona at 1:6400. The highest overall serogroup specific seroprevalence was to Bratislava at 47.5% (**Table 4-3**). When divided into seroreactivity to the single highest reciprocal titer out of the seroreactive horses, Bratislava was still the most common (30.5%), followed by Grippotyphosa (25.4%), with equally high reciprocal titers to more than one serovar seen in 19.7% of the horses (**Table 4-4**). To further assess horses and serovars with reciprocal titers ≥800 , the number of each horse out of 204 with a titer equal to each provided serial dilution was counted. Bratislava had the most horses with reciprocal titers ≥800 (**Table 4-5**).

Table 4-3: Overall seroprevalence and seroprevalence for each individual serovar determined by serum MAT (reciprocal titer ≥ 100). Number of seropositive horses (157) out of the total 204, the maximum titer, and percentage of horses that are seroreactive reported. Bratislava was the most common, followed by Icterohaemorrhagiae. The population had an overall seroreactivity (positive to at least one serovar) of 77%.

Serovar	# of Positive Horses	Max. Titer	Seroprevalence
Canicola	25	800	12.5%
Pomona	26	6400	13.0%
Grippotyphosa	72	3200	35.0%
Icterohaemorrhagiae	84	25,600	42.0%
Hardjo	27	1600	13.5%
Bratislava	97	3200	47.5%
		OVERALL	77%

Table 4-4: Highest reciprocal titers for each serovar of seropositive horses (n=157). Seroreactivity defined as serovar with highest reciprocal titer for all horses with at least one reciprocal titer ≧ 100 (157/204).Bratislava (30.5%) and Grippotyphosa (25.4%) were most common. Equally high titers of one or more serovars were seen in 19.7% of the population. B=Bratislava; C=Canicola; G=Grippotyphosa; H=Hardjo; I=Icterohaemorrhagiae; P=Pomona.

Highest Reciprocal Titer, Serovar (s)	n/157	%
Bratislava	48	**30.5**
Canicola	2	1.3
Grippotyphosa	40	**25.4**
G, B	6	3.8
Icterohaemorrhagiae	28	17.8
I, B	10	6.4
Pomona	5	3.2

Other possible combinations found: (C, G), (C, I), (C, I, B), (C, I, H, B), (G, I), (G, I, B), (H, B), (I, H, B), (P, B), (P, I)

Table 4-5: Number of horses (n/204) with reciprocal MAT titers of ≥ 800. Bratislava had the most horses with reciprocal titers ≥800 (18/204), followed by Pomona (7/204).

	800	1,600	3,200	6,400	12,800	25,600	Total
Canicola	1	0	0	0	0	0	**1**
Pomona	2	0	3	2	0	0	**7**
Grippotyphosa	2	2	1	0	0	0	**5**
Icterohaemorrhagiae	4	2	0	0	0	1	**7**
Hardjo	1	1	0	0	0	0	**2**
Bratislava	10	5	3	0	0	0	**18**

To further analyze potential risk factors, modified Wald tests were performed to determine whether the risk factors highlighted in **Table 4-1** were associated with a horse having a reciprocal MAT titer of ≥100 or ≥800. No significant correlations were detected when the cut-off was classified as test-positive reciprocal MAT titer of ≥800 cut-off, however season (*p =0.004*) and rainfall in the past week (*p=0.035*) were significantly associated the odds of being MAT-positive using a reciprocal MAT titer of ≥100 cut-off. Multiple logistic regression using the same variables showed that the summer and fall seasons, as well as rainfall in the last seven days were significantly associated with the odds of horses having a reciprocal MAT titer of ≥100 (**Table 4-6**). Horses who experienced rainfall within the past 7 days had 3.4 times (95% CI: 0.123; 0.927) greater odds to have a reciprocal MAT titer of ≥100 than those who did not experience rain. Horses that were sampled in the summer or fall had 8.46 and 4.10 times greater odds, respectively, to have a reciprocal MAT titer of ≥100 than those collected in the spring (**Table 4-7**). Only 20.9% of horses with access to ponds, stream, and creeks were seronegative.

Table 4-6: Logistic regression model of horses with one or more reciprocal MAT titers ≥100 and potential risk factors for leptospirosis exposure. Note: * represents p-value <0.05 and ** represents p-value <0.01.

HOUSING (ref stable)	**Estimate**	**Std. error**	**Z value**	**P-value**
Pasture	-0.7328	0.7442	-0.985	0.32480
Mixed	-0.8026	0.7402	-1.084	0.27821
Dry Lot	-1.1193	0.7953	-1.407	0.15932
PRIMARY WATER SOURCE (ref city)				
Rural	-0.4095	0.4957	-0.826	0.40881
Well	-0.7718	0.5407	-1.427	0.15349
Pond	-1.4722	1.3598	-1.083	0.27894
Other	13.0547	1017.3132	0.013	0.98976
CONTACT W CATTLE, SHEEP, DOGS (ref yes)				
No	0.2367	0.4918	0.481	0.63033
RAIN PAST 7 DAYS (ref yes)				
No	**-1.0870**	**0.5159**	**-2.107**	**0.03512***
SEASON (ref spring)				
Summer	**2.1356**	**0.6803**	**3.139**	**0.00169****
Fall	**1.4112**	**0.4706**	**2.999**	**0.00272****
Winter	1.0238	0.5727	1.788	0.07384

Table 4-7: Odd Ratios (95% confidence interval) of season from multivariate model of seroreactivity defined as a reciprocal MAT titer of ≥ 100.

Reference \	**Spring**	**Summer**	**Fall**	**Winter**
Spring		8.46 (2.23,32.11) **	4.10 (1.63,10.31) **	2.78 (0.91, 8.55)
Summer			0.48 (0.14, 1.71)	0.33 (0.09, 1.27)
Fall				0.68 (0.25, 1.87)

*Note: * represents p-value less than 0.05; ** represents p-value less than 0.01.*

Chapter 5 - Discussion

The objectives of the present study were to determine whether apparently healthy horses were shedding leptospiral bacteria in their urine and acting as potential carriers using urine PCR. Our secondary objectives were to determine the seroprevalence of leptospirosis in horses in the Central Midwest and to identify potential risk factors for equine exposure that could be used as a component of a prevention and control strategy for human and other animal exposure.

In the present study, there was a urinary shedding rate of 1% from asymptomatic horses. The overall prevalence from this population was expected to be low, based on previous studies from other species in non-endemic areas. In dogs, between 1.5-8% of urinary leptospiral shedding from asymptomatic canids has been reported (Harkin et al., 2003; Rojas et al., 2010; Llewellyn et al., 2016). Interestingly, in our study, one PCR positive horse had extremely high MAT titers, consistent with active infection (OIE 2012); however, was not displaying any outward signs of clinical infection, indicating that subclinical infection can potentially be a real issue with disease spread and zoonosis. Since both seronegative and seroreactive animals can shed the bacteria, direct detection of leptospires in urine (by culture or PCR) is an important tool for earlier leptospiral detection, a successful control program, and may better guide vaccination protocols.

In our study, 77% of horses were seropositive (reciprocal MAT titer $\geq$ 100) to at least one of the 6 serovars tested, which is consistent with the previously reported seroprevalence for the Midwestern US (76.2%) (Zoetis LLC 2015). The panel of serovars analyzed in this study included locally occurring serovars found in dogs, cattle, and horses After infection, antibodies (IgG and IgM) can persist for years or even during the entire lifespan of an animal (Levett 2001).

As Blatti and others concluded from a similar study (2011), we also suspected that the high seroprevalence in these healthy horses suggests that horses are often exposed to or may be carriers of *Leptospira* spp. and never develop clinical signs. This is most likely due to exposure to host-adapted serovars of *Leptospira* spp., leading to an inapparent infection in their respective hosts. In contrast, some horses may be seronegative but PCR positive. This scenario may be due to being infected by a serovar not detected on that specific MAT, potential immunosuppression and an inadequate antibody response, testing an infected animal where the serovars are localized to an immunologically protected site, or testing prior to seroconversion (Harkin et al. 2003a). Previous clinical leptospirosis infection or vaccination leading to the development of antibody production in our sample population is unlikely, as any of these horses were excluded prior to sampling and analysis.

In a study by Blatti and others from 2011, risk factors for increased seroprevalence were increasing age ($p = 0.006$), being a mare ($p = 0.001$), being a pony ($p = 0.028$), increasing duration spent on pasture per day, as well as seasonal variation in seropositivity with the prevalence being higher in summer and autumn, and lower in winter and spring ($p = 0.003$). While we did appreciate increased seroprevalence in the summer and fall seasons, we also saw increased seroprevalence with reports of rain in the previous week, and we must interpret these results with caution. We did not experience significant flooding during the study period, and yet most horse owners (155/204; 75.9%) reported rain. The exact amount of rain in each location for each seropositive horse was not documented. Summer and autumn having higher seroprevalence seems to be a frequently reported risk factor, as we saw in our study. Thus, we are confident reporting that horses are 8.4 times as likely in the summer and 4.1 times as likely in the fall to be seroreactive compared with horses in the spring. The increased seroprevalence in spring and fall

is likely due to longer survival and infectivity of leptospires in the environment in warm and humid climate conditions. Pond water as the primary water source was low at 1.5%, but 31.8% of the horses had creeks, ponds, or streams on the farm that they lived. Only 20.9% of horses with reported ponds, stream, and creek on their property were seronegative, however it was unknown how many horses had direct contact with these water sources. We expected that water sources, increased time spent on pasture, and contact with potential leptospirosis reservoir species (dogs, cattle, sheep) would potentially have a significant effect on seroprevalence based on the biology of the bacteria, however these results were not significant in our study. In another study by Hamond and others (2013), as well as one by Wangdi and others (2013), it was also found that different types of water sources did not have any influence on the status of horses being seropositive to *Leptospira* spp., which could be an interesting topic to explore further. No significant findings were appreciated in our study population with regards to risk when the positive MAT titer was defined as the reciprocal of ≥ 800. This could potentially be due to smaller sample size (only 14.7% of horses), this population being considered healthy and not having active or clinical leptospirosis, or there truly being no increased risk of having a reciprocal titer of greater than or equal to 800 with the variables evaluated.

Bratislava was the most commonly observed serovar in this study: 47.5% with all reactive serovars of every horse included (reciprocal titers of ≥100), 30.5% as only the highest reciprocal titer (≥100) from seropositive horses, and 18 horses having a reciprocal titer of ≥800. In a study in dogs in Switzerland, serovars Australis and Bratislava, both belonging to serogroup Australis, were also associated with about half of the seroreactors. In Switzerland, infections with this serogroup have been known to cause acute clinical infections, and are suspected to be transmitted by hedgehog reservoir hosts (Delaude et al. 2017). While many previous reports

from the US have implicated Pomona as the most common serovar, finding Bratislava in our study was not that surprising. In a study in South Africa, the most common serovar in all three provinces analyzed was Bratislava, and other studies performed around the world have reported the seropredominance of this serovar in their surveys in horses (Baverud et al., 2009; Hamond et al. 2013; Turk et al. 2013). Interestingly, in the current study, we noted that after Bratislava, depending on how we defined seroreactivity had a profound effect on which serovar was the second most commonly identified. In each model, this changed from Icterohaemorrhagiae to Grippotyphosa when comparing most positive titers to the highest titers, and finally when looking at titers ≥800, Pomona was the second most common. Looking at the most represented serovar in different ways could potentially skew the way we interpret geographical differences. A potential downside to the current equine vaccine is that it is made from serovar Pomona, which may not be the most pathogenic equine serovar seen in every area, and as previously discussed, very little cross protection exists. Further, some horses had titers to multiple serovars. This may represent multiple infection of different strains or different cross-reactions between serovars from the same serogroups, which is a reported problem with the MAT (Barwick et al. 1998a; Hamond et al. 2013).

One limitation of this study was that we only collected one, free catch urine sample from each horse. Some reports have said that shedding of leptospires in urine may be intermittent, therefore some potential carriers may have been missed (Hamond et al. 2014). Multiple samples from each horse over time may be something to consider in the future, however this may be difficult in field conditions. Further, as Hamond and others (2014) suggested in a similar study using urine PCR, we did not check for potential PCR inhibitors in clinical samples of urine and some has been stored for up to 48 hours at 4ºC; Therefore, their presence cannot be ruled out as

the cause of MAT positive (high titers)/PCR-negative results, particularly in those horses who had reciprocal MAT titers of greater than or equal to 800 which could indicate active infection. We also did not check for potential contaminating bacteria and fungi, which may lead to a false positive PCR result. However, our PCR (23S rRNA based), unlike the 16S rRNA-based PCR, does not seem to detect non-pathogenic leptospiral DNA as frequently, similar to the *LipL32* based assay (KR Harkin-personal communication, unpublished data; Fink et al. 2015).

In conclusion, the results of this study show that Central Midwestern horses are commonly exposed to pathogenic *Leptospira* spp. with exposure being most common to serovars belonging to Bratislava. Depending of evaluation method used, Grippotyphosa, Icterohemorrhagiae, and Pomona were also serovars of interest. Rain in the horse's environment during the previous week, as well as seasonality (summer and fall) seem to be potential risk factors for seropositivity (reciprocal MAT titer $\geq$ 100). Based on our findings, the risk of apparently healthy horses contributing to the spread of pathogenic *Leptospira* spp. in the environment appears low (1%).

Chapter 6 - Future Directions

Geographic/Epidemiology Studies

With the lack of specificity given with current serology and PCR techniques as well as the concerns with serovar cross-reactivity, more serovar specific tests and serotyping methods are important, particularly identifying the more pathogenic serovars. In a recent study by Arent and other from 2017, the evolution of molecular typing techniques is described, which highlighted the heterogeneity within serovars and may facilitate analysis of these subpopulations by host and geographical location, as well as potentially identify new pathogenic serovars earlier. Another potential study would be not only looking at domestic species, but also known wildlife reservoir species to better assess the possibility that domestic species are becoming infected more commonly via wildlife contamination, though this could be quite difficult in a field setting.

Further exploring the United States with studies similar to our own may also be of benefit for better understanding the epidemiology of leptospirosis and to better advise on prevention and control of the disease. This could also lead to the creation of a more efficacious vaccine. Because serovars seem to have the potential to be regionally distributed and the currently available vaccine is monovalent, a potential sequelae to these studies would be the ability to develop a multivalent vaccine composed of multiple serovars to provide greater protection over a larger geographic area.

With global climate change and extreme weather events such as cyclones and floods occurring more frequently and with greater intensity, it may also be beneficial to perform these studies in areas that hold the highest risk for leptospirosis outbreaks.

PCR Studies

To date, we are not aware of any studies in horses which correlate the infecting serovar identified by culture or serovar specific PCR and MAT serogroup reactivity. In this study we did not perform culture or molecular typing of *Leptospira* spp. on the two PCR positive horses. Various reports have commented on the low sensitivity and return rate on urine culture. Currently, molecular typing tools or serovar specific PCR using pulse field gel electrophoresis, multilocus sequence typing, multispacer sequence typing, VNTR, and RED are not readily available for commercial use, though these methods would have the potential of being able to compare MAT seroreactivity to the acutely infecting serovar or the serovar being shed by a carrier. Hopefully, in the future we can add to the serologic data obtained through this study with molecular typing of *Leptospira* spp. derived from PCR positive urine. However, due to the very low rate of shedding that we saw (1%), this part of the project was not pursued at this time.

Clinical Disease Studies

This study was performed on apparently healthy horses. It would be interesting to pursue similar experiments using sample populations of horses with clinical signs consistent with leptospirosis such as ERU, acute renal failure, abortions, and pulmonary hemorrhage, and assess the positive predictive value of such testing. This data could be useful for herd management and developing vaccination protocols.

Human Studies

Humans frequently interact with horses, therefore it is a very real concern that there could be potential zoonotic spread from horses and their urine to handlers, stable hands, owners, and

veterinarians. In a number of canine studies, dog ownership has been identified as a risk factor for human leptospirosis, as well as studies concerning meat workers and farmers demonstrating 22% and 74% case incidence, respectively; Therefore, it would be a valid assumption that this would also be a risk factor for equine owners (Jansen et al., 2005; Douglin et al., 1997; Fang et al. 2015; Trevejo et al., 1998).

Chapter 7 - References

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