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AN ASSESSMENT OF THE CARRIER STATE AND A NOVEL MARKER OF *LEPTOSPIRA* AND ABORTION IN CENTRAL KENTUCKY HORSES

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AN ASSESSMENT OF THE CARRIER STATE AND A NOVEL MARKER OF *LEPTOSPIRA* AND ABORTION IN CENTRAL KENTUCKY HORSES

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

AN ASSESSMENT OF THE CARRIER STATE AND A NOVEL MARKER OF *LEPTOSPIRA* AND ABORTION IN CENTRAL KENTUCKY HORSES

Leptospirosis is a reemerging zoonotic infection of worldwide importance and affects all mammals. The bacterium is transmitted to animals and humans by urine, fetal membranes and body fluids. *Leptospira* shedding in the urine contaminates both soil and water, exposing both humans and animals to the bacterium. Leptospirosis in horses can cause abortion and is one of the etiologies of equine recurrent uveitis which can lead to blindness. Equine leptospiral abortion in Central Kentucky is primarily caused by serovar Pomona, with occasional cases attributed to serovar Grippotyphosa. There are a few reports in the literature attributing abortion to serovar Bratislava in the United States. Interestingly, Bratislava has the highest seropositivity in the horse in the United States.

Two studies were conducted that are included in this dissertation. The first was to determine the prevalence of leptospirosis in horses located in Central Kentucky submitted for necropsy to the University of Kentucky Veterinary Diagnostic Laboratory. Heart, vitreous humor, kidney and urine samples were collected for microagglutination testing (MAT) and real-time PCR (qPCR). Heart blood and vitreous were tested using MAT for serovars Grippotyphosa, Pomona and Bratislava. Kidney, vitreous and urine samples were tested for pathogenic *Leptospira* by qPCR. MAT test results for heart blood indicated an increased seroprevalence for Bratislava as compared to Grippotyphosa and Pomona. Three horses had positive titers for serovar Bratislava in vitreous and heart blood samples. All urine samples tested negative by qPCR, and only one kidney sample had a weak positive result. Four vitreous samples tested positive for leptospirosis by qPCR, but all samples were negative upon MAT testing. No samples with positive MAT titers were positive by qPCR for any of the samples tested. Females were more likely to have positive MAT titers and were considerably older than males. MAT titers in females were also significantly higher as compared males. Finally, there was widespread seroprevalence in horses, regardless of the reason for necropsy submission. This suggests that

exposure to *Leptospira* on Central Kentucky horse farms is common and the risk of exposure to humans and other animals is possible.

The second study evaluated the enzyme heme oxygenase-1 (HO-1) and its potential as a marker for abortion in pregnant mares with elevated MAT titers. HO-1 is the rate-limiting step in the breakdown and degradation of heme to carbon monoxide, free iron and biliverdin, which is converted to bilirubin. Increased levels of HO-1 and its' by-products are upregulated during inflammation and sepsis. HO-1 together with its by-products are essential in protecting the body from both increased inflammation and in reducing the risk of sepsis in humans, mice and rats. HO-1 together with its by-products are also essential in helping maintain pregnancy in humans, mice and rats. HO-1 in the serum of horses has not been previously investigated. The presence of HO-1 in the serum of healthy non-pregnant mares (NPM), pregnant mares throughout pregnancy (PMOT), pregnant mares ≥7 months pregnant with and without placentitis (PM) and pregnant mares with high MAT titers (MATS) was investigated. HO-1 levels in both NPM and PMOT were lower than PM, and significantly lower than MATS. Pregnancy alone increased HO-1, the same has been shown in other mammals and humans. Additionally, mares pregnant with elevated leptospiral titers had significantly higher HO-1 levels compared to other mares. This indicates that both pregnancy and high MAT titers increase the animals' HO-1 response. Further investigation of HO-1 in horses is needed to ascertain its' importance during infection and/or pregnancy and potential therapeutics.

KEYWORDS: Leptospirosis, Horses, Serovars, Heme Oxygenase-1, Pregnancy

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June 30, 2021
Date

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Chapter One

Literature Review

1.1 Introduction

Leptospirosis is a zoonotic infection of global importance. Once thought to be contained, it has emerged as one of the most widespread zoonotic diseases in the world [1-3]. It is readily propagated from animal to animal and animal to man; however, human-to-human transmission is rare. Leptospirosis is a major public health concern in South America and South East Asia and is now considered a re-emerging disease in most developed countries [2, 4]. An estimated 1 million people are infected worldwide annually, leading to over 59,000 deaths [5].

Leptospira spp. are found in over 160 mammals worldwide, including dogs, cats, ruminants, horses and sea lions [6-8]. Multivalent vaccines only exist for dogs, swine and cattle. A monovalent vaccine for serovar Pomona became available for the horse in the United States (US) in the fall of 2015. Vaccine availability is limited to a small number of species, allowing the spread of this disease in other mammals and humans in many countries, which can lead to a public health crisis. Appendix 1 contains two tables. Table One is a list of available vaccines, serovars and species by pharmaceutical company available in the US. Table Two is a list of available vaccines, serovars and species by pharmaceutical company available outside the US.

The prevalence and economic impact of leptospirosis in humans are not well documented. However, leptospirosis is estimated to infect millions of

humans annually with case fatality rates ranging from 5 to 25% [9]. The highest occupational risk for contracting the disease either through direct contact (urine or body fluids) or indirect contact (contaminated water or soil) are veterinarians, farmers, slaughterhouse workers, butchers and sewer workers [3, 10-12]. Many outbreaks have been related to floods caused by high rainfall events. Clinical illness in both animals and humans can result in economic losses through lost wages, medical and veterinary bills, loss of animals and quality of life.

1.2 Leptospirosis

Leptospirosis is caused by a gram-negative bacterial spirochete in the order Spirochaetales, family Leptospiraceae and genus *Leptospira*. Leptospirosis was first described in 1886 by Adolph Weil and demonstrated by A.M. Stimson in 1907. The first isolation in pure culture was achieved by Japanese scientists, led by Ryokichi Inada in 1914 [13]. Leptospires are 0.1-0.2 μm in diameter and range from 6-25 μm in length [14]. There are both pathogenic and saprophytic species [6, 12].

Spirochaetales contain three families: *Brachyspiraceae*, *Leptospiraceae* and *Spirochaetaceae* [7, 14]. Both *Borrelia* and *Treponema* (cause of Lyme disease and syphilis respectively) also belong to the *Spirochaetales* order. Leptospires are unlike other members of the order in that they contain terminal hooks. They are highly motile by virtue of two periplasmic flagella located at each end, which allow the organism to burrow into the tissue [6]. Leptospires are obligate aerobes and demonstrate characteristics of both gram-positive and

gram-negative bacteria [6, 14]. The outer envelope is composed of an antigenic mucopeptide and a lipopolysaccharide (LPS). The LPS is very similar to those found in other gram-negative bacteria. However, it has a significantly lower endotoxic activity compared to typical gram-negatives.

Visualization of the organism is only possible by dark-field or phase-contrast microscopy. This is because the bacteria are extremely thin, also influencing the poor gram-staining characteristics of leptospires. [6, 14]. Further, leptospires are difficult to grow in culture and require several additives for enrichment [6, 14, 15]. The pathogenic species are heat sensitive; they can tolerate temperatures between 0^0-25^0C but not well above 42˚C. They are inactivated at temperatures below freezing or above 55˚C. Saprophytic *Leptospira* are ubiquitous in fresh water and soil [16, 17]. Only pathogenic leptospires have the genetic capability of utilizing cobalamin through autotrophy [18]. The autotrophic ability of pathogenic leptospires possibly allows it to infect mammals despite the host's ability to sequester cobalamin [18, 19]. Pathogenic leptospires also have a complete folate biosynthetic pathway whereas saprophytes do not [18]. This allows pathogenic leptospires the ability to survive in a nutrient poor locale within the host.

The incidence of leptospirosis in humans is underestimated due to several factors. First, the disease mimics many commonly occurring viral and bacterial infections such as influenza, Dengue, rickettsial infections, viral hepatitis, brucellosis and malaria [15, 20]. Second, leptospiral clinical infections can be short and self-limiting, with typical symptoms of fever, headache, nausea,

vomiting and abdominal pain [6, 9]. Early in the disease presentation, misdiagnosis is common. For subacute and chronic cases, liver and kidney failure (16-40% of cases), and respiratory complications (range from 20-70%) can occur. Other reasons for underestimation of the disease are general lack of clinical curiosity by healthcare professionals and a shortage of diagnostic laboratories with validated methods for leptospiral testing. Finally, human infections commonly occur in developing countries that are unlikely to have diagnostic laboratories for rapid disease confirmation.

Leptospirosis is known to occur extensively in various mammalian species; however, it is typically confirmed in cattle, swine and dogs [6, 14, 21]. Bats have been diagnosed with leptospirosis but their ability to transmit leptospirosis to humans and other mammals is considered rare [22-24]. In humans and animals, the portal of entry for the bacteria are cuts and abrasions in the skin, conjunctiva and mucous membranes. Another portal of entry is via ingestion of contaminated water or food [9, 12]. While the potential for birds as carriers of leptospires has been investigated, early research studies did not yield evidence of leptospiral infections [25]. However, a recent article demonstrated five different types of birds in Botswana with 27.8% renal positivity by PCR [26]. Further studies are needed to characterize the leptospiral carrier state of birds as they could provide another mode of transmission of leptospirosis.

Animals can become chronic carriers of leptospires once they have recovered from the acute phase of the infection. This allows the disease to perpetuate in nature posing a risk to humans and other animals [6, 21].

Leptospires can survive and propagate in renal tubules, can remain viable for years and are passed by urine into water and soil. In some species such as bovine and swine, shedding is intermittent, making carriers difficult to detect [6, 9, 15, 21]. In humans, leptospires in the urine typically present the first week after clinical signs are seen [6]. Leptospires can be difficult to identify by culture or molecular methods due to inhibitory substances and the acidic pH of the urine in some species, whereas alkaline urine can foster the persistence of leptospires [9]. Table 1.1 lists the urine pH of several species. Urine also tends to be 'contaminated' with multiple microorganisms which may inhibit isolation of leptospires [27].

Table 1.1: Urinary pH Range by Species

Species	Urinary pH Range*
Bovine	7.0-8.4
Ovine	7.5-8.5
Caprine	7.5-8.5
Equine	7.6-9.0
Canine	5.5-7.0
Rabbit	8.2
Humans	4.6-8

*[28]

Leptospires are trophic for renal tubules but they can also remain viable in the genital tract, brain and eye. Indirect modes of transmission of leptospirosis is by contact with contaminated urine, water or soil, making the risk of environmental exposure dependent on various factors. A temperate climate and a slightly alkaline pH, moderate to excessive rainfall can increase survival time of leptospires in the environment [15, 29, 30]. Other factors such as pH of soil can range from 4.0-8.0 depending on vegetation, moisture and soil age [31]. Inhibitors in soil and water such as competition with other microorganisms and

fungi can reduce the survival time [31, 32]. Survival time in the environment range from hours to months depending on the conditions [15, 32].

1.3 Diagnostics

Due to non-specific clinical signs, a leptospirosis diagnosis requires laboratory confirmation. The classical method is aerobic culture of the organism from spinal fluid, uterine fluid, aborted fetuses, kidney, placenta, blood or urine. Leptospires can also be isolated from liver, spleen and lung specimens. The culture medium, Ellinghausen-McCullough-Johnson-Harris (EMJH), is commonly used to isolate leptospirae from animal samples. Selective inhibitors such as antibiotics, bovine serum albumin and Tween 80 can be utilized to help promote the growth of leptospires. EMJH base media and enrichment additives are listed in Appendix 2. EMJH media contains either 10-15% rabbit serum or 1% bovine serum albumin along with long-chained fatty acids, vitamins B1 and B12 are required for growth. Typically, the generation time of saprophytic leptospirae is five hours, whereas pathogenic strains require about twenty hours [33]. The pH of the media is maintained at between 6.8-7.4 with an incubation temperature of 28-30^{o}C. Some leptospirae can take anywhere from a few days to a month to grow. Primary cultures grow very slowly and should be retained for 13 weeks with no growth to be considered negative [12, 14]. Once growth is established in culture, the organism can stay viable for up to ten years in Korthof's medium (10% hemolyzed rabbit serum, 0.08% Neopeptone and 0.14% NaCl buffered to pH7.2) [9, 16]. Certain species require additional additives for growth such as

glycerol, pyruvate and carbon dioxide [6, 14]. A new culture medium, Hornsby-Alt-Nally (HAN) has been developed for growth at 37°C instead of 30°C [34]. The medium has only been tested with two strains (strains IC:20:001 and HB15B203) of *Leptospira*, and further testing is needed with other strains. The advantage of this medium is growth at 37°C which is more compatible with *in vivo* temperatures and laboratory instrumentation [34]. The formulation for this culture medium is listed in Appendix 2. Culture and identification is difficult and time-consuming, especially if samples are not taken within the first week after clinical signs occur [7]. Further, samples should be collected prior to antibiotic therapy. Treatment with antibiotics can reduce the viability of leptospiral and other organisms present in the specimen making the organism more difficult to isolate. Antibiotics can also reduce serological titers when given early in the infection of dogs [6]. Due to the difficulty of culturing leptospirae, most diagnostic laboratories do not stock EMJH culture media.

Other laboratory methods are used in both humans and animals for diagnosing leptospirosis, all with limitations. The microscopic agglutination test (MAT) is considered the 'gold standard' for serological testing because of its high sensitivity and specificity, 92% and 95% respectively. However, this method requires a high level of technical proficiency, leading to low precision across laboratories [6, 7, 15, 35, 36]. Further, the MAT method requires the use of live organisms, adding a slight risk to laboratory workers that handle them.

MAT antibodies are not detectable until six or more days after exposure/infection occurs, with peak titers occurring typically by the fourth week

after exposure. Paired serum samples (acute and convalescent) with a four-fold increase in serum titers after 10 days, indicate an active leptospiral infection, with or without clinical signs [6]. Titers of greater than ≥800 in endemic areas along with symptoms of disease in humans are indicative of current or recent exposure or infection. In a seven-year study, patients diagnosed with leptospirosis were monitored in an endemic area of Barbados [37]. Blood samples for MAT titers were acquired during the acute and convalescent phases, and then annually for seven years. High titers (1600-3200) were observed up to 4 years post-convalescence. Seroprevalence was detected in most samples during the entire study (Table 1.2). This study indicates humans can be seropositive for an extended period. Additional environmental exposure (quantity and number of exposures in a timeframe) can increase the seropositivity. Titers of ≥800 can sometimes indicate active disease. Therefore, additional tests are needed to confirm cases of leptospirosis. Additional papers have shown similar seroprevalence persistence [38, 39].

Table 1.2: MAT Titers During Acute and Convalescent Phases in Humans*

Serogroup	Maximum MAT Titer (per cent seroprevalence)	% Titers ≥ 800 End of Study	Maximum MAT Titer End of Study	% Seropositive End of Study
Ballum	25,600 (90%)	0	400	60%
Icterohaemorrhagiae	51,200 (86.8%)	0	400	75%
Autumnalis	51,200 (95%)	8.7	800	60.9%
Australis	51,200 (54.9%)	2.3	800	16.3%

*[37]

Interestingly, some serovars generate low titers of 100 or less even when infection is present, especially in nonendemic areas [33, 40]. In general, the serovar with the highest titer is considered to be the infecting serovar whereas serovars that have lower titers are thought to be cross-reactions [6]. Cross reactions in humans with other bacteria have occurred with MAT in patients with syphilis, relapsing fever, Lyme disease and legionellosis [40]. Cross reactivity in animals to other bacteria has not been reported [41, 42].

Vaccinated dogs can have an increased titer from 3-6 months following vaccination [6, 43]. Titers in vaccinated dogs are rarely over 300, however titers of 3200 have been seen. Post-vaccination titers can increase (anamnesis) when natural environmental exposure occurs [43]. In one study, when post-vaccination titers were measured in cattle, titers as high as 12,800 were seen and remained high (800) 4 months after vaccination [44].

Other serological tests used are the immunoglobulin M (IgM)-enzyme-linked immunosorbent assay (ELISA), IgM dot blot, indirect hemagglutination assay (IHA), IgM dot-ELISA dipstick, macroscopic slide agglutination test, immunofluorescence and radioimmunoassay. All these tests have their limitations such as: varying degrees of sensitivity/specificity for early diagnosis (IgM-ELISA and dot blot, IHA); lack of laboratories capable of performing these tests especially in developing countries (RIA and immunofluorescence) and some detection only to genus level (IgM). The use of the IgM based tests have become readily accepted as alternatives in laboratories for aiding in confirming a

diagnosis of leptospirosis, especially with the introduction of kit-based diagnostics [6, 15, 33, 40, 45, 46].

Globally, the use of PCR for diagnosis of leptospirosis is becoming common. But, as with the MAT method, technicians must be highly trained and undergo regular proficiency testing to assure accurate results. In addition, the cost of equipment, kits, and hardware maintenance is high, limiting the use of these methods in developing countries. PCR-based analysis is used for early, efficient and rapid diagnosis, based on either the 16S ribosomal ribonucleic acid (rRNA) or 23S rRNA [15, 33, 47-50]. Several methods such as PCR, real-time PCR (qPCR) and nested PCR isolate deoxyribonucleic acid (DNA) using blood, urine or serum for detection, although tissue and cerebrospinal fluid can be used. All these methods utilize primers specific to pathogenic *Leptospira.* However, they differ on the quantification and sensitivity parameters. PCR has been commercially available for over three decades [51]. PCR is widely used to detect leptospires and is more economical compared to qPCR, however not as sensitive and more time consuming [52, 53] . Nested-PCR uses two sets of primers in successive runs, where the second set of primers amplifies an area within the first product [54]. Nested-PCR is more sensitive than PCR but takes more time and increases risk of false positives through amplification of contaminants. Real-time quantitative PCR calculates fluorescent signals after each cycle to determine amount of signal at specific times [51]. qPCR is more sensitive, reproducible and requires less time than both PCR and nested-PCR [51, 55].

The disadvantage of using the 16s or 23s rRNA is it will detect both the non-pathogenic and pathogenic *Leptospira* along with any other bacteria. Specific primers to the LipL32 outer membrane protein, which only pathogenic *Leptospira* have, is necessary to determine whether active infection is present. The disadvantage of PCR based methods is the need to have specific primers for each individual serovar, especially when desired for epidemiologic studies. Multilocus sequencing (MLST) is a PCR based method used in differentiating serogroups for epidemiological studies [56]. MLST uses PCR and sequencing to determine targeted genes. MLST is robust especially in determining specificity and sensitivity [55, 57]. A drawback to MLST is high-cost equipment needed along with the necessary expertise in running and evaluating the testing data. Additionally, MLST research has been focused on pathogenic *Leptospira* with limited knowledge of the biodiversity found in the environment [58]. An emerging molecular analysis is whole-genome sequencing (WGS) for both epidemiological and strain typing [58]. WGS utilizes MLST along with core genome and as this method is further developed it will lead to a more comprehensive characterization of *Leptospira*. In 2018 the genus *Leptospira* was updated with the addition of 35 new species and divided into three phylogenetic clusters correlating to their pathogenicity: saprophytic, intermediate and pathogenic [59]. In these three clades there are 13 pathogenic species, 11 intermediate species containing over 260 serovars, whereas the saprophytic group contains 11 species and over 60 serovars. The use of WGS restructured the *Leptospira* genus in 2019 with the addition of 31 new species [58, 60]. Two major clusters of pathogenic species

were further divided into 2 subclusters called P1 and P2. The P2 subcluster now contains the intermediate species and the saprophytes, which were further divided and named S1 and S2.

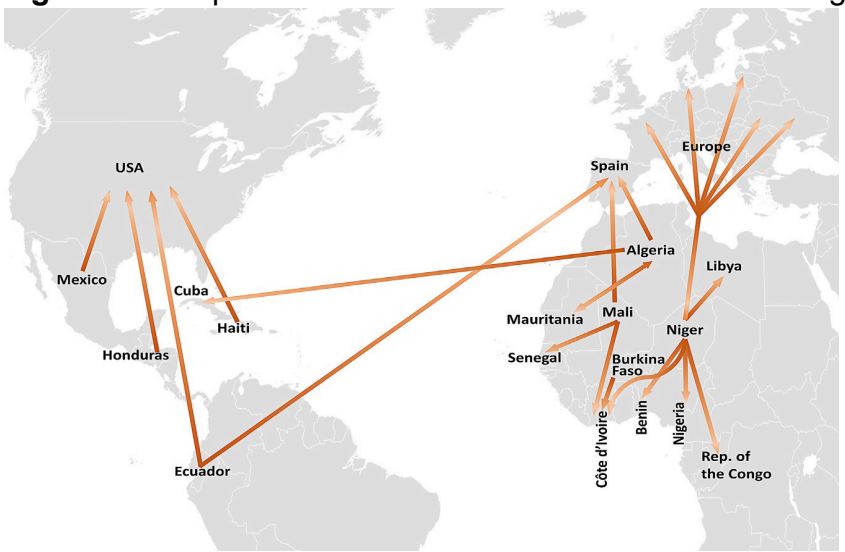
Proper collection and storage of samples for successful isolation of DNA is crucial. Whole blood or plasma are superior to serum for detection of leptospires by PCR. This is due to the organism 'sticking' to either/or white blood cells (WBC) and red blood cells (RBC) [47]. Only EDTA or sodium citrate should be used as an anti-coagulant as heparin will interfere with downstream PCR and sequencing. Ideally, urine samples should not be collected until the second or third week after suspicion of a *Leptospira* infection (i.e. compatible signs and test results) are detected, when shedding in urine is most likely [47]. Once collected, it must be kept on ice until it can either be refrigerated or frozen to decrease enzymatic breakdown.

Additional molecular methods such as pulse-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA, amplified fragment length polymorphism (RFLP), multi-locus variable number of tandem repeats analysis and multilocus sequence typing have shown promising results [15, 33]. The use of PFGE is considered the gold standard for the molecular identification of leptospires. All these tests require very specific training, can be labor intensive, and are typically performed only in larger diagnostic or research laboratories. However, the use of molecular techniques in conjunction with MAT has become the gold standard for the diagnosis of leptospirosis.

1.4 Environmental Effects on Incidence of Leptospirosis in Mammals

Evidence is mounting that select zoonotic diseases might be exacerbated by climate change [61, 62]. Earth's land and oceanic temperatures are increasing which has impact on the microbiome of ecosystems. Microbes can adapt to higher temperatures. Increasing environmental temperatures may select for heat tolerant microbes [61]. Climate change is thought to be a factor leading to increased spread of zoonotic infections. Microbes adapt and migrate with humans and animals to new environments [63]. When people move within a country or migrate to a new one, they are carrying microbes along with them and are also exposed to 'foreign' microbes at the new location. Examples of climate change include alterations in precipitation patterns, leading to increased wildfires, hurricanes and tornadoes [62]. Often, people are forced to relocate because of climate change. For example, in the western US, wildfires and flooding in the southeast have forced people to move (Figure 1.1) [64-66].

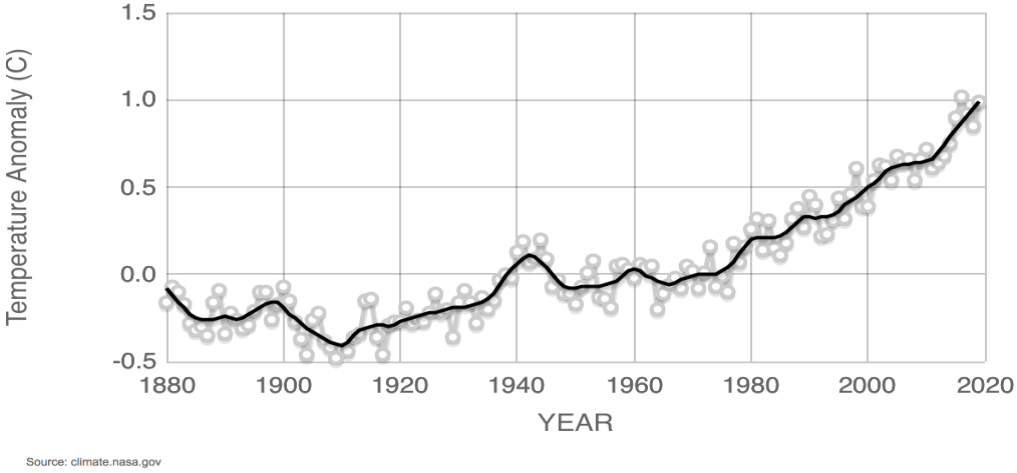
Figure 1.1: Population Movement Based on Climate Change Issues



Booth, M. Adv Parasitol. 100: 39-126, 2018.

Globally, temperatures have increased 1.70F since 1890 [67]. Figure 1.2 depicts the global rise in temperature. The previous ten years have been the warmest recorded since 1884.

Figure 1.2: Global Temperature Rise from 1880-2020



Precipitation, on average, has increased globally over the past century. The total area of Arctic ice has decreased overall by ~13.4% since 2010, increasing ocean levels [67]. These environmental changes have promoted zoonotic infections to be endemic in locations previously not reported. Further, microbes will adapt to higher temperatures and increased precipitation, encouraging mutations posing increased risk for humans [61, 63]. The increase in global temperatures over the last 30 years, has doubled the number of survival days in the environment for Vibrio spp. [68].

Environmental conditions (precipitation, temperature, soil pH, soil microorganisms, bodies of water, and wildlife) play a part in the survival of leptospires in the environment [17, 30, 32, 69, 70]. Increases in both

precipitation and temperature have been implicated in increased incidence of leptospirosis in both animals and humans [6, 15, 30]. In a 16 year retrospective study of weather data, precipitation was found to increase the incidence of leptospirosis in horses more so than temperature [30]. Both precipitation and temperature combined resulted in increased incidence above precipitation alone [30]. In the US, there has been increased rainfall and temperatures in the Northeast, along with major flooding events and heatwaves in the Midwest over the past 20 years. The average annual precipitation worldwide has increased at a rate of 0.08 inches/decade since 1901 [71]. In the Bluegrass region of Kentucky, a record 76 inches of rain was recorded in 2018 [72]. It is well known that *Leptospira* flourish in temperate climates with moderate to increased rainfall [30].

In a 2019 article from the Pasteur Institute, leptospirosis was the only bacterial disease predicted to have increased incidence/prevalence related to climate change across Europe [73]. This can be seen by increases in global flooding destroying crops and increasing rodent populations, leading to outbreaks of leptospirosis [74].

There has been insufficient research in the survival of leptospires in soil. Recent studies have shown soil may have a significant role in the persistence of leptospires in the environment [17, 32]. Leptospires can survive for long periods of time in moist soil with organic matter [75]. Research has demonstrated a higher incidence of leptospires in soil compared to adjacent reservoirs of fresh water [69, 75]. Various soil samples have a greater diversity and concentration

of both non-pathogenic and pathogenic *Leptospira* compared to water [59, 60, 76]. The complexity of soil and extensive growth period of *Leptospira in vitro* has made it difficult to culture the organism, but with the advent of multi-sequencing genomics additional information is being discovered [17]. Genomics has advanced knowledge regarding leptospires in the environment. It is now believed leptospires are retained in the soil and with increased rainfall are flushed into adjacent waterways and standing water [17, 32, 77]. Soil nutrients also may influence the survival of leptospires in the soil. One study demonstrated that soil with high levels of copper, iron and manganese had a higher concentration of *Leptospira* than in soil with lesser concentrations of these elements [78].

Seasonality helps to determine the species detected in the environment. In Chile, *L. kirschneri* was more common in the spring, whereas *L. interrogans* occurred more often in the winter [79]. Leptospiral species prevalence could be due to seasonality changes in wildlife populations. A study in New Caledonia found species changes in the black rat, from *L. interrogans* in the winter season to *L. borgpetersenii* during the hot rainy season [80].

Biofilms in conjunction with climate change may also be important in the survival of *Leptospira* in the environment. The first description of *Leptospira* biofilms was in 2008 [4]. *In vitro* experiments found saprophytic leptospires take 5 days or less to form biofilms, compared to an average of 20 days for pathogens. Additionally, saprophytic leptospires were able to form biofilms at 21, 30 and 37^{o}C whereas pathogenic occurred only at 30 and 37^{o}C, in liquid culture.

The ability of leptospires to form biofilms in nature allows them to survive in a multitude of environments. Studies have shown a communal relationship with other organisms in soil and aquatic biofilms [81-83]. Leptospires were once thought to exist in nature alone however biofilms allow them to exist in a protective environment for longer periods of time [4, 59]. The protection of biofilms increases the time for animals and humans to become exposed to leptospires. *Leptospira* biofilms have also be shown to occur *in vivo* [84, 85]. Further investigations are needed to ascertain the importance of biofilms regarding the pathogenesis of leptospirosis.

1.5 Epidemiology of Leptospirosis

Leptospirosis is found in every continent, except for the earth's polar regions [86]. Detecting and tracking outbreaks is difficult because of the complexity and cost of making a definitive diagnosis of leptospirosis. In addition, clinical presentations vary, physicians may lack suspicion and knowledge regarding the pathogenesis and epidemiology of the disease.

In temperate climates, leptospirosis occurs more often between June and early fall. In the United States and Canada, it occurs more frequently in late summer through January [6, 87, 88]. In the semitropical portions of the United States, an increase of canine leptospirosis if often seen more in late summer and early fall [6]. Outbreaks of leptospirosis increase in warmer temperatures following periods of heavy rainfall and when flooding occurs [11, 15, 89]. Leptospires are unable to replicate outside the host, however they can live and

replicate in invertebrates and insects, though the importance of this as it relates to the transmission of leptospirosis is unknown [6, 90]. Leptospirosis is designated as a reportable disease by the European Union [91]. Because of its ubiquitous nature, surveillance for both human and animal cases of leptospirosis is recommended throughout the world. [20].

There are two categories to describe hosts of leptospirosis, maintenance and accidental [6]. In animals, a maintenance host generally means that the infection is endemic and is transmitted from animal to animal through direct contact [11, 89]. Leptospires are usually acquired at a young age, with lifelong shedding of the organism in the urine common [6]. The degree of shedding tends to increase with age. Rodents, and domestic animals such as dogs, cows and pigs, are considered as key maintenance animals. Some serovars have more than one primary host. However, serovars such as Icterohaemorrhagiae are primarily associated with rats and Hardjo with cattle [92].

Accidental hosts, which includes humans, are infected by indirect contact with the bacteria in water (either through drinking or recreational exposure), soil or through direct contact of urine or reproductive fluids, often during occupational exposure [6] [11, 21].

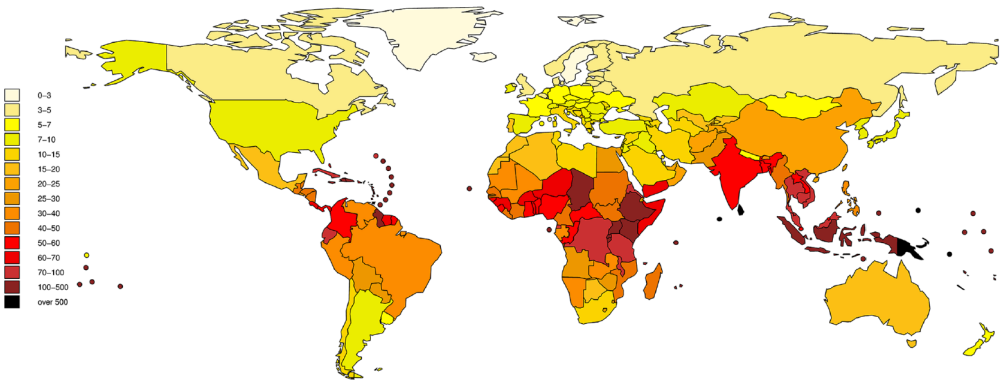
There are various factors that influence the maintenance of leptospires in the environment and the spread of leptospirosis in both humans and animals. It is no surprise that a higher incidence is found in developing countries. Factors such as age, socioeconomic status, access to clean water, crowding, medical care, prior health status, nutrition and lack of diagnostic tools, along with lack of

rapid and accurate laboratory identification all play a role in prevalence and spread of the disease [6]. Many physicians are not well-trained in zoonotic diseases such as leptospirosis and often will not consider leptospirosis in their differential diagnosis list [20]. The incidence of leptospirosis for animals and humans is higher in developing countries with an extensive agricultural base. Whereas in developed countries, agriculture may have a role, but to a lesser extent.

In 2008, an outbreak in Sri Lanka resulted in 3,825 cases (January-August 2008) of leptospirosis in humans with 33 deaths reported in a three-week period [93]. In October 2009, an outbreak of leptospirosis occurred in the Philippines after heavy rains from a tropical storm [94]. There were over 2,000 human cases with 167 deaths within a month after the flooding had occurred. In Ireland there was an increase in human leptospirosis cases, 22 occurring in 2007 and 30 cases in 2008 with 17 hospitalizations and one death [95, 96]. The cases in Ireland occurred primarily through agricultural exposure and participation in watersports. In the previous 5 years there have been 96 postings on ProMed-Mail regarding cases of leptospirosis throughout the world [97]. In 2014, 239 active military members became ill after exposure to pond water on a marine base in Okinawa, Japan [98]. In September 2017 after Hurricane Maria in Puerto Rico, outbreaks occurred in both humans and domestic animals [99]. Increased cases in Puerto Rico are thought to be linked to lack of access to clean drinking water. During the months of June-August 2018 an outbreak occurred in northern Israel [100]. Over 600 people were diagnosed with leptospirosis, and 146

hospitalized with a history of swimming in both natural springs and streams. The global burden of leptospirosis cases in humans in 2015 is depicted in Figure 1.3.

Figure 1.3: **Human Global Burden of Leptospirosis (DALYs*/100,000/year)**



*Disability Adjusted Life Years
[101]

In developed countries, exposure can occur during recreational activities and in urban areas with high rodent populations [6, 21, 102]. Table 1.3 lists several human outbreaks of leptospirosis in the past two decades in the US. Urbanization can lead to an increased prevalence of leptospirosis. In a retrospective study conducted in Indiana 1997-2002, increased prevalence of leptospirosis was detected in dogs in once rural areas which been had become urbanized over the past 10 years [92]. Outbreaks of leptospirosis occurred in Illinois in 1998 after a triathlon and again in 2005 in Florida. The triathlon involved a trek through a marshy area [103]. In one case, an infant contracted the disease after nursing a mother who was infected with leptospirosis. There have also been several cases of transplacental leptospirosis with subsequent abortion in humans [104, 105].

Table 1.3: **Recent Outbreaks of Leptospirosis in Humans in US**

Year	City/Area	Numbers Affected	Possible Cause
1993-1998	Baltimore[1]	3/3 (100%)	Rats
1997-2002	Indiana[2]	19/30 (63.3%)	Multiple exposures
1998	Illinois[3]	52/474 (11%)	Triathlon
1974-1998	Hawaii[4]	353 cases	Multiple exposures
2005	Florida[5]	14/31 (45%)	Triathlon

[1][106]; [2][92]; [3][107]; [4][108]; [5][103]

Humans or animals that have been exposed or have recovered from leptospirosis develop an acquired immune response which can help protect against future infections of the same serovar. Immunity generated by exposure to one serovar is generally not cross-protective for other serovars [6, 7].

Recent studies have shown seroconversion in humans to *Leptospira* positive animals. A study conducted in 2010 demonstrated seroconversion to leptospires by practicing equine veterinarians and horse farm workers located in Central Kentucky for the first time [30]. Veterinary students in Trinidad showed a higher seroconversion to *Leptospira* than non-veterinary students [109]. In New Zealand veterinarians attending a conference volunteered to be tested for leptospiral antibodies. Over 5% of those tested showed a seroconversion [110].

1.6 Clinical Leptospirosis in Humans

Leptospirosis in humans mimic flu or flu-like diseases such as dengue and malaria [15, 35, 111]. Presentation of the disease in humans range from asymptomatic to severe. Typically, symptoms begin within a few days after exposure, but can take up to four weeks to occur [12, 14]. Fever tends to be biphasic and may last up to nine days, followed by several afebrile days before the fever reappears. Once the leptospires enter the body, they soon move into the blood stream and infect multiple organ systems such as liver, spleen, kidneys, central nervous system, genital tract and eyes, causing various lesions [14, 36]. These lesions are thought to be caused by leptospiral toxin(s) or toxic cellular components that damage the endothelium of small blood vessels, triggering localized ischemia in organs. This leads to hepatocellular damage, renal tubular necrosis and myositis. In severe cases hemorrhage can occur along with platelet insufficiency and jaundice.

Leptospirosis manifests itself in two distinct phases. The first phase involves fever, chills, vomiting and/or diarrhea, myalgia and headache. A rash may occur on the skin and palate [14]. Chronic uveitis may also occur typically a year after the initial infection [112]. Symptoms usually abate within several days but may continue for 3 weeks or longer. Relapses can occur and often lead to kidney and/or liver failure, myocarditis, mucosal hemorrhage or meningitis [6, 12, 14]. The second phase is referred to as Weil's disease named after Adolph Weil, a German physician who first described leptospirosis. Mild to severe illnesses in humans are caused by serovars Hardjo, Grippotyphosa or Pomona. However,

serovars Copenhageni and Icterohaemorrhagiae are typically the ones that result in the most severe illness. The severity of the disease depends on the organ systems infected. The serovars that cause illness vary from country to country, making it difficult to develop a generic vaccine to protect humans from leptospirosis. In addition, new serovars can emerge. One study revealed the emergence of new serovars on the islands of American Samoa [113]. These serovars (Hebdomadis, LT 751, LT 1163) are suspected to have caused chronic leptospirosis with systemic illness. Mortality can be as high as 25% in untreated cases within two weeks of symptoms. However, deaths have been reported as early as five days subsequent to initial symptoms in severe cases [114].

In 1994, leptospirosis was removed from the Center for Disease Control (CDC) list of notifiable disease in the United States since incidence in humans had been declining [115, 116]. Furthermore, testing for leptospirosis was not readily available by medical laboratories throughout the US. As a result, many states were not reporting and likely underestimating prevalence. In addition other emerging diseases required placement on the notifiable disease list, taking the place of leptospirosis [117]. In the late 1990s, outbreaks of recreational human leptospirosis in the US prompted the Council of State and Territorial Epidemiologists to reinstate leptospirosis as a notifiable disease in 2012 [20, 108]. Leptospirosis was placed back on CDC list of notifiable diseases in 2013. Leptospirosis outbreaks are posted as part of the National Outbreak Reporting System on the CDC's website [118]. Increased incidence of human leptospirosis outbreaks has been seen over the past several decades, mostly occurring at

recreational lakes [115, 119]. In the 1980s and 1990s outbreaks also occurred in poor metropolitan areas of Detroit and Baltimore [6, 115, 117, 119].

Human leptospirosis is treated using antibiotics such as penicillin, erythromycin or doxycycline, and most effective when given early in the course of the disease [6, 12, 114, 120]. Even though the acute disease can be self-limiting, 5-10% of cases lead to severe illness, thus most physicians initiate treatment with antibiotics to try and clear the infection. Numerous studies have shown the duration of the disease is decreased when antibiotics are administered following the onset of symptoms [6, 114]. Managing symptoms through supportive care such as bed rest, analgesics for headaches and muscle pain, antipyretics for fever and intravenous fluids where indicated, will help in minimizing disease progression, leading to a positive outcome.

1.7 Animals-Epidemiology and Geographic Distribution of Leptospirosis

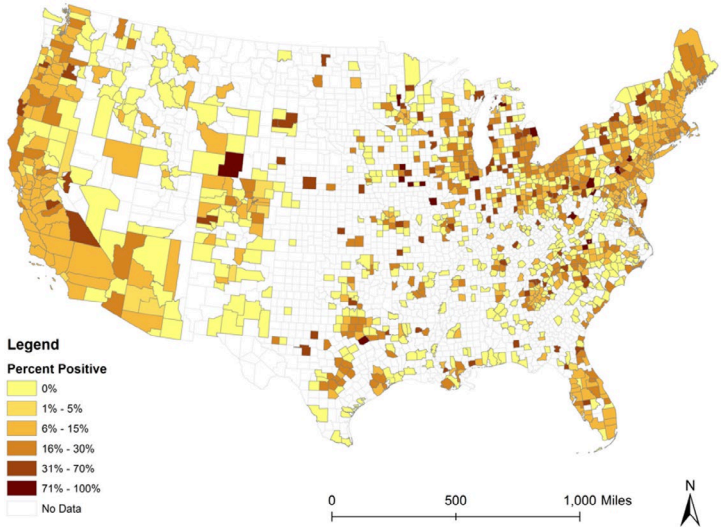
Leptospiral infections in animals present as a peracute, acute, subacute, or chronic infections, with a range of signs from gastrointestinal, respiratory, ocular, reproductive, renal and hepatic [6]. The signs and duration vary dependent on species.

1.7.1 Clinical Leptospirosis in Canines

The classic presentation is vomiting, diarrhea, fever, jaundice, uremia secondary to renal failure, disseminated intravascular coagulation (DIC),

hemorrhage and death [6, 121]. The incidence of canine leptospirosis in the US has increased over the past 25 years [122]. Increased incidence has been reported in Ohio, Florida, Michigan, Texas, Illinois, New York, California, Massachusetts and most recently Utah in 2019. In September 2019, Utah reported over 12 dogs diagnosed with leptospirosis [123]. A study published in 2017 shows the number of positive canine MAT tests in the US between 2000-2014 (Figure 1.4) [122]. Positive MAT tests increased from 8.7% to 12% from 2002 through 2004.

Figure 1.4: **Results of Positive Canine MAT Tests by County in US Between 2000-2014 (Counties without indicated data had no MATs submitted to reference laboratories)***



*[122]

The increase in cases could potentially be due to decreased vaccination for leptospirosis in dogs [124]. In 2011 the American Veterinary Medical Association, (AVMA) reported <4% of dogs had been vaccinated against leptospirosis [122]. Conceivably, with the increased use of dog parks and daycare, this may increase the risk of exposure, especially when dogs are not

routinely being vaccinated. The urbanization of rural areas increases contact of humans, dogs and other domestic animals to wildlife, many of which can shed leptospires into the environment throughout their lives [92].

The combination of climate change, low vaccination rate, and urbanization of rural areas may be increasing the occurrence of leptospirosis in dogs. Asymptomatic chronic carriers may be shedding leptospires in their urine, contaminating the environment [125]. In canines, treatment is supportive care, along with antimicrobial therapy [121]. Penicillin, amoxicillin and doxycycline are the drugs of choice for treating leptospirosis in dogs.

1.7.2 Clinical Leptospirosis in Felines

Clinical leptospirosis occurs infrequently in cats. One study showed 1/172 (0.5%) urine samples positive by PCR [126]. Additional studies have shown a range of positivity by MAT from 0%-9.3% [127-131]. However, positive titers for serovars such as Canicola, Icterohaemorrhagiae, Bataviae, Javanica and Pomona have been seen clinically in cats throughout the world [6, 29, 126, 127, 132, 133]. Identification of leptospires in cats is mainly from kidney and urine samples [6, 42]. Seroprevalence in cats has been reported as high as 18% in the British Isles, Australia 6% and in the US 5% [128, 133]. Shedding of pathogenic leptospires in feline urine also occurs in feral and wild cats, but the role in transmission to humans or other animals is unknown [126, 134]. Transmission from other animals have been shown in two separate studies with the potential transmission of serovar Pomona from dairy cows to a cat [135, 136].

In one study the cat presented with hematuria, the cat's urine was both culture and qPCR positive. The MAT titer was 6400 for both Pomona and Autumnalis. Exposure to rodents is thought to result in increased titers to either serovars Copenhageni or Ballum [135]. Therefore, the increased titers to Pomona and Autumnalis were a result of exposure to domestic animals. Previous work has also shown cats contract serovars Ballum and Copenhageni when exposed to rodents [128]. Research indicates that practicing veterinarians should always consider leptospirosis as a rule-out in cats with renal disease and/or access to the outdoors [135, 137, 138]. Cats have been shown to shed *Leptospira* in their urine but more research is needed to determine shedding frequency and duration [139]. Interestingly cats can have a negative MAT titer but still be shedding *Leptospira* in their urine [128]. Therefore, tests in addition to MAT such as PCR testing for urinary shedding need to be carried out to determine positivity in cats. In addition, cats infected by leptospires can present with no clinical signs. [135, 137]. This appears to be a global phenomenon which may also be due to under reporting of leptospirosis in cats [126, 137-139].

1.7.3 Clinical Leptospirosis in Livestock

1.7.3.1 Ruminants

In 1935, bovine leptospirosis was first reported in Russia [140]. Clinical signs were decreased milk production and abortions. Reports of bovine leptospirosis soon followed in Palestine. Two different serotypes had been identified, in Europe *L. bovis* and in US, *L pomona*, both had also caused illness

in humans. Blood in the milk of dairy cows was frequently seen in mild disease [140]. In the early 1950s it was believed leptospires shed in urine from infected animals was crucial to spreading the disease. Research conducted at the time indicated beef and dairy cattle can shed the bacterium for up to two years [141]. Another study determined cattle can shed *Leptospira* in their urine for only 70 days [142]. Recovery and shedding of *Leptospira* in another study was found to occur for up to 90 days [143]. The first documented case of bovine leptospirosis in the US was in 1947 by Baker and Little and subsequently identified in 1950 by Goehenour and Yager [143]. Up to 70% of US bovine herds were seropositive at this time. The first vaccine was developed in 1953 by Charles York [143]. The introduction of a bacterin and subsequent vaccine in the 1950s was followed by a decline in the incidence of bovine leptospirosis [144, 145].

Serovar Hardjo in bovine and sheep can be transmitted directly through sexual contact, urine exposure, infected placenta or uterine infection and do not seem to be impacted by levels of rainfall and is difficult to control by routine management practices [146]. In ruminants, incidental infections occur more often by indirect transmission by contaminated surfaces, feed and water [146]. Most infections in ruminants tend to be subclinical. However, exceptions to this occur in the very young presenting with meningitis, renal disease, hematuria, jaundice, hemoglobinuria and death [146]. Older animals can also present with these signs, but occurrence is rare. Pregnant or lactating females can present with agalactia or reproductive damage from either a host-maintained or incidental leptospiral infection in both bovine and sheep [146].

Clinical findings in cattle vary with the serovar causing the infection. Abortion caused by Pomona in cattle will occur 1-6 weeks after acute infection whereas serovar Hardjo abortion occurs 4-12 weeks after initial signs [146]. A chronic result of infection by serovar Harjo is infertility, however this can be controlled by vaccinating the herd [146]. In utero leptospiral infections can cause abortions, stillbirths, and weak offspring, mostly caused by serovars Pomona or Hardjo. Abortion is frequently caused by serovar Pomona (up to 50%) compared to serovar Hardjo (3-10%) [146]. When abortions occur, the fetus and placenta typically contain a heavy load of leptospires resulting in risk of exposure to farm workers and veterinarians. Shedding of *Leptospira* in the urine is common and can aid in transmission of the disease to other mammals and humans. Exposure to *Leptospira* in humans and other mammals can occur after contact with stock ponds and slow-moving streams contaminated through urinary shedding of leptospires by cattle. Economic losses due to reproductive failures on cattle and dairy farms, along with decreased milk production can be high [10]. In dairy cows, quality and production of milk can be affected [147]. Financial loss due to human illness can also occur due to lost wages and medical costs. Finally, serovar Hardjo in both bovines and caprines has been shown to persist in the mammary glands [146].

In sheep, leptospirosis is more common in countries with large ovine populations, such as New Zealand and Australia. Although infections in other countries do occur. Leptospiral abortions in sheep and goats generally occur late in gestation [146]. Abortion is mainly caused by serovar Hardjo, although

serovars Pomona, Bratislava, Grippotyphosa, Sejroe, Autumnalis and Ballum have also been implicated [146, 148]. Abortion, infertility, neonatal mortality and reduced milk production are common in small ruminants [148, 149]. Systemic infections pyrexia, anemia and hemorrhagic syndromes are seen, but rarely [148, 149]. Sheep are considered carriers of serovar Hardjo [86, 148]. Leptospiral infections in sheep are still considered rare when compared to bovine and swine, however the seroprevalence appears to be increasing [148]. Ongoing farm management is critical in containment of leptospirosis in small ruminant herds [148]. Isolation of infected animals and timely vaccination are critical. In 2011, 80% of small ruminants were located in developing countries and 70% of these were residing in tropical environments [148]. Management is critical in these areas since leptospirosis is more common in tropics [6, 13, 15, 150]. Increased incidence is seen in the last several weeks of gestation or postpartum in sheep [146]. Occurrence of leptospirosis in goats ranges from 2.6% in Spain to 31% in Brazil [149]. Literature regarding leptospirosis in goats is limited compared to other ruminant species and swine. This could be because losses through production and diseases in goats are not seen as a significant concern in developed countries compared to developing countries [86].

Vaccines are available for bovines (worldwide) and small ruminants in some countries, such as Australia, but not licensed in the US (Appendix One).

1.7.3.2 Swine

Leptospirosis in swine is common. The first reported isolation of *Leptospira* serovar Hardjo in swine was in 1983 [151]. Serovar Hardjo is typically not isolated from swine, however both serovars Bratislava and Pomona are common [9]. Seroprevalence is dependent on location. Leptospires are commonly found in swine all over the world with symptoms ranging from sub-clinical to abortion [152-154].

Leptospirosis in swine occurs worldwide [86, 155]. Reproductive losses caused by serovars Canicola, Pomona, Icterohaemorrhagiae, Grippotyphosa, Bratislava and Tarassovi can cause significant losses through abortion [86]. In young animals, signs such as jaundice, renal failure, hematuria and hemorrhage from acute infection occur and can be fatal [86]. Serovar Pomona is a common cause of infection in young pigs. Leptospirosis has been detected in feral swine throughout the US, presenting a threat to other animals and humans [155]. Serovar Bratislava was the prominent serovar detected in a nationwide study of feral swine [155]. In domestic swine serovar Bratislava is the major cause of reproductive failure in Europe and occurrences in the US are on the increase [86, 156, 157]. The complete genome sequence for serovar Bratislava from a pig (strain PigK151) was published in 2015. Until then, a complete genome sequence had only been completed on human *Leptospira* serovars Lai and Copenhageni [158]. A vaccine with serovars Bratislava, Canicola, Grippotyphosa, Pomona, Hardjo and Icterohaemorrhagiae is available

specifically for breeding swine [159]. A vaccine without serovar Bratislava is also available [160].

The successful treatment of leptospirosis for cattle, sheep and swine involves the use of tetracycline, oxytetracycline, amoxicillin and enrofloxacin [147].

1.7.4 Sea Lions

The occurrence of leptospirosis is not limited to mammals on land. The first report of leptospirosis in sea lions was in 1970 off the coast of California [161]. Only adult males (2-8 years old) were involved with an overall mortality of 40%. An outbreak occurred the same year off the coast of Oregon, with 2% mortality [161]. MAT titers from sea lions beached in California in 1971, showed high titers to Pomona (100,000) and cross-reaction to Autumnalis (10,000). Since 1971, numerous outbreaks have occurred in both sea lions and otters [8, 162-164]. An outbreak in 2004 along the coast from southern California to Washington showed high prevalence of serovar Pomona [163]. A high morbidity (65%) and mortality rate (73%) in the California coast sea lions was detected. A majority of those infected were juveniles (71%) and 92% of infections were in males [163]. The 2004 outbreak occurred throughout the year, with the highest number of cases between July-December. Surveillance of the California coastline continued through 2007. No outbreaks occurred between 2005-2006, however another outbreak happened in 2007 [163]. Morbidity and mortality were 63% and 74% respectively, and 58% were juveniles [8, 163]. Outbreaks in sea

lions off the coast of California have been on the increase with the second largest outbreak occurring in 2018, over 200 were diagnosed with 70% mortality [165].

Clinical signs of illness in the sea lions include fever, emaciation, polydipsia, reluctance to use hind flippers, and dehydration [161, 163, 166]. High mortality occurs every 3-5 years as they migrate along the Pacific coast [8, 166]. Leptospirosis in sea lions appears to be endemic with cyclic outbreaks caused by asymptomatic carriers shedding leptospires and exposing younger animals for long periods of time [166]. Asymptomatic carriers are more common among older adults and have been shown to shed leptospires post-infection for up to 154 days [8]. The cyclic nature of outbreaks is believed to be due to the interaction of birth rates and herd immunity. Where young animals are infected during outbreaks, many become carriers that spread leptospires to incoming young adults for years, and spread with the migration of males to Alaska and back [8]. In addition, there is possible involvement of other animals contributing to the infection in sea lions [163]. The difference in males vs females is likely due to yearly migration north of males, while females and pups stay by the rookeries in Southern California [163, 164]. This also poses a risk of transmission to humans and other animals coming in contact with sea lions.

1.8 History of Leptospirosis in Horses

The first experimentally induced leptospirosis in horses occurred in 1930 after exposure to *Leptospira icterohaemorrhagiae* [167]. In 1948, the first evidence of antibodies to *Leptospira* in horses (22/291, 7.5%) was reported [168,

169]. The first recorded isolation of *Leptospira pomona* by aerobic culture in horses in the United States (US) was achieved in 1952 [170]. In 1953 a horse was diagnosed with leptospirosis after becoming ill, with no other animals on the farm sick nor had increased titers [171]. The source of the *Leptospira* was never found. Prior to 1952, the horse had not yet been considered to be susceptible to leptospirosis, and thus any possible systemic infections were never diagnosed. In addition, abortions in the horse caused by *Leptospira* were also not well defined.

Prior to 1950, leptospirosis in horses was associated with uveitis, not clinical presentations. In 1950s the only evidence linking recurrent uveitis to leptospirosis, was increased titers [172].

Since then, global studies have revealed increased seroprevalence of leptospires in the horse, including the US. The presence of specific serovars varied depending on the location/country and percent positivity. A study in New York showed 30% of horses tested were seropositive to *L. pomona, icterohemorrhagieae*, or *Canicola* [173]. Whereas a study in Illinois showed prevalence to serovars Pomona, Canicola and Grippotyphosa (19.5%, 9.5% and 2.5% respectively) [174]. In 1957 several case studies of leptospirosis were reported in California. Several mares aborted, and serological agglutination was to *Leptospira Pomona,* the organism was never isolated [175].

1.8.1 Equine Leptospirosis

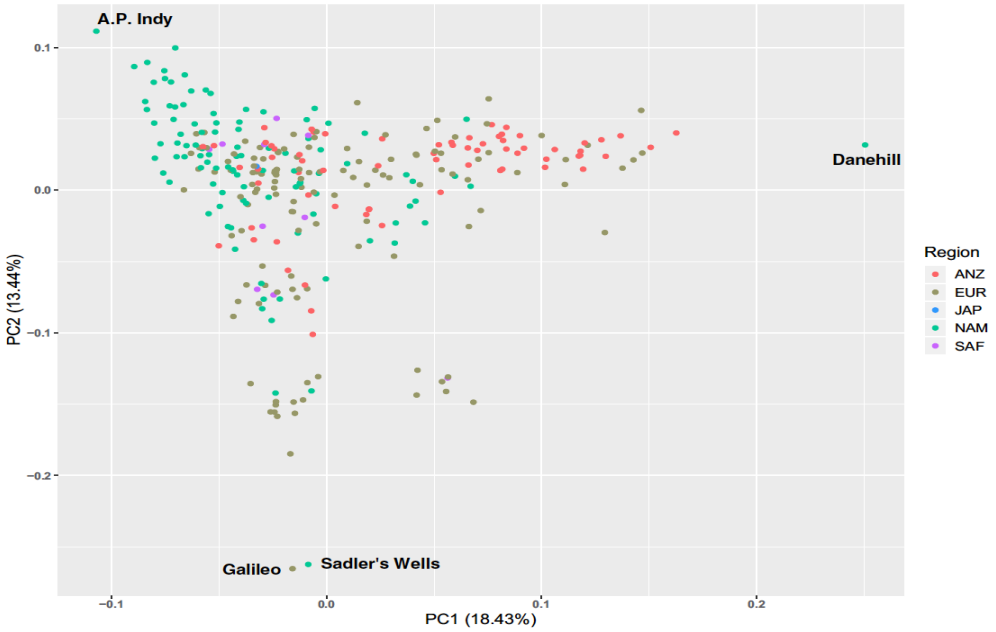
Horses are thought of as incidental hosts for most serovars of leptospirosis, except for *L. interrogans* serovar (sv) Bratislava [121]. *L. interrogans* (sv) Bratislava, has caused abortions in horses, especially in Ireland [86, 176, 177]. A 1998 serological study in New York revealed a high prevalence of both *L. interrogans* (sv) Autumnalis and *L. interrogans* (sv) Bratislava, with a smaller percent of five other serovars occurring in the equine population throughout the state [178, 179]. Serovar Bratislava was first isolated from a hedgehog in 1953. The hedgehog is considered the maintenance host for this serovar in Europe [180]. In swine, Bratislava causes abortion and neonatal death [181]. Swine are considered a maintenance host for this serovar in both Europe and the US. Dogs are also a maintenance host for serovar Bratislava, as both clinical illness and abortion have been seen in that species [86]. Domestic and wild dogs may pose a risk for horse exposure [182]. Exposure and seroprevalence for Bratislava in humans has also occurred [183].

Since the 1980s, horses in Europe have been considered maintenance hosts for serovar Bratislava, although clinical infections have occurred [177, 184]. However, the association of Bratislava with disease in horses is still not conclusive [86, 87, 157, 185, 186]. Serovar Bratislava has never been isolated aerobic culture from horses in the US due to the fastidious nature of this organism [9]. There is evidence that the strains detected by multiple-locus variable-number tandem repeat analysis (MLVA) testing and sequencing among

animals, are not the same (pigs, horses, cattle, sheep, dogs, hedgehogs and sea lions) [157, 187, 188].

Transmission of serovar Bratislava in mammals can occur through exposure to infected urine, placenta, wildlife and possibly via breeding [9, 185, 186]. The differences in clinical syndromes caused by serovar Bratislava in Europe and North America could be affected by equine genetics, strain differences and/or environment. Clinically, Bratislava cases in Europe included abortion, equine recurrent uveitis (ERU), jaundice, fatigue and respiratory distress. However, clinical cases related to serovar Bratislava have not been recognized in North America [177, 185]. Horses are genetically homogeneous. Thoroughbreds from Europe and North America do not cluster together due to differences in stallions used for breeding as seen in Figure 3.1 [189, 190]. Slight genetic diversity may contribute to differences seen between Europe and North America pertaining to serovar Bratislava**.** Environmental pressure to strains of serovar Bratislava may also contribute to these differences. Strains of serovar Bratislava circulating in Ireland and the European continent also differ [187]. In Europe, serovar Bratislava is considered both a non-pathogenic resident and a pathogen in the horse. In the US it is only considered a non-pathogenic resident or commensal organism. Further research is needed to better understand the pathogenesis of these strains.

Figure 1.5: Principal Component Plot of Global Genetic Relatedness in Thoroughbred Horses*



*McGivney et al 2020

In North America, transmission of *Leptospira* to horses is believed to be mainly through contact with wildlife; however, contact with contaminated feed, water and/or soil infected with *Leptospira* does occur. Skunks are thought to be the maintenance host for serovar Kennewicki, Hardjo is maintained by cattle, and Grippotyphosa by raccoons [176]. Additional transmission may occur through semen and mare's milk. Male horses could be involved in the spread of leptospirosis. Both swine and cattle have been shown to pass *Leptospira* through their semen [191, 192]. Stallions have also been shown to harbor leptospiral DNA [186, 193, 194]. *Leptospira* have been detected in the milk of female mammals during lactation in rats, bovine and humans [104, 195, 196]. Thus, another potential point of transmission could be through the mares' milk, though to date there has been no published reports of such transmission.

In horses, leptospirosis has several clinical syndromes. In pregnant mares, abortion, stillbirth or premature foaling is common. In yearlings recurrent uveitis (Moon Blindness) and renal failure are frequent [197]. Other rare systemic clinical manifestations seen in horses are fever, anorexia, lethargy, respiratory distress, encephalitis, jaundice and liver failure. Several studies have reported increases in the seroprevalence within the past twenty years in horses [185, 198-200]. In Sweden, a study looking at 2000 horses over a period of a year found a substantial number were positive for both *L. interrogans* serovars Bratislava and Icterohaemorrhagiae using MAT (16.6% and 8.3% respectively), a majority of titers occurred at 1:100 [185]. A study from Brazil also showed a high seroprevalence of Icterohaemorrhagiae along with *L*. *interrogans* (sv) Castellonis and *L*. *interrogans* (sv) Djasiman [201]. Both, the Swedish and Brazilian studies demonstrated higher titers in older animals vs. yearlings. This has also been observed in other studies [200, 202]. A study conducted in 2011 by Carter et al showed seroprevalence in horses throughout the United States and one Canadian Province with a range from 10% to 30% depending on the region [203]. These studies also showed a high percent of animals with low titers to numerous serovars, which indicates exposure, but not necessarily disease. MAT cross reactivity is common and is caused by the persistence of IgM and IgG antibodies [15, 37]. Persistent titers can remain for years after infection and/or additional exposure without presence of infection. Unless blood titers are checked repeatedly throughout the year, the chance of detecting a highly significant titer could be elusive. A survey of veterinary diagnostic laboratories in the United

States was conducted to determine the number of cases, serovars and abortions seen in both horses and cattle [204]. Test results indicated a much higher detection rate in cattle compared to horses except for one laboratory. In horses, penicillin plus dihydrostreptomycin has been used effectively to treat leptospirosis [176].

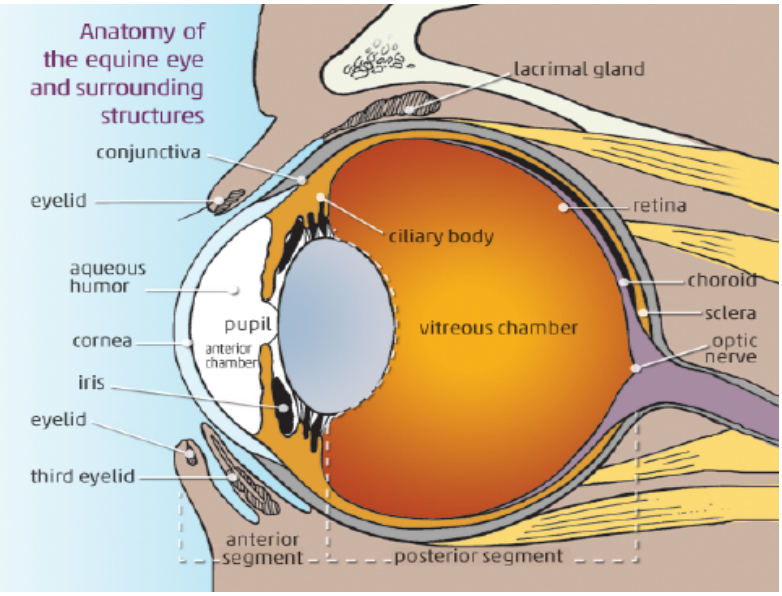
1.8.2 Equine Recurrent Uveitis

Chronic uveitis (aka moon blindness or equine recurrent uveitis) caused by leptospires is a common problem and is one of the leading causes of blindness in horses [2, 185, 198, 202, 205]. Uveitis is inflammation of the vascular tissue in the eye, specifically the iris, ciliary body (anterior uvea) and choroid (posterior uvea) [112, 206, 207]. These are part of the uveal tract (Figure 1.6). Between the peripheral vasculature and the inner structure of the eye, there are two barriers (blood/aqueous and blood/retinal barriers) to prevent cells and larger molecules from entering the eye. The blood/aqueous barrier consists of the iris and ciliary body barrier and blood/retinal barrier comprises the choroid [112]. The barriers allow the eye to be immune privileged (limiting an immune response), until one or both of those barriers are disrupted. Disruption of one or both barriers allow leakage through the vasculature causing an immune response, leading to uveitis. The eye is also protected from an immune response through decreased antigen presenting cells and a lack of lymphatics [112]. Without these protections, a mounted immune response could lead to

irreversible damage. The theory that ERU is caused by an immune mediated response, first appeared in the literature in the 1980s [208-210].

Uveitis can be caused by trauma, infection (either local or systemic), neoplasia or can be idiopathic [206, 211]. Uveitis can either be acute with sudden onset, chronic (never healed) or recurrent, which is referred to as ERU.

Figure 1.6: Anatomy of the Equine Eye



[212]

There are three forms of ERU. The first form comprises an intraocular inflammatory process which reoccurs months after the initial diagnosis of uveitis, and with each occurrence becomes more severe [112, 206]. This is considered the classic form of ERU. The second form of ERU is called insidious and is typically seen in Appaloosas horses [206]. This includes a low-grade inflammatory process which persists with no outward signs, although damage to the eye continues. The third type is posterior and is seen more in Warmblood horses. Inflammation is mainly in the vitreous, retina and choroid [211]. ERU is

more common in the US (2-25%), and some European countries (8-10%) in the general equine population. However, occurrence in Britain (0.3%) is much lower when compared to other countries [2, 206, 213]. Reasons for discrepancies could be due to the type of breeds and/or genetics of the horses in Britain compared to other countries. Environmental factors may also contribute to the differences. Numerous studies have shown a predilection of ERU to occur more frequently in Appaloosas and Warmblood horses compared to other breeds [2, 214, 215]. In both Appaloosas and German Warmblood horses, genetic risk factors have shown to contribute to a higher percent of ERU. Additionally, these two breeds show an association with the MHC-I haplotype ELA-A9 and ERU suggesting a genetic component [216-218].

The contributing causal factors of ERU are elusive. However, in the mid-twentieth century a correlation between ERU and leptospirosis was proposed. There was no concrete evidence showing a relationship between the two and why, until recently [172, 219].

The suspicion that ERU was related to *Leptospira* was first suggested in the 1940s and later substantiated in the 1950s [169, 170, 220]. In one study, horses with recurrent iridocyclitis (ERU) had 85% (223/263) agglutinating antibodies to *Leptospira*. Initial reports indicated *L. pomona* as the principal cause of both leptospirosis and ERU. However, serovars Canicola and Icterohaemorrhagiae have also been associated with both leptospiral infections and uveitis [173].

Currently, leptospirosis is considered the primary bacterial infection leading to ERU [112, 221]. Uveitis can occur early after a few months or years after leptospiral infection [220, 222, 223]. Serovar Pomona is still considered the most common cause of ERU. Serovars Grippotyphosa and Bratislava have recently been implicated [224, 225].

In 1985 antigenic correlations between the cornea and *Leptospira* were discovered [210]. In 2005, two novel lipoproteins were localized in pathogenic *Leptospira interrogans,* but not in saprophytic *Leptospira*. Both reacted with the ocular components of horses, signifying a role in equine uveitis [205]. These two lipoproteins, referred to as leptospiral recurrent uveitis associated proteins A and B (LruA and LruB) were identified in the inner membrane of only pathogenic *Leptospira*. Increased levels of both IgA and IgG to both LruA and LruB were detected in fluid from horses with uveitis but not in normal eyes (controls) [205]. Furthering the belief that uveitis was triggered by an immune response, the study showed an antisera specific reaction by ocular components to both LruA and LruB. Both LruA and LruB have also been found in humans with leptospiral induced uveitis [213, 226]. A study published in 2010 also demonstrated the relationship of *Leptospira* to ERU [224]. An additional novel protein was also discovered, leptospiral recurrent uveitis associated protein (LruC) and published in 2012. LruC is also considered to be associated with ERU [227].

Vimentin, α-crystallin B and β-crystallin B2 have been shown to be critical for preserving lens clarity. LruA was found to be associated with both vimentin and α-crystallin B when they cross-reacted with LruA antiserum [224]. LruB

antiserum was found to cross-react with β-crystallin B2. In one study, eye fluid was collected from ERU eyes which contained significantly higher levels of antibodies recognizing vimentin, α-crystallin B and β-crystallin B2, when compared to controls [224]. This study further demonstrates the immune relevant reactions occurring following a leptospiral infection and subsequent ERU.

For a localized antibody reaction in the eye to *Leptospira* to be conclusive, there has to be 4x the MAT titer in the eye compared to MAT serum titers. This is referred to as the Goldmann-Whitmer coefficient (C-value) [228]. A titer 4x higher in the eye fluid, implicates a leptospiral induced uveitis has occurred. Blindness may not occur for months or even years after infection with leptospirosis. Treatment with either doxycycline or tetracycline for one month may minimize uveitis but rarely will prevent the progression to blindness [229].

In Kentucky, the occurrence of leptospirosis in horses has been well documented, especially in the past several decades, with over 25 articles compared to less than 20 in the remainder of the US during the same timeframe. The most common serovar found in Kentucky is Kennewicki (serogroup Pomona), however the occurrence of several other serovars is not uncommon [87, 230]. Interestingly, for the first time in 30 years, the University of Kentucky Veterinary Diagnostic Laboratory 2019-2020 did not diagnose any leptospiral abortions in horses. The first vaccine licensed for horses (https://www.zoetisus.com/products/horses/lepto-eq-innovator/index.aspx) which

came on the market in 2015 may have had a role in the decline of cases, especially in the Central Kentucky Bluegrass region.

Prevention of leptospirosis is multi-faceted. Prevention in animals includes wildlife control, avoiding feed contamination, clean water, vaccination, isolation of sick animals and proper farm management practices. In humans, early identification and monitoring of outbreaks and occurrences, hygienic practices in the workplace, avoidance of wading or swimming in water contaminated by animal urine will also help decrease the occurrence of leptospirosis. Improved awareness by physicians of the symptoms and risks especially in high-risk areas assist in diagnosis and prevention. Combined, all can help facilitate the prevention of leptospirosis.

The studies designed for this dissertation were to answer several different questions pertaining to equine leptospirosis. To address the first question in Chapter Two, testing methods offered by laboratories of the American Association of Veterinary Laboratory Diagnosticians for leptospirosis were examined. A survey was designed to understand if there is enough testing capacity offered and the types of testing offered by veterinary diagnostic laboratories in the United States for leptospirosis. In Chapter Three the seroprevalence of leptospirosis in Central Kentucky was investigated in the general equine population submitted for necropsy regardless of reason for submission. The aim of this study was to determine the percent positivity in kidneys, vitreous humor and urine by real-time PCR; seroprevalence of serovars Pomona, Grippotyphosa and Bratislava; and the effect of age and gender on

seropositivity. Chapter Four investigated the effects of pregnancy and/or infection on the levels of heme oxygenase-1 (HO-1) in serum of horses. The aims of the study were to determine if pregnancy and/or infection (specifically leptospirosis) upregulates HO-1 in the same manner seen in other mammals, and if HO-1 is affected more by pregnancy compared to infection.

Chapter Two

Survey of Leptospiral Testing Capabilities in the US by Veterinary Diagnostic Laboratories

2.1 Introduction

The Centers for Disease Control and Prevention defines a zoonosis as a *disease spread between animals and people.* Table 2.1 illustrates some recent outbreaks of zoonotic diseases in the world. One of the most notable zoonotic disease pandemics in history, COVID-19, is occurring at the time of this writing and was declared a global health emergency in January 2020 by the World Health Organization (WHO). Caused by Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2), this agent has led to a global public health crisis, causing high morbidity and mortality in humans. At least 6 of 10 infectious diseases in humans originate in animals and 3 out of 4 emerging diseases are zoonotic [61, 231]. As a result, zoonotic disease prevention and management is one of the highest priorities of public agencies throughout the globe.

Table 2.1: Zoonotic Outbreaks*

Disease	Vector	Year	Country	Vaccine
Ebola	Fruit Bat; nonhuman primate	1976; 2014-2016; ongoing	Africa	Trials
Salmonella	Mammals Turtles	2006-present	Global	No
Yellow Fever	Primates to mosquitoes to humans	1647 (1st outbreak) 2000-2020	Africa and South America	Yes
Lassa Fever	*Mastomys* rodents	1969	Africa	Yes
H1N1 (swine flu)	Swine	2009	Global	No
SARS	Possibly bats and/or civet cats	2003-2004 2020 to present	China, Japan, Hong Kong, Taiwan, Canada	No
MERS-CoV	Dromedary camels	2012-2019	United Kingdom, Ireland, Austria, Turkey, Middle East (Saudi Arabia, Qatar, UAE, Oman, Bahrain)	No
SARS CoV-2	Unknown Bats?	2019 to present	Worldwide	Yes

*[232]

A One Health approach, with full integration of animal health, human health, and the environment is needed to aid in the prevention of future outbreaks and pandemics and the related economic losses. In the case of food production animals, a One Health approach aids in the production of a wholesome and plentiful food supply and reduces economic losses related to morbidity and mortality [233]. Optimal animal health is realized through effective

vaccination programs for diseases such as brucellosis, leptospirosis, rabies and many other diseases. Vaccination affords protection against leptospiral infections. However, because of serovar diversity worldwide, a vaccine to protect against all pathogenic serovars in all species is currently not feasible. The large number of pathogenic *Leptospira* serovars (over 300) throughout the world, along with vaccine development and side effects, are reasons for the implausibility of one vaccine for a single species.

Good communication and coordination among human health, animal health, and environmental health professionals' disciplines can help prevent future outbreaks of serious diseases. For example, a 2005 Q-fever outbreak in the Netherlands could have been largely avoided through better communication, decreasing economic losses and human illnesses [233]. Partnerships formed by members of the allied health professions have enabled strong surveillance for West Nile Virus, saving human and animal lives and conserving precious funds [233]. It is hoped that the One Health philosophy will continue to grow and thrive to serve public health, animal health, and environmental health.

In the US prior to the 1990s, leptospirosis was sporadic in animals. However, in the past 30 years, the incidence of clinical leptospirosis has risen, especially in dogs [88, 122, 234, 235]. Since the 1950s, many peer-reviewed scientific articles have reported leptospiral seroprevalence in equine and bovine populations [30, 140, 170, 199, 200, 203].

There is a wide range of seroprevalence in horses based on location of premises. A 2009 study conducted in Brazil showed a low seropositivity (8%) from 1200 equine serum samples [236]. A study in 2013-2014 conducted in Ethiopia found that the location and age of cart horses made a difference in seroprevalence [237]. Ethiopia has the largest equine population in Africa, estimated at 1.91 million and an even larger donkey population (6.75 million) [237]. Depending on the region in Ethiopia, 184/418 seropositive horses (titers≥ 100) ranged from 39.8% to 62.1%. In Europe, the seroprevalence has a broad range, from 1.5% in Italy to 72.2% in Ireland [238]. Another study published in 2016 found a seroprevalence of over 29% of 747 serum samples from horses in Italy [239]. A study in Ireland found overall seroprevalence of 72.2% in horses with serovar Bratislava the highest (59.1%) [177]. A complete list of serovars detected in the above studies is listed in Table 2.2. The differences in seroprevalence among these studies could be due to location of the horses not only between countries, but also within a country and sample size. This also may be true for the differences in the US. A study published in 2012 showed the highest seroprevalence in the Western and Southeastern sections of the US to serovar Bratislava [203]. Two recent studies showed increased seroprevalence to serogroups Australis and Grippotyphosa in Kansas and serovars Bratislava and Canicola in Colorado [199, 200].

Table 2.2 Equine *Leptospira* Seroprevalence in Different Countries*

Paper	Year of Study	Country	Species	Serovars Detected*	Seroprevalence range of *Leptospira* spp.
Siqueira et al; 2019	2009-2010	Brazil	Equine	C, B, H, P, He, Pa, V, Ca, A, J, W, Py, Br	2.1% (Autumnalis-serogroup) to 53.6% (Bratislava-serovar)
Tsegay et al; 2016	2013-2014	Ethiopia	Equine	Br, D, To, Pom, M, G, Ar, Bu, K, Ca, Cy, J, B, Z, He, H, Au Ce I, Sz, Pa, R, Sh, Ta	0.2% (Hebdomadis-serovar) to 34.5% (Bratislava-serovar)
Tagliabue et al; 2016	2010-2011	Italy	Equine	Br, C, I, Gr	2.9% (Grippotyphosa-serovar) to 78.5% (Bratislava-serovar)
Ellis et al; 1983	1979-1980	Ireland	Equine	Br, H, C, Py, Ba, Ca	1.7% (Canicola-serovar) to 59.1 (Bratislava-serovar)

* A=Autumnalis; Au=Australis; Ar=Arboreae; B=Bataviae; Br=Bratislava; Bu=Bulgarica; Ca=Canicola; Ce=Celledoni; C=Copenhageni; Cy=Cynopteri; D=Djasiman; G=Grippotyphosa; H=Hardjobovis; He=Hebdomadis; I=Icterohaemorrhagiae; J=Javanica; K=Kremastos; M-Medanensis; Pa=Pana; P=Pomona; Py=Pyrogenes; R=Robinsoni; Sh=Shermani; Sz=Szwajizak; Ta=Tarassovi; To=Topaz; V= Valbuzzi; Z=Zanon

In many areas of the world, testing for *Leptospira* is not performed, negating surveillance in areas where leptospirosis may be emerging and/or prevalent. Diagnostic monitoring for the presence of leptospirosis in animals is critical to limit the spread of the diseases to both animals and humans. The aim of the survey done for this dissertation was to assess the availability of leptospirosis testing offered by member laboratories of the American Association of Veterinary Laboratory Diagnosticians (AAVLD), the extent they are utilized, and to better understand if there is enough testing capacity offered by these laboratories.

2.2 Materials and Methods

2.2.1 Survey

A survey was sent to 60 AAVLD laboratory directors in early October 2015. The survey requested the laboratories to report all the leptospirosis test methods offered (e.g., MAT, PCR, FA), serovars measured, and the average number of both equine and bovine cases per year (e.g., abortions, uveitis, chronic renal or systemic leptospirosis) (Appendix 3).

2.2.2 Statistics

Descriptive statistics (sample size, averages, percent) and graphs were determined using Microsoft Office Excel 2016 for MAC.

2.3 Results

Seventeen of the 60 AAVLD/NAHLN laboratories (28%) responded to the survey. Only 13/17 (76.5%) of responding laboratories offered one or more tests for leptospirosis. One laboratory had discontinued testing in the past year due to lack of submissions and interest. Table 2.3 includes the laboratories and serovars offered by the responding laboratories in the study. A current listing and map of all AAVLD accredited laboratories and leptospiral tests available are in Appendix 4 and 5 respectively. Table 2.4 includes the number of laboratories testing for the following serovars by MAT: Pomona, Hardjo, Icterohaemorrhagiae, Grippotyphosa, Canicola, Bratislava, Autumnalis, Sejroe and Copenhageni. Responses from the 17 laboratories indicate 76.5% are testing for similar serovars. The serovars commonly analyzed by MAT were Pomona, Hardjo, Icterohaemorrhagiae, Grippotyphosa, Canicola, and Bratislava. Serovar Bratislava was tested in 10/13 laboratories (76.9%). One laboratory tested for Bratislava specifically in bovine samples only.

To determine whether *Leptospira* was the cause of an abortion in either horses or cattle, 10/17 (58.8%) utilized PCR while 4/17 (23.5%) ran FA tests. Regarding the diagnosis of Equine Recurrent Uveitis, 7/17 (41.2%) used PCR and/or MAT for analysis. Ten of the seventeen respondents (58.8%) do not test eye fluid for the presence of leptospires.

A total of 10,376 bovine samples from 13 laboratories were submitted for *Leptospira* MAT testing. The range of bovine cases submitted for testing was 10-3774/year (Table 2.5 and Figure 2.1). A total of 4415 equine samples were

tested, the lowest in one state was 1/year and highest was 2731/year. The average number of abortions caused by *Leptospira* in bovine was 2.4/year and in equine 1/year (Figure 2.2).

Table 2.3: MAT Serovars Offered by Responding Laboratories

Laboratory	MAT Serovars Offered-Equine and/or Bovine
1	Pomona, Hardjo, Icterohaemorrhagiae, Grippotyphosa, Canicola, Autumnalis, Bratislava
2	Icterohaemorrhagiae, Hardjo, Pomona, Grippotyphosa, Canicola, Bratislava
3	Pomona, Icterohaemorrhagiae, Canicola, Grippotyphosa, Hardjoprajitno, Bratislava, Sejroe
4	Bratislava, Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, Pomona
5	Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, Pomona, Bratislava
6	Bratislava, Canicola, Pomona, Grippotyphosa, Icterohaemorrhagiae, Hardjo
7	Autumnalis, Bratislava, Canicola, Grippotyphosa, Icterohaemorrhagiae, Hardjo, Pomona
8	Not Offered
9	Bratislava, Canicola, Grippotyphosa, Hardjo, Copenhageni, Pomona
10	Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, Pomona
11	Grippotyphosa, Hardjo, Icterohaemorrhagiae, Pomona, Sejroe (Canicola and Bratislava also included for Bovine panel)
12	Bratislava, Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, Pomona
13	Canicola, Autumnalis, Bratislava, Hardjo, Icterohaemorrhagiae, Pomona, Grippotyphosa
14	Not Offered
15	Not Offered
16	Discontinued
17	Grippotyphosa, Pomona, Hardjo, Icterohaemorrhagiae, Canicola

Table 2.4: Laboratory Analysis of Serovars by MAT*

Serovar	Number of Laboratories Testing
Pomona	13/17 (76.5%)
Hardjo	13/17 (76.5%)
Icterohaemorrhagiae	13/17 (76.5%)
Grippotyphosa	13/17 (76.5%)
Canicola	13/17 (76.5%)
Bratislava	13/17 (76.5%)
Autumnalis	3/17 (17.7%)
Sejroe	2/17 (11.8%)
Copenhageni	1/17 (5.9%)

*Microagglutination Test

PCR targeting pathogenic *Leptospira* was the principal test used to determine leptospiral abortions. Of those labs responding to the survey, 25% of laboratories do not test for leptospirosis and 62% did not test eye fluid by PCR.

Table 2.5: Total Number of Samples-Average Bovine and Equine (2014/2015)

Laboratory (previous number)	MAT Diagnostic Test	Total Samples/year	Bovine Samples/year	Equine Samples/year
1	Yes	4259	3211	1048
2	Yes	1346	1165	181
3	Yes	3992	3774	218
4	Yes	271	235	36
5	Yes	160	150	10
6	Yes	260	250	10
7	Yes	110	92	18
8	Yes	24	10	14
9	Yes	583	505	78
10	Yes	123	76	47
11	Yes	689	688	1
12	Yes	85	62	23
13 (17)	Yes	2889	158	2731
14 (8)	No			
15 (14)	No			
16 (15)	No			
17 (16)	Discontinued			

Figure 2.1: Average MAT Bovine and Equine Leptospiral Submissions (2014/2015)

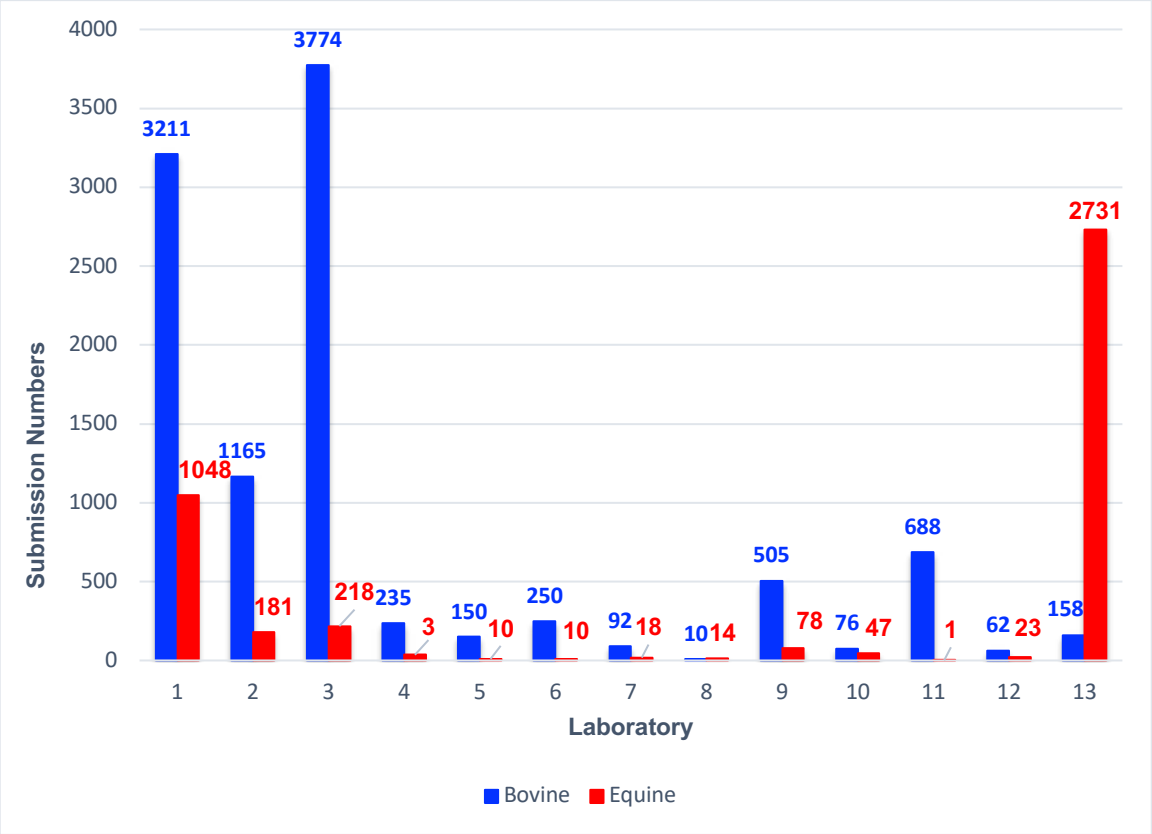
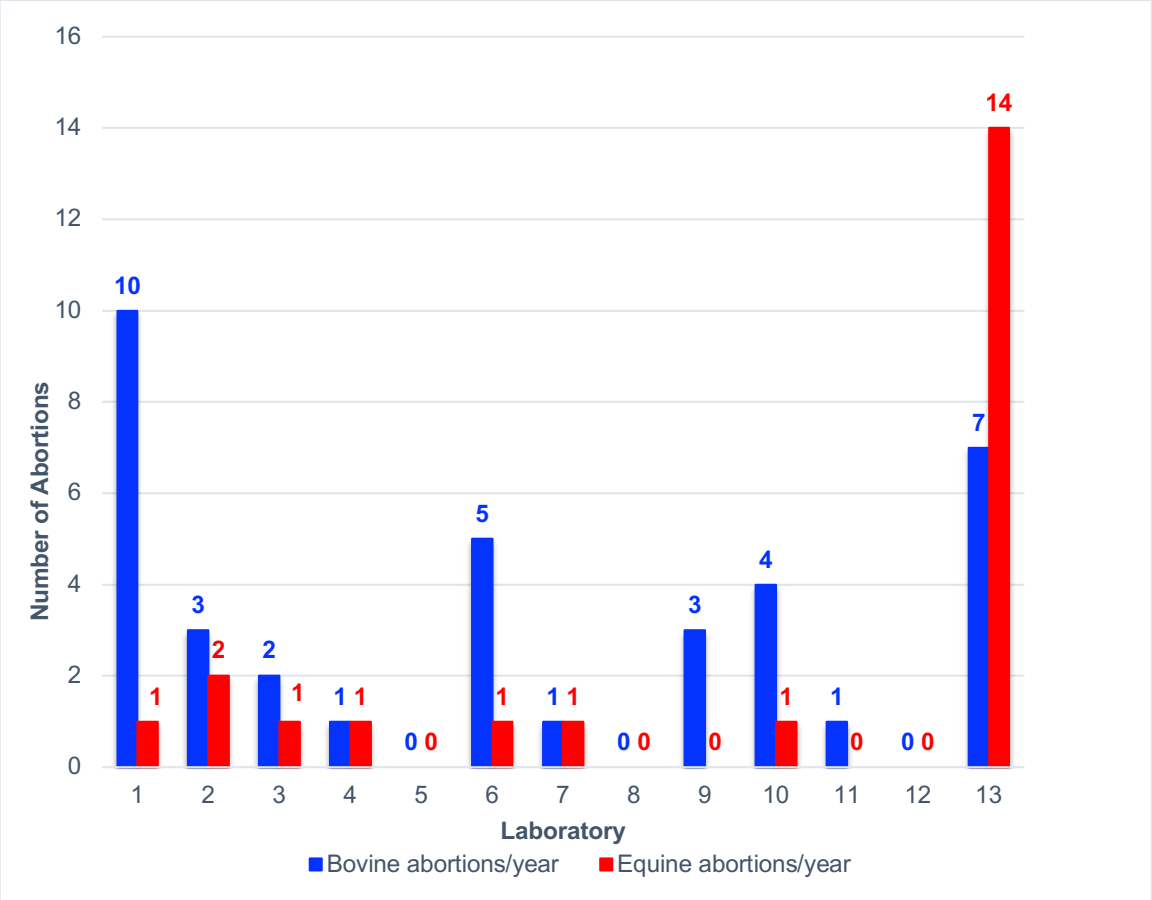


Figure 2.2: Average Bovine and Equine Leptospiral Abortions (2014/2015)



2.4 Discussion

Only 28% of AAVLD accredited laboratories responded to the survey. The low number of respondents makes it difficult to determine the extent of leptospiral testing throughout the US. A high percent of the responding laboratories tested for serovar Bratislava. Previous US studies have shown a high local seroprevalence for serovar Bratislava in horses [199, 203]. One study showed a seroprevalence of 30.5% for titers≥100 where another study showed 31.6% with titers≥ 200 to serovar Bratislava [199, 203]. A high seroprevalence does not

necessarily translate to either clinical disease or abortion in horses, Bratislava is rarely reported as a pathogenic serovar [86]. However, it has been identified by MAT in horses with clinical infections and MAT and culture in aborted fetuses [86, 184]. Bovine seroprevalence of serovar Bratislava is infrequently reported and ranges from <1% to ~23% [240-243]. There are few reports in the US of seroprevalence to Bratislava in cattle [243, 244]. However, abortions in seropositive bovines in Spain and Brazil have occurred [240, 242, 245]. A 1997 study in Spain found 25.4% of cattle tested had a seroprevalence to Bratislava, with 9.7% aborting from 32 farms [240]. The study in Brazil found 15.74% seroprevalence to Bratislava in cattle from 15 farms [242].

Four of the laboratories responding to the survey did not offer leptospirosis testing. Presently, three laboratories are now offering MAT and PCR testing. The decision to begin offering leptospirosis testing at a veterinary diagnostic laboratory may be driven by evidence of increased leptospiral prevalence in the US and across the globe. From January 2014 through October 2020, there were 114 ProMed-mail reports of leptospirosis, 50 of which were animal outbreaks (https://promedmail.org/promed-posts/). During this same timeframe, 20 reports of leptospirosis outbreaks in animals occurred in the US. Between January 2010-January 2015 there were only 6 reports in ProMed-mail of leptospirosis in animals in the US while the number of reports in the world increased by 33.3% and in the US 70%. There appears to be an increase in reporting of animal leptospirosis in the United States and globally. However, this may not represent

an increase in the incidence of clinical human or animal leptospirosis geographically.

A high percent (77%) of laboratories that responded to the survey offer testing for *Leptospira* by PCR. A small percent of laboratories tested eye fluid for *Leptospira*, therefore many cases of Equine Recurrent Uveitis may remain undiagnosed. Most diagnostic testing for uveitis is likely done pre-mortem at veterinary clinics and hospitals, rather than on postmortem examinations (personal communication-Dr. Craig Carter, UKVDL).

Samples submitted to veterinary diagnostic laboratories are not random and do not reflect a true incidence of a disease confirmed in specimens sent from the field. Practicing veterinarians submit specimens to veterinary diagnostic laboratories and request testing for leptospirosis because of clinical suspicion as a possible cause of morbidity or morbidity in an animal or group of animals. Furthermore, the risk of exposure to *Leptospira* by veterinarians and animal owners may not be considered and may be overlooked. Considering the low numbers of leptospiral testing in Figures 4 and 5, it appears laboratories that do offer testing services for leptospirosis are being underutilized. In addition, veterinarians and animal owners may decide not to request a necropsy on animals dying on the farm for economic reasons or lack of curiosity.

There are 60 AAVLD laboratories in various states throughout the nation, with some states operating multiple laboratories). Every state except Idaho, Nevada, Alaska, Rhode Island, Massachusetts and Maine have an AAVLD laboratory. Two states refer leptospiral testing out of state. Seven states (14%)

do not run MAT titer. Since 12% of the states do not test for leptospirosis there is a likelihood that some clinical cases are not detected. Current information reported from ProMED-mail and journal articles, indicates cases of leptospirosis have increased over the past ten years in both humans and animals [73, 150, 246].

It is currently accepted that climate change can increase the risk of future pandemics [63, 64]. Climate change has a role in emerging a re-emerging zoonotic pathogens which can lead to high morbidity and mortality in both animals and humans. This is also true for leptospirosis as new serovars are emerging and infections with increased incidence in both animals and humans [234, 246]. There is a critical need for more veterinary diagnostic laboratories to offer high quality and timely testing for leptospirosis throughout the US. When animals present with clinical signs of leptospirosis, it is important that the disease be ruled-in or ruled-out in a laboratory, with follow-up to be sure that humans exposed to the animal(s) are not also infected. A follow-up study is recommended to confirm the total availability of leptospirosis testing in the US, including commercial laboratories, to assure the timely and accurate identification of *Leptospira*-related disease in both animals and humans.

This study and other recent studies suggest a lack of reporting positive titers and/or diagnoses for clinical leptospirosis around the US. This is likely due to lack of awareness of those laboratories that offer testing or the lack of availability of high-quality testing offered by diagnostic laboratories. This can also be explained by lack of clinical suspicion of leptospirosis by producers,

practicing veterinarians, and diagnostic laboratory staff [29, 199, 244, 247]. Finally, both veterinary and human laboratories should work closely to aggregate data on confirmed cases of leptospirosis to provide much needed medical situational awareness of this widespread disease which can cause severe illness and can be fatal [248]. To be sure, increased testing and diagnosis of leptospirosis in clinical veterinary medicine would likely translate to improved animal health, public health, in the spirit of One Health.

Chapter Three

Assessment of the Carrier State of *Leptospira* in 500 Juvenile and Adult Horses in the Bluegrass Region of Kentucky

3.1 Introduction

Leptospirosis has been identified on every continent except Antarctica [14]. Leptospirosis can affect mammals, insects and birds [6, 26]. Leptospirosis in livestock is common and can be linked to leptospirosis in horses. Horses grazing pastures once occupied by livestock can pose a risk of exposure to *Leptospira*. Serovar Hardjo is the primary serovar in both bovine and ovine in the US but has rarely been a problem in horses. However, transmission of other serovars such as Bratislava can occur [249-251].

In Central Kentucky seroprevalence of Bratislava has been detected since the early 1990s [252, 253]. Serovars Bratislava and Grippotyphosa have been shown to cause abortion, but serogroup Pomona serovar Kennewicki is currently the predominant agent causing abortion in Kentucky [87, 253]. In Kentucky, serovar Bratislava is not included in the panels that are routinely requested by clients at the University of Kentucky Veterinary Diagnostic Laboratory (UKVDL) (personal communication-Dr. Craig Carter, UKVDL).

One of the objectives of this study was to determine the seroprevalence of Bratislava in the general equine population. Leptospirosis serology testing in Central Kentucky is run mainly on pregnant mares to monitor titer levels in their herds. In Central Kentucky many farms begin MAT surveillance in October,

every two to four weeks through March until all mares foal (personal communication-Dr. Craig Carter, UKVDL). Many equine veterinarians practicing in the Bluegrass Region of Kentucky use these titers to decide whether to provide prophylactic antibiotic treatment for mares to prevent abortion (personal communication-Dr. Stuart Brown, formerly of Hagyard Equine Medical Institute). Unless non-pregnant or male horses are sick, monitoring MAT titers are not routinely done. Therefore, it is essential to determine the prevalence of serovar Bratislava to help to understand the role is may play regarding clinical disease in horses. Because of the widespread seroprevalence of serovar Bratislava, it is important to determine the risk of exposure and potential of illness in mammals, including humans.

More than 90 peer-reviewed articles have been published in the scientific literature over the past 10 years investigating leptospiral seroprevalence in horses. To date there have been no studies published evaluating the postmortem *Leptospira* carrier state in horses. Previous papers have focused on necropsy studies in aborted fetuses but not in juvenile and/or adult horses. Papers published in the past 40 years studying the causes of abortion vary depending on the locale. Over 45 of the previous studies were undertaken in Kentucky and the British Isles. Donahue et al (1991) published the first extensive article on equine leptospirosis in Kentucky from a diagnostic laboratory perspective [252]. The study found 15/584 (2.5%) equine abortions/stillbirths over a one-year period were due to leptospirosis. A paper in 1993 investigated two foaling seasons (1988 and 1989) in Kentucky [254]. Over 1200 aborted

fetuses, placentas and stillborn foals were examined to determine the etiology ranging from infectious disease, twinning and/or dystocia. More than 40% were aborted after 300 days. Abortions in mares with placentitis occurred on average, at day 276 [254]. Out of the number of abortions due to placentitis, 37 of 236 (15.67%) were caused specifically by *Leptospira* [255]. This was the first evidence of *Leptospira spp*. causing an increased number of abortions in Kentucky. Another study examined fetuses, stillborn foals and placentas over a five year period [253]. A total of 71/2945 (2.4%) abortions caused by *Leptospira* were identified. Serovar Pomona was detected more often 40/47 (85.1%) in fetuses. Serovars Bratislava and Grippotyphosa were detected in 2 and 4 aborted fetuses respectively.

The primary aim of this study is to determine the carrier state of *Leptospira* in horses submitted to the University of Kentucky Veterinary Diagnostic Laboratory for necropsy. Another aim of this study was to ascertain the prevalence of leptospires in heart blood, kidney, vitreous humor and urine by both MAT and qPCR. An additional aim was to determine if age, gender and reason for submission has a role in seroprevalence.

3.2 Materials and Methods

3.2.1 Study Design

Kidney, vitreous humor, heart blood and urine (when available) samples were collected from 500 horses (6 months or older) brought to the UKVDL for necropsy. Horses were enrolled within 24 hours after either euthanasia or death.

Study candidates were not excluded based on sex, status of pregnancy, age or breed. Clinical, diagnostic and vaccination history for each horse was captured from the accession form and final diagnostic reports when available. Necropsies were performed by board certified veterinary pathologists.

3.2.2 Sample Collection and Processing

3.2.2.1 Heart Blood

Heart blood was collected from either the left or right ventricle utilizing a 16-gauge needle, placed in a sterile container, centrifuged and serum was removed. Serum was stored at 4C prior to analysis. Leptospiral antibody titers for serovars Pomona, Grippotyphosa and Bratislava were measured by MAT. MAT was performed in accordance with the methodology of the National Veterinary Services Laboratories (Ames, IA) and the OIE (World Organization for Animal Health, 6th edition) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Procedure. The reference antigens were propagated in polysorbate-80 bovine albumin media for 4-8 days and diluted to working strength on the day of the test. Serum was screened at a 1:50 dilution (in phosphate buffered saline) in a volume of 50µl in wells of a 96-well flat bottom plate containing 50µl of diluted antigen. The final serum dilution was therefore 1:100. The plate was incubated at room temperature for 1½ hours. After incubation the plates were examined for agglutination using dark-field microscopy. A reaction was deemed positive when 50% of the antigen was agglutinated. Control sera from horses with known titers were run concurrently

on each day of testing. Sera that demonstrated at least 50% agglutination at the screening dilution of 1:100 was serially diluted with their respective antigen to determine an endpoint titer.

3.2.2.2 Kidneys

A square 2.5 cm x 1 cm section was removed from the caudal and cranial cortex regions of both kidneys, further sectioned, placed in a sterile container and frozen at -80C until analyzed. qPCR for *L. interrogans* was performed utilizing a validated method at UKVDL on the ABI 7500 Fast instrument (Life Technologies/Thermo Fisher Scientific, Carlsbad, CA) (Erol et al 2014). See DNA/PCR section below. Gross and histopathological studies were performed on all kidneys as part of the routine necropsy.

3.2.2.3 Vitreous Humor

Vitreous humor samples were harvested from both eyes by inserting an 18-gauge needle 1 cm caudal to the limbus and aspirating 2 ml from the central vitreal body. Samples were stored at 4C prior to analysis. MAT was performed as stated in Heart Blood section. Following MAT analysis, remaining vitreous sample was stored at -20C pending further evaluation. Real-time PCR for *L. interrogans* was performed as stated below.

3.2.2.4 Urine

Urine samples were extracted from the bladder when present (postmortem changes often fully express all urine from the bladder). Cystocentesis was

performed using a 14g needle, removing up to 10ml when possible, of urine (when present) was removed placed in a sterile container and frozen at -80C until analyzed. Urine samples were processed by two methods [47]. For method one, urine was thawed, vortexed with 200ul removed and placed in a sterile container for further DNA extraction. For method two, a second sample (1ml) was placed in a separate sterile microcentrifuge tube, centrifuged at 12,000g for 10 minutes at 4C. Supernatant was removed and the pellet was reconstituted with 200µl phosphate buffered saline (PBS). DNA was extracted from both methods at the same time.

3.2.3 DNA Extraction/PCR Analysis: Kidney, Vitreous Humor and Urine Samples

DNA extraction from all samples was performed using Qiagen DNeasy Blood and Tissue Kit (Carlsbad, CA) following the manufacturer's protocol. Briefly a small section of kidney (~25mg) was removed placed in a MagNa Lyser Green Bead Tube (Roche Life Science, Indianapolis, IN) with ATL Buffer and proteinase K solution (Qiagen) and homogenized for 40 seconds using a Roche MagNA Lyser Instrument. Phosphate buffered saline (PBS) and a sample from a *Leptospira interrogans* culture was used as negative and positive controls respectively. Both positive and negative controls were processed with each group of samples. Samples/controls were incubated at 56C for one hour, continuously mixed on a rotating platform and then centrifuged for 5 minutes at room temperature, 200ul of supernatant was removed and processed using

QIAcube Nucleic Acid Purification Instrumentation (Qiagen) according to manufacturer's protocol. DNA extracted samples were stored at -20C until analyzed by qPCR. DNA processing of both vitreous humor and urine samples were processed exactly as kidney samples, using 200ul from each sample.

Real-time PCR was carried out using primers and protocol from an established procedure [47, 256]. Primers used targeted the LipL32 gene found only in pathogenic *Leptospira*. Briefly 2.5µl of DNA sample was added to 22.5µl of QuantiFast Pathogen PCR +IC (internal control) Kit (Carlsbad, CA) and 0.4µmol/l each of forward (LipL32-45F [CCCGCGTCCGATTAG]) and reverse (LIPL32-286R [GAACTCCCATTTCAGCGATT]) primers and probe LipL32-189P (6-FAM AAAGCCAGGACAAGCGCCG-BHQ1). A negative (sterile water) and positive control (*Leptospira interrogans* DNA) were included for amplification. The ABI 7500 Fast qPCR instrumentation (Applied Biosystems-Thermo Fisher Scientific, Foster City, CA) was used for amplification. The amplification cycle was one cycle at 95C (5 minutes), 45 cycles of denaturing at 95C (15 seconds), extension-detection (30 seconds) at 58C. A Ct 30±3 was considered positive for the internal control, following manufacturers guidelines. Samples were considered positive with Ct<45.

3.2.4 Animal and Weather Data Collection

The following data elements were collected from the necropsy forms submitted for each animal if filled out by the submitter, including breed, age, sex, weight, necropsy results, county/state living at time of death, date of death.

Weather data was obtained from Midwestern Regional Climate Center (https://mrcc.illinois.edu) for foaling seasons from June 2015 through May 2019 for the Central Bluegrass region of Kentucky. The Central Bluegrass region for this study, included the counties of Fayette, Bourbon, Woodford and Scott. Average monthly temperature and precipitation were acquired.

3.2.5 Statistical Analysis

The association between sex, age, breed, MAT, renal lesions (with or without the presence of *Leptospira*), and PCR was calculated using multiple linear regression. Distribution Analysis was performed on study population by gender and population by breed. Contingency Analysis and analysis by Fisher's Exact Test were used to analyze study population by age, combined results by gender by titer and age brackets, and MAT heart blood results by gender. One-way analysis was used to determine breed differences by gender and age. Differences between gender of MAT titers≥1600 was analyzed using fit least squares of titer bracket by sex, age bracket and serovar. Differences within females and pregnancy was determined by Contingency Analysis and further analyzed by Bowker's Test and Cochran Armitage Trend Test. All data was analyzed using JMP 13.2 for MAC (SAS Institute Inc. Cary, NC). P-values ≤0.05 were considered statistically significant.

3.3 Results

Stratification by gender, breed and age are found in Tables 3.1, 3.2 and 3.3 respectively. Representative breeds submitted in the study can be found in

Figure 3.1. There was no statistical difference in the study population based on gender. There were 29 (10.5%) females pregnant or had aborted at the time of death.

There were significantly more Thoroughbreds submitted compared to other breeds which was anticipated. According to the 2012 census of horses in Kentucky (Table 3.4), the number of Thoroughbreds in the state and in the Central Kentucky region was much higher (68.1%) when compared to other breeds [257].

Table 3.1: Study Population by Gender

Gender	Number	Pregnant
Female	277 (55.4%)	29 (10.5%)
Male	208 (41.6%)	
Unknown	15 (3%)	

Three age brackets were established as follows: bracket 1≤ 1 year; bracket 2 is 2 to 12 years; bracket 3≥13 years. Age brackets were chosen to include foals and yearlings together, the additional two brackets obtained by averaging the age spread of the horses submitted. Age bracket two had the highest percent of submissions followed by brackets three and one (46.8%, 21.8% and 12.6% respectively).

Figure 3.1: Representative Breeds

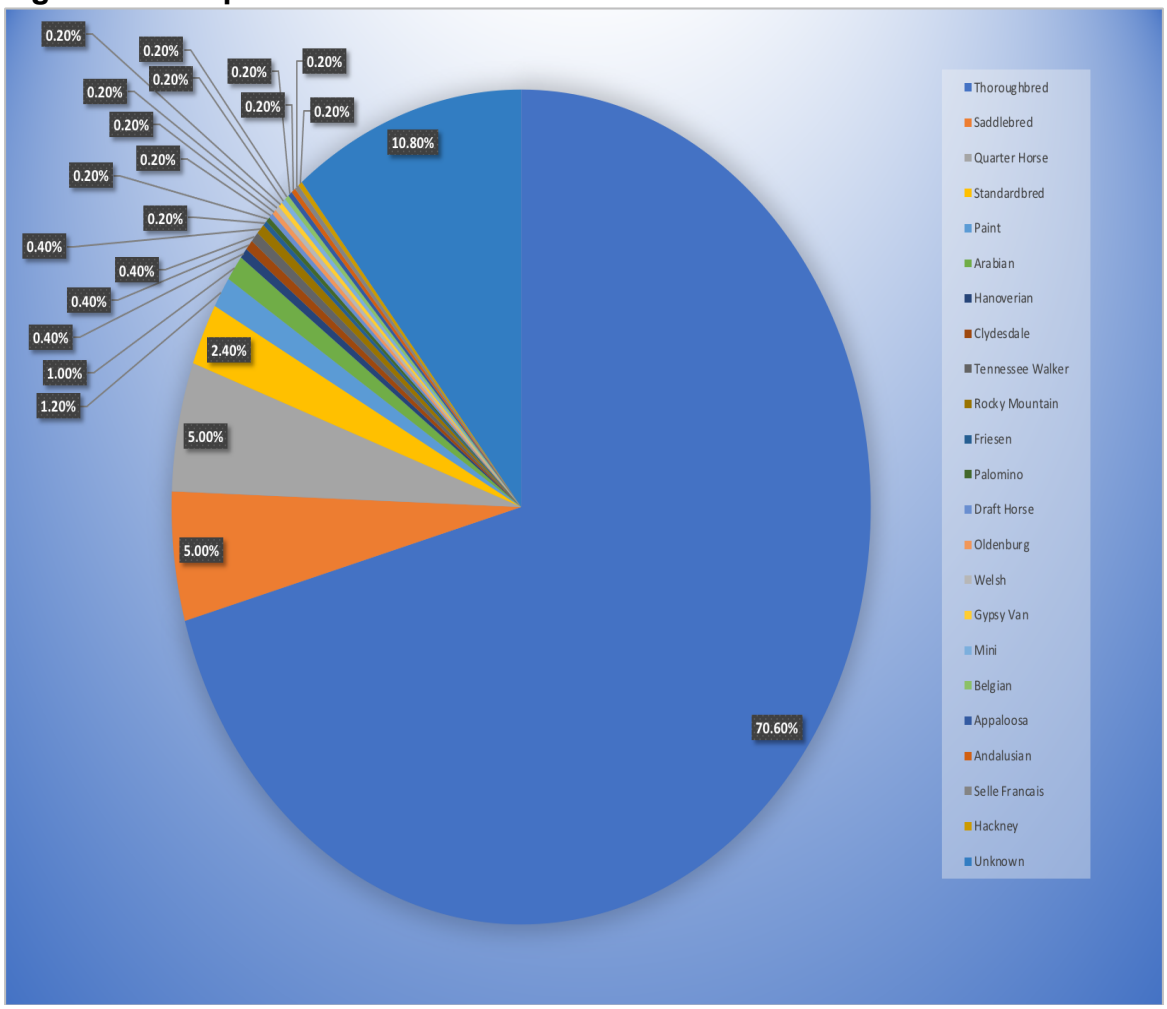


Table 3.2: Study Population by Breed

Breed	Number
Thoroughbred	353 (70.6%)
Saddlebred	25 (5%)
Quarter Horse	25 (5%)
Standardbred	12 (2.4%)
Other	31 (6.2%)
Unknown	54 (10.8%)

Table 3.3: Study Population by Age Stratification

Age	Age Bracket	Total Number	Female	Male
≤ **1 year**	1	76 (15.2%)	25 (5.0%)	51+ (10.2)
2-12 years	2	231 (46.2%)	123 (24.6%)	108 (21.6%)
≥**13 years**	3	109 (21.8%)	70++ (14%)	39 (7.8%)
Unknown			59	10

+ p<0.0001 significantly more younger males
++ p<0.0001 significantly more older females

Table 3.4: 2012 Equine Survey-Central Kentucky Counties*

County	Total Equine Population	TB	SB	QH	SDB	Other
Fayette	24,600	17,510	1240	960	400	1090
Bourbon	13,000	9330	980	380	580	360
Scott	7,000	3170		690	330	680
Woodford	11,900	8440	670	570	670	310
Total	44,800	38450	2890	2600	1980	2440

*2012 Kentucky Equine Survey (Thoroughbred state total=54,000)
TB-thoroughbred; SB-Standardbred; QH-Quarter Horse; SDB-Saddlebred

The groupings for breed and sex are listed in Table 3.5. Standardbreds were under-represented compared to Saddlebreds, Quarter horses and Thoroughbreds. Therefore, they were combined with other known breeds which were present in very low numbers and unidentified breeds, all were placed into one group labeled *Other*. The mean age for both Thoroughbred ($p<0.0001$) and quarter horses ($p<0.417$) was significantly higher in females than their respective males. The mean age of thoroughbred males was significantly younger ($p<0.0001$) when compared within to all other groups.

Table 3.5: Breed Statistics by Gender and Age

Breed	N	Mean Age	Fe*	Mean Age	M*	Mean Age	Total Missing
Thoroughbred	353 (70.6%)	7.4	177	9.6^{+}	125	4.3^{++++}	51
Saddlebred	25 (5%)	9	11	7.3	11	111.4	3
Quarter Horse	25 (5%)	10.2	6	14.8^{++}	17	9.1	2
Other	97 (19.4%)	11.8	24	11	45	12.7	28

*Fe (female), M (male)
+$p<0.0001$
++$p<0.0417$
+++$p<0.0001$ within

There was a significant difference ($p<0.001$) in the number of older animals (≥13 years) with titers when compared to younger animals (Table 3.6). Older animals with titers also had significantly higher titers ($p<0.001$) when compared to younger animals. There was a significantly higher number of

females with titers (p<0.0001) compared to males (Table 3.7). Females additionally had significantly higher titers (p<0.001) compared to males.

Table 3.6: Combined Female and Male Titer Results by Age

Age	Negative	Titer=100	Titer≥ 200
≤ 1 year	67	5	9
2-12 years	163	20	49*
≥13 years	54	11	44**

Both * and **p<0.0001 to age≤ 1 year

Table 3.7: Gender by Age and Titer Bracket

Age Bracket	Titer Bracket	Female	Male
1	0	19 (6.9%)	44 (21.2%)
1	1	1 (0.4%)	3 (1.4%)
1	2	5 (1.8%)	4 (1.9%)
2	0	77 (27.8%)	84 (40.4%)
2	1	8 (2.9%)	12 (5.8%)
2	2	38* (13.7%)	11 (5.3%)
3	0	31 (11.2%)	23 (11.1%)
3	1	5 (1.8%)	6 (2.9%)
3	2	34* (12.3%)	10 (4.8%)
Total Number with titer		91**	46

*p<0.0005
**p<0.0001
Titer Brackets: 0=negative; 1=100; 2≥200

3.3.1 MAT Heart Blood

Serovar results for heart blood and results by gender can be found in Tables 3.8 and 3.9 respectively. There was a significantly higher number of horses ($p<0.0001$) with titers to serovar Bratislava compared to Pomona and Grippotyphosa. Females had significantly higher number of titers to Bratislava ($p<0.0001$) compared to males, and within females ($p<0.0001$). Females also had a significantly higher number of titers ≥200 compared to males ($p<0.0001$). Results within males revealed significantly more males with serovar Bratislava compared to both Pomona and Grippotyphosa ($p<0.0001$). Titers ≥100 seroprevalence for Bratislava increased to 25.6%. Both serovars Pomona and Grippotyphosa only increased slightly to 3% and 5% respectively comparing titers ≥100 to titers ≥200.

Table 3.8: Serovar-Prevalence in Heart Blood≥200

Serovars	Major (N=497*	Minor (N=497) *	Same (N=497) *
Pomona	14 (2.8%)	22 (4.5%)	8 (1.6%)
Grippotyphosa	21 (4.2%)	12 (2.4%)	2 (0.4%)
Bratislava	90 (18.1%) **	17 (3.5%)	9 (1.8%)

*3 titers were not analyzed
** $p<0.0001$

Table 3.9: Heart Blood Serovars by Gender

Serovar/Titer Bracket	Female	Male
Pomona	15	1
Grippotyphosa	19	5
Bratislava	82* (29.6%)	41** (29.7%)
Negative	162	160
Titer=100	19	21
Titer≥200	96***	26

*p<0.0001
**p<0.0001
***p<0.0001

Both sex and age had significant effects on serovar (Tables 3.10-3.12). Older animals had significantly greater titers (p<0.0001) compared to younger animals regardless of sex. Females were significantly (p<0.0002) more likely to have a titer regardless of serovar compared to males.

Table 3.10: Combined Female and Male Positive Serovars by Age

Age	Bratislava	Grippotyphosa	Pomona	Negative
≤ 1 year	9	5	0	56
2-12 years	49*	13	7	164
≥13 years	46**	2	8	53

*p<0.0001
**p<0.0001

Table 3.11: Serovar Results for Female by Age

Age	Total Female	Female Bratislava	Female Grippotyphosa	Female Pomona	Female Negative
≤ 1 year	25	2 (0.9%)	4 (1.8%)	0	19 (8.7%)
2-12 years	123	30 (13.8%)*	9 (4.1%)	7 (3.2%)	77 (35.3%)
≥13 years	70	31 (14.2%)*	2 (0.9%)	7 (3.2%)	30 (13.8%)

*p<0.0006

Table 3.12: Serovar Results for Male by Age

Age Bracket	Total Male	Male Bratislava	Male Grippotyphosa	Male Pomona	Male Negative
≤ 1 year	51	6	1	0	44
2-12 years	107	19*	4	0	84
≥13 years	39	15*	0	1	23

*p<0.01

There were a significantly higher number of Thoroughbreds with titers to Bratislava compared to all other breeds (p>0.0001) (Table 3.13). Females of both Thoroughbreds and horses in Other breed category had a significantly higher seroprevalence to Bratislava compared to males (both p>0.0001).

Table 3.13: Serovar Bratislava Results by Sex and Breed

Breed/Serovar	Female	Male
Other/Bratislava	20^ (4.1%)* (7.2%)** (48%)*** (22%)****	12 (2.5%)* (5.8%)** (24%)*** (13.2%)****
Thoroughbred/Bratislava^	57^ (11.8%)* (20.6%)** (26%)*** (16.6%)****	21 (4.3%)* (10.1%)** (16%)*** (6.1%)****
Saddlebred/Bratislava	2 (0.4%)* (0.7%)** (17%)*** (8.3%)****	6 (1.2%)* (2.9%)** (50%)*** (25%)****
Quarter Horse/Bratislava	3 (0.6%)* (1.1%)** (43%)*** (12%)****	2 (0.4%)* (0.9%)** (11%)*** (8%)****

*485 in each group, 15 animals' unknown sex not included
**within sex
*** within total of breed+sex
****within total of breed
^p<0.0001

The prevalence of titers ≥1600 are listed in Table 3.14. Serovar Bratislava occurred more often regardless of gender. Females had a significantly higher number of titers ≥1600 compared to males (p=0.0003).

Table 3.14: Serovar Titers ≥1600

Serovar	Total*	Female	Male
Bratislava	18 (58.1%)	12* (38.7%)	5 (16.1%)
Pomona	5 (16.1%)	5 (16.1%)	
Grippotyphosa	8 (25.8)	6 (19.4%)	1 (3.2)
Average Age		15.1 years	12.6 years

*Two samples gender unknown
*p=0.0003

3.3.2 Pregnancy

There were 29/277 (10.5%) females presented as, in-foal or complications from abortion for necropsy. There was no significant difference between age of non-pregnant (NP) and pregnant mares (P) (9.8 and 12.1 years respectively). The titer bracket distribution of NP compared to P-mares are in Figures 3.2 and 3.3 respectively. A larger percent of NP-mares (61%) were negative (bracket zero) compared to 34% of P-mares. There was no difference between NP and P-mares in titer bracket one (titers=100) with 6% and 10% respectively. Titer bracket two (titer≥200) comprised 32% NP and 55% of P-mares. P-mares had significantly higher titers compared to NP-mares ($p<0.0318$) (Figure 3.4). Both NP and P-mares had a significantly higher number of positive titers to serovar Bratislava ($p<0.0079$) compared to the other serovars. Non-pregnant mares with titers ≥1600 regardless of serovar verged on significance ($p<0.067$) compared to P mares (Figure 3.5). The number of non-pregnant and pregnant mares with titers ≥1600 was low (18 and 5 respectively). A greater number of samples is needed for any conclusions to be reached.

Figure 3.2: Distribution of Non-Pregnant Mares by Titer Brackets

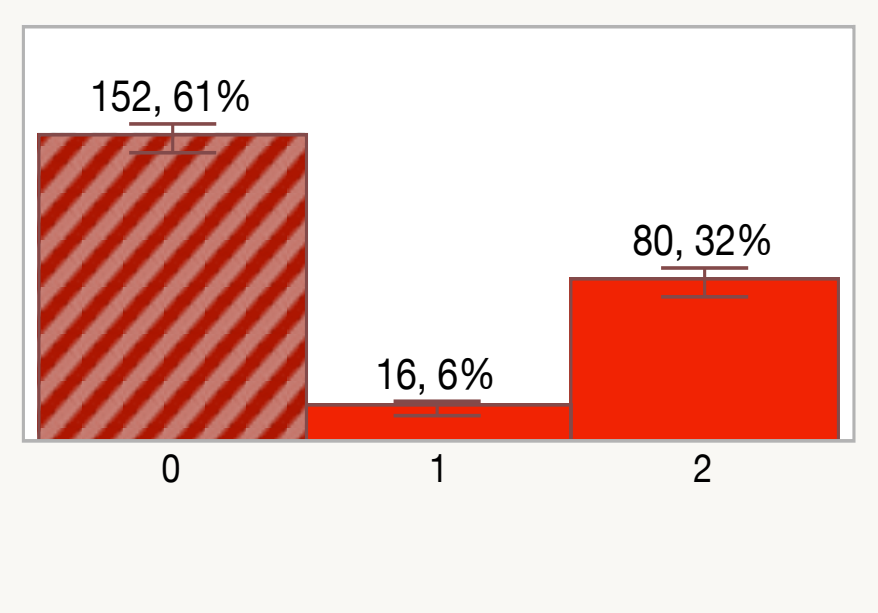


Figure 3.3: Distribution of Pregnant Mares by Titer Brackets

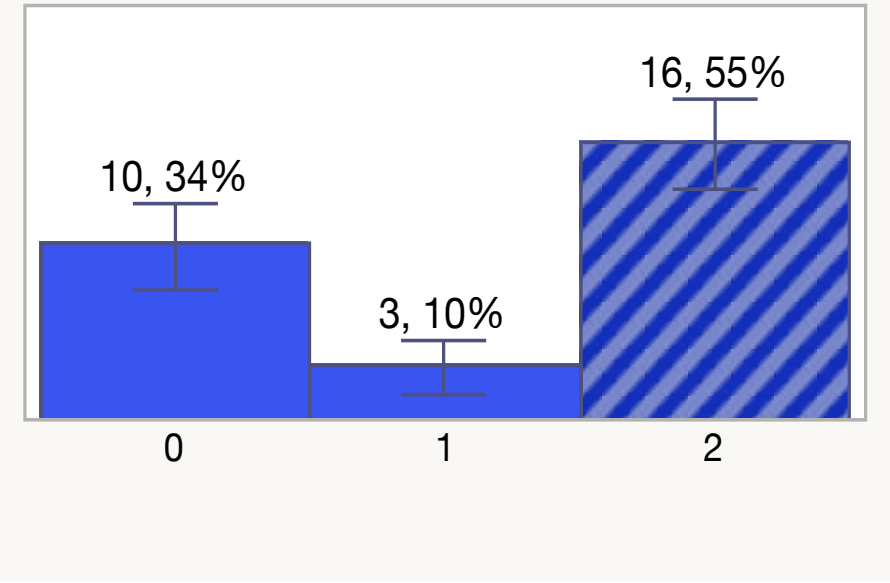
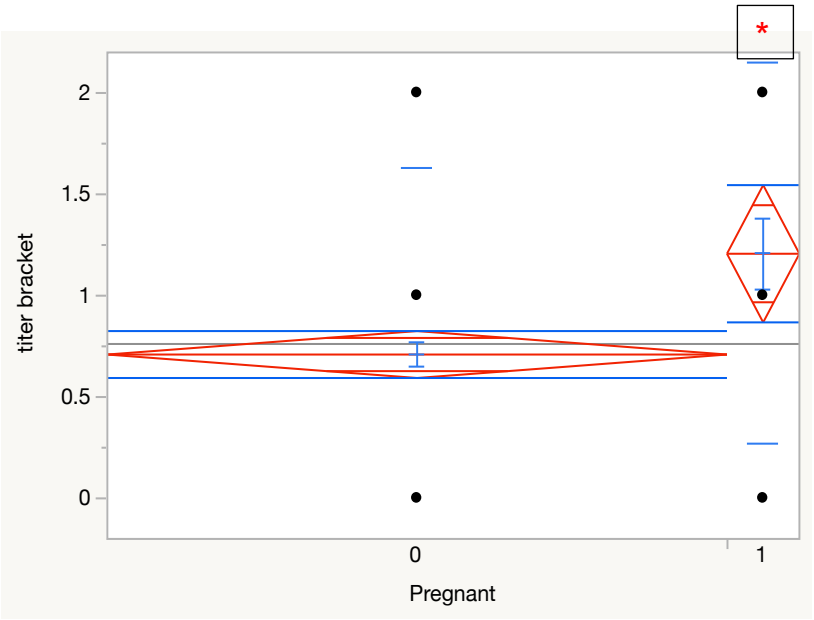
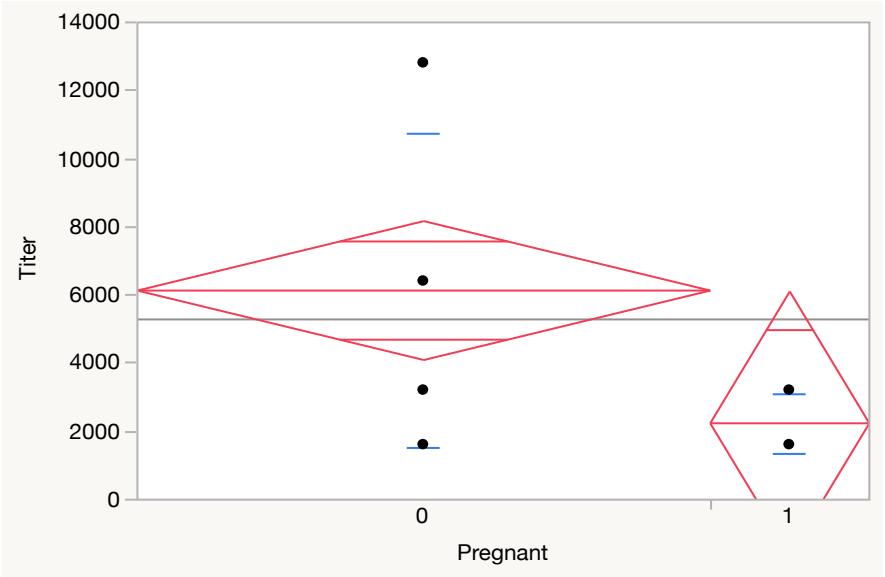


Figure 3.4: Analysis of Titer Brackets by Pregnancy



*p<0.0318

Figure 3.5: Female Serovar Titers ≥1600 by Pregnancy



p<0.067

3.3.3 Vitreous

Serovars and titers detected in vitreous samples and corresponding heart blood titers are listed in Table 3.15. There were 4 vitreous samples with titers ≥100. Three out of four had titers to Bratislava in both the heart and vitreous samples. Only one vitreous sample had a titer to Pomona, the heart sample was negative.

Table 3.15: Vitreous Humor and Heart Blood Titers

Serovar	Vitreous		Heart
	N	Titer	Titer
Bratislava	1	6400	6400
	1	200	12,800
	1	100	1600
Pomona	1	100	0

3.3.4 Real-Time PCR Testing

There were 496 kidney samples tested by qPCR, one sample was positive. A total of 73 urine samples were tested, all were negative. Only 3/455 vitreous samples were positive by qPCR, none had elevated titers.

3.3.5 Precipitation and Temperature

No differences were seen with time of year, titers and increases in temperature and/or precipitation (data not shown).

3.4 Discussion

The objective of this study was to determine the seroprevalence of *Leptospira* in the general population of horses submitted to the VDL for necropsy regardless of sex, age, breed or pregnancy status. The VDL receives serum samples from farms beginning in October through March to monitor leptospiral titers in pregnant mares. Titer evaluations are performed to detect rising titers, which could lead to abortion and assisting in the decision whether or not to employ a prophylactic antibiotic treatment. Titers are rarely evaluated on non-pregnant mares or male horses. Leptospiral titers may be analyzed on other horses if uveitis, ERU, renal disease or septicemia are occurring (personal communication-Dr. Craig Carter, UKVDL).

The current study found no significant differences between the number of female and male submissions to the VDL. Looking at equine submissions between 2010-2020 to the VDL, other years have shown the same pattern (Appendix 4). The number of Thoroughbreds was significantly higher when compared to all other breeds (known and unknown) combined. This was a predictable outcome for the Central Kentucky region. The number of Thoroughbreds submitted mirrors a survey of the equine industry which showed approximately 68% of horses in Central Kentucky area are Thoroughbreds [257].

The majority of horses were in three breed groups (70.6% Thoroughbred, 5% Saddlebred, 5% Quarter horses). The mean age of male to female Thoroughbreds was significantly lower. Examining age brackets between gender, there was a significantly higher number of males ≤ 1 year compared to

females and higher number of females 13 years compared to males. Regarding breed submissions, males had a significantly higher number of young Thoroughbreds submitted for fractures, compared to any other breed. Males also had a significantly higher number of fractures compared to females. Males in this study consisted of 24% of total submissions in age brackets 1 and 2. A significantly higher number of males were submitted for fractures and Wobbler Syndrome. One reason for increased number of young males with fractures might be an escalation in aggressiveness leading to accidents in paddock areas (personal communication-Dr. Jennifer Janes, UKVDL). Wobbler Syndrome has been shown to be significantly higher in males compared to females [258].

The number of titers ≥ 200 were seen in older horses. Published research shows as a horse ages, titers appear to become established and environmental exposure to *Leptospira* over many years increases this risk [41, 237]. Healthy horses as they age (>7 years) have been shown to have increased titers compared to younger horses (<3 years) [185, 202, 259]. One study showed an increase in seroprevalence by 10% for every year a horse ages [202].

Combining females and males there were no significant difference between the number of horses with titers in age brackets 2 and 3. However, when separating the genders, significant differences can be seen. There was a significantly higher number of females with titers compared to males. Previous research has shown this, and could be due to females pastured together more often than males [260]. This allows females to have increased exposure to wildlife and each other, potentially increasing exposure to *Leptospira.* Females

had significantly higher titers compared to males. A reason behind a higher number of females with increased titers is age stratification. Males had a significantly higher number in age bracket 1 compared to females. Horses in age bracket 1 had either no titer or very low titers. Females had a significantly higher number in age bracket 3 compared to males.

Differences between gender are observed in serovar prevalence. There were significantly more horses with titers to serovar Bratislava compared to both Pomona and Grippotyphosa. These results are similar to previous studies in both North America and Europe. As early as 1981 serovar Bratislava was found to be the most prevalent serovar (31.7%) in a random group of horses for export from England [261]. Others in Canada, US and Europe have also found this serovar to be more prevalent [177, 259, 262]. The horse is thought to be the maintenance host for serovar Bratislava, although subclinical infections are thought to be prominent [86, 186]. Clinical infections caused by serovar Bratislava have occurred [177]. A study in Croatia found over 28% of serum samples from horses had titers to serovar Bratislava [263]. Recently a study published in 2018 showed over 47% of horses in the Midwest (specifically Kansas, Missouri, and Nebraska) had titers of ≥ 100 to serovar Bratislava [199].

A significantly higher number of females had titers to Bratislava compared to males, and within females. Females also had a significantly higher number of titers ≥200 compared to males to serovar Bratislava. Considering gender and age there was a significant effect on serovar. Females were significantly more likely to have a titer to all three serovars. Once again, this phenomenon may be

due to the way females are grouped together. Males are typically either alone or grouped in very small numbers.

The high incidence of serovar Bratislava in this study has been previously documented in Kentucky. On one Kentucky horse farm, a 53% seroprevalence of Bratislava was observed [264]. In another study, horses from three Central Kentucky farms had a 33% seroprevalence to Bratislava [230]. A study conducted in 2011 analyzed 50 serum samples for leptospiral titers submitted to the VDL for reasons other than leptospirosis [203]. These samples were part of a larger study evaluating the prevalence of equine leptospirosis in the US and one province of Canada. The study demonstrated 38/39 (97.4%) of samples in Kentucky with a titer ≥100 to Bratislava, with only one sample having a titer with Grippotyphosa. The study separated out geldings from stallions, which demonstrated 75% of stallions had negative titers compared to 87.5% geldings with a titer ≥100. There were no significant differences between gender regardless of separating out geldings from stallions or grouping them together. There was no difference between gender and serovar prevalence. The outcomes between the 2011 and this study may be due to the differences in breeds represented. The present study had significantly more Thoroughbreds (70.6%) compared to only 8.5% in the 2011 study. Both studies had no significant differences in the number of females vs males. Another difference was the time of year the samples were taken. All samples in 2011 were collected in April compared to samples taken throughout the year in the current study. Typically, in the Central Kentucky area the highest number or horses with titers

occurs between the months of October and March [30, 87, 254]. When looking at age in the 2011 study (not age bracket) females (mean=12.4) are significantly older compared to all males (p=0.0155) (mean=8.9).

The current study had significantly more female Thoroughbreds with a seroprevalence to Bratislava compared to males and all other breeds. Also, females in the Other category had a significantly higher seroprevalence to Bratislava compared to males. A study published in 2009 showed similar results [185]. However, in the 2009 study, geldings and stallions were analyzed separately. If they had grouped all males, it appears there would have not been a difference. The suggestion was mares spend more time out in the field together compared to males (especially stallions) therefore exposing them to increased risk of exposure to *Leptospira*.

Racoons have long been associated with the spread of *Leptospira* [265, 266]. Racoons in the state of Kentucky are legally hunted for their pelts. However, over the past several decades hunting racoons has decreased dramatically due to the value of the pelt plummeting (personal communication-Dr. Matthew Springer, University of Kentucky). The number of raccoons in the state have increased dramatically directly related to decreased hunting. Several studies have shown raccoons have a high percent of seroprevalence to serovar Bratislava. A study in Indiana found over 38% seroprevalence to serovar Bratislava in raccoons [266]. A second study in Illinois found over 28% seroprevalence to serovar Bratislava in raccoons [29]. One can speculate this may contribute to the increased occurrence of leptospiral titers and possibly

clinical leptospirosis not only in horses but other mammals. A study conducted in Kentucky isolated *Leptospira interrogans* serovar Pomona type Kennewicki in 4/14 raccoons [267].

Leptospirosis in horses is endemic in Kentucky. Veterinarians practicing in Kentucky consider titers ≥1600 indicative of a possible infection or a major exposure. In this study, females had a significantly higher number of titers ≥1600 and neither age nor serovar had a significant impact on the level of titers. Pregnant mares had significantly higher titers (not titer brackets) after taking into consideration unequal variances. There were no other significant differences within the female group. Mares are immune compromised in the last trimester of pregnancy, therefore titers may increase during this time period, after multiple exposures to *Leptospira* environmentally and/or by direct contact with other animals. Stress caused by increased leptospiral titers and transplacental infection may increase chances for abortion [86].

Only 4 horses had positive titers in the vitreous, none considered to have an active infection according to the Goldmann-Whitmer coefficient. The low number of vitreous samples with a titer is not surprising since none of these animals were euthanized because of blindness or ERU. However, there were 14 vitreous samples with a green fluorescent color indicating they had recently had an ophthalmological exam. All fourteen horses had negative titers in both their heart and vitreous samples. A high percent of horses in this study were Thoroughbreds. Although they can present with ERU, they have not been found

to have a genetic link like Appaloosas [214, 216]. Also, a lower percent of Thoroughbreds present with ERU compared to other breeds [214, 217].

Urine samples were collected from 16% of animals, all were negative by PCR. Identification of leptospires through culture or PCR from urine samples is difficult because of the sensitivity of the organism to pH and enzymatic breakdown in the urine. Additionally, all samples were taken from deceased horses resulting in a collection of urine which might reduce detection by PCR. Studies have shown positive results for *Leptospira* by PCR in urine upon collection from live horses especially after abortions caused by leptospirosis [167, 268, 269].

There are a number of limitations to this study. Samples were taken at the time of death. In addition, there were no paired serum sample collections to determine whether horses with titers ≥1600 might indicate a current recent leptospiral infection (fourfold rise in titer). Multiple serum titers are critical in determining leptospirosis and its course. Only 73/500 (14.6%) urine samples were analyzed, all negative by PCR. The low number along with only one sample from each horse makes it difficult to draw conclusions about shedding of leptospires in the horse. A small section of kidney was taken for analysis. Lack of isolation from the kidney could have been due to missing potential tubule(s) containing the organism from the section taken at necropsy and subsequent DNA isolation.

3.5 Conclusions

Horses submitted for necropsy between May 2016 to January 2019 have evidence of exposure to *Leptospira* (elevated MAT titers) regardless of the reason for submission. There was a higher seroprevalence of serovar Bratislava compared to both Pomona and Grippotyphosa. This has been seen previously in Kentucky [203, 230]. Further research is needed to determine the strains of Bratislava in the US and their differences, if any, to Europe. Females compared to males appear to have an increased risk of having significantly higher MAT titers. This may be due to housing of mares together compared to males which tend to be either alone or in smaller numbers. This could also be specific to Central Kentucky. Further research is needed to ascertain whether this is occurring only in this region or is common throughout the US and/or globally. Older horses are more likely to have a titer for *Leptospira* and also have higher titers. This has been documented in multiple papers [41, 185, 202, 237, 259]. PCR results were negative for pathogenic *Leptospira* in urine, with a low number of positive kidney and vitreous samples. This suggests that the risk of leptospiral shedding of the organism in urine by horses in Central Kentucky is minor and possibly indicates that horses clear leptospiral infections quickly. Results also suggest the risk of humans/animals acquiring a leptospiral infection from horses without an active infection is low. However, only one sample was analyzed/animal with no previous or follow up samples for comparison in order to determine the occurrence of an active infection. Therefore, further research is needed to confirm these findings.

Chapter Four

Heme Oxygenase-1: A Novel Marker for Equine Leptospiral Abortion?

4.1 Introduction

Heme oxygenase (HO) is an enzyme with three known mammalian isoforms: HO-1, HO-2 and HO-3 [270-273]. HO-1 is found throughout the body but is highly expressed in the liver, spleen and places with high red blood cell (RBC) turnover [270, 274]. HO-1 concentrations are very low or undetectable under normal conditions, but highly inducible under inflammatory conditions and stress. HO-2 is expressed constitutively in mammals [272, 274]. HO-2 is highly expressed in many tissues such as kidney, liver, testes, spleen, and the nervous and cardiovascular systems [274, 275]. HO-2 is thought to be neuroprotective in the brain and play an important role in male reproduction [275]. HO-2 is also thought to be involved in the maintenance of basal heme metabolism. Unlike HO-1, information about HO-2 is limited due to the lack of selective chemical probes to study the enzyme [275]. HO-3 is thought to be a pseudogene derived from HO-2 transcripts and has only been identified in rat brain [273]. The function of HO-3 is still unknown.

HO-1 deficiency in humans was first noted in a young boy with a multitude of developmental disturbances, including hemolytic anemia, low bilirubin, abnormal coagulation, intravascular hemolysis, severe growth impedance along

with many other maladies all traced back to HO-1 deficiency [276]. A HO-1 deficiency in mice was associated with a number of problems including abortion, increased inflammatory responses, lymphadenopathy and splenomegaly [277]. Deficiency in HO-1 in mammals leads to the inability to respond to inflammatory processes. The body's failure to respond with a proper immune response can lead to an exaggerated inflammatory response [277]. In cases of organ transplantation, it will lead to rejection. The importance of HO-1 in both adaptive and general oxidative stress in mammalian cells was confirmed in the mid-1980s [278]

The increasing interest and importance of HO-1 can be appreciated by the number of articles published over the past 30 years. In 1992 there were 8 articles published (PubMed), since 1993 there have been well over 10,000.

4.2 Heme Oxygenase-1

HO-1 is upregulated by numerous chemicals, inflammatory cytokines and is found in various tissues, bone marrow and cells [279-281]. It has been detected in various mammals including humans, rats, mice, swine, sheep and bovine [282-288]. HO-1 is an enzyme involved in the biosynthesis of heme and is the first and rate-limiting step of the oxidative degradation of heme. The by-products of the reaction produce carbon monoxide (CO), free iron and heme precursor biliverdin (BV), which subsequently is converted to bilirubin (BR) [278, 279, 289]. HO-1 and HO-2 can degrade heme into its by-products, however only HO-1 can be induced by a large number of chemicals and drugs, such as metals,

eicosanoids, aspirin, statins and niacin [290]. The by-products of HO-1 are anti-inflammatory, reducing organ injury. Additionally, they assist in maintaining heme at normal levels [291]. HO-1 is involved in vascular tone, anti-inflammatory processes, anti-apoptotic responses, responding to and reducing oxidative stress leading to decreased tissue damage in numerous organ systems [288].

4.3 Hemoglobin

Hemoglobin (Hb) is a protein found in red blood cells [292]. In humans approximately 1% of Hb is degraded daily. The main function of Hb is the storage and transport of oxygen [293, 294].

There are differences in equine Hb as compared to humans and ruminants. Horse Hb has a lower binding affinity to oxygen than humans, but higher than ruminants [295, 296]. Oxygen affinity is important for the quick release and delivery of oxygen to tissues and is pH dependent [293, 296].

4.4 Equine Red Blood Cells

The average life span of RBCs in the horse is 140-150 days compared to 120 days in humans [292, 297]. Red blood cells have many roles, the main one is carrying O_2 from the lungs throughout the body to tissues, organs, muscles and to carry CO_2 back to the lungs for exhalation. RBCs in humans and horses both release and rapidly uptake amino acids, which represent 15-17% of their

total content [297]. This indicates another role for RBCs, transporting amino acids throughout the body.

Equine RBCs vary among breeds [298]. There are key differences in RBCs between hot-blooded and cold-blooded breeds. Hot-blooded horses include Arabian, Standardbred, Quarter horses, Thoroughbreds and Appaloosas [298]. Cold-blooded breeds include ponies, hackneys, draft horses and donkeys. Table 4.1 shows RBC differences in hot- and cold-blooded equine breeds and humans.

Table 4.1: RBC Differences Between Hot- and Cold-Blooded Horses

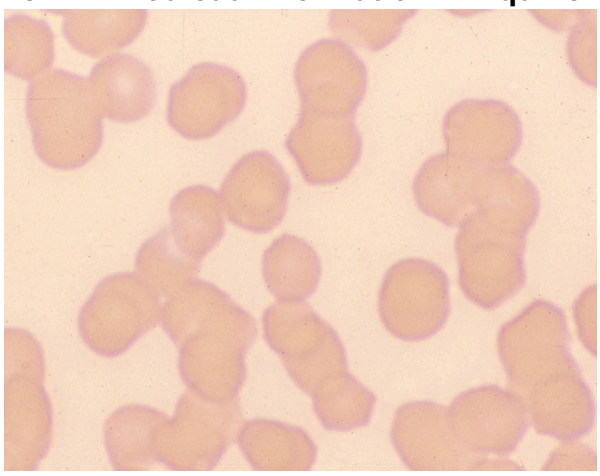
Parameter	Unit	Hot-Blooded Horses	Cold-Blooded Horses	Humans	
				Female	Male
RBC	$_X10^{12}$/L	8.2-12.2	5.5-9.5	4.2-5.4	4.6-6.2
Hemoglobin	g/L	130-170	80-140	115-160	140-180
PCV	percent	32-48	24-44		
MCV	fL	36-50	40-48	80-95	80-95
MCH	pg	13-19	12-17		
MCHC	g/L	330-390	320-380	300-340	300-340
Diameter	μm	5.7	5.7	6.2-8.2	6.2-8.2

PCV=packed cell volume
MCV=mean corpuscular volume
MCH=mean corpuscular hemoglobin
MCHC=mean corpuscular hemoglobin concentration

RBCs in the horse are unique in forming a rouleaux configuration [298]. The RBCs aggregate together linearly, with the number of rouleaux formations attributed to the number of RBCs in circulation. Figure 4.1 shows RBC rouleaux formation in horses. Normally, RBCs have a strong negative charge and repel each other [298]. However, in horses the charge is weak, causing them to stack together into the rouleaux formation and have the propensity to aggregate in

anticoagulated blood samples. Increased rouleaux formation can be seen in certain diseases such as hyperproteinemia [298].

Figure 4.1: Rouleaux Formation in Equine RBC*



*[298]

4.5 HO-1: Physiology and Immune Response

HO-1 is crucial for an oxidative stress response in humans [278]. HO-1 can act as an agonist or antagonist depending on conditions and upregulation upon stimulation [278]. Stimulation can be an inflammatory process or trauma (protective). One indicator of HO-1 trauma is bruising of the skin. When trauma occurs blood capillaries break releasing RBCs [278]. The RBCs will lyse, a red/brown color on the skin occurs caused by hemoglobin/met-hemoglobin. The yellow/green color is then generated by the release of biliverdin.

During oxidative stress HO removes free heme decreasing the over production of reactive oxygen species (ROS) [299]. If left unchecked, free heme will undergo autooxidation to produce superoxide and hydrogen peroxide with

further development to ROS. HO-1 is well known to demonstrate cytoprotective effects upon stress related conditions [277, 278, 300]. When oxidative injury occurs, be it from infection, cardiovascular event, trauma, inflammation or organ transplants, HO-1 is modulated. HO-1 breaks down heme releasing CO and biliverdin, which execute their own antioxidant/anti-inflammatory properties concurrently with HO-1 downregulating pro-inflammatory cytokines [277, 301, 302].

Both tissue and cell damage/injury occur through lipid peroxidation and free radical formation if heme is left unchecked by HO [291, 303]. HO-1 metabolizes high amounts of heme, elevating by-products and leading to tissue damage [303]. Major damage can occur in the kidney by heme overload. The heme proteins can form a precipitant in the renal tubules, compromising the filtration system of the kidneys and potentially leading to renal failure [303].

A number of transcription factors control HO-1 including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), c-Jun NH2-terminal protein kinase (JNK) and activator protein 1 (AP-1) (Appendix 6) [303]. These transcription factors can be activated by iron, heme and free radicals. Free iron in the ferrous form is a strong pro-oxidant and one of the breakdown products of heme. The ferrous form is used in a number of processes to facilitate free radical formation, and in the Fenton reaction [303]. The Fenton reaction drives the production of free radicals. Fenton reaction can also stimulate the production of ferritin which acts as an antioxidant by binding to iron and releasing it in a precise manner [304]. Ferritin is an iron storage protein found in many cells and tissues

in mammals, and in bacteria [304, 305]. Ferritin also has cytoprotective properties [299]. The amount of ferrous iron produced is dependent on the amount and availability of heme and induction of apoferritin [303, 306]. Apoferritin is a protein when bound to iron, forms ferritin. HO-1 control of heme prevents program cell death of parenchyma cells by co-expression of ferritin H chain (FTH) [304]. FTH controls the pro-oxidant effect of Fe after its' release from the protoporphyrin IX ring of heme. Transcription factor JNK is continually activated by the intracellular accumulation of free radicals; however FTH controls JNK activation and in a feedback mechanism JNK activation inhibits FTH, controlling the cellular overload of Fe [304]. All of this centers on availability and regulation of HO-1.

HO-1, like nitric oxide, can be detrimental by unchecked degradation of heme and accumulation of its by-products [291]. HO-1 can downregulate nitric oxide synthase (NOS), and NOS can up and down regulate HO through multiple pathways including NF-kB [291, 307]. Dysregulation of either HO or NOS can lead to detrimental effects such as tissue and vascular damage.

Interleukin-10 (IL-10) is a cytokine that influences multiple cell types with a wide range of effects [301, 308]. Notably IL-10 has the ability to inhibit pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor alpha (TNFα). IL-10 has also been shown to induce HO-1, and there appears to be a synergistic effect between IL-10 and HO-1 [302]. When HO-1 is restricted, the inhibitory effect of IL-10 on pro-inflammatory cytokines is reduced. HO-1 can be induced by TNFα, IL-1β, IL-6,

IL-8 and IL-10 [271]. HO-1 along with its by-products has also been shown to be important in not only the maintenance of pregnancy in humans, but also in protection against eclampsia and uterine contractility [274, 302]. IL-10 is also important in maintaining pregnancy.

4.6 Pregnancy

4.6.1 Humans

HO-1 has been well documented regarding the vasculature of the uterus in humans, mice and rats [271, 281, 284, 309]. HO-1 supports and stabilizes the implantation of the embryo at conception and has a protective effect throughout pregnancy [281, 285, 309, 310]. HO-1 protects the placenta in immune tolerance/suppression between the fetus and mother, decreasing oxidative stress during pregnancy [311].

HO is found in the basal plate, chorionic villi, fetal membranes and the chorionic plate during pregnancy in humans [282, 288]. HO-1 has also been identified in human vascular endothelium, syncytiotrophoblast and cytotrophoblast cells. The regulation of HO in the placenta is partly dependent on localized amounts of glucose and oxygen [312]. An *in vitro* study showed increases in HO-1 after cells were deprived of glucose and oxygen for 24 hours, and subsequently exposed to low concentrations of both [312]. The blood vessels in the placenta are not innervated, consequently vascular tone is controlled by localized soluble factors such as CO [312]. The placenta can overcome low oxygen by undertaking anaerobic glycolysis, however this will

increase oxidative stress. In order to maintain homeostasis, levels of HO will increase to protect the placenta from by-products of anaerobic glycolysis [312]. Preeclampsia (PE) can also cause complications in blood flow to the uterus and placenta, causing a deficiency in both glucose and oxygen, thereby increasing oxidative stress [312].

HO-1 and breakdown products of heme, namely CO and biliverdin, are important for maintenance throughout pregnancy [311]. There is an increased concentration gradient between the maternal and fetal circulation of biliverdin and bilirubin further indicating a role of HO [313]. Biliverdin cannot cross the placental barrier like bilirubin, however both the placenta and uterus (pregnant) can reduce biliverdin to bilirubin for transfer [313]. Once transfer occurs, bilirubin can serve as an antioxidant. During pregnancy there is increased oxidative stress with trophoblast cells in contact with the maternal vascular system [285]. The trophoblast cells carry oxygen and have the ability to cross the placenta barrier to the growing fetus [314]. Low levels of HO can lead to increased levels of heme at the fetal-maternal interface increasing an inflammatory response.

The occurrence of fatty acids (FA), specifically polyunsaturated FA, have been found in the plasma of human umbilical cord, increasing chances of oxidation and fetal injury [288]. The amount of FA in fetal circulation is lower when compared to an adult. The polyunsaturated FAs are susceptible to oxidation placing the fetus at increased risk of oxidative damage [288].

Both nitric oxide (NO) and HO have been found in humans, rats and mice at implantation, and are essential in trophoblast attachment and maintenance

[281, 288]. Increased oxidative stress in the fetus initiates an increase in both iNOS and HO-1 [315].

HO-1 is a stress response protein upregulated at pregnancy [282]. The critical period in pregnancy is the implantation of the trophoblast cells into the uterine wall. The uterine wall is extremely hypoxic with 1-2% oxygen, little vasculature, and an increased immune response during pregnancy [282]. In humans HO-1 and exhaled CO levels are lower in the placenta of women with PE compared to normal pregnancies [311, 316].

Several studies have shown a decrease in HO and spontaneous abortions in humans [314, 317]. Levels of HO-1 and HO-2 were decreased in trophoblasts from aborted fetuses compared to trophoblasts from terminated normal pregnancies. In a case study of 3 deleterious pregnancy outcomes, a homozygous deletion was found in the HMOX1 gene (HO-1) leading to a frameshift and a premature termination codon [318]. This mutation was carried by both parents and to abortion and/or termination of each pregnancy.

4.6.2 Mice/Rats

Studies in mice have demonstrated the importance of HO-1 in maintaining pregnancy from early development through birth [319, 320]. In these models, as HO-1 decreased circulating levels of t-helper cell-1 (TH-1), while pro-inflammatory IL-1β, TNFα and IFNγ, increased [283]. In mouse models of abortion, levels of HO-1 in the placenta have been shown to decrease along with circulating IL-10 [310, 321, 322]. Supplementing abortion prone mice with HO-1

adenoviral vector has been shown to decrease levels of pro-inflammatory cytokines [281, 313, 322]. In both mice and rats a Th1 response triggers an elevation of IL-2, TNFα and IFNγ cytokines, and the loss of the fetus may occur [283, 323]. When a Th2 response occurs, increases in IL-4 and IL-10 have been shown to protect and maintain the pregnancy through birth. In HO-1 knockout mice abortion will occur even if Th2 cytokines have been upregulated [283]. HO-1 and its by-products are essential for maintaining pregnancy, protecting against eclampsia and uterine contractility in humans and other mammals [281, 313]. HO-1 has also been shown to play an important role in bacterial infections that cause abortion, such as *Listeria monocytogenes* [324].

The equine immune response during pregnancy has been well documented [325-327]. The importance of HO-1 in the horse is yet to be determined. Preliminary data (presented below) shows an increased level of both HO-1 and IL-10 in pregnant mares, and mares with increased MAT titers to *Leptospira*. The studies below were undertaken to determine the normal serum levels of HO-1 in the horse. By establishing the basal levels of HO-1 we hope to determine changes, if any, when horses become ill or pregnant, to avert potential serious life-threatening disease and/or abortion.

4.7 Materials and Methods

4.7.1 Pilot Study

In 2015 there were no published papers referencing serum HO-1 levels in horses. Therefore, a preliminary study was undertaken to determine if HO-1 could be measured. The literature indicates HO-1 is upregulated in pregnancy and/or infections in humans, rats and mice. The farm chosen for the study recently had cases of leptospirosis confirmed by University of Kentucky Veterinary Diagnostic Laboratory in pregnant mares.

4.7.1.1 Study Design

Blood samples were collected from 9 mares by the resident veterinarian on one farm for four consecutive weeks during 2015. The first blood samples taken were 20-40 days after 6 mares were bred. Three mares included in the study were not pregnant and had been bred 3 months before the study but came up empty at day 28 post breeding (30+days prior to first sample). Samples were submitted to the VDL and analyzed for leptospirosis by microagglutination test (MAT), centrifuged and serum was removed. Serum was stored at 4C prior to MAT analysis. Samples were stored at -20C after MAT analysis. One additional sample was taken 294 days from day one. MAT was performed in accordance with the methodology of the National Veterinary Services Laboratories (Ames, IA) and the OIE (World Organization for Animal Health, 6th edition) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Procedure. The reference antigens were propagated in polysorbate-80 bovine albumin media for 4-8 days

and diluted to working strength on the day of the test. Serum was screened at a 1:50 dilution (in phosphate buffered saline) in a volume of 50µl in wells of a 96-well flat bottom plate containing 50µl of diluted antigen. The final serum dilution was therefore 1:100. The plate was incubated at room temperature for 1½ hours. After incubation the plates were examined for agglutination using dark-field microscopy. A reaction was deemed positive when 50% of the antigen was agglutinated. Control sera from horses with known titers were run concurrently on each day of testing. Sera that demonstrated at least 50% agglutination at the screening dilution of 1:100 was serially diluted with their respective antigen to determine an endpoint titer. *Leptospira* reference serovars used included Canicola, strain Hond Utretcht IV; Copenhageni (Icterohemorragica reference) strain M-20; Grippotyphosa, strain Andaman; Hardjo, strain Hardjoprajtino; and Pomona, strain Pomona.

Serum samples were also analyzed by Enzyme-Linked Immunosorbent Assay **(**ELISA) for the following: Equine IL-10, Catalog#GR106003 (Genorise Scientific Inc., Glen Mills, PA; https://www.genorise.com) and Equine HO-1, Catalog#MBS019561 (MyBioSource, San Diego, CA; https://www.mybiosource.com). ELISA tests were carried out following manufacturer's instructions; both standards and samples were run in duplicate. The sensitivity of the IL-10 assay is 2pg/ml with a detection range of 6-2000pg/ml. The intra and inter-assay coefficient of variation is 5% and 8% respectively. The sensitivity of the HO-1 ELISA kit is 0.1ng/ml and range of

detection is 0.625-20ng/ml. Both the intra- and inter-assay coefficient of variation is less than 15% for HO-1 assay.

4.7.1.2 Statistics

Statistical analysis was carried out using student's t-test and least squares fit (JMP v12, SAS, Cary, NC). Confidence levels were set at 95%.

Funding: This study was supported by a grant from the Southeast Center for Agricultural Health and Injury Prevention, via NIOSH/CDC Cooperative Agreement U500H007547.

4.8 Results

MAT, IL-10 and HO-1 data are listed in Tables 4.2 and 4.3 respectively. The primary serovar identified was Pomona. There was a significant difference in HO-1 ($p<0.002$) levels in *Leptospira* positive compared to negative mares and pregnancy alone showed a significant difference in HO-1($p<0.02$), but not in IL-10 concentrations (Figure 4.2).

Table 4.2: MAT Pilot Study Results

Animal	Pomona Day 1	Pomona Day 7	Pomona Day 21/Day 14*	Pomona Day 28	Pomona Day 294
Mare 1[P]	Negative	102,400	51,200	51,200	1600
Mare 2[P]	Negative	102,400	102,400	102,400	1600
Mare 3[P]	Negative	NA	204,800	NT	1600
Mare 4[P]	Negative	51,200	51,200	51,200	1600
Mare 5	Negative	12,800	51,200	12,800	100
Mare 6	100	100	100	NT	ND
Mare 7	1660	3200	1600	NT	ND
Mare 8[P]	400	400	800	NT	ND
Mare 9[P]	100	100	100	NT	ND

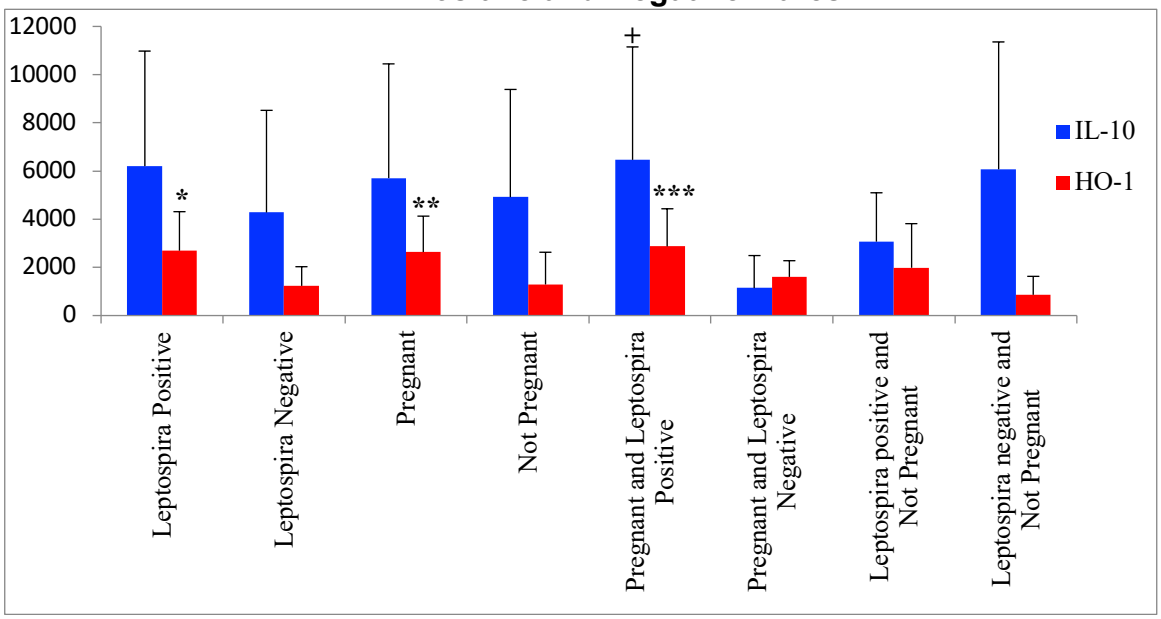
P=Pregnant; NT=not tested; ND=Non-detectable
*Day 21 for mares 1-5 and Day 14 for mares 6-9

Table 4.3: IL-10 and HO-1 Pilot Study

Animal	Day 1		Day 7		Day 21/Day 14*		Day 28		Day 294	
	IL-10	HO-1	IL-10	HO-1	IL-10	HO-1	IL-10	HO-1	IL-10	HO-1
Mare 1[P]	9.6	3.9	11.9	2.3	15.3	3	11.9	3.2	16.9	1.3
Mare 2[P]	0.66	4.4	0.98	4.3	2.9	5	3.7	3.9	4	1.7
Mare 3[P]	9.4	4.5	10.6	3.6	10.6	4.2	10.9	3.9	5.1	BDL
Mare 4[P]	4.2	3.6	3	3	4.4	3.5	3	BDL	0.91	BDL
Mare 5	2.5	0.86	6.4	5.2	1	1.2	3.4	1.3	2.1	1.4
Mare 6	8	1.1	9.6	1.4	10.3	1.4	NT	NT	14.4	BDL
Mare 7	BDL	2	4.1	1	BDL	BDL	NT	NT	2.2	BDL
Mare 8[P]	BDL	3.4	3.3	1.4	3.3	0.66	NT	NT	2.6	2.4
Mare 9[P]	BDL	2.6	3.5	1.8	5.2	1.5	NT	NT	2	0.91

BDL=below detectable limit; P=pregnant; NT=not tested
*Day 21 for mares 1-5 and Day 14 for mares 6-9
IL-10 and HO-1 reported in ng/ml

Figure 4.2: Pilot Study Levels of IL-10 and HO-1 Levels in *Leptospira* Positive and Negative Mares



IL-10 and HO-1 reported in pg/ml
*p<0.002; **p<0.009; ***p<0.04; +p<0.04

There was no significant difference in either IL-10 or HO-1 levels in barren mares regardless of *Leptospira* state. When comparing HO-1 levels in pregnant mares over time, there was a significant difference between day 1 and 7 ($p<0.03$), and day 1 and 28 ($p<0.04$). Additionally, HO-1 at day 294 in the pregnant mare group was significantly different in all the days (day 1 $p<0.0002$; day 7 $p<0.002$; day 14 $p<0.01$) except for days 28 and 294. There is a significant correlation on both day 1 ($p<0.003$) and day 21 ($p<0.02$) in pregnant horses, as MAT levels increased so did HO-1 levels, when compared to non-pregnant mares. There were no significant correlations detected for IL-10 to MAT levels in either group.

This is the first study to show a response to HO-1 in both pregnant/non-pregnant mares, with/without increased leptospiral titers. The significance of this

protein in either pregnancy or infection in horses is yet to be determined. However, this pilot study has shown HO-1 could be detected and differences do appear to occur with pregnancy and leptospiral infection. This study laid the foundation for the larger study which follows.

4.9 Determination of HO-1 Levels in Horses Located in Central Kentucky

4.9.1 Materials and Methods

4.9.1.1 Study Design

The pilot study showed HO-1 could be measured in equine serum, this larger study was undertaken to identify the following:

1. Determine HO-1 levels in healthy non-pregnant mares in Central Kentucky
2. Determine HO-1 levels in mares in three groups:

4.9.1.2 Animals

4.9.1.2.1 Group One

The following samples were submitted to the University of Kentucky Veterinary Diagnostic Laboratory (VDL) for leptospiral screening titers. All serum samples were fresh and from pregnant mares except for two samples from foals.

1. **MAT titers of ≥25,600 to either serovar Pomona or Grippotyphosa**: 64 mares fit this requirement and 1 sample from each mare was analyzed except for the following:

    a. 9 mares had two samples within one month, both analyzed
    b. 2 samples were foals

4.9.1.1.2 Group Two

Serum samples for Group Two were provided by Dr. Barry Ball, University of Kentucky, Professor of Veterinary Science. All serum samples in Group Two had been frozen at -20^{o}C.

4.9.1.2.2.1 Control: 51 healthy mares not pregnant, located on the University of Kentucky's Research Farm were designated as the control group. One sample from each mare was analyzed. Sample times varied from February 2013 to February 2018, with the majority of samples collected between the months of May-July.

4.9.1.2.2.2 Field: 44 pregnant mares were included in this group. These samples were collected from horses located on farms in Central Kentucky. Samples analyzed were collected from 7 months of pregnancy till foaling or abortion. Twenty pregnant mares were separated into two groups of 10, designated as field with and without supplement (Table 4.4). The supplement given was Altrenogest, a synthetic progestin. The remaining 24 pregnant mares had a number of different conditions listed in Table 4.5. One sample from each mare in this group were analyzed at the following time points of pregnancy:

c. 7 months
d. 9 months
e. After foaling/abortion

Table 4.4: Field Samples

Group	Number of Mares
Field	
No Supplement	10
Supplement	10
Case	24

Table 4.5: Field Samples-Case

Diagnosis	Number of Cases
Umbilical torsion	1
Placentitis	2
Bacterial Placentitis	2
Nocardioform Placentitis	2
Mild acute placentitis	1
Mild focal placentitis	2
Inconclusive	1
Viral Placentitis-EHV-1	1
Mild placentitis-dystocia	2
Idiopathic	1
Separation	1
Minimal placentitis/amnionitis	1
Chronic focal bacterial placentitis-Nocardioform	1
Placenta previa-foal dead separation	1
Focal, chronic placentitis	1
Bacterial and mycotic yeast placentitis	1
Minimal focal suppurative placentitis	1
Focal severe bacterial placentitis	1
Amnionitis and funistis	1

4.9.1.2.2.3 Pregnant mares over time: 18 healthy mares located on the University of Kentucky's Research Farm, were included in this group. All mares carried to full term. One serum sample from each mare at the following time points of pregnancy was analyzed:

f. Initial sample day 15 -April 2012

g. 75 days-June 2012

h. 250 days-November 2012

i. 310 days-January 2013

j. 325 days-January 2013

k. 340 days-January 2013

4.9.1.2.3 Group Three

1. A group of pregnant mares that had aborted due to leptospirosis were also analyzed. **Leptospiral Positive Mares who aborted**: 34 mares had aborted because of confirmed leptospiral infection (samples provided by Dr. John Timoney, Professor University of Kentucky Veterinary Science Department). One sample from each mare was analyzed.

4.9.2 Serum Sample Processing

4.9.2.1 MAT Analysis

Fresh blood samples for MAT were submitted to the University of Kentucky's Veterinary Diagnostic Laboratory (Group One), centrifuged and serum was removed. Serum was stored at 4C prior to MAT analysis. Samples were stored at -20^{O}C after MAT analysis. Serum samples were analyzed for leptospirosis using MAT. MAT was performed in accordance with the methodology of the National Veterinary Services Laboratories (Ames, IA) and the

OIE (World Organization for Animal Health, 6th edition) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Procedure. The reference antigens were propagated in polysorbate-80 bovine albumin media for 4-8 days and diluted to working strength on the day of the test. Serum was screened at a 1:50 dilution (in phosphate buffered saline) in a volume of 50µl in wells of a 96-well flat bottom plate containing 50µl of diluted antigen. The final serum dilution was therefore 1:100. The plate was incubated at room temperature for 1½ hours. After incubation the plates were examined for agglutination using dark-field microscopy. A reaction was deemed positive when 50% of the antigen was agglutinated. Control sera from horses with known titers were run concurrently on each day of testing. Sera that demonstrated at least 50% agglutination at the screening dilution of 1:100 were serially diluted with their respective antigen to determine an endpoint titer. *Leptospira* reference serovars used included Grippotyphosa, strain Andaman and Pomona, strain Pomona.

4.9.2.2 HO-1 Analysis

Serum samples for HO-1 analysis were acquired as followed: Frozen serum samples for control, field samples and pregnant mares over time (Group Two) were provided by Dr. Barry Ball, University of Kentucky, Professor of Veterinary Science, Lexington, Kentucky (samples collected between 2013-2018); frozen serum samples from leptospiral positive mares who aborted (Group Three) were provided by Dr. John Timoney, University of Kentucky Professor of Veterinary Science, Lexington, Kentucky (samples collected between 1991-

1998). Fresh serum samples submitted to UKVDL between November 2017 and January 2018 were also analyzed.

Samples for HO-1 analysis were processed as follows: samples were analyzed by ELISA for the following: Equine HO-1, Catalog#MBS019561 (MyBioSource, San Diego, CA; https://www.mybiosource.com). ELISA was carried out following manufacturer's instructions; both standards and samples were run in duplicate. The sensitivity of the HO-1 ELISA kit is 0.1ng/ml and range of detection is 0.625-20ng/ml. Both the intra- and inter-assay coefficient of variation is less than 15%.

4.9.3 Statistical Analysis

One of the primary reasons for this study was to determine the normal levels of HO-1 in horses in order to identify changes related to stressors such as pregnancy and illness. A power analysis for one-way independent ANOVA was performed to determine the number of animals needed for a significant difference at $p=0.05$, 216 animals were included with a total of 340 samples analyzed for HO-1. All HO-1 samples were analyzed using ELISA kits from the same lot number.

UKVDL samples were analyzed using a one-way ANOVA for differences between MAT serovars, and serovar/HO-1 results. The effect of serovar and titer was determined by Standard Least Squares. Nine samples taken a month apart were analyzed using Matching Pair.

Field samples were analyzed using one-way ANOVA for non-pregnant control mares and time of estrous. Field samples overtime were analyzed using MANOVA. Tukey's Means Comparison was used to analyze any differences amongst field samples and between leptospiral survey samples. All samples were analyzed using JMP v13.2 (SAS, Cary, NC). Significance was set to $p \leq 0.05$.

4.10 Results

4.10.1 Group One: UKVDL Leptospiral Survey Samples

Results for HO-1 are in Table 4.6. There were no differences seen with serovar/MAT and HO-1 outcome.

Table 4.6: UKVDL Leptospiral Survey

Serovar	Number of Samples	Effect of Serovar	Effect of Titer	HO-1 (μg/ml)
Pomona	62	None	None	8.3
Grippotyphosa	3	None	None	5.5

*p=0.0437

Serovar Pomona had significantly higher titers compared to serovar Grippotyphosa ($p<0.0437$). Additionally, 9 animals had serum analyzed a month from the initial sample. Seven of these were mares and 2 were foals. Eight had elevated titers to serovar Pomona (two foals and 6 mares), and 1 to Grippotyphosa. Results in Table 4.7.

Table 4.7: Matched Paired MAT/HO-1

Horse Number	Serovar	MAT Titer First Second*	HO-1** First Second*
1	Pomona	25600 12800	7.0 10.5
2	Grippotyphosa	25600 12800	3.7 5.7
3	Pomona	51200 12800	3.3 5.8
4	Pomona	51200 12800	5.8 3.4
5	Pomona	51200 12800	3.4 6.4
6-Foal	Pomona	102400 51200	47*** 7.4
7	Pomona	204800 102400	7.4 8.3
8-Foal	Pomona	204800 12800	43*** 6.4
9-Mare Aborted	Pomona	102400 204800	19.6 11.9

*Taken one month after first sample
**ng/ml
***Above standard curve, both removed prior to statistical analysis

Using Matching Pair analysis within and among pairs by titer and HO-1, a significant difference was seen ($p=0.0411$). As titer went down HO-1 went up in 5/7 mares, all were still pregnant at the time of the second blood collection. One mare (#9) had an increased MAT titer, but HO-1 decreased. The second sample was taken after she aborted. MAT titers and HO-1 levels for both foals decreased. There was no change looking at differences by serovar overtime. A

Oneway analysis and using Tukey-Kramer HSD Means Comparisons of HO-1 by titer showed a significant difference between mares with MAT titers of 102,400 and 51,200 (p=0.0412).

4.10.2 Group Two

Results for control mares (healthy and not pregnant) can be found in Table 8. The mean HO-1 levels for control mares were 5.6μg/ml. There were 12/39 (23.5%) samples above the standard curve, excluded from statistical analysis and not included in Table 4.8. There were no significant differences in HO-1 in mares who were in estrous and those who were not.

Table 4.8: Control Mare Field Samples

Sample Number	Days in Estrous	HO-1*
1		3
3		8.1
4		6.7
5		1.7
6		2
7		11.4
8		0.85
10		1.8
11		1
12		3.6
13	12	0.44
15		3.2
16		2.9
17		8.3
18		4.5
20	6	4.1
21	8	4.5
22	3	15
23	2	9.2
25	12	1.7
26	12	3.1
27	0	1.8
28		4
29	1	8.5
30	12	3.4
33		2.3
34		2.9
35		2.2
36	8	7.2
37	14	13
38	6	19.7
39	12	4.2
41	6	4.9
44	6	2.8
45	9	3.5
48	12	17.6
49	10	17.9
50	6	3.1
51		3

*ng/ml

Samples were acquired from mares in the Field group (pregnant with and without supplement, and case) over a period of four months, starting in December of 2013 through May 2014 (actual start date of samples varied from mare to mare). Comparison of means for initial, midway and the last blood sample taken (time of foaling/abortion) are in Table 4.9. Comparing mares in this group only (those with and without supplement) to case mares, there were no significant differences over time between or within groups using MANOVA.

Table 4.9: Field Samples Over Time-HO-1*

Group	All Samples	Initial HO-1	Middle HO-1	Last HO-1
Control	6.6			
NS		6.2	7.3	8.1
S		5.6	6.3	6.4
Case	6.8	7.6	6.1	5.7

NS=No supplement
S=Supplement
*ng/ml

Mean HO-1 results for field samples (no supplement, supplement and case) of all samples taken over 4 months are in Table 4.10. There were no significant differences between groups.

Table 4.10: Field Study No Supplement, Supplement and Case HO-1

Group	HO-1*
No Supplement	7.2
Supplement	6.1
Case	6.8

*ng/ml

Results for mares pregnant over time are in Table 4.11. There were 18 mares in this group. Blood samples were drawn on the following days of

gestation: 15, 75, 250, 310, 325 and 340 depending on time of foaling. Results were averaged according to the day of sample collection. There was no significant difference regardless of days of gestation.

Table 4.11: Mares Pregnant Over Time-HO-1

Day of Gestation	HO-1*
15	5.1
75	5.7
250	5.5
310	5
325	4.9
340	6.2

*ng/ml

Results for control, pregnant overtime and field samples (combined no supplement/supplement) can be found in Table 4.12. There were no significant differences.

Table 4.12: Control, Pregnant Overtime and Field Sample

Group	HO-1*
Control	4.7
Pregnant Overtime	5.4
Field	
Control-NS+S	6.6
Case	6.8

*ng/ml

Results from control, pregnant mares overtime, field samples (no supplement, supplement and case) and leptospirosis survey samples can be found in Table 4.13. There was a significant difference between leptospirosis survey samples and control ($p<0.0017$) and pregnant overtime ($p<0.0011$).

Table 4.13: Field, Pregnant Overtime and *Leptospira* Survey

Group	HO-1*
Not Pregnant (Control)	4.7
Pregnant Overtime	5.4
Field	
No Supplement	7.2
Supplement	6.1
Case	6.8
Leptospirosis Survey Samples	8.3**+

*ng/ml
**p<0.0017 to Not Pregnant (Control)
+p<0.0011 to Pregnant OT

All field (no supplement and supplement) carried to term. The average days of gestation in the field group with no supplement was 333.4 days compared to 328.4 days in the supplement group. Field case mares had 6 abortions (2 never made it to start of study, no blood samples were taken). A total of 18/24 mares in the case group carried to full term. The average gestation was 342 days, although including 6 mares who aborted the average gestation was 332.3 days. Comparing gestation days (including all case mares and those that aborted) there was no statistical difference within the field group. However, if the 6 mares who aborted using Tukey-Kramer HSD means comparisons are excluded, there is a significant difference between case/supplement (p=0.0067).

Table 4.14: HO-1 Levels: Field, Pregnant Overtime, Leptospirosis Survey and Leptospirosis/Mares Aborted

Group	HO-1*
Not Pregnant (Control)	4.7
Pregnant Overtime	5.4
Field	
No Supplement	7.2
Supplement	6.1
Case	6.8
Leptospirosis/Mares Aborted	5.2
Leptospirosis Survey Samples	8.3**+#

*ng/ml
*p<0.0016 to Not Pregnant (Control)
+p=0.001 Pregnant OT
#p=0.0232 Leptospirosis/Mares Aborted

4.10.3 Group Three

When samples from mares who had been identified with leptospirosis and aborted were compared to all others there was a significant difference only for leptospirosis survey samples (p<0.0232) results are in Table 4.14.

4.11 Discussion

The role of HO-1 in horses has not previously been elucidated. A pilot study was conducted in 2015 to determine if HO-1 levels could be detected in the serum of horses. HO-1 levels in non-pregnant mares were significantly lower compared to pregnant mares. These results are consistent with literature in both humans, rats and mice showing an increase in HO-1 levels during pregnancy [282, 299, 317]. The last sample taken (day 295) was after foaling and shows a decrease in HO-1 similar to what has been shown in other mammals [313, 328]. These data concur with HO-1 levels in other mammals that are pregnant and

have underlying maladies such as PE, hypertension and other disease entities [318, 328, 329].

The first part of this project examined HO-1 levels in pregnant mares screened for leptospirosis through UKVDL. By the time the second study was initiated an equine leptospiral vaccine, specific for serovar Pomona had been on the market for two years. Data have shown MAT titers post-vaccination in horses are around 1600, but titers up to 6400 have been seen [330]. Because of this, horses submitted were required to have titers ≥25,600. Regardless of serovar or MAT titer, HO-1 mean level was the same for Pomona and Grippotyphosa. Sixty-four horses were included in this part of the study and only 9 had an additional sample analyzed for comparison. The small sample size (N=9) makes it difficult to conclude if there truly is no difference between the dominate serovar and HO-1 levels. The host and its response to the prevailing serovar will determine the severity of infection by MAT [331]. Differences in host response have been seen depending on infective serovar [86, 157, 185]. The HO-1 response to bacterial virulence can be detrimental to the host (Vasil 1999; Chung 2008). A larger study is needed to determine the extent to which the infective serovar affects HO-1 levels.

Seven mares and two foals had two samples analyzed a month apart. MAT levels in 8/9 animals decreased over one month, however only 4/9 showed a decrease in HO-1 levels. Decreased levels in 3/4 were the foals and a mare who had aborted. These three show a similar trend seen in other mammals. Both animals and humans have shown a decrease in HO-1 right before and after

abortion has occurred [283, 285, 314]. HO-1 has been shown to decrease overtime after infections [332, 333]. The small subset of animals (N=9) and smaller number who showed a decrease in HO-1 (N=3), makes it impossible to determine if this is the same in horses. A larger study is needed to determine if this is also true in horses. The second MAT titer analyzed from the mare who aborted increased while HO-1 decreased, showing pregnancy may influence HO-1 levels more than infection in horses. Increases in HO-1 levels in 5/9 mares whose MAT titers decreased also shows the influence pregnancy has on HO-1 levels. This has been seen in other pregnant mammals with bacterial infections [324, 334, 335].

Estrous cycle in Group Two-healthy nonpregnant mares showed no differences in HO-1 levels compared to mares not in estrous. These results are different compared to other mammals such as mice and rats. In both rats and mice HO-1 has been shown to increase during estrous [336, 337]. In mice higher levels of HO-1 occur during metestrus [337]. Mares in the field group were divided into 3 groups (control no supplement, control supplement and case). Control mares with or without supplement and case mares showed no significant differences in HO-1 levels over a period of 20 weeks. This concurs with results seen in the leptospiral survey group. Pregnancy seems to play a greater role in HO-1 levels than infection. A majority of mares in the case group (79%) had placentitis, regardless their HO-1 levels were very similar to mares who had no known health issue.

Mares pregnant overtime also showed no significant differences regardless of the day of gestation. Nor were there any differences comparing this group to the field mares, this strengthens the concept HO-1 levels are influenced more by pregnancy. Case mares who aborted did not show any decrease in HO-1 levels prior to abortion, seen in other mammals.

The leptospirosis survey samples had significantly higher HO-1 when compared to non-pregnant and pregnant overtime mares. The significantly higher HO-1 in leptospiral survey mares maybe due to the type of bacterial infection in addition to pregnancy. The length of gestation of mares in the leptospiral survey group were unknown, which could also have influenced HO-1 levels. Both field case and leptospiral survey mares had significantly higher HO-1 levels when compared to nonpregnant mares. In other mammals HO-1 is upregulated during infection [274, 279, 338]. Increased HO-1 levels have been shown in infections caused by *Corynebacterium*, *Pseudomonas*, *Listeria*, *Acinetobacter* and others [339-341]. Bacteria, viruses and fungi also have heme oxygenase, including those bacteria previously mentioned [339, 342]. The HO found in bacteria is needed for the breakdown of heme and uptake of iron. Iron is essential for the bacteria's pathogenicity, virulence, colonization and survival [341]. The host attempts to limit free iron by the use of iron-binding molecules, however bacteria have developed ways to circumvent the limited availability. Some bacteria have outer membrane receptors for ferrous iron such as *Campylobacter jejuni* [341]. Other bacteria, such as *Pseudomonas*, secrete siderophores that bind ferrous iron and can transport it back into the bacteria

[343]. Still others acquire iron by the degradation of heme through heme oxygenase. *Leptospira* have HO and require it for the breakdown of heme *in vivo* and to establish colonization in the host [344-346]. *Leptospira* can bind to heme through the hemin-binding protein (HbpA) [347-349]. Heme oxygenase gene in pathogenic *Leptospira* (hemO) is also required for host pathogenicity [345]. *Leptospira* acquire iron from heme needed for growth *in vivo* from the host [350]. Both case and leptospiral survey groups had higher levels of HO-1. This could be due to additional breakdown of heme by the bacteria or viral infections ongoing in these mares. Specifically, the leptospiral survey group had significantly higher levels of HO-1 than any other group analyzed. *Leptospira* have been shown to be one of the leading causes of placentitis leading to abortion in central Kentucky [255]. *Leptospira* cause a consistent diffuse placentitis compared to any other causes of placentitis. Large numbers of spirochetes can be found throughout the placenta and this diffuse placentitis originates from hematogenous [255]. It is possible with the increased flux of blood caused by *Leptospira* in the placenta, host HO-1 is also upregulated, and a feedback loop occurs by *Leptospira* causing increased bleeding and spirochetes utilizing heme, thereby increasing their proliferation. A similar feedback loop has been proposed in *Pseudomonas* [351]. The upregulation of HO-1 and cytokines by pregnant mares with leptospiral infection may also protect from abortion.

In *Pseudomonas* and *Neisseria* infections, heme oxygenase (HemO) have a smaller solvent-accessible surface when compared to human HO. Both have less than 15% homology to human HO-1 [270]. Small antimicrobial molecules

against this site may have potential to inhibit these bacteria from pathogenicity and proliferation. In Pseudomonas's HemO an additional binding site has been identified, where a small molecule with a lipophilic group can possibly bind the hydrophobic pocket [270]. The binding would impair the HemO stability/activity of the enzyme by stopping the initial hydroxylation of the heme group [352]. Recently the use of a gallium(III)-salophen has shown promise to inhibit both the HO and iron uptake in *Pseudomonas* [351]. Similar structures are now being investigated with *Leptospira* [346]. The crystal structure of *Leptospira* HemO shows several key areas used to block the enzyme activity. By targeting these differences found only in bacterial heme oxygenase may be one method to stop progression of infection.

HO-1 in other mammals is upregulated in the placenta under normal pregnancy [311, 353]. HO-1 gene (HMOX1) has been detected in the placenta of mares [354]. HMOX1 in the equine placenta is upregulated and appears to stay constant through gestation. A study evaluating gene expression in the equine placenta from 45 days gestation to 10 months showed no difference in HMOX1 during this time period [354].

Serum HO-1 levels in this study ranged from 0.44ng/ml to 47ng/ml. Non-pregnant mares had the lowest HO-1 average (4.7ng/ml). HO-1 levels in humans have been shown to range from below 0.5ng/ml to 10.1ng/ml [355-357]. HO-1 average serum levels in pregnant healthy women in one study was 1.9ng/ml, while non-pregnant controls levels averaged 3.5 [355]. Another study showed average HO-1 levels in healthy pregnant women was 1.8ng/ml [356].

There can be several reasons for the differences between HO-1 levels seen in humans vs horses in this study. ELISA kits from different manufacturers were used which can lead to discrepancies. Also, the differences in RBC and hemoglobin between humans and horses can be contributing factors. Rouleaux formation, increased number of RBCs and lower oxygen binding in horses may all be contributing factors in higher HO-1 levels compared to humans.

The mare's gestation is unique compared to other mammals, including humans. Relaxin is produced in the mare's placental trophoblast cells and concentrations increase in late gestation and increase further during labor [358, 359]. Whereas in swine the corpora lutea is the major site of relaxin during pregnancy [360]. Breed differences of relaxin have been seen in horses during pregnancy. Relaxin in Thoroughbreds was undetectable until day 80, levels rise till day 175 then decline to day 225 and increase until foaling [359]. Whereas in Standardbreds increases occurred till day 150 and slowly declined until foaling. Relaxin in humans and mares is important in for vasodilation and maintaining the placenta during pregnancy [361, 362]. In women and dogs, lower levels of relaxin occur when symptoms of approaching miscarriage during early pregnancy are seen [360]. The mare has a specialized area between the yolk sac and chorioallantois forming the chorionic girdle made up of trophoblastic cells, which is visible ~gestation day 25 [363]. The chorionic girdle transports nutrients and hormones between the uterus and placenta. Trophoblast cells in humans contribute to HO-1 levels in the placenta [353]. The HO-1 levels in this study tended to show an increase till mid-late gestation followed by a decrease and an

increase towards foaling. This is not what has been shown in other mammals. The HO-1 levels seem to follow relaxin levels shown in pregnant mares. The increase in trophoblasts between the yolk sac and chorioallantois may also be contributing to these differences HO-1 levels in mares. Vascular endothelial growth factor (VEGF) has been shown to require HO-1 activity [309]. Relaxin has also been shown to be strongly associated with VEGF [364-366]. Both relaxin and HO-1 are critical in regulation of angiogenesis during pregnancy and are regulated by MAPK and p38 pathways [309, 365]. Relaxin gene (RLN) in the horse has been shown to be highly upregulated from 45 days to 10 months of gestation [354]. The importance of both HO-1 and relaxin in angiogenesis increases their association during pregnancy in mares. Further studies are needed to ascertain the connection between relaxin and HO-1 in mares.

Limitations of this study include the use of several studies from different years. Not having a study from start to finish carried out is subject to additional variations, including sample handling and storage and variation of parameters in studies.

This study showed HO-1 in the serum of horses is upregulated in both mares pregnant with significant leptospiral MAT titers and with placentitis. Further studies are needed to determine HO-1 progression before, during and after normal pregnancy and any complications during pregnancy. Additional studies are also needed to ascertain the increase in HO-1 levels in mares who are pregnant and have high MAT titers to *Leptospira*, and complications that may

occur if HO-1 levels decline. The importance of HO-1 in infection in horses and pregnancy in the mare requires further research.

Chapter 5

Discussion and Future Studies

5.1 Discussion and Future Studies

Leptospirosis in mammals continues to cause global morbidity, mortality, animal welfare concerns, and economic losses. Developing countries, which can least afford the economic losses, are the most affected. Developed countries including the United States (US) have seen an increase in the past 20 years in morbidity, especially among domestic animals. The low incidence of leptospirosis in humans in the US led to CDC removing it from the notifiable disease list in 1990s. However, leptospirosis was placed back on the list in 2013 due to a rise in both animal and human cases.

Compared to developing countries, the US has typically reported low numbers of leptospiral cases for both animals and humans. A concern is that leptospirosis is not being considered in the differential diagnosis list. In many parts of the world, leptospirosis is considered endemic, therefore the diagnosis and tracking of outbreaks are routine. The value of a near-real-time monitoring system for disease outbreaks has been underscored by the recent COVID-19 pandemic. Because leptospirosis is a zoonotic disease, monitoring incidence in animals is critical. Improved understanding of the epidemiology and clinical presentation of leptospirosis by both veterinarians and physicians will aid in successfully identification of cases.

The changing climate, including increasing temperature and precipitation, favors the increased presence of leptospires in the environment [150]. Several

recent peer-reviewed scientific articles have cited climate change as an accelerator of active clinical cases of leptospirosis [73, 246]. Therefore, tracking and confirming diagnoses is increasingly more important, not only for leptospirosis, but other endemic and emerging microbial diseases. Even with the advent of vaccines, animal cases have been on the rise. There are some data suggesting that leptospirosis vaccines are not widely utilized. For example, in dogs the vaccine can cause a mass to form at the injection site, causing some owners and veterinarians to want to avoid the vaccine. Other possible causes of perceived decreased effectiveness of the vaccines are serovar drift, increased testing, urbanization and climate change.

Chapter Two examined the frequency of leptospiral diagnosis in equine and bovine cases by AAVLD laboratories in the US. The number of submissions for leptospiral testing in 17 states varied by a wide margin, from 1 up to 2731/year. This discrepancy likely reflects the difference in testing from state to state or lack of centralized reporting of the disease. As has been mentioned, the inconsistency in the number of cases tested and diagnosed in the US may reflect the lack of suspicion of cases by veterinarians, meaning the incidence may be higher. More active surveillance for the disease over time would provide a clearer representation of leptospirosis in animals. Additionally, educating the general public about leptospirosis, risk of infection, and preventive measures (e.g. vaccines), would increase awareness and could lead to decreasing infections in both animals and humans.

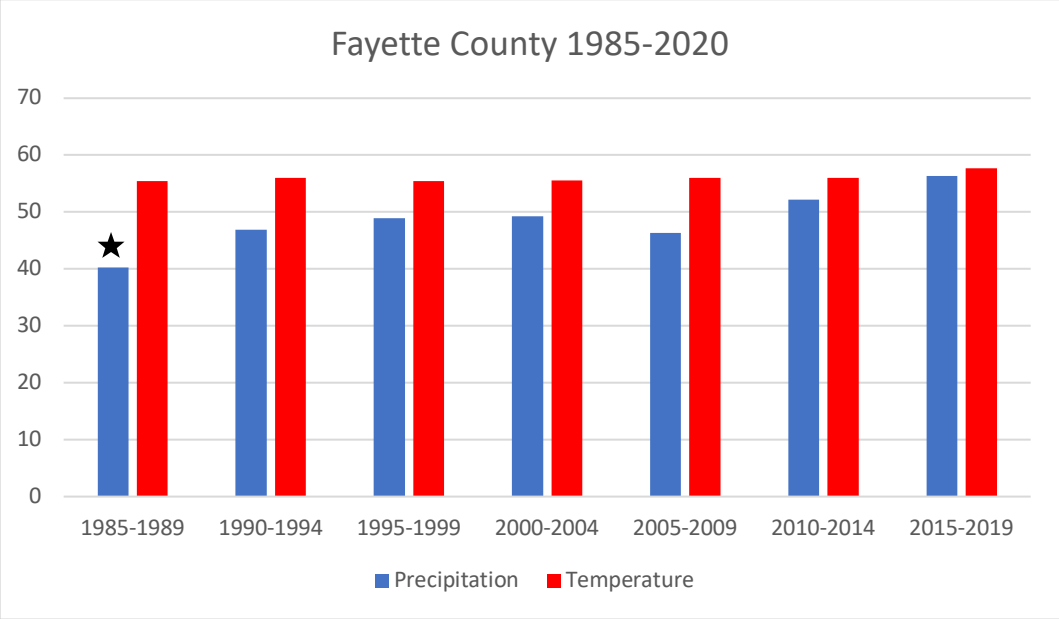
Equine leptospirosis continues to be a problem in Central Kentucky. However, with the advent of a vaccine in 2015 an apparent decrease in the number of abortions has been seen. However, controlled studies need to be done on horse farms to determine the ability of the vaccine to prevent and control equine leptospirosis. Chapter Three emphasized leptospiral seroprevalence in the general equine population of Central Kentucky. Over 25% of horses tested have a positive titer ≥200, suggesting that horse exposure to *Leptospira* is common in Central Kentucky. A significantly greater number of females had titers compared to males. Separating out females in pastures may help eliminate exposure/re-exposure. Decreasing exposure to wildlife can also help mitigate exposure and risk for becoming infected by *Leptospira*.

Since 2009, 89 leptospiral abortions have been confirmed by the UK Veterinary Diagnostic Laboratory, most of which occurred in Central Kentucky [367]. Serovar Pomona str. kennewicki is the main cause of equine abortions in Central Kentucky, with Grippotyphosa playing a minor role. Seroconversion to serovar Bratislava has been seen in Central Kentucky since the mid-1980s [87]. Identification of serovar Bratislava by culture has never been reported in the US equine population yet the serovar has been isolated frequently in Ireland and Great Britain [157, 177]. Early papers published from samples collected in Central Kentucky from aborted fetuses showed an occasional abortion caused by serovar Bratislava diagnosed by MAT [254]. One plausible reason the strain of serovar Bratislava seen in the US hasn't been isolated could be it is transient in horses and does not colonize any organs. Increased titers to the serovar which

occur often could be from repeated environmental exposure only. Bratislava may not colonize the kidneys therefore short or long-term shedding is not seen. A recent paper looking at colonization of serovar Bratislava could not isolate the organism after repeated inoculations, however increased titers were seen [368]. One reason for this outcome could have been the strain used was isolated from a pig in 1989 with repeated freeze/thawing. The swine strain used may not be able to colonize the horse at all and increased titers showed seroconversion only, which is frequently seen. The study didn't start collecting samples until day 3 possibly missing the time of shedding. To truly know whether serovar Bratislava is a problem in horses the organism needs to be isolated and studied to determine its' ability to colonize in an organ. Studies looking at shedding should start at day zero (inoculation day) to see if the organism is passing through immediately (between days 0-3) indicating an immune response but no colonization. The horse has long thought to be host adapted to serovar Bratislava; however recent genotyping has found this may not be true [157].

Since 1985 the change in precipitation and temperature in Central Kentucky has been significant. Looking at 5-year increments, precipitation/temperature has changed from 40.3"/55.4^{o}F in 1985-1989 to 56.3"/57.7^{o}F in 2015-2019, Figure 5.1. Temperature was not seen as significant by statistical analysis, but a change in over 2^{o}F is considered significant in this period of time [67]. This change maybe causing environmental pressure on serovars seen globally and in Central Kentucky.

Figure 5.1: Five-Year Averages for Temperature and Precipitation in Fayette County, Kentucky 1985-2020



★p=0.0446 between 1985-1989 and 2015-2019

Changes in serovar prevalence on samples submitted to a diagnostic laboratory have occurred in the state over time from Icterohaemorrhagiae/Bratislava/Grippotyphosa/Pomona (49.1%, 76.3%, 20.1% and 15.9% respectively) in 1989/1990 to Bratislava/Grippotyphosa/Pomona (18.1%, 4.2% and 2.8% respectively) in this study (Table 1) [230].

Table 5.1: Serovar Prevalence in Central Kentucky

Serovar	%Prevalence 1989/1990	%Prevalence 2016/2019
Icterohaemorrhagiae	49.1	
Bratislava	76.3	18.1
Grippotyphosa	20.1	4.2
Pomona	15.9%	2.8%

Isolation and molecular identification of Bratislava in horses has been reported but not in the US [184, 188]. Reproductive problems and poor health in horses has been associated with serovar Bratislava [184, 369]. Further studies in the US need to be undertaken looking at reproductive problems and poor health, and

their possible association with serovar Bratislava. Exposure and increased titers to serovar Bratislava may not cause abortion but could be associated with reproductive and general poor health in horses.

Methods to attempt to detect and prevent leptospiral abortion was the focus of the research in Chapter Four. Heme oxygenase-1 (HO-1) and its' role in pregnancy and infection has been studied in humans and several other mammalian species, but until now, not in horses. HO-1 in horses does not fully follow what occurs in other mammals. This study showed it is significantly upregulated in horses who have elevated leptospiral MAT titers and are pregnant. Pregnant horses had increased HO-1 levels as compared to non-pregnant mares and increased further in mares who are pregnant and have placentitis. Compared to other mammals, HO-1 in mares in this study didn't decrease prior to foaling. The decrease in other mammals appears to be part of the preparation of the placenta for labor. Why this doesn't occur in horses requires further research. HO-1 in the mare could be closely tied to relaxin which also does not decrease prior to foaling but increases similar to HO-1. Further studies are indicated in the role HO-1 contributes not only to pregnancy, but also with infection in the horse.

Leptospirosis continues to remain a challenge for the equine industry in the US but also globally in humans and animals. It appears that the changing climate will increase these challenges as this bacterium continues to change and adapt to its environment, potentially making it more difficult to prevent infection[60].

Appendix 1

Leptospiral Vaccines

Table One: US Leptospiral Vaccines

Species	Zoetis	Boehringer Ingelheim	Elanco	Merck
Bovine	Hardjo Pomona ICT* Canicola Grippotyphosa	Hardjo Pomona ICT Canicola Grippotyphosa	Hardjo Pomona ICT Canicola Grippotyphosa	Hardjo Pomona ICT Canicola Grippotyphosa
Ovine				
Caprine				
Swine	Hardjo Pomona ICT Canicola Grippotyphosa Bratislava	Hardjo Pomona ICT Canicola Grippotyphosa		
Canine	Pomona ICT Canicola Grippotyphosa	Pomona ICT Canicola Grippotyphosa	Pomona ICT Canicola Grippotyphosa	Pomona ICT Canicola Grippotyphosa
Equine	Pomona			

*ICT=Icterohaemorrhagiae

Table Two: Non-US Leptospiral Vaccines

Species	Virbac (New Zealand)	Zoetis (Australia)	Hipra (Brazil)	M-Laboratorios Microsules
Bovine	Hardjo, Pomona Copenhageni	Hardjo Pomona	Hardjo Wolffi Icterohaemorrhagiae Bratislava Pomona	Hardjo Wolffi Icterohaemorrhagiae Pomona Canicola Copenhageni Grippotyphosa Tarassovi Pyrogenes Bataviae
Ovine		Hardjo Pomona		Wolffi Icterohaemorrhagiae Pomona Canicola Copenhageni Grippotyphosa Tarassovi Pyrogenes Bataviae
Caprine		Hardjo Pomona		Wolffi Icterohaemorrhagiae Pomona Canicola Copenhageni Grippotyphosa Tarassovi Pyrogenes Bataviae
Swine				Wolffi Icterohaemorrhagiae Pomona Canicola Copenhageni Grippotypohsa Tarassovi Pyrogenes Bataviae

Appendix 2

BD/DIFCO EMJH Base Medium*

Ingredient	Amount/L
Albumin	
Polysorbate 80	
Disodium phosphate (buffering agent)	1gram
Monopotassium phosphate (buffering agent)	0.3gram
Sodium chloride	1gram
Thiamine (growth factor)	0.005gram
Ammonium chloride (nitrogen source)	

pH 7.5±0.2

*https://legacy.bd.com/ds/technicalCenter/inserts/S1368JAA(201107).pdf?_ga=2.167973857.1115642891.1605379874-2065563281.1605379874

BD/BBL Leptospira Enrichment Media**

Ingredient	Purpose
Long-chain fatty acids	Carbon and energy source
Thiamine B1)	Growth and energy
Cyanocobalamin (B12)	
Lyophilized rabbit serum	Hemoglobin

**https://www.bd.com/en-us/support/bd-life-sciences-diagnostic-systems-package-inserts

HIMEDIA

EMHJ Base Medium

Ingredient
Disodium hydrogen orthophosphate (buffering)
Monopotassium phosphate (buffering)
Sodium chloride (osmotic equilibrium and essential ions)
Ammonium chloride (essential nutrients for growth)
Thiamine

pH (25^{O}C) 7.5 ± 0.2

Himedia: Leptospira Enrichment Media (add 1vial/180ml sterile water then add to base media)***

Ingredients
Calcium chloride
Magnesium chloride
Zinc sulphate
Copper sulphate
Ferrous sulphate
Vitamin B_{12}
Bovine serum albumin
Polysorbate 80
Glycerol
Distilled water

Final pH at 25^{O}C is 7.2 ± 0.2

***http://www.himedialabs.com/TD/FD066.pdf (enrichment website)

+http://himedialabs.com/TD/M1009.pdf (base media)

Horsnby and Nally Media[++]

HAN media

BSA	10g	
Thiamine chloride	1ml,	0.5g/100ml water
Calcium chloride	1ml,	3g/100ml
Magnesium chloride		1ml, 3g/100ml
Zinc sulphate		1ml, 0.4g/100ml
Hemin		10ml, 6.52mg/100ml
Vitamin B12		1ml, 0.02g/100ml
Tween 80		12.5ml, 10ml/100ml
Glycerol		1ml, 10% solution in water
Ammonium Chloride		1ml, 25% solution in water
Disodium phosphate		1g
Monopotassium phosphate		0.3g
Sodium chloride		1g
Sodium pyruvate		0.1g
5-Fluoruracil		100ug/ml

Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 200ml

pH 7.4Made up to 1L and filtered sterilized

[++]Hornsby, R.L., D.P. Alt, and J.E. Nally, Isolation and propagation of leptospires at 37 degrees C directly from the mammalian host. Sci Rep, 2020. 10(1): p. 9620.

APPENDIX 3

Survey Sent to AAVLD Accredited Laboratories

1. Does your lab run MAT for leptospirosis?
    - If YES to #1 what serovars can/does your lab run
    - If YES to #1, on average, about how many serum samples does your lab receive per year for MAT testing
        - **Break out numbers for bovine and equine samples**
2. On abortion necropsies, does your lab run an FA or PCR to rule-in/out leptospiral abortion?
3. On average, how many lepto abortions are confirmed in your laboratory each year
    - Break out numbers for bovine and equine samples
4. On equine uveitis cases, does your lab run a FA or PCR on aqueous humor to rule-in/out a leptorspiral etiology?
5. On average, how many equine recurrent uveitis cases does your lab diagnose each year?

   Would your lab be willing to participate in a necropsy-based study to assess the carrier state of leptospires in healthy adult horses?

APPENDIX 4

AAVLD Accredited Laboratories*

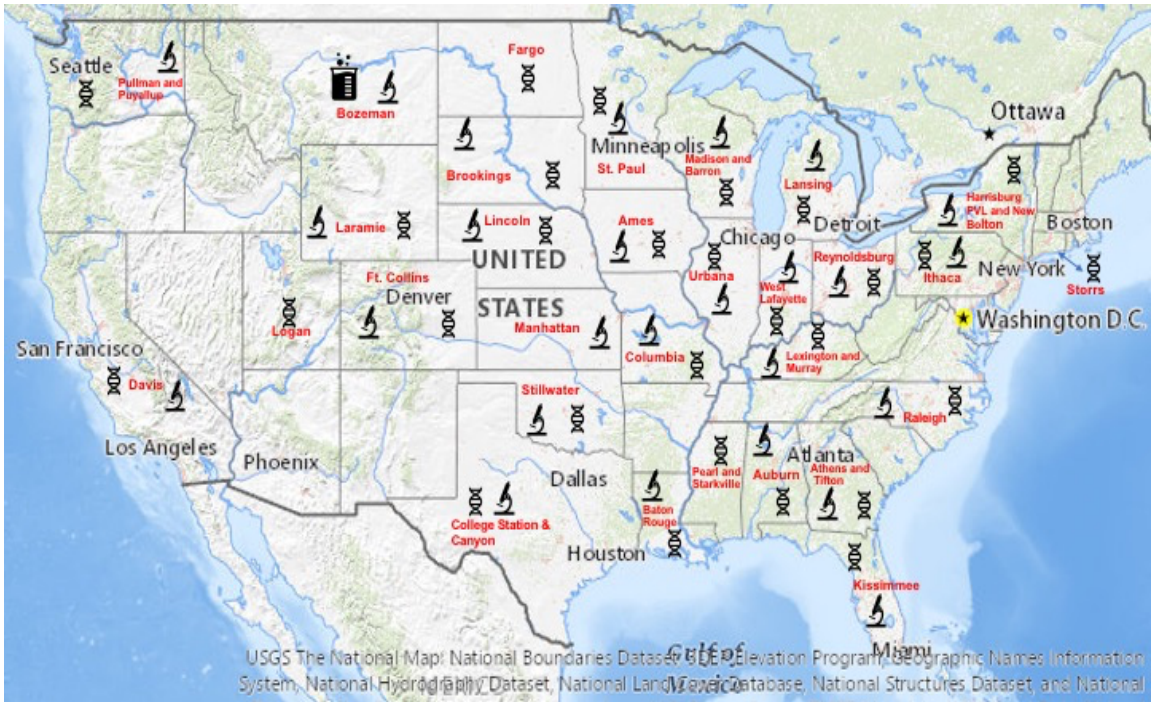
State	Serovar	PCR	FA	IHC
Alabama	Canicola, Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae	Yes		
Arizona	**Send Out**			
California	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae	Yes		
Colorado	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae	Yes		
Connecticut		Yes		
Florida	Canicola, Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae	Yes		Yes
Georgia-Athens	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae		Yes	
Georgia-Tifton	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae, Autumnalis			
Illinois	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae, Autumnalis	Yes		Yes
Indiana	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae, Autumnalis	Yes		
Iowa	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae	Yes		Yes
Kansas	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae			
Kentucky-Lexington	Canicola, Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae	Yes	Yes	
Kentucky-Murray	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae, **(Other)**	Yes		
Louisiana	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae	Yes		
Michigan	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae	Yes		
Minnesota	Canicola, Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae, Bratislava	Yes		Yes
Mississippi-Starkville/Pearl	Starkville-Refer to TVMDL Pearl-PCR	Yes		
Missouri	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae	Yes		
Montana	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae, Autumnalis			Yes

Nebraska	Canicola, Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae, Bratislava	Yes		Yes
New York-Ithaca	Canicola, Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae/Copenhageni	Yes	Yes	
North Carolina-Raleigh	Canicola, Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae, Autumnalis, Bratislava	Yes		Yes
North Dakota		Yes		Yes
Ohio	Canicola, Hardjoprajitno, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae	Yes		Yes
Oklahoma	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae	Yes		
Pennsylvania-New Bolton	Canicola, Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae (either Autumnalis or Bratislava); Canine-does all 7	Yes	Yes	
Pennsylvania-PSU		Yes	Yes	
Pennsylvania-PVL	Canicola, Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae (either Autumnalis or Bratislava); Canine-does all 7	Yes	Yes	
South Dakota	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae	Yes		
Texas-College Station	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae, Autumnalis, Sejroe	Yes		
Texas-Canyon	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae, Autumnalis, Sejroe			
Utah-Logan		Yes		
Virginia-Blacksburg	Nothing listed on test/fee pdf (lcrvth@vt.edu)			
Washington-Pullman	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae (Canine additionally Autumnalis)	Yes	Yes	
Washington-Puyallup	Avian only			
Wisconsin-Madison	Canicola, Hardjo, Pomona, Bratislava, Grippotyphosa, Icterohaemorrhagiae (will also run Autumnalis and Sejroe)	Yes		
Wisconsin-Barron	Probably Madison only but didn't list			
Wyoming	Canicola, Hardjo, Pomona, Grippotyphosa, Icterohaemorrhagiae	Yes		

*Current-2021

APPENDIX 5

Map of AAVLD Locations and Leptospiral Tests



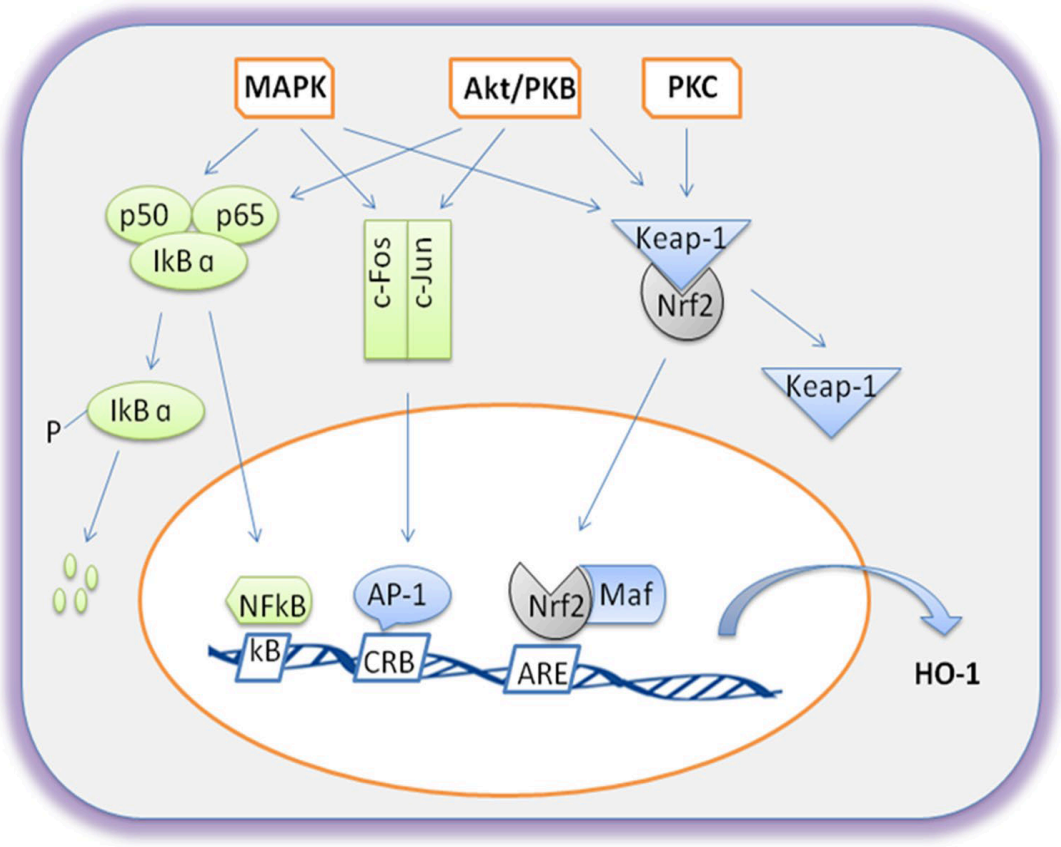
Microagglutination Test (MAT)

Polymerase Chain Reaction (PCR)

Immunohistochemistry (IHC)

APPENDIX 6

Induction Pathways of HO-1*



AP-1, Nrf2 and NF-κB are localized in cytosol under normal conditions. Under stress, they are activated and translocate to the nucleus. In the nucleus they bind specifically to the DNA sequence which leads to the HO-1 transcription.

*Waza, A. A.: Z. Hamid, S. Ali, S. A. Bhat, and M. A. Bhat, M. *A review on heme oxygenase-1 induction: is it a necessary evil.* Inflamm Res, 2018. **67**: p. 579-588.

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VITA

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PROFESSIONAL EXPERIENCE

USDA-ARS-FAPRU, Lexington, KY — January 2007-February 2020
Biological Science Technician, Microbiology

University of Kentucky, Lexington, KY — April 2005-January 2007
Research Associate, Molecular and Cellular Biochemistry

University of Kentucky, Lexington, KY — January 2004-March 2005
Research Analyst, Center on Aging

Laura's Lean Beef, Lexington, KY — May 2003-August 2003
Scientific Consultant

BD Biosciences San Diego, CA — March 2002-June 2003
(formerly Lexington, KY)
Research Associate III

University of Kentucky, Lexington, KY — February 1988-March 2002
Research Analyst, Department of Surgery

University of Kentucky, Lexington, KY — 1987-1988
Principal Laboratory Technician, Department of Animal Science

University of Kentucky, Lexington, KY — 1983-1986
Graduate Student and Teaching Assistant, Department of Animal Science

Michigan State University, East Lansing, MI — September 1979-July 1983
Veterinary Technologist, Supervisor, Veterinary Medical School

EDUCATION

Doctoral Candidate — August 2013-present
Gluck Equine Center
University of Kentucky
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MPH, Master of Public Health, Epidemiology — 2010

University of Kentucky
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MS, Master of Science, Microbiology 1986
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BS, Bachelor of Science 1979
University of Kentucky
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Morehead State University
Morehead, Kentucky

Pre-veterinary medicine (45 credit hours) 1974-1975
Purdue University
West Lafayette, Indiana

HONORS

Delta Epsilon Iota Academic Honor Society 2009-Present
Honorary Diplomate of American Veterinary
Epidemiology Society 2019

GRANTS

Research Activity Grant, University of Kentucky College of Agriculture 2009
Southeast Center for Agricultural Health and Injury Prevention, Via NIOSH/CDC Cooperative Agreement (U50OH007547), University of Kentucky,
Serological Survey for Potential Occupational Exposure to Leptospirosis in Kentucky Horse Farm Workers and Veterinarians

Southeast Center for Agricultural Health and Injury Prevention, Via NIOSH/CDC Cooperative Agreement (U50OH007547), University of Kentucky, Equine Immune Response to Leptospiral Infection 2015

Zoetis, Florham Park, NJ, Detection of Leptospiral DNA and Antibodies in Horses Submitted to the University of Kentucky Veterinary Diagnostic Laboratory for Necropsy 2015

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ABSTRACTS

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PROFESSIONAL PRESENTATIONS

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metabolism in vascular smooth muscle cells. 79th Clinical Congress of the American College of Surgeons Surgical Forum, San Francisco, CA (1993).
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meeting of the Association for Academic Surgery, Milwaukee, WI (2001).
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22. Davis BE, Lawrence LM, Flythe MD, Hayes SH, Gellin GL, Strasinger LA, Brummer M, Fowler AL. Effects of antibiotics on the microflora and fermentative characteristics of equine feces. Presented at the University of Kentucky 2nd Annual College of Agriculture Animal and Food Sciences Graduate Associates Poster Symposium, Lexington, KY, 2012.
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25. Gellin G. Serological Survey for Potential Occupational Exposure to Leptospirosis in Kentucky Horse Farm Workers and Veterinarians. Presented at the 62nd Annual James Steele Conference on Diseases in Nature Transmissible to Man, San Antonio, TX, 2012.
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